

ETHNIC DIFFERENCES IN ENDOTHELIAL FUNCTION AND  
MONOCYTE SUBSETS IN HEART FAILURE

by

EDUARD SHANTSILA

A thesis submitted to

The University of Birmingham for the degree of

DOCTOR OF PHILOSOPHY

Primary Care Clinical Science

School of Health & Population Sciences

The University of Birmingham

January 2012

UNIVERSITY OF  
BIRMINGHAM

**University of Birmingham Research Archive**

**e-theses repository**

This unpublished thesis/dissertation is copyright of the author and/or third parties. The intellectual property rights of the author or third parties in respect of this work are as defined by The Copyright Designs and Patents Act 1988 or as modified by any successor legislation.

Any use made of information contained in this thesis/dissertation must be in accordance with that legislation and must be properly acknowledged. Further distribution or reproduction in any format is prohibited without the permission of the copyright holder.

## **Abstract**

**Introduction and Aims:** The progressive nature of heart failure (HF) is reflected by its complex pathophysiology, featured by imbalance of damaging and reparative factors. The overall aim was to assess the implication of endothelial (dys)function, monocyte subsets, different types of endothelial progenitors and plasma microparticles in subjects with HF. A special focus was an investigation of possible ethnic differences in these parameters.

**Methods:** Parameters of vascular function, monocyte subsets, endothelial progenitors, and cellular microparticles were compared between South Asian subjects with systolic HF, and those with heart disease without HF and healthy controls. Ethnic differences in HF were assessed in three ethnic groups: South Asians, Whites, and African-Caribbeans. Additionally, leukocyte counts were compared between subjects with HF with reduced or preserved ejection fraction, whose outcome (mortality) was recorded during follow-up.

**Results:** South Asian subjects with HF had significantly impaired micro- and macrovascular endothelial function, reduced levels of endothelial progenitors, and monocytes with reparative potential, but increased levels of microparticles. In HF patients, a high count of monocyte microparticles was associated with low ejection fraction.

There were significant ethnic differences in characteristics of microvascular endothelial function, counts of CD14<sup>++</sup>CD16<sup>+</sup> and CD14<sup>+</sup>CD16<sup>++</sup> monocytes and monocyte-derived endothelial progenitors. On multivariate analysis, a high monocyte count was a significant predictor of death in HF with preserved ejection fraction unlike in those with systolic HF.

**Conclusions:** Significant impairment of microvascular endothelial function is present in South Asian subjects with HF. High monocyte count is an independent predictor of death in HF with preserved ejection fraction. The value of the tested biological markers as therapeutic targets should be explored in future studies.

## **Dedications**

*This thesis is dedicated to  
my parents Cheslaw and Teresa Shantsila, and to  
my wife Alena Shantsila  
for their love, endless support and encouragement.*

## **Acknowledgments**

Firstly, I would like to thank my Supervisors Professor Gregory Lip and Dr Paramjit S Gill for giving me an opportunity to undertake this PhD. I am most grateful for all their invaluable encouragement, guidance, and support over the last four years.

I also want to thank all colleagues from the Centre for Cardiovascular Sciences at City Hospital for their help and assistance, particularly Luke Tapp, Benjamin Wrigley, Silvia Montoro-Garcia, Deirdre Lane, Jeetesh Patel, Khoo Chee, Suresh Krishnamoorthy, Stavros Apostolakis, Balu Balakrishnan, Anthony Jaipersad, Girish Dwivedi, and senior colleagues Professor D Gareth Beevers, and Dr Robert MacFadyen.

Special thanks to Dr Andrew D Blann for his introduction to the world of experimental and laboratory research, his patient support in the elaboration and validation of the research tools and statistical advice.

I am very grateful to Ronnie Haynes, Susan Cartwright, Barbara Philpotts and other members of the ASCOT team for providing excellent facilities for clinical part of the study.

A big thank also goes to my colleagues from Belarus – Professor Viktor Snezhitskiy, Professor Vladimir Pyrochkin, Dr Alexander Pyrochkin and other Belarussian colleagues who provided generous help and support for this study.

## Table of Contents

CHAPTER I. INTRODUCTION .....	21
CHAPTER II. LITERATURE REVIEW .....	29
2.1. Heart failure in South Asians .....	29
2.1.1. Search strategy .....	29
2.1.2. Prevalence of heart failure .....	30
2.1.3. Aetiology of heart failure .....	34
2.1.4. Clinical characteristics of heart failure .....	37
2.1.5. Drug management of heart failure .....	38
2.1.6. Hospital admissions .....	38
2.1.7. Prognosis .....	39
2.1.8. The developmental origins of adult cardiovascular disease .....	41
2.1.9. Conclusions .....	44
2.2. Endothelial function in heart failure .....	46
2.2.1. Pathophysiological insights on endothelial dysfunction .....	46
2.2.2. Clinical evidence of endothelial dysfunction .....	50
2.2.3. Endothelial dysfunction of the coronary circulation .....	52
2.2.4. Systemic nature of endothelial dysfunction .....	53
2.2.5. Endothelial dysfunction and clinical outcome .....	54
2.2.6. Blood markers of endothelial dysfunction .....	56

2.2.7. Pharmaceutical agents, nutritional supplements and endothelial function .....	58
2.2.8. Exercise and endothelial function.....	64
2.2.9. Genetic predisposition of endothelial dysfunction .....	66
2.2.10. The endothelium and thrombotic risk.....	68
2.2.11. Conclusions and future directions.....	74
2.3. Monocytes in heart failure.....	76
2.3.1. Inflammation and heart failure .....	76
2.3.2. Monocyte activation .....	80
2.3.3. Monocyte-endothelium and monocyte-myocardial interactions .....	82
2.3.4. Monocytes and inflammation and plaque destabilisation.....	86
2.3.5. Monocyte scavenger receptors.....	88
2.3.6. Monocyte counts in myocardial infarction and heart failure.....	90
2.3.7. Drug treatment and monocyte activity.....	91
2.3.8. Monocyte subsets.....	93
2.3.9. Angiogenesis and heart failure .....	96
2.3.10. Monocytes and angiogenesis .....	98
2.3.11. Endothelial progenitors in heart failure .....	100
2.3.12. Monocytes as endothelial progenitor cells.....	103
2.3.13. Monocyte-platelet aggregates .....	107
2.3.14. Conclusions.....	111
2.4. Circulating microparticles in cardiovascular disease .....	114

2.4.1. Definition of microparticles.....	114
2.4.2. Endothelial cell microparticles – a marker of vascular damage/dysfunction ...	115
2.4.3. Platelet microparticles – beyond a marker of platelet activation.....	119
2.4.4. Leukocyte microparticles – regulators of cellular interactions.....	123
2.4.5. Microparticles and atherogenesis.....	125
2.4.6. Microparticles and (athero)thrombosis.....	127
2.4.7. Microparticles in heart failure.....	130
2.4.8. Conclusions.....	131

### CHAPTER III. ETHNIC DIFFERENCES IN ENDOTHELIAL AND VASCULAR

FUNCTION IN HEART FAILURE .....	132
3.1. Introduction to the chapter.....	132
3.2. Aims and hypotheses.....	136
3.3. Study population.....	138
3.3.1. Interethnic comparisons in heart failure .....	138
3.3.2. Comparison of heart failure subjects to controls without hear failure.....	141
3.4. Methods .....	143
3.4.1. Assessment of macrovascular endothelial function: flow-mediated dilation of brachial artery .....	143
3.4.2. Assessment of microvascular endothelial function: laser Doppler flowmetry .	147
3.4.3. Assessment of arterial stiffness.....	149
3.4.4. Echocardiography .....	151



3.4.5. Flow cytometry .....	151
3.4.6. Statistical analysis and power calculation .....	156
3.5. Results .....	158
3.5.1. Endothelial function and arterial stiffness in heart failure.....	158
3.5.2. Monocyte subsets and monocyte-platelet aggregates in heart failure .....	167
3.5.3. Endothelial progenitor cells in heart failure .....	174
3.6. Discussion.....	182
3.6.1. Endothelial function and arterial stiffness .....	182
3.6.2. Monocyte subsets.....	186
3.6.3. Microparticles derived from monocytes and platelets.....	188
3.6.4. Endothelial progenitor cells.....	189
3.6.5. Potential limitations .....	192
3.7. Conclusions .....	194
 CHAPTER IV. DEFINITION AND CHARACTERISATION OF HUMAN MONOCYTE SUBSETS.....	 195
4.1. Introduction .....	195
4.2. Aims and hypotheses.....	196
4.3. Study population.....	197
4.3.1. Overall comments .....	197
4.3.2. Immunophenotypic characterisation.....	198
4.3.3. Effects of physical exercise .....	198

4.3.4. Diurnal variation .....	199
4.3.5. Time-delay analysis .....	199
4.4. Methods: Flow cytometry.....	200
4.4.1. Equipment and software .....	200
4.4.2. Absolute count of monocyte subsets .....	200
4.4.3. Expression of surface antigens on monocyte subsets .....	204
4.4.4. Assessment of intracellular activation of NFκB pathway .....	206
4.4.5. Monocyte phagocytic activity.....	209
4.4.6. Cell sorting and stimulation experiments .....	210
4.4.7. Statistical analysis.....	212
4.5. Results .....	213
4.5.1. Discrimination of monocyte subsets.....	213
4.5.2. Immunophenotypic characteristics of monocyte subsets.....	215
4.5.3. Bone marrow monocytic cells .....	216
4.5.4. Functional characteristics of monocyte subsets.....	219
4.5.5. Effects of physical exercise .....	222
4.5.6. Diurnal variation .....	225
4.5.7. Time-delay analysis .....	227
4.6. Discussion.....	230
4.7. Postscript .....	238
4.8. Conclusions .....	238

CHAPTER V. BLOOD LEUKOCYTES IN PATIENTS WITH HEART FAILURE: IMPACT ON PROGNOSIS.....	240
5.1. Introduction .....	240
5.2. Aims and hypotheses.....	241
5.3. Methods .....	242
5.3.1. Study population.....	242
5.3.2. Echocardiography .....	242
5.3.3. White blood cell count.....	243
5.3.4. Statistical analysis.....	243
5.4. Results .....	244
5.4.1. Demographic and clinical characteristics .....	244
5.4.2. Predictors of left ventricular ejection fraction .....	246
5.4.3. Blood cells and mortality.....	247
5.4. Discussion.....	249
5.5. Limitations.....	250
5.6. Conclusions .....	251
CHAPTER VI. SUMMARY AND OVERALL CONCLUSIONS .....	252
6.1. Thesis summary.....	252
6.2. The study limitations .....	255
6.3. Overall conclusion.....	256
6.4. Future research and implication for practice .....	257

APPENDICES.....	262
Appendix 1. Standard Operating Procedure 103 ‘Flow-mediated dilation’ .....	262
Appendix 2. Standard Operating Procedure 107 ‘Measurement of microvascular endothelial function’ .....	267
Appendix 3. Standard Operating Procedure 197 ‘Enumeration of monocytes sub-populations by flow cytometry’ .....	270
Appendix 4. Standard Operating Procedure 190 ‘Enumeration of microparticles by flow cytometry’ .....	280
Appendix 5. Standard operating procedure 201 ‘Monocyte subsets, monocyte-platelet aggregates by flow cytometry’ .....	288
Appendix 6. Standard Operating Procedure 206 ‘Intracellular I-Kappa-Kinase Beta (IKK $\beta$ )’ .....	298
Appendix 7. Standard Operating Procedure 206 ‘Assessment of monocyte phagocytic activity by Flow Cytometry’ .....	308
Appendix 8. List of the study publications.....	314
LIST OF REFERENCES .....	317

## List of illustrations

Figure 1. Mechanisms and effects of endothelial function in heart failure.....	48
Figure 2. Regulation of haemostasis by the vascular endothelium.....	70
Figure 3. Implication of monocytes in acute coronary syndrome.....	87
Figure 4. Endothelial microparticles in atherogenesis and atherothrombosis.....	116
Figure 5. Platelet microparticles in atherogenesis and atherothrombosis.....	121
Figure 6. Analysis of monocyte subpopulations by flow cytometry.....	153
Figure 7. Analysis of CD34+KDR+ endothelial progenitor cells.....	154
Figure 8. Ethnic differences in flow mediated dilation in heart failure.....	161
Figure 9. Ethnic differences in microvascular endothelial function in heart failure.....	161
Figure 10. Ethnic differences in endothelial microparticles in heart failure.....	163
Figure 11. Ethnic differences in CD14++CD16+ monocytes (Mon2) in heart failure.....	170
Figure 12. Ethnic differences in CD14+CD16+ monocytes (Mon3) in heart failure.....	170
Figure 13. Platelet microparticles in heart failure subjects vs. control groups.....	172
Figure 14. CD34+ monocytes in heart failure vs. control groups.....	176
Figure 15. KDR+ monocytes in heart failure vs. control groups.....	177
Figure 16. CD34+KDR+ endothelial progenitor cells (EPCs) in heart failure vs. control groups.....	178
Figure 17. Gating strategies and presentation of monocyte subsets.....	201
Figure 18. Gating strategies and presentation of bone marrow monocytes.....	202
Figure 19. Schematic illustration of monocyte isolation by magnetic sorting.....	211
Figure 20. The dynamics of monocytes and monocyte-platelet aggregates after the exercise.....	224
Figure 21. The diurnal variation of Mon2 monocyte subset.....	225

Figure 22. Effect of the delay in the sample processing on monocyte-platelet aggregate  
number.....229

## List of tables

Table 1. Published studies on ethnic differences in heart failure that included South Asian patients .....	31
Table 2. Features of heart failure in South Asians vs. Whites .....	45
Table 3. Effect of endothelial dysfunction on clinical outcomes in heart failure .....	55
Table 4. The role of plasma markers of endothelial function in heart failure.....	57
Table 5. Clinical studies on the effects of drug treatment and nutritional supplements on endothelial function in heart failure .....	59
Table 6. Clinical studies reporting effects of statins on endothelial function in heart failure .....	63
Table 7. Effects of exercise on endothelial function in heart failure subjects .....	67
Table 8. Cytokines/chemokines produced by monocytes/macrophages in response to vascular inflammation.....	79
Table 9. Studies on CD14 gene polymorphism .....	81
Table 10. Studies on MCP-1 gene polymorphism .....	85
Table 11. Effects of drug treatment on monocytes .....	92
Table 12. Studies assessing circulating endothelial progenitor cells in heart failure.....	101
Table 13. Monocyte tissue factor expression and monocyte-platelet aggregates in patients with cardiovascular risk factors and cardiovascular disorders.....	110
Table 14. The influence of circulating microparticles in patients with cardiovascular risk factors and cardiovascular disease .....	118
Table 15. Demographic, clinical and echocardiographic characteristics of subjects with heart failure .....	139

Table 16. Demographic, clinical and echocardiographic characteristics of subjects with heart failure .....	142
Table 17. Intra-observer variability of measurements of macrovascular endothelial function (brachial artery) with ultrasound.....	145
Table 18. Inter-observer variability of measurements of macrovascular endothelial function (brachial artery) with ultrasound.....	146
Table 19. Inter-assay variability of parameters of microvascular endothelial function assessed by laser Doppler flowmetry.....	148
Table 20. Intra- and inter-assay variability of measurement of pulse wave velocity .....	150
Table 21. Characteristics of endothelial and vascular function in South Asian subjects with heart failure compared to controls.....	159
Table 22. Parameters of endothelial and vascular function in subjects with heart failure.	160
Table 23. Univariate linear regression analysis of predictors of parameters of endothelial function and arterial stiffness .....	165
Table 24. Multivariable analysis of predictive value of ethnicity for parameters of endothelial function and arterial stiffness .....	166
Table 25. Predictive value of statins for parameters of endothelial function and arterial stiffness (univariate regression analysis) .....	166
Table 26. Monocytes, monocyte-platelet aggregates and microparticles in South Asian subjects with heart failure compared to controls.....	168
Table 27. Monocytes, monocyte-platelet aggregates and microparticles in subjects with heart failure .....	169
Table 28. Predictive value of ethnicity on monocyte subsets, and microparticles .....	173



Table 29. Endothelial progenitors and monocyte expression of VEGF receptor 1 in South Asian subjects with heart failure compared to controls .....	175
Table 30. Endothelial progenitors and monocyte expression of VEGF receptor 1 in subjects with heart failure .....	179
Table 31. Predictive value of the endothelial progenitors and monocyte angiogenic characteristics for cardiac geometry and function.....	180
Table 32. Predictive value of ethnicity on endothelial progenitors and monocyte angiogenic characteristics .....	181
Table 33. Intra-assay variability of measurement of monocyte subsets and monocyte-platelet aggregates .....	203
Table 34. Inter-assay variability of measurement of monocyte subsets and monocyte-platelet aggregates .....	203
Table 35. Intra- and inter-assay variability of measurement of monocyte surface expression of CCR2 .....	205
Table 36. Intra- and inter-assay variability of measurement of intracellular monocyte IKK $\beta$ levels .....	208
Table 37. Intra-assay variability of measurement of monocyte phagocytic activity .....	209
Table 38. Comparative characteristics of monocyte subsets .....	214
Table 39. Comparative characteristics of bone marrow and peripheral blood monocytes	218
Table 40. Summary of comparative characteristics of the 3 monocyte subsets.....	219
Table 41. Baseline values and mean fold change of MFI after LPS stimulation of monocyte subsets .....	221
Table 42. Comparison of the response to LPS stimulation between monocyte subsets ....	222
Table 43. Effect of exercise on monocyte subsets and monocyte-platelet aggregates .....	223

Table 44. Diurnal variation of monocyte subsets and monocyte-platelet aggregates.....	226
Table 45. Effects of delayed sample processing on monocyte subsets and monocyte-platelet aggregates.....	228
Table 46. Demographic and clinical characteristics of patients with reduced and normal ejection fraction.....	245
Table 47. Predictive value of the study variables for left ventricular ejection fraction.....	246
Table 48. Predictors of all cause death in patients with heart failure with preserved ejection fraction.....	247
Table 49. Logistic regression analysis for predictors of all cause death in patients with heart failure with reduced ejection fraction.....	248

## List of abbreviations

<b>AC</b>	– African-Caribbean
<b>ACE</b>	– angiotensin converting enzyme
<b>ACS</b>	– acute coronary syndrome
<b>BD</b>	– Becton Dickinson, Oxford, United Kingdom
<b>BNP</b>	– brain natriuretic peptide
<b>CAD</b>	– coronary artery disease
<b>CRP</b>	– C-reactive protein
<b>CRT</b>	– cardiac resynchronisation therapy
<b>CV</b>	– coefficient of variability
<b>EDTA</b>	– ethylene-diamine tetra-acetic acid
<b>EF</b>	– ejection fraction
<b>EMP</b>	– endothelial microparticles
<b>eNOS</b>	– endothelial nitric oxide synthase
<b>EPC</b>	– endothelial progenitor cells
<b>FGF</b>	– fibroblast growth factor
<b>FMD</b>	– flow-mediated dilation
<b>GM-CSF</b>	– granulocyte-macrophage colony-stimulating factor
<b>GP</b>	– glycoprotein
<b>GTN</b>	– glycerol trinitrate
<b>HF</b>	– heart failure
<b>HFpEF</b>	– heart failure with preserved ejection fraction
<b>HFrfEF</b>	– heart failure with reduced ejection fraction

<b>ICAM</b>	– intercellular adhesion molecule
<b>IDCM</b>	– idiopathic dilated cardiomyopathy
<b>IKK</b>	– inhibitory $\kappa$ B kinases
<b>IL</b>	– interleukin
<b>iNOS</b>	– inducible nitric oxide synthase
<b>KDR</b>	– kinase domain receptor, VEGF receptor 2
<b>LFA</b>	– lymphocyte function-associated antigen
<b>LPS</b>	– lipopolysaccharide
<b>LV</b>	– left ventricular
<b>LVSD</b>	– left ventricular systolic dysfunction
<b>MCP</b>	– monocyte chemottractant protein
<b>M-CSF</b>	– macrophage colony-stimulating factor
<b>MFI</b>	– median fluorescent intensity
<b>MI</b>	– myocardial infarction
<b>MMP</b>	– monocyte-derived microparticles
<b>MNCs</b>	– mononuclear cells
<b>MOMC</b>	– monocyte-derived multipotential cells
<b>Mon1</b>	– CD14 <sup>++</sup> CD16 <sup>–</sup> (CCR2 <sup>+</sup> ) monocytes
<b>Mon2</b>	– CD14 <sup>++</sup> CD16 <sup>+</sup> (CCR2 <sup>+</sup> ) monocytes
<b>Mon3</b>	– CD14 <sup>+</sup> CD16 <sup>+</sup> (CCR2 <sup>–</sup> ) monocytes
<b>MPA</b>	– monocyte platelet aggregates
<b>NADPH</b>	– nicotinamide adenine dinucleotide phosphate
<b>NF<math>\kappa</math>B</b>	– nuclear factor $\kappa$ B
<b>NIHF</b>	– non-ischaemic heart failure

<b>NO</b>	– nitric oxide
<b>NOS</b>	– nitric oxide synthase
<b>NYHA</b>	– New York Heart Association
<b>PAI-1</b>	– plasminogen activator inhibitor type 1
<b>PCI</b>	– percutaneous coronary intervention
<b>PMP</b>	– platelet microparticles
<b>PWV</b>	– pulse wave velocity
<b>ROS</b>	– reactive oxygen species
<b>SA</b>	– South Asian
<b>SDF</b>	– stromal cell-derived factor
<b>SR-AI</b>	– scavenger receptor class A type I
<b>TF</b>	– tissue factor
<b>TLR</b>	– Toll-like receptor
<b>TNF</b>	– tumour necrosis factor
<b>t-PA</b>	– tissue-type plasminogen activator
<b>VCAM</b>	– vascular cell adhesion molecule
<b>VEGF</b>	– vascular endothelial growth factor
<b>VEGFR1</b>	– vascular endothelial growth factor receptor 1
<b>vWF</b>	– von Willebrand factor

## CHAPTER I. INTRODUCTION

Heart failure (HF) is a common cardiovascular disorder with a complex aetiology and pathogenesis. In the countries members of the European Society of Cardiology there are at least 15 million patients who have symptomatic HF.<sup>1-4</sup> Also there could be a similar number of subjects with asymptomatic left ventricular (LV) dysfunction with the overall prevalence of HF estimated to be about 4% of the population.<sup>1</sup> However even these high numbers may underestimate the real scale of the problem. Recent guidelines of the European Society of Cardiology highlight that the diagnosis of HF is not always reported by clinicians, being considered as a condition secondary to the other disorders.<sup>1</sup> The worldwide prevalence of HF is still growing with the ageing population, and better immediate survival of patients with acute coronary events.<sup>2-3</sup>

HF is often associated with substantial disability and poor quality of life. At present about 5% of all acute hospital admissions are related to HF, and about 2% of national health budgets are attributable to the costs associated with HF management.<sup>4</sup> Despite improvements in available therapies and revascularization, the mortality of patients with HF remains high. Available data suggest that about half of HF patients die within 4 years of the diagnosis, with even worse prognosis (i.e. approximately 40% within 1 year) in those admitted to hospital with acute decompensation of HF.<sup>3, 5-6</sup>

Evidence exists that there may be epidemiological and pathophysiological differences between patients with systolic HF from different ethnic groups.<sup>7</sup> People of South Asian (SA) origin (i.e. those originating from India, Pakistan, and Bangladesh) represent one of

the world biggest ethnic groups and the largest ethnic minority group in the United Kingdom.<sup>8</sup> Importantly, there are significant differences in the pattern of cardiovascular morbidity in SA patients.<sup>7</sup> For example, SA patients with coronary artery disease (CAD) have 40-50% higher mortality compared to CAD patients from the white population.<sup>9-10</sup> Given that CAD is a major cause of HF this might partly account for the higher prevalence of HF in SAs.<sup>11</sup> HF in SAs develops at a younger age and results in 60% more hospitalizations compared to subjects of other ethnic origins.<sup>12</sup> Additionally, SAs have been under-represented in majority of clinical trials on HF.<sup>13</sup> However, treatments effective in white subjects may not be equally effective in patients from other ethnic groups, which may affect their prognosis.<sup>14-15</sup> Given the limited data available on the pathogenesis of HF in SAs this high risk community has received a specific focus in the thesis. However the possibility of ethnic differences in mechanisms of HF was assessed in the major ethnic groups represented in Birmingham, that are whites, SAs and African-Caribbeans.

Persistently high morbidity and mortality in patients with HF is likely to reflect the complex pathophysiology of the disorder. HF is no longer regarded as a purely cardiac problem, but rather a systemic disorder associated with various, initially adaptive and consequently detrimental, neurohormonal and inflammatory compensatory processes.<sup>16</sup> The knowledge of the pathophysiology and management of HF has significantly improved during the last decades. However despite modern medical interventions targeting neurohormonal activation, mortality and morbidity related to HF remains considerably high.<sup>16</sup> An approach aiming comprehensive neurohormonal blockade has reached its limits of effectiveness and new treatment targets have to be identified in relation to the complex pathophysiological nature of HF.

HF is a functional consequence of a wide range of disorders. A number of diverse and potentially modifiable pathological pathways contribute to the development and progression of HF. These mechanisms include structural and functional cardiac abnormalities, but also vascular damage and endothelial dysfunction, and inflammation.

The prominent regulatory activity of the vascular endothelium in HF was discovered about two decades ago, and its assessment in different aspects of cardiovascular medicine in general and in HF particularly has been the focus of intense research.<sup>17-19</sup> Indeed, various aspects of endothelial function are affected in HF including vasomotor, haemostatic, antioxidant and inflammatory activities. Differences also exist in the pattern of endothelial function depending on aetiology, severity and stability of HF in individual patients. Accumulating evidence also suggests a prognostic value of different measures of endothelial dysfunction in HF. Furthermore, a number of therapeutic interventions and regular exercise can improve endothelial function in HF.<sup>20-21</sup> Healthy subjects of SA origin have been shown to have abnormal endothelial function compared with white subjects.<sup>22</sup> However the status of endothelial/vascular function in SAs with HF and ethnic differences in these parameters in HF have not been explored. These issues were addressed in this study aiming to employ a holistic approach to the problem with micro- and macrovascular endothelial function addressed as well as arterial stiffness assessed in the study populations.

Inflammation is an important mechanism of endothelial dysfunction and part of ischaemia-induced myocardial damage. Inflammation has also been shown to be implicated in the



progression of HF.<sup>23-24</sup> Monocytes are a type of blood cells which is actively involved in various inflammatory processes in addition to their phagocytic capacity and the role in the innate immunity.<sup>25-26</sup> These cells are equipped with various scavenger receptors (e.g., scavenger receptor class A type I [SR-AI, CD204], haptoglobin [CD163]) for removal of biological substances from the circulation and tissues.<sup>27-28</sup> SR-AI is involved in lipid accumulation (by macrophages) and atherogenesis, but there are emerging data to implicate this receptor in a number of reparative processes, such as clearance of apoptotic cells, attenuation of oxidative stress and inflammation.<sup>29-33</sup> The number of monocytes expressing SR-AI is increased in patients with acute coronary syndrome (ACS), but limited data are available on monocyte SR-AI expression in HF.<sup>34-35</sup>

Monocytes can also be considered as the largest pool of the circulating progenitor cells, with potential to migrate into tissues and differentiate into macrophages and dendritic cells. Additionally, monocytes include a proportion of pluripotent progenitors with impressive developmental plasticity.<sup>36-37</sup> Monocytes are actively involved in angiogenesis and repair and appear to constitute a major subset of endothelial progenitor cells.<sup>38-40</sup> This functional diversity is partly attributable to the existence of several monocyte subsets.

Indeed, mice have been shown to include a specific monocyte subset able to repopulate damaged myocardial tissue in a model of myocardial infarction (MI) and this subset was associated with a better myocardial recovery and angiogenesis.<sup>41</sup> However, humans do not have exact equivalents of mouse monocyte markers and subsets, which makes direct comparisons between mouse and human monocytes problematic.<sup>25</sup> Until recently, human monocytes were described as two subsets, on the basis of the surface expression of CD14

and CD16. They included the CD14<sup>++</sup>CD16<sup>-</sup> subset (comprises about 85% of total monocytes) and the CD14<sup>+</sup>CD16<sup>+</sup> subset (usually <15% of total monocytes).<sup>42</sup> Importantly, whilst mouse data on Ly-6C<sup>hi</sup> monocytes appear to concord with those on human CD14<sup>++</sup>CD16<sup>-</sup> subset, studies on human CD16<sup>+</sup> monocytes demonstrate little, if any agreement with mouse Ly-6C<sup>lo</sup> monocytes. This raises the possibility that the profile and functions of human monocyte subsets have more differences to their mouse counterparts than currently known.<sup>41-43</sup>

“In previous research I have observed that among CD16<sup>+</sup> monocytes there were two different populations not formally recognised at that time.<sup>40</sup> These two subsets differed by their expression of CD14 (i.e., those expression high or low levels of the marker), and were defined as CD14<sup>++</sup>CD16<sup>+</sup> and CD14<sup>+</sup>CD16<sup>+</sup> cells, respectively.<sup>40, 44-45</sup> This definition of monocyte subsets based on three populations was used for the assessment of the implication of monocyte subsets in HF. More recently the existence of these three human monocyte subsets has been accepted in the consensus on monocyte nomenclature.<sup>46</sup> According to this concensus monocyte subsets should be defined as ‘classical’ CD14<sup>++</sup>CD16<sup>-</sup>, ‘intermediate’ CD14<sup>++</sup>CD16<sup>+</sup>, and ‘non-classical’ CD14<sup>+</sup>CD16<sup>+</sup> populations. Additionally it was suggested that addition of CCR2 as an additional marker might improve the subset discrimination in the future, which was confirmed by my work.

Circulating cellular microparticles, which are small submicron anucleoid phospholipid vesicles released from different cells (e.g., platelets, endothelial cells, leukocytes) have recently emerged as important messengers of cellular signals.<sup>47</sup> Indeed, microparticles carry surface proteins and contain cytoplasmic material of the parental cells which mediate

numerous biological effects of microparticles on the target cells.<sup>48</sup> Circulating microparticles have been implicated in various cardiovascular disorders, partly due to their prothrombotic and proinflammatory properties.<sup>47, 49</sup>

However only scarce data are available at present on the involvement of individual monocyte subsets, monocyte-derived angiogenic cells and plasma microparticles in HF and information on possible ethnic differences in these parameters is lacking. Accordingly these aspects of HF pathophysiology have been addressed in this thesis (Chapter III).

Although the role of different monocyte subsets in cardiovascular pathology is increasingly recognised, advances in the field are hampered by the methodological limitations, particularly in relation to definition of minor monocyte subsets. Discrimination of CD16-expressing monocytes based on their CD14/CD16 density is problematic due to a substantial overlap in density of these receptors on their surface. This makes accurate characterization of phenotype and functional status of individual subsets difficult, and results in almost unavoidable contamination with other subsets upon isolation for in vitro experiments. Thus there is a pressing need for additional markers or a new highly specific marker, which would allow unambiguous discrimination of individual monocyte subsets.

The problem related to definition of monocyte subsets is further amplified by ability of monocytes to rapidly change their phenotype during their activation, maturation and differentiation towards macrophages, dendritic and other cells. Thus there is a possibility of up- or down-regulation on CD14 or CD16 expression on individual subsets, which may lead to their wrong assignment to another subset. Although this problem is perhaps less

important for analysis of fresh whole blood samples, adding an additional marker or discovery of constitutively expressed parameter(s) is desirable for in vitro experiments.

Accordingly in a separate part of my thesis (Chapter IV) I aimed to identify markers which would allow researchers to unequivocally define human monocyte subsets. In that chapter I also aimed to shed some further light on the phenotype and functionality of monocyte subsets. Additionally, the effect of diurnal variation, physical exercise and delay in sample preparation has been addressed in this part of the study.

The clinical importance of HF with preserved ejection fraction (HFpEF) —as contrasted to HF with reduced ejection fraction (HFrEF)— is increasingly acknowledged and this condition is considered as an emerging problem of cardiovascular medicine.<sup>50</sup> The prevalence of HFpEF is growing rapidly and it is expected to become a dominant form of HF in the near future.<sup>51</sup> Despite the increasing appreciation of the problem and recognition of the comparably poor outcome of the two forms of HF, the pathophysiological mechanisms of HFpEF are still poorly understood.<sup>52-53</sup> Specifically, very scarce information is available on the role of monocytes and other leukocytes in HFpEF.

From clinical diagnostic point of view these two forms of HF can be clearly discriminated, and have different and mutually exclusive diagnostic criteria (i.e., LVEF <50% in HFrEF vs.  $\geq$ 50% in HFpEF according to the current guidelines)<sup>1</sup>. Patients with systolic HF predominantly have ischemic background, whilst HFpEF is more linked to hypertension. Of note, treatments well established in systolic HF have not necessarily been effective in HFpEF. However there is a certain overlap between these conditions. In fact, features of

diastolic dysfunction (a hallmark of HFpEF) are present in majority of subjects with systolic HF. Also the currently used cut-off level for 'normal' LVEF is rather consensus-based and signs of mild abnormalities of systolic cardiac function have been reported in HFpEF.<sup>52</sup> Although limited data are currently available on the rate of progression from HFpEF to HFrEF, it is apparent that a fraction of patients with HFpEF can consequently develop HFrEF.

Also, it is still debated on how closely pathological mechanisms of HFpEF are related to those with HFrEF. The role of inflammation has been suggested for both conditions, but possible difference between the two disorders in monocytes and other leukocytes has not yet been assessed. I aimed to address these issues in a separate study presented in Chapter V. In that study levels of blood leukocytes and their impact on outcome in HFrEF and HFpEF has been evaluated in another population with a high risk of cardiovascular disorders in Belarus.

## CHAPTER II. LITERATURE REVIEW

### 2.1. Heart failure in South Asians

#### 2.1.1. Search strategy

Published data for this review were identified by searches of PUBMED (from 1980 - ); reference lists from relevant articles as well as authors personal lists. Searches were concentrated on the following key words: (a) 'heart failure' AND one of the following terms 'South Asian', 'South Asians' – , 'India' – , 'Indian' – , 'Pakistan' – , 'Pakistani' , 'Bangladesh', 'Bangladeshi' .

(b) 'left ventricular systolic dysfunction' AND one of the following terms 'South Asian' , 'South Asians' , 'India' , 'Indian' , 'Pakistan' , 'Pakistani' , 'Bangladesh' , 'Bangladeshi' .

Total number of identified manuscripts after removal of duplicates – 131.

Manual analysis of these publications selected original clinical studies relevant to pathogenetic, diagnostic, prognostic and therapeutic aspects of systolic HF (left ventricular [LV] ejection fraction [EF] <50% was used as a threshold) and included data on patients of SA origin identified 13 relevant studies.<sup>12, 54-66</sup> Analysis of references included in these publications identified additional 7 papers.<sup>66-72</sup>

### 2.1.2. Prevalence of heart failure

Data on the prevalence of HF amongst SAs in Europe is sparse with majority of studies evaluating hospital admission rates.

Population-based studies on HF prevalence have limited number of participants from the Indian subcontinent. For example, in the Echocardiographic Heart of England Screening study 1.8% of 6286 randomly selected patients (3960 responders) aged  $\geq 45$  years with LVEF $<40\%$ .<sup>66</sup> Among patients with LVSD 47% were asymptomatic; 4% of the screened population had mildly impaired LV function with EF 40-50%. However, SAs only formed 0.7% of the sample and no data were provided to characterize HF by ethnic group.

Galasko et al. assessed LV function in 734 patients aged  $\geq 45$  years from 7 geographically and socioeconomically representative general practices in Harrow and included 216 non-White subjects, mainly of SA origin (Table 1).<sup>54</sup> LVSD with EF  $<45\%$  was documented in 3.3% of SAs with no differences seen between the SA and White ethnic groups.

In a hospital based study by Blackledge et al. the crude annual admission rates/ 10 000 population were significantly higher for both SA men (161 vs.101 for white men) and SA women (144 vs. 93 for white women).<sup>12</sup> Differences in crude incidence rates (first admission) were less noticeable (56 vs. 44 for SA and white men, and 43 vs. 41 for SA and white women, respectively), indicative of the high rates of repeated hospital admissions related to HF attributable to SA origin.<sup>12</sup> In this study, SAs were on average 8 years younger.

**Table 1. Published studies on ethnic differences in heart failure that included South Asian patients**

Author Year	Study design	Subjects	HF prevalence/ incidence	HF assessment	Results
Lip <sup>72</sup> (1997) UK	Hospital based. Prospective/ 1 centre	348 with HF	16% of SAs. Proportion of SAs in the catchment area is not reported	Clinically, 23% had EchoCG at admission, plus 7.7% 3 months prior admission	SAs were younger than the whites 68±11 vs. 75±11 years, respectively). No difference in symptoms or duration of in-hospital stay. Trend towards lower mortality in SAs. Diabetes was more common in SAs.
Singh <sup>55</sup> (2005) Canada	Hospital based. Retrospective/2 centres	827 with HF: 99 SAs, others – whites	12% of SAs. The proportion of SAs in hospital catchment area is not reported	Clinically	SAs were younger than Whites (69±12 vs. 75±13 years). SAs were younger at their first HF hospitalization (67±14 vs. 74±19 years). SAs had lower BMI, more diabetes (57% vs. 39%), less smokers (24% vs 41%). No differences in hypertension, dyslipidemia, prior MI, aetiology. Similar in-hospital mortality.
Liew <sup>73</sup> (2006) UK	Hospital based. Cross-sectional, observational	2640 with acute MI	29% of SAs. The proportion of SAs increased from 22% in 1988-92 to 37% in 1998-2002. The proportion of SAs in the catchment area is not reported	Clinically	Decline in LVSD from 33.2% to 26.5% during 15 years in both whites and SAs. No difference in LVSD or mortality between the two ethnic groups.
Lip <sup>74</sup> (2004) UK	Hospital based. Cross-sectional	103 with congestive HF	33% of SAs. Proportion of SA in the catchment area is not reported	Clinically + EF<35%	Different perception of HF in SAs, less knowledge on the diagnosis, but equally good adherence to treatment.
Newton <sup>67</sup> (2005) UK	Hospital based. Cross-sectional observational	176 SAs, 352 matched Whites	Not provided	Clinically + EchoCG	HF was less advanced in SAs. Diabetes and hypertension were more prevalent in SAs. Survival was better in SAs than in Whites.



Author Year	Study design	Subjects	HF prevalence/ incidence	HF assessment	Results
Seow <sup>65</sup> (2007) Singapore	Hospital based. Observational study	225 with HF	At 5 years 67.5% died (70% for cardiovascular causes).	Clinically +EchoCG (EF $\leq$ 40%)	Higher proportion of Malay and Indian patients than Chinese. Ethnicity was not a significant predictor of cardiovascular mortality or all-cause mortality.
Blackledge <sup>12</sup> (2003) UK	Hospital based. Historical cohort study	5789 with HF	10% of SAs. The proportion of SAs in the catchment area is not reported	Clinically	SAs compared with whites were 8 years younger; had more prior MI (10.1% vs. 5.5%), diabetes, hypertension, but less atrial arrhythmias. SAs had lower in-hospital case fatality (13% vs 19%); better 30 days, 1 year, and 2 years survival; 38% lower risk of death, more often undergone revascularisation procedures.
Patel <sup>75</sup> (2007) UK	Hospital based. Cross-sectional study	54 of SAs with HF, 47 Whites with HF		Clinically	Plasma levels of leptin in HF are higher in SAs than in whites.
Jafary <sup>76</sup> (2007) Pakistan	Hospital based. Retrospective cohort study	197 SAs with LVSD	Not reported	Clinically + EchoCG	Median follow-up 379 days 27.5% died (92.5% of cardiovascular causes), 37.2% required rehospitalisation.
Joshi <sup>68</sup> (1999) India	Hospital based. Prospective study	125 SAs with congestive HF	Not reported	Clinically	Aetiology: rheumatic heart disease 52.8%, CAD 9.6%, hypertension 9.6%, CAD with hypertension 8%, other 18.4%. Non-compliance with treatment was a leading precipitant of admission (49.6%).
Ng <sup>56</sup> (2003) Singapore	Hospital based. Retrospective observational	15774 admissions with HF (1064 died)	9.8% of Indians (4.9% of Indians among those who died)	Clinically	Hospital admissions for congestive HF were 35% higher in Malays and Indians than in Chinese; mortality was 3.5 times higher in Malays, then in Indians and Chinese.

Author Year	Study design	Subjects	HF prevalence/ incidence	HF assessment	Results
Galasko <sup>54</sup> (2005) UK	Community based. Cohort	734	29% are non-whites, mainly SAs; 5.5% had probable LVSD and 3.5% definite LVSD	By EchoCG: LVSD as LVEF <50%	No ethnicity-related difference in prevalence of HF. Non-white patients had a higher prevalence of CAD and significant LVSD than white patients (100% vs. 56%) and a trend towards less alcoholic cardiomyopathy.
Davis <sup>63</sup> (2002) UK	Community based. Epidemiological	1062 with CAD, hypertension, or diabetes	Non-white patients: MI 1,6%, angina 2,2%, hypertension 5,4%, diabetes 11,1%	EchoCG: (i) mild LVSD (EF 40-50%); (ii) severe LVSD (EF <40%)	Ethnicity was not predictive of LVSD.
Davies <sup>66</sup> (2001) UK	Community based. Epidemiological	3960	0.7% of SAs, 3% - non-whites	LVSD with EF <40%, HF clinically	Similar prevalence of LVSD in white and non-white patients (3%)

EchoCG – echocardiography

compared to Whites with a higher proportion of men (56.5%).<sup>12</sup> This was also the case for one study from Canada.<sup>55</sup> In a multiethnic population in Singapore (Chinese 77%, Malay 14%, Indians 8%) the incidence of hospital admissions for congestive HF was significantly higher (by about 35%) in both Malays and Indians than in Chinese.<sup>56</sup>

### **2.1.3. Aetiology of heart failure**

CAD is a major cause of HF amongst SAs.<sup>54, 76-77</sup> Indeed, SAs develop more extensive MI at a younger age than the general population.<sup>78</sup> A history of MI, prior to the first admission for HF (10.1%) or the presence of acute MI at time of the first admission for HF (18.8%) was much more common in SAs (10.1% and 18.8%, respectively) than in white subjects (5.5% and 10.7%, respectively).<sup>55</sup>

The high rate of CAD among SAs corresponds to higher prevalence of cardiovascular risk factors,<sup>57</sup> diabetes,<sup>12</sup> and possibly hypertension.<sup>55, 79-80</sup> They also display a characteristic dyslipidaemia pattern with relatively normal total cholesterol, low HDL cholesterol and high triglycerides.<sup>55</sup> In a recent analysis of 1668 consecutive South East Asian patients with congestive HF (EF 28±12%) in Singapore, 50.2% were found to be diabetic and 65% had ischaemic cardiomyopathy.<sup>81</sup> Diabetes was identified as an independent predictor of ischaemic cardiomyopathy (odds ratio=1.85, p=0.01) and all-cause mortality (odds ratio=1.70, p=0.01) in patients of SA origin.<sup>81</sup> In contrast, smoking is less common among SAs with HF compared to other ethnic groups.<sup>56</sup>

A non-ischaemic aetiology of HF is perhaps less typical for SAs. For example, 18% of white patients and none of the SA subjects have been reported to have alcoholic cardiomyopathy as the primary aetiology of LVSD.<sup>54</sup> This corresponds to a significantly lower frequency of heavy alcohol intake found among SAs.<sup>58, 82</sup> Additionally, atrial fibrillation is about twice as common in Whites than in SA patients (31% vs. 15%), although SAs are more likely to experience ventricular arrhythmias.<sup>56, 67</sup> Accordingly, white subjects with HF more frequently have atrial arrhythmias, both before or at the time of admission for HF.<sup>55</sup>

Of note, the pattern of HF aetiology in SAs appears to be different between SAs living in India and those who have migrated to the UK. In a small study of 125 consecutive patients admitted to the Government Medical College in Nagpur (India) with the clinical diagnosis of HF, Joshi et al.<sup>68</sup> found that rheumatic heart disease was the most common aetiology (52%) followed by ischaemic and/or hypertensive heart disease (27%), thus reflecting the impact of socio-economical differences on the profile of HF morbidity. However, a predominantly ischaemic aetiology (i.e., 77%) was reported in Pakistan.<sup>76</sup>

Of note, the approach used to determine aetiology of HF is important for comparison of the results from different studies. However such details often omitted in the manuscripts reviewed and this should be considered during interpretation of their findings.

Are ethnic differences associated with HF in SA patients related to any specific genetically determined defect? At present there is no definitive answer to this question, but polygenetic differences are most likely. For example, a deletion of 25 bp in the gene

encoding cardiac myosin binding protein C, a key constituent of the thick filaments localized to doublets in the C-zone of the A-band of the sarcomere, is common in Indians (2% to 8% of the population), and causes disorganization of sarcomeric structure; there is an association with inheritable cardiomyopathies and an increased risk of HF in Indian populations (odds ratio = 6.99,  $p < 0.05$ ).<sup>59-60, 83</sup>

In the subanalysis of the large (about 30000 participants) ethnic population-based the London Life Sciences Prospective Population (LOLIPOP) study, echocardiograms were analysed in a sub-set of 458 healthy individuals. Compared to Whites, SAs had significantly higher body mass index, fasting triglycerides and lower HDL-cholesterol, whilst blood pressure and fasting glucose levels did not differ significantly between the two groups. Although LVEF was similar in both groups, Whites had significantly greater left atrial volumes, LV mass and volumes even after indexation for body size. However, apparently healthy SAs had significantly lower mitral annular systolic velocity (8.9 cm/s vs. 9.5 cm/s,  $p < 0.001$ ) and a higher E/E' ratio (7.9 vs. 7.0,  $p < 0.001$  compared to Whites) thus indicating subtle features of reduced systolic and diastolic cardiac performance.<sup>61</sup> Additionally, SAs have a greater degree of concentric remodelling with significantly higher relative wall thickness. Multivariate models confirm that ethnicity remains independently associated with differences in LV volume and mass as well as in left atrial volume and relative mass index. It is still a matter of speculation on which specific genetic, environmental (e.g., increase stress) or behavioral (e.g., cultural) factors promote these differences as well as whether these differences are associated with a higher risk of clinically significant HF.<sup>61</sup>

Finally, albumin excretion is much higher in SAs than in Whites independently of older age, hypertension and diabetes.<sup>84</sup> Patients with HF of any cause are known to have elevated homocysteine levels, a risk factor for CAD that may contribute to an unfavourable prognosis, and several studies in the UK and other western countries have reported increased homocysteine levels amongst SAs compared to Whites or African-Caribbeans.<sup>62,</sup>  
<sup>85</sup> However, Sosin et al. have recently demonstrated that there were no inter-ethnic differences in homocysteine levels nor in serum folate or B12 concentrations.<sup>69</sup>

#### **2.1.4. Clinical characteristics of heart failure**

Despite the substantially more frequent hospital admissions and higher prevalence of CAD, SA patients with HF more often have preserved LV systolic function than Whites (38% vs. 23%, respectively).<sup>67</sup> Severe LVSD was found in 28% of Whites compared to 18% of SA patients.<sup>58</sup> Accordingly, a higher prevalence of diastolic dysfunction in SAs has been suggested but not well documented.<sup>63</sup>

One may speculate that higher hospitalization rates among SAs may be associated with co-morbidities such as diabetes mellitus. Even despite presenting at a younger age, SAs have more high-risk features at hospital discharge.<sup>12, 81</sup> For example, SA patients with HF had significantly lower levels of hemoglobin and higher levels of urinary albumin excretion.<sup>67,</sup>  
<sup>84</sup> Renal damage may partly be associated with a higher prevalence of diabetes mellitus in SAs and represent an additional cardiovascular risk factor. SA patients with mild-to-moderate congestive HF also have higher plasma leptin levels compared to white patients, which were associated with insulin resistance.<sup>75</sup> In contrast, serum adiponectin levels

showed no ethnic variation, but were associated with congestive HF and a previous history of MI, irrespective of ethnic origin.<sup>75</sup>

### **2.1.5. Drug management of heart failure**

There is a dearth of specific data on effectiveness of different drugs for HF in the SA population. An analysis of 59 randomized controlled trials in HF revealed marked ethnic variations of patients included, with under-representation of SA subjects.<sup>13</sup> Importantly, there was no significant improvement in the representation of the ethnic minorities in large clinical trials during the last two decades. Thus, the applicability of recommendations developed on white patients to all other ethnic groups can be debated. In clinical practice, SAs have been reported to receive beta-blockers, calcium antagonists (i.e., reflecting more prominent hypertensive status) and nitrates more often than white patients, whilst loop diuretics were less frequently required at admission.<sup>67</sup> Rates of discharge prescription of diuretics, beta-blockers and renin–angiotensin system antagonists did not differ significantly.<sup>67</sup> The rate of revascularisation procedures appears to be higher among SA patients, reflecting the higher prevalence of CAD.<sup>67</sup>

### **2.1.6. Hospital admissions**

Elderly SAs aged between 60 and 79 years have a five-fold higher risk of presenting with HF at hospital than their white counterparts.<sup>70</sup> Of note, hospital admissions for congestive HF were about 35% higher in Indians than in Chinese, but mortality was similar in Indians and Chinese.<sup>56</sup>

The exact reasons for more hospital admissions in SA patients with HF in Europe are not clear but may be attributable to more pronounced diastolic HF, adverse co-morbidity (e.g., hypertension, diabetes mellitus, and CAD) or poorer compliance with treatment, as well as other reasons.<sup>12, 57</sup> Indeed, only 50.4% of patients with HF in Nagpur (India) were compliant with their prescribed diet or drug therapy.<sup>86</sup> Also, 64.7% of Indo-Asians felt that God/fate controlled their health rather than reliance on doctors.<sup>74</sup> In this study, the majority of Indo-Asians (61.8%) were not aware of their primary diagnosis of HF and 3 times more often than whites did not have enough information about their drugs.

#### **2.1.7. Prognosis**

Despite a higher age-adjusted prevalence, greater hospitalization rate and worse risk factor profile, SA patients with HF appear to have much better prognosis than white patients. Unadjusted in-hospital case fatality rates are lower in SA patients than in Whites (i.e., 13% vs. 19%).<sup>12, 67</sup> Indeed, the 30 day, one year, and two year survival rates are also consistently better amongst SAs.<sup>12, 67</sup> Multivariate analysis has also confirmed an independent association of SA ethnicity with better prognosis in HF.<sup>12, 67</sup> Indeed, the better prognosis in SA patients may be associated with a lower rate of significant impairment of LV contractility, but when patients with preserved LV function were analyzed separately similar outcomes were registered irrespective of ethnic origin.

Among factors affecting outcome, age is the most important with a 44% increase in the risk of death per decade of life. Lower systolic blood pressure, renal impairment and



history of stroke were the strongest predictors of fatality rate in SAs.<sup>12</sup> A diagnosis of concomitant acute MI was associated with a much poorer event free survival.<sup>12</sup> Higher creatinine and lower hemoglobin levels at admission were each associated with adverse outcomes in SAs, although the strength of such association was statistically weaker than for white patients.<sup>67</sup> High glucose concentrations at admission were associated with poor outcome and remained independently predictive of adverse events among HF patients discharged without any treatment for diabetes.<sup>67</sup>

Interestingly, no significant relation was found between social deprivation and outcome for patients with HF in the UK. In fact, patients living in the most disadvantaged areas had lower mortality that may reflect the free health service in the UK with ability to provide additional support and more often hospitalizations to socially deprived patients.<sup>12</sup> In contrast to data from the United Kingdom, a retrospective sequential chart review of SAs and non-SA Whites in Canada hospitalized with a primary diagnosis of congestive HF showed in-hospital mortality to be similar in the ethnic groups.<sup>55</sup>

In Singapore, mortality from congestive HF was 3.5 times higher in Malays, but was about the same in Indians and Chinese. Over the period from 1991 to 1998, mortality from HF declined in both Chinese and Indians, but rose in Malays.<sup>56</sup> Another study of 225 patients with HF with LVEF <40% found proportionally more Malay and Indian patients admitted for HF than Chinese patients; of this cohort, 67.5% of discharged patients died at 5 years and predictors of mortality were female gender, aged  $\geq 70$  years, renal impairment, NYHA class III or IV, and lack of treatment with angiotensin converting enzyme inhibitors.<sup>65</sup>

Prognosis in SA patients with HF failure may also differ in relation to health service provision in different countries. For example, data from a tertiary care hospital in Pakistan reported that 27.5% patients admitted with HF died and 52% experienced a combined event of death or repeat hospitalization for HF during 1-year follow-up.<sup>76, 81</sup>

#### **2.1.8. The developmental origins of adult cardiovascular disease**

During the last decades accumulating data led to introduction of a paradigm of the developmental origins of adult disease. The concept of epigenetic modifications was originally developed by Barker and colleagues based on epidemiological links found between lower birthweight and a higher risk of cardiovascular death and type 2 diabetes in adulthood.<sup>87-88</sup> The concept, also known as the thrifty phenotype model states that the fetus can trigger *in utero* adaptations to survive environmental stresses, such as maternal undernutrition.<sup>89</sup> Restriction of the fetal growth is one of many responses, which can involve some organs or the whole body. However, the presumable benefits in the deprived environment can negatively affect the organism wellbeing in an enriched environment in the adulthood. Initial epidemiological associations were confirmed by the followed research.<sup>90</sup>

It is important to note that any associations between the birth size and future morbidity are continuous across all birth weights and do not have specific cut-off values, thus indicating applicability of the hypothesis to babies born with normal size.

The model has received a support from extensive mechanistic clinical and experimental findings. For example, a prospective study has demonstrated that children born with lower weight more often developed insulin resistance in the future.<sup>91-92</sup> The concept is in agreement with experimental studies in which manipulation of animal maternal nutrition caused long-term effects on the offspring biology.<sup>93</sup> In animal maternal undernutrition models the offspring showed impaired metabolic homeostasis with insulin resistance, hypertension and obesity, as well as features of endothelial dysfunction.<sup>94-96</sup> Even brief periods of maternal undernutrition may result into long-term health consequences.<sup>97</sup>

The Barker's model was consequently advanced by Gluckman and colleagues who suggested that many of the adaptive responses made by the fetus were produced not for immediate advantage, but in expectation of the future postnatal environment (either favourable or unfavourable, based on maternal-derived signals).<sup>98-99</sup> The concept declares that the risk of adulthood morbidity reflects the degree of mismatch between the environment the organism is exposed to during the plastic (i.e., fetal and neonatal) phase of life as opposed to the environment the organism is exposed to in the post-plastic stage. The greater the degree of mismatch is the higher the risk of disease. Thus the Gluckman's hypothesis also accommodates the postnatal components of the development.<sup>100</sup> However, the relative contribution of the fetal and the postnatal periods of the human development are still under debates, and require further research.<sup>100</sup> Of interest, an animal model of prenatal maternal undernutrition revealed a synergistic effect with postnatal exposure to a fat rich diet resulting in insulin and leptin resistance, obesity and hypertension.<sup>95</sup>

Of importance, the concepts of developmental origins are not based on the role of the birth size per se, whilst the birth size can act as one of indicators of an unfavourable fetal environment.<sup>101</sup> The developmental plasticity appears to be a tightly regulated phenomenon by which one genotype can lead to a range of phenotypes. The processes of developmental plasticity embrace the commitment of cells to specific lineages, tissue differentiation and growth, at least partly via epigenetic changes in gene expression. It has been demonstrated that the environmental factors may modulate fetal gene expression by two different mechanisms: (i) by transiently and reversibly affecting gene expression (i.e., a normal mechanism of gene promotion and repression), or (ii) via chemical modification of the DNA or chromatin (i.e., epigenetic modification), for example via DNA methylation or histone acetylation.<sup>102-103</sup> Epigenetic gene modification may mediate changes in hypothalamic–pituitary–adrenal axis, glucocorticoid sensitivity, mitochondrial function, and (anti) oxidative capacity. This can consequently contribute to the higher risk of coronary heart disease, stroke, diabetes, hypertension, and heart failure.<sup>104</sup>

These models of epigenetic changes and developmental origin of adult disease perhaps can partly explain some ethnic differences in cardiovascular morbidity and pathogenesis. Indeed, very high prevalence of insulin resistance/diabetes, dyslipidemia, and CAD in subjects of SA origin can be partly attributable to the developmental gene modulation in less developed countries with suboptimal nutrition. These speculations are supported by the evidence of impaired endothelial function, seen even in apparently healthy SA subjects, and increased leptin levels in HF patients from this ethnic group.<sup>22, 75</sup>

### 2.1.9. Conclusions

There are substantial ethnic differences for systolic HF, which tend to occur at a younger age in SAs and requires more hospital readmissions (Table 2), and could perhaps be partly explained by developmental restrictions (e.g., undernutrition). In contrast, survival for such patients appears to be significantly better than for Whites, which might be associated with different patterns of HF (e.g., lower rate of severe LVSD). A high prevalence of hypertension and diabetes amongst SAs may predispose to diastolic HF (i.e., HFpEF). Additionally, the lack of convincing data on clinical effectiveness of currently recommended treatments stems from underrepresentation of this ethnic group in pharmaceutical clinical trials and racial inequity of access to the health service should also be considered.<sup>105</sup>

Certain heterogeneity in the published data is likely to reflect differences in health service systems, differences in study populations (i.e., primary care vs. hospital-based), variations in the employed diagnostic thresholds for HF, differences in HF aetiology, etc. Admittedly, current knowledge on specific prognosis in SAs with HF is often based upon in-hospital cohorts, and more longitudinal community-based studies are needed to establish more accurate picture of ethnicity-related prognostication in HF patients. Although HF is an important cause of cardiovascular morbidity and mortality, there are still little data on the prevalence of systolic HF amongst the minority ethnic communities in Europe.

**Table 2. Features of heart failure in South Asians vs. Whites**

	South-Asians vs. Whites	Study
Prevalence	Similar	Galasko <sup>54</sup>
Age of presentation	Lower	Blackledge <sup>12</sup> Singh <sup>55</sup>
Ischaemic aetiology of HF	More common	Galasko <sup>54</sup>
History of myocardial infarction prior to the first HF admission	Higher	Blackledge <sup>12</sup>
Diabetes in HF patients	More common	Blackledge <sup>12</sup>
Atrial fibrillation in HF patients	Less common	Newton <sup>67</sup>
Hospital re-admissions	More common	Blackledge <sup>12</sup> Newton <sup>67</sup> Sosin <sup>70</sup>
Age-adjusted mortality	Lower	Blackledge <sup>12</sup>

Of importance, the pathophysiological mechanisms of the ethnic differences in systolic HF are not clear at present. These differences may be partly linked to the abnormalities in vascular endothelial dysfunction in HF (the review of the literature on the role of endothelium in HF is provided in Section 1.2.). Also the role of immune system in pathogenesis of different cardiovascular disorders is progressively appreciated. Among blood cells monocytes could be of particular interest as they could contribute to detrimental processes (e.g., inflammation, thrombosis, formation of atherogenic ‘foam’ macrophages, atherosclerotic plaque progression and destabilization) but they also seem to be crucial for cardiovascular repair. The literature on the role of monocytes in cardiovascular disorders/conditions relevant to HF development and progression is discussed in Sections 2.3 and 2.4.

## **2.2. Endothelial function in heart failure**

### **2.2.1. Pathophysiological insights on endothelial dysfunction**

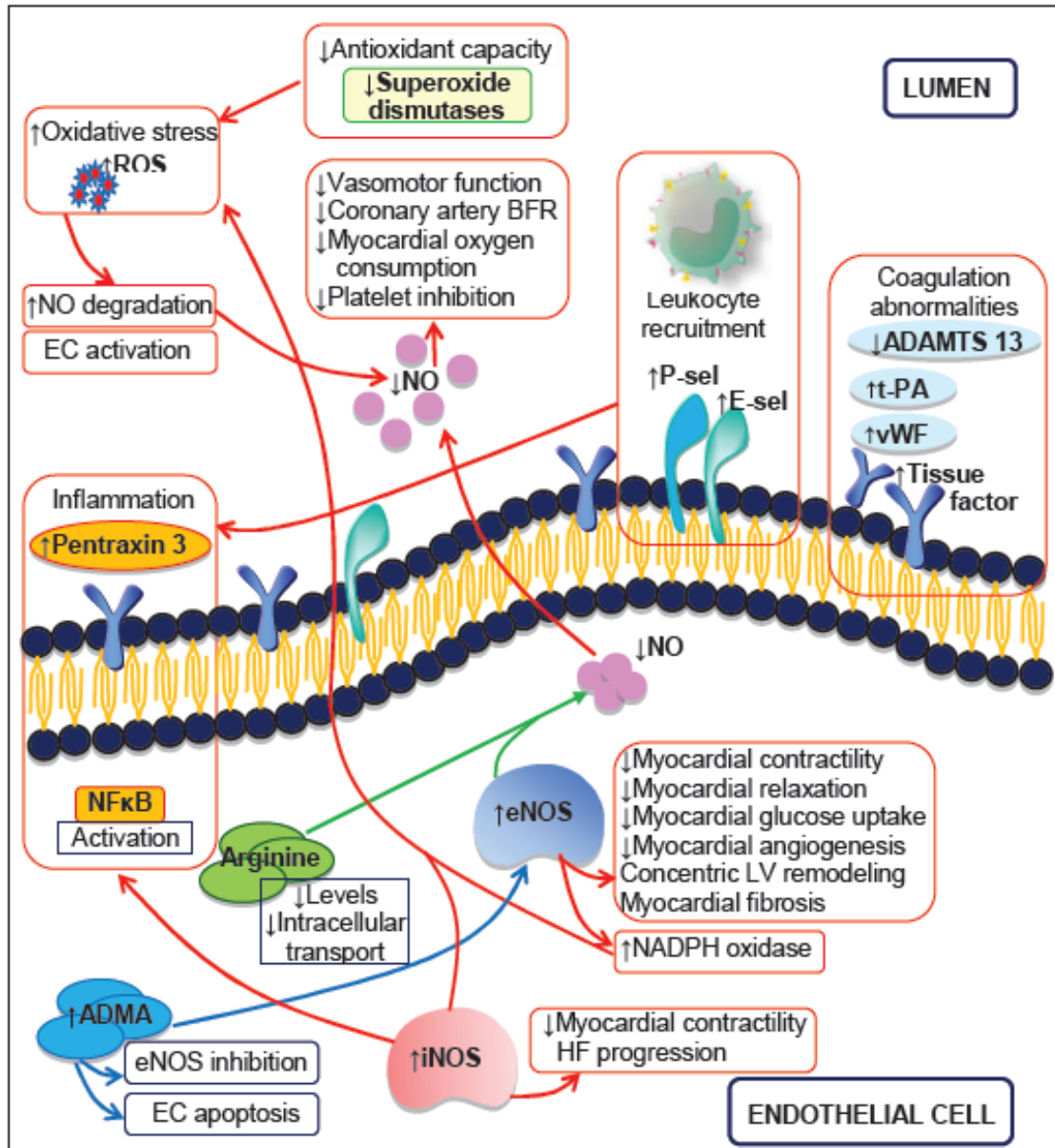
Although the term ‘endothelial dysfunction’ is used throughout the manuscript there is a continuum of the quiescent endothelial ‘activation’, endothelial ‘dysfunction’, and endothelial ‘damage’.<sup>106-107</sup> Endothelial ‘activation’ usually refers to physiological response to various stimuli (including inflammatory cytokines), such as bleeding, infection, etc. aiming to preserve homeostatic stability of the host (protective changes). Endothelial ‘activation’ may involve increased expression and shedding of some surface adhesion molecules, release of von Willebrand factor (vWF) and fibrinolytic factors. A crucial aspect of ‘activation’ is that it is reversible upon cessation of the activating agent(s). In contrast, endothelial ‘dysfunction’ refers to the situations of sustained excessive (e.g., increased reactive oxygen species [ROS] production) or depressed (e.g., impaired vasodilation) endothelial performance. Given that endothelial ‘dysfunction’ could follow chronic ‘activation’ (such as by prolonged and inappropriate activation by inflammatory cytokines), there is a certain overlap between the two states. In terms of blood pressure control, a major pathological feature of endothelial dysfunction (in the context of cardiovascular disorders) is a functional deficiency of endothelial NO synthase (eNOS). This leads to the reduced bioavailability of nitric oxide [NO] and is usually detected by impaired vasomotor responses. Whereas dysfunction may be reversible, endothelial ‘damage’ refers to the extreme degree of endothelial dysfunction characterized by premature apoptosis/death of endothelial cells. Increased shedding of the circulating

endothelial cells and high plasma concentrations of vWF are considered markers of endothelial damage. Such damage is unlikely to be reversible.<sup>106</sup>

Although the endothelium serves as a critical regulator of different aspects of vascular biology, such as haemostasis and inflammation, its ability to produce NO is pivotal for the different endothelial-dependent functions related to the development and progression of HF. In addition to the regulation of the haemodynamics, NO acts as a potent modulator of myocardial oxygen consumption in the failing heart.<sup>108-109</sup> Reduced availability of NO in HF stems either from its reduced production by eNOS or accelerated NO degradation by ROS (Figure 1).<sup>110</sup> Down-regulation of constitutively expressed eNOS by the endothelium is a characteristic feature of endothelial dysfunction and its endomyocardial expression in patients with dilated nonischaemic cardiomyopathy was linearly correlated with LV contractility.<sup>111</sup> Paradoxically, the chronic production of NO by inducible NO synthase (iNOS) in HF exerts deleterious effects on ventricular contractility and circulatory function.<sup>112</sup>

Mice lacking eNOS have abnormal cardiac NO production, impaired myocardial glucose uptake and pathologic concentric LV remodelling whilst eNOS overexpression reduced severity of HF.<sup>113-115</sup> Reduced eNOS activity led to the hypertrophic growth of cardiomyocytes in vitro and eNOS deficiency in mice was associated with impaired myocardial angiogenesis.<sup>116-117</sup> Whilst eNOS mRNA is reduced in LV tissue of patients with end-stage HF, iNOS mRNA is up-regulated and associates with impaired myocardial relaxation.<sup>118</sup> iNOS is located primarily and invariably in the endothelium and vascular





**Figure 1. Mechanisms and effects of endothelial function in heart failure**

ADMA, asymmetric dimethylarginine, BFR, blood flow reserve, EC, endothelial cell, E-sel – soluble E-selectin, HF, heart failure, NO, nitric oxide, eNOS, endothelial NO synthase, NFκB, nuclear factor κB, P-sel – soluble P-selectin, iNOS, inducible NO synthase, ROS, reactive oxygen species, t-PA, tissue type plasminogen activator

smooth muscle cells of the myocardial vasculature and its expression appears to be associated with the condition of HF per se rather than related to the aetiology of HF.<sup>119</sup>

ROS causes endothelial dysfunction by accelerating NO inactivation. A family of multi-subunit NADPH oxidases is a major contributor to the increased superoxide anion production and oxidative stress in HF.<sup>120</sup> Depression of the endothelial function and eNOS activity may directly lead to the increased NO release, thus maintaining a vicious circle of oxidative stress. In a rat model of diastolic HF, the increase in cardiac eNOS expression by the eNOS enhancer, AVE3085 (which is an activator of eNOS transcription) was accompanied by the reduction in NADPH oxidase, attenuation of cardiac hypertrophy, fibrosis and diastolic dysfunction.<sup>121</sup>

Antioxidant capacity is also diminished in HF. For example, in animal experiments activity of different superoxide dismutase isoforms are present in the failing myocardium whilst gene transfer of extracellular superoxide dismutase significantly improved endothelial function.<sup>122-124</sup> Increased xanthine-oxidase and reduced extracellular superoxide dismutase activity is closely associated with increased vascular oxidative stress in HF patients, indicating that an increased oxidative burden and loss of vascular oxidative balance as possible contributors to endothelial dysfunction in HF.<sup>125</sup>

There are *in vivo* and *in vitro* data showing an impaired arginine (eNOS substrate) transport in human HF.<sup>126</sup> Additionally, circulating levels of asymmetric dimethylarginine (an endogenous inhibitor of eNOS) and circulating proinflammatory cytokines are increased in HF, further contributing to endothelial dysfunction and accelerated apoptosis

of endothelial cells.<sup>127</sup> A strong correlation has been demonstrated between eNOS down-regulation and endothelial apoptosis.<sup>128</sup> Of interest, the beta-blocker carvedilol suppresses the caspase cascade and excessive human umbilical vein endothelial cell apoptosis induced by the serum of HF patients or addition of tumour necrosis factor- $\alpha$  (TNF $\alpha$ ).<sup>129</sup>

Inflammatory changes are common in patients with HF, with numerous studies reporting high concentrations of cytokines (e.g., TNF- $\alpha$  and interleukin [IL]-6) and C-reactive protein (CRP).<sup>130-134</sup> These biomarkers strongly correlate with HF severity and are strongly and independently predictive of mortality.<sup>130, 132-135</sup> In an animal model, as elsewhere, inflammation is not an innocent bystander but is rather an active participant in HF development and progression.<sup>136</sup> A dysfunctional endothelium also facilitates a pro-inflammatory status in HF by release of various inflammatory factors, such as pentraxin 3. Endothelial activation of nuclear factor  $\kappa$ B (NF $\kappa$ B), a key proinflammatory transcriptional factor, is more prominent in patients with severe HF undergoing heart transplantation.<sup>137</sup> Of note, pentraxin 3 levels independently predict cardiac death or HF re-hospitalization.<sup>138</sup> However, despite the strong evidence of a detrimental impact of inflammation on HF outcome, data on specific biological therapies against TNF- $\alpha$  (i.e., using etanercept and infliximab) have been generally disappointing.<sup>139-140</sup>

### **2.2.2. Clinical evidence of endothelial dysfunction**

Direct evidence of impairment of endothelial dysfunction in the genesis of haemodynamic abnormalities in HF has been provided by the infusion of NG-monomethyl-L-arginine, an inhibitor of NO production, to volunteers with HF.<sup>141</sup> Administration of the NG-

monomethyl-L-arginine increased median pulmonary and systemic vascular resistances and arterial pressure, characteristic features of the HF syndrome. Numerous studies have demonstrated peripheral endothelium-dependant vasomotor abnormalities in HF assessed by brachial artery flow-mediated dilation (FMD) or forearm blood flow changes in response to acetylcholine.<sup>142-144</sup> However, many such studies show weak, if any, correlations between these measures of endothelial dysfunction and clinical parameters of HF severity, cardiac contractility or wedge pressure.<sup>144-146</sup>

Although the presence of endothelial dysfunction has been uniformly shown in ischaemic HF, the evidence is less robust for non-ischaemic HF (NIHF). Both endothelial-dependent and endothelial-independent vasodilation are progressively depressed with increasing clinical severity in HF related to valvular heart disease, making it difficult to be precise on the cause of the vascular dysfunction.<sup>147</sup> In one small study, HF of non-ischaemic aetiology was not associated with abnormalities in FMD, which is in contrast to ischaemic HF.<sup>148</sup> In other studies, patients with ischaemic systolic HF have more prominent endothelial dysfunction compared with those with non-ischaemic HF of the same severity.<sup>142, 149</sup>

Although endothelial dysfunction is a feature of HF of any aetiology, multiple co-factors typical of ischaemic HF (e.g., atherosclerosis, diabetes, etc.) seem to contribute to systemic endothelial impairment per se. In contrast, patients with NIHF could be expected to have more localised endothelial dysfunction of cardiac vasculature. The evidence supporting this concept will be discussed further below.

### 2.2.3. Endothelial dysfunction of the coronary circulation

Significant impairment of coronary endothelial function has been observed in patients with LV dysfunction.<sup>150</sup> Significant impairment of coronary endothelial function is also present in most patients with stable idiopathic dilated cardiomyopathy (IDCM) despite normal epicardial coronary arteries, and profound coronary endothelial dysfunction was observed in acute-onset IDCM.<sup>151-152</sup> Of note, the abnormalities of coronary blood flow reserve in IDCM correlated with cardiac geometry, NT-pro-BNP levels, but not with FMD of peripheral arteries.<sup>153-154</sup> These findings indicate that in contrast to patients with an atherosclerosis-related HF aetiology, many patients with NIHF tend to have localised (i.e., coronary/endocardial) rather than systemic endothelial dysfunction. Coronary endothelial impairment in IDCM involves both resistance and conductance vessels, but may vary substantially in its severity, possibly reflecting individual pathological features of the disease (e.g., inflammatory activity).<sup>155</sup>

In one study, the coronary response to acetylcholine correlated with the subsequent improvement in LVEF during a 7 month follow-up period.<sup>152</sup> Furthermore, coronary endothelial dysfunction was independently associated with impaired cardiac relaxation in 160 patients with normal LVEF in the absence of occlusive CAD.<sup>156</sup> Also, coronary endothelial dysfunction in CAD has been associated with future progression of myocardial diastolic dysfunction.<sup>157</sup> These data are supported by findings showing that altered myocardial NO balance contributes to hypertrophy-mediated myocardial ischaemia and a transition to HF.<sup>158</sup> Both eNOS activity and expression in cardiac tissue were reduced in

ischaemic HF whilst iNOS was increased in human failing hearts.<sup>158-159</sup> Thus, coronary endothelial perturbations may be central to the pathogenesis of HF.

#### **2.2.4. Systemic nature of endothelial dysfunction**

Accumulating evidence indicates a systemic nature of endothelial dysfunction in systolic HF. Venous endothelial cells in dogs with decompensated HF show evidence of inflammatory activation secondary to high vascular stress and peripheral blood flow congestion.<sup>160</sup> On a background of CAD, impaired venous endothelial function in ischaemic HF has been associated with elevated CRP levels and over 2-fold higher NADPH-dependent superoxide generation.<sup>161</sup> Endothelium-dependent venodilation by dorsal hand veins was also significantly reduced in chronic HF, but particularly so in acute decompensated HF and its improvement correlated with the 6-minute walk test distance.<sup>162</sup> In contrast, Nightingale et al. reported preserved forearm venous endothelial function in patients with chronic NIHF despite arterial endothelial dysfunction.<sup>163</sup>

Resting exhaled NO, a marker of pulmonary endothelial NO release is increased in HF, indicating that NO output plays a counter-regulatory role in the impaired blood flow seen in chronic HF.<sup>164</sup> However, HF patients have significantly reduced ability to increase NO release during exercise, which parallels any oxygen deficit and is consistent with the presence of endothelial dysfunction. The systemic nature of endothelial dysfunction in HF is also supported by reports of defective endothelium-dependent dilatory response of the microvascular segment.<sup>165-166</sup> Also, patients with HF have reduced capillary density that is inversely related to maximal oxygen consumption.<sup>167</sup> Indeed, systemic levels of the natural

eNOS inhibitor, asymmetric dimethylarginine, are increased in HF patients and independently predict a reduced effective renal plasma flow.<sup>168</sup>

Systemic vascular dysfunction in HF also involves impaired arterial elastic properties.<sup>169</sup> These changes in HF seem to be secondary to arterial remodelling with hypertrophy of vascular wall layer seen in severe chronic HF.<sup>170</sup> Indeed, the vascular wall hypertrophy correlates with both impaired FMD and the arterial response to GTN, thus suggesting that increased arterial stiffness may affect the vascular dilatory response irrespective of endothelial function.<sup>170</sup> However, an analysis of the local arterial elastic characteristics (e.g., distensibility, compliance) in HF shows their correlation with forearm FMD, but not with the response to GTN, indicating that the increased arterial rigidity may be a feature or consequence of endothelial dysfunction.<sup>171</sup>

### **2.2.5. Endothelial dysfunction and clinical outcome**

The clinical evidence of vasomotor endothelial dysfunction in HF comes from prospective outcome studies. Although these studies are relatively small (Table 3) all invariably show an independent significant association of endothelial dysfunction with a negative outcome in HF, ranging from mild HF (NYHA class I) with relatively preserved myocardial contractility to advanced HF (NYHA class IV) with severely depressed LV function.<sup>172-173</sup>

Both FMD and the arterial response to acetylcholine are significant predictors of a high risk of unfavourable events and their predictive value does not seem to be affected by HF

**Table 3. Effect of endothelial dysfunction on clinical outcomes in heart failure**

Study	Study population	NYHA /Ejection fraction	Measure of endothelial function	Follow-up duration	Outcome	Results*
Shechter <sup>173</sup>	82 (100% IHF)	IV /22±3	FMD	14 months	Death	HR (median FMD) 2.04; 95% CI 1.09-5.1, p=0.03
de Berrazueta <sup>174</sup>	242 (38% IHF)	I-IV /36±13	FBF in response to ACH (VOP)	5 years	Composite of death, heart attack, angina, stroke, NYHA class IV, or hospitalization due to HF	HR [Exp(B)] 0.67; SE 0.18, p=0.01
Heitzer <sup>172</sup>	289 (56% IHF)	I /41±7	FBF in response to ACH (VOP)	4.8 years	Composite of death from cardiac causes, hospitalization due to HF, heart transplantation.	HR 0.96; 95% CI 0.94-0.98, p=0.007
Katz <sup>175</sup>	149 (33% IHF)	II-III /25±1	FMD	28 months	Death or urgent transplantation	HR (1% decrease in FMD) 1.20; 95% CI 1.03-1.45, p=0.027
Katz <sup>175</sup>	110 (56% IHF)	II-III /25±1	Exhaled NO production	13 months	Death or urgent transplantation	HR 1.31; 95% CI 1.01-1.69, p=0.04
Fischer <sup>176</sup>	67 (64% IHF)	II-III /47±10	FMD	46 months	Composite of cardiac death, hospitalization due to HF, or heart transplantation	HR [Exp(B)] 0.665; SE 0.18, p=0.01
Kübrich <sup>177</sup>	185 heart transplant recipients (32% IHF)	75±10	Coronary vasomotor function	60 months	Composite of death, progressive HF, MI, percutaneous or surgical coronary revascularisation	RR 1.97; CI 1.1-3.6, p=0.028

\*For all studies in the table the endothelial function was an independent predictor of outcome; ACH, acetylcholine, CI, confidence interval, FBF, forearm blood flow, FMD, flow-mediated dilation, HF, heart failure; HR, hazard ratio, IHF, ischaemic heart failure, NO, nitric oxide, NYHA, New York Heart Association, VOP, venous occlusion plethysmography, RR, relative risk, SE, standard error.



aetiology. Epicardial endothelial dysfunction is also an independent predictor of cardiovascular events and death in patients after cardiac transplantation.<sup>177</sup> Also, FMD significantly predicts the likelihood of response to cardiac resynchronisation therapy (CRT), independently of QRS duration, LVEF, or LV dyssynchrony and the improvement in FMD 3-months following CRT positively correlates with an increase in 6-minute walk distance.<sup>178</sup>

#### **2.2.6. Blood markers of endothelial dysfunction**

Most studies showed significant up-regulation of plasma markers of endothelial activation (e.g., E-selectin) and damage (e.g., vWF) in chronic HF (Table 4). However, most of these studies included patients of mixed aetiology (predominantly ischaemic) with healthy individuals serving as controls. Thus it is difficult to differentiate precisely the role of HF per se and the influence of co-morbidities (e.g., atherosclerosis, diabetes) as a driving force for the up-regulation of these markers. Indeed, Kistorp et al. observed increased levels of E-selectin and vWF in HF patients with diabetes but not in those without diabetes.<sup>179</sup> The impact of co-morbidities may also explain lack of correlation between vWF/E-selectin and parameters of HF severity (e.g., BNP levels, LVEF, 6-minute walk test or NYHA class) and no difference between patients with stable and decompensated HF.<sup>180-181</sup> Although vWF (but not E-selectin) levels were predictive of future adverse cardiovascular outcomes in stable HF, the role of co-morbidities is in question, especially given that BNP was of no predictive value in the study cohort.<sup>180</sup> Another index of endothelial damage, circulating endothelial cells have been reported to be increased in HF, with no differences between acute and chronic HF.<sup>143, 182</sup> Nonetheless, levels of the circulating endothelial cells

**Table 4. The role of plasma markers of endothelial function in heart failure**

Study	Study population	EF, % (inclusion criteria/actual)	Aetiology	Controls	Marker	Results
Kistorp <sup>179</sup>	195 CHF with diabetes 147 CHF without diabetes	≤45 /30±8 in diabetic group, 30±8 in non-diabetic group	74% IHF in diabetic group, 51% IHF in non-diabetic group	116 healthy	E-selectin vWF	↑ diabetes group ↔ in non-diabetic ↑ diabetes group ↔ in non-diabetic
Chong <sup>180</sup>	35 with AHF 40 with CHF	≤40 /30 [21-33] in AHF, 30 [29-33] in CHF	63% IHF in AHF group, 83% IHF in CHF group	32 healthy	E-selectin vWF	↑ in AHF and CHF ↔ AHF vs. CHF ↑ in AHF and CHF ↔ AHF vs. CHF
Vila <sup>183</sup>	59 CHF	Not specified	Not specified	59 healthy	vWF thrombospondin-1	↑ ↓
Chong <sup>181</sup>	137 CHF	≤45 /30 [25-35]	61% IHF	106 healthy	E-selectin vWF	↔ ↔
Chong <sup>182</sup>	30 with AHF 30 with CHF	≤40 /30 [22-32] in AHF group, 30 [29-34] in CHF	70% IHF in AHF group, 80% IHF in CHF group	20 healthy	E-selectin vWF CECs	↑ in AHF and CHF ↔ AHF vs. CHF ↑ in AHF and CHF ↔ AHF vs. CHF ↑ in AHF and CHF ↔ AHF vs. CHF
Leyva <sup>184</sup>	39 CHF	Not specified/ 22±12 in IHF, 26±16 in DCM	59% IHF	16 healthy	E-selectin	↑ (↔ IHF vs. DCM)
Chong <sup>143</sup>	30 CHF	<40 /31 [29-35]	77% IHF	20 healthy	vWF sTM CECs	↑ ↔ ↑

AHF, acute heart failure, CECs, circulating endothelial cells, CHF, chronic heart failure, DCM, dilated cardiomyopathy, EF, ejection fraction, IHF, ischaemic heart failure, sTM, soluble thrombomodulin, vWF, von Willebrand factor, ↑, increased, ↓, decreased, ↔, no changes

correlate with other markers of endothelial damage/dysfunction (FMD, plasma vWF, E-selectin) and BNP levels but not with LVEF or NYHA class.<sup>143, 182</sup>

Endothelin-1 is produced by the endothelium and vascular smooth muscle cells, and is the most potent known vasoconstrictor.<sup>185</sup> Endothelin-1 overexpression has been shown to parallel with other features of endothelial dysfunction and to be implicated in the pathogenesis of various cardiovascular disorders.<sup>185</sup> Unsurprisingly, plasma endothelin-1 levels are increased in HF patients.<sup>186</sup> Endothelin-1 promotes production of asymmetric dimethylarginine in experimental HF, which explains correlation of endothelin-1 levels with parameters of endothelial dysfunction in patients with dilated cardiomyopathy.<sup>187-188</sup> Inhibition of the endothelin-1 pathway significantly improves vasomotor endothelial function and survival in rats with HF.<sup>189</sup> Endothelium-mediated dilation in HF patients also significantly improves after 3 weeks of treatment with low (but not high) doses of endothelin A receptor blocker, LU 135252.<sup>190</sup> However clinical trials on the endothelin receptor(s) blockers in HF were disappointing,<sup>191</sup> but beta-blockers and statins have been shown to reduce endothelin-1 production thus possibly contributing their beneficial effects in HF.<sup>192-195</sup>

### **2.2.7. Pharmaceutical agents, nutritional supplements and endothelial function**

Excessive activation of the renin-angiotensin system disrupts NO downstream signalling.<sup>196</sup> Accordingly, all available studies indicate that pharmacological blockade of renin-angiotensin-aldosterone axis (e.g., angiotensin converting enzyme [ACE] inhibitors or spironolactone) improves endothelial function and reduces vWF in HF (Table 5).

**Table 5. Clinical studies on the effects of drug treatment and nutritional supplements on endothelial function in heart failure**

Author	Study	HFpatients	EF, %	Treatment*	Duration	Results
Boman <sup>197</sup>	R, DB, controlled	267 (53% IHF)	25±7	Carvedilol 25mg bd or metoprolol 50mg bd	1 year	↓vWF by carvedilol ↔vWF by metoprolol
Hornig <sup>198</sup>	R, PC	40 (34% IHF)	~25	Quinaprilat, enalaprilat, ia	Acute effects	↑FMD by quinaprilat ↔FMD by enalaprilat
Hryniewicz <sup>199</sup>	R, PC, DB	64 (52% IHF)	25±1	Ramipril 10mg or sildenafil 50mg or combination	Acute effects (1-4 h)	↑FMD (with all 3 treatments)
Drakos <sup>200</sup>	NR	11		Enalapril 10-30mg bd	4-8 weeks	↑FMD with higher doses
Tavli <sup>201</sup>	NR	30 (100% IHF)	25±5	Cilazapril 5mg	3 days	↑FMD
Gibbs <sup>202</sup>	NR	40 (80% IHF)	30	Lisinipril 10mg od, or BB (bisoprolol 5 mg or carvedilol 25mg od)	6 months	↓vWF
Poelzl <sup>203</sup>	NR	33 (40% IHF)	~24	Optimised doses of various ACEI and BB	3 months	↑FMD in responders defined by functional capacity
Farquharson <sup>204</sup>	R, DB, PC, crossover	10 (100% IHF)	31±6	Spironolactone 50mg daily	1 month	↑FBF in response to ACH (VOP)
Macdonald <sup>205</sup>	R, DB, PC, crossover	43 (67% IHF)	<25	Spironolactone 12.5-50 mg daily	3 months	↑FBF in response to ACH (VOP)
Abiose <sup>206</sup>	NR	20	24±9	Spironolactone	8 weeks	↑FMD
Farquharson <sup>207</sup>	R, PC, DB	10 (100% IHF)	20±8	Amiloride 5mg od	1 month	↔FBF in response to ACH
Belardinelli <sup>208</sup>	R, PC, DB	51 (100% IHF)	33±5	Trimetazidine 20mg tid	4 weeks	↑RA response to ACH (US)
Ito <sup>209</sup>	NR	12 NIHF	34	Vitamin C 1g, iv	Acute effects	↔FMD
Hornig <sup>210</sup>	PC	15 (20% IHF)	21	Vitamin C 0.25g, ia; 1g bd, oral	Acute effects 4 weeks	↑FMD
Ellis <sup>211</sup>	R, PC, DB	10		Vitamin C 2g, iv	Acute effects	↑FMD
Ellis <sup>212</sup>	PC	40 NIHF	<35	Vitamin C 2g, iv; 2g bd, oral	Acute effects 1 month	↑FMD

Author	Study	HFpatients	EF, %	Treatment*	Duration	Results
Erbs <sup>213</sup>	NR	18 (50% IHF)	25±4	Vitamin C 0.5g, ia	Acute effects	↑RA response to ACH (US)
George <sup>214</sup>	R, PC, DB	30		Allopurinol 300 od or bd	4 weeks	↑FBF in response to ACH (VOP) (more with 300mg bd)
Doehner <sup>215</sup>	DB, PC, crossover	14 (79% IHF)	~23	Allopurinol 300 mg od	1 week	↑FMD
Hambrecht <sup>216</sup>	R	40 (40% IHF)	19±3	L-arginine 8 g daily	4 weeks	↑FBF in response to ACH (VOP)
Hirooka <sup>217</sup>	NR	20 NIHF	~43	L-arginine 50 mg, ia	Acute effects	↑FBF in response to RH (VOP)
Chin-Dusting <sup>218</sup>	R, PC, DB	20 (60% IHF)	21	L-arginine 20 g daily	4 weeks	↔FBF in response to ACH (VOP)
Paul <sup>219</sup>	R, PC, DB	22 (100% IHF)	27±7	Methyltetrahydrofolate, iv	Acute effects	↔PWA (salbutamol-mediated changes AI) ↓ADMA
Napoli <sup>220</sup>	R, DB, PC	16 (31% IHF)	<40	Growth hormone (4 IU, sc every other day)	3 months	↑FBF in response to ACH (VOP)
Fichtlscherer <sup>221</sup>	Controlled	18 (50% IHF)	25±1	Etanercept 25 mg, sc, single dose	7 days	↑FBF in response to ACH (VOP)
Fuentes <sup>222</sup>	R, PC, DB	22 (41% IHF)		800mg magnesium oxide bd	3 months	↑small artery elasticity index
George <sup>214</sup>	R, PC, DB	26		Probenecid 1g daily	4 weeks	↔FBF in response to ACH
Patel <sup>223</sup>	NR	19 (100% IHF)	27±2	Dobutamine 3 ug/kg/min, iv	72 h	↑FMD for ≥2 weeks
Freimark <sup>224</sup>	Controlled	20 (100% IHF)		Dobutamine, 3.5 ug/kg/min, iv, 5 h twice a week	4 months	↑FMD
Schwarz <sup>225</sup>	R, PC	31 NIHF	19±7	GTN 10 <sup>-9</sup> mol/L, ia	20 min or 12h	↑ FBF in response to ACH
Guazzi <sup>226</sup>	DB, PC	16 (63% IHF)	≤45	Sildenafil 50 mg	Acute effects	↑FMD

\*oral administration unless indicated otherwise. ACH, acetylcholine, AI, augmentation index, DB, double blind, FBF, forearm blood flow, ia, intraarterial, FMD, flow-mediated dilation, IHF, ischaemic heart failure, iv, intravenous, NR, randomised, PC, placebo controlled, PWA, pulse wave analysis, R, randomised, RA, radial artery, sc-subcutaneously, VOP, venous occlusion plethysmography

However, the ability to restore endothelial function varies between individual ACE inhibitors or depends on their dose (e.g., higher doses are needed for enalapril).<sup>198, 200</sup> In one study, responder status determined by improvement in physical capacity was linked to the improvement in endothelial function with ACE inhibitors.<sup>203</sup> In the African-American HF Trial (A-HeFT) improvement in morbidity, mortality, and functional status in HF was linked to the restoration in endothelial function.<sup>227</sup>

These clinical findings are supported by experimental data showing that ACE inhibition normalizes NO-dependent dilatation in HF models. Beneficial effects of ACE inhibitors on the endothelium in HF are mediated via different mechanisms including eNOS up-regulation, inhibition of endothelial apoptosis and reduced production of vasoconstrictor prostanoids.<sup>228-230</sup>

Pleiotropic (i.e., lipid-independent) properties of statins have attracted substantial attention of researchers and their endothelial effects were extensively studied. Statins have helped to maintain coronary NO production and activity in pacing-induced cardiomyopathy, markedly improved endothelium-dependent vasorelaxation, and enhanced myocardial neovascularisation.<sup>231-232</sup> Treatment with statins parallels with improvement of LV function and survival via eNOS-mediated mechanisms.<sup>232-233</sup> In the Controlled Rosuvastatin Multinational Trial in Heart Failure (CORONA) trial, therapy with 10mg of atorvastatin had no effect on the mortality.<sup>234</sup> This controversy may stem from a number of factors which include the choice of a specific statin and its dose, the population studied or, alternatively, longer (almost 3 years) follow-up duration in the CORONA trial.

In all but one clinical studies statins resulted in a marked improvement of endothelial function, irrespective of HF aetiology (Table 6). However, the treatment of NIHF with the highest doses of atorvastatin (80 mg daily) failed to improve endothelial function.<sup>235</sup> Endothelial effects of the statins do not seem to depend of their lipid-lowering properties and they can improve endothelial function even in HF individuals with normal lipid levels.<sup>21, 236</sup> Lipid-independent effects of statins on the endothelium are also supported by failure of ezetimibe to affect endothelial function despite similar reduction in LDL.<sup>237</sup>

Statins seem to have no effect on asymmetric dimethylarginine levels but they significantly reduce oxidative stress in HF patients.<sup>194, 238</sup> Simvastatin increases extracellular superoxide dismutase activity, a potential contributor to the statin-induced improvement of endothelial function, but this is not seen with ezetimibe therapy.<sup>239</sup> However, a direct causal relation between antioxidant properties of statins and their positive endothelial effects in human HF is more difficult to prove and recovery of endothelial function with atorvastatin alone was more potent than with its combination with an antioxidant, vitamin E.<sup>240</sup>

Given that endothelial NO is produced from L-arginine, supplementation with this aminoacid has been used as a therapeutic option to improve endothelial function in HF. Also L-arginine might reduce endogenous NO inhibitor asymmetric dimethylarginine, known to be increased in HF.<sup>127</sup> For example, rats with HF had diminished plasma L-arginine levels and chronic supplementation with low (but not high) L-arginine doses improved aortic endothelium-dependent relaxation *ex vivo*, but did not affect haemodynamics *in vivo*.<sup>241</sup> Similarly, in human studies, a lower (e.g., 8 g daily), but not

**Table 6. Clinical studies reporting effects of statins on endothelial function in heart failure**

Author	Study	Number of HF patients	Mean EF, %	Treatment*	Duration	Results
Erbs <sup>194</sup>	R, PC	42 (27% IHF)	30±1	Rosuvastatin 40mg od	12 weeks	↑FMD ↑CD34+KDR+ EPCs
Guonari <sup>237</sup>	R, DB crossover	22 (100% IHF)	30±1	Rosuvastatin 10mg od or ezetimibe 20mg od	4 weeks	↑FMD with rozuvasatin ↔FMD with ezetimibe
Bleske <sup>235</sup>	R, PC, DB	15 NIHF	25±9	Atorvastatin 80 mg	12 weeks	↔FMD
Strey <sup>193</sup>	R, DB, PC, crossover	23 NIHF	30±8	Atorvastatin 40 mg od	6 weeks	↑FMD ↓endotehlin-1
Young <sup>238</sup>	R, PC, DB	24 (17% IHF)	31±8	Atorvastatin 40 mg od	6 weeks	↑FMD ↔ADMA
Castro <sup>242</sup>	R, PC	38	27±12	Atorvastatin 20 mg od	8 weeks	↑FMD
Strey <sup>21</sup>	R, PC, crossover	240 NIHF	<40	Atorvastatin 40 mg od	6 weeks	↑FBF in response to ACH (VOP)
Tousoulis <sup>236</sup>	R, controlled	38 (66% IHF), cholesterol levels <220 mg/dl	≤35	Atorvastatin 10mg od	4 weeks	↑FBF in response to RH
Tousoulis <sup>240</sup>	R, controlled	38 (100% IHF)	~25	Atorvastatin 10mg od or atorvastatin 10mg od plus vitamin E 400 IU/day	4 weeks	↑ FBF in response to RH in both treatment groups, but more in atorvastatine alone group
Landmesser <sup>239</sup>	R	20 (33% IHF)	23	Simvastatin 10mg od or ezetimibe 10mg od	4 weeks	↑ RA FMD ↑EPC ('early' EPC colonies) (for both with simvastatin but not ezetimibe)

\*oral administration unless indicated otherwise. ACH, acetylcholine, ADMA, asymmetric dimethylarginine, DB, double blind, EPC, endothelial progenitor cell, FBF, forearm blood flow, ia, intraarterial, IHF, ischaemic heart failure, NIHF, non-ischaeamic heart failure, PC, placebo controlled, R, randomised, RA, radial artery, VOP, venous occlusion plethysmography



higher (20 g daily) dose of L-arginine have been shown to improve endothelial function.<sup>216, 218</sup> In a placebo-controlled study, 6 week treatment with oral L-arginine hydrochloride (5.6-12.6 g daily) significantly increased distances during a 6-minute walk test, improved arterial compliance and reduced circulating levels of endothelin-1.<sup>243</sup> Thus, whilst the restoration of reduced L-arginine levels may be beneficial in some patients, excessive L-arginine consumption is unlikely to provide any additional benefits to the endothelium. Moreover a significant increase in urea and aspartate transaminase levels was observed in patients who received high L-arginine doses.<sup>218</sup>

Vitamin C inhibits endothelial cell apoptosis in congestive HF and studies in this area have shown an improvement in endothelial function (Table 5).<sup>244</sup> Nevertheless given rather unsuccessful results of antioxidant vitamin supplementation in clinical trials, they should not be routinely recommended in HF patients.<sup>245</sup> Still, healthy diet habits are essential to maintain an appropriate antioxidant status.<sup>246</sup> Although the number of other medications (e.g., allopurinol, sildenafil, etanercept, growth hormone, etc.) have been shown to have positive effects on endothelial function, it is currently difficult to speculate on clinical relevance of these observations (Table 5).

### **2.2.8. Exercise and endothelial function**

Physical exercise has gained acceptance as a beneficial intervention for patients with HF. According to a systematic review of randomized controlled trials, exercise might slow the pathophysiological progression of HF.<sup>247</sup> In chronic HF, regular exercise inhibits neurohormonal stimulation, the production of proinflammatory cytokines, reduces levels of

natriuretic peptides, systemic vascular resistance and attenuates the oxidative burden.<sup>247</sup> These favourable effects parallel improvement in symptoms, exercise capacity and quality of life, whilst patients who do not improve their exercise capacity significantly after an exercise training programme have a poorer prognosis.<sup>248</sup>

The shear stress associated with exercise is a critical endothelial stimulus, commonly employed to assess endothelial function (i.e., FMD). Accordingly it is natural to expect an effect of physical training on the endothelial functional performance. Indeed, regular aerobic training of patients with ischaemic and non-ischaemic systolic HF is associated with restoration of the vasodilatory endothelial-dependent function (Table 7). In contrast, training of isolated muscle groups (e.g., handgrip training) does not seem to have any substantial effect on systemic endothelial function.<sup>249</sup> Physical training in HF has been shown to up-regulate eNOS gene expression, accelerate L-arginine transport, and increase basal endothelial NO formation.<sup>250-254</sup> Additionally, in patients with HF, exercise training increases vascular endothelial growth factor (VEGF) and mobilises endothelial progenitors, presumably an indicator of the desired endothelial regeneration.<sup>255-256</sup>

Intensity of exercise training may also be of importance. Whereas acute bouts of exercise have been reported to enhance production of pro-inflammatory cytokines and markers of endothelial damage in HF, these effects are not seen when moderate exercise is performed regularly.<sup>257-258</sup> Even in healthy individuals undergoing moderate-intensity exercise, but not mild- or high-intensity exercise, there is a significant augmentation of acetylcholine-induced vasodilation.<sup>259</sup> Indeed, improvement of FMD after the course of training paralleled reverse LV remodelling, prominent improvement in LVEF and reduction in NT-

pro-BNP levels, although a causative relationship between these processes is difficult to establish.<sup>20</sup> Also it is still not clear whether exercise-related improvement of endothelial function has any independent effect on clinical outcomes.

### **2.2.9. Genetic predisposition of endothelial dysfunction**

In the Genetic Risk Assessment of HF (GRAHF) sub-study of the African-American HF Trial, eNOS genotype differed between white and black patients with HF.<sup>260</sup> In black patients the -786T allele was associated with lower LVEF.<sup>260</sup> The prevalence of allele Glu298 was significantly higher in HF patients as genotype Glu298Glu, being associated with an increased prevalence of hypertension.<sup>261</sup> Also HF patients homozygous for eNOS promoter polymorphism (thymidine to cytosine transition [T(-786)C]) were found to have a more advanced cardiac autonomic imbalance (i.e., abnormal heart rate variability).<sup>262</sup>

In the GRAHF study, the Glu298Asp polymorphism was associated with reduced effectiveness of fixed-dose combination of isosorbide dinitrates and hydralazine which only improved the composite score of survival, hospitalization, and quality of life in Glu298Glu allele carriers.<sup>260</sup> McNamara et al. also confirmed poorer event-free survival in HF patients with Asp298 variant of eNOS, particularly in those with NIHF.<sup>263</sup>

**Table 7. Effects of exercise on endothelial function in heart failure subjects**

Author	Study design	HF (n)	Mean EF, %	Exercise programme	Duration	Results
Wisloff <sup>20</sup>	R, controlled	27 (100% IHF)	29	Treadmill: (i) Aerobic interval training: four 4-minute intervals at 90-95% PHR, with 3 min active pauses (walking at 50-70% PHR); (ii) Moderate continuous training (walked at 70-75% PHR for 47 minutes) – 3 dpw	12 weeks	↑FMD (more with aerobic interval training than moderate continuous training)
Miche <sup>249</sup>	NR	42 (77% IHF)	23	Weekly, cycle ergometer training (3 times), a 6 min walk (twice) and muscle strength training (twice)	4 weeks	↔FMD (irrespectively of the presence diabetes)
Sarto <sup>256</sup>	NR	22 (64% IHF)	31	Cycle ergometer (55 min) – 3dpw	8 weeks	↑CD34+KDR+EPC ↑EC-CFU
Bank <sup>264</sup>	NR	7	23	Handgrip exercise (30 min) – 4dpw	4-6 weeks	↔FBF in response to ACH (↑ in healthy controls)
Parnell <sup>25</sup>	Controlled	21 (30% IHF)	22	Walking, light hand weights and cycling (50–60% of maximal HR) plus home-base programme – 3dpw	8 weeks	↑L-arginine transport ↑FBF in response to ACH
Kobayashi <sup>265</sup>	R, controlled	28 (46% IHF)	31	Cycle ergometer training (in two 15min sessions per day) – 2-3tpw	3 months	↑FMD in tibial arteries ↔FMD in brachial arteries
Linke <sup>266</sup>	R, controlled	22 (45% IHF)	25	Cycle ergometer (6 times a day for 10 min, at 70% peak oxygen consumption)	4 weeks	↑RAD in response to ACH
Hornig <sup>267</sup>	NR	12	21	Handgrip training (70% of the maximal workload for 30 min) – daily	4 weeks	↑RAD in response to ACH
Hambrecht <sup>252</sup>	R, controlled	20 (35% IHF)	24	Cycle ergometer (6 times daily for 10 min at 70% PHR) – first 3 weeks, followed by twice daily (40 min in total) – 5dpw	6 months	↑Femoral artery blood flow in response to ACH ↑basal NO formation

ACH, acetylcholine, EC-CFU, endothelial cell colony forming units, EF, ejection fraction, EPC, endothelial progenitor cells, FBF, forearm blood flow, FMD, flow-mediated dilation, dpw, times per week, HF, heart failure, PHR, peak heart rate, IHF, ischaemic heart failure, NO, nitric oxide, NR, non-randomized, RAD, radial artery dilation, R, randomised

## **2.2.10. The endothelium and thrombotic risk**

### ***2.2.10.1. Prothrombotic state in heart failure***

Chronic HF is increasingly recognised as a syndrome which confers a considerable prothrombotic risk. Whilst the annual incidence of venous thromboembolism is about 0.1% in general population, prospective observational studies have shown that thromboembolic events would occur in 1.7-2.7% of patients with HF each year.<sup>268</sup> Indeed, a retrospective analysis of the Studies of Left Ventricular Dysfunction (SOLVD) trials reported that the annual incidence of thrombotic events (including strokes, pulmonary emboli and peripheral emboli) in patients with ventricular dysfunction and sinus rhythm was 1.8% in men and 2.4% in women.<sup>269</sup> Furthermore, patients with HF generally suffer more severe strokes that are associated with high mortality rates.<sup>270</sup>

However, even this high prevalence of thromboembolic events may just represent the tip of the iceberg. Indeed, more than 30% of patients with HF, irrespectively of its aetiology, may experience ‘silent’ asymptomatic thromboembolic strokes detected by brain magnetic resonance imaging.<sup>271</sup>

In addition to the risk of venous thromboembolism, patients with HF also have a high [and often underestimated] risk of arterial thrombosis. The risk of sudden cardiac death, often attributable to acute arterial thrombosis, is 6 - 9 fold higher in those with HF compared to the general population.<sup>272</sup> In the Assessment of Treatment with Lisinopril and Survival [ATLAS] study, fresh coronary thrombus was found in more than 30% of such sudden

death cases. Both acute MI and unstable angina are independent risk factors for death in patients with congestive HF.<sup>273</sup>

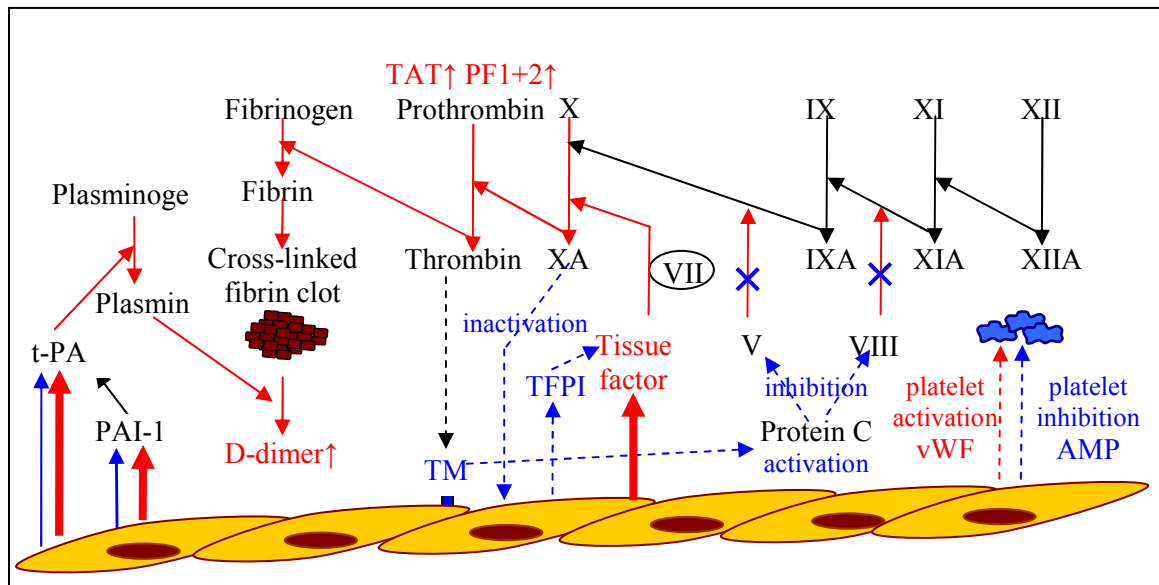
Why does HF confer a prothrombotic state? HF is associated with abnormalities of flow (low cardiac output, impairment of intracardiac haemodynamics, dilated cardiac chambers, stasis of blood in peripheral vascular beds, etc), vascular wall (endothelial damage/dysfunction), and abnormalities of blood constituents (e.g., platelets and haemorrhology). Thus HF fulfils all criteria of Virchow's triad of characteristics of a prothrombotic state, which promote the risk of thrombosis and, unsurprisingly, the risk of thromboembolic complications rises accordingly to the degree of LV contractility impairment.<sup>269</sup> Given that endothelial dysfunction is commonly seen in HF pathophysiology, the contribution of the endothelium to thrombosis may be especially important in patients with HF.

#### ***2.2.10.2 Vascular endothelium and the maintenance of blood fluidity***

The lumen surface of the healthy endothelium is both anticoagulant and antithrombotic. In addition to being a passive barrier, endothelial cells directly and actively participate in the prevention of venous and arterial thrombosis by regulation of platelet activity and the balance of procoagulant factors.

Endothelial cells maintain blood fluidity via different anticoagulant pathways (Figure 2). One of the most important is the protein C/protein S pathway.<sup>274</sup> Stimulation of the endothelial cell receptor thrombomodulin by thrombin activates protein C, and activated protein C then inhibits coagulation factors VIIIa and Va. To be effective, protein C must

form a complex with protein S, which is synthesized by endothelial cells.<sup>275</sup> The interaction of thrombin with thrombomodulin also inhibits thrombin-mediated platelet activation and ultimately, clot formation.<sup>276</sup> Additionally, healthy endothelium inactivates thrombin by expressing on their surface heparin-like glycosaminoglycans associated with large amounts of antithrombin.<sup>277-278</sup> Furthermore, endothelial cells possess receptors that mediate endocytosis and help to eliminate from the circulation factor Xa, responsible for conversion on prothrombin into thrombin<sup>279</sup>.



**Figure 2. Regulation of haemostasis by the vascular endothelium**

cAMP – cyclic adenosine monophosphate; NO – nitric oxide; PAI-1 – plasminogen activator inhibitor type 1; PF1+2 – prothrombin fragments 1+2; TAT – thrombin-antithrombin III complex; TFPI – tissue factor pathway inhibitor; TM – thrombomodulin; t-PA – tissue-type plasminogen activator; vWF – von Willebrand factor. Blue lines reflect activities of normal endothelium, red lines show changes associated with dysfunctional endothelium

In the quiescent state, endothelial cells do not express tissue factor (TF), the key activator of extrinsic pathway of coagulation. In contrast, normal endothelium produces TF pathway inhibitor to inactivate TF synthesised by other cells.<sup>280</sup> The endothelium also participates in fibrinolysis by releasing tissue-type plasminogen activator (t-PA) and urokinase, thus promoting formation of plasmin able to digest the fibrin network.<sup>281</sup> Although endothelial cells also produce plasminogen activator inhibitor type 1 (PAI-1) the balance of t-PA and PAI-1 is normally maintained in favour of thrombosis prevention.<sup>282</sup>

The antiplatelet properties of the endothelium are largely mediated by prostacyclin and NO, which prevents platelet activation by the increase in the cyclic adenosine monophosphate contained within platelets. Additionally, endothelial cells possess on their luminal surface enzymes (ectonucleotidases) which are able to hydrolyse adenosine triphosphate and adenosine diphosphate, both potent platelet activators.<sup>283</sup>

### ***2.2.10.3. Endothelium and thrombogenic alterations***

The prominent antithrombotic characteristics of healthy endothelial cells are changed dramatically in the damaged or dysfunctional endothelium. Indeed, endothelial abnormalities are typical amongst patients with HF and represent one of the major pathophysiological pathways implicated in the development and progression of HF. For example, impaired FMD, increased numbers of circulating endothelial cells shed off vascular wall, raised plasma levels of vWF (a marker of endothelial damage) are all consistently reported in patients with HF irrespectively of its aetiology (i.e., whether ischaemic or non-ischaemic).<sup>143</sup> Persistent exposure to various cytokines, chemokines and



proinflammatory stimuli shifts the balance towards a prothrombotic endothelial cell phenotype. Endothelial damage as assessed by plasma vWF levels is predictive of adverse outcomes in patients with HF.<sup>180</sup>

Endothelial impairment results in dysregulation of all major elements of coagulation system. Endothelial activation shifts the balance in favour of platelet aggregation and clot formation through the suppression of anticoagulant mechanisms and stimulation of procoagulant pathways. Inflammatory cytokines are abundant in patients with HF (e.g. TNF- $\alpha$ , IL-1 $\beta$ ) and these modify endothelial physiology and may trigger endothelial expression of TF.<sup>284</sup> Active endothelial production of TF results in downstream activation of factor Xa and leads to cleavage of prothrombin to form active thrombin. Indeed, the cytokine-mediated expression of TF by endothelial cells and inflammatory cells acts as one of the primary initiators of thrombosis in different pathological conditions.<sup>285</sup> In acute HF, elevation of TF levels is significantly correlated with inflammatory burden and is much higher in those who died during a 6-month follow-up period.<sup>286</sup> TF levels have also been demonstrated to be predictive of poor prognosis in chronic HF.<sup>287</sup>

Activation of thrombin also promotes development of thrombosis via fibrin formation, as well as via a number of fibrin-independent effects. Indeed, thrombin may activate platelets, thus enhancing their surface expression of P-selectin and increasing production of platelet activation factor. Through protease-activated receptors presenting on endothelial cells, thrombin also contributes to endothelial cell activation, vWF release, possible TF production, change in endothelial cell shape and increase in endothelial permeability.<sup>288</sup> All together, these changes promote inflammation and a procoagulant state in HF.

Indeed, thrombin activity is substantially increased in patients with HF as demonstrated by elevated plasma levels of thrombin-antithrombin complex and prothrombin fragments 1+2.<sup>289</sup> In a follow-up of 214 subjects with HF, thrombin-antithrombin complex levels were significantly associated with increased risk of death, even after adjustment for other prognostic factors (e.g., age, gender, traditional cardiovascular risk factors, NYHA class, systolic LV function, renal failure, haemoglobin).<sup>290</sup> Of note, these prothrombotic markers can be significantly reduced by an anticoagulant, warfarin.<sup>291</sup> However, the clinical utility of long-term anticoagulation has not been confirmed as yet in large clinical trials, such as the Warfarin and Antiplatelet Therapy in Heart Failure(WATCH) trial.<sup>292</sup>

Platelet abnormalities in HF are also well described and are at least partly attributable to the endothelial cell impairment.<sup>293</sup> Indeed, a dysfunctional endothelium initiates the expression of platelet-activating factor which promotes platelet adhesion to endothelial cells and up-regulates production of vWF.<sup>293</sup> The latter is constitutively expressed and stored in Weibel–Palade bodies within endothelial cells and physiologically facilitates the binding of platelets to exposed extracellular matrix components of damaged vascular wall. Impaired endothelial cells also release excessive amounts of vWF, further promoting platelet adhesion and activation. Also, patients with HF have increased concentrations of  $\beta$ -thromboglobulin, platelet surface P-selectin and CD63P.<sup>294</sup> More recently, endothelium-derived fractalkine was also found to be a contributor to platelet adhesion and activation.<sup>295</sup>

### **2.2.11. Conclusions and future directions**

Endothelial abnormalities are a common feature of HF. Various aspects of the endothelial function can be affected, including vasomotor, haemostatic, antioxidant and inflammatory activities. Substantial differences exist in the pattern of endothelial function depending on aetiology, severity and stability of HF in individual patients. There is considerable evidence that in the majority of patients with ischaemic aetiology of HF, endothelial dysfunction is systemic in its nature and involves arteries, conductance vessels and microvascular beds, coronary, pulmonary and peripheral vessels. There is also minor evidence of venous endothelial dysfunction in HF. The pattern of endothelial dysfunction is more heterogeneous in NIHF, with fewer features of systemic abnormalities. In fact many subjects with NIHF have functionally preserved endothelium in peripheral arteries with endothelial dysfunction seen only in coronary vessels. It is possible that coronary endothelial dysfunction in these patients mirrors local pathological processes in the heart and may reflect their activity.

Endothelial dysfunction undoubtedly has a significant prognostic value in HF but its clinical application is hampered by methodological limitations. Some of these methods are invasive and some (e.g., FMD) are very operator-dependent. A number of medications (including ACE inhibitors and statins) and regular physical activity has been shown to improve endothelial function in HF, but there are still no pharmaceutical agents specifically targeting the vascular endothelium. In an animal model of HF, endothelial dysfunction was successfully corrected by tyrosine phosphatase 1B inhibitors but their effects have not yet been tested in human clinical trials.<sup>296</sup>

Major qualitative differences exist between macro- and micro-vascular endothelial cells. The latter have distinct morphology, patterns of specific expression of adhesion molecules and they vary in their responsiveness towards agonists.<sup>297</sup> For example, endothelial cell thickness varies from  $<0.1\mu\text{m}$  in capillaries to  $1\mu\text{m}$  in the aorta. In stable conditions, TF plasma inhibitor is primarily associated with the microvascular endothelium, while protein C receptor is predominantly expressed on the luminal surface of large vessels, and vWF is not only expressed by macrovascular but also lung microvascular endothelial cells.<sup>298-300</sup> eNOS activity is also higher in the endothelium of arteries from the renal medulla compared with glomeruli and peritubular capillaries.<sup>276</sup> Admittedly, only limited data are available on the performance of the microvascular endothelium in HF.

Moreover, ethnicity is known to affect the risk of HF development. For example, it has been shown that patients of SAs origin more often (for about 40%) suffer from CAD with higher mortality rates when compared to white population.<sup>301</sup> Indeed, endothelial damage/dysfunction may be an important contributor to the ethnicity-related adverse outcomes in HF. Of note, eNOS gene polymorphisms among SA population are associated with the risk of arterial hypertension and aneurysmal subarachnoid haemorrhage.<sup>302-303</sup> Even healthy SAs have diminished endothelial activity and reduced endothelial progenitor cell numbers and function, but increased CRP levels when compared with Whites.<sup>22, 304</sup> However, the role of ethnicity in the pathophysiology of HF is still grossly understudied with limited data available on possible ethnic differences in endothelial activity in relation to the risk of thrombotic and thromboembolic complications.

## **2.3. Monocytes in heart failure**

### **2.3.1. Inflammation and heart failure**

It is widely recognised that inflammation is directly implicated in almost every aspect of pathological cardiovascular conditions including HF.<sup>24, 305</sup> Circulating levels of inflammatory cytokines are correlated with short-term and long-term outcomes and, according to some reports, with the response to treatment.<sup>131, 305-307</sup> Abnormal cytokine activity, particularly increased concentrations of IL-6 and TNF- $\alpha$  are detected from early stages of HF and appear to have value as potential markers for diagnosis, risk stratification, and prognosis. TNF- $\alpha$  has been shown to be involved in HF progression as a factor promoting myocardial dysfunction and adverse cardiac remodelling.<sup>308</sup> According to animal studies there is an increased cardiac expression of TNF- $\alpha$  in failing myocardium associated with progressive LV dysfunction and dilatation.<sup>309-310</sup> Case-control studies revealed up-regulation of inflammatory cytokines (e.g., TNF- $\alpha$ ) in both myocardium and peripheral monocytes in patients with HF and they have indicated that abnormally high circulating cytokine levels predict increased mortality rates.<sup>135, 311</sup> In fact, pro-inflammatory shift is obvious from initial stages of HF even before neurohormonal activation could be detected, which is a hallmark of more advanced stages of HF. For example, significantly risen TNF- $\alpha$  concentrations were seen in subjects with mild HF (NYHA II) when compared to normal individuals.<sup>130</sup> Furthermore, a linear relationship has been shown between TNF- $\alpha$  levels and HF functional status.<sup>130, 305</sup> It is hypothesized that sustained increase in cytokines results in cardiomyocyte apoptosis, changes in monocyte phenotype transition and activation of matrix metalloproteinases contributing to cardiac hypertrophy and adverse LV remodelling.<sup>139-140</sup> However, reported attempts to utilize

inflammatory molecules, such as TNF- $\alpha$  as therapeutic targets for management of severe systolic LV dysfunction were rather disappointing and led to an increase in mortality.<sup>312-313</sup>

This controversy could be partly due to the fact that despite its many detrimental effects, the cytokines also play an essential regulative role in protective adaptive processes aiming to contradict acute or chronic ischaemic injury.<sup>234, 314</sup> The cutoff cytokine concentration associated with the shift from protective to largely deleterious effects is subtle and it is likely to fluctuate based on many (patho)physiological variables or even between different organs and tissues of the same body. Admittedly, at present the role that TNF- $\alpha$  and other cytokines play in HF has not yet been fully clarified and warrants further investigation.

Chronic oxidative stress has been also linked to the establishment and progression of HF. Experimental and clinical studies documented an increased generation of ROS, including superoxide, hydrogen peroxide, and hydroxyl radicals in the failing heart. Recent findings suggest that the number of different pathways could lead to increased ROS production in HF, which include NADPH and xanthine oxidase pathways, alteration of NOS and the mitochondrial electron transport chain.<sup>315</sup> Experimental data reveal abnormal activation of NADPH oxidase in phagocytes in response to neurohormonal activation which promotes apoptosis of cardiomyocytes and excessive activation of matrix metalloproteinases accompanied by unfavourable remodelling of the failing LV. Furthermore, the critical negative consequence of the oxidative burden in HF is its impact on endothelial function.<sup>176</sup> According to clinical studies on HF subjects with severe endothelial dysfunction and diminished NO availability the degree of the endothelial dysfunction significantly predicts the risk of negative clinical outcomes.<sup>316</sup> Consequently ROS have

been suggested as a possible target of pharmaceutical agents with known pleiotropic anti-oxidative and anti-inflammatory properties. Several studies have indicated significant role of the antioxidant effects of statins on cardiac remodelling, endothelial function in subjects with HF.<sup>317-318</sup>

Of note, monocytes represent both a major cellular source and one of the main cellular targets of inflammatory cytokines (Table 8). However, the role of monocytes in HF is not restricted to cytokine signalling. Growing evidence implicates these cell into various cardiovascular disorders associated with HF. Following migration to tissues and under appropriate stimulation, monocytes differentiate into different types of macrophages. Macrophages are mobile phagocytes specialized in elimination, via various forms of endocytosis, invading microorganisms, and debris of cellular, extracellular, and foreign origin.<sup>318</sup>

Monocytes are implicated in atherogenesis, the pathological process underlying CAD and ischaemic cardiomyopathy.<sup>23</sup> Their initially protective inflammatory activity may later result in chronic arterial damage, endothelial dysfunction and massive release of various cytokines, chemokines and growth factors by circulating monocytes and monocyte-derived tissue macrophages. As the result, monocytes significantly contribute to smooth muscle cell migration into the arterial intima and formation and progression of atherosclerotic lesions.<sup>23</sup>

Ischaemia-induced cardiac damage and consequent myocardial remodelling are paralleled by intense monocyte infiltration of the damaged tissue, inflammation and impairment of

**Table 8. Cytokines/chemokines produced by monocytes/macrophages in response to vascular inflammation.**

Cytokine	Major action in relation to vascular inflammation
TNF $\alpha$ <sup>135, 306, 308-310, 312</sup>	Induces inflammation Induces apoptotic cell death Potent chemoattractant for neutrophils
IL-1 $\alpha/\beta$ <sup>307</sup>	Induces chemokine/cytokine expression Promotes the expression of adhesion molecules on endothelial cells
IL-6 <sup>130, 305</sup>	Induces chemokine/cytokine production Major mediator of the acute phase response
IL-8 <sup>307</sup>	Neutrophil activation and chemotaxis SMCs proliferation and migration
IL-10 <sup>130, 305</sup>	Anti-inflammatory cytokine Down-regulates the expression of Th1 cytokines. Inhibits NF $\kappa$ B activity
IL-12 <sup>317</sup>	Induces cell-mediated immunity T cell stimulating factor Induces the production of TNF- $\alpha$ and INF- $\gamma$
IL-18 <sup>130, 305, 307</sup>	Induces cell-mediated immunity Induces INF- $\gamma$ production
MIP 1 $\alpha/\beta$ <sup>130, 305, 307</sup>	Granulocyte activation and chemotaxis Induce the synthesis and release of pro-inflammatory cytokines
MCP-1 <sup>130, 305, 307</sup>	Monocyte activation and chemotaxis Induces SMC accumulation and migration

TNF, tumour necrosis factor  $\alpha$ ; IL, interleukin; INF, interferon; MIP, monocyte inflammatory protein; MCP, monocyte chemoattractant protein; SMC, smooth muscle cells.



LV contractility.<sup>318-319</sup> For example, Maekawa et al. reported a strong association between peripheral monocytois, LV dysfunction and LV aneurysm formation after MI.<sup>320</sup> Accordingly, inhibition of monocyte activation is a tempting therapeutic target in the prevention and management of ischaemia-related HF.

### **2.3.2. Monocyte activation**

Before their involvement in the pathogenesis of ACS or HF, monocytes are undergoing phenotypic transformation, leading to their activation. CD14, which is a monocyte endotoxin receptor (coupled with with Toll-like receptor [TLR]-4) that binds lipopolysaccharides (LPS) and initiates monocyte activation. Activated monocytes interact with endothelium triggering an inflammatory cytokine cascade.<sup>321-322</sup> This process is enhanced by high levels of CRP or heat shock proteins.<sup>323-329</sup>

Indeed, circulating TLR4-expressing monocytes are ~2.5-fold increased in ACS patients and the density of CD14 on the monocyte surface is much higher in patients with myocardial necrosis.<sup>330-332</sup> However it should be kept in mind that that TLR4-mediated responded may be reduced by aging.<sup>333</sup> In some (but not all) studies, the CD14 gene polymorphism has been associated with a history of ACS or MI, indicating its potential role in genetic predisposition to the development of ACS and atheromatous plaque vulnerability (Table 9).<sup>334-335</sup> Monocytic TLR4 overexpression in acute MI, demonstrated both in the circulation and on ruptured plaque, has been associated with high levels of IL-6 and TNF- $\alpha$ .<sup>336</sup> These markers remained elevated for at least 2 weeks after MI onset were related to the development of HF.<sup>337</sup>

**Table 9. Studies on CD14 gene polymorphism**

Study	Patients	Polymorphism analysed	Results
Hubacek <sup>338</sup>	178 patients with MI and 135 control subjects	-260 C/T	The frequency of the T allele was higher in MI patients, with higher density of the CD14 receptor the T/T homozygotes
Shimada <sup>335</sup>	81 MI patients, 43 CAD patients, 83 healthy controls	-260 C/T	The frequencies of T allele and T/T homozygotes in MI patients were higher than in controls and in patients with angina without prior MI
Heesen <sup>339</sup>	95 healthy blood donors	-260 C/T	No differences in CD14 density and soluble CD14 levels.
Arroyo-Espliguero <sup>334</sup>	194 ACS survivors, 140 CAD patients without ACS history, 94 patients with normal coronary arteries	-260 C/T	Patients with a prior ACS had higher frequency of the T/T genotype than CAD patients without prior ACS. No differences between CAD patients without prior ACS and controls.
Longobardo <sup>340</sup>	213 MI survivors, 213 healthy controls	-260 C/T	No association with acute MI
Unkelbach <sup>341</sup>	2228 patients after diagnostic coronary angiography	-159 C/T	No association with MI or CAD in the whole cohort. In low coronary risk patients (normotensive nonsmokers) increased risk for MI in T allele homozygotes
Koch <sup>342</sup>	793 MI patients, 998 CAD patients, 340 healthy controls	-159 C/T	Gene polymorphism was not associated with CAD or MI
Agema (REGRESS trial) <sup>321</sup>	759 patients with CAD	-159 C/T	No association with CAD severity
Rechciński <sup>343</sup>	57 MI survivors	-159 C/T	No differences in the first MI age or the number of cardiovascular risk factors

### 2.3.3. Monocyte-endothelium and monocyte-myocardial interactions

On being activated, monocytes modify their phenotype, thus enhancing their interaction with endothelial cells and ability to infiltrate arteries and cardiac tissue. The recruitment of circulating monocytes to the vascular subendothelial tissue occurs via a tightly regulated multi-step process mediated by a series of cell surface adhesion molecules. Endothelial cell activation also results in the up-regulation of a number of endothelial cell adhesion molecules, including E-selectin, vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1), mediated by the pro-inflammatory cytokines (e.g., IL-1 $\beta$ , TNF- $\alpha$ ).<sup>344</sup>

In the first step, activated vascular endothelial cells at the site of atherosclerosis express P-selectin that mediates the tethering and rolling of circulating monocytes. P-selectin binds to P-selectin glycoprotein ligand-1 and other glycosylated ligands on monocytes.<sup>345-346</sup> E-selectin is also inducibly expressed at sites of atherosclerosis.<sup>347</sup> P- and E-selectins allow the capturing and rolling of leukocytes on the endothelium. They play an important role in the signalling pathway through P-selectin glycoprotein ligand-1 in which they activate integrins and induce monocyte activation.<sup>348</sup> An ex vivo model of isolated carotid arteries showed that blocking P-selectin with anti-P-selectin antibodies resulted in the reduction of monocyte rolling and attachment to the endothelium.<sup>349</sup> Integrins support both rolling and adhesion of leukocytes. Very late antigen-4 ( $\alpha 4\beta 1$  integrin) is a monocyte integrin closely related to atherosclerosis.<sup>350</sup>  $\alpha 4\beta 1$  mediates interaction with VCAM-1 that is induced on cytokine-stimulated endothelium.<sup>351</sup> *In vitro* experiments on monocyte flow along cytokine-activated endothelial cells (i.e., under shear stress) suggested the involvement of P-selectin,

L-selectin, VCAM-1, and very late antigen-4 in monocyte recruitment to the endothelium.<sup>352-353</sup>

Increased monocyte expression of Mac-1 (CD11b/CD18), lymphocyte function associated antigen-1, and very late antigen-4 promotes monocyte attachment to the endothelium.<sup>354-356</sup>

Monocyte-associated levels of ICAM-1, VCAM-1, and L-selectin are also elevated from the early stages of ACS (either with or without myocardial necrosis).<sup>356-357</sup> Such overproduction of adhesion molecules in ACS may be associated with diminished monocyte expression of peroxisome proliferator-activated receptor- $\gamma$ .<sup>358</sup> Also, monocytes possess receptors facilitating their localisation at sites of injured myocardium. The ACS-related up-regulation of monocyte fibronectin receptor very late antigen-5 may be involved in their migration to tissues via interaction with fibronectin, the latter being an important component of cardiac extracellular matrix.<sup>359</sup>

Injured heart muscle also promotes monocyte recruitment. Indeed, acute MI is associated with the rapid induction of mononuclear cell chemoattractants (such as MCP-1 and monocyte colony stimulating factor [M-CSF]) that promote monocyte infiltration into the injured area, as well as monocyte differentiation to macrophages and proliferation of the latter, all critical processes for effective healing of the infarcted area.<sup>360</sup>

MCP-1 (gene name CCL2, receptor gene name CCR2) is the most important chemokine that regulates migration and infiltration of monocytes/macrophages. Ischaemia rapidly stimulates infiltration of infarcted area by MCP-1-positive macrophages, partly via M-CSF expression by mature cardiac resident macrophages.<sup>361-362</sup> MCP-1 production by cultured

human cardiac cells has been shown to be upregulated by inflammatory cytokines and downregulated by hypoxia.<sup>363</sup> Cardiac overexpression of MCP-1 induces macrophage infiltration, neovascularization, expression of the wide range of cytokines and chemokines (e.g., TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-8, IL-10, transforming growth factor- $\beta$ ), and the accumulation of cardiac myofibroblasts, thereby affecting LV remodelling.<sup>361-362, 364-366</sup>

Of note, plasma MCP-1 levels are increased as early as 3 hours after the onset of chest pain, reach their maximum at 24 hours and remain elevated at least for 7 days.<sup>364, 366</sup> Interestingly, the majority of studies on MCP-1 genetic polymorphism failed to find any association with the risk of MI (Table 10). However, in the Framingham Heart Study Offspring Cohort, the MCP-1-2578G allele was significantly related to a higher prevalence of MI (with an adjusted odds ratio 2.0).<sup>367</sup>

Stress-induced expression of MCP-1 contributes significantly to the development of coronary collaterals during the early phase of acute MI.<sup>368</sup> Nonetheless, excessive MCP-1 production may have adverse effects. In 2270 patients with ACS enrolled in the OPUS-TIMI-16 trial, MCP-1 levels were independently associated with an increased risk of death or MI during 10 months of follow-up.<sup>369</sup> Similarly, MCP-1 levels measured in 4244 post-ACS patients in the A to Z trial were predictive of the recurrence of cardiovascular events (hazard ratio 2.16) and mortality after 4 months (hazard ratio 1.76).<sup>370</sup> Interestingly, MCP-1 levels appear to have little prognostic importance in patients with stable CAD.<sup>371</sup>

**Table 10. Studies on MCP-1 gene polymorphism**

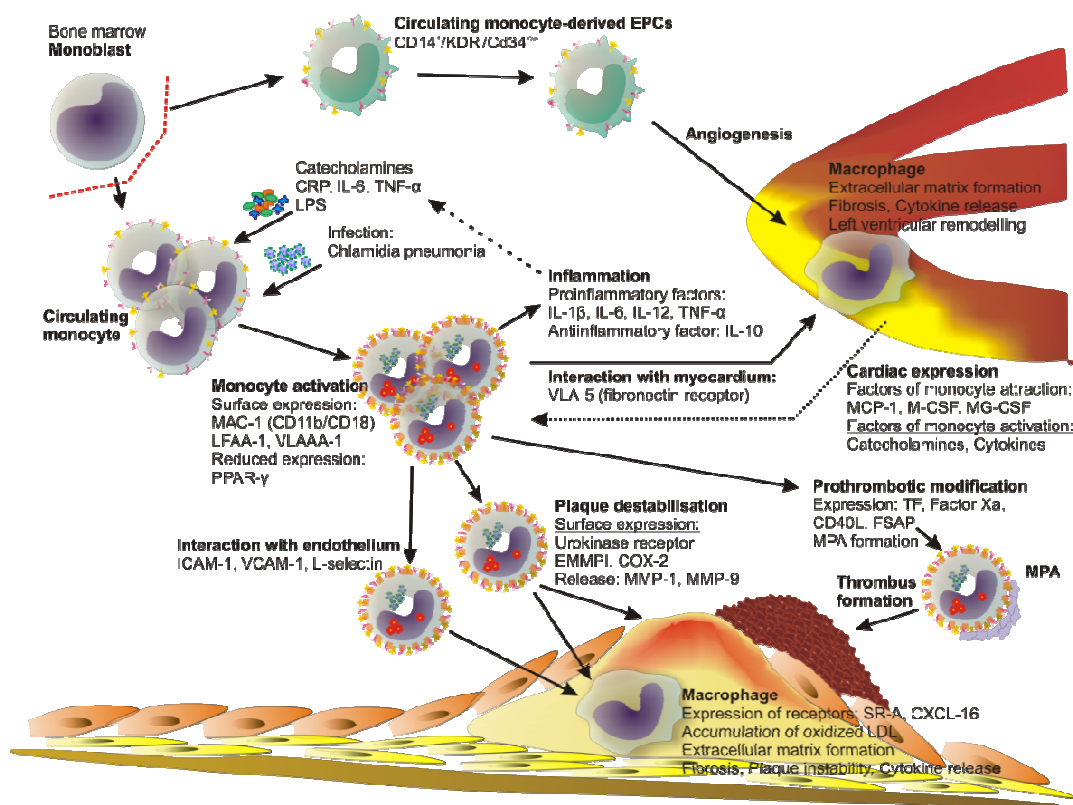
Study	Patients	Polymorphism analysed	Results
Iwai <sup>372</sup>	2,266 subjects, including 34 MI survivors	-2138 A/T	No significant association with MI or atherosclerosis
Jemaa <sup>373</sup>	319 MI patients, 467 healthy controls	-2518 G/A	Patients with MI had significantly higher frequency of the AG+GG genotypes compared to controls
McDermott <sup>367</sup> (Framingham Heart Study Offspring Cohort)	1797 subjects	-2518 G/A	The MCP-1-2578G allele was associated with higher serum MCP-1 levels and higher MI prevalence
Bjarnadottir <sup>374</sup>	460 MI survivors, 1842 disease free controls	-2518 G/A, -2076 A/T plus -190 G/A polymorphism of CCR2 gene	No difference in the frequencies of any of the polymorphisms studied between the cases and the controls
Cermakova <sup>375</sup>	139 MI patients, 359 healthy controls	-2518 G/A	No relationship was observed between circulating MCP-1 levels and carriage of the G allele

MCP-1 may be a potential target for intervention, but data from preclinical data have been controversial. In experimental studies, a genetically determined lack of MCP-1 synthesis or anti-MCP-1 therapy were associated with more favourable myocardial remodelling, better survival and contractility preservation, attenuated interstitial fibrosis and reduced infarct size, but also with delayed replacement of injured cardiomyocytes with granulation tissue and defective macrophage differentiation.<sup>362, 376-377</sup> In contrast, two rodent studies show that cardiac MCP-1 overexpression or myocardial MCP-1 injection reduced infarct area and scar formation and prevented LV dysfunction after MI.<sup>365, 378</sup> Of note, MCP-1 treatment stimulated neovascularization independently from the involvement of bone marrow-derived endothelial progenitors.<sup>365, 378</sup> Thus the time and extent of MCP-1 availability may be of importance.

#### **2.3.4. Monocytes and inflammation and plaque destabilisation**

Monocytes are actively involved in initiation of the inflammatory cascade in ACS and HF (Figure 3). Activated monocytes promote the synthesis of proinflammatory molecules, such as IL-6 and TNF- $\alpha$ , partly mediated by TLR4 stimulation and Mac-1 expression – as discussed above.<sup>328</sup> Unstable angina and MI are also associated with increased levels of TLR4, and downstream molecules, such as IL-12 and B7-1.<sup>330</sup> The anti-inflammatory cytokine IL-10 is also up-regulated in response to TNF- $\alpha$  in ACS, suggesting a control mechanism for inflammation.<sup>379</sup>

Catecholamines are directly involved in the regulation of IL-10 expression in monocytes, but not in T-cells, after acute stressful conditions.<sup>379</sup> Furthermore, circulating monocytes in



**Figure 3. Implication of monocytes in acute coronary syndrome.**

LFAA – lymphocyte function associated antigen, VLAAA – very late after activation antigen, PPAR - peroxisome proliferator-activated receptor, COX – cyclooxygenase, MG-CSF – monocyte-granulocyte colony stimulating factor, FSAP – factor VII activating protease

ACS produce equal amounts of  $\text{TNF-}\alpha$ , but less  $\text{IL-10}$  (after stimulation with LPS *in vitro*) as compared with healthy controls.<sup>380-381</sup> Thus, the production of proinflammatory cytokines is not counterbalanced by anti-inflammatory cytokines such as  $\text{IL-10}$ .

Monocytes are involved in the destabilization of atherosclerotic plaques by their production of matrix metalloproteinases. Indeed, catecholamines potentiate LPS-induced synthesis of matrix metalloproteinase 1 and matrix metalloproteinase 9 in circulating monocytes and monocyte-derived macrophages.<sup>382</sup> Oxidized LDL also stimulates



monocyte expression of the urokinase receptor and consequent urokinase-mediated matrix metalloproteinase 9 generation.<sup>383</sup> The scavenger receptor CD204 and a chemokine, CXCL 16 are responsible for the uptake of oxidized LDL and phosphatidylserine, and transforming the macrophage into a foam cell.

In other studies, increased monocyte expression of extracellular matrix metalloproteinase inducer and cyclooxygenase-2 enhance the production of matrix metalloproteinase 1 and matrix metalloproteinase 9 in acute MI.<sup>384-385</sup> In animal models of acute MI, strong monocyte myeloperoxidase activity correlates with progressive LV dilation and LV function impairment.<sup>386</sup> Also, human monocytes stimulated with TNF- $\alpha$  release angiotensin II, which further stimulates matrix metalloproteinase 1 synthesis.<sup>387</sup>

### **2.3.5. Monocyte scavenger receptors**

Various scavenger receptors represent an important part of the monocyte receptor system, which is involved in detecting of different substances and their elimination from blood and tissues (e.g., accumulation of lipids into the atherosclerotic plaques).<sup>388-389</sup>

The main scavenger receptors expressed on circulating monocytes express are SR-AI (CD204) and CD36. These receptors have a high-affinity to oxidized phospholipid components of the oxidized LDL.<sup>390</sup> Mice studies showed absence of lipid accumulation in aortas of mice that lacked both CD36 and apoE.<sup>391</sup> Scavenger receptor-dependent lipid accumulation and progression of atherosclerosis is supported by findings of an abundance of oxidized phospholipids that serve as binding partners for CD36/CD204 in the plaque region of blood vessels, along with absence of oxidized phospholipids in the nonplaque

region. Recent experimental studies suggest that CD36 has both a proatherogenic and a prothrombotic role in the cardiovascular system. The enrichment of oxidized phospholipids in the plaque allows CD36 to penetrate the plaque, whereas removal of CD36 decreases the progression of atherosclerosis.<sup>392</sup>

Macrophage/foam cells produce cytokines that activate neighbouring smooth muscle cells, resulting in extracellular matrix formation, fibrosis, and plaque instability.<sup>393</sup> In one study 12,625 genes were analysed in peripheral blood mononuclear cells in ACS patients in the acute and chronic stable phases.<sup>394</sup> Gene expression profiles revealed that amongst different immune response factors and the receptor activity markers, CD204 was the most markedly increased in the acute phase of ACS. Recurrence of cardiovascular events was significantly lower in the 'CD204 low' group compared to patients with high CD204 expression.<sup>394</sup> The number of CD204-expressing monocytes is much higher amongst patients with ACS compared to those with chronic CAD and this was associated with a higher rate of residual mural thrombus in acute MI patients.<sup>34</sup>

Of interest emerging data also indicate that CD204 may be implicated in a number of reparative processes, such as clearance of apoptotic cells, attenuation of oxidative stress and inflammation.<sup>29-33</sup> However, information on monocyte CD204 expression in HF not available at present.

### **2.3.6. Monocyte counts in myocardial infarction and heart failure**

Recent data show that monocyte counts correlate with mortality in patients with acute MI.<sup>395</sup> Peripheral monocytosis occurs 2 to 3 days after MI onset, reflecting the mobilisation of monocytes and macrophages into the necrotic myocardium and this is associated with the extent of myocardial damage, creatine kinase release and fibrinogen levels.<sup>396-397</sup> An increase in procoagulant activity of circulating monocytes after successful PCI in MI has been correlated with an increase in systemic IL-6 levels.<sup>398</sup>

How do monocytes relate to clinical parameters and prognosis post-MI? Monocyte counts on the first few days after acute MI treated with primary PCI correlated with markers of effective myocardial reperfusion, such as myocardial blush Grade 2-3 and ST-segment resolution on the ECG.<sup>399</sup> Amongst all white blood cells, only monocytes were significantly and independently associated with the contractile recovery of the infarcted area at 6 months.<sup>399</sup> Of note, patients with MI complicated by HF or LV aneurysm had much higher peak monocyte counts than those without these complications. Indeed, pre-discharge left ventriculography revealed that peak monocyte count negatively correlated with LVEF and positively correlated with LV end-diastolic volume (indicating their role in myocardial remodelling) and cardiac events, including readmission for HF, recurrent MI and cardiac deaths (including sudden deaths).<sup>320</sup> In another study, peripheral monocytosis was associated with non-recovery of LV performance in patients with LV dysfunction complicating acute MI.<sup>400</sup>

Interestingly, patients who experienced hostility and stressful events in ACS often had more pronounced monocytosis.<sup>401</sup> In the study by Horne et al., 3227 stable subjects monocyte count was independent predictor of future MI development or death.<sup>402</sup>

Despite the well recognised contribution of monocytes to the pathogenesis of different cardiovascular disorders scarce information is available on their numbers in patients with HF.

### **2.3.7. Drug treatment and monocyte activity**

The significant role of monocytes in cardiovascular disorders makes them a potential target for pharmaceutical treatment (Table 11). However only a limited number of studies is currently available on effects of drug therapy on monocyte characteristics, with the most of the agents tested being routinely used in ACS and/or in HF.

*Anticoagulants.* Abciximab (but not heparin) reduced platelet mass attached to monocytes in acute MI patients through the reduction of Mac-1 expression but did not affect the number of MPAs.<sup>403</sup> Combination of reteplase and abciximab was much more effective in MPA reduction than reteplase alone.<sup>404</sup> Implantation of heparin-coated stents in ACS accelerates normalisation of platelet-leukocyte aggregate levels compared to balloon angioplasty alone.<sup>405</sup>

**Table 11. Effects of drug treatment on monocytes**

Drug	Species	Disorder/Model	Effects on monocytes
Abciximab <sup>403</sup>	Human	MI	Reduced Mac-1 expression and platelet mass attached to monocytes; no effect on MPA number
Reteplase and abciximab <sup>404</sup>	Human	MI	Reduced MPA number more effectively than reteplase alone
Heparin-coated stents <sup>405</sup>	Human	ACS	Restored MPAs quicker compared to angioplasty alone
Captopril, idrapril, fosinopril, losartan <sup>406</sup>	Human	Healthy volunteers	Decreased monocyte TF expression
Enalapril <sup>407</sup>	Human	MI	Reduced MCP-1 levels
Perindopril, candesartan <sup>408</sup>	Human	MI	No effects on MCP-1 levels
Clopidogrel <sup>409</sup>	Human	ACS	Decreased MPA number
Atorvastatin <sup>410</sup>	Human	ACS	Reduced MCP-1 levels
Atorvastatin <sup>411</sup>	Human	MI	Reduced monocyte cyclooxygenase-2
Cerivastatin <sup>383</sup>	Human	Healthy volunteers	Decreased expression of urokinase receptor on monocytes
Celecoxib <sup>385</sup>	Human	MI	Reduced monocyte secretion of IL-6 and matrix metalloproteinase 9
Dobutamine <sup>412</sup>	Human	Monocyte culture	Inhibited LPS-induced production of MCP-1
Eplerenone <sup>413</sup>	Rat	MI	Prevented LV dilation and improved LV function via mineralocorticoid receptor blockade

*ACE inhibitors and angiotensin II receptor antagonists.* Captopril, idrapril, fosinopril and losartan decreased monocyte TF expression *in vitro*.<sup>406</sup> In a small randomized, double-blind, placebo-controlled study 4 week treatment with enalapril (5 mg daily) significantly reduced MCP-1 levels in MI patients.<sup>407</sup> However, no effect of perindopril or candesartan on MCP-1 levels was found in acute MI.<sup>408</sup>

*Antiplatelet drugs.* Both loading dose (300mg) and continuous administration of clopidogrel decreased MPA numbers in ACS.<sup>409</sup>

*Statins.* Atorvastatin reduced MCP-1 levels in ACS and monocyte cyclooxygenase-2 expression in acute MI.<sup>410-411</sup> Another statin, cerivastatin was able to decrease expression of urokinase receptors on monocytes.<sup>383</sup>

*Other drugs.* *In vitro* studies celecoxib dramatically reduced monocyte secretion of IL-6 and matrix metalloproteinase 9 and dobutamine successfully inhibited LPS-induced production of MCP-1.<sup>385, 412</sup> In animal MI models, eplerenone effectively prevented LV dilation and improved LV function via mineralocorticoid receptor blockade.<sup>413</sup>

### **2.3.8. Monocyte subsets**

Circulating monocytes are not a homogeneous population of cells - whether defined phenotypically and functionally - and include several subpopulations, which play complex roles in atherogenesis, each with their own potential for promotion of plaque formation. A substantial part of our knowledge on the role of monocyte subsets in atherogenesis derives from studies of mouse monocytes which include two major subsets: Ly-6C<sup>hi</sup> monocytes and Ly-6C<sup>lo</sup> monocytes.

Counts of Ly-6C<sup>hi</sup> monocytes are increased dramatically in hypercholesterolemic apoE-deficient mice consuming a high-fat diet, actively adhere to activated endothelium, infiltrate atherosclerotic lesions, and become lesional macrophages.<sup>414</sup> Ly-6C<sup>lo</sup> monocytes enter atherosclerotic lesions less frequently, but they are prone to developing into plaque cells expressing the dendritic cell-associated marker CD11c, and appear to have enhanced

atherogenic activity, indicating that phagocyte heterogeneity within plaques is linked to distinct sub-populations of infiltrating monocytes.

Plaque recruitment of Ly-6C<sup>lo</sup> cells is CCR5 (but not CX3CR1)-dependent whilst Ly-6C<sup>hi</sup> monocytes unexpectedly required CX3CR1 (normally expressed at low levels on these cells) in addition to CCR2 and CCR5 to accumulate within plaques.<sup>415</sup> Genetic deletion of CCR2, CX3CR1 or their ligands markedly reduces atherosclerotic lesion size in animal models of atherosclerosis.<sup>416</sup> Moreover, double deletion of CX3CL1 and CCR2 expression in mice has an additive effect and dramatically reduces macrophage accumulation in the artery wall and development of atherosclerosis.<sup>416</sup> Recently, a triple inhibition of CCL2, CX3CR1, and CCR5 in hypercholesterolemic mice led to a marked and additive 90% reduction in atherosclerosis.<sup>417</sup> Of interest, in this study the lesion size strongly correlated with the absolute number of circulating monocytes, but particularly so with the Ly-6<sup>lo</sup> subset.<sup>417</sup>

Oxidized LDL promotes conversion of CD11c<sup>-</sup> monocytes to CD11c<sup>+</sup> cells and a high fat diet significantly increases the proportion of Ly-6C<sup>lo</sup> CD11c<sup>+</sup> monocytes in apoE-deficient mice resulting in an increased proportion of circulating CD11c<sup>+</sup> monocytes with abundant lipids in the cytoplasm and 'foam' cell phenotype.<sup>418</sup> This formation of CD11<sup>+</sup> circulating 'foam' cells was associated with CD204 expression on their surface and was accompanied by abundant accumulation of CD11c<sup>+</sup> cells in the atherosclerotic plaques.<sup>418</sup>

Whilst human monocytes do not have direct equivalents of markers used for discrimination of mouse monocyte subsets, it is clear that monocyte subpopulations also exist in humans.

At present, human monocyte subsets are characterised by surface expression of CD14 (LPS and apoptotic cell receptor) and CD16 (Fc  $\gamma$  receptor type III) with about 85% of circulating monocytes being CD14<sup>++</sup>CD16<sup>-</sup> cells and about 15% of monocytes being represented by minor CD16<sup>+</sup> populations (normally below 15%). CD14<sup>++</sup>CD16<sup>-</sup> cells monocytes are considered to be counterparts of the Ly-C6<sup>hi</sup> mouse monocytes and CD14<sup>+</sup>CD16<sup>+</sup> cells – of the Ly-C6<sup>lo</sup> mouse subset.<sup>25</sup>

A proportion of CD14<sup>+</sup>CD16<sup>+</sup> monocytes has been previously shown to positively correlate with serum cholesterol and triglyceride levels, and negatively correlate with HDL cholesterol levels in patients with hypercholesterolemia and they are increased in those with developed CAD.<sup>419-420</sup> Also, CD14<sup>++</sup>CD16<sup>-</sup> monocytes from patients with familial hypercholesterolemia preferentially take up native LDL whereas CD14<sup>+</sup>CD16<sup>+</sup> monocytes exhibit an increased uptake of oxidized LDL via CD36, an increased expression of CD11c and macrophage markers and a higher adherence to activated endothelial cells in response to oxidized or native LDL stimulation.<sup>421</sup> Given that recent data show that mouse Ly-6C<sup>lo</sup> monocytes (analogous of CD16<sup>+</sup> human monocytes) ‘patrol’ arterial endothelial surface, it seems possible that CD16<sup>+</sup> monocytes exert a specific role in the handling of oxidized LDL deposits on the vessel wall.<sup>418, 422</sup> Indeed, monocytes isolated from patients with CAD also have increased CX3CR1 expression.<sup>423</sup>

However, the acknowledgment of the substantial role of monocytes and their subsets in atherogenesis raises important issue of the interactions of these cells with recognised cardiovascular risk factors. At present it is commonly accepted that cardiovascular risk



factors exert atherogenic properties mainly through the damage of vascular wall and modulation of lipid metabolism.

The presence and number of cardiovascular risk factors is significantly and differentially associated with counts of monocyte subsets.<sup>424</sup> There is a significant correlation between the 'classical' inflammatory CD14<sup>++</sup>CD16<sup>-</sup> monocytes and the number of risk factors present in patients with CAD suggesting novel mechanisms linking cardiovascular risk factors and chronic inflammation in atherosclerosis.<sup>424</sup>

Despite the growing evidence of important and diverse roles of monocyte subsets in cardiovascular disorders only scarce data are available in relation to HF. Recently Barisione et al. suggested that in a small study that CD14<sup>++</sup>CD16<sup>+</sup> cells was increased in 30 patients with chronic stable HF compared with 26 healthy controls.<sup>425</sup> However, the study participants differed not only by the presence of HF but also by the presence of CAD, hypertension, diabetes, and other morbidities commonly seen in patients with HF and which could drive the between the two groups. Clearly more data are needed in relation to this area.

### **2.3.9. Angiogenesis and heart failure**

Angiogenesis is a physiological process characterised by the growth of new blood vessels from preexisting ones.<sup>426</sup> More recently the term arteriogenesis has been additionally introduced, defined as an outgrowth of pre-existing arterioles into large conductance

collateral arteries.<sup>426</sup> Both processes have been implicated in the pathophysiology of various cardiovascular disorders including CAD and HF.<sup>426</sup>

Among of the pro-angiogenic factors vascular endothelial growth factor (VEGF) is perhaps best characterized and featured by high (as compared to other growth factors) specificity for the vascular endothelium.<sup>427</sup> VEGF triggers a potent signalling cascade within endothelial cells and represents a major contributor to angiogenesis, inducing accelerated formation of the capillary network in affected tissues. In *in vitro* studies stimulation with VEGF led to proliferation of bovine capillary endothelial cells followed by formation of tubular structures.<sup>427</sup>

Abnormal levels of angiogenic factors, such as VEGF have been reported in HF patients.<sup>287, 428</sup> VEGF levels are reduced in congestive HF but they are up-regulated in acute HF.<sup>429-430</sup> VEGF did not independently predict outcome in chronic HF, and a pathophysiological role of angiogenic factors in human HF is unclear.<sup>287</sup> However, treatment with VEGF reduced myocardial apoptosis, promoted capillary growth, preserved myocardial contractility and prolonged survival in animal studies.<sup>431-432</sup>

More recently, the angiopoietins, another family of growth factors specific for the vascular endothelium has been characterised.<sup>433</sup> The angiopoietins are growth factors acting through interaction with their surface cellular receptors, Tie-1 and Tie-2.<sup>434</sup> These receptors are tyrosine kinases similar to VEGF receptors.<sup>434</sup> However the actions of the angiopoietins appear to be quite different from those of VEGF, although they act in a complementary and coordinated way, playing a key role in vascular development. Angiopoietin-1 is also a

chemotaxin and - in conjunction with VEGF - recruits endothelial cells to initiate and accelerate angiogenesis. In the presence of VEGF angiopoietin-2 promotes a rapid increase in capillary diameter, remodelling of the basal lamina and new vessel growth. In contrast, if VEGF is inhibited, angiopoietin-2 leads to endothelial cell death and vessel regression.<sup>428, 434</sup> Chong et al. demonstrated increased levels of VEGF, angiopoietin-2 and Tie-2, but normal levels of angiopoietin-1, in both stable and decompensated HF thus indicating a potential role for these angiogenic factors in the pathophysiology of HF.<sup>312</sup>

Increased levels of angiogenic factors in HF cannot be considered as a direct proof of enhanced angiogenesis in HF per se. Further evidence of abnormal angiogenesis in HF is coming from animal models indicating that impaired angiogenesis may lead to HF.<sup>435</sup> For example, STAT3-deficiency in mice was associated with reduced density of myocardial capillary network leading to enhanced interstitial fibrosis, dilation of cardiac chambers and premature death.<sup>435</sup> Also VEGF was able to improve collateral circulation, facilitating recovery of global and regional cardiac contractility in a model of temporal coronary artery occlusion.<sup>436</sup>

### **2.3.10. Monocytes and angiogenesis**

Current data suggest a potential implication of monocytes in vascular tissue repair. It has been shown that macrophage depletion markedly delays wound healing, impairs cardiac remodelling and increases mortality after myocardial injury, thus identifying the macrophages as key players in myocardial healing.<sup>437</sup> Indeed, in animal models the lack of macrophages was accompanied by premature development of myocardial dysfunction.<sup>438</sup>

In contrast, appropriate macrophage activation might be important for cardiovascular repair, removal of apoptotic/necrotic tissues and other debris, and thus monocyte/macrophage depletion may not necessarily be an effective approach for the prevention of HF.<sup>438</sup> Indeed, these studies revealed a favourable role of monocytes in the prevention of excessive ischaemia-induced myocardial remodelling, probably due to their role in angiogenesis.

Angiogenesis stimulated by tissue hypoxia is mediated by activation of expression of hypoxia-inducible factor-1 $\alpha$  and it usually results into the development of new capillaries.<sup>439-440</sup> Angiogenesis is a tightly regulated process that occurs in the context of a fine-tuned balance between pro-angiogenic and anti-angiogenic factors.<sup>440</sup> In various cardiovascular injury models, including MI and stroke angiogenesis positively correlates with macrophage levels at the site of damage.<sup>440-441</sup> These findings are supported by the observation of an up-regulated DNA synthesis by endothelial cells at sites of macrophage accumulation in damaged tissues. Additionally, macrophages isolated from sites of tissue injury have been shown to induce angiogenesis *in vitro*, similarly to media taken from the macrophage culture.<sup>442-443</sup> Taken together, the available data imply that activated macrophages are able to influence angiogenesis, mainly via expression of pro-angiogenic factors (e.g., VEGF) and orchestrating activity of other cell types involved in angiogenesis.<sup>444-445</sup> Finally emerging data suggest that some monocyte populations have a potential to directly differentiate into endothelial cells, which is discussed in the following section.

### **2.3.11. Endothelial progenitors in heart failure**

According to a changing paradigm, in addition to endothelium-resident cells, endothelial-progenitor cells (EPCs) derived from the bone marrow play a significant role in angiogenesis and endothelial repair.<sup>38</sup> EPCs are defined as circulating cells of myeloid origin sharing stem cell surface markers (e.g., CD34) and an endothelial marker (usually VEGF receptor 2 [KDR]).<sup>39</sup> The surface marker CD133 is detected on an immature subset of EPCs and it was suggested as an additional marker of human angioblast-like EPCs to distinguish these from mature endothelial or monocytic cells. Accordingly, the combined phenotype of CD34+CD133+KDR+ cells was used in many studies alongside to CD34+KDR+ definition of EPCs.<sup>39</sup>

Conflicting reports have been published on the numbers of circulating CD34+ haematopoietic progenitors in HF with some studies observing reduction or no change in their levels irrespectively of HF aetiology (Table 12).<sup>446</sup> HF severity rather than its aetiology appears to be the main factor affecting levels of CD34+ cells. Indeed, CD34+ cells have been shown to be increased in mild HF and depressed in severe HF compared to HF free controls.<sup>447</sup> Others studies confirmed a significant inverse correlation between CD34+ and CD34+CD133+ counts and NYHA functional class and their progressive reduction from NYHA class I-II to NYHA class III HF independently of aetiology.<sup>448-449</sup>

**Table 12. Studies assessing circulating endothelial progenitor cells in heart failure**

Study	Study design and definition of heart failure	Definition of EPCs	Outcome
Valgimigli <sup>449</sup>	Case-control, observational Objective ventricular impairment [NYHA I-IV]	CD34+CD133 +KDR+	↑ EPCs in early stages of HF
Nonaka-Sarukawa <sup>447</sup>	Case-control observational [NYHA I-IV]	CD34 +	↓ EPC in advanced HF
Michowitz <sup>450</sup>	Prospective observational Primary end point: survival [NYHA II-IV]	‘Early’ EPC colonies (flk-1+, Tie-2+, CD31+)	The number of colonies was an independent predictor of mortality
Geft <sup>451</sup>	Case-control observational [NYHA I-IV]	CD34 +	Number of apoptotic EPCs positively correlated with NYHA class
Fritzenwanger <sup>448</sup>	Case-control, observational [NYHA I-IV]	CD34 + CD133 +	Number of EPCs inversely correlated with NYHA class.

NYHA, New York Heart Association; EPC, endothelial progenitor cells; VEGF, vascular endothelial growth factor, HF, heart failure; EC, endothelial cell.

It is likely that a reduction in CD34+ cell levels is secondary to HF rather than an element of HF pathogenesis itself and it probably reflects bone marrow depression and anaemia common in severe HF. These hypotheses are supported by observations that implantation of ventricular assist devices was associated with a transient increase in CD34+ cells in parallel with a reduction in BNP levels.<sup>452</sup> Also CD34+ cell numbers in HF were not affected by physical exercise testing, and transcatheter transplantation of CD34+ cells into

patients with a history of an anterior MI did not have any effect on endothelial function.<sup>453-454</sup>

Numbers of 'early' EPCs, counted in culture are reduced in HF, being inversely related to NYHA class, but not to VEGF, NT-pro-BNP or CRP levels.<sup>449-450</sup> The migratory activity of 'early EPCs' is significantly impaired in HF (particularly of ischaemic aetiology), correlate with endothelial dysfunction and can be normalised by physical exercise.<sup>446, 453</sup> Functional impairment of circulating progenitors in HF is also supported by increased numbers of 'late' apoptotic progenitors, particularly in severe HF, which correlated inversely with LVEF and positively with NYHA class.<sup>451</sup> In 107 patients with chronic HF, 'early EPCs' were independent predictors of all-cause mortality.<sup>450</sup> NT-pro-BNP dose-dependently increased the numbers, proliferative and functional capacity of human 'early' EPCs *in vitro*.<sup>455</sup> Systemic BNP administration to mice led to a significant increase in bone marrow Sca-1/Flk-1+ endothelial progenitors and improvement in blood flow and capillary density in the ischaemic limbs.<sup>455</sup>

CD34+KDR+ cells seem to be only significantly reduced in patients with very severe HF.<sup>449, 453</sup> Indeed, numbers of CD34+KDR+ cells in HF are unaffected by physical exercise or implantation of ventricular assist devices.<sup>452-453</sup> However, in one randomized study 3 months treatment with high doses of rosuvastatin increased CD34+KDR+ cells and their integrative capacity, which paralleled a significant improvement in FMD and LVEF.<sup>194</sup> Additionally, implantation of CD133+ cells in the infarcted zone in 7 candidates for cardiac transplantation was associated with improvement of NYHA class, LVEF, reduction in NT-pro-BNP levels, and risk of sudden death by 24 months after treatment.<sup>456</sup>

However, the last study was small, lacked a control group and two of the seven patients died during the period of the observation – thus, larger controlled trials are essential to draw any robust conclusion on clinical perspectives of such therapies in HF.

In summary, the biological and pathological roles of altered production of angiogenic factors are still not entirely clear. They may reflect a degree of compensatory angiogenesis in patients with ischaemic HF but it is more plausible that these changes reflect an ongoing process of vascular remodelling in response to the haemodynamic changes. The potential of these pathways as therapeutic targets is still to be determined.

#### **2.3.12. Monocytes as endothelial progenitor cells**

Admittedly, the origin as well as the phenotypic and functional characteristics of EPCs remains poorly understood. Several cell types obtained either from bone marrow, peripheral blood or tissue-resident stem cells have potential to differentiate into endothelial cells, with CD14<sup>+</sup> myeloid monocytic cells being a common source of EPCs. Moreover, EPC phenotype may change during the process of maturation, which begins in the bone marrow and continues in the circulation. Early in the circulation EPCs lose CD133 and they increasingly express endothelial markers. More recently EPCs were re-defined according to their *in vitro* angiogenic potency as endothelial cell colony-forming units (with limited potential for proliferation), and endothelial colony-forming cells (with almost unlimited ability to proliferate in culture).<sup>457</sup> The authors concluded that endothelial colony-forming cells are the ‘true’ EPCs able to differentiate into functional endothelial cells and form perfused vessels *in vivo*. It becomes apparent that EPCs represent a



heterogeneous population in terms of origin and their angiogenic properties. This heterogeneity should be cautiously taken into consideration in future cell therapy studies and studies aiming to identify novel pharmacological target molecules related to angiogenesis.

Much attention has also been attracted to the capacity of monocytes to act as potential EPCs.<sup>458-459</sup> Circulating monocytes can differentiate into different types of phagocytes, such as macrophages, dendritic cells, osteoclasts, microglia and Kupffer cells.<sup>460</sup> Until recently, the differentiation potential of monocytes was believed to be restricted to cells with phagocytic or antigen-presenting properties. However, several lines of evidence suggest that circulating CD14<sup>+</sup> monocytes are able to (trans)differentiate into various non-phagocytes, including cells of mesodermal and neuroectodermal lineages.<sup>459</sup> Curiously, this phenomenon was probably first described almost 150 years ago, in 1867 when Cohnheim and co-workers showed that peripheral blood monocytes participated in tissue renewal in different organs.

The ability of circulating cells of hematopoietic origin to differentiate into endothelial cells at sites of vascular remodelling was initially reported by Crosby et al.<sup>461</sup> who demonstrated that blood-derived cells represent ~10% of endothelial cells in mice neovasculature developed in response to ischaemia. At almost the same time another group revealed that under appropriate *in vitro* conditions CD14<sup>+</sup> cells can differentiate into endothelial-like cells with characteristic endothelial features.<sup>462</sup> Also macrophages were shown to infiltrate the myocardium in response to cardiac MCP-1 overexpression and to form erythrocyte-containing vascular-like tubes.<sup>463</sup>

Injection of human CD14<sup>+</sup> cells improves healing and vascularisation of the ischaemic limbs in diabetic mice.<sup>464</sup> Transplantation of CD14<sup>+</sup>KDR<sup>+</sup> cells into balloon-injured femoral arteries of nude mice significantly contributed to efficient reendothelialization, whilst CD14<sup>+</sup>KDR<sup>-</sup> monocytes failed to produce any effect.<sup>465</sup> Experimental work shows that monocytes may include subpopulations of multipotent cells capable of *in vitro* differentiation into various somatic cell types.<sup>37</sup> These cells, but not ‘classical’ monocytes, were able to successfully restore LV function after experimental MI.<sup>466</sup>

The number of circulating CD14<sup>+</sup>KDR<sup>+</sup> cells has been found to be increased in acute MI. CD14<sup>+</sup>KDR<sup>+</sup> but not CD14<sup>+</sup>KDR<sup>-</sup> cells stimulated organization of human microvascular endothelial cells into capillary-like structures.<sup>467</sup> Human activated macrophages delivered to rats early after MI accelerated vascularization, tissue repair, and improved cardiac remodelling and function.<sup>468</sup> In mice, M-CSF reduced the infarct area and improved LV remodelling after MI through the recruitment of CXCR4<sup>+</sup> cells into the infarcted myocardium.<sup>469</sup>

However, it was noted that cells of the monocytic origin lacked some endothelial cell antigens and could themselves be subsequently colonized by endothelial cell. Indeed, Harraz et al. reported that CD14<sup>+</sup>CD34<sup>+</sup> monocytes have the potential to be incorporated into the vascular endothelium in mouse ischaemic limbs and to transdifferentiate into endothelial cells (or other cells types) depending on the environmental cues.<sup>470</sup> Furthermore, under angiogenic stimulation (e.g., with VEGF) monocytes can develop an endothelial phenotype with expression of specific surface markers and even to form tubular-like structures *in vitro*, thus suggesting possible implication in vasculogenesis.<sup>471</sup>

Finally, Kuwana et al. described a primitive cell population termed as monocyte-derived multipotential cells (MOMC) and they introduced a concept of the multipotential nature of circulating monocytes.<sup>472</sup> This cell population contains progenitors that can differentiate into several distinct mesenchymal cell types, including bone, cartilage, fat, skeletal and cardiac muscle cells, as well as neurons. MOMCs can be generated *in vitro* by culturing circulating CD14<sup>+</sup> monocytes on fibronectin in the presence of soluble factors derived from circulating CD14<sup>+</sup> cells. MOMCs express several endothelial markers, including vascular endothelial cadherin and VEGF type 1 receptor, and have the ability to take up acetylated LDL. More recently, the same group have demonstrated that transplantation of the MOMCs promoted the neovessel formation with over 40% of the newly formed vessels incorporated human endothelial cells derived from MOMCs.<sup>473</sup> These findings indicate that human MOMCs can proliferate and differentiate along the endothelial lineage in a specific permissive environment and thus could represent an autologous transplantable cell source for therapeutic vasculogenesis.

These data clearly demonstrate the intimate relationship between monocytes and endothelial cells suggesting that specific monocyte population(s) may be recruited for vasculogenesis and may represent a population of endothelial precursors. However, it remains to be determined how EPCs are implicated in the pathophysiology of HF and how can EPC activity be manipulated in beneficial ways.

### 2.3.13. Monocyte-platelet aggregates

The adhesion of leukocytes to platelets deposited at the site of vascular injury may represent an important mechanism by which leukocytes contribute to haemostasis and thrombosis. Indeed, the risk of graft occlusion after reconstructive surgery amongst patients with peripheral vascular disease is much higher in those with more active leukocyte-platelet adhesion in the early postoperative period.<sup>474</sup> The number of monocyte-platelet aggregates (MPA) is also markedly increased after hip and knee arthroplasty.<sup>475-476</sup> Such an increased formation of MPAs has been associated with high levels of P-selectin, CD40 ligand, TF and Mac-1 expression on monocytes; a correlation with beta-thromboglobulin levels suggests a role of MPAs in augmenting blood coagulability after major joint surgery.<sup>477</sup> Patients with essential thrombocythemia, a disorder characterised by a high risk of thrombosis, also had increased MPA levels.<sup>478</sup>

MPAs form when platelets are activated and undergo degranulation. Activated platelets express on their surface high levels of P-selectin that binds to the leukocyte receptor, P-selectin glycoprotein ligand-1.<sup>479</sup> *In vivo* data confirm that P-selectin augments platelet-leukocyte aggregation.<sup>479</sup> In experimental models, the infusion of recombinant human P-selectin glycoprotein ligand-1 reduced myocardial reperfusion injury in an animal model of vascular injury.<sup>480</sup> Platelet derived microparticles, which are shed from platelets upon their activation, also interact with monocytes in a mechanism dependent upon CD62P, similar to that seen with MPA formation.<sup>481</sup> Additionally, platelet adhesion to monocytes may occur through calcium independent mechanisms, involving neither P-selectin glycoprotein ligand-1 nor P-selectin.<sup>482</sup> Thrombospondin is also reported to be involved in the cross-link

between platelets and monocytes in early vascular injury via an interaction with glycoprotein IV on the surface of both cells.<sup>483</sup> In fact, MPAs is a good marker of platelet activation (e.g., in ACS) being even superior to surface P-selectin expression as the latter is rapidly released into circulation by degranulated platelets whilst MPAs continues to be detected in the circulation.<sup>484</sup>

An important question relates to the biological role of MPAs. They may simply reflect the physiological mechanism of elimination of activated platelets from the circulation and just represents a stage of platelet adherence to monocyte prior to their phagocytosis. As early as in 1966, Poole<sup>485</sup> demonstrated the phagocytosis of platelets by monocytes in organised arterial thrombi using electron microscopy. That means that high levels of MPAs seen in ACS may perhaps reflect disproportionately high number of activated platelets in the circulation in relation to the relatively small area of local vascular injury. Alternatively, monocytes may be attracted to remove excessive activated platelets.

However, the role of MPAs might be even more complicated. Elstad et al. have suggested that P-selectin may prime monocytes to increase platelet-activating factor synthesis, thus representing a vicious circle leading to progressive and uncontrolled platelet activation.<sup>345</sup> Additionally, conjugation with platelets activates expression of Mac-1 on monocytes, which amplifies interactions with platelets via fibrinogen bivalently linking Mac-1 with its platelet glycoprotein IIb/IIIa.<sup>486-487</sup> The importance of MPAs for the vascular thrombosis is further supported by the observation that monocytes constitute 16% of platelet thrombus-bound leukocytes, which represents an almost 4-fold enrichment as compared with their proportion in circulating blood.<sup>488</sup>

The conjugation of leukocytes with platelets is promoted by different conditions associated with inflammation and endothelial dysfunction (e.g., diabetes).<sup>489-490</sup> Incubation of human blood with CRP doubles MPA formation and injection of LPS to mice increased MPA count 4-fold.<sup>491</sup> MPAs—but not platelet aggregates with lymphocytes and neutrophils—are increased in patients with diabetes. Indeed, more severe diabetes (for example, complicated by proliferative retinopathy and nephropathy) is associated with an even higher numbers of MPAs.<sup>490</sup> The increased association of monocytes with platelets accords with the data of enhanced of thromboxane A<sub>2</sub> by monocytes in diabetic patients.<sup>492</sup> The number of circulating MPAs increases with age and correlates with reduced synthesis of NO by platelets.<sup>493</sup>

Not surprisingly, MPA formation is increased in patients with CAD (Table 13). Patients with stable CAD have increased circulating MPAs compared with controls (15.3% vs.6.3%, respectively).<sup>494</sup> However, destabilisation of atherosclerotic plaques complicated by atherothrombosis results in a profound (3-fold) increase in total and TF-positive MPAs found in blood of ACS patients compared to stable CAD.<sup>483, 495</sup> For example, Furman et al. examined 61 patients with acute MI and compared them with 150 control subjects and reported that intensive formation of MPAs in MI starts very early and may be detected within 4 hours of symptom onset, even prior the increase in creatinine kinase levels.<sup>496</sup> However, utility of MPA count for diagnostic purposes in ACS has to be tested with direct comparison to cardiac troponins, and it has important limitations relevant to a labour intensive procedure for MPA enumeration by flow cytometry and the lack of exclusive cardiac specificity.

**Table 13. Monocyte tissue factor expression and monocyte-platelet aggregates in patients with cardiovascular risk factors and cardiovascular disorders**

Study	Condition	Controls	Monocyte effects
Puccetti <sup>497</sup>	Hypercholesterolemia	Healthy	Increased TF expression
Ichikawa <sup>498</sup>	Diabetes mellitus	Healthy	Increased TF expression
Kaplar <sup>490</sup>	Diabetes mellitus with angiopathy	Diabetes mellitus without angiopathy	Increased MPAs
Hölschermann <sup>499</sup>	Smoking pre-menopausal women	Non-smoking pre-menopausal women	Increased TF expression
Furman <sup>494</sup>	Stable CAD	Healthy	Increased MPAs
Kälsch <sup>500</sup>	Unstable angina	Stable CAD	No effect on TF binding and MPAs
Brambilla <sup>495</sup>	Acute coronary syndrome	Stable CAD, healthy	Increased TF-positive MPAs
Sarma <sup>482</sup>	Acute coronary syndrome	Stable CAD	Increased MPAs
Furman <sup>496</sup>	Myocardial infarction	Stable CAD	Increased MPAs
Freeburn <sup>501</sup>	Myocardial infarction	Stable CAD	Increased MPAs
Vieira <sup>502</sup>	Deep vein thrombosis	Healthy	Increased TF expression
Hölschermann <sup>503</sup>	Cerebral venous thrombosis (women)	Healthy	Increased TF expression
Arellano-Rodrigo <sup>504</sup>	Essential thrombocythemia with history of thrombosis	Healthy	Increased TF expression, increased MPAs
Kornberg <sup>478</sup>	Polycythemiavera	Healthy	Increased TF expression
Bunescu <sup>476</sup>	Hip arthroplasty	Prospective study	Increased MPAs

CAD – coronary artery disease, MPA – monocyte-platelet aggregates, TF – tissue factor

Given the clear association of MPAs with the onset of atherothrombosis, these may perhaps be appealing targets for antithrombotic treatment. However, only limited data are available on the effects of medical therapy on MPA levels. Of note, anticoagulation has little impact on monocyte-platelet interactions.<sup>505</sup> In a recent randomized study of 60 patients undergoing percutaneous coronary intervention (mostly with stable CAD) anticoagulation with a combination of unfractionated heparin and eptifibatid was associated with higher MPA counts compared with bivalirudin, but this difference disappeared after pretreatment with clopidogrel.<sup>479</sup> In one *in vitro* study, the formation of MPAs was markedly enhanced in the presence of glycoprotein IIb/IIIa antagonist, MK-852.<sup>506</sup> In contrast to anticoagulants, antiplatelet therapy reduced MPAs by 46% within 4 days.<sup>505</sup> This effect has been correlated with a concomitant decrease in monocyte Mac-1 expression.<sup>505</sup>

#### **2.3.14. Conclusions**

There is a convincing evidence that monocytes possess high developmental plasticity, and are able to trans-differentiate into other non-phagocytic cell types under diverse microenvironments. Recent literature —mostly based on *in vitro* data— supports the hypothesis that monocytes include a population of potent progenitor cells actively involved in postnatal vasculogenesis, although, it is not certain whether monocytes can become fully functional endothelial cells. There is also a convincing evidence to support a significant role of monocytes in angiogenesis —a significant step in the process of tissue repair— following ischaemic myocardial injury. Increased monocyte activation seen at the sites of post-ischaemic injury is more likely to reflect an attempt for tissue repair rather than a



deteriorating immune overreaction. Thus, induction rather than inhibition of monocyte activation might be a potential therapeutic target.

However the available data do not sufficiently establish a causative relation between altered monocyte-derived pathways and progression of HF. It is not clear whether this is a pathway leading to HF or a consequence of the systemic nature of HF. In the first case, mobilization of monocytes could be a potential therapeutic target whilst in the second case, monocyte number and function could be an excellent disease marker. Moreover it must be elucidated whether monocytes can differentiate to a fully functional endothelial cells or their contribution in angiogenesis and/or vasculogenesis is restricted to production of vascular growth factors. There is also a need to identify appropriate stimuli to lead monocyte differentiation to progenitor cells, and such stimuli have to be cell-specific and organ specific in order to limit their action towards the desired direction. Brain natriuretic peptides, granulocyte-colony stimulating factor and erythropoietin that have been used up to now as progenitor cell activators are neither cell nor organ specific.

There is a big gap in research due to the lack of *in vivo* evidence supporting the ability of monocytes/macrophages infiltrating the dysfunctional vascular wall/myocardium to act as potent progenitor cells. Perhaps, certain stimuli are required for monocytes/macrophages to trans-differentiate into functional progenitor cells. Such stimuli might not be present in the dysfunctional tissue and may need to be externally applied. Alternatively, only a subset of monocytes may possess a capacity to trans-differentiate into progenitor cells. In my opinion it will be some time before monocyte-derived progenitor cells-based therapies can be applied in clinical practice. Nonetheless, much progress has been made during the last

years in the understanding of monocyte role in cardiovascular disease. I believe that their presence in sites of vascular or myocardial injury is not a catastrophic immune overreaction but a desperate attempt for tissue repair.

Finally, anti-inflammatory treatment-strategies for the management of HF are now more than appealing theories; however, the initial trials that tested therapies targeting inflammation in the overall HF population have had disappointing results. As it has been underlined in a recent statement issued by the Translational Research Committee of the Heart Failure Association of the European Society of Cardiology, the most successful trials have been those where small very carefully selected groups of patients were treated. Indeed, the idea of a common inflammatory pathway that underlies all different forms of HF appears to be unlikely.<sup>507</sup> It is probably more realistic to design specific anti-inflammatory approaches for different types and/or stages of HF. Thus determination of the specific inflammatory pathways in different forms of HF is essential. The use of specific inhibitors of inflammation appears to be a promising treatment option; however, sufficient knowledge of basic mechanisms, detailed pre-clinical studies and justified patient selection for clinical trials are required for the development of successful anti-inflammatory approaches in the management of HF.

## **2.4. Circulating microparticles in cardiovascular disease**

### **2.4.1. Definition of microparticles**

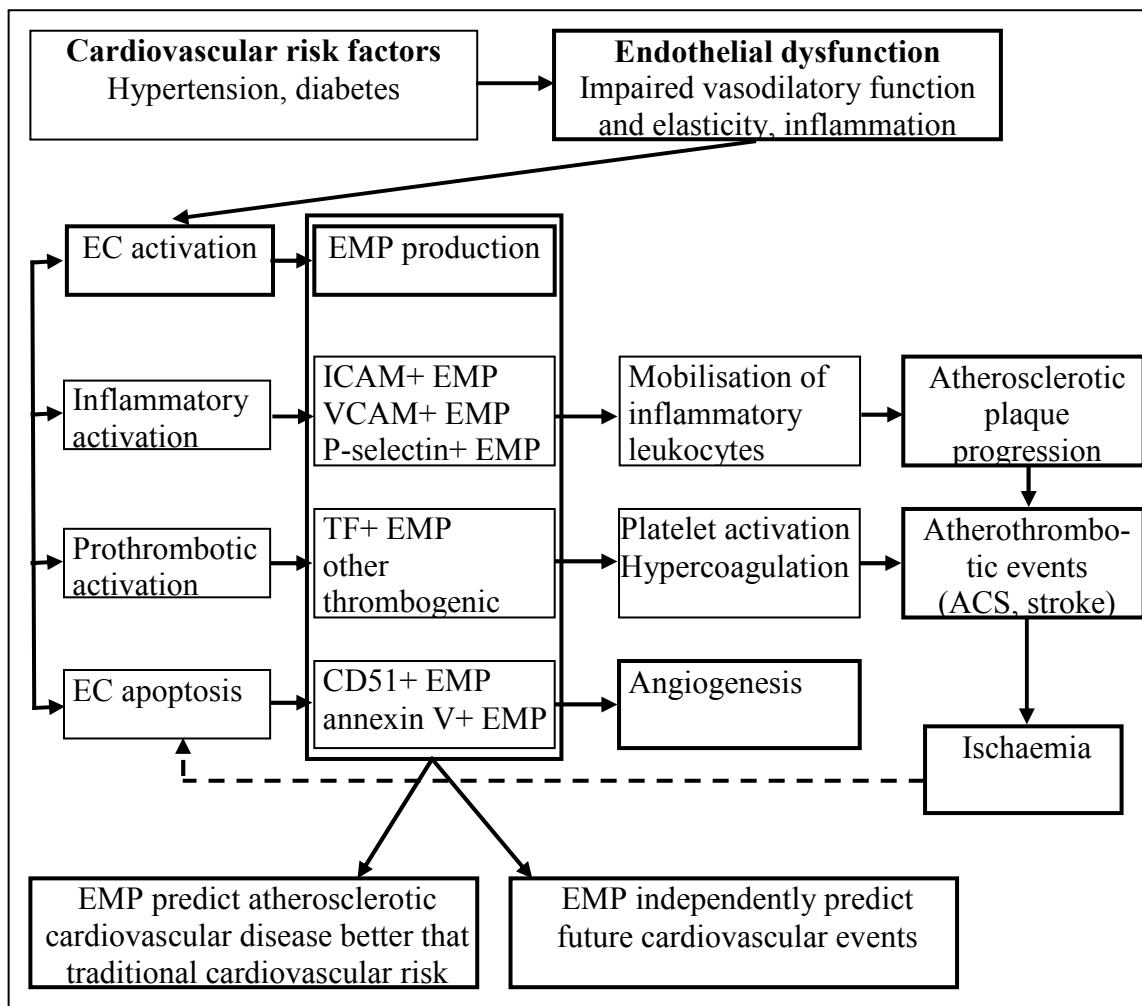
The extremely complex and multifactorial nature of many cardiovascular disorders, including atherogenesis and HF is well recognised.<sup>508</sup> For example, in atherogenesis in addition to accumulation of lipids by ‘foam’ cells, plaque formation and progression, there are numerous interactions between various cell types inside the vascular wall (e.g., macrophages, smooth muscle cells) and in the blood (e.g., leukocytes and platelets). These interactions orchestrate alterations of the extracellular matrix, the migration of leucocytes through endothelium, inflammatory burden, plaque neovascularisation, growth and destabilisation, and ultimately, the development of atherothrombotic complications.<sup>508</sup> One relatively recent advance in this area is the discovery of circulating microparticles and their strong association with endothelial damage, platelet activation, hypercoagulability, and regulation of inter-cellular interactions, which links the presence of cardiovascular risk factor, atherogenesis and thrombosis.<sup>509-510</sup>

Microparticles are defined as small (0.1 – 1 µm diameter) anucleoid phospholipid vesicles released from different cells, platelets, erythrocytes, leukocytes, endothelial cells.<sup>511-513</sup> Microparticles carry surface proteins and include cytoplasmic material of the parental cells. Microparticle membrane includes negatively charged phospholipids, mainly phosphatidylserine. Microparticles are distinguished from exosomes (which are smaller vesicles [40-100 nm] derived from endoplasmic membranes), and apoptotic bodies (which are larger particles [ $>1.5$  µm] that contain nuclear components).<sup>514</sup>

#### **2.4.2. Endothelial cell microparticles – a marker of vascular damage/dysfunction**

Circulating endothelial cell microparticles (EMPs) can be identified and measured by detection of antigens constitutively expressed by mature endothelial cells (e.g., CD31 [platelet-derived growth factor], CD105 [endoglin], CD144 [vascular endothelium cadherin], CD146, etc).<sup>515</sup> During the process of vesiculation, EMPs may also acquire inducible endothelial markers such as CD54 [ICAM-1], CD62E [E-selectin] and CD106 [VCAM-1], TF and annexin V [an apoptotic marker]). The presence of these antigens on the microparticle surface affects their biological activity and may serve as a basis of EMP classification: for example, CD54+, CD62E+ and CD106+ EMPs predominantly reflect inflammatory endothelial cell activation, whilst TF+ EMPs reflect prothrombotic changes of endothelial cells, and annexin V+ EMPs characterise endothelial apoptosis.<sup>516</sup> However, the expression of these antigens may not be exclusive and at present it is unclear if the process of microparticle formation occurs randomly from the surface of dysfunctional endotheliocytes or whether it is actively regulated so that EMP immunophenotype may be different from the antigen profile of the ‘maternal’ cells. Additionally, analysis of the antigenic composition of EMP using a proteomic approach reveals the presence of the wide range of proteins, such as metabolic enzymes, proteins involved in adhesion and fusion processes, cytoskeleton associated proteins and nucleosome indicating a potential role of EMPs as messengers of signals of dysfunctional endothelium.<sup>517</sup>

Indeed, irrespective of their antigen characteristics, EMP values reflect the presence of endothelial dysfunction (Figure 4). There are strong associations between high levels of EMPs (CD31+, CD51+, CD144+) and various vascular structural and functional



**Figure 4. Endothelial microparticles in atherogenesis and atherothrombosis.**

Cardiovascular risk factors and resulting endothelial dysfunction are accompanied by release of microparticles from endothelial cells. The pattern of the circulating EMPs appears to reflect functional state of parental endotheliocytes and various EMP subtypes trigger specific pathophysiological responses. For example, endothelium may release microparticles expressing adhesion molecules facilitating recruitment of inflammatory cells, whilst microparticles from apoptotic endothelial cells seem to promote angiogenesis. Different types of EMPs play their own unique roles in the development and progression of atherosclerosis and their levels are independent predictors of atherosclerotic disease and future cardiovascular events. ACS – acute coronary syndromes, CRP – C-reactive protein, EC – endothelial cells, EMP – endothelial microparticles, ICAM – intracellular adhesion molecule, TF – tissue factor, VCAM – vascular cell adhesion molecule

abnormalities, for example, impairment of FMD, accelerated pulse wave velocity, and high CRP levels.<sup>518-520</sup> Annexin V+CD31+ EMPs correlate with vasodilatory endothelial dysfunction and have been shown to have a potential as a marker of coronary endothelial function independent of classical cardiovascular risk factors.<sup>521-522</sup> Furthermore, therapeutic improvement of endothelial function has been associated with a reduction of circulating EMP levels.<sup>523</sup>

Patients with different cardiovascular risk factors have significant up-regulation of EMPs before the development of CAD. For example, various EMP types (CD144+, CD31+, CD51+ and annexin V+) are increased in diabetic patients, and appear to be good predictors of CAD (Figure 4). Also, EMP numbers in diabetes were closely correlated with the presence of non-calcified coronary plaques more prevalent in patients with ACS.<sup>524</sup> Significant correlation of HbA1c and CD31+ and CD51+ EMPs indicates that poor glycaemia control may directly promote vesiculation from endothelial cells.<sup>518</sup>

Another line of evidence on the pathophysiological links between EMP formation and vascular dysfunction/damage is provided by studies on hypertension, both systemic and pulmonary (Table 14). Indeed, hypertension causes increased shear stress and direct mechanical damage of arteries. EMPs appear to be very sensitive to hemodynamic changes in hypertension, and their numbers are increased even in mild hypertension and rise further in proportion to blood pressure elevation, even in the presence of multiple risk factors.<sup>525</sup> Moreover, EMP generation is strongly associated with abnormalities of arterial elastic properties in hypertension.<sup>526</sup> Persistent arterial elastic abnormalities correlate to EMPs even after blood pressure normalisation.<sup>527-528</sup> In contrast, pulmonary hypertension is

**Table 14. The influence of circulating microparticles in patients with cardiovascular risk factors and cardiovascular disease**

<b>Condition</b>	<b>Endothelial MP</b>	<b>Platelet MP</b>	<b>Leukocyte MP</b>
Hypertension	↑CD31+CD42- MP <sup>525</sup>	↑CD41+ MP <sup>525</sup>	NA
Hypercholesterolaemia	NA	↑CD42a+CD42b+ MP <sup>529</sup>	NA
Diabetes	↑CD144+ MP <sup>516, 521</sup> ↑CD51+ MP <sup>518</sup> ↑CD31+CD42- MP <sup>518</sup>	↑CD41+ MP <sup>530</sup> ↑CD42a+ MP <sup>531</sup> ↑CD42b+ MP <sup>532</sup> ↑CD42b+CD61+MP <sup>533</sup> ↑CD31+CD42+ MP <sup>518</sup> ↑CD42a+ MP in those with retinopathy <sup>534</sup>	↑CD14+ MP <sup>531</sup> ↑annexin V+ MP <sup>531</sup> ↑CD45+ MP <sup>518</sup> ↑CD14+ MP in patients with retinopathy <sup>535</sup> ↑annexin V+ in patients with retinopathy <sup>535</sup>
Smoking	NA	↔CD61+ MP <sup>536</sup>	NA
Obesity	↑CD31+CD42b-MP <sup>537</sup>	↑CD31+CD42b+MP <sup>537</sup>	NA
Coronary artery disease	↑CD51+ MP <sup>538</sup> ↑CD31+CD42- MP <sup>538</sup> ↑CD144+ MP <sup>519</sup> ↑CD31+annexin+ MP in patients with LV dysfunction <sup>521</sup> CD31+annexin V+ MP correlated with endothelial dysfunction <sup>522</sup>	↑CD62P+ MP <sup>539</sup> ↑CD63+ MP <sup>539</sup> ↑CD61+ MP (after PCI) <sup>540</sup>	NA
Peripheral artery disease	NA	↑CD42a MP <sup>541-542</sup> ↑CD62P+ MP <sup>539</sup> ↑CD63+ MP <sup>539</sup>	NA
Carotid atherosclerosis	CD105+ MP associated with inward carotid remodelling <sup>543</sup>	↑CD41+CD61+ MP <sup>544</sup>	↑CD11a+ MP in subclinical atherosclerosis <sup>545</sup>
Acute coronary syndrome	↑CD31+CD42- MP <sup>538</sup> ↔CD51+ MP <sup>538</sup> CD54+ MP were associate with persistent coronary occlusion <sup>546</sup>	↑CD41+CD61+ MP <sup>547</sup> ↑CD41+ MP <sup>539</sup> ↑CD41+ MP <sup>539</sup> ↑CD41+ MP <sup>548</sup>	CD15+ MP were associate with persistent coronary occlusion <sup>546</sup>
Stroke	↑CD54+ MP <sup>549</sup> ↑CD105+ MP <sup>549</sup> ↑CD144+ MP <sup>549</sup> ↑Annexin V+ MP <sup>549</sup> ↑CD62E+ MP <sup>550</sup>	↑CD41+CD61+ MP <sup>551</sup> ↑CD42b+ MP <sup>552</sup>	NA

MP – microparticles, NA – data not available, ↑ - increased, ↔ - not changed.

associated with the rise of microparticles of different origin (eg, from platelets, endothelial cells, leucocytes), but only EMPs predict the hemodynamic severity of pulmonary hypertension.<sup>553</sup> In one study, elevated CD62E+ EMPs but not leukocyte microparticles predicted adverse clinical events (ie, combination of death and re-admission for right HF or worsening of right HF symptoms) in patients with pulmonary hypertension during 12-month follow-up.<sup>554</sup>

Additionally, EMPs may represent a ‘link’ between endothelial dysfunction and arterial thrombosis. Microparticles released from endothelial cells *in vivo* have been shown to carry TF on their surface.<sup>555</sup> Although *in vivo* role of this phenomenon has not yet been assessed and EMPs are unlikely to compete with monocyte-derived microparticles as a main microparticle-related source of circulating TF.<sup>556</sup> EMPs generation is stimulated by plasminogen activator inhibitor-1 and may trigger platelet aggregation via von Willebrand factor.<sup>557-558</sup> A recent prospective study of 488 consecutive patients with various cardiovascular risk factors demonstrated during a mean follow-up of three years that EMP count is a significant (but weak) independent predictor of future cardiovascular events (hazard ratio: 1.35, 95% CI: 1.09 to 1.65,  $p = 0.005$ ).<sup>559</sup>

### **2.4.3. Platelet microparticles – beyond a marker of platelet activation**

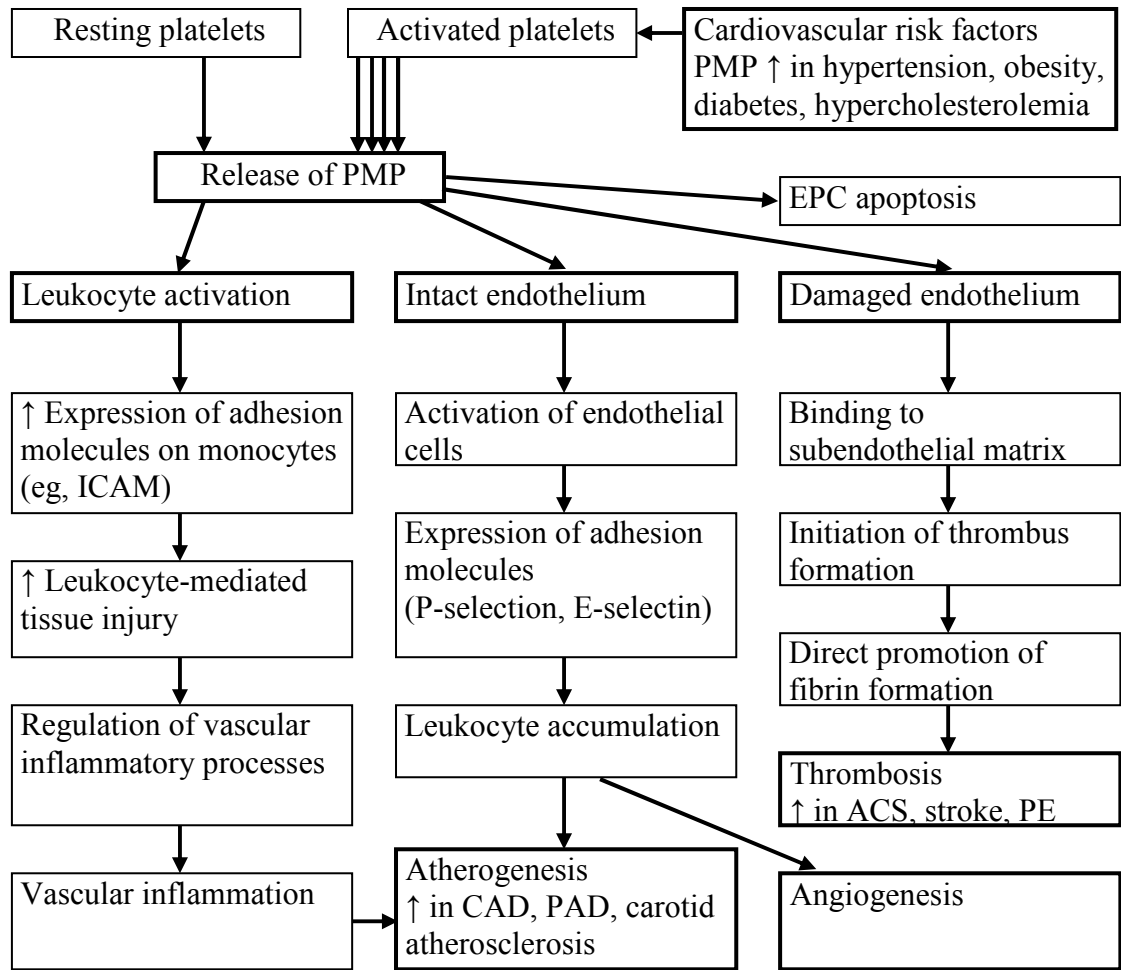
Platelets microparticles (PMPs) may also be generated by megakaryocytes, and are typically identified by the expression of constitutive platelets markers such as CD41, CD42b or CD61.<sup>511, 560-562</sup> PMPs express various antigens from the platelet surface such as glycoproteins I, IIa, IIb, IIIa, vWF, P-selectin, thrombospondin and chemokine receptors



(e.g., CXCR4).<sup>511, 560-562</sup> Immunophenotypic PMP characteristics, their phospholipid composition and cholesterol content may therefore reflect the functional state of platelets shedding microparticles as well as nature of a stimulator of their release.<sup>563</sup>

About 25% of the procoagulant activity of stimulated platelet suspensions is associated with microparticles released upon platelet activation and their surface may be approximately 50- to 100-fold more procoagulant than the surface of activated platelets per se.<sup>564-565</sup> Low amounts of PMPs are continuously shed from platelets but this process is highly accelerated following platelet activation or intensive physical activity.<sup>566-568</sup> Release of PMPs appears to be an active process with different platelet receptors (e.g., tetraspanin,  $\alpha_{IIb}\beta_3$  integrin) implicated into its regulation.<sup>569</sup> PMPs bind to the subendothelial matrix acting as a catalytic centre for further platelet binding at sites of endothelial injury.<sup>570</sup> For example, Siljander and colleagues showed that PMPs are involved in the formation of fibrin fibrils.<sup>571</sup> Thrombogenic properties of PMPs have since been confirmed in experimental studies and high PMP levels are strongly associated with different thrombotic conditions (Figure 5). In a mouse model of venous thrombosis, for example, the injection of microparticles increased the extent of thrombosis, whereas leukocyte microparticles were negatively correlated with thrombus weight.<sup>572</sup> PMPs were also significantly increased in patients with acute pulmonary embolism and were the main source of procoagulant microparticles.<sup>573</sup> In valvular atrial fibrillation, which carries a very high risk of thromboembolism, the numbers of PMPs are more than 3-fold increased.<sup>574</sup>

Interestingly, the biological roles of PMP spread beyond their participation in thrombotic processes.<sup>575</sup> PMPs actively interact with endothelial and blood cells and they are



**Figure 5. Platelet microparticles in atherogenesis and atherothrombosis.**

ACS – acute coronary syndromes, CAD – coronary artery disease, EPC – endothelial progenitor cells, ICAM – intracellular adhesion molecule, PAD – peripheral artery disease, PE – pulmonary embolism, PMP – platelet microparticles.

intimately involved in the regulation of their function.<sup>576</sup> For example, PMPs can activate EMPs, trigger up-regulation of cell adhesion molecules on them, induce apoptosis of endothelial progenitors, and can activate and aggregate neutrophils *in vitro*.<sup>577-579</sup> Arachidonic acid released from PMPs can activate ICAM-1 on monocytes, as well as the adhesion molecules, P- and E-selectins on endothelial cells.<sup>560, 577, 580-582</sup> Also, PMPs participate in the regulation in leukocyte interactions and amplify leukocyte-mediated tissue injury in thrombotic and inflammatory disorders.<sup>579, 583</sup> PMPs have been shown to contain active caspase 3 and seem to serve as a messenger triggering apoptosis of macrophages.<sup>584</sup> They may also participate in the regulation of inflammatory responses and induce angiogenesis *in vitro*, probably through the activation of endothelial cells.<sup>585-587</sup> PMPs differ in size which can affect the pattern of plasma membrane receptors, adhesion molecules, chemokines, growth factors, protein composition and membrane function.<sup>588</sup>

PMPs are significantly up-regulated in patients with different cardiovascular risk factors (e.g., hypertension, hyperlipidemia, diabetes, obesity) and a combination of these risk factors appears to have a cumulative effect on PMP production.<sup>525, 530, 536-537, 589</sup> PMP seem not to be elevated in patients who smoke.<sup>537</sup> In diabetes, PMPs may be associated with the high rate of thrombotic events, atherosclerosis and retinopathy, because PMP levels correlated with platelet activation (e.g., platelet P-selectin expression).<sup>530, 532-534</sup> Unfortunately, there is only limited information on the effect of cardiovascular treatment on PMP levels. In one study, eicosapentaenoic acid but not pitavastatin reduced PMPs in hyperlipidemic and diabetic patients.<sup>529</sup>

#### 2.4.4. Leukocyte microparticles – regulators of cellular interactions

Microparticles are also released by all major types of white blood cells: monocytes, granulocytes and lymphocytes. TF-expressing monocyte-derived microparticles (MMP) constitute the second largest pool of thrombogenic microparticles in addition to PMPs.<sup>381, 590-591</sup> Exposure to endotoxemia increases procoagulant microparticles (predominantly TF+ monocyte microparticles) *in vivo* and *in vitro*.<sup>592-595</sup> MMPs bind to activated platelets triggering the formation of fibrin.<sup>381, 590</sup> Experimental data also show that active generation of MMPs results in disruption of endothelial cell integrity and increases endothelial thrombogenicity (e.g., increased endothelial expression of TF and reduced expression of anticoagulant TF pathway inhibitor and thrombomodulin).<sup>593</sup> Procoagulant TF-bearing leukocyte microparticles are incorporated into evolving thrombi, suggesting their role in thrombus development and propagation.<sup>572</sup> Moreover, granulocytes and monocytes transfer TF-positive microparticles to platelets, thus enhancing their procoagulant activity and initiating clot formation.<sup>572</sup> Leukocyte microparticles can also activate platelets by Akt phosphorylation and P-selectin interaction.<sup>596</sup>

Non-thrombotic regulatory functions of leukocyte microparticles are also recognised. Gasser et al. have reported that leukocyte microparticles may specifically adhere to monocytes and that endothelial cells are involved in cell signalling and inflammatory responses.<sup>597</sup> Polymorphonuclear cell microparticles can induce IL-6 release from endothelial cells suggesting their involvement in the pro-inflammatory transformation of the endothelium.<sup>598</sup> Stimulated T cells generate microparticles that induce the production of TNF- $\alpha$  and IL-1 $\beta$  by monocytes, in keeping with a role in the inflammatory

processes.<sup>599</sup> The mutual regulatory effects of microparticle-mediated signals are reflected by correlation between levels of microparticles of different origin. For example, monocyte microparticles are increased in diabetes and correlate with PMPs, as well as with markers of platelet activation (CD62P, CD63, P-selectin).<sup>535</sup> Elevated microparticles of different origin including PMPs, EMPs, and monocyte microparticles, are increased in patients with spontaneous deep vein thrombosis and correlate with D-dimer levels, a marker of fibrin turnover.<sup>600</sup>

Microparticles released from neutrophils carry selectins, integrins, complement regulators on their surface, Fc-receptors typical of parental cells, and contain enzymes able to degrade the extracellular matrix, such as matrix metalloproteinase 9 and elastase.<sup>597</sup> The pattern of microparticles generated by monocytes (and probably by other cells) depends on the stimuli that produced them.<sup>601</sup> However, not only blood- or endothelial cell-derived microparticles may be found in the circulation. For example, tumour-derived TF-bearing microparticles were also associated with venous thromboembolism in cancer patients and may be essential in the pathogenesis of cancer-associated thrombosis.<sup>602-606</sup>

Taken together, the currently available data indicate the presence of various regulatory roles of the different types of circulating microparticles that may be involved in thrombosis, endothelial perturbations and inflammation, processes related to the development and progression of cardiovascular disorders.

#### 2.4.5. Microparticles and atherogenesis

EMPs are significantly increased in patients with CAD.<sup>521, 539</sup> Although formation of microparticles might be a passive shedding of membrane microvesicles from dysfunctional endothelial cells, they may represent a potentially clinically relevant pathway involved in activation of various physiological reparative processes. For example, experimental muscular ischaemia triggers an intensive generation of apoptotic annexin V+ microparticles by endothelial cells.<sup>607</sup> Microparticles isolated from ischaemic muscles induce a more potent differentiation of bone marrow mononuclear cells into endothelial-like cells compared to microparticles from non-ischaemic limbs.<sup>607</sup> Microparticles from apoptotic endothelial cells and endothelial cells activated by inflammatory factors accelerate differentiation of bone marrow mononuclear cells and enhance their *in vivo* angiogenic capacity.<sup>607</sup> However, Koga et al. have shown that high levels of EMPs were good predictors for CAD, even stronger compared to traditional risk factors.<sup>519</sup> This discrepancy may either stem from inability of microparticle-mediated angiogenesis to adequately balance progressive ischaemia or the presence of other not currently known mechanisms than link excessive budding of endothelial cells and atherogenesis.

However some available data show that EMPs may at least partly be responsible for prothrombotic state typical of CAD. Indeed, microparticles released by dysfunctional endothelium bind platelets and promote platelet activation in patients with coronary atherosclerosis.<sup>608</sup> Additionally, local endothelial damage during percutaneous coronary interventions is associated with temporary platelet activation and accompanied by prompt (within about 15 minutes) shedding of PMPs, especially in those undergoing coronary

stenting.<sup>609</sup> Importantly, this process occurs even despite optimal antiplatelet therapy and the lack of classical signs of platelets activation (e.g., normal soluble P-selectin levels).<sup>609</sup>

The systemic nature of atherosclerosis is reflected by microparticle perturbations observed in patients with different plaque location. Patients with carotid plaques have higher levels of large PMPs than subjects without carotid atherosclerosis, even after adjustments for cardiovascular risk factors.<sup>610</sup> High levels of CD11a+ leukocyte microparticles and CD105+ EMPs were associated with inward carotid remodelling in individuals with the greatest intima-media thickness even before atherosclerosis was detectable.<sup>543</sup> PMP levels significantly correlate with both intima-media thickness and the presence of intracranial stenosis, but cannot predict subclinical atherosclerosis burden in asymptomatic subjects.<sup>552,545</sup> PMP shedding is also amplified in patients with peripheral artery disease closely following a degree of platelet hyperreactivity.<sup>539, 541</sup> In patients with an arteriosclerotic arterial obliterans PMPs are further increased by vascular surgery in parallel with levels of proinflammatory cytokines (e.g., IL-6).<sup>542</sup>

Microparticles are also produced within atherosclerotic plaques themselves, predominantly by cells of leukocytic origin.<sup>611</sup> Demonstration of the presence of immunoglobulins encapsulated inside plaque-derived microparticles suggests their potential immunomodulating role. The vast majority (more than 90%) of the immunoglobulin G-containing microparticles were CD14+ indicating their monocyte/macrophage origin.<sup>611</sup> Although detailed biological roles of plaque-derived microparticles are poorly understood they may probably serve as regulatory messengers involved in orchestration of immune/inflammatory responses in an atherosclerotic plaque. Additionally, microparticles

isolated from human atherosclerotic lesions express the CD40 ligand, activate endothelial cells and appear to stimulate intra-plaque neovascularization, plaque progression and destabilization.<sup>612</sup> Furthermore, certain types of microparticles may promote vascular inflammation and expression of inflammatory cytokines (e.g., TNF- $\alpha$ ) by endothelial cells.<sup>613</sup> Importantly, even stable human atherosclerotic plaques shed membrane microparticles with procoagulant potential (mainly of monocytic and lymphocytic origin) in the process of apoptosis.<sup>614</sup> However, although these microparticles are likely to facilitate atherogenesis and are associated with a prothrombotic state much more data are needed to establish their pathophysiological and clinical significance. Important issues are to establish links between different types of microparticles, biological mechanisms of their release and clinically relevant consequences directly attributed to their activity.

#### **2.4.6. Microparticles and (athero)thrombosis**

PMPs are associated with ACS and their levels are substantially increased in ACS patients with or without of myocardial necrosis. PMPs have also been reported to be independent predictors of thrombotic events.<sup>547-548, 615</sup> However, being involved in the initiation of the atherothrombotic cascade their levels do not reflect the severity of myocardial damage and similar PMP levels were seen in patients with unstable angina and MI.<sup>546</sup> Indeed, PMP elevation in acute atherothrombosis parallels with co-existing activation of platelets which peaks during the first 1-2 days after the event.<sup>544, 616</sup>

In contrast to PMPs, the number of EMPs progressively increases from patients with unstable angina to those with acute MI and further from patients with uncomplicated MI



course to those with recurring MI.<sup>538</sup> Considering the relation between EMP formation, endothelial dysfunction and atherogenesis, it is interesting to see that high EMP levels in ACS are associated with the presence of ‘high-risk’ angiographic lesions including eccentric plaque location, multiple irregular plaques as well as lesions with thrombi.<sup>617</sup> Of note, being linked to the different parameters marking ‘high-risk’ lesions EMP levels do not directly correspond the severity of coronary stenosis and, in fact, higher in patients with moderate stenosis than in those with severe lesions. One may speculate that maximal release of microparticles by the endothelium may occur at the stage of active plaque formation and remodelling and remit following the stabilisation of mature plaques. In fact this explanation would correspond to the known higher vulnerability of relatively small atherosclerotic plaques.<sup>618</sup> Active intraplaque generation of CD105+ EMPs, TF+ microparticles and CD11a+ microparticles and their relevance to the plaque destabilisation is supported by the observation that their concentrations are significantly higher within the occluded coronary artery than in the peripheral circulation.<sup>619</sup> Furthermore, in a study of 123 patients with ST-elevation MI, coronary occlusion prior to angiography was associated with the highest EMP numbers.<sup>546</sup> Although the details of the role of EMPs in the development of atherothrombotic events are still poorly understood the interaction between EMPs is probably involved. Indeed, the levels of EMP aggregates with platelets are diminished during the first hours of MI and return to values close to those observed in stable CAD at approximately 48 h after the onset of MI.<sup>608</sup> The coincidence of the temporal dynamics of EMP-platelet aggregates with the time-frame of excessive platelet activation on the background of increased numbers of free EMPs indirectly indicates their preferable participation in platelet clot formation. Increased EMPs were also predictive of recurrent MI compared to those with an uncomplicated MI course.<sup>538</sup>

Some controversy exists about dynamics of TF+ microparticles (mainly of monocytic origin) in ACS. One study showed no apparent increase in TF+ microparticles.<sup>620-621</sup> However, Huisse et al. reported that failure of thrombolysis with tenecteplase to restore coronary circulation in patients with ST-elevation MI was associated with high levels of TF+ microparticles, which correlated with the levels of thrombin-antithrombin complexes.<sup>622</sup> This controversy is likely to reflect the background antithrombotic therapy and the clinical status of patients included in the studies. Of note, a prospective study of 286 patients with ACS (a mean follow up of 3.26 years) revealed that plasma TF activity (mainly attributed to circulating TF+ microparticles) was an independent predictor of survival, with hazard ratio of 9.27 (95% confidence interval 1.24-69.12, p = 0.03).<sup>623</sup>

Accumulating data also indicate that microparticles are involved in the development of cerebrovascular events. Microparticle numbers correspond to the severity, lesion volume and outcome in patients with stroke.<sup>624</sup> PMPs are prominently increased in patients with cerebral vaso-occlusive conditions, especially during transient ischaemic attacks, in those with lacunar infarcts, small vessel disease, and multi-infarct dementia.<sup>552</sup> The degree of PMP up-regulation in acute-phase cerebral infarction may vary substantially, depending on its aetiology and it is more prominent in small-vessel occlusions and large-artery atherosclerosis when compared to patients with cardioembolic strokes.<sup>552</sup> However, even among the last category of patients (e.g., among patients with prosthetic heart valves) PMP elevation is significantly associated with the development of adverse events.<sup>625</sup> Although only limited data are currently available on the role of microparticles in stroke, increased levels of PMP in acute stroke seem to persist in the convalescent phase of the disease.<sup>626</sup>

Cerebral ischaemic episodes and infarct volumes in acute stroke are also significantly associated with high levels of EMPs.<sup>549</sup> Various types of EMPs appear to play differential role in the pathogenesis of different forms of cerebrovascular atherosclerosis. CD62E+ EMPs dominate in patients with extracranial stenosis whilst CD31+ and CD31+anexin V+ EMPs represent a predominant type of EMPs in those with intracranial stenosis.<sup>550</sup> High levels of CD105+ and CD144+ EMPs on admission with stroke are significantly associated with negative clinical outcomes.<sup>549</sup> However, not all types of EMPs allowed discriminating acute ischaemic stroke from conditions mimicking stroke and details of the specific roles of different subsets of EMPs in the development of stroke is a subject of future research.<sup>627</sup>

#### **2.4.7. Microparticles in heart failure**

Only scarce information is currently available on the role of microparticles in HF. CD144+ EMP are significantly up-regulated in Japanese subjects with systolic HF being progressively higher in subjects with more severe forms of the disease.<sup>628</sup> A 30-month follow-up of these patients revealed that high EMP count was significantly and independently associated with higher incidence of a composite endpoint of cardiovascular events (MI, stroke, re-hospitalization for HF, and cardiovascular death).

Bulut et al. reported that reduction in total and endothelial microparticles after immunoabsorption in 13 patients with severe HF paralleled improvement in endothelial function and LVEF.<sup>629</sup> Also cardiac transplantation in subjects with terminal HF was followed by significant reduction in CD62+ EMP.<sup>630</sup> However the causal relationship

between the decrease in circulating microparticles and other favourable cardiovascular changes is still to be established.

Data on the implications of other microparticle types (e.g., PMP or MMP) in HF are scanty but may be of potential clinical interest.

#### **2.4.8. Conclusions**

Most of the currently available clinical studies on human microparticles are observational in their nature with only few studies assessing prognostic role of different microparticle types and effects of interventions. These data prove associations of microparticle count with different cardiovascular disorders but do not necessarily imply direct pathophysiological links. Robust experimental studies based on the evaluation of the delivery of different microparticles types in various models of cardiovascular disorders are often missing and are highly desirable. Nevertheless microparticles are undoubtedly involved in pathogenesis of cardiovascular disorders and highlight potential areas of investigation rather than provide definitive answers to numerous questions. More research is clearly needed to shed further light on this intriguing area.

## **CHAPTER III. ETHNIC DIFFERENCES IN ENDOTHELIAL AND VASCULAR FUNCTION IN HEART FAILURE**

### **3.1. Introduction to the chapter**

As it was discussed in the Literature review section, HF remains a common pathological condition associated with significant ethnic differences in clinical presentation, course and prognosis.<sup>631-632</sup> Subjects of SA origin represent a large ethnic group featured by a unique and unfavourable pattern of high prevalence of CAD, diabetes, associated with development of HF at younger age and an increased risk of HF-related hospital admissions.<sup>8, 301</sup> Indeed, HF in SAs develops at a younger age and results in 60% more hospitalizations compared to subjects of white origin.<sup>12</sup> In addition, there are pathological differences between subjects with HF from different ethnic groups although the pathophysiological mechanisms of such differences are still poorly understood.<sup>7,9</sup>

Given that prevalence of systolic LV impairment does not seem to vary substantially among different ethnic groups it is reasonable to speculate that the known clinical and prognostic ethnicity-related differences in HF may be partly attributable to extracardiac factors, such as endothelial (dys)function, systemic inflammation, and capacity of the reparative mechanisms.<sup>633</sup> Indeed, the endothelium plays a critical role in the regulation of vascular tone, recruitment of leukocytes (and other inflammatory processes), maintenance of the balance between the pro- and anticoagulant states, and many other pathophysiological processes.<sup>191</sup> Impairment of endothelial function is associated with negative outcome in various cardiovascular disorders but it seems particularly important in

subjects with HF. Indeed, impairment of endothelial response to hyperaemia or acetylcholine is a significant independent predictor of negative outcomes (including death, and re-hospitalization) in HF, irrespectively of its aetiology.<sup>172-176, 178</sup> Additionally EMPs have recently emerged as a novel marker of the endothelial (dys)function, which strongly reflects the degree of endothelial activation and damage and EMP levels have been shown to be up-regulated in subjects with HF.<sup>49, 634</sup> Although previous research has mostly focused on subjects with clinically apparent cardiovascular morbidity, it was also shown that even healthy SAs had worse parameters of endothelial function compared to healthy white subjects.<sup>22</sup> However, the state of endothelial function in SAs with HF has not been specifically studied.

The progressive nature of HF reflects a complex pathophysiology of the disorder, with features of an imbalance between inflammatory and reparative processes.<sup>305</sup> Monocytes play important roles in both.<sup>25</sup> Monocytes are not uniform and include subsets with profoundly different functional properties which might be implicated in both HF progression and repair (discussed in more details in the Chapter II).<sup>635</sup>

There are scarce data on the specific monocyte subsets in HF. In a small study, Mon2 subset was increased in European patients with chronic stable HF compared with healthy controls and their counts correlated with worsening NYHA class and reduced LV contractility.<sup>425</sup> However in addition to the presence of HF per se, the influence of co-morbidities and cardiovascular risk factors needs to be considered and dictates the need for an appropriate ‘disease controls’ group which was missing the that study.

The reparative properties of monocytes may also be relevant in HF. Scavenger receptor class A type I (SR-AI, also CD204) is involved in lipid accumulation (by macrophages) and atherogenesis, but there are emerging data that implicate this receptor in a number of reparative processes, such as clearance of apoptotic cells, attenuation of oxidative stress and inflammation.<sup>29-33</sup> The number of monocytes expressing SR-AI is increased in patients with ACS, but data on monocyte SR-AI expression in HF are not available at present.<sup>34-35</sup> Circulating levels of MPAs have been shown to be a good marker of monocyte activation, and they are up-regulated in various cardiovascular and thrombotic disorders.<sup>636</sup> Also, circulating cellular MMP and PMP are increasingly recognised as important messengers of cellular signals in cardiovascular disorders.<sup>637</sup> However, scarce data are currently available on the levels of MPAs, MMPs and PMPs in HF.

Continuous cardiac and vascular remodelling in HF reflects the need for adaptation to haemodynamic changes, as well as to the ischaemic background common in HF subjects. The role of EPCs in angiogenesis and vascular remodelling is progressively acknowledged and accumulating data indicate their relevance in the context of HF.<sup>38</sup>

EPC counts have been shown to be abnormal in HF subjects and depend upon HF severity.<sup>447, 449</sup> A large proportion of EPCs has been found to be of monocytic origin.<sup>38</sup> Monocytes include cells able to acquire an endothelial phenotype in culture.<sup>462</sup> Indeed, cultures of 'early' EPCs are largely comprised of monocytes (e.g., over 97% them are CD14+ cells) and the colony formation is strictly dependent upon monocytes presence.<sup>638-</sup>  
<sup>641</sup> A proportion of circulating CD14+ monocytes express low levels of CD34 and these CD14+CD34+ cells were able to improve carotid re-endothelialization after balloon injury

in animals.<sup>642</sup> Also, potent angiogenic properties were demonstrated for monocytes expressing KDR (VEGF receptor type 2).<sup>465</sup> However, the numbers of CD34+ and KDR+ angiogenic monocytes, total or attributable to individual monocyte subsets have not been studied in HF.



### **3.2. Aims and hypotheses**

The aims of this thesis were to assess possible ethnic differences in endothelial function, monocyte subsets, EPCs and plasma microparticles in systolic HF and to investigate the status of these parameters in SA patients with HF vs. control subjects with normal LV contractility.

In this thesis, I hypothesised the presence of ethnic differences in endothelial macro- and micro-vascular function between HF subjects of SA, white and African-Caribbean (AC) origin. Macrovascular function was quantified by FMD of the brachial artery in response to reactive hyperaemia, whilst arterial stiffness was assessed by quantification of the pulse wave velocity (PWV). Microvascular function was quantified by forearm laser Doppler flowmetry using skin iontophoresis with acetylcholine and sodium nitroprusside. I also contrasted these measures of localised endothelial function by measuring the numbers of EMPs, which may arise from any vascular bed.<sup>637</sup>

I also hypothesised that there would be significant HF-associated changes and ethnic differences in monocyte subsets (and their expression of SR-AI), MMPs and PMPs, which may account for some of the observed clinical ethnicity-related differences in HF.<sup>12, 54</sup> To test this hypotheses I studied ethnic differences in monocytes subsets, MMPs, PMPs in HF subjects of SA, AC and white origin. The SA patients with HF were also compared against matched 'disease controls' and 'healthy controls' of SA origin.

The next hypothesis of this thesis was that SA subjects with HF would have abnormal parameters of endothelial progenitors compared with matched patients with preserved LV contractility and secondly, there would be significant ethnic differences in these parameters between HF subjects of SA, AC and white ethnic origin.

### **3.3. Study population**

#### **3.3.1. Interethnic comparisons in heart failure**

Fifty subjects of SA origin were compared with 50 age- and sex-matched Whites, and 28 subjects of AC ethnic origin with HF. All HF subjects had LVEF <40% confirmed by echocardiography (described below) and were clinically stable at the time of assessment with no hospitalisation or therapy changes in the preceding 3 months. Subjects with atrial fibrillation, cancer, rheumatoid arthritis, or haemodynamically significant valvular heart disease, or taking hormone replacement therapy, were excluded.

Subjects with HF from different ethnic groups were generally well matched for demographic features (Table 15). The majority of the patients had an ischaemic aetiology of HF (82% of SAs, 80% of Whites, 79% of ACs,  $p=0.71$ ). A higher proportion of subjects of AC origin had a history of hypertension compared to Whites ( $p=0.012$ ) and mean diastolic blood pressure was higher in ACs ( $p=0.04$ ). Whites had less diabetes than both SAs and ACs ( $p<0.001$ ). Additionally ACs had higher body mass index ( $p=0.01$ ) and lower leucocyte counts ( $p=0.02$ ). The higher prevalence of diabetes associated with increased body mass index in ACs could reflect more features of metabolic syndrome, compared to other ethnic groups. The three ethnic study groups were matched for most medications (Table 15), with the exception of statins which were more commonly taken by SAs.

**Table 15. Demographic, clinical and echocardiographic characteristics of subjects with heart failure**

	South Asian	White	African-Caribbean	p
Age, years	66.5±11.1	68.8±8.91	69.3±10.3	0.42
Male, %	88	84	86	0.85
NYHA class: I/II/III, %	22/38/40	24/44/32	8/46/46	0.20
Past Medical History				
Ischaemic aetiology, %	82	80	79	0.71
Previous myocardial infarction, %	66	70	46	0.08
Previous stroke, %	8	6	7	0.90
Hypertension, %	72	58‡	86	0.03
Diabetes, %	62*	30‡	64	0.001
Smoking, %	14	16	10	0.93
Chronic obstructive pulmonary disease, %	26	20	18	0.47
ICD/CRTP/CRTD	6	7	4	0.93
Systolic BP, mm Hg	130±19.4	130±18.1	136±22.8	0.34
Diastolic BP, mm Hg	74.7±11.1†	74.7±9.67‡	80.5±10.6	0.04
Body mass index, kg/m <sup>2</sup>	26.9±4.57†	27.7±4.03	30.4±6.80	0.01
Haemoglobin, g/dl	13.2±1.62	13.9±2.13	12.9±1.72	0.08
Leukocytes, 10 <sup>6</sup> /ml	7.20±1.89†	7.32±2.07‡	6.03±2.29	0.02
Creatinine, µmol/l	102±29.7†	99±29.8‡	125±39.3	0.003
Glomerular filtration rate, ml/min/1.73m <sup>2</sup>	67.7±20.8	64.7±18.5	64.4±22.2	0.74
Glucose, mmol/l	6.84±2.27	5.46±0.99	6.91±3.50	0.16
Total cholesterol, mmol/l	4.00±1.03	4.16±1.05	4.04±1.01	0.82
HDL cholesterol, mmol/l	1.22±0.42	1.27±0.46	1.39±0.41	0.52
Echocardiographic parameters				
LV end diastolic volume, ml	159±52.9	162±59.0	201±99.9	0.11
LV end systolic volume, ml	111±48.4	115±48.6	150±75.4	0.07
LV ejection fraction, %	30.5±11.3	29.7±7.68	30.3±7.26	0.94
E/E' ratio	11.7±8.00	12.4±4.86	13.9±6.12	0.60
Left atrium diameter, mm	4.06±0.83	4.40±0.72	4.46±1.20	0.28
Interventricular septum, mm	11.8±2.4	11.5±3.2	13.5±4.7	0.16
Posterior wall, mm	9.4±2.3*	9.3±2.3‡	12.6±5.9	0.004

	South Asian	White	African-Caribbean	p
Medications				
Aspirin, %	86	92	96	0.55
Clopidogrel, %	14	14	17	0.99
Warfarin, %	10	4	4	0.36
ACE inhibitors, %	84	80	89	0.45
Loop diuretics, %	62	72	93	0.06
Thiazide diuretics, %	4	16	12	0.16
Spironolactone, %	26	26	43	0.25
Digoxin, %	12	16	7	0.53
Nitrates, %	26	26	25	0.96
Statins, %	84*	64	79	0.02
Calcium channel blockers, %	18	18	18	0.99
Beta-blockers, %	56	68	57	0.52

BP - blood pressure, E/E' ratio - early filling (E) to early diastolic mitral annular velocity (E') ratio, LV – left ventricular. Values expressed as Mean±Standard Deviation. \*p<0.05 between SAs and Whites, †p <0.05 between SAs and ACs, ‡ p <0.05 between Whites and ACs, \*p<0.05 between SAs and Whites. ICD/C RTP/CRTD – Implantable cardioverter-defibrillator/cardiac resynchronisation therapy-pacemaker/cardiac resynchronisation therapy-defibrillator.

### **3.3.2. Comparison of heart failure subjects to controls without heart failure**

Fifty SA subjects with HF were compared with two age- and sex- matched control groups of 40 SA ‘disease controls’ (subjects with CAD diagnosed during elective coronary angiography but with normal LV systolic function [LVEF >50%]) and 40 ‘healthy controls’ with no vascular, metabolic, neoplastic or inflammatory disease revealed by careful history, examination and routine laboratory tests.

The groups were well-matched for age, sex, body mass index and smoking (Table 16). ‘Disease controls’ and HF subjects had lower haemoglobin and HDL-cholesterol, a higher leukocyte count and creatinine than the ‘healthy controls’.

All study subjects fasted for 12 hours and abstained from smoking, alcohol, tea and coffee for 24 hours prior to the study. The subjects were advised to omit their medications on the study day and the evening before, as a more prolonged treatment omission was deemed unethical. All scans were performed in a quiet, darkened, temperature controlled room after resting for 15-20 minutes. Ethical approval was granted by the local research ethics committee and written informed consent was obtained from all participants.

**Table 16. Demographic, clinical and echocardiographic characteristics of subjects with heart failure**

	Healthy controls	Disease controls	Heart failure	p value
Age, years	61.3±13.7	64.2±9.52	66.5±11.1	0.11
Male, %	80%	90%	88	0.76
Clinical parameters				
Systolic BP, mm Hg	134±14.5	134±19.5	130±19.4	0.54
Diastolic BP, mm Hg	80.0±8.88†	75.0±13.7	74.7±11.1	0.03
Body mass index, kg/m <sup>2</sup>	25.9±4.08	26.2±3.66	26.9±4.57	0.71
Smoking, %	12	10	14	0.71
Haemoglobin, g/dl	14.3±1.49*†	13.3±2.02	13.2±1.62	0.006
Leucocytes, 10 <sup>6</sup> /ml	6.25±1.17*†	7.58±1.52	7.20±1.89	0.001
Creatinine, µmol/l	83.9±22.4†	91.2±28.3	102±29.7	0.02
Glomerular filtration rate, ml/min/1.73m <sup>2</sup>	75.5±15.0	75.8±17.6	67.7±20.8	0.11
Glucose, mmol/l	5.56±1.30	6.86±2.59	6.84±2.27	0.22
Total cholesterol, mmol/l	4.63±1.09	4.06±1.00	4.00±1.03	0.06
HDL cholesterol, mmol/l	1.52±0.37*†	1.18±0.31	1.22±0.42	0.016
Echocardiographic parameters				
LV end diastolic volume, ml	89.1±32.4†	109±21.6‡	159±52.9	0.00
LV end systolic volume, ml	34.5±17.2†	44.9±11.7‡	111±48.4	0.00
LV ejection fraction, %	62.2±10.9†	58.6±7.00‡	30.5±11.3	0.00
E/E' ratio	8.50±3.50	10.2±3.97	11.7±8.00	0.16
Left atrium diameter, mm	3.64±0.42	3.88±0.62	4.06±0.83	0.12

BP - blood pressure, E/E' ratio - early filling (E) to early diastolic mitral annular velocity (E') ratio, LV – left ventricular. Values expressed as Mean±Standard Deviation. \*p<0.05 between healthy controls and disease controls, †p <0.05 between healthy controls and heart failure group, ‡ p <0.05 between disease controls and heart failure group.

### **3.4. Methods**

#### **3.4.1. Assessment of macrovascular endothelial function: flow-mediated dilation of brachial artery**

FMD was assessed according to the published guidelines.<sup>643</sup> High resolution ultrasound scanning of the right brachial artery was performed with a 10 MHz probe 3-5cm above the antecubital fossa, with subjects lying flat and the probe kept in the same position throughout the study. Anterior to posterior wall diameters (leading edge to leading edge) were recorded simultaneously with synchronization by the R-wave on electrocardiogram. For every stage the arterial diameter was calculated as an average of 5 measurements of 3 consecutive cardiac cycles. Endothelium-dependent dilation was assessed by response to reactive hyperaemia (i.e., FMD). The sphygmomanometer cuff placed around the right upper arm was inflated to 30-40mmHg above the systolic blood pressure for 5 minutes following by prompt deflation and recording of brachial artery images for 5 minutes with the maximal response used for the analysis. Once the baseline brachial artery diameter and flow was restored, the endothelium-independent dilation was assessed 3 minutes after sublingual administration of 0.4mg glycerol trinitrate (GTN, Nitrolingual®Hohenlockstedt, Germany). The endothelium-dependent and endothelium-independent responses were estimated as a percentage of the brachial diameter changes compared to baseline levels (Standard Operating Procedure 103 'Flow-mediated dilation').

For the purpose of the technique validation and assessment of intra-observer variability I repeated measurements of flow-mediated- and GTN-mediated dilation on 5 different days



(in addition to the original measurement made on the day of the study, i.e., 6 measurements in total). This was done on two study subjects. Intra-observer coefficients of variability (CV) for FMD were 2.30%, and 2.28% for the two subjects with average value 2.29%. Intra-observed CV for GTN-mediated dilation was 2.19%, and 2.96% for the two subjects with average value 2.58% (Table 17).

For the assessment of inter-observer CV the measurements of the parameters were performed by another member of the department experienced with the technique who was unaware of the subjects' details. Measurements done on 10 subjects showed inter-observer CV 2.36% for FMD and 3.48% for GTN-mediated dilation (Table 18).

**Table 17. Intra-observer variability of measurements of macrovascular endothelial function (brachial artery) with ultrasound**

Subject	Day	Baseline diameter, mm	Diameter after reactive hyperaemia, mm	FMD, %	Diameter after GTN, mm	GTN-mediated dilation, %
Subject 1	Day 1	3.56	4.31	21.01	3.90	9.55
	Day 2	3.57	4.33	21.3	3.91	9.52
	Day 3	3.54	4.31	21.8	3.89	9.89
	Day 4	3.56	4.32	21.3	3.91	9.83
	Day 5	3.57	4.3	20.4	3.91	9.52
	Day 6	3.54	4.31	21.8	3.87	9.32
	Mean±SD	3.56±0.01	4.31±0.01	21.3±0.49	3.90±0.02	9.61±0.21
	CV, %	0.38	0.24	2.30	0.41	2.19
Subject 2	Day 1	5.42	6.05	11.6	6.05	11.6
	Day 2	5.43	6.04	11.2	6.04	11.2
	Day 3	5.43	6.06	11.6	6.06	11.6
	Day 4	5.44	6.05	11.2	6.06	11.4
	Day 5	5.43	6.03	11.0	6.03	11.0
	Day 6	5.41	6.04	11.6	6.06	12.0
	Mean±SD	5.43±0.01	6.05±0.01	11.4±0.26	6.05±0.01	11.5±0.34
	CV, %	0.19	0.17	2.28	0.21	2.96
<i>Average CV, %</i>		<i>0.29</i>	<i>0.21</i>	<i>2.29</i>	<i>0.31</i>	<i>2.58</i>

CV, coefficient of variability; FMD, flow-mediated dilation; GTN, glycerol trinitrate.

**Table 18. Inter-observer variability of measurements of macrovascular endothelial function (brachial artery) with ultrasound**

Subject	Baseline diameter, mm	Diameter after reactive hyperaemia, mm	FMD, %	Diameter after GTN, mm	GTN-mediated dilation, %
Operator 1					
1	4.8	5.25	9.37	5.27	9.8
2	4.36	4.78	9.63	4.85	11.24
3	4.43	4.78	7.9	4.81	8.58
4	3.56	4.31	21.01	3.9	9.55
5	5.42	6.05	11.62	6.05	11.62
6	4.66	4.97	6.65	4.94	6.04
7	4.13	4.47	8.23	4.9	18.64
8	4.55	4.87	7.03	4.92	7.03
9	4.4	4.82	9.55	4.86	10.45
10	4.38	4.56	4.11	4.63	5.71
Operator 2					
1	4.73	5.18	9.51	5.19	9.73
2	4.34	4.77	9.91	4.86	11.98
3	4.5	4.88	8.44	4.84	7.56
4	3.59	4.4	22.56	3.96	10.31
5	5.1	5.72	12.16	5.74	12.55
6	4.68	4.99	6.62	4.96	5.98
7	4.1	4.43	8.05	4.83	17.80
8	4.58	4.9	6.99	4.92	7.42
9	4.4	4.83	9.77	4.84	10.00
10	4.4	4.57	3.86	4.64	5.45
Inter-observer CV, %	0.90	0.99	2.36	0.84	3.48

CV, coefficient of variability; FMD, flow-mediated dilation; GTN, glycerol trinitrate.

### **3.4.2. Assessment of microvascular endothelial function: laser Doppler flowmetry**

Assessment of microvascular endothelial function has been performed using Laser Doppler perfusion monitor (DRT system with MIC1-e iontophoresis controller, Moor Instruments, Axminster, UK) with upper forearm iontophoresis (0.1mA for 60 sec.) of 1% acetylcholine (Sigma-Aldrich, Dorset, UK) to evaluate endothelial-dependent response, and 1% sodium nitroprusside (Sigma-Aldrich, Dorset, UK) to evaluate endothelium-independent response.<sup>644</sup> Maximum percentage changes in perfusion were calculated using mean baseline perfusion (during 1 minute of baseline scanning) and maximum perfusion during the tests, and the analysis was performed off-line using software provided by the equipment manufacturer (Standard Operating Procedure 107 'Measurement of microvascular endothelial function').

For the purpose of the method validation, the study has been repeated on different days in two healthy subjects (Subject 1 and Subject 2) (Table 19). Inter-observer CV of the response to acetylcholine was 9.79% in Subject 1 and 12.5%, with average 11.2%; CV of the response to sodium nitroprusside was 12.4% in Subject 1 and 22.6% in Subject 2, with average 17.5%. Intra-assay variability (blinded analysis of stored data) gave the same results due to automatic nature of analysis of the stored data. A relatively high inter-observer variability in response to acetylcholine and sodium nitroprusside is apparently due to substantial physiological variability of the skin blood flow, also reported by other authors.<sup>645</sup> In contrast, the baseline values of the skin perfusion showed high variability (which is commonly acknowledged in literature) due to its high sensitivity to numerous factors (e.g., some variations in temperature, blood pressure, etc).

**Table 19. Inter-assay variability of parameters of microvascular endothelial function assessed by laser Doppler flowmetry**

		Perfusion before Ach, PU	Perfusion after Ach, PU	Response to Ach, %	Perfusion before SNP, PU	Perfusion after SNP, PU	Response to SNP, %
Subject 1	Day 1	12.9	116.6	804	12.4	121.1	877
	Day 2	24.2	211.7	775	8	70.7	784
	Day 3	30.5	276.8	808	31.5	230.8	633
	Day 4	26.3	256.9	877	11	100.3	812
	Day 5	15.9	172	982	26.2	253.8	869
	Mean± SD	22.0± 7.34	207± 64.8	849.2± 83.1	17.8± 10.4	155± 81.8	795± 98.6
	CV, %	33.4	31.3	9.79	58.4	52.8	12.40
Subject 2	Day 1	21.6	321.1	1387	18.2	139.7	668
	Day 2	15.2	201.7	1227	12.2	71.6	487
	Day 3	16.4	227.4	1287	16.5	122.1	640
	Day 4	17.1	217.8	1174	33	213.2	546
	Day 5	15.9	172	982	26.3	253.8	865
	Mean± SD	17.2± 2.53	228± 56.1	1211± 151	21.2± 8.33	160± 73.0	641± 145
	CV, %	14.7	24.6	12.50	39.3	45.6	22.60
<i>Average CV, %</i>		<i>24.1</i>	<i>28.0</i>	<i>11.2</i>	<i>48.9</i>	<i>49.2</i>	<i>17.5</i>

Ach, acetylcholine; CV, coefficient of variability; MPAs, monocyte-platelet aggregates; PU, perfusion units; SD, standard deviation; SNP, sodium nitroprusside.

### **3.4.3. Assessment of arterial stiffness**

Carotid-femoral PWV, carotid-radial PWV, and aortic augmentation index were measured using Sphygmocor device (Sphygmocor, Atcor medical, Sydney, Australia). Radial artery waveforms were recorded over 10 seconds using a high-fidelity hand-held applanation tonometer to perform pulse wave analysis and calculate aortic augmentation index using an inbuilt modern Sphygmocor CVMS software system (Version 8). Carotid-femoral PWV (predominantly arteries of elastic type) and carotid-radial PWV (predominantly arteries of muscular type) were obtained by making sequential ECG-gated tonometer recordings at the carotid, femoral, and radial arteries. The straight-line distances between the sternal-notch and waveform measurement sites were determined, and path length taken as the difference between the two distances (according to the manufacture recommendations).

Intra-observer CV for PWV was calculated on the basis of four consecutive measurements done on the same day, which were performed on two different healthy subjects twice on each. My average intra-observer CV was 5.29% (Table 20). Inter-observer CV for PWV was calculated on the basis of five measurements performed on different days in two healthy subjects. My inter-observer CV was 6.58% and 2.73% for the two subjects, with average CV 4.66%. Intra-observer CV for the aortic augmentation index was 7.99%. Inter-observer CV for the aortic augmentation index was 5.29% and 11.12% for the two subjects, with average CV 8.21%.

**Table 20. Intra- and inter-assay variability of measurement of pulse wave velocity**

<b>Intra-assay variability assessment</b>				
	Subject 1		Subject 2	
	Day 1	Day 2	Day 1	Day 2
Measurement 1	10.5	9.8	8	7.8
Measurement 2	9.3	9.2	8.6	8.3
Measurement 3	9	9	7.8	7.7
Measurement 4	10.4	9.5	8.7	7.5
Mean±SD	9.80±0.76	9.38±0.35	8.28±0.44	7.83±0.34
Intra-assay CV, %	7.76	3.73	5.34	4.34*
<b>Inter-assay variability assessment</b>				
	Subject 1		Subject 2	
Day 1	10.5		8.4	
Day 2	9		8	
Day 3	9.6		8	
Day 4	9.8		7.8	
Day 5	9		8.1	
Mean±SD	9.58±0.63		8.06±0.22	
<i>Inter-assay CV, %</i>	6.58		2.73*	

Pulse wave velocity units – m/sec; \*Average intra-assay CV, 5.29; Average inter-assay CV, % 4.66

### **3.4.4. Echocardiography**

Cardiac function was assessed by two-dimensional echocardiography (Phillips iE33 ultrasound machine, Bothel, WA, USA) according to the current guidelines.<sup>646-648</sup> Modern off-line QLAB software [Xcelera, Phillips Ultrasound Quantification Module, USA] was used for quantification of parameters of cardiac structure and function. LV volumes and LVEF were measured using the modified Simpson's biplane method.<sup>648</sup> For diastolic function assessment, transmitral flow was obtained in the apical 4-chamber position, where the pulsed Doppler sample volume cursor was placed in parallel with the blood flow direction. Mitral early inflow velocity (E and A) was measured by conventional Doppler measurement technique. Early diastolic lateral mitral annular velocity (E') was measured by Tissue Doppler imaging with E/E' ratio calculated as a parameter of diastolic function. Left atrial diameter, thickness of posterior wall and interventricular septum were measured in M-mode.

### **3.4.5. Flow cytometry**

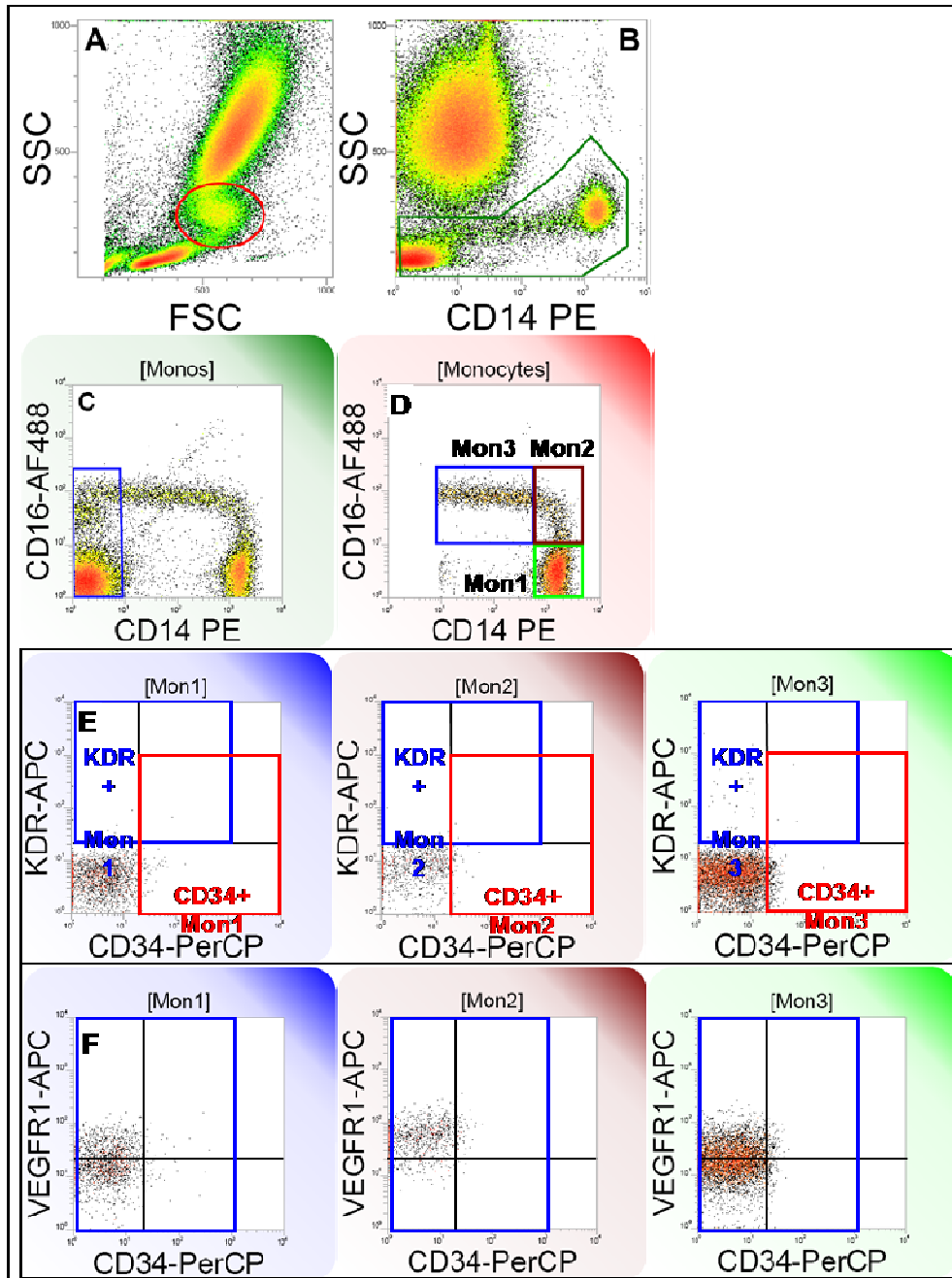
#### ***3.4.5.1. Quantification and characterisation of monocyte subsets, monocyte-platelet aggregates and endothelial progenitors***

Flow cytometric analysis was performed using the BD FACSCalibur flow cytometer (Becton Dickinson, Oxford, UK [BD]). Following 20 minutes of supine rest, blood samples were collected into ethylene-diamine tetra-acetic acid (EDTA)-containing tubes. Within 1 hour 100µl of blood was incubated with fluorochrome-conjugated antibodies for



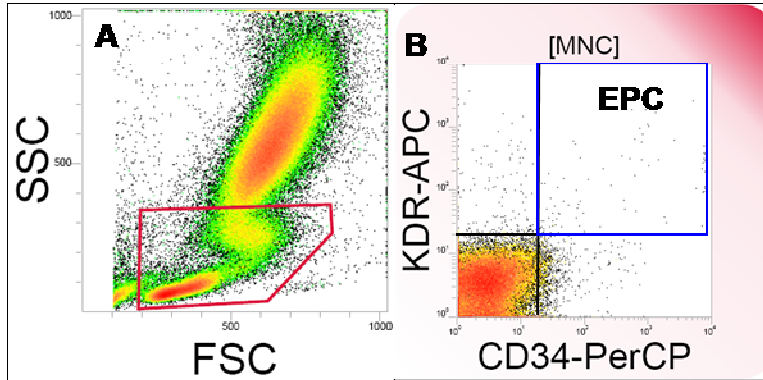
15 minutes in the dark. Red blood cells were lysed with 2ml of BD lysing solution® for 10 minutes, washed in PBS, followed by immediate flow cytometric analysis. Anti-CD16-Alexa Fluor 488 (clone DJ130c, AbDSerotec, Oxford, UK) and anti-CD14-PE (clone MφP9, BD) antibodies were used to define monocyte subsets. Anti-CD42a-PerCP (clone Beb1, BD) antibodies were used to characterize monocyte interaction with platelets and anti-CD204-APC (clone 351520, R&D Systems, Abingdon, UK [R&D]) antibodies were used to assess CD204 expression on monocyte subsets. Monoclonal antibodies for CD204 were conjugated to APC using LL-APC-XL conjugation kit (Innova Biosciences, UK). Anti-CD34-PerCP antibodies (clone 351520, BD), anti-KDR-APC antibodies clone ,R&D Systems, Abingdon, UK [R&D]) and anti-VEGF receptor 1 (VEGFR1)-APC antibodies (R&D) were used to enumerate CD34+KDR+ EPCs, CD34+ and KDR+ monocytes.

Monocyte subsets were defined as CD14<sup>++</sup>CD16<sup>-</sup> cells (Mon1), CD14<sup>++</sup>CD16<sup>+</sup> cells (Mon2) and CD14<sup>+</sup>CD16<sup>+</sup> cells (Mon3) (Figure 6). Absolute counts of individual monocyte subsets and EPCs (cells/ $\mu$ l) were calculated using total monocyte count obtained with haematoanalyser (Bayer Advia 120, Siemens, Newbury, Berks, UK) and proportions of individual monocyte subsets determined by flow cytometry. MPAs were defined as events positive to both monocyte markers (as above) and a platelet marker CD42a (glycoprotein IX). ‘Classical’ EPCs were defined as CD34<sup>+</sup>/KDR<sup>+</sup> mononuclear cells (i.e., monocytes plus lymphocytes) (Figure 7). Monocyte-derived EPCs were defined as CD34<sup>+</sup> and KDR<sup>+</sup> monocytes (total and attributable to the individual subsets).SR-AI and VEGFR1 expression was measured by its median fluorescent intensity (MFI) on individual monocyte subsets



**Figure 6. Analysis of monocyte subpopulations by flow cytometry**

Analysis of monocyte subpopulations by flow cytometry A. Selection of monocyte cluster on the basis of forward and side scatter optic properties (aiming to include all monocytes in a sample); B. Exclusion of granulocytes; C. Exclusion of lymphocytes; D. Selected monocytes and their subsets: Mon1 (CD14<sup>++</sup>CD16<sup>-</sup> monocytes), Mon2 (CD14<sup>++</sup>CD16<sup>+</sup> monocytes), and Mon3 (CD14<sup>+</sup>CD16<sup>++</sup> monocytes); E. Individual monocyte subsets are gated according to their CD34 and KDR expression for enumeration of KDR<sup>+</sup> and CD34<sup>+</sup> monocyte subsets; F. VEGF receptor 1 expression is assessed on individual monocyte subsets.



**Figure 7. Analysis of CD34+KDR+ endothelial progenitor cells**

A. Selection of mononuclear cells (i.e., monocytes plus lymphocytes) on the basis of forward and side scatter optic properties; B. Gating of the CD34+KDR+ endothelial progenitor cells (EPCs)

The analysis was performed according to the approved SOP (Standard Operating Procedure 197 ‘Enumeration of monocytes sub-populations by flow cytometry’). In order to obtain intra-assay CV for the method I was given 6 fresh blood samples taken from 2 volunteers whilst I was blinded to any information on the mixture of the 6 samples provided. The blind samples were given and CV calculations were done by Dr Andrew D Blann, Consultant Clinical Scientist and Consultant Statistician responsible for the laboratory quality control in the department. The analysis showed that intra-assay CV derived from the results on percentage of the different subpopulations of monocytes (i.e., Mon1, Mon2, Mon3) gave a mean CV of 4.75%. The CV of the monocyte count on the Advia was 6.65%. CV for the cumulative analysis of monocyte subset enumeration was 6.8%. CVs of sub-analyses of the Mon1, Mon2 and Mon3 populations according to their VEGFR1/CD34 expression were variable: 8.5% for Mon1, 9.0% for Mon2, and 17.9% for Mon3. Higher variability for the analysis of minor monocyte subsets is attributable to the

scarcity of these subsets, and partial overlap between the two subsets when their traditional (as above) definition was used (i.e., on the basis of CD14 and CD16 expression only). My further work aiming to develop an unequivocal and more robust approach of enumeration of the monocyte subsets and further validation of the protocol is provided in Chapter IV.

#### ***3.4.5.2. Quantification of plasma microparticles***

A venous blood sample was taken from the antecubital vein into citrated vacutainers (Becton Dickinson, Oxford, UK). Samples were centrifuged at 2880g for 15min and plasma was stored at -70°C. Quantification of microparticles was performed by flow cytometry (FACSCalibur, Becton Dickinson, Oxford, UK). For batched analysis 50 µl of defrosted citrated plasma were incubated with fluorochrome-labeled monoclonal anti-CD144-PE antibody (clone 123413, R&D Systems, Abingdon, UK), anti-CD14-PerCP antibody (clone 134620, R&D) and anti-CD42b-APC (clone HIP1, BD) for 20 minutes in the dark at room temperature. After that 25 µl of CytoCount beads (DakoCytomation, Glostrup, Denmark) and 200 µl of phosphate buffered saline were added. Acquired events were plotted according to their forward and side scatter characteristics in log-scales and gated to include events smaller than 1 µm (defined using latex microbeads, Sigma-Aldrich, Dorset, UK), but to exclude electronic noise. The size of the region was kept the same for all study samples.

The EMPs were defined as CD144+ events, MMPs were defined as CD14+ events and PMPs as CD42+ events in the microparticle gate. The degree of positivity to the individual markers was defined using species-, immunoglobulin-, protein mass-matched isotype

controls (clone 20116, R&D Systems, Abingdon, UK). A minimum of 100,000 microparticles were collected for analysis per sample. Absolute counts of the microparticles were obtained using their proportion to count beads and expressed as the numbers of events per  $\mu\text{l}$  of plasma. The analysis was performed according to the approved Standard Operating Procedure 197 'Enumeration of microparticles by flow cytometry'. Intra-observer and inter-observer variability of microparticle analysis was 3.5% and 11.7%, respectively.

#### **3.4.6. Statistical analysis and power calculation**

Data are expressed as mean  $\pm$  standard deviation (SD) for normally distributed data; or median with first and third quartiles (Q1-Q3) for non-normally distributed data. Study data were analysed by one way analysis of variance (ANOVA). A post-hoc Tukey test was performed to assess inter-group differences where appropriate. Log transformation of non-normally distributed variables was performed prior to ANOVA. A  $\chi^2$  test was used for the comparison of the categorical variables between three groups. When such comparisons showed significant differences pair-wise  $\chi^2$  tests were performed to establish any differences between individual groups. A p-value (two tailed) of  $<0.05$  was considered statistically significant. SPSS 17 (SPSS, Inc, Chicago, Illinois, USA) statistical software was used to perform the statistical analyses. Multivariable linear regression analysis has been performed in order to determine clinical and demographic factors associated with the study parameters in HF subjects. Regression analysis was started from univariate analysis in order to assess unadjusted predictive value of the demographic and clinical variables and ethnicity for parameters of vascular and cardiac function, and monocytes/microparticles.

Those variables which showed a significant predictive value on the univariate analysis were consequently included into a stepwise multivariate regression model.

On the basis on previous work of the department on the study parameters (arterial stiffness, endothelial damage/dysfunction) a sample size of 25 subjects in each group would have an 80% power to detect a difference of 0.5 standard deviations in FMD.<sup>649</sup> For further confidence I aimed to recruit 50 subjects per group for the inter-ethnic comparisons and 40 subjects per each control group.

## **3.5. Results**

### **3.5.1. Endothelial function and arterial stiffness in heart failure**

#### ***3.5.1.1. Flow-mediated dilation***

##### *(a) Comparison of HF subjects vs. controls.*

SAs with HF had significantly lower FMD compared to ‘disease controls’ and ‘healthy controls’ ( $p=0.033$  and  $p<0.001$ , respectively)(Table 21). No significant difference in the baseline diameter of brachial artery and endothelium-independent response to GTN was observed between the groups ( $p=0.53$ ).

##### *(b) Inter-ethnic comparisons in HF.*

SAs and ACs with HF had similar values of FMD ( $p=0.98$ ), which were significantly lower compared to Whites ( $p<0.001$  and  $p=0.01$ , respectively)(Table 22, Figure 8). No significant difference in the baseline diameter of brachial artery and endothelium-independent response to GTN was observed between the groups ( $p=0.77$ ).

#### ***3.5.1.2. Microvascular function***

SAs with HF had significantly lower endothelium-mediated microvascular response to acetylcholine compared to ‘disease controls’ and ‘healthy controls’ ( $p<0.001$ , for both)(Table 21). SAs had significantly lower response to acetylcholine compared to Whites and ACs ( $p<0.001$ )(Table 22, Figure 9). There was no significant difference in response

toacetylcholine between subjects of white and AC origin (p=0.88). There was no ethnic difference in baseline skin perfusion (p=0.81).

**Table 21. Characteristics of endothelial and vascular function in South Asian subjects with heart failure compared to controls**

	Healthy controls (n=40)	Disease controls (n=40)	Heart failure (n=50)	p
Flow mediated dilation				
BA diameter, mm	4.08±0.73	4.35±0.52	4.16±0.68	0.19
BA FMD, %	11.7±4.65*†	7.22±3.69‡	4.76±4.78	<0.001
BA response to GTN, %	12.1±4.33	11.3±4.18	11.0±5.25	0.53
Microvascular function				
LD baseline, PU	26.2 (17.6-35.6)	24.1 (19.1-34.3)	20.8 (14.5-27.3)	0.07
LD acetylcholine, %	543±254*†	390±290‡	129±93.1	0.001
LD sodium nitroprusside, %	350 (163-706)	252 (108-517)	258 (169-452)	0.26
Arterial stiffness				
PWV (femoral), m/sec	7.19±2.08*	7.83±2.85	8.63±3.11	0.06
PWV (radial), m/sec	7.61±1.40	7.51±1.08	7.81±1.45	0.69
Augmentation index, %	28.8±12.1	26.0±11.0	25.1±14.3	0.59
Endothelial microparticles				
EMP, per µl	9470 (6107-11025)†	9724 (5831-11136)‡	10929 (6225-19600)	0.04

BA - brachial artery, EMP – endothelial microparticles, FMD - flow-mediated dilation, GTN – glycerol trinitrate, LD – laser Doppler, PWV – pulse wave velocity, PU – perfusion units. Values expressed as Mean±Standard Deviation or Median (Q1-Q3). \*p<0.05 between healthy controls and disease controls (p=0.07 for EMPs), †p <0.05 between healthy controls and HF group, ‡ p <0.05 between disease controls and HF group (p=0.08 for EMPs).



**Table 22. Parameters of endothelial and vascular function in subjects with heart failure**

	South Asian (n=50)	White (n=50)	African-Caribbean (n=28)	p
Flow-mediated dilation				
BA diameter, mm	4.16±0.68	4.15±0.68	4.47±0.84	0.14
BA FMD, %	4.76±4.78*	8.49±4.63‡	4.55±3.56	<0.001
BA response to GTN, %	11.0±5.25	11.5±4.73	10.9±4.93	0.77
Microvascular function				
LD baseline, PU	20.8 (14.5-27.3)	19.2 (15.2-30.1)	19.8 (15.4-25.5)	0.81
LD acetylcholine, %	129±93.1*†	265±151	281±159	<0.001
LD sodium nitroprusside, %	258 (169-452)	249 (152-493)	273 (109-744)	0.67
Arterial stiffness				
PWV (femoral), m/sec	8.63±3.11	8.11±3.11	9.35±2.33	0.23
PWV (radial), m/sec	8.15 (6.60-8.72)	7.10 (6.30-7.60)	7.70 (6.90-8.87)	0.88
Augmentation index, %	25.1±14.3†	25.9±14.1‡	15.2±16.5	0.02
Endothelial microparticles				
EMP, per µl	10929 (6225-19600)†	8527 (6984-10818)	8786 (6415-11381)	0.04

BA - brachial artery, EMP – endothelial microparticles, FMD - flow-mediated dilation, GTN – glycerol trinitrate, LD – laser Doppler, PWV – pulse wave velocity, PU – perfusion units. Values expressed as Mean±Standard Deviation or Median (Q1-Q3). \*p<0.05 between SAs and Whites (p=0.075 for EMPs), †p <0.05 between SAs and ACs, ‡ p <0.05 between Whites and ACs.

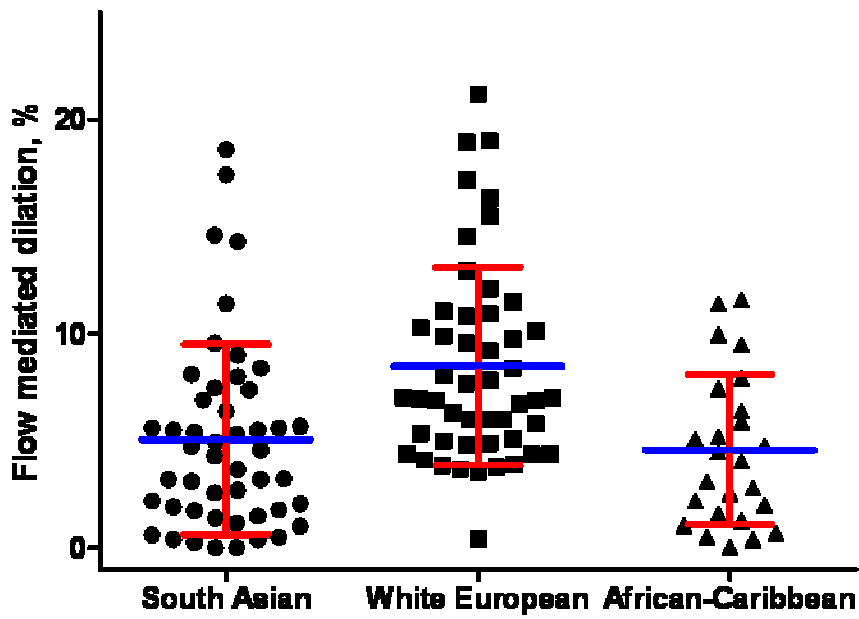


Figure 8. Ethnic differences in flow mediated dilation in heart failure

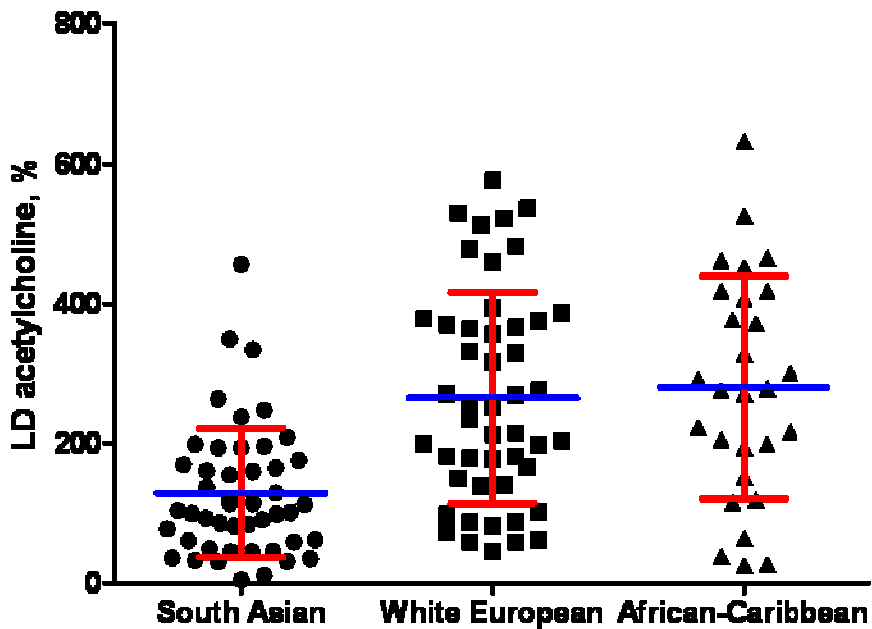


Figure 9. Ethnic differences in microvascular endothelial function in heart failure  
LD acetylcholine – response to iontophoresis of acetylcholine measure by laser Doppler

### **3.5.1.3. Arterial stiffness**

#### *(a) Comparison of HF subjects vs. controls.*

Carotid-femoral PWV was significantly higher in SAs with HF compared to healthy individuals ( $p=0.048$ )(Table 21). No significant difference in carotid-radial PWV and augmentation index was found.

#### *(b) Inter-ethnic comparisons in HF.*

No significant ethnic difference in PWV (carotid-femoral and carotid-radial) in HF subjects was evident (Table 22). Aortic augmentation index was significantly lower amongst AC subjects compared to other ethnic groups ( $p=0.044$  compared to SAs;  $p=0.033$  compared to Whites).

### **3.5.1.4. Endothelial microparticles**

#### *(a) Comparison of HF subjects vs. controls.*

The median EMP count was significantly different among the three SA groups (Kruskal Wallis test,  $p=0.04$ ) but not significantly different between the individual groups (posthoc tests,  $p=0.08$  and  $p=0.07$  between ‘healthy controls’ vs. HF subjects, and ‘healthy controls’ vs. ‘disease controls’, respectively) (Table 21).

#### *(b) Inter-ethnic comparisons in HF.*

EMPs were significantly different between the three HF ethnic groups (Kruskal Wallis test,  $p=0.04$ ) but were not significantly different on post-hoc testing for the individual inter-

group comparisons (SAs vs. ACs [p=0.07] and SAs vs. Whites [p=0.10]) (Table 22, Figure 10).

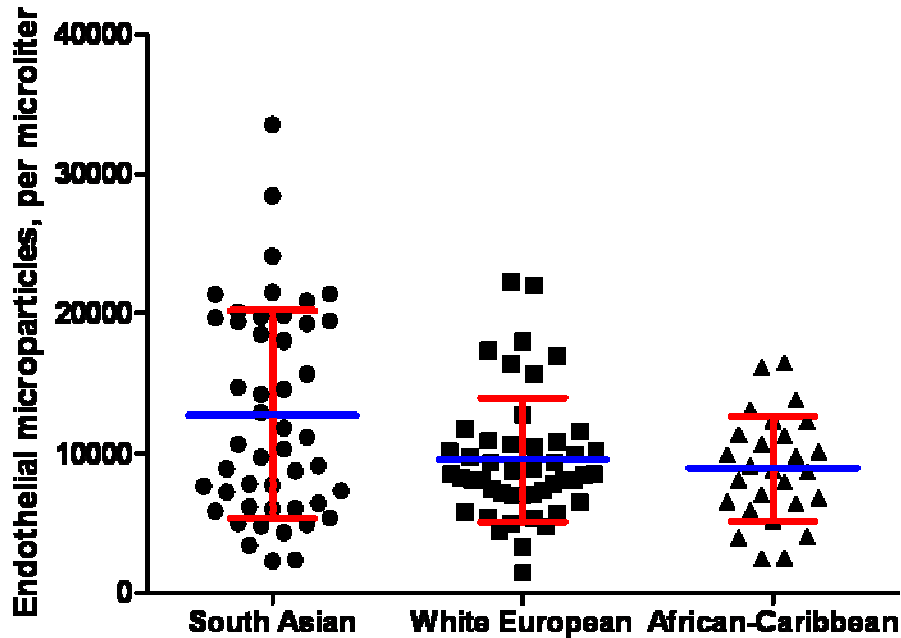


Figure 10. Ethnic differences in endothelial microparticles in heart failure

### 3.5.1.5. Multivariable analysis

Using linear regression analysis, FMD in HF subjects (n=128) was significantly associated with age, NYHA class, history of hypertension, glucose and total cholesterol levels, and carotid-femoral PWV (Table 23). The microvascular endothelial function in HF significantly correlated with diabetes, glucose and HDL levels, smoking and ethnicity. Carotid-femoral PWV was significantly associated with age, diabetes, systolic and diastolic blood pressure, glomerular filtration rate, glucose levels, and FMD. LVEF was the only parameter significantly correlated with carotid-radial PWV. Ethnicity and NYHA

class were associated with aortic augmentation index. Ethnicity was not correlated with the carotid-femoral PWV, carotid-radial PWV. On univariate analysis, EMP count was significantly associated with ethnicity, LVEF and systolic blood pressure.

After adjustment for other significant clinical and demographic variables in a multivariable analysis, ethnicity was significantly associated with microvascular endothelial function (i.e., response to acetylcholine) (Table 24). Although a significantly higher proportion of SA patients with HF received statins compared with Whites, statin use was not significantly associated with parameters of endothelial function and arterial stiffness (Table 25).

**Table 23. Univariate linear regression analysis of predictors of parameters of endothelial function and arterial stiffness**

Predictor	$\beta$	R <sup>2</sup>	p
<b>Flow-mediated dilation</b>			
Age	-0.29	0.08	0.001
NYHA class	0.23	0.04	0.011
History of hypertension	-0.19	0.04	0.038
Glucose	-0.48	0.23	0.004
Total cholesterol	0.24	0.06	0.03
Carotid-femoral PWV	-0.22	0.05	0.018
Diabetes	-0.15	0.02	0.099
<i>Ethnicity</i>	<i>0.39</i>	<i>0.15</i>	<i>0.005</i>
<b>Laser Doppler, response to acetylcholine</b>			
Diabetes	-0.21	0.04	0.02
Glucose levels	-0.43	0.18	0.01
HDL cholesterol	0.27	0.07	0.039
Smoking	0.28	0.08	0.002
<i>Ethnicity</i>	<i>0.42</i>	<i>0.18</i>	<i>&lt;0.001</i>
<b>Carotid-femoral PWV</b>			
Age	0.38	0.14	<0.001
Diabetes	0.29	0.08	0.002
Systolic blood pressure	0.35	0.12	<0.001
Diastolic blood pressure	0.19	0.04	0.042
Glomerular filtration rate	-0.20	0.04	0.046
Glucose	0.032	0.10	0.067
Flow-mediated dilation	-0.22	0.05	0.018
<i>Ethnicity</i>	<i>0.07</i>	<i>0.01</i>	<i>0.46</i>
<b>Carotid-radial PWV</b>			
Ejection fraction	-0.36	0.13	0.007
<i>Ethnicity</i>	<i>-0.006</i>	<i>0.00</i>	<i>0.96</i>
<b>Augmentation index</b>			
NYHA III class	-0.27	0.07	0.013
<i>Ethnicity</i>	<i>-0.24</i>	<i>0.06</i>	<i>0.028</i>
<b>Endothelial microparticles</b>			
Ejection fraction	-0.23	0.05	0.04
Systolic blood pressure	-0.21	0.04	0.02
<i>Ethnicity</i>	<i>0.25</i>	<i>0.07</i>	<i>0.005</i>

PWV, pulse wave velocity

**Table 24. Multivariable analysis of predictive value of ethnicity for parameters of endothelial function and arterial stiffness**

Parameter	$\beta$	R <sup>2</sup>	p
Flow-mediated dilation	-0.06	0.39	0.49
Laser Doppler, response to acetylcholine	0.49	0.41	0.003
Endothelial microparticles	0.20	0.23	0.06
Augmentation index	-0.21	0.09	0.35

**Table 25. Predictive value of statins for parameters of endothelial function and arterial stiffness (univariate regression analysis)**

Parameter	$\beta$	R <sup>2</sup>	p
Flow-mediated dilation	-0.044	0.00	0.63
Laser Doppler, response to acetylcholine	-0.33	0.00	0.72
Pulse wave velocity (femoral)	0.13	0.02	0.16
Pulse wave velocity (radial)	0.074	0.01	0.51
Augmentation index	0.14	0.02	0.21
Endothelial microparticles	0.12	0.01	0.20

### **3.5.2. Monocyte subsets and monocyte-platelet aggregates in heart failure**

#### ***3.5.2.1. Monocyte subsets and monocyte-platelet aggregates***

##### *(a) Comparison of HF subjects vs. controls.*

SAs with HF had lower numbers and percentage of Mon3 compared to ‘disease controls’ and ‘healthy controls’ (p=0.036 and p=0.014, respectively for Mon3 numbers; p=0.07 and p=0.005 for Mon3 percentage)(Table 26). No significant difference in Mon1, Mon2 and MPA counts was found between the study groups (p=0.90, p=0.78 and p=0.67, respectively).

##### *(b) Inter-ethnic comparisons in HF.*

Whites had significantly higher Mon2 counts and percentages compared with SAs and ACs (p=0.004 and p=0.002, respectively for Mon2 counts, p=0.006 and p=0.009, respectively for Mon2 percentages)(Table 27, Figure 11). ACs had the highest count of Mon3 (p=0.015 vs. SAs and p=0.052 vs. Whites) and the highest percentages of Mon3 (p<0.001 vs. SAs and p<0.001 vs. Whites)(Figure 12). No significant differences in Mon1 counts were observed between the study groups. No significant difference in Mon1, and MPAs was found between the study groups (p=0.20, p=0.73, respectively).



**Table 26. Monocytes, monocyte-platelet aggregates and microparticles in South Asian subjects with heart failure compared to controls**

	Heart failure	Disease controls	Healthy controls	p
<b>Monocyte subsets</b>				
Mon1, per $\mu$ l	342 $\pm$ 118	333 $\pm$ 125	329 $\pm$ 139	0.90
Mon2, per $\mu$ l	18.0 (9.37-31.7)	15.7 (12.7-30.6)	22.4 (15.2-28.9)	0.78
Mon3, per $\mu$ l	26.8 (15.6-43.7)* $\dagger$	34.0 (25.9-41.0)	36.9 (26.2-60.7)	0.019
Mon1, %	85.0 $\pm$ 8.27	84.1 $\pm$ 6.64	81.4 $\pm$ 9.56	0.18
Mon2, %	4.73 (2.95-8.14)	5.04 (3.18-8.17)	5.20 (4.09-7.84)	0.73
Mon3, %	7.47 (4.49-10.9)* $\dagger$	9.31 (7.52-11.3)	9.91 (6.33-15.9)	0.023
MPA, per $\mu$ l	61.2 (35.5-123)	70.0 (39.6-121)	65.9 (44.1-114)	0.67
<b>CD204 expression</b>				
CD204 (all monocytes), MFI	11.5 (8.33-26.0)	11.3 (9.18-16.6)	29.9 (9.26-39.0)	0.30
CD204 (Mon1), MFI	10.5 (7.82-26.0)	10.4 (8.50-14.9)	33.5 (9.02-42.8)	0.15
CD204 (Mon2), MFI	41.5 (25.3-55.5)	48.5 (31.5-72.3)	50.5 (35.4-58.2)	0.30
CD204 (Mon3), MFI	26.5 $\pm$ 18.3	25.9 $\pm$ 12.3	23.9 $\pm$ 15.5	0.78
CD204 (Mon1), %	22.2 (9.46-66.6)	20.2 (14.1-37.7)	71.2 (22.4-82.3)	0.14
CD204 (Mon2), %	74.4 $\pm$ 19.8	76.6 $\pm$ 19.3	81.6 $\pm$ 14.9	0.25
CD204 (Mon3), %	53.9 $\pm$ 21.5	53.1 $\pm$ 18.3	51.4 $\pm$ 22.9	0.89
<b>Microparticles</b>				
Monocyte MPs, per $\mu$ l	1106 (583-2816)* $\dagger$	602 (449-1085)	457 (303-622)	0.001
Platelet MPs, per $\mu$ l	36368 (25524-55991)* $\dagger$	28851 (25294-37091) $\ddagger$	23141 (16725-28749)	0.001

MFI, median fluorescent intensity, MP, microparticles.\*p<0.05 between HF and disease control groups,  $\dagger$ p <0.05 between HF and healthy control groups,  $\ddagger$  p <0.05 between disease controls and healthy controls.

**Table 27. Monocytes, monocyte-platelet aggregates and microparticles in subjects with heart failure**

	South Asians	Whites	African-Caribbeans	p
<b>Monocyte subsets</b>				
Mon1, per $\mu$ l	342 $\pm$ 118	344 (256-446)	282 (185-397)	0.20
Mon2, per $\mu$ l	18.0 (9.37-31.7)*	27.3 (18.8-57.8)‡	19.9 (9.67-38.3)	0.003
Mon3, per $\mu$ l	26.8 (15.6-43.7)†	27.6 (18.8-47.2)‡	43.9 (26.065.9)	0.026
Mon1, %	87.2 (80.6-91.5)	85.3 (81.1-87.0)	82.0 (77.4-85.6)	0.10
Mon2, %	4.73 (2.95-8.14)*	7.32 (5.63-10.8)‡	5.29 (2.69-9.97)	0.005
Mon3, %	7.47 (4.49-10.9)†	7.51 $\pm$ 3.94‡	13.8 $\pm$ 7.38	<0.001
MPA, per $\mu$ l	61.2 (35.5-123)	69.6 (41.8-133)	55.8 (40.1-150)	0.73
<b>CD204 expression</b>				
CD204 (all monocytes), MFI	11.5 (8.33-26.0)	10.4 (8.15-37.0)	11.3 (8.24-54.0)	0.88
CD204 (Mon1), MFI	10.5 (7.82-26.0)	8.94 (7.41-39.4)	10.7 (7.06-62.4)	0.87
CD204 (Mon2), MFI	41.5 (25.3-55.5)	29.5 (21.6-57.6)	35.6 (19.2-62.0)	0.58
CD204 (Mon3), MFI	26.5 $\pm$ 18.3†	16.6 (10.8-24.4)	14.5 (8.88-23.9)	0.08
CD204 (Mon1), %	22.2 (9.46-66.6)	15.9 (9.66-71.2)	24.8 (10.3-81.9)	0.77
CD204 (Mon2), %	74.4 $\pm$ 19.8	69.9 $\pm$ 18.2	72.1 $\pm$ 20.9	0.54
CD204 (Mon3), %	53.9 $\pm$ 21.5†	46.8 $\pm$ 20.5	38.8 $\pm$ 16.9	0.014
<b>Microparticles</b>				
Monocyte MPs, per $\mu$ l	1106 (583-2816)*	761 (566-987)	890 (664-1482)	0.054
Platelet MPs, per $\mu$ l	36368 (25524-55991)*†	28288 (24783-34986)	30045 (23201-40674)	0.016

MFI, median fluorescent intensity, MP, microparticles.\*p<0.05 between SAs and Whites, †p <0.05 between SAs and ACs (p=0.057 for platelet MPs), ‡ p <0.05 between Whites and ACs.

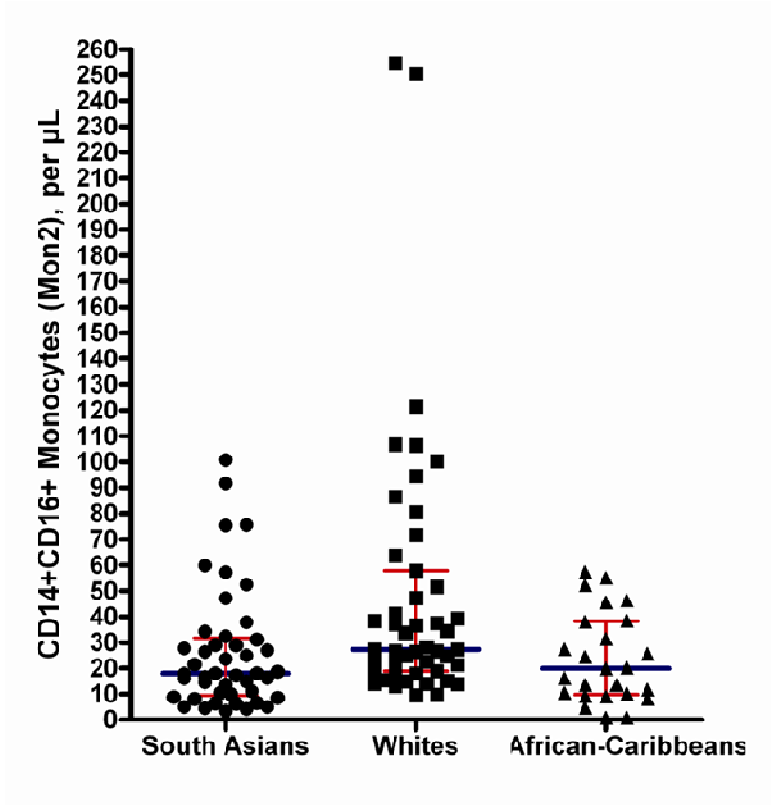


Figure 11. Ethnic differences in CD14++CD16+ monocytes (Mon2) in heart failure

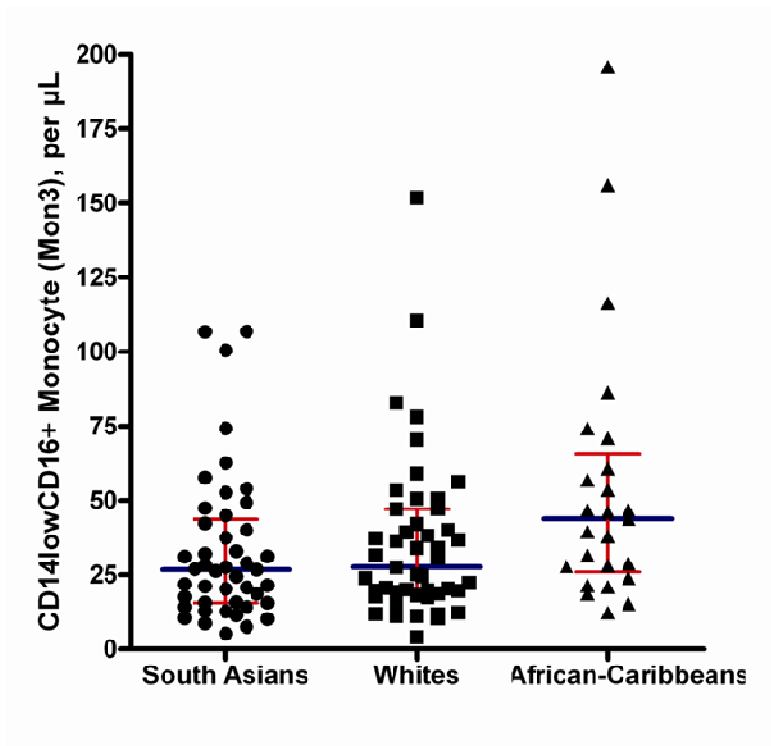


Figure 12. Ethnic differences in CD14+CD16+ monocytes (Mon3) in heart failure

### **3.5.2.2. Monocyte CD204 expression**

#### *(a) Comparison of HF subjects vs. controls:*

HF subjects had similar monocyte CD204 expression when compared with controls (Table 26).

#### *(b) Inter-ethnic comparisons in HF.*

SAs had significantly higher proportion of Mon3 expressing CD204 compared with ACs ( $p=0.011$ ) and similar trend compared with Whites ( $p=0.088$ ) (Table 27).

### **3.5.2.3. Monocyte-derived and platelet-derived microparticles**

#### *(a) Comparison of HF subjects vs. controls.*

MMP count was significantly increased in the HF group compared to ‘disease controls’ and ‘healthy controls’ ( $p=0.014$  and  $p=0.001$ , respectively) (Table 26). PMPs were incrementally increased from ‘healthy controls’ to ‘disease controls’ ( $p=0.016$ ) and further from ‘disease controls’ to patients with HF ( $p=0.035$ ) (Figure 13).

#### *(b) Inter-ethnic comparisons in HF.*

SA subjects had more MMPs than Whites ( $p=0.008$ ). There was a non-significant trend towards higher PMP levels in SAs compared with Whites ( $p=0.079$ ) and ACs ( $p=0.057$ ) (Table 27).

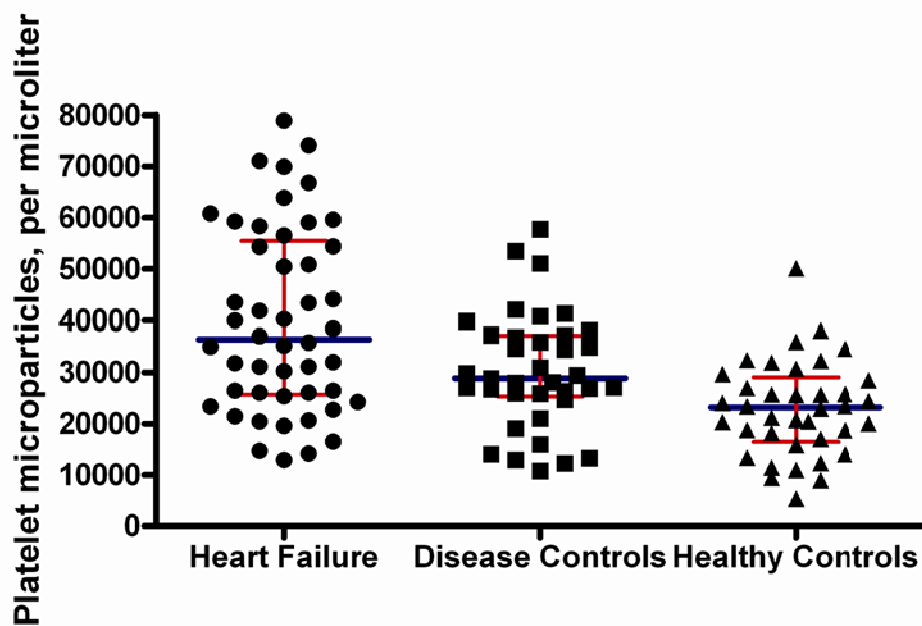


Figure 13. Platelet microparticles in heart failure subjects vs. control groups

#### 3.5.2.4. Multivariate analysis

On univariate linear regression analysis, ethnicity was predictive of Mon2 and Mon3 in HF patients (Table 28). After the adjustment for age and diabetes in multivariate analysis, white origin was independently predictive of high Mon2 counts, whilst AC origin was independently predictive of high Mon3 count and percentages and low granulocyte counts. SA origin was predictive of high MMP and PMP counts.

High MMP count was significantly predictive of LVEF in HF patients, before and after adjustment for age ( $\beta$  -0.23 for both;  $p=0.035$  and  $p=0.042$ , respectively).

**Table 28. Predictive value of ethnicity on monocyte subsets, and microparticles**

Parameter	Compa- rative ethnicity	Unadjusted			Adjusted*		
		$\beta$	R <sup>2</sup>	p	$\beta$	R <sup>2</sup>	p
Mon2, per $\mu$ l	Whites	0.29	0.09	0.005	0.25	0.11	0.02
	ACs	-0.29		0.77			-0.05
Mon3, per $\mu$ l	Whites	0.42	0.07	0.68	0.094	0.10	0.38
	ACs	0.28		0.007			0.31
Mon2, %	Whites	0.24	0.06	0.016	0.19	0.09	0.081
	ACs	-0.12		0.90			-0.04
Mon3, %	Whites	-0.04	0.20	0.65	0.01	0.23	0.93
	ACs	0.43		<0.001			0.48
MMPs, per $\mu$ l	Whites	-0.37	0.12	<0.001	-0.36	0.12	<0.001
	ACs	-0.25		0.009			-0.25
PMPs, per $\mu$ l	Whites	-0.27	0.08	0.007	-0.30	0.09	0.005
	ACs	-0.27		0.007			-0.27

ACs, African-Caribbeans, MMPs, monocyte microparticles, PMP, microparticles.

\*Adjusted for age and diabetes. B, coefficient of regression, MMP, monocyte microparticles, PMP, platelet microparticles, SE, standard error,  $\beta$ , adjusted coefficient of regression.

### **3.5.3. Endothelial progenitor cells in heart failure**

#### ***3.5.3.1. Endothelial progenitor cells in heart failure in South Asians***

SAs with HF had significantly reduced total numbers of CD34+ monocytes compared to ‘disease controls’ (p=0.015) and ‘healthy controls’ (p=0.003) (Table 29, Figure 14). Also, CD34+ Mon1 were reduced in HF compared with ‘disease controls’ (p=0.037); CD34+ Mon2 were lower in HF compared to ‘disease controls’ (p=0.02) and showed similar trend when compared to ‘healthy controls’ (p=0.06). The CD34+ Mon3 counts were not significantly different between the three groups.

A different trend was seen for the numbers of KDR+ monocytes. Total KDR+ monocytes and KDR+ Mon1 were increased in ‘disease controls’ compared with ‘healthy controls’ (p=0.021 and p=0.005, respectively); however, KDR+ Mon1 counts were reduced in HF subjects (p=0.036 vs. ‘disease controls’) (Figure 15). There were no significant differences in KDR+ Mon2 and KDR+ Mon3 between HF and control groups.

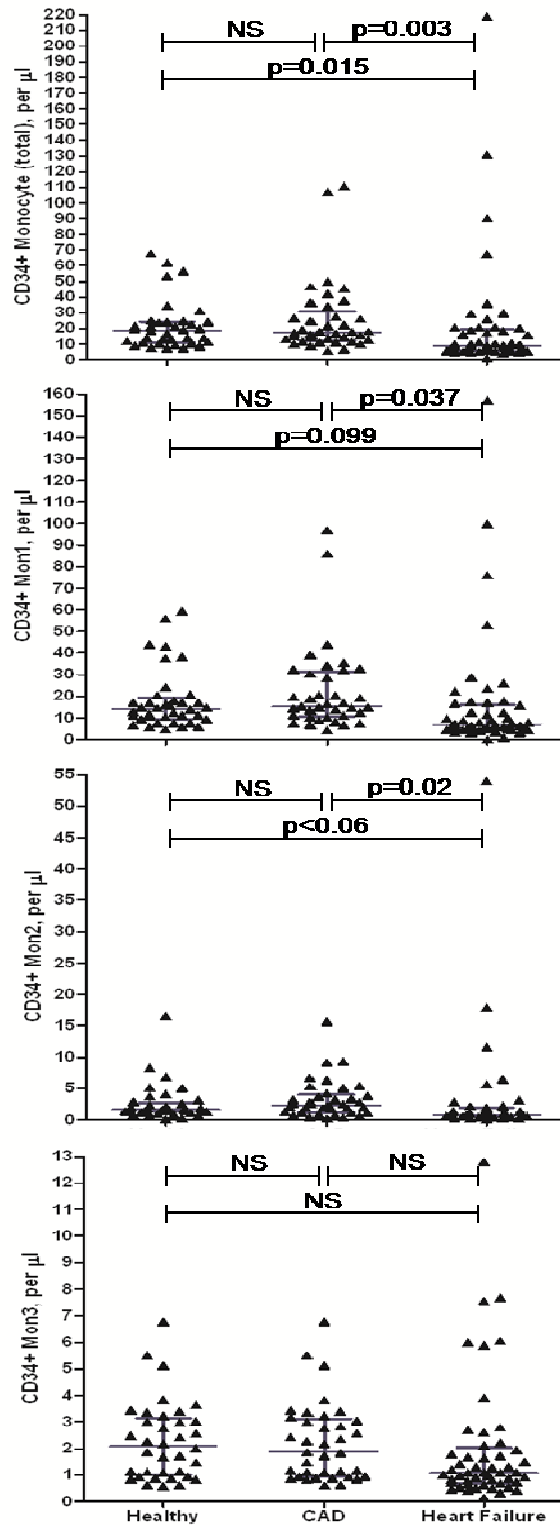
VEGFR1 expression on Mon1 and Mon2 was significantly reduced in HF compared to ‘disease controls’ (p=0.035, and p=0.001, respectively), whilst VEGFR1 expression on Mon3 was not significantly different. Levels of CD34+KDR+ EPCs were reduced in HF vs. ‘healthy controls’ (p<0.0001) and ‘disease controls’ (p=0.018) (Figure 16).

**Table 29. Endothelial progenitors and monocyte expression of VEGF receptor 1 in South Asian subjects with heart failure compared to controls**

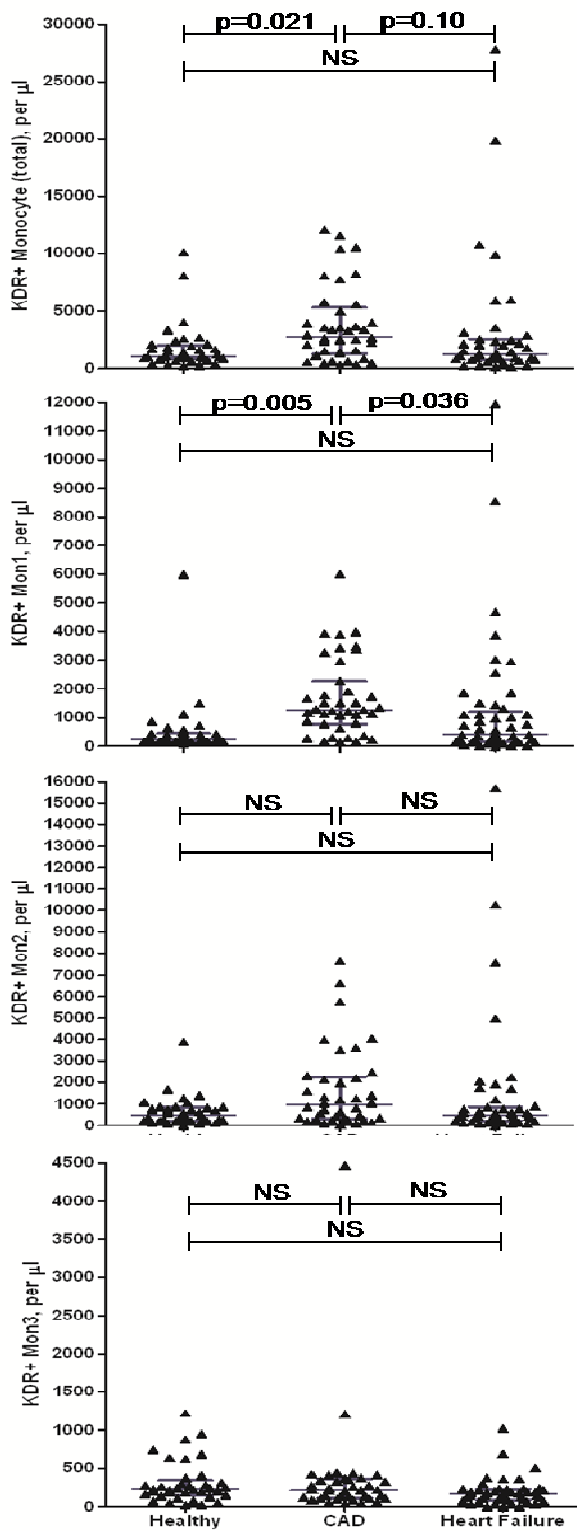
	Heart failure	Disease controls	Healthy controls	p
CD34+ monocytes, per $\mu$ l	9.37 (6.20-20.7)* <sup>†</sup>	18.0 (12.8-36.9)	19.0 (11.7-24.2)	0.015
CD34+ Mon1, per $\mu$ l	7.35 (4.79-17.2)*	14.8 (10.1-32.3)	13.0 (7.65-18.6)	0.012
CD34+ Mon2, per $\mu$ l	0.82 (0.54-1.97)* <sup>†</sup>	2.12 (1.09-4.08)	1.69 (1.08-3.36)	0.031
CD34+ Mon3, per $\mu$ l	1.15 (0.71-2.33)	1.96 (1.23-2.30)	2.02 (0.92-3.27)	0.14
KDR+ monocytes, per $\mu$ l	1190 (767-2531)*	3068 (1009-6246) <sup>‡</sup>	993 (744-1935)	0.021
KDR+ Mon1, per $\mu$ l	410 (161-1503)*	1234 (340-3296) <sup>‡</sup>	275 (169-578)	0.004
KDR+ Mon2, per $\mu$ l	469 (242-1200)	915 (255-2730)	417 (201-770)	0.17
KDR + Mon3, per $\mu$ l	162 (77.5-231)	219 (104-382)	229 (138-517)	0.24
VEGFR1 (Mon1), MFI	12.6 $\pm$ 5.82*	16.2 $\pm$ 6.54	14.4 $\pm$ 5.93	0.045
VEGFR1 (Mon2), MFI	30.9 $\pm$ 14.4*	46.0 $\pm$ 21.5	36.0 $\pm$ 15.6	0.001
VEGFR1 (Mon3), MFI	11.3 $\pm$ 3.34	13.5 $\pm$ 5.31	11.3 $\pm$ 4.18	0.055
CD34+KDR+ EPC, per $\mu$ l	915 (441-1580)* <sup>†</sup>	1567 (902-2574)	2292 (1066-5775)	0.001

EPC, endothelial progenitor cells, KDR, kinase domain receptor, MFI, median fluorescent intensity, VEGFR1, vascular endothelial growth factor receptor 1. \*p<0.05 between HF and disease control groups, <sup>†</sup>p <0.05 between HF and healthy control groups (p=0.06 for CD34+ Mon2), <sup>‡</sup> p <0.05 between disease controls and healthy controls.



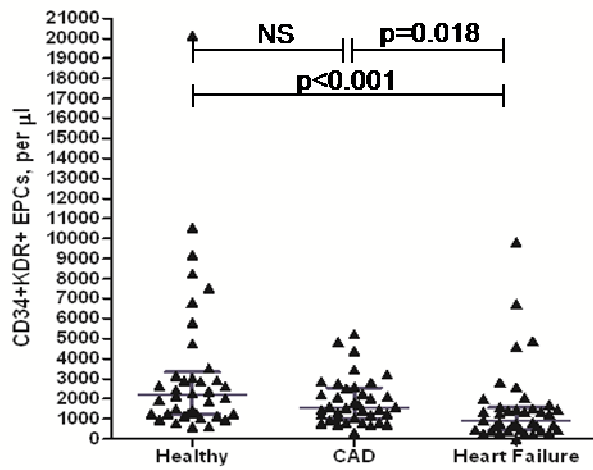


**Figure 14. CD34+ monocytes in heart failure vs. control groups**  
 CAD, coronary artery disease ('disease' controls)



**Figure 15. KDR+ monocytes in heart failure vs. control groups**

CAD, coronary artery disease ('disease' controls)



**Figure 16. CD34+KDR+ endothelial progenitor cells (EPCs) in heart failure vs. control groups**

CAD, coronary artery disease ('disease' controls)

### 3.5.3.2. Inter-ethnic comparisons in heart failure

Whites had significantly less KDR+ Mon3 than ACs (p=0.033), but significantly more CD34+ Mon2 than both SAs (p=0.001) and AC (p=0.048). There were no ethnic differences in other study parameters (Table 30).

**Table 30. Endothelial progenitors and monocyte expression of VEGF receptor 1 in subjects with heart failure**

	South Asians	Whites	African-Caribbeans	p
KDR+ monocytes, per $\mu$ l	1190 (767-2531)	1603 (623-4668)	2454 (856-6549)	0.46
KDR+ Mon1, per $\mu$ l	410 (161-1503)	565 (196-1958)	1018 (427-3938)	0.30
KDR+ Mon2, per $\mu$ l	469 (242-1200)	758 (283-2078)	661 (284-1623)	0.50
KDR + Mon3, per $\mu$ l	162 (77.5-231)	96.8 (29.5-185)‡	215 (98.5-643)	0.01
CD34+ monocytes, per $\mu$ l	9.37 (6.20-20.7)	14.1 (8.96-42.0)	12.0 (7.41-27.7)	0.10
CD34+ Mon1, per $\mu$ l	7.35 (4.79-17.2)	10.5 (6.45-27.6)	9.11 (4.70-19.5)	0.27
CD34+ Mon2, per $\mu$ l	0.82 (0.54-1.97)*	2.38 (1.12-5.70)‡	1.37 (0.56-2.73)	<0.001
CD34+ Mon3, per $\mu$ l	1.15 (0.71-2.33)	1.23 (0.73-2.34)	2.53 (1.05-4.07)	0.05
VEGFR1 (Mon1), MFI	12.6 $\pm$ 5.82	15.2 $\pm$ 9.07	15.5 $\pm$ 8.22	0.21
VEGFR1 (Mon2), MFI	30.9 $\pm$ 14.4	33.9 $\pm$ 18.9	36.7 $\pm$ 21.4	0.43
VEGFR1 (Mon3), MFI	11.3 $\pm$ 3.34	12.0 $\pm$ 9.56	11.5 $\pm$ 4.01	0.88
CD34+KDR+ EPC, per $\mu$ l	915 (441-1580)	1229 (697-1810)	1673 (583-2874)	0.29

EPC, endothelial progenitor cells, KDR, kinase domain receptor, MFI, median fluorescent intensity, VEGFR1, vascular endothelial growth factor receptor 1. \*p<0.05 between SAs and Whites, ‡ p <0.05 between Whites and ACs.

### 3.5.3.3. Regression analysis

On univariate linear regression analysis, endothelial progenitors were not significantly associated with LVEF or E/E' (Table 31). On univariate analysis, VEGFR1 expression by Mon2 showed a trend towards predictive value for LVEF ( $p=0.054$ ), but after adjustment for ethnicity this parameter was significantly predictive of LVEF ( $\beta=-0.25$ .  $p=0.039$ ). After adjustment for ethnicity and age, this was of borderline significance ( $\beta=-0.24$ .  $p=0.051$ ).

**Table 31. Predictive value of the endothelial progenitors and monocyte angiogenic characteristics for cardiac geometry and function**

	Ejection fraction			E/E'		
	$\beta$	$R^2$	p	$\beta$	$R^2$	p
EPC	-0.17	0.03	0.17	-0.06	0.00	0.68
CD34+ monocytes	-0.06	0.00	0.65	0.06	0.00	0.63
CD34+ Mon1	-0.06	0.00	0.60	0.07	0.01	0.59
CD34+ Mon2	-0.07	0.01	0.58	-0.02	0.00	0.91
CD34+ Mon3	0.07	0.01	0.57	0.12	0.01	0.38
KDR+ monocytes	-0.16	0.03	0.20	-0.04	0.00	0.79
KDR+ Mon1	-0.16	0.03	0.20	-0.01	0.00	0.97
KDR+ Mon2	-0.16	0.03	0.21	-0.05	0.00	0.72
KDR + Mon3	-0.15	0.02	0.25	-0.06	0.00	0.69
VEGFR1 (Mon1)	-0.21	0.04	0.081	0.06	0.00	0.66
VEGFR1 (Mon2)	-0.23	0.05	0.054	-0.03	0.00	0.82
VEGFR1 (Mon3)	-0.13	0.02	0.29	0.01	0.00	0.96

EPC, endothelial progenitor cells, KDR, kinase domain receptor, VEGFR1, vascular endothelial growth factor receptor 1.

On univariate regression analysis, AC ethnicity was predictive of the numbers of KDR+ Mon3 in HF (p=0.03) (Table 32). On multivariate analysis, AC ethnicity remained independently predictive of KDR+ Mon3 count even after adjustment for age, HF aetiology and history of diabetes ( $\beta=0.021$ , p=0.045).

**Table 32. Predictive value of ethnicity on endothelial progenitors and monocyte angiogenic characteristics**

	South Asians			Whites			African-Caribbeans		
	$\beta$	R <sup>2</sup>	p	$\beta$	R <sup>2</sup>	p	$\beta$	R <sup>2</sup>	p
EPC	-0.14	0.02	0.22	0.11	0.01	0.30	0.04	0.00	0.71
CD34+ monocytes	-0.15	0.02	0.17	0.13	0.02	0.19	-0.02	0.00	0.88
CD34+ Mon1	-0.12	0.01	0.28	0.09	0.01	0.37	0.01	0.00	0.93
CD34+ Mon2	-0.12	0.02	0.27	0.14	0.02	0.15	-0.08	0.01	0.43
CD34+ Mon3	-0.12	0.01	0.29	0.12	0.01	0.23	-0.03	0.00	0.76
KDR+ monocytes	-0.10	0.01	0.37	0.05	0.00	0.61	0.08	0.01	0.46
KDR+ Mon1	-0.09	0.01	0.44	-0.02	0.00	0.82	0.17	0.03	0.10
KDR+ Mon2	-0.11	0.01	0.35	0.12	0.01	0.25	-0.05	0.00	0.65
KDR + Mon3	-0.10	0.01	0.40	-0.05	0.00	0.62	0.22	0.05	0.03
VEGFR1 (Mon1)	-0.17	0.03	0.12	0.10	0.01	0.30	0.08	0.01	0.40
VEGFR1 (Mon2)	-0.09	0.01	0.40	0.03	0.00	0.79	0.10	0.01	0.30
VEGFR1 (Mon3)	-0.05	0.00	0.66	0.05	0.00	0.62	-0.01	0.00	0.89

EPC, endothelial progenitor cells, KDR, kinase domain receptor, VEGFR1, vascular endothelial growth factor receptor 1.

## **3.6. Discussion**

### **3.6.1. Endothelial function and arterial stiffness**

This study shows for the first time significant ethnic differences in endothelial function in a cohort of 128 subjects with systolic HF. The White ethnic group had significantly higher values of FMD of the brachial artery compared to other ethnic groups, with no difference seen between SA and AC subjects.

The impact of endothelial dysfunction on pathogenesis and outcome of systolic HF is well recognised. Numerous studies have demonstrated peripheral endothelium-dependent vasomotor abnormalities in HF as assessed by brachial artery FMD or forearm blood flow changes in response to acetylcholine.<sup>142-144</sup> Direct evidence for endothelial dysfunction in the genesis of haemodynamic abnormalities in HF has been provided by the infusion of NG-monomethyl-L-arginine, an inhibitor of NO production, into volunteers with HF.<sup>141</sup> Also, there is an independent significant association of endothelial dysfunction with a negative outcome in HF, whether in mild HF (NYHA class I) with relatively preserved myocardial contractility or in more advanced HF (NYHA class IV) with severely depressed LV function.<sup>172-173</sup> The predictive value of FMD for the outcome does not seem to be affected by HF aetiology.

In this study I found that SAs with HF had significantly worse macrovascular endothelial function than HF-free control subjects, a finding that accords with well recognised macrovascular endothelial abnormalities in Whites with HF.<sup>175</sup> However, in the inter-

ethnic comparison non-White ethnic groups had differences in the proportion of patients having diabetes mellitus, a potent contributor to endothelial dysfunction.<sup>650</sup> When the study results were adjusted for important clinical and demographic co-variables, such as age, NYHA class, diabetic status, ethnicity was no longer associated with FMD in HF patients. This analysis indicates that ethnicity-related differences in co-morbidities/risk factors accompanying HF rather than ethnicity itself may determine the status of *macrovascular* endothelial dysfunction.

In contrast to robust evidence of *macrovascular* endothelial dysfunction in HF, only limited data are available on the status of *microvascular* endothelial function in such subjects.<sup>651-652</sup> In my study, I show significant impairment of the cutaneous vascular response to acetylcholine in SAs with HF. I also show for the first time that SAs with HF have significant impairment of microvascular endothelial function compared to other ethnic groups, with SA ethnicity being associated with microvascular endothelial dysfunction, even after adjustment for age, presence of hypertension and diabetes, blood pressure and glucose levels. It might be plausible to speculate that these abnormalities of endothelium-dependant regulation of microvascular flow could account for the unexplained discrepancy between the distinctly more prominent HF symptoms in SA subjects (e.g., much higher hospitalisation rate) despite relatively preserved LV contractility as compared to white HF subjects.<sup>12, 67</sup>

Most but not all studies have shown the ability of statins to improve FMD in subjects with systolic HF.<sup>193, 235, 238, 242</sup> Of note, the study with the longest controlled period of treatment with statins in HF (i.e., for 12 weeks) failed to significantly improve FMD.<sup>235</sup> These



observations suggest that beneficial effects of statins on vasomotor endothelial function in HF may wear off during longer-term treatment. Also, there are no published data on the effects of statins on microvascular endothelial function in HF patients. In my study, treatment with statins was not associated with parameters of macrovascular (i.e., FMD), and microvascular (i.e., response to acetylcholine) endothelial function. Thus, despite a higher proportion of SA subjects with HF received statins compared with Whites, this is unlikely to significantly affect the ethnic differences seen in the study, particularly given that Whites had higher FMD than SAs.

In contrast to measures of endothelial function, parameters of arterial stiffness did not differ between SA subjects with HF and matched 'disease controls'. No significant ethnic difference in PWV was observed, but aortic augmentation index was slightly lower in ACs. Ethnicity was associated with differences in the aortic augmentation index (but not in PWV). However, its significance failed to be independent of other factors affecting aortic stiffness.

EMPs have emerged as a novel marker of endothelial dysfunction related to the pathogenesis of various cardiovascular disorders.<sup>637</sup> Recently, EMPs were shown to be of significant predictive value for negative clinical outcomes in HF.<sup>628</sup> However, I was unable to identify any data on EMP count in subjects with systolic HF as compared to individuals with normal cardiac contractility. In this study, I observed a trend towards increased values of EMPs in subjects with HF compared to control groups as well as increased EMP values in SAs with HF compared with other ethnic groups. Whilst ethnicity tended to be associated with EMP count in HF on linear regression, this was not significant after the

adjustments ( $p=0.06$ ). It is therefore unclear whether ethnicity affects EMPs as a marker of endothelial dysfunction, but given the strong trend towards higher EMPs in SAs with HF observed in this study, further investigations are probably warranted.

It has been previously shown that subjects of SA origin present with symptoms of congestive HF on average 8 years earlier than Whites.<sup>12</sup> Additionally SAs with HF have about 60% more hospital admissions in general and 5-fold higher risk of presenting with HF at hospital among the elderly without signs of more severe LV impairment.<sup>12, 70</sup> A higher prevalence of diastolic dysfunction in SAs has been suggested but not well documented.<sup>63</sup> Accordingly it is unclear at present which factors could be responsible for more HF symptoms in SAs.

It is increasingly recognised that endothelial dysfunction could be a significant contributor to the development of HF with preserved EF and it may also affect the symptoms of systolic HF.<sup>1</sup> Even healthy SAs have worse parameters of endothelial function compared to healthy Whites.<sup>22</sup> The results of the present study show that SA ethnicity is associated with more severe endothelial dysfunction, even after adjustment for the cardiovascular risk factors including diabetes. It is thus tempting to speculate that more prominent endothelial dysfunction may be at least partly responsible for the more symptomatic status in SAs with HF. Further research would be needed to prove the concept.

Despite the higher age-adjusted prevalence, greater hospitalization rate and worse risk factor profile, SA subjects with HF appear to have better prognosis than Whites. The 30 day, 1-year, and 2-year survival rates are consistently better amongst SA.<sup>12, 67</sup>

Multivariable analysis has also confirmed an independent association of SA ethnicity with more favourable prognosis in HF.<sup>12, 67</sup> Again it is plausible that better prognosis in SAs may reflect the situation that earlier presentation with HF due to symptoms associated with vascular disease related to endothelial dysfunction leads to earlier and timely initiation of the treatment. Additionally the study results identify microvascular endothelial dysfunction as a potential target for the therapeutic interventions in SAs with HF.

### **3.6.2. Monocyte subsets**

This study shows for the first time significant ethnic difference in monocyte subsets in HF. The counts and proportion of Mon3, which have been suggested to have a reparative potential were significantly higher in HF subjects of AC origin.<sup>41</sup> These observations may reflect an implication of monocyte subsets in the ethnicity-related differences in pathogenesis and clinical outcomes of HF.

The progressive nature of HF reflects an imbalance between detrimental (e.g., inflammation) and reparative processes.<sup>305</sup> Monocytes are actively involved in both processes by release a diverse range of cytokines and angiogenic factors orchestrating recruitment and activity of other cell types.<sup>25, 653</sup> Individual monocyte subsets have been shown to be featured by different profiles of the cytokines and growth factors produced. For example, Mon1 actively produce various inflammatory cytokines, Mon3 have clearly attenuated inflammatory properties, whilst Mon2 shows the highest of all monocytes expression of IL-10 and receptors to angiogenic factors (the differences between monocyte subsets are addressed in Chapter IV of the thesis).<sup>635</sup>

The findings of higher Mon3 levels in AC subjects with HF could account for reported ethnicity-related differences in HF. Although some studies showed an increased net mortality in Black vs. White patients with HF this difference disappears after adjustment for worse medication adherence and lower socio-economic status among Blacks.<sup>654-655</sup> Despite a higher prevalence of cardiovascular disease and risk factors for HF, Black patients have similar prevalence of reduced LVEF as compared with Whites.<sup>656-657</sup> However, the mortality after hospitalization for HF was 32% lower for Blacks during short-term follow-up and 16% lower during long-term follow-up than for Whites. It is possible that these observations may reflect 'better' intrinsic protective/reparative potential in this ethnic group, which may be partly attributable to higher Mon3 levels seen in AC subjects in my study. These hypotheses are perhaps further supported by the reported reparative properties of this subset.<sup>635</sup>

In this study I also show significant ethnic difference in SR-AI expression on Mon3 subset, with higher levels seen in SA subjects. These findings may offer a further explanation why SA subjects with HF have a better prognosis than Whites despite a higher rate of an ischaemic aetiology, a recognised risk factor of adverse prognosis, and on average 8-years younger age of presentation with HF.<sup>12, 67</sup> Indeed, emerging data indicate that SR-AI is critical for the number of reparative processes, such as clearance of apoptotic cells, attenuation of oxidative stress and inflammation.<sup>29-33</sup>

In contrast to the previous report of increased Mon2 levels in European patients with HF (compared with healthy controls), there were no significant HF-related changes in this

monocyte subset in subjects of SA origin.<sup>425</sup> Nonetheless, my results indicate significant ethnic variations for this subset with white subjects having the highest values, which possibly explain the discrepancy.

A feature of HF is the continuous cardiac remodelling associated with adaptation to the shifts in haemodynamics, tissue ischaemia and endothelial dysfunction.<sup>658</sup> Mon2 has the maximal (of all monocytes) expression of receptors to angiogenic factors, such as angiopoietin and VEGF. In patients with stroke, the Mon2 subset is increased whereas Mon3 is decreased, but both subsets were positively associated with a favourable prognosis (in contrast to Mon1).<sup>44</sup> Contrary, a recent small study showed a negative correlation between Mon2 count and LVEF and other parameters of HF severity, but this was not seen in our larger study population of SA patients with HF.<sup>425</sup> Thus, the clinical relevance of Mon2 subset should be explored further taking into account ethnic origin of the study population.

### **3.6.3. Microparticles derived from monocytes and platelets**

It has been shown that patients with systolic HF have increased levels of EMPs which independently predicted future risk of cardiovascular events.<sup>628</sup> In this study I show for the first time that patients with HF also have significantly increased levels of both MMPs and PMPs. Initially PMPs attracted attention of researchers and clinicians as markers and contributors of thrombosis.<sup>637</sup> However, their biological roles apparently spread far beyond their participation in thrombotic processes. For example, PMPs regulate activity of endothelial and other blood cells.<sup>576</sup> They can activate endothelial cells and neutrophils and

amplify leukocyte-mediated tissue injury in thrombotic and inflammatory disorders.<sup>577, 579,</sup>  
<sup>583</sup> Also, excessive generation of MMPs leads to disruption of endothelial integrity and prothrombotic activation of the endothelium.<sup>593</sup>

The present study indicates that although both MMPs and PMPs are increased in HF, only MMPs levels are independently and negatively predictive of LVEF. Although detailed mechanisms of such association are to be clarified in the future, several factors are likely to be implicated. Firstly, MMPs can impair endothelial function as discussed above. Secondly, high MMP levels parallel monocyte activation and may reflect an accompanying excessive inflammatory burden.<sup>47</sup> Thirdly, microparticles are increasingly acknowledged as messengers of biological information and direct regulators of various (patho)physiological processes and may thus be directly implicated in the pathogenesis of HF.<sup>48</sup>

#### **3.6.4. Endothelial progenitor cells**

In this study, I show *for the first time* significant abnormalities in monocyte-derived CD34+ and KDR+ EPCs in patients with systolic HF. The study also demonstrates the presence of significant ethnic differences in some populations of monocyte-derived EPCs, which may be related to the observed ethnic differences in clinical characteristics and prognosis in HF.

EPCs play a significant role in endothelial repair and angiogenesis.<sup>38</sup> Given that ischaemic aetiology is dominant among patients with systolic HF and local tissue hypoperfusion is

common in HF subjects irrespective of aetiology it is not surprising that abnormal numbers and activity of endothelial progenitors were seen in HF subjects.<sup>449</sup> The origin as well as the phenotypic and functional characteristics of EPCs still remains insufficiently understood and several cell types derived from the bone marrow, peripheral blood and tissue-resident cells may obtain endothelial phenotype under appropriate stimulation.<sup>38</sup> However CD14<sup>+</sup> myeloid monocytic cells have been shown to be a major type of circulating angiogenic cells.<sup>640</sup>

Despite similar numbers of total monocytes in the study groups, the numbers of CD34<sup>+</sup> monocytes were 2-fold lower in HF subjects than in both control groups with normal LV contractility. Importantly, these changes were only attributable to Mon1 and Mon2 subsets. The changes in CD34<sup>+</sup> monocytes were similar to that seen in circulating CD34<sup>+</sup>KDR<sup>+</sup> EPCs, which were also reduced in HF patients. The pattern of changes of KDR<sup>+</sup> monocytes was different from that seen for CD34<sup>+</sup> monocytes. Of note, KDR<sup>+</sup> Mon1 were 4.5-fold increased in patients with CAD with normal LVEF ('disease controls') than in healthy volunteers.

How could the differences seen between the CD34<sup>+</sup> and KDR<sup>+</sup> cells be explained? CD14<sup>+</sup>KDR<sup>+</sup> cells support vascular repair via release of angiogenic factors rather than through differentiation into functional endothelial cells per se. Indeed, transplantation of CD14<sup>+</sup>KDR<sup>+</sup> monocytes (but not CD14<sup>+</sup>KDR<sup>-</sup> cells) into murine balloon-injured femoral arteries significantly promoted reendothelialization.<sup>465</sup> However, CD14<sup>+</sup>KDR<sup>+</sup> cells *in vitro* stimulated the organization of human microvascular endothelial cells into capillary-

like structures rather conversion to endothelial cells themselves.<sup>467</sup> Also these cells were able to improve LV function after experimental MI.<sup>466</sup>

In contrast to KDR+ monocytes, CD34+ monocytes were reported to incorporate into the endothelium of blood vessels in ischaemic tissues and to transdifferentiate into endothelial cells.<sup>470</sup> This accords with *in vitro* findings that under angiogenic stimulation (e.g., with VEGF) monocytes can develop a typical endothelial phenotype with expression of specific surface markers.<sup>471</sup> Thus different types of monocyte-derived angiogenic cells may have different implications for vascular repair (for example, by providing an appropriate proangiogenic environment or by differentiation to endothelial cells). At present, it is difficult to confidently speculate on whether the down-regulation of monocyte-derived EPCs in HF patients is related to the pathogenesis of HF per se, or whether it is secondary to HF and reflects bone marrow depression common in severe HF.

In my study, counts of monocyte-derived EPCs did not correlate with parameters of cardiac geometry and function, raising the question of the clinical importance of these cells. However, higher VEGFR1 expression on the Mon2, a subset with a reported reparative potential showed some association with lower LVEF.

The study also highlights for the first time apparent differences between three major monocyte subsets in terms of their contribution to the pool of monocytic angiogenic cells in HF. Whilst HF was associated in with down-regulation of CD34+ and KDR+ monocytes derived from ‘classical’ Mon1 and CD34+ Mon2, endothelial progenitors derived from ‘non-classical’ monocytes Mon3 were not significantly affected. These observations



provide further evidence of differential roles of monocyte subsets in cardiovascular disorders.<sup>659-660</sup> Although detailed pathological implication and prognostic value of the abnormalities in monocyte-derived EPCs seen in this study are still to be clarified, they may potentially become novel therapeutic targets in the future. In this respect, the preliminary clinical success of transplantation of even unselected bone marrow mononuclear cells gives basis for some cautious optimism.<sup>661</sup>

Although the main focus of this thesis were subjects of SAs origin, significant ethnicity-related differences in characteristics of cardiovascular health have also been reported for Black Africans and ACs.<sup>662-664</sup> The pathophysiological background of these differences could be partly related to the worse profile of inflammatory markers and vascular dysfunction seen in even in relatively healthy AC subjects. Indeed, ACs with no vascular disease had higher fasting insulin, TNF- $\alpha$ , and IL-6 levels, greater carotid intima-media thickness and attenuated small vessel reactivity compared with Whites.<sup>665</sup> In accordance with previous data AC subjects in my study also tended to have higher blood pressure.<sup>665</sup>

### **3.6.5. Potential limitations**

One limitation is the relatively small number of AC subjects. There were difficulties in finding AC subjects that met strict inclusion criteria and many of them were reluctant to participate in this research.

This study is also limited by its cross-sectional design and it did not aim to assess clinical and prognostic implications of the found ethnic differences in endothelial function. The

study control groups were of SA origin aiming to focus on this high-risk ethnic group. However it is difficult to speculate whether the observations (both positive and negative) could be characteristic of HF in general, particularly given the number of significant ethnic differences in HF patients reported above.

The healthy controls were defined from careful clinical history and examination, but as angiography was not performed in these healthy subjects (for ethical reasons), there is a possibility that some of them could have subclinical CAD.

Admittedly ethnic minority groups are also heterogeneous and significant differences can be seen even within these groups (e.g., depending on environmental factors). For example, ACs from the UK had greater carotid intima media thickness, decreased endothelium dependent vasodilatation, higher homocysteine and lower folate levels than age- and sex-matched ACs in Jamaica.<sup>666-670</sup> This was associated with higher insulin resistance despite no significant differences in body mass index or the waist to hip ratio, suggesting a role of a nutritionally enriched environment, as a significant contributor to cardiovascular morbidity irrespectively of ethnicity.

### **3.7. Conclusions**

There are significant ethnicity-related differences in both micro- and macrovascular endothelial function in systolic HF. SA ethnicity is associated with microvascular endothelial dysfunction in this disorder.

Patients with systolic HF of SA origin have significantly reduced numbers and proportions of Mon3, a monocyte subset with reparative potential, and increased levels of PMPs, MMPs, and EMPs. Among different types of microparticles, only MMPs were independently predictive of reduced LVEF.

Circulating counts of monocyte-derived EPCs are significantly altered in HF, but their levels are not significantly associated with echocardiographic parameters of cardiac geometry and function.

There are significant ethnicity-related differences in monocyte-derived EPCs, monocyte subsets, their expression of scavenger receptor SR-AI in patients with systolic HF.

The study observations may contribute to understanding of the role of the endothelium, different monocyte populations and blood microparticles in the complex pathophysiology of HF. Additionally these findings may provide some insight to the known clinical ethnicity-related differences in HF and could be useful for developing future strategies of HF management.

## CHAPTER IV. DEFINITION AND CHARACTERISATION OF HUMAN MONOCYTE SUBSETS

### 4.1. Introduction

Until recently, monocytes were divided into two subsets: ‘classical’ CD14<sup>+</sup>CD16<sup>-</sup> monocytes and ‘non-classical’ CD14<sup>low</sup>CD16<sup>+</sup> subsets. However it was observed that the immunophenotypic pattern of CD16<sup>+</sup> cells includes both cells with low and high CD14 surface levels and that the expression of important surface antigens varies between these cells (sometimes defined as CD14<sup>+</sup>CD16<sup>+</sup> and CD14<sup>++</sup>CD16<sup>+</sup> respectively).<sup>40, 44-45</sup> However, because there is an overlap between these two subsets when they are defined just by CD14 and CD16 expression, an unequivocal discrimination and accurate characterisation of these subsets is problematic. As a result only scarce data are currently available on biological and functional characteristics of the individual monocyte subsets.

Given that the work reported in this part of the thesis was done when a substantial part of data from Chapter 3 had been collected, the newly developed protocol of monocyte analysis could not be used retrospectively for subjects investigated in the previous chapter. Thus the new protocol on monocyte flow cytometric characterisation is described in this chapter.

## **4.2. Aims and hypotheses**

The aim of this part of the study was to develop an approach of objective discrimination of the three human monocyte subsets, and to provide some insight on the phenotypic, and functional properties of the monocyte subsets and to assess effects of the diurnal variation and exercise on monocyte subsets and MPAs.

I also hypothesized that monocyte subsets and MPAs would be affected by physical exercise, and undergo diurnal circadian variations.

### **4.3. Study population**

#### **4.3.1. Overall comments**

Assessment of blood monocytes has been done on young healthy volunteers. The risk of presence of subclinical atherosclerosis in healthy volunteers was considered low based on lack of symptoms in young active individuals, profile of risk factors for atherosclerosis, lack of any suggestive signs or symptoms during the exercise (in 12 participants). Additionally a fraction of those volunteers undergone measurement of carotid intima-media thickness (a surrogate parameter for early atherosclerosis) as part of a parallel study ongoing in the department.

The study was approved by the Warwickshire Research Ethics Committee (reference number 08/H1211/23, dated 04.09.2008). Additional amendments have been granted by the same committee to allow substudies on monocyte subsets in healthy volunteers (amendment 1, dated 12.11.2008, amendment 3, dated 24.04.2009, and amendment 5, dated 14.05.2009).

The immunophenotypic analysis of bone marrow cells was performed on anonymised samples provided by the Clinical Immunology Service (Professor M.T. Drayson). These samples were the residue of normal bone marrow taken and sent for routine clinical testing for lymphoma staging; these residues would normally have been incinerated. All participants signed an informed consent for analysis of their bone marrow cell immunophenotype and the Clinical Immunology Service has ethics approval for use of these samples for research and development

### **4.3.2. Immunophenotypic characterisation**

Peripheral vein blood samples were collected from 20 carefully selected non-fasting healthy volunteers (aged  $30.3 \pm 6.4$  years, 8 male, body mass index  $23.2 \pm 2.9$  kg/m<sup>2</sup>) between 11.00 and 12.00h, which were processed within 30 minutes of collection. Bone-marrow samples were obtained from 11 individuals with suspected peripheral lymphomas (otherwise healthy and with no bone-marrow involvement).

### **4.3.3. Effects of physical exercise**

The effects of exercise on the dynamics of total monocyte count, monocyte subsets and MPAs, as well as monocyte expression of CD14, CD16, CCR2 were assessed in 12 healthy volunteers (age  $35.3 \pm 7.62$  years, 8 male). 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.<sup>671</sup> 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. In this pilot exercise, the highest monocyte counts were seen at 15 minutes and lowest at 1 hour and these time-points were selected for the for the rest of the participants. All participants were asked to abstain from other strenuous physical activity for the period of the study.

#### **4.3.4. Diurnal variation**

Possible diurnal variations in monocyte subsets and MPAs were assessed in 16 healthy volunteers (age  $36\pm 8.7$  years, 7 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).

#### **4.3.5. Time-delay analysis**

Effects of delay in sample preparation on parameters of monocyte subsets and MPAs were assessed in 12 samples. The samples were reprocessed at 1, 2, and 4 hours after collection in order to quantify temporal changes in the study parameters once ex vivo (4 samples in total). The samples were kept at room temperature with slow rotation.



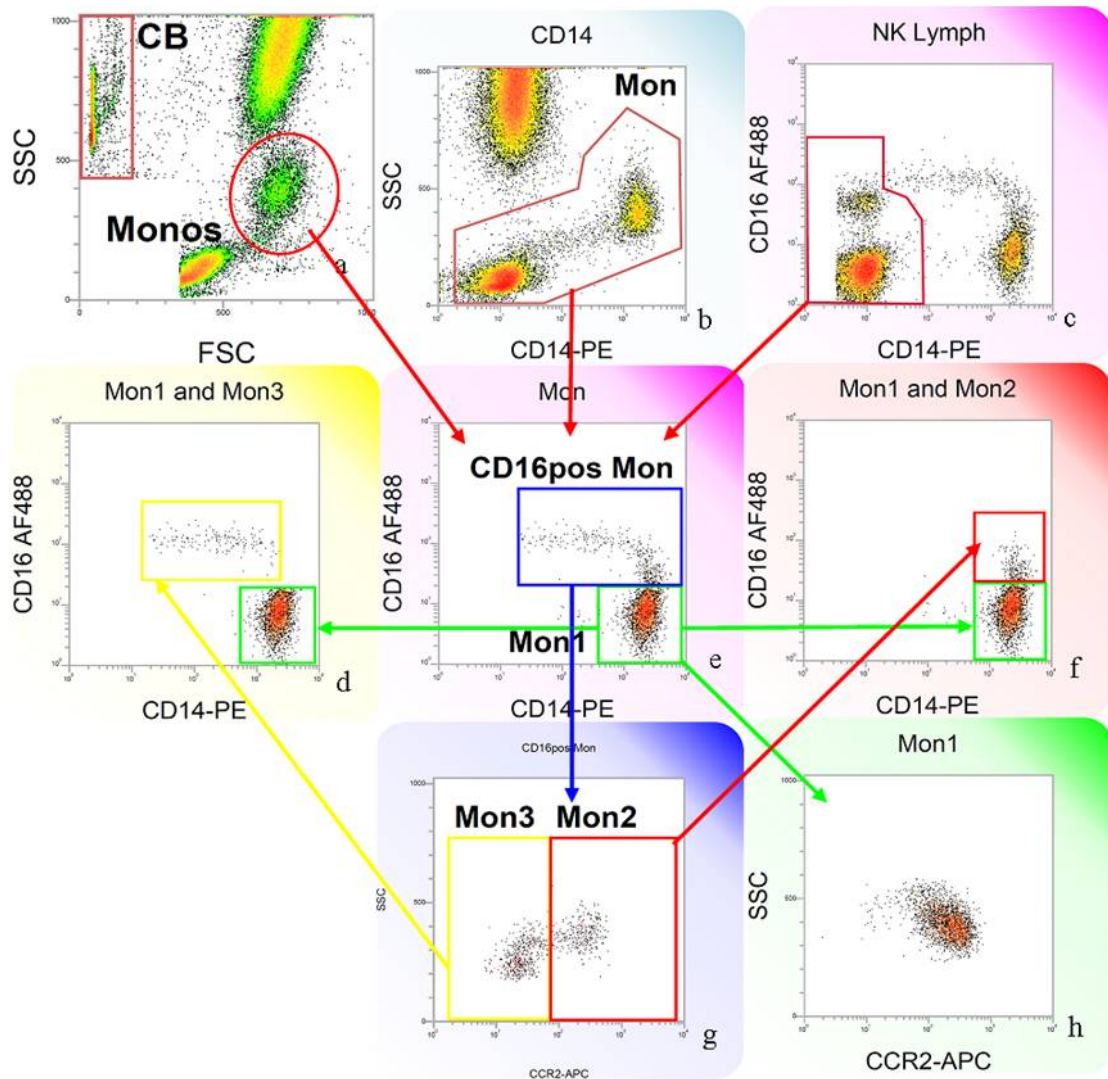
## **4.4. Methods: Flow cytometry**

### **4.4.1. Equipment and software**

Flow cytometric analysis was performed using the BD FACSCalibur flow cytometer (Becton Dickinson, Oxford, UK [BD]). VenturiOne, Version 3.1 software (Applied Cytometry, Sheffield, UK) was employed for data analysis.

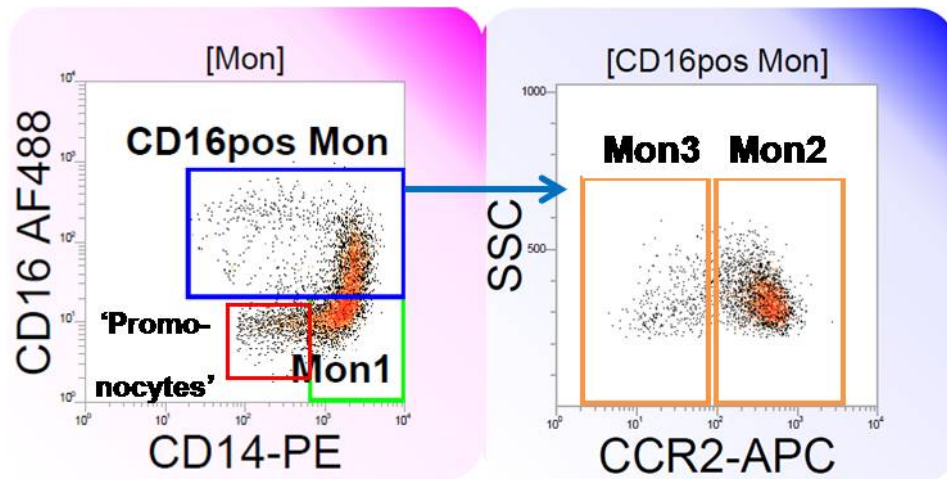
### **4.4.2. Absolute count of monocyte subsets**

Mouse anti-human monoclonal fluorochrome-conjugated antibodies anti-CD16-Alexa Fluor 488 (clone DJ130c, AbDSerotec, Oxford, UK), anti-CD14-PE (clone MφP9, BD), anti-CD42a-PerCP (clone Beb1, BD) and anti-CCR2-APC (clone 48607, R&D) were mixed with 50µl of fresh EDTA-anticoagulated whole blood or bone marrow in BD TruCount tubes (BD) containing a strictly defined number of fluorescent count beads. After incubation for 15 minutes red blood cells were lysed by 450µl of BD lysing solution® (BD) for 15 minutes followed by dilution in 1.5 ml of PBS and immediate flow cytometric analysis. Monocytes were selected by gating strategies based on forward and side scatter properties to select monocytes (Figure. 17a), side scatter properties versus CD14 expression to exclude granulocytes (Figure. 17b), and ungated CD14 versus CD16 expression to exclude natural killer lymphocytes (Figure. 17c). Monocyte subsets were defined as CD14<sup>++</sup>CD16<sup>-</sup>CCR2<sup>+</sup> cells (Mon1), CD14<sup>++</sup>CD16<sup>+</sup>CCR2<sup>+</sup> cells (Mon2) and CD14<sup>+</sup>CD16<sup>+</sup>CCR2<sup>-</sup> cells (Mon3) (Figures 17 and 18).



**Figure 17. Gating strategies and presentation of monocyte subsets**

(a) selection on monocyte region on forward scatter (FSC) vs. side scatter (SSC) plot, (b) separation of mononuclear cells from granulocytes, (c) selection of CD14<sup>-</sup> lymphocytes to separate CD16<sup>+</sup>CD14<sup>-</sup> natural killer lymphocytes from CD16<sup>+</sup> monocyte subsets, (e) separation of monocyte subsets, that must correspond to regions Monos on (a) and Mon on (b) but must not include lymphocytes from (c), (g) separation of CD16<sup>+</sup> monocyte subsets on the basis of their CCR2 expression, (h) CCR2 expression of Mon1 subset, (d) and (f) location of on Mon3 and Mon2, respectively, on CD14 vs. CD16 plot. Mon 1 – CD14<sup>++</sup>CD16<sup>-</sup>CCR2<sup>+</sup> monocytes, Mon 2 – CD14<sup>++</sup>CD16<sup>+</sup>CCR2<sup>+</sup> monocytes, Mon 3 – CD14<sup>+</sup>CD16<sup>+</sup>CCR2<sup>-</sup> monocytes.



**Figure 18. Gating strategies and presentation of bone marrow monocytes**

(a) selection on monocyte region on forward scatter (FSC) vs. side scatter (SSC) plot, (b) separation of CD16+ monocyte subsets on the basis of their CCR2 expression.

Absolute counts of monocyte subsets (cells/ $\mu$ l) were obtained by calculating the number of monocytes proportional to the number of count beads according to the manufacturers recommendations. MPAs were defined as events positive to both monocyte markers (as above) and the platelet marker CD42a (glycoprotein IX) (Figures 17 and 18).

My intra-assay CV was performed on blood samples taken from two healthy subjects (four samples from each) (Table 33). Intra-assay CV was: total monocyte count – 2.31%, Mon1 – 1.91%, Mon2 – 5.23%, Mon3 – 8.29% (average for monocyte counts – 4.44%), MPAs – 6.11%. My inter-assay CV was performed on blood samples taken from one healthy subject on six consequent days (Table 34). Inter-assay CV was: total monocyte count – 3.36%, Mon1 – 3.68%, Mon2 – 13.01%, Mon3 – 9.16% (average for monocyte counts – 7.30%), MPAs – 8.28%. The method is described in the Standard operating procedure 201 ‘Monocyte subsets, monocyte-platelet aggregates by flow cytometry’.

**Table 33. Intra-assay variability of measurement of monocyte subsets and monocyte-platelet aggregates**

		Total monocytes (per $\mu$ l)	Mon1 (per $\mu$ l)	Mon2 (per $\mu$ l)	Mon3 (per $\mu$ l)	MPA (per $\mu$ l)
Subject 1	Sample 1	470	397	28.7	44.4	38.7
	Sample 2	459	390	29.0	40.6	33.3
	Sample 3	454	383	26.9	43.9	35.2
	Sample 4	447	381	28.4	37.7	35.5
	Mean $\pm$ SD	457 $\pm$ 9.85	387 $\pm$ 7.32	28.6 $\pm$ 0.96	42.2 $\pm$ 3.13	35.4 $\pm$ 2.20
	CV, %	2.16	1.89	3.37	7.42	6.24
Subject 2	Sample 1	213	169	10.8	33.1	21.0
	Sample 2	211	163	12.7	35.8	21.5
	Sample 3	222	169	12.3	40.7	23.8
	Sample 4	221	170	11.7	39.3	21.5
	Mean $\pm$ SD	217 $\pm$ 5.34	169 $\pm$ 3.25	12.0 $\pm$ 0.85	37.6 $\pm$ 3.44	21.5 $\pm$ 1.28
	CV, %	2.46	1.92	7.09	9.17	5.97
<i>Intra-assay CV (average), %</i>		2.31	1.91	5.23	8.29	6.11

CV, coefficient of variability; MPAs, monocyte-platelet aggregates; SD, standard deviation.

**Table 34. Inter-assay variability of measurement of monocyte subsets and monocyte-platelet aggregates**

	Total monocytes (per $\mu$ l)	Mon1 (per $\mu$ l)	Mon2 (per $\mu$ l)	Mon3 (per $\mu$ l)	MPA (per $\mu$ l)
Day 1	470	397	28.7	44.4	38.7
Day 2	447	384	26.1	36.6	32.5
Day 3	443	380	25.3	37.7	31.2
Day 4	455	398	21.7	35.0	35.1
Day 5	456	387	31.0	37.8	36.7
Day 6	425	360	30.5	35.2	37.5
Mean $\pm$ SD	449 $\pm$ 15.1	384 $\pm$ 14.1	27.2 $\pm$ 3.54	37.8 $\pm$ 3.46	35.3 $\pm$ 2.92
<i>Inter-assay CV, %</i>	3.36	3.68	13.0	9.16	8.28

CV, coefficient of variability; MFI, media fluorescent intensity, MPAs, monocyte-platelet aggregates; SD, standard deviation.

#### 4.4.3. Expression of surface antigens on monocyte subsets

For analysis of surface antigens, 100µl of whole blood or bone marrow was incubated with mouse anti-human monoclonal fluorochrome-conjugated antibodies for 15 minutes in the dark. Red blood cells were lysed with 2ml of BD lysing solution® for 10 min, washed in PBS followed by immediate flow cytometric analysis. Anti-CD16-Alexa Fluor 488 (clone DJ130c, AbDSerotec, Oxford, UK) and anti-CD14-PerCP-Cy5.5 (clone M5E2, BD) were used for definition of monocyte subsets into CD14<sup>++</sup>CD16<sup>-</sup> monocytes (Mon1), CD14<sup>++</sup>CD16<sup>+</sup> monocytes (Mon2) and CD14<sup>+</sup>CD16<sup>+</sup> monocytes (Mon3). PE-conjugated antibodies were used against TLR4 (clone 285219, R&D), integrin β2/CD18 (ICAM receptor), clone 212701, R&D Systems Europe Ltd, Abingdon, UK [R&D]), CXCR4 (SDF-1 receptor), clone 12G5, R&D), CD34 (clone 8G12, BD), VEGF receptor 1 (clone 49560, R&D), ferritin (monoclonal antibody by AbdSerotec, Oxford, UK, clone F317B2 conjugated to PE using LL-PE-XL conjugation kit from Innova Biosciences, UK), CD64 (clone 276426, R&D). APC-conjugated antibodies were used against IL6 receptor (clone 17506, R&D), integrin α4/CD49d (VCAM-1 receptor), clone 7.2R, R&D), CD163 (clone 215927, R&D), KDR (VEGF receptor 2, clone 89106, R&D), Tie2 (clone 83715, R&D), CD115 (clone 61708, R&D), CD204 (clone 351520, R&D) and ApoB (clone 369717, R&D). Monoclonal antibodies for last two antigens were conjugated to APC using LL-APC-XL conjugation kit [Innova Biosciences, UK].

My inter-assay CV for a surface marker expression (CCR2) on Mon1 – 3.69%, on Mon2 – 4.15%, on Mon3 – 4.07% (average for monocyte CCR2 expression – 3.97%). Inter-assay CV for a surface marker expression (CCR2) on Mon1 – 10.24%, on Mon2 – 4.85%, on

Mon3 – 4.86% (average for monocyte CCR2 expression – 6.65%)(Table 35). The method is described in more details in Standard operating procedure 201 ‘Monocyte subsets, monocyte-platelet aggregates by flow cytometry’.

**Table 35. Intra- and inter-assay variability of measurement of monocyte surface expression of CCR2**

		CCR2 (Mon1) MFI	CCR2 (Mon2) MFI	CCR2 (Mon3) MFI
<b>Intra-assay variability assessment</b>				
Subject 1	Sample 1	140	106	15.1
	Sample 2	135	107	15.1
	Sample 3	129	106	15.8
	Sample 4	122	97.0	14.1
	Mean±SD	132±7.76	106±4.73	15.1±0.68
	CV, %	5.88	4.46	4.53
Subject 2	Sample 1	86.5	85.8	13.8
	Sample 2	85.8	91.3	12.9
	Sample 3	88.1	92.0	12.8
	Sample 4	85.1	85.7	13.6
	Mean±SD	86.1±1.28	88.6±3.40	13.2±0.48
	CV, %	1.50	3.84	3.61
<i>Intra-assay CV (average), %</i>		<i>3.69</i>	<i>4.15</i>	<i>4.07</i>
<b>Inter-assay variability assessment</b>				
Day 1		140	106	15.1
Day 2		116	102	15.3
Day 3		114	108	15.0
Day 4		123	97.7	13.5
Day 5		106	98.0	15.4
Day 6		110	96.3	15.1
Mean±SD		118±12.1	101±4.92	14.9±0.73
<i>Inter-assay CV, %</i>		<i>10.2</i>	<i>4.85</i>	<i>4.86</i>

CV, coefficient of variability; MFI, median fluorescent intensity, MPAs, monocyte-platelet aggregates; SD, standard deviation.

#### 4.4.4. Assessment of intracellular activation of NFκB pathway

Stimulation of monocyte receptors (including Toll-like receptors and CD14) by a variety of stimuli results in activation of the nuclear factor κB (NFκB) pathway. Phosphorylation and disintegration of IκBs by the specific inhibitory κB kinases (IKK) is followed by translocation of cytoplasmic NFκB into the nucleus. Here, it binds to the promoter region of various genes, including cytokines (e.g., TNF-α, and IL-1β), cyclooxygenase-2, inducible NO-synthase, and matrix metalloproteases, thereby activating their transcription.<sup>672-673</sup>

The IKK complex consists of two catalytic subunits of which IKKβ, but not IKKα, plays an essential role in NFκB activation mediated by lipopolysaccharides, TNFα, or IL-1. IKKβ is 20-fold more active than IKKα.<sup>674-675</sup> Because the activated (translocated into nuclei) form of NFκB is identical to its resting cytoplasmic form, determination of NFκB total levels inside a cell is not informative. Several methods of NFκB assessment in isolated nuclei are associated with multistep procedures of cell isolation and preparation which are likely to cause some degree of artificial activation.<sup>676</sup> For this study, I developed a method of direct measurement of intracellular levels of IKKβ as a surrogate cytoplasmic marker of activation of NFκB pathway, aiming to assess proinflammatory shift in monocyte phenotype and function.

To assess intracellular levels of IKKβ, 100μl of fresh whole blood was incubated with monoclonal mouse-antihuman antibodies against CD16-Alexa Fluor 488 (clone DJ130c, AbDSerotec, Oxford, UK) and CD14-PerCP-Cy5.5 (clone M5E2, BD) for 15 min

following by lysing of red blood cells with 2ml of BD PharmLyse™ for 10 min and washing in staining buffer. The resulting pellet was resuspended in Fixation/Permeabilization solution (BD) for 20 minutes and, following centrifugation, in 2 ml of BD Perm/Wash™ buffer for 10 minutes. Following further centrifugation the pellet was incubated for 30 minutes with monoclonal mouse anti-human APC-conjugated (LL-APC-XL conjugation kit [Innova Biosciences, UK]) antibodies against IKK $\beta$  (clone 10A9B6, Abcam, Cambridge, UK), washed and resuspended in 200 $\mu$ l of 2% PBS/2% paraformaldehyde solution (PharmFix, BD) for immediate flow cytometric analysis.

Intra-assay CVs for the method were 5.52% for Mon1, 5.75% for Mon2, and 4.60% for Mon3 (Table 36). Inter-assay CVswere9.2% form Mon1, 9.7% for Mon2, 11.4%for Mon3 (Standard operating procedure 202 ‘Intracellular I-Kappa-Kinase Beta (IKK $\beta$ )’).



**Table 36. Intra- and inter-assay variability of measurement of intracellular monocyte IKK $\beta$  levels**

		Mon1, MFI	Mon2, MFI	Mon3, MFI
<b>Intra-assay variability assessment</b>				
Subject 1	Sample 1	66.28	65.6	60.47
	Sample 2	72.47	74.71	63.24
	Sample 3	76.24	73.11	63.8
	Mean $\pm$ SD	71.7 $\pm$ 4.1	71.1 $\pm$ 4.0	62.5 $\pm$ 2.3
	CV, %	5.7	5.6	2.3
Subject 2	Sample 1	77.6	81.0	80.0
	Sample 2	69.6	70.3	67.5
	Sample 3	70.9	73.9	74.4
	Mean $\pm$ SD	72.7 $\pm$ 3.51	75.1 $\pm$ 4.4	74.0 $\pm$ 5.1
	CV, %	4.8	5.9	6.9
<i>Intra-assay CV (average), %</i>		5.25	5.75	4.60
<b>Inter-assay variability assessment</b>				
Day 1		59.8	63.1	65.3
Day 2		55.4	57.6	54.7
Day 3		59.4	73.7	60.6
Day 4		71.1	74.9	70.7
Day 5		70.1	75.3	78.7
Day 6		64.3	66.4	68.0
Mean $\pm$ SD		63.4 $\pm$ 5.8	68.5 $\pm$ 6.6	68.0 $\pm$ 7.8
<i>Inter-assay CV, %</i>		9.2	9.7	11.4

CV, coefficient of variability; MFI, media fluorescent intensity.

#### 4.4.5. Monocyte phagocytic activity

The phagocytic activity of monocyte subsets was assessed in 15 healthy individuals using novel commercially available pHrodoE.Coli BioParticles Phagocytosis Kit for Flow Cytometry (Invitrogen, California, UK). The assay is based on the fluorescence of pHrodo™ E. coli BioParticles® conjugates, which are inactivated, unopsonized Escherichia coli filled with the fluorochrome with minimal fluorescence at neutral pH (eg, in blood) that dramatically increases in acidic condition (i.e., inside phagocytome). The assay was run according to the manufacture recommendations with CD16-Alexa Fluor 488 (as above) and CD14-APC (clone MφP9, BD) used to discriminate monocyte subsets. Expression of the surface markers, IKKβ levels and phagocytic activity are presented as median fluorescent activity (MFI).

Intra-assay CVs for the method are for Mon1 - 5.4%, for Mon2 - 7.1%, for Mon3 - 1.4% (average for the three monocyte subsets – 4.63% (Table 37. Standard operating procedure 206 ‘Assessment of monocyte phagocytic activity by Flow Cytometry’).

**Table 37. Intra-assay variability of measurement of monocyte phagocytic activity**

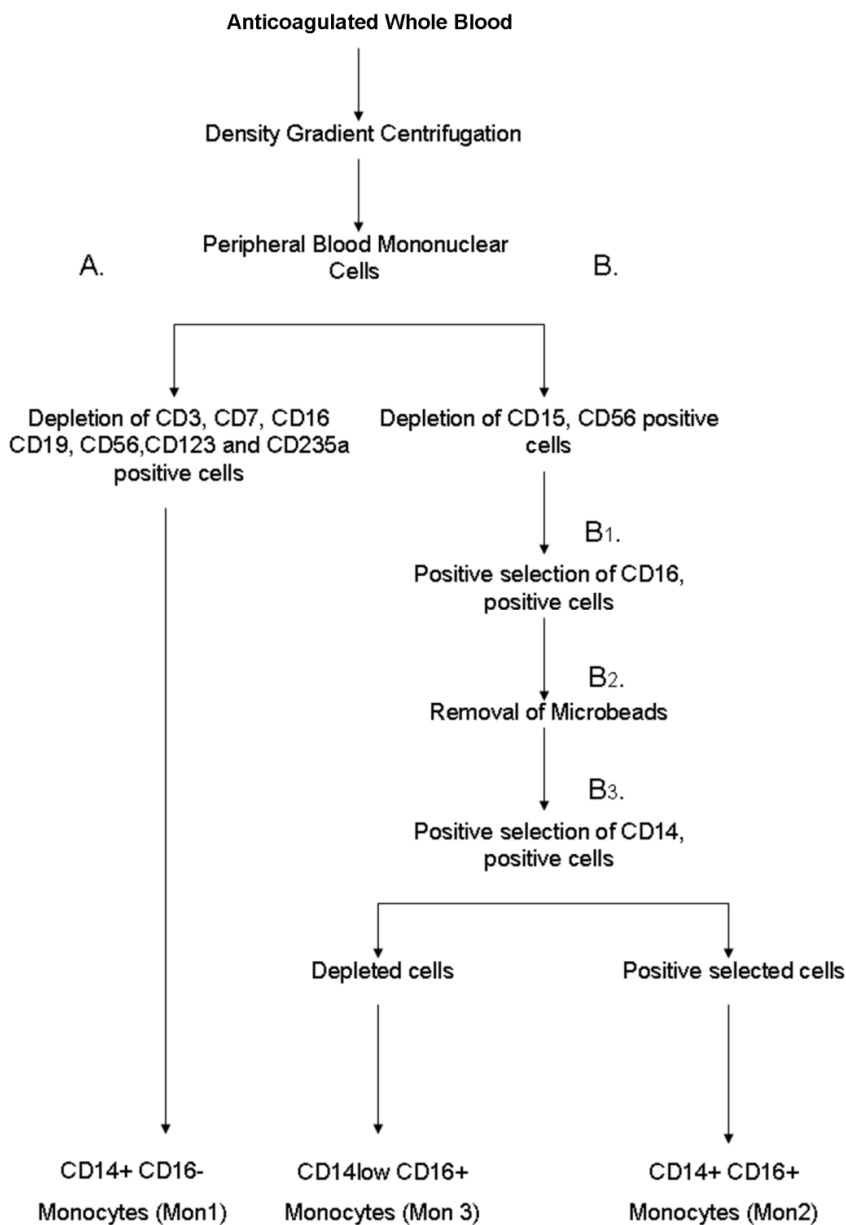
Subsets	Sample 1	Sample 2	Sample 3	Intra-assay CV*
Mon1, MFI	155.38	139.49	147.22	5.4%
Mon2, MFI	155.38	137.00	138.24	7.1%
Mon3, MFI	56.23	54.74	55.73	1.4%

Average intra-assay CV is 4.63%

#### 4.4.6. Cell sorting and stimulation experiments

Thirty ml of citrate-anticoagulated blood were collected from 3 healthy volunteers. Peripheral blood mononuclear cells were isolated by Ficoll-Paque density gradient centrifugation, as previously described.<sup>677</sup> Monocyte subsets were further isolated by magnetic sorting of the PBMC fraction using VarioMACS (MiltenyiBiotec, Düsseldorf, Germany) and direct or indirect labeling with microbead-conjugated antibodies. The whole procedure is schematically illustrated (Figure 19).

Monocyte subsets obtained by cell sorting were resuspended at a density of  $1 \times 10^6$  cells/ml, in culture medium consisting of RPMI-1640 supplemented with 2 mM L-glutamine, 100 µg/ml gentamycin and 100 µg/ml penicillin. Cell suspensions were incubated in gently shaking polystyrene tubes, at 37°C, in a 5% CO<sub>2</sub> humidified atmosphere, in the presence or absence of 1 µg/ml LPS endotoxin. Cells were harvested 24 hours post stimulation. Intracellular staining for 6 inflammatory markers (IL-1β, MCP-1, IL-6, IL-10, TNF-α and IKKβ) was performed as described above. Allophycocyanin (APC) conjugated antibodies were used for intracellular staining of IL-10, TNF-α and IKKβ while phycoerythrin (PE) conjugated markers were utilized for the intracellular staining of IL-1 β, MCP-1 and IL-6 (all BD, Oxford, UK). The MFI of stained cells was assessed by the FACScalibur cytometer (BD, Oxford, UK). Experiments were conducted in triplicates and mean values were compared. Optimal time points and LPS concentrations were retrieved through a set of preliminary experiments where 2, 4 and 24 hours of stimulation and 0.1, 1 and 10 µg/ml of LPS were evaluated.



**Figure 19. Schematic illustration of monocyte isolation by magnetic sorting**

After density gradient centrifugations cells were either processed for CD14<sup>++</sup>CD16<sup>-</sup> isolation (root A) or CD14<sup>++</sup>CD16<sup>+</sup>/CD14<sup>+</sup>CD16<sup>+</sup> isolation (root B). For root A, Monocyte Isolation Kit I was used (MiltenyiBiotec, Dusseldorf, Germany). For step B<sub>1</sub>, CD16<sup>+</sup>Monocyte isolation Kit (MiltenyiBiotec, Dusseldorf, Germany) was used with the following modification: instead of CD16-microbeads, CD16-FITC conjugated antibody was used (MiltenyiBiotec, Dusseldorf, Germany). Cells were then indirectly labelled with anti-FITC microbeads and CD16 presenting cells were positively selected. Anti-FITC microbeads were removed (step B<sub>2</sub>) using the anti-FITC multisort Kit (MiltenyiBiotec, Dusseldorf, Germany) in order cells to be relabelled (step B<sub>3</sub>) with CD14 microbeads (MiltenyiBiotec, Dusseldorf, Germany).

#### **4.4.7. Statistical analysis**

Data are expressed as mean $\pm$ SD for normally distributed parameters and median (interquartile range) for non-normally distributed parameters. Statistical significance of differences between monocyte subsets was determined by ANOVA with Tukey's post-hoc test for normally distributed parameters and Friedman test with Dunns post-hoc test or Tukey's post-hoc test of arithmetically transformed variables for non-normally distributed parameters. For comparisons of values in the presence or absence of endotoxin stimulation, paired-sample t-test was utilized.

Statistical significance of the changes in different monocyte subsets and MPAs during diurnal variation analysis, exercise test and time-delay analysis was assessed using repeated measures ANOVA. Greenhouse-Geisser correction was applied when sphericity assumption was not met. Pairwise comparisons were made using Sidak's adjustment. Friedman test was utilized for non-normally distributed parameters. A value of  $p < 0.05$  (two-tailed) was considered statistically significant. Statistical analysis was performed with SPSS18.0 software and GraphPad Prism 4.0 software (La Jolla, CA, USA). A power calculation determined the need for at least 12 subjects in order to have 80% power to detect changes of  $\geq 0.5$  standard deviation, based upon our preliminary work on monocyte subsets and the assessment of biological fluctuations in circulating blood cells.

## 4.5. Results

### 4.5.1. Discrimination of monocyte subsets

During the screening of various parameters I have found that two distinct populations of CD16<sup>+</sup> monocytes can be reliably discriminated on the basis of their expression of CCR2 (MCP-1 receptor) (Figure 17). CD14<sup>++</sup>CD16<sup>+</sup> monocytes were positive for CCR2 (MFI 152.5±55.6) whilst CD14<sup>+</sup>CD16<sup>+</sup> cells expressed minimal if any levels of CCR2 (MFI 18.4±1.90,  $p < 0.0001$ ) (Table 38). CD14<sup>++</sup>CD16<sup>-</sup> monocytes had maximal density of CCR2 on their surface (MFI 241.5 ±49.2,  $p < 0.0001$  vs. the 2 other monocyte subsets).

To simplify further characterisation of monocyte subsets in the manuscript, I classified them as Mon1 (CD14<sup>++</sup>CD16<sup>-</sup>CCR2<sup>+</sup>), Mon2 (CD14<sup>++</sup>CD16<sup>+</sup>CCR2<sup>+</sup>) and Mon3 (CD14<sup>+</sup>CD16<sup>+</sup>CCR2<sup>-</sup>) subsets. On the CD14 vs. CD16 plot, there is a small area which may include proportions of both Mon2 and Mon3 subsets (Figures. 17d vs. 17f). Accordingly, CCR2 is essential for determining the absolute count of the three monocyte subsets. However, the two markers (CD14 and CD16) were enough to characterise surface antigen profile, providing that a small gap was left between Mon2 and Mon3 regions to prevent contamination from another subset.

In healthy subjects, the Mon1 subset constituted 84.6±5.77% of all monocytes (360.1±87.0 cells/μl); whilst Mon2, 5.90±3.40% (24.5±14.8 cells/μl); and Mon3, 9.51±3.74% (38.9±12.7 cells/μl) (Table 38). Although all Mon2 express CD16, CD16 density on this subset was 3.5-fold lower compared to Mon3 ( $p < 0.0001$ ). Mon2 represented the monocyte subset with largest size (on the basis of forward scatter

**Table 38. Comparative characteristics of monocyte subsets**

Parameter	Mon1	Mon2	Mon3	p
Count, cell/ $\mu$ l	360.1 $\pm$ 87.0	24.5 $\pm$ 14.8	38.9 $\pm$ 12.7	
% of all monocytes	84.6 $\pm$ 5.77	5.90 $\pm$ 3.40	9.51 $\pm$ 3.74	
Aggregates with platelets, %	10.4 $\pm$ 2.30*	16.0 $\pm$ 5.65 <sup>‡</sup>	11.95 $\pm$ 2.56	<0.001
Forward scatter	696 $\pm$ 20.0* <sup>†</sup>	715 $\pm$ 20.3 <sup>‡</sup>	673 $\pm$ 18.6	<0.001
Side scatter	360 $\pm$ 82.1 <sup>†</sup>	357 $\pm$ 29.9 <sup>‡</sup>	279 $\pm$ 27.0	<0.001
CCR2, MFI	241.5 $\pm$ 49.2* <sup>†</sup>	152.5 $\pm$ 55.6 <sup>‡</sup>	18.4 $\pm$ 1.90	<0.001
CD14, MFI	1737 $\pm$ 296 <sup>†</sup>	1820 $\pm$ 400 <sup>‡</sup>	146 $\pm$ 63.8	<0.001
CD16, MFI	7.02 $\pm$ 1.02* <sup>†</sup>	37.0 $\pm$ 5.94 <sup>‡</sup>	128.1 $\pm$ 49.5	<0.001
CD64, MFI <sup>§</sup>	369.9 $\pm$ 91.5 <sup>†</sup>	349.5 $\pm$ 106.8 <sup>‡</sup>	61.2 $\pm$ 45.4	<0.001
CD115+, %	77.5 (37.6-87.4)* <sup>†</sup>	99.1 (93.5-99.8) <sup>‡</sup>	87.2 (58.3-93.6)	<0.001
CD115, MFI	13.6 $\pm$ 6.24* <sup>†</sup>	34.8 $\pm$ 14.9 <sup>‡</sup>	20.1 $\pm$ 9.89	<0.001
CD204+, %	24.5 $\pm$ 14.9* <sup>†</sup>	56.9 $\pm$ 16.7 <sup>‡</sup>	61.1 $\pm$ 14.29	<0.001
CD204, MFI	5.84 $\pm$ 2.32* <sup>†</sup>	11.5 $\pm$ 3.91	14.6 $\pm$ 6.66	<0.001
CD163+, %	98.6 $\pm$ 1.27 <sup>†</sup>	99.7 $\pm$ 0.32 <sup>‡</sup>	70.3 $\pm$ 13.7	<0.001
CD163, MFI	201.8 $\pm$ 79.1* <sup>†</sup>	289.2 $\pm$ 90.3 <sup>‡</sup>	24.3 $\pm$ 14.9	<0.001
Integrin $\beta$ 2, MFI <sup>§</sup>	53.8 $\pm$ 18.8* <sup>†</sup>	100.2 $\pm$ 26.5 <sup>‡</sup>	57.8 $\pm$ 21.3	<0.001
Integrin $\alpha$ 4+, %	74.7 (59.8-82.2)* <sup>†</sup>	94.7 (91.1-96.7) <sup>‡</sup>	99.1 (98.2-99.4)	<0.01
Integrin $\alpha$ 4, MFI	14.4 $\pm$ 3.12* <sup>†</sup>	30.2 $\pm$ 8.07 <sup>‡</sup>	58.0 $\pm$ 6.78	<0.001
CXCR4+, %	91.0 (79.3-96.2)* <sup>†</sup>	95.6 (92.0-97.9) <sup>‡</sup>	51 (42.9-58.9)	<0.01
CXCR4, MFI	21.8 (16.0-39.3)* <sup>†</sup>	31.3 (24.8-47.5) <sup>‡</sup>	9.95 (8.40-12.6)	<0.01
Tie2+, %	25.1 $\pm$ 5.46	53.7 $\pm$ 8.87	31.9 $\pm$ 13.9	<0.001
Tie2, MFI	6.2 $\pm$ 0.75* <sup>†</sup>	10.4 $\pm$ 1.67 <sup>‡</sup>	7.0 $\pm$ 2.24	<0.001
VEGFR1+, %	18.2 (14.5-52.0)	69.6 (62.3-85.3)	28.3 (20.75-33.23)	<0.001
VEGFR1, MFI	6.15 (5.65-9.88)* <sup>†</sup>	14.1 (12.0-19.6) <sup>‡</sup>	5.50 (4.90-6.28)	<0.001
KDR+, %	5.00 (3.38-7.23)* <sup>†</sup>	8.25 (5.65-10.9) <sup>‡</sup>	2.80 (2.23-4.58)	<0.001
CD34+, %	0.45 (0.2-1.0)* <sup>†</sup>	1.45 (0.83-3.3)	2.40 (1.8-3.7)	<0.001
Ferritin+, %	9.58 $\pm$ 7.41* <sup>†</sup>	18.8 $\pm$ 9.94 <sup>‡</sup>	4.0 $\pm$ 2.55	<0.001
Ferritin, MFI	4.8 $\pm$ 0.96* <sup>†</sup>	5.7 $\pm$ 1.24 <sup>‡</sup>	2.9 $\pm$ 0.51	<0.001
ApoB+, %	28.8 $\pm$ 14.7* <sup>†</sup>	46.7 $\pm$ 17.5 <sup>‡</sup>	28.4 $\pm$ 10.4	0.001
ApoB, MFI	6.3 $\pm$ 2.32* <sup>†</sup>	9.3 $\pm$ 3.37 <sup>‡</sup>	6.3 $\pm$ 1.56	0.001
TLR4+, %	18.4 (14.3-32.2) *	63.2 (46.4-75.2) <sup>‡</sup>	21.4 (18.4-29.9)	<0.001
TLR4, MFI	6.50 (5.60-7.70) * <sup>†</sup>	12.4 (8.93-15.9) <sup>‡</sup>	5.20 (4.53-6.18)	<0.001
IL-6 receptor, MFI <sup>§</sup>	62.3 $\pm$ 10.0 <sup>†</sup>	58.9 $\pm$ 8.95 <sup>‡</sup>	30.6 $\pm$ 5.92	<0.001
IKK $\beta$ , MFI	61.1 $\pm$ 16.7 <sup>†</sup>	47.7 $\pm$ 9.70	42.6 $\pm$ 5.91	0.013
Phagocytic activity, MFI	101.0 $\pm$ 25.9 <sup>†</sup>	90.3 $\pm$ 20.9 <sup>‡</sup>	35.0 $\pm$ 13.5	<0.001

\* Significant difference between Mon1 and Mon2 (p<0.05); <sup>†</sup> Significant difference between Mon1 and Mon3 (p<0.05); <sup>‡</sup> Significant difference between Mon2 and Mon3 (p<0.05).

<sup>§</sup> Apparently all monocytes expressed this marker.

characteristics) followed by Mon1, with Mon3 representing the smallest subset ( $p < 0.05$  for all subsets, Table 38). Mon1 and Mon2 subsets had similar granularity, which was significantly higher compared to Mon3 ( $p < 0.0001$ ).

#### **4.5.2. Immunophenotypic characteristics of monocyte subsets**

The Mon2 subset had statistically significant differences in surface expression of various receptors compared to other subsets. They had the highest density of TLR4 (more than 2-fold compared to other subsets), integrin  $\beta 2/CD18$  (ICAM receptor, about 2-fold higher compared to other subsets), CXCR4, Tie2, CD163, KDR, VEGF receptor 1 (more than 2-fold), CD115 (macrophage colony-stimulating factor (M-CSF) receptor) (for all these parameters,  $p < 0.05$ ). The Mon2 subset also had the highest density of ferritin and ApoB on their surface ( $p < 0.05$  compared to other subsets). Additionally, the Mon2 subset had the highest proportion of aggregates with platelets ( $16.0 \pm 5.65\%$ , compared to  $10.4 \pm 2.30\%$  of Mon1 and  $11.95 \pm 2.56\%$  of Mon3).

Given that Mon1 comprised about 85% of all monocytes, most circulating MPAs were derived from this subset. Mon1 and Mon2 subsets expressed similar levels of CD14, IL6 receptor, CD64, which were much higher than on the surface of Mon3 ( $p < 0.0001$ ). The density of surface expression of integrin  $\alpha 4/CD49d$  (VCAM-1 receptor) and CD204 (SR-AI) were lowest on Mon1, significantly higher on Mon2 and maximal on Mon3. Mon3 included the highest (about 2.4%) proportion of cells expressing CD34.



### 4.5.3. Bone marrow monocytic cells

Compared to the blood, the total monocyte count in bone marrow was increased 3-fold ( $420 \pm 88.4$  cell/ $\mu$ L vs.  $1294 \pm 832$  cell/ $\mu$ L, respectively,  $p < 0.001$ ), whilst the count of bone marrow Mon2 was >10-fold higher compared to blood Mon2 ( $24.5 \pm 14.8$  cell/ $\mu$ L in the blood vs.  $276 \pm 363$  cell/ $\mu$ L in the bone marrow,  $p < 0.001$ ) (Table 39). Accordingly, as compared to the blood the proportion of Mon2 in bone marrow was 2.5-fold higher ( $5.90 \pm 3.40\%$  vs.  $15.6 \pm 15.4\%$ , respectively,  $p < 0.001$ ), the proportion of Mon1 lower ( $84.6 \pm 5.77\%$  vs.  $75.6 \pm 15.6\%$ , respectively,  $p = 0.001$ ) and of Mon3 very similar ( $9.51 \pm 3.56\%$  vs.  $8.80 \pm 3.73\%$ , respectively,  $p = 0.97$ ) (Table 39). In addition to the three subsets defined above, bone marrow uniformly included a population of CD14+CD16- cells, located on the plots closely to Mon1 and not seen (at any significant numbers) in the blood (Figure 18). This population included on average  $229 \pm 154$  cells/ $\mu$ L of bone marrow and constituted  $15.7 \pm 7.47\%$  in relation to the total count of other monocyte subsets. Although the exact functional role of these cells is not clear, given their exclusivity to bone marrow, they might represent a pool of monocyte progenitors undergoing maturation.

All three bone marrow monocyte subsets expressed more integrin  $\beta 2$ /CD18 (especially Mon2) and CD204 (especially Mon3) and less IL-6 receptor and CXCR4 than their circulating counterparts (Table 39). In the bone marrow, Mon1 expressed less TLR4; bone marrow Mon2 carried less CD14 and more CD16, than blood monocytes. A higher proportion of Mon3 expressed CD34. Bone marrow and blood monocytes expressed similar levels of VEGF receptor 1 (all subsets) and TLR4 (Mon2 and Mon3).

Although expression of angiogenic markers, Tie2 and KDR in bone marrow were significantly higher on all three monocyte subsets, the maximal levels of these parameters were typical of Mon2. Tie2 was expressed on 64.7%, and KDR on 58.6% of bone marrow Mon2. Compared to peripheral blood, the bone marrow Mon2 and Mon3 included larger proportions of CD34+KDR+ cells commonly defined as endothelial progenitors (0.2% vs. 1.69% for Mon2 and 0.6% vs. 7.21% for Mon3, respectively).

For all three bone marrow subsets, the proportion of monocytes carrying the surface platelet marker CD42a (i.e., MPAs) was about 3-fold higher than in peripheral blood. Again, the bone marrow Mon2 had the highest proportion of MPAs (45.3±19.6%) compared to bone marrow Mon1 (33.9±19.2%) and bone marrow Mon3 (32.3±20.7%,  $p<0.001$ ). The rate of aggregation with platelets for monocyte progenitors was much lower compared to other subsets (12.1 [10.4-14.6]%).

**Table 39. Comparative characteristics of bone marrow and peripheral blood monocytes**

	Mon1			Mon2			Mon3		
	Blood	Bone marrow	p	Blood	Bone marrow	p	Blood	Bone marrow	p
Count, cell/ $\mu$ L	360 $\pm$ 87.0	980 $\pm$ 406	0.001	24.5 $\pm$ 14.8	276 $\pm$ 363	<0.001	38.9 $\pm$ 12.7	126 $\pm$ 94.8	<0.001
% to 3 all subsets	84.6 $\pm$ 5.77	75.6 $\pm$ 15.6	0.001	5.90 $\pm$ 3.40	15.6 $\pm$ 15.4	<0.001	9.51 $\pm$ 3.56	8.80 $\pm$ 3.73	0.97
Aggregates with platelets, %	10.4 $\pm$ 2.30	33.9 $\pm$ 19.2	<0.001	16.0 $\pm$ 5.65	45.3 $\pm$ 19.6	0.002	11.95 $\pm$ 2.56	32.3 $\pm$ 20.7	<0.001
CCR2, MFI	241.5 $\pm$ 49.2	153 $\pm$ 65.7	0.27	152.5 $\pm$ 55.6	143 $\pm$ 67.2	0.64	18.4 $\pm$ 1.90	17.8 $\pm$ 4.42	0.09
TLR4, MFI	6.50 (5.60-7.70)	5.29 (3.75-7.33)	0.037	12.4 $\pm$ 6.46	9.23 $\pm$ 5.14	0.93	5.20 (4.53-6.18)	5.02 (3.18-6.44)	0.28
Interleukin-6 receptor, MFI	62.3 $\pm$ 10.0	47.9 $\pm$ 26.1	0.024	58.9 $\pm$ 8.95	40.0 $\pm$ 19.4	0.020	30.6 $\pm$ 5.92	21.0 $\pm$ 8.71	0.07
Integrin $\beta$ 2, MFI	53.8 $\pm$ 18.8	327 $\pm$ 135	0.003	100.2 $\pm$ 26.5	451 $\pm$ 136	0.001	57.8 $\pm$ 21.3	167 $\pm$ 80.2	0.18
Integrin $\alpha$ 4, MFI	14.4 $\pm$ 3.12	17.6 $\pm$ 6.57	<0.001	30.2 $\pm$ 8.07	38.2 $\pm$ 10.4	<0.001	58.0 $\pm$ 6.78	52.3 $\pm$ 37.5	<0.001
CXCR4, MFI	21.8 (16.0-39.3)	8.90 (7.85-21.5)	0.005	31.3 (24.8-47.5)	15.1 (10.6-21.0)	0.001	9.95 (8.40-12.6)	7.79 (5.18-17.2)	0.17
CD34, MFI	2.80 (2.50-3.00)	2.10 (1.91-2.43)	<0.001	2.60 (2.50-3.15)	1.81 (1.66-2.15)	0.003	2.30 (2.20-2.50)	2.24 (1.51-2.95)	0.20
KDR, MFI	3.38 $\pm$ 0.91	5.00 $\pm$ 2.47	0.002	4.23 $\pm$ 1.42	12.1 $\pm$ 10.1	0.003	3.00 (2.75-3.45)	7.39 (4.98-15.4)	<0.001
KDR+, %	5.00 (3.38-7.23)	22.9 (2.27-27.5)	0.041	8.25 (5.65-10.9)	58.6 (17.3-73.3)	0.001	2.80 (2.23-4.58)	35.2 (26.0-58.5)	<0.001
VEGFR1, MFI	7.68 $\pm$ 2.89	8.47 $\pm$ 2.42	0.48	15.7 $\pm$ 4.84	14.1 $\pm$ 4.38	0.85	5.50 (4.90-6.28)	5.79 (4.06-8.00)	0.93
CD204, MFI	5.84 $\pm$ 2.32	13.7 $\pm$ 15.3	0.006	11.5 $\pm$ 3.91	37.7 $\pm$ 26.7	<0.001	14.6 $\pm$ 6.66	47.5 $\pm$ 27.5	<0.001
Tie2, MFI	6.19 $\pm$ 0.75	6.63 $\pm$ 3.26	0.005	10.4 $\pm$ 1.67	13.4 $\pm$ 5.93	<0.001	7.0 $\pm$ 2.24	10.3 $\pm$ 2.72	0.83
Tie2+, %	25.1 $\pm$ 5.46	33.0 $\pm$ 22.1	0.004	53.7 $\pm$ 8.87	64.7 $\pm$ 24.6	0.001	31.9 $\pm$ 13.9	50.8 $\pm$ 10.4	0.22

Mon1, CD14+++CCR2+ monocytes, Mon2, CD14++CD16+CCR2+ monocytes, Mon3, CD14+CD16+CCR2- monocytes, MFI, median fluorescence intensity, VEGFR, vascular endothelium growth factor receptor 1, TLR4, Toll-like receptor-4. \*Significant difference (p<0.05) between bone marrow and blood monocyte subsets.

#### 4.5.4. Functional characteristics of monocyte subsets

Mon1 had significantly higher levels of IKK $\beta$  (MFI 61.1 $\pm$ 16.7) compared to the two other monocyte populations ( $p < 0.05$ ). IKK $\beta$  levels were similar in Mon2 and Mon3 subsets (MFI 47.7 $\pm$ 9.70 and 42.6 $\pm$ 5.91, respectively). Mon1 and Mon2 had much higher phagocytic activity compared with Mon3 ( $p < 0.001$ , Tables 38 and 40). There was no significant difference in the phagocytic activity of Mon1 and Mon2.

Stimulation with 1  $\mu$ g/ml LPS produced dissimilar alterations of the intracellular phenotype in monocyte subsets [Tables 41 and 42]. Mon1 and Mon2 had a comparable degree of LPS-stimulated TNF $\alpha$  production ( $p = 0.16$ ). Mon1 had a higher LPS-stimulated increase in expression in IL1 $\beta$  ( $p < 0.001$ ), IL6 ( $p = 0.001$ ), MCP-1 ( $p < 0.001$ ) and IKK $\beta$  ( $p < 0.01$ ), whilst Mon2 had maximal increase in IL10 production among all monocyte subsets ( $p < 0.05$ ). LPS had no effect on the intracellular expression of cytokines and IKK $\beta$  in Mon3.

**Table 40. Summary of comparative characteristics of the 3 monocyte subsets**

	Mon1	Mon2	Mon3
Size (forward scatter)	++	+++	+
Granularity (side scatter)	++	++	+
CD14	++	++	+
CD16	-	+	++
CCR2	+++	++	+
TLR4	+	++	+
IL6 receptor	++	++	+
integrin $\beta$ 2/CD18	++	++	+
integrin $\alpha$ 4/CD49d	+	++	+++
CXCR4	++	+++	+
VEGF receptor 1	++	+++	+
Tie2	+	++	+

CD163	++	+++	+
CD115	+	++	+
CD64	++	++	+
CD204	+	++	+++
Apolipoprotein B	+	++	+
Ferritin	++	+++	+
Aggregation with platelets	+	++	+
NFκB	++	+	+
Phagocytic activity	++	++	+

TLR4 - Toll-like receptor 4, IL6 – interleukin 6, VEGF – vascular endothelial growth factor, NFκB – nuclear factor κB.

**Table 41. Baseline values and mean fold change of MFI after LPS stimulation of monocyte subsets**

Monocyte Subset	Intracellular marker	Baseline MFI	Post stimulation MFI	Proportional change in MFI	P
Mon1	TNF- $\alpha$	7.9 $\pm$ 0.4	13.3 $\pm$ 0.9	1.7 $\pm$ 0.02	0.002
	IL-1 $\beta$	16.0 $\pm$ 1.5	254.4 $\pm$ 15	15.9 $\pm$ 0.6	0.001
	IL-6	12.8 $\pm$ 1.2	21.1 $\pm$ 2.4	1.7 $\pm$ 0.04	0.007
	IL-10	124.5 $\pm$ 12.6	178.4 $\pm$ 15.9	1.4 $\pm$ 0.02	0.001
	MCP-1	32.8 $\pm$ 3.5	67.2 $\pm$ 9.7	2.0 $\pm$ 0.07	0.011
	IKK $\beta$	14.4 $\pm$ 0.5	28.9 $\pm$ 1.0	2.0 $\pm$ 0.01	0.000
Mon2	TNF- $\alpha$	11.8 $\pm$ 0.8	18.9 $\pm$ 0.7	1.6 $\pm$ 0.07	0.001
	IL-1 $\beta$	6.4 $\pm$ 0.1	8.1 $\pm$ 0.1	1.3 $\pm$ 0.03	0.002
	IL-6	6.3 $\pm$ 0.1	7.9 $\pm$ 0.1	1.3 $\pm$ 0.02	0.000
	IL-10	59.2 $\pm$ 3.4	115.2 $\pm$ 7.2	1.9 $\pm$ 0.02	0.002
	MCP-1	5.9 $\pm$ 0.02	7.8 $\pm$ 0.1	1.3 $\pm$ 0.02	0.001
	IKK $\beta$	19.9 $\pm$ 1.2	33 $\pm$ 1.4	1.7 $\pm$ 0.09	0.003
Mon 3	TNF- $\alpha$	20.4 $\pm$ 0.3	18.8 $\pm$ 0.3	0.92 $\pm$ 0.03	0.029
	IL-1 $\beta$	7.5 $\pm$ 1.2	6.8 $\pm$ 0.2	0.92 $\pm$ 0.13	0.426
	IL-6	6.5 $\pm$ 0.1	6 $\pm$ 0.7	0.93 $\pm$ 0.11	0.446
	IL-10	125.9 $\pm$ 3.7	104.5 $\pm$ 43.1	0.83 $\pm$ 0.35	0.489
	MCP-1	6.3 $\pm$ 0.1	6 $\pm$ 0.1	0.95 $\pm$ 0.02	0.041
	IKK $\beta$	28.8 $\pm$ 4.9	24.3 $\pm$ 0.3	0.86 $\pm$ 0.13	0.257

IL, interleukin ,MFI, median fluorescence intensity, MCP-1, monocyte chemoattractant protein-1, TNF, tumor necrosis factor, p values baseline vs. post stimulation values.

**Table 42. Comparison of the response to LPS stimulation between monocyte subsets**

Intracellular marker	Proportional change MFI			p		
	Mon 1	Mon 2	Mon3	Mon1 vs. Mon2	Mon1 vs. Mon3	Mon2 vs. Mon3
TNF- $\alpha$	1.7 $\pm$ 0.02	1.6 $\pm$ 0.07	0.92 $\pm$ 0.03	0.16	<0.001	<0.001
IL-1 $\beta$	15.9 $\pm$ 0.6	1.3 $\pm$ 0.03	0.92 $\pm$ 0.13	<0.001	<0.001	0.845
IL-6	1.7 $\pm$ 0.04	1.3 $\pm$ 0.02	0.93 $\pm$ 0.11	0.001	<0.001	0.005
IL10	1.4 $\pm$ 0.02	1.9 $\pm$ 0.02	0.83 $\pm$ 0.35	0.07	0.034	0.002
MCP-1	2.0 $\pm$ 0.07	1.3 $\pm$ 0.02	0.95 $\pm$ 0.02	<0.001	<0.001	<0.001
IKK $\beta$	2.0 $\pm$ 0.01	1.7 $\pm$ 0.09	0.86 $\pm$ 0.13	<0.01	0.001	<0.001

IL, interleukin, MFI, median fluorescence intensity, MCP-1, monocyte chemoattractant protein-1, TNF, tumor necrosis factor, p values baseline vs. post stimulation values. NB. 1 = no effect.

#### 4.5.5. Effects of physical exercise

The participants of this substudy performed incremental treadmill exercise for 13.1 $\pm$ 2.03 minutes (Bruce protocol), equivalent to VO<sub>2</sub>max 46.8 $\pm$ 8.40 mL/kg/min. Total monocyte count and numbers of Mon1 increased significantly by 15 minutes after the exercise (p=0.021 and p=0.009, respectively) followed by a significant reduction at 1 hour (p=0.012 and p=0.055, respectively) (Table 43, Figure 20). Although Mon2 and Mon3 numerically followed the same trend, only the reduction in Mon3 by 1 hour after the exercise reached the level of significance (p=0.008). No significant changes in relative proportions of the monocyte subsets were detected (Table 43).

The total MPA count and numbers of MPAs associated with Mon1 dropped significantly by 15 minutes after exercise (p=0.044 and p=0.040, respectively) with even lower numbers seen by 1 hour (p=0.014 and p=0.024, respectively) when compared to the pre-exercise values (Table 43). The reduction in MPAs associated with Mon2 reached the level

significance by 1 hour after the exercise ( $p=0.035$ ). No significant changes in MPAs associated with Mon3 were observed.

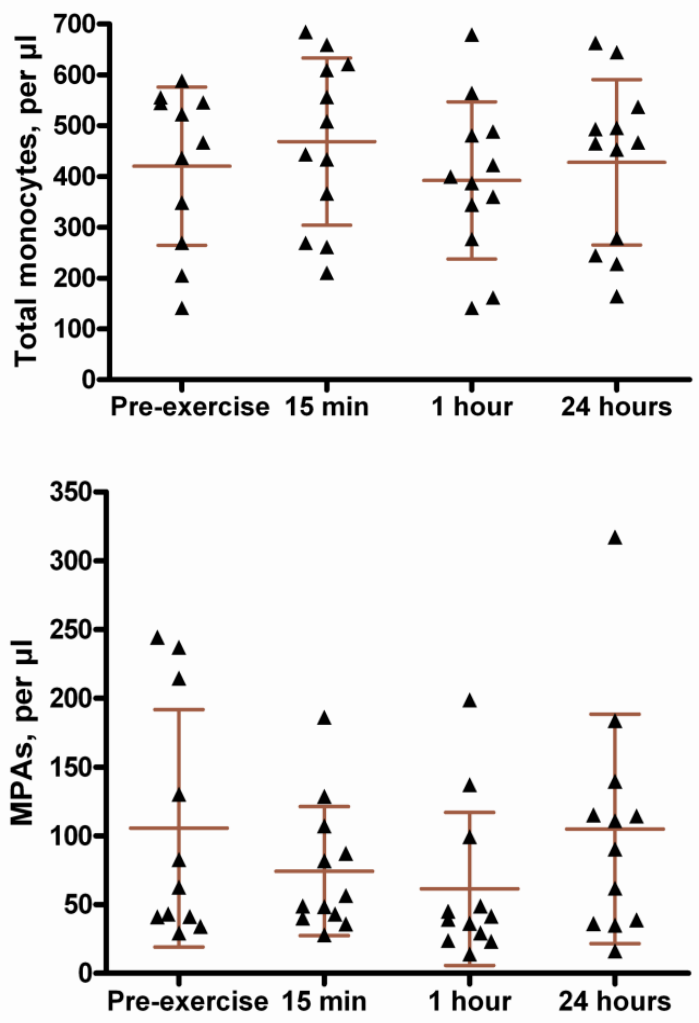
Expression of CD14 on Mon1 reduced significantly by 1 hour after exercise ( $p=0.013$ ), with no significant changes seen in CD14 expression on Mon2 and Mon3. Physical exercise did not affect CD16 and CCR2 expression on any monocyte subsets. Monocyte and MPA parameters measured at 24 hours after the exercise did not differ significantly from baseline values.

**Table 43. Effect of exercise on monocyte subsets and monocyte-platelet aggregates**

	Pre-exercise	15 min	1 hour	24 hours	p
Total monocytes, cells/ $\mu$ l	420 $\pm$ 47.0	478 $\pm$ 51.0*	397 $\pm$ 48.6†	444 $\pm$ 48.1	0.013
Mon1, cells/ $\mu$ l	348 $\pm$ 39.4	397 $\pm$ 42.4*	337 $\pm$ 42.8†	374 $\pm$ 40.7	0.032
Mon2, cells/ $\mu$ l	25.9 $\pm$ 6.40	26.3 $\pm$ 6.81	20.2 $\pm$ 6.76	22.6 $\pm$ 5.26	0.079
Mon3, cells/ $\mu$ l	46.2 $\pm$ 6.05	55.3 $\pm$ 6.31	39.2 $\pm$ 6.08†	48.1 $\pm$ 7.20	0.008
Mon1, %	83.0 $\pm$ 1.69	81.8 $\pm$ 1.69	84.7 $\pm$ 1.98	83.9 $\pm$ 1.73	0.29
Mon2, %	5.92 $\pm$ 1.12	5.70 $\pm$ 1.00	4.98 $\pm$ 1.20	5.31 $\pm$ 1.05	0.29
Mon3, %	11.1 $\pm$ 0.92	12.5 $\pm$ 1.03	10.4 $\pm$ 1.27	10.7 $\pm$ 1.16	0.12
MPA, per $\mu$ l	106 $\pm$ 26.0	73.7 $\pm$ 14.8*	57.9 $\pm$ 17.2*	104 $\pm$ 26.4	0.021
MPA with Mon1, per $\mu$ l	81.2 $\pm$ 22.3	52.1 $\pm$ 11.8*	40.4 $\pm$ 11.8*	80.6 $\pm$ 21.8	0.019
MPA with Mon2, per $\mu$ l	12.1 $\pm$ 4.01	8.90 $\pm$ 2.57	7.86 $\pm$ 3.41*	9.59 $\pm$ 3.14	0.16
MPA with Mon3, per $\mu$ l	12.3 $\pm$ 3.14	12.2 $\pm$ 2.85	9.74 $\pm$ 3.08	13.9 $\pm$ 3.85	0.23
CD14 (Mon1), MFI	1365 $\pm$ 63.7	1252 $\pm$ 60.8	1169 $\pm$ 62.6*	1336 $\pm$ 48.8	0.006
CD14 (Mon2), MFI	1254 $\pm$ 131	1072 $\pm$ 97.0	1068 $\pm$ 103	1195 $\pm$ 112	0.17
CD14 (Mon3), MFI	173 $\pm$ 17.0	150 $\pm$ 12.5	152 $\pm$ 19.1	167 $\pm$ 13.1	0.094
CD16 (Mon1), MFI	11.9 $\pm$ 0.50	11.9 $\pm$ 0.51	11.6 $\pm$ 0.57	12.0 $\pm$ 0.45	0.53
CD16 (Mon2), MFI	74.1 $\pm$ 5.63	75.6 $\pm$ 4.88	73.0 $\pm$ 5.95	70.3 $\pm$ 4.50	0.86
CD16 (Mon3), MFI	147 $\pm$ 15.7	146 $\pm$ 14.3	135 $\pm$ 13.6	144 $\pm$ 13.8	0.34
CCR2 (Mon1), MFI	123 $\pm$ 5.67	125 $\pm$ 7.44	106 $\pm$ 8.34	119 $\pm$ 15.1	0.25
CCR2 (Mon2), MFI	97.0 $\pm$ 3.77	98.9 $\pm$ 3.34	92.5 $\pm$ 5.19	99.0 $\pm$ 8.27	0.58
CCR2 (Mon3), MFI	13.6 $\pm$ 0.48	13.9 $\pm$ 0.55	12.8 $\pm$ 0.35	13.9 $\pm$ 0.62	0.30

\* $p<0.05$  compared with the pre-exercise values, † $p<0.05$  compared with values 15 min. after exercise (except,  $p=0.055$  for Mon1)





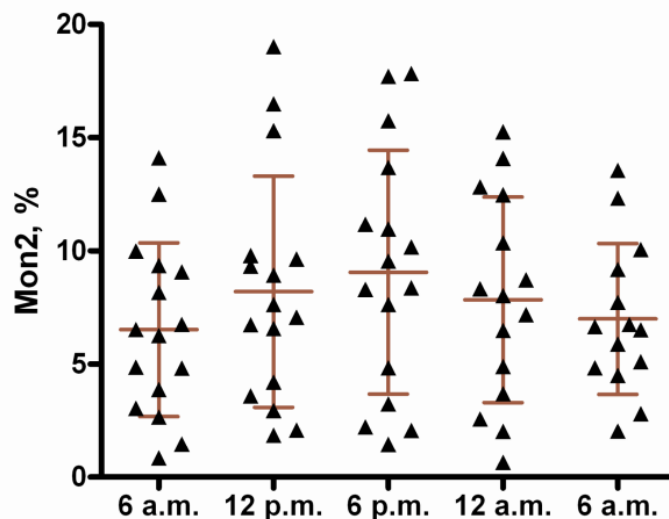
**Figure 20. The dynamics of monocytes and monocyte-platelet aggregates after the exercise**

MPA, monocyte-platelet aggregates

#### 4.5.6. Diurnal variation

Significant diurnal variations have been observed in the numbers and relative proportions of Mon2, which peaked at 6pm and were lowest at 6am (p=0.011 and p=0.001, respectively) (Table 44, Figure 21). Moreover, surface expression of CCR2 on Mon2 followed the same pattern. No significant diurnal variation was observed in monocyte expression of CD16 and CD14.

There were trends towards variations in total monocyte and Mon1 counts (peak at 12am., nadir at 12pm., p=0.003 and p=0.002, respectively; pairwise comparisons were not significant) and Mon3 counts (peak at 12am, nadir at 6am, p=0.076).



**Figure 21. The diurnal variation of Mon2 monocyte subset Mon2, CD14++CD16+CCR2 monocytes**

**Table 44. Diurnal variation of monocyte subsets and monocyte-platelet aggregates**

	6 a.m.	12 p.m.	6 p.m.	12 a.m.	6 a.m.	p
Total monocytes, cells/ $\mu$ l	477 $\pm$ 118	450 $\pm$ 91.7	467 $\pm$ 109	543 $\pm$ 147	466 $\pm$ 121	0.003
Mon1, cells/ $\mu$ l	407 $\pm$ 113	367 $\pm$ 93	379 $\pm$ 109	448 $\pm$ 143	389 $\pm$ 109	0.002
Mon2, cells/ $\mu$ l	30.5 $\pm$ 15.5	37.0 $\pm$ 21.4	42.1 $\pm$ 21.4*	40.9 $\pm$ 18.4*	31.9 $\pm$ 18.1	0.011
Mon3, cells/ $\mu$ l	39.5 $\pm$ 14.6	45.6 $\pm$ 13.7	45.5 $\pm$ 14.2	50.8 $\pm$ 14.1	44.7 $\pm$ 12.8	0.076
Mon1, %	84.9 $\pm$ 5.00	81.0 $\pm$ 7.43	80.4 $\pm$ 7.63	75.7 $\pm$ 21.0	83.1 $\pm$ 5.10	0.24
Mon2, %	6.84 $\pm$ 3.91	8.67 $\pm$ 5.35*	9.61 $\pm$ 5.57*	8.19 $\pm$ 4.38	7.07 $\pm$ 3.45	0.001
Mon3, %	8.30 $\pm$ 2.32	10.3 $\pm$ 2.91	10.0 $\pm$ 3.08	9.91 $\pm$ 4.00	9.87 $\pm$ 2.96	0.093
MPA, per $\mu$ l	78.9 $\pm$ 43.8	96.8 $\pm$ 48.2	77.4 $\pm$ 45.7	100 $\pm$ 38.0	91.2 $\pm$ 50.3	0.083
MPA with Mon1, per $\mu$ l	66.1 $\pm$ 39.2	80.2 $\pm$ 43.1	62.4 $\pm$ 41.4	81.5 $\pm$ 35.4	77.3 $\pm$ 41.2	0.13
MPA with Mon2, per $\mu$ l	8.54 $\pm$ 5.78	12.0 $\pm$ 9.21	10.6 $\pm$ 5.71	13.6 $\pm$ 9.51	11.1 $\pm$ 11.4	0.13
MPA with Mon3, per $\mu$ l	9.48 $\pm$ 18.2	4.60 $\pm$ 2.77	4.40 $\pm$ 3.63	5.32 $\pm$ 3.62	4.37 $\pm$ 2.92	0.38
CD14 (Mon1), MFI	1071 $\pm$ 194	1187 $\pm$ 194	1053 $\pm$ 282	1162 $\pm$ 152	1193 $\pm$ 205	0.061
CD14 (Mon2), MFI	564 $\pm$ 419	626 $\pm$ 442	599 $\pm$ 439	639 $\pm$ 440	633 $\pm$ 443	0.18
CD14 (Mon3), MFI	89.4 $\pm$ 48.8	89.4 $\pm$ 33.1	91.7 $\pm$ 35.4	98.3 $\pm$ 40.1	98.9 $\pm$ 40.0	0.28
CD16 (Mon1), MFI	11.5 (9.56-1276)	11.8 (10.4-1406)	12.1 (9.50-1234)	12.8 (10.5-1456)	11.7 (10.2-1460)	0.13
CD16 (Mon2), MFI	40.5 $\pm$ 30.0	40.2 $\pm$ 24.6	40.8 $\pm$ 24.9	39.1 $\pm$ 23.8	40.5 $\pm$ 26.0	0.50
CD16 (Mon3), MFI	171 $\pm$ 60.6	176 $\pm$ 61.2	175 $\pm$ 71.0	168 $\pm$ 64.6	177 $\pm$ 53.9	0.39
CCR2 (Mon1), MFI	159 $\pm$ 29.5	171 $\pm$ 31.9	163 $\pm$ 43.9	163 $\pm$ 39.5	162 $\pm$ 34.0	0.64
CCR2 (Mon2), MFI	113 $\pm$ 17.8	119 $\pm$ 20.0	123 $\pm$ 19.8	116 $\pm$ 17.3	112 $\pm$ 15.9	0.048
CCR2 (Mon3), MFI	15.9 $\pm$ 1.41	16.0 $\pm$ 1.92	16.2 $\pm$ 1.40	16.1 $\pm$ 1.29	16.0 $\pm$ 1.96	0.79

MPA, monocyte-platelet aggregates, MFI, median fluorescent intensity. \* $p$ <0.05 compared with the baseline values (except,  $p$ =0.051 for CCR2 (Mon2)), † $p$ <0.05 compared with previous time-point.

#### **4.5.7. Time-delay analysis**

A delay of up to 2 hours in sample analysis after venipuncture did not significantly affect counts and relative proportions of monocyte subsets (Table 45). By 4 hours, the proportion of Mon2 significantly increased ( $p=0.004$ ) and this was driven by a reduction in the proportion of Mon1 ( $p=0.001$ ). Mon3 counts (but not percentage) also tended to increase ( $p=0.021$ , with no pairwise significant differences).

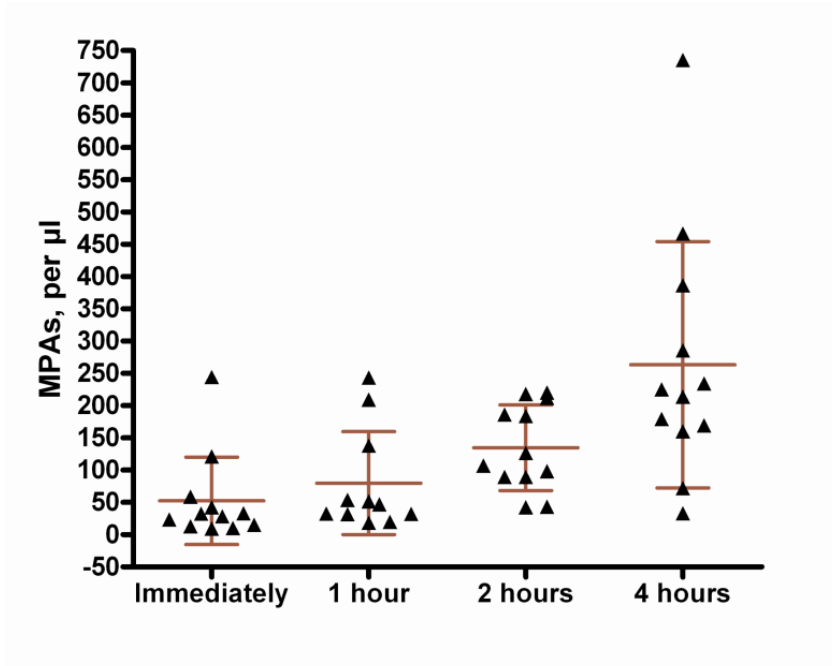
MPA counts were more sensitive to the delay in sample processing. There was a numerical trend towards an increase by 1 hour, and MPAs significantly increased by 2 hours and further by 4 hours ( $p=0.025$  and  $p=0.016$ , respectively) (Figure 22). A similar trend was seen for MPAs associated with all individual monocyte subsets, with MPA numbers approximately doubling by 2 hour and further doubling by 4 hours (Table 45).

CD14 and CD16 expression on Mon1 significantly increased by 2 hours ( $p=0.031$  and  $p=0.002$ , respectively) and further by 4 hours ( $p=0.006$  and  $p=0.007$ , respectively). Monocyte CCR2 expression was not affected by delay in sample preparation (Table 45).

**Table 45. Effects of delayed sample processing on monocyte subsets and monocyte-platelet aggregates**

	Immediately	1 hour delay	2 hour delay	4 hour delay	p
Total monocytes, cells/ $\mu$ l	386 $\pm$ 60.4	401 $\pm$ 63.3	406 $\pm$ 76.8	416 $\pm$ 65.7	0.58
Mon1, cells/ $\mu$ l	330 $\pm$ 52.0	339 $\pm$ 52.1	339 $\pm$ 62.8	325 $\pm$ 49.7*	0.84
Mon2, cells/ $\mu$ l	24.3 $\pm$ 6.72	34.8 $\pm$ 10.1	31.5 $\pm$ 9.40	50.9 $\pm$ 13.8*	0.042
Mon3, cells/ $\mu$ l	31.8 $\pm$ 5.92	34.3 $\pm$ 6.83	38.0 $\pm$ 7.65	40.1 $\pm$ 8.29	0.021
Mon1, %	86.6 $\pm$ 1.32	85.5 $\pm$ 1.55	84.4 $\pm$ 1.49	79.7 $\pm$ 2.73	0.014
Mon2, %	5.58 $\pm$ 0.99	5.82 $\pm$ 1.10	6.33 $\pm$ 1.06	10.7 $\pm$ 1.81*	0.004
Mon3, %	8.50 $\pm$ 1.04	8.71 $\pm$ 1.03	9.28 $\pm$ 1.17	9.54 $\pm$ 1.22	0.32
MPA, per $\mu$ l	52.0 $\pm$ 21.4	79.8 $\pm$ 24.0	137 $\pm$ 20.8*	271 $\pm$ 59.8*	0.02
MPA with Mon1, per $\mu$ l	35.9 $\pm$ 14.6	58.3 $\pm$ 17.9	104 $\pm$ 16.4*	201 $\pm$ 45.1*	0.03
MPA with Mon2, per $\mu$ l	7.58 $\pm$ 4.05	9.74 $\pm$ 4.26	14.7 $\pm$ 3.90	38.9 $\pm$ 11.4*	0.006
MPA with Mon3, per $\mu$ l	8.50 $\pm$ 2.94	11.7 $\pm$ 3.30	18.3 $\pm$ 3.37*	31.0 $\pm$ 7.80*	0.008
CD14 (Mon1), MFI	950 $\pm$ 119	1205 $\pm$ 75.3	1464 $\pm$ 83.1*	1967 $\pm$ 186*	<0.005
CD14 (Mon2), MFI	1084 $\pm$ 145	1302 $\pm$ 73.3	1503 $\pm$ 98.3	1903 $\pm$ 315	0.067
CD14 (Mon3), MFI	169 $\pm$ 18.2	185 $\pm$ 21.8	209 $\pm$ 21.3	276 $\pm$ 51.0	0.096
CD16 (Mon1), MFI	13.2 $\pm$ 0.53	13.6 $\pm$ 0.56	14.1 $\pm$ 0.57*	17.3 $\pm$ 0.86*	0.001
CD16 (Mon2), MFI	59.1 $\pm$ 1.65	58.3 $\pm$ 1.37	59.2 $\pm$ 2.29	61.4 $\pm$ 4.08	0.58
CD16 (Mon3), MFI	167 $\pm$ 13.6	166 $\pm$ 19.6	185 $\pm$ 13.4	203 $\pm$ 22.3	0.087
CCR2 (Mon1), MFI	121 $\pm$ 16.7	129 $\pm$ 14.9	125 $\pm$ 12.9	146 $\pm$ 12.9	0.30
CCR2 (Mon2), MFI	103 $\pm$ 7.51	105 $\pm$ 4.17	104 $\pm$ 6.11	110 $\pm$ 3.78	0.56
CCR2 (Mon3), MFI	15.1 $\pm$ 0.74	14.7 $\pm$ 0.44	13.3 $\pm$ 0.63	14.3 $\pm$ 0.86	0.23

MPA, monocyte-platelet aggregates, MFI, median fluorescent intensity. \*p<0.05 compared with the baseline values (except, p=0.067 for Mon2).



**Figure 22. Effect of the delay in the sample processing on monocyte-platelet aggregate number**

MPA, monocyte-platelet aggregates

## 4.6. Discussion

In this section I show that three human monocyte subsets can be reliably discriminated on the basis of varying expression of CD14, CD16 and CCR2 and they comprise three phenotypically and functionally distinct subsets.

Mon1 is characterised by high expression of CD14, IL6 receptor, CD64, CCR2 and CD163, but low expression of VCAM-1 receptor and CD204 and absent CD16. Mon2 is characterised by maximal expression of ICAM-1 receptor, Tie2, CXCR4, CD163, VEGF receptor 1, KDR, ferritin, ApoB and CD115. Compared to Mon1, Mon2 have up-regulated CD16, VCAM1 receptor and CD204. In contrast, Mon3 had maximal expression of CD16, VCAM1 receptor and CD204, but diminished levels of CD14, IL6 receptor, CD64, CCR2 and CD163. On the basis of these findings, Mon1 appear to correspond to Ly-6C<sup>hi</sup> mouse monocytes and Mon3 to Ly-6C<sup>lo</sup> monocytes.<sup>25</sup> However, it is not clear which mouse monocyte subset may correspond to the human Mon2.

Important questions arise about the developmental origins and functional differences between the three monocyte populations. Experimental findings show that the two mouse monocyte subsets do not arise from distinct lineages. Rather, Ly-6C<sup>lo</sup> monocytes (Mon3 analogue) are a product of the Ly-6C<sup>hi</sup> monocyte subset (Mon1 analogue).<sup>678</sup> In this study I show that all three human monocyte subsets are already present in bone marrow, although the relative proportion of the Mon2 subset is much higher in bone marrow than in blood.

In accordance with previous studies showing that inflammatory activity of Mon3 is significantly lower compared to 'classical' monocytes (CD14<sup>++</sup>CD16<sup>-</sup>), I observed significantly lower levels of IKK $\beta$  in Mon3 compared to Mon1. Intriguingly, the observation that IKK $\beta$  levels in Mon2 are similar to Mon3 and significantly lower compared to Mon1 suggests that the functional role of Mon2 is distinct from pro-inflammatory Mon1.

Monocyte subpopulations significantly differed in their ability to produce cytokines in response to LPS. Mon1 had the highest LPS-stimulated expression of IL1 $\beta$ , IL6, MCP-1 and IKK $\beta$ , whilst Mon2 exerted the maximal of all subset increases in IL10 expression. In accordance with previous data, Mon3 were apparently unresponsive to LPS.<sup>679-680</sup> Of note, in it was previously shown that CD14<sup>+</sup>CD16<sup>+</sup> monocytes (i.e., Mon3) could produce inflammatory cytokines in response to viral rather than bacterial stimulation.<sup>680</sup> These observations further confirm significant functional differences of the three monocyte subsets. Recently it has been demonstrated that in a similar manner to mice Ly-6C<sup>lo</sup> monocytes, the human CD14<sup>+</sup>CD16<sup>+</sup> subset 'patrols' the endothelium in a lymphocyte function-associated antigen (LFA)-1 dependent manner.<sup>680</sup> Given the relatively small levels of CD18 ( $\beta$ -chains) expressed by Mon3 in our study, it is likely that  $\alpha$ -chain of the ICAM-1 receptor (ie, LFA-1, CD11a) is critical for the 'patrolling' activity. However, expression of LFA-1 on monocyte subsets has not been assessed in this study, which should be considered as a study limitation.

Divergent directions of phenotypic change on Mon2 compared to Mon1 (including increase in size, increased expression of various markers such as ICAM-1 receptor, Tie2,



CXCR4, CD163, VEGF receptor 1, KDR, ferritin and ApoB) and Mon3 (reduced size and granularity, down-regulation of most markers discussed above, but specific maximal up-regulation of CD204, VCAM-1receptor and CD16) may indicate that Mon2 and Mon3 represent developmentally independent subsets rather than different stages of monocyte maturation. Although only few studies separately analysed CD14<sup>++</sup>CD16<sup>+</sup> and CD14<sup>+</sup>CD16<sup>+</sup>monocytes, dynamics in their counts appear to be discordant.<sup>44-45</sup> The CD14<sup>++</sup>CD16<sup>+</sup> subset is increased, whereas CD14<sup>+</sup>CD16<sup>+</sup> cells are decreased in numbers after stroke, although both subsets (in contrast to CD14<sup>++</sup>CD16<sup>-</sup> monocytes) were positively associated with a favourable prognosis.<sup>44</sup> CD14<sup>++</sup>CD16<sup>+</sup> but not CD14<sup>+</sup>CD16<sup>+</sup> monocytes, are increased in severe asthma.<sup>45</sup> CD14<sup>++</sup>CD16<sup>+</sup> are associated negative prognosis in chronic kidney disease.<sup>681</sup>

These clearly distinct characteristics of monocyte populations raise the important question as to what the role may be of this ‘mysterious’ Mon2 subset? To address this, we must consider them in context of previous findings. Expression of a marker seen on macrophages (CD16) and high density of M-CSF receptor (CD115) may indicate their transformation towards tissue-resident cells (e.g., macrophages). Mon2 uniformly express maximal levels of receptors to proangiogenic factors (Tie2 [an angiopoietin receptor], KDR, CXCR4 and VEGF receptor 1). It has been reported that CD14<sup>+</sup> cells include a subset of pluripotent cells able to differentiate into various cell lineages (e.g., epithelial, neuronal, neuronal and hepatic) under appropriate microenvironments and that multipotent cells of monocytic origin successfully restored LV function after experimental MI.<sup>37, 466</sup> Bone marrow and peripheral blood-derived CD14<sup>+</sup>Tie2<sup>+</sup>CD34<sup>-</sup> cells (thus, phenotypically corresponding to Mon2) were shown to transdifferentiate into endothelial lineage cells and

contribute to neovascularisation *in vivo* in response to MCP-1.<sup>642</sup> Of note the similarity of Mon2 to bone marrow monocytic cells and their higher proportion in bone marrow may imply their direct bone marrow origin rather than their development from Mon1.

M-CSF treatment of infarcted mice significantly reduced infarct size and improved LV function.<sup>469</sup> Immunohistochemistry revealed that M-CSF increased macrophage infiltration and neovascularization of infarcted myocardium. This paralleled the mobilization of CXCR4+ cells into the peripheral circulation and their homing into the infarct area which showed marked SDF-1 expression.<sup>469</sup> These effects were abrogated by the CXCR4 antagonist AMD3100.

Interesting parallels can be drawn in relation to Mon2 which expressed maximal levels of the unique M-CSF receptor (CD115) and CXCR4. Also in accordance with these findings, cardiac MCP-1 overexpression in mice with bone marrow replaced by Tie2/LacZ mice was associated with effective prevention of cardiac dysfunction and remodelling after MI, by myocardial mobilisation of Tie2 monocytes.<sup>365</sup> These angiogenic/reparative MCP-1 responsive (CCR2+Tie2+) monocytes probably correspond to the Mon2 subset. Of note, no increase in CD34+KDR+ EPCs was observed in this model.<sup>365</sup> These cells exhibit the potential to differentiate *in vitro* into cells with endothelial characteristics and significantly contributed to efficient re-endothelialization in a mouse model of vascular injury.<sup>465</sup> Taken together, although these various studies employed different markers to describe angiogenic/reparative monocytes, all of them appear to correspond to the Mon2 subset.

Of importance, Tie2-expressing peripheral blood monocytes are selectively recruited to spontaneous and orthotopic tumours, to promote angiogenesis in a paracrine manner, and they account for most of the proangiogenic activity of myeloid cells in tumours.<sup>682-683</sup> High Mon2 expression of CD163 (a scavenger receptor known to exert anti-inflammatory properties) and high surface density of ferritin (iron-binding protein involved in control of oxidative burden). Taken together with previous data on high IL10 expression on CD14<sup>++</sup>CD16<sup>+</sup> monocytes the findings may indicate that the reparative properties of Mon2 may even go beyond their putative angiogenic potential.<sup>684-688</sup>

Interestingly there was an unexpected observation of a higher rate of MPAs in bone marrow than in peripheral blood. Generally such aggregates are believed to reflect platelet activation, an unlikely scenario in bone marrow.<sup>636</sup> Although exact significance of this phenomenon is unclear it might indicate the role of platelets in monocyte maturation in bone marrow. However, these observations could be biased by the slightly longer processing time of bone marrow samples than blood samples.

Also this study shows for the first time the presence of significant diurnal variation in the Mon2 subset. The highest levels of this subset were observed at 6pm, possibly reflecting increased day-time activity of the participants. Also this variation may partly be related to the 'biological clock', e.g., being affected by well recognised and clinically important variations in glucocorticoid levels. Indeed, glucocorticoids (e.g., cortisol) play a significant role in regulation of vascular reactivity and inflammation.<sup>689-691</sup> Glucocorticoids modulate different aspects of monocyte structure, metabolism and function (predominantly inhibitory effects).<sup>692-694</sup> These effects are mediated by corticosteroid receptors expressed

by monocytes/macrophages.<sup>695</sup> Acute administration of glucocorticoids has also been reported to reduce circulating monocyte levels.<sup>696</sup> Apparently scarce information is available on mechanisms of cortisol-mediated down-regulation of monocyte mobilization from bone-marrow and regulation of their homing to tissues, which might be partly related to the cortisol-mediated effects on adhesion molecules.<sup>697</sup> However, in my study the pattern of diurnal monocyte variation did not strictly follow the classical pattern of cortisol variation (i.e., peak levels in the early morning and lowest levels at about midnight - 4 am).<sup>698</sup> This suggests the presence of other regulatory mechanisms of monocyte mobilisation to the circulation. For example, sleep per se and irrespectively of diurnal variation was shown to down-regulate proportions of IL-10 producing monocytes (i.e., predominantly Mon2), which appear to correspond to my data showing a significant reduction of Mon2 at night time (6 am) as compared with day-time (6 pm,  $p=0.001$ ).<sup>699</sup>

Although Mon1 and Mon3 showed a trend towards diurnal variation, their changes were relatively small and are thus unlikely to have a significant biological role, at least in healthy volunteers.

Another novel observation in this study is the prompt increase in monocyte count and the contrasting reduction in MPAs following exercise. One possibility is that some MPAs could disaggregate under enhanced flow/shear stress conditions seen during exercise. However, this is perhaps unlikely given that MPA numbers continued to decrease further by 1 hour after the exercise, when baseline haemodynamics should be restored. An alternative explanation is that the exercise facilitated preferential migration to tissues of

monocytes aggregated with platelets. This is supported by published data showing the role of platelets in the regulation and acceleration of monocyte homing.<sup>700-701</sup>

The study also shows significant post-exercise reduction in CD14 expression on Mon1. CD14 is intimately involved in monocyte antibacterial and inflammatory responses.<sup>26</sup> Thus, this observation may shed further light on the potential mechanism for the beneficial/anti-inflammatory effects of exercise but also their immunodepressive effects.<sup>702</sup>

Monocyte subsets appear to respond differentially to physical activity. In the study by Simpson et al., the proportion of CD14<sup>++</sup>CD16<sup>+</sup> monocytes increased by 27% immediately after exercise, but reduced by 49% after just one hour, indicating fairly prominent dynamics of the 'proinflammatory' CD16<sup>+</sup> monocytes.<sup>703</sup> Moreover, monocyte expression of surface receptors differs between various subsets.<sup>703</sup> Also, moderate-intensity treadmill exercise predominantly increased CD16<sup>+</sup> monocytes, and revealed significant inter-subset differences and significant changes in CD62L, CD11b, CXCR2, and HLA-DR expression.<sup>704</sup> Indeed, physically active individuals had a lower percentage of CD14<sup>++</sup>CD16<sup>+</sup> monocytes, a lower unstimulated monocyte production of TNF- $\alpha$  and lower CRP levels compared to sedentary individuals.<sup>705</sup> The advantage of the present study is its accurate and objective discrimination of the three monocyte subsets which further increases our knowledge on immediate monocyte responses to exercise in healthy individuals.

Finally, the time-delay substudy shows that a delay in sample processing of up to 2 hours has little effect on the accuracy of monocyte subset counting. More prolonged delays tend

to shift monocyte proportions from Mon1 to Mon2 (and possibly Mon3). These findings are also of interest from the point of view of monocyte biology, given that they may highlight a propensity for ‘classical’ monocytes (Mon1) to express CD16, a typical feature of the minor monocyte subsets. It is unclear whether these changes reflect Mon1 differentiation to macrophages (known to express CD16) or their ability to differentiate into other monocyte subsets. In order to answer this question, more detailed analysis of isolated Mon1 will be required.<sup>706</sup> In contrast to monocyte subsets, analysis of MPA counts should not be performed later than one hour from sample collection as further delay significantly affect the results due to a progressive increase in MPA numbers. Analysis of monocyte CD14 and CD16 expression should also be performed within one hour.

Of note, the number of participants in this study was relatively small, leaving a possibility of “significant” changes/differences detected by chance alone. This needs to be kept in mind interpreting the results, and further research with more mechanistic insight into monocyte subset functionality would be useful.

## **4.7. Postscript**

After I had completed the immunophenotypic part of the study with the manuscript being under submission a consensus on monocyte subset nomenclature was published. Interestingly, the consensus agreed about the existence of the three monocyte subsets defined in the study.<sup>42, 46</sup>

## **4.8. Conclusions**

In conclusion, in this chapter of the thesis I demonstrate that human monocytes include three major functionally and phenotypically different subsets which can be unequivocally discriminated on the basis of their CCR2 expression. These subsets are defined as CD14<sup>++</sup>CD16<sup>-</sup>CCR2<sup>+</sup> cells (Mon1), CD14<sup>++</sup>CD16<sup>+</sup>CCR2<sup>+</sup> cells (Mon2) and CD14<sup>+</sup>CD16<sup>+</sup>CCR2<sup>-</sup> cells (Mon3). Thus, previous monocyte work might need to be re-evaluated in the light of this newly characterised Mon2 cell population.

Mon2 undergoes significant diurnal variations. A single episode of exercise causes a temporal increase in monocytes with a contrasting significant reduction in MPA numbers. Analysis of monocyte subset counts should be performed within 2 hours of blood sampling whereas measurement of MPAs and monocyte CD14 and CD16 expression should be performed within 1 hour.

The functional roles of the Mon2 subset need further investigation, both in health and in diseases in which monocytes play a pivotal role, such as atherosclerosis. Also, Mon2 may

correspond to a population of angiogenic/pluripotent progenitor monocytes, as described in previous studies.



## **CHAPTER V. BLOOD LEUKOCYTES IN PATIENTS WITH HEART FAILURE: IMPACT ON PROGNOSIS**

### **5.1. Introduction**

HF with preserved ejection fraction (HFpEF) is an emerging problem of cardiovascular medicine.<sup>50</sup> Whilst the prevalence of HF with reduced ejection fraction (HFrEF) has been relatively stable over the last two decades, the prevalence of HFpEF is growing rapidly and it is expected to become a dominant form of HF in the near future.<sup>51</sup> Despite the increasing appreciation of the problem and recognition of the comparably poor outcome of the two conditions, the pathophysiological mechanisms of HFpEF are still poorly understood.<sup>52-53</sup>

Different types of leukocytes play important roles in the pathogenesis and prognosis of HFrEF via their contribution to inflammation, extracellular matrix remodelling, and reparatory processes.<sup>660</sup> In a number of studies, relative lymphopenia, and particularly T-helper deficit were strong predictors of higher risk of death in severe HFrEF and acute decompensated HF.<sup>707-713</sup> In patients with moderate-to-severe chronic HFrEF, low lymphocyte count was a much stronger predictor of mortality than LVEF.<sup>714</sup> Lymphocyte abnormalities, such as increase in Th17 population and reduction in Treg lymphocytes has been shown in HFpEF but the impact of lymphocyte counts on mortality in HFpEF is not known.<sup>715</sup>

In contrast to lymphocytes, neutrophils are often increased in HFrEF and show features of distinct proinflammatory activation.<sup>716-717</sup> In a retrospective analysis of patients from the Studies Of Left Ventricular Dysfunction (SOLVD), a relative neutrophil elevation posed an increased risk of all-cause and cardiovascular mortality.<sup>713, 718-719</sup> However, only scarce data are available on the impact of neutrophils on pathophysiology and outcome in HFpEF. Also, only limited data are currently available on the role of monocytes in HF. Of note, in patients after acute MI high monocyte count was associated with poor recovery of LVEF.<sup>400</sup> However, the impact of monocytes on outcome in stable HF and their role in HFpEF is unclear.

## **5.2. Aims and hypotheses**

In this study I aimed to assess possible differences in neutrophils, lymphocytes and monocytes between patients with HFrEF and HFpEF and establish the effect of their numbers on the risk of death in HF patients.

I hypothesised that:

1. There are significant differences in neutrophils, lymphocytes and monocytes between patients with HFrEF and HFpEF;
2. Counts of the leucocytes predict the risk of death in patients with HFrEF and HFpEF.

## **5.3. Methods**

### **5.3.1. Study population**

A total number of 1019 patients referred the Cardiology Clinic of the Grodno Regional Clinical Hospital in 2008-2009 with HF either with HFrEF (n=856) or with HFpEF (n=163) have been included into the study. The diagnosis of HFrEF or HFpEF has been established according to the current guidelines of the European Society of Cardiology.<sup>1</sup> Exclusion criteria for inclusion in the study were life expectancy less than one year due to non-cardiac causes (e.g., cancer), and terminal HF (NYHA class IV). All included patients were clinically stable and treated (>3 months) before the recruitment, and under regular outpatient follow up.

The patients were followed for at least one year with median (inter-quartile range [IQR]) follow-up duration of 17 (14-20) months. The endpoint of any-cause death has been registered at the end of the period. The study was performed in accordance with Declaration of Helsinki and local ethical regulations.

### **5.3.2. Echocardiography**

All subjects underwent routine M-mode, B-mode, and Doppler transthoracic echocardiography. Images of parasternal long axis, short axis (at aortic valve level, mitral leaflet level, papillary muscle level and apex), apical 4-chamber, apical 5-chamber, apical 2-chamber and apical 3-chamber views were acquired. The relevant American Society of

Echocardiography (ASE) guideline recommendations were used for image acquisition and analysis.<sup>646, 648, 720</sup> LVEF was measured using the modified Simpson's biplane method.<sup>648</sup> The diastolic functional assessment was performed by evaluating E/A ratio, a ratio of early and late mitral inflow velocities.

### **5.3.3. White blood cell count**

The white blood cells (neutrophils, lymphocytes, monocytes) were measured in the Central laboratory of the Grodno Regional Clinical Hospital using an automatic haematocytometer (Micros 60 Horiba, ABX Diagnostics, France). Inter- and intra-assay CVs were <5%.

### **5.3.4. Statistical analysis**

Data are expressed as mean  $\pm$  standard deviation (SD) for normally distributed variables; or as median and IQR for non-normally distributed variables. Comparisons between two groups (i.e., patients with EF <50% vs.  $\geq$ 50%) were performed using T-test (for normally distributed parameters) or Mann-Whitney (for non-normally distributed parameters). Predictive value of the study parameters for LVEF was assessed using linear regression analysis, and factors predictive for death were assessed with logistic regression analysis. Predictive value of white blood cells for death was calculated per 1000 granulocytes, 200 monocytes and 1000 lymphocytes. A p-value of <0.05 was considered statistically significant. SPSS 18 (SPSS, Inc, Chicago, Illinois, USA) statistical software was used to perform the statistical analyses.

## **5.4. Results**

### **5.4.1. Demographic and clinical characteristics**

The study included 1019 consecutive patients with HF either HFrEF (n=856, age 55 [49-64], 81% males) or HFpEF (n=163, age 58 [51-65], 56% males) (Table 46). Patients with HFrEF had lower body mass index (p=0.017), higher proportion of males (p<0.001), patients with atrial fibrillation (p<0.001), subjects with history of previous MI (p=0.002), and lower proportion of patients with history of hypertension (p<0.001). As expected patients with HFrEF had higher NYHA class than patients with HFpEF (p<0.001).

Patients with HFrEF as compared with patients with HFpEF had significantly higher neutrophil counts (p=0.004) and granulocytes percentage (0.018) and significantly lower lymphocyte percentage (p=0.004) and platelet numbers (p=0.001)(Table 46).

**Table 46. Demographic and clinical characteristics of patients with reduced and normal ejection fraction**

	EF <50% (n=163)	EF ≥50% (n=858)	p
Age, years	55 (49-64)	58 (51-65)	0.066
Male, n (%)	132 (81)	480 (56)	<0.001
Ishaemic aetiology, n (%)	119 (73)	631 (74)	0.89
NYHA I (%)	36 (22)	654 (76)	<0.001
NYHA II (%)	97 (60)	192 (22)	
NYHA III (%)	30 (18)	12 (1)	
Atrial fibrillation, n (%)	97 (60)	248 (29)	<0.001
Diabetes mellitus, n (%)	8 (5)	82 (9)	0.055
Hypertension, n (%)	115 (71)	754 (88)	<0.001
BMI, kg/m <sup>2</sup>	27 (25-31)	29 (26-32)	0.017
History of MI, n (%)	50 (31)	168 (20)	0.002
Neutrophils, per μL	4140 (3498-5293)	3933 (3247-4760)	0.004
Neutrophils, %	65 (59-69)	63 (58-68)	0.018
Monocytes, per μL	208 (80-378)	188 (73-354)	0.48
Monocytes, %	3.0 (1.0-6.0)	3.0 (1.0-6.0)	0.85
Lymphocytes, per μL	1936 (1562-2358)	1908 (1567-2277)	0.45
Lymphocytes, %	29 (24-33)	30 (26-34)	0.004

#### 5.4.2. Predictors of left ventricular ejection fraction

Using univariate analysis, significant clinical predictors of reduced LVEF in the whole study population were advanced age, male sex, history of hypertension and atrial fibrillation (Table 47). Among leukocytes, high neutrophil count and percentage and low lymphocyte percentage were associated with reduced LVEF. These parameters remained significantly associated with reduced LVEF after adjustment for the clinical predictors above (Table 47).

**Table 47. Predictive value of the study variables for left ventricular ejection fraction**

Parameter	Unadjusted		
	R <sup>2</sup>	β	p
Age	0.01	0.09	0.006
Male sex	0.07	-0.25	<0.001
Atrial fibrillation	0.04	0.21	<0.001
Ischaemic aetiology	0.00	0.01	0.66
Diabetes mellitus	0.00	-0.001	0.98
Hypertension	0.04	0.20	<0.001
Body mass index	0.00	0.06	0.072
Neutrophils, count	0.02	-0.13	<0.001
Neutrophils, %	0.01	-0.08	0.014
Monocytes, count	0.00	0.002	0.13
Monocytes, %	0.00	-0.02	0.46
Lymphocytes, count	0.00	-0.02	0.59
Lymphocytes, %	0.01	0.10	0.001
	<b>Adjusted for age, sex, atrial fibrillation, hypertension</b>		
Neutrophils, count	0.14	-0.09	0.003
Neutrophils, %	0.14	-0.07	0.019
Monocytes, count	0.13	-0.03	0.33
Monocytes, %	0.13	-0.02	0.13
Lymphocytes, count	0.13	0.02	0.56
Lymphocytes, %	0.14	0.09	0.004

### 5.4.3. Blood cells and mortality

During the follow-up period, 41 deaths (4.02%) occurred (28 deaths in HFpEF and 13 deaths in HFrEF). Using univariate logistic regression analysis, a history of hypertension was the only significant clinical predictor for mortality in patients with HFpEF (Table 48). Amongst the leukocytes, high monocyte count was the only significant predictor of death in HFpEF on univariate analysis ( $p=0.01$ ). On multivariate analyses, after adjustment for age, LVEF, and hypertension, high monocyte count still significantly predicted death ( $p=0.012$ ). The results in HFpEF indicate a 41% increase in risk of death per rise by every 200 monocytes (Table 48). Additionally, monocytosis (i.e., monocyte count above 800 cells/ $\mu$ l) was associated with a 441% increase in risk of death compared to patients with normal monocyte levels (i.e.,  $\leq 800$  per ul, odds ratio 5.41 [95% CI 1.50-19.6],  $p=0.01$ ,  $R^2=0.022$ ). We were unable to identify significant predictors for mortality in patients with HFrEF among the study parameters (Table 49).

**Table 48. Predictors of all cause death in patients with heart failure with preserved ejection fraction**

Parameter	Unadjusted			
	R <sup>2</sup>	Odds ratio	95% CI	p
Age	0.000	1.01	0.98-1.05	0.52
Sex	0.014	2.01	0.88-4.62	0.10
Ejection fraction	0.006	0.97	0.92-1.03	0.27
Hypertension	0.021	0.35	0.15-0.85	0.02
Neutrophils, per 1000 cells	0.008	1.16	0.95-1.41	0.15
Monocytes, per 200 cells	0.026	1.42	1.09-1.84	0.01
Lymphocytes, per 1000 cells	0.006	1.40	0.78-2.53	0.27
	<b>Adjusted for hypertension, age, and ejection fraction</b>			
Monocytes, per 200 cells	0.057	1.41	1.08-1.84	0.012



**Table 49. Logistic regression analysis for predictors of all cause death in patients with heart failure with reduced ejection fraction**

Parameter	Unadjusted			
	R <sup>2</sup>	Odds ratio	95% CI	p
Age	0.004	0.99	0.94-1.04	0.59
Sex	0.002	1.77	0.20-2.96	0.70
Ejection fraction	0.026	0.96	0.91-1.02	0.31
Granulocytes, per 1000 cells	0.002	1.06	0.81-1.39	0.69
Monocytes, per 200 cells	0.001	0.92	0.52-1.62	0.77
Lymphocytes, per 1000 cells	0.000	0.95	0.42-2.11	0.89

## 5.4. Discussion

This study shows for the first time that high monocyte count is a significant independent predictor of mortality in patients with HFpEF. The study also shows significant differences between HFpEF and HFrEF in the proportions of leukocytes.

Despite better LV contractility the clinical prognosis in HFpEF is poor and similar to the prognosis in HFrEF.<sup>50</sup> However, the pathophysiology of HFpEF is still poorly understood given that this group of patients is rather heterogeneous with leading factors driving the symptoms being increased myocardial stiffness, delayed myocardial relaxation, and impaired endothelial function.<sup>721</sup> It is uncertain whether cardiomyocyte dysfunction or impaired extracellular matrix properties should be considered as a primary pathogenic factor of HFpEF.<sup>722-723</sup>

The observed impact of monocytes on outcome in HFpEF is perhaps not unexpected. Monocytes are immune and pro-inflammatory cells with plethora of other biological roles.<sup>659-660, 724</sup> These cells play a key role in the regulation of myocardial extracellular matrix turnover.<sup>659</sup> Moreover, monocytes do contribute to and orchestrate tissue remodelling by release of various matrix metalloproteases, cytokines and growth factors.<sup>660</sup> It has been previously shown that high monocyte levels were associated with a higher risk of future MI in stable CAD and in those with MI, high monocyte numbers were strongly associated with increased risk of unfavourable outcomes.<sup>320, 396, 402</sup> This study expands the field by demonstrating of the independent negative impact of monocytosis (i.e., monocyte count over 800 per  $\mu\text{l}$ ) on outcome in patients with HFpEF.

In my study, monocytes were not significantly predictive of death in HFrEF. This finding is consistent with the increasing appreciation that HFrEF and HFpEF are two different clinical entities with different pathogenesis.<sup>50</sup> Indeed, features of the primary cardiomyocyte dysfunction dominate in HFrEF, whilst abnormalities of the cardiac elastic properties and pathological changes of the extracellular matrix are perhaps pivotal in HFpEF.<sup>50</sup>

In agreement with previous reports, I observed that neutrophil and lymphocytes were associated with impairment of LV contractility, but I was unable to establish their significant role for the outcome.<sup>707-713, 719</sup> This may be due to the fact that subjects with predominantly mild-to-moderate HF were included in my study in contrast to the predominance of moderate-to-severe or acute decompensated HF subjects in most previous studies. The present study also shows for the first time that patients with HFrEF have significantly higher values of neutrophils and percentage of lymphocytes than subjects with HFpEF.

## **5.5. Limitations**

The leukocyte counts were obtained using a routine haematological analysis, which did not provide information on specific monocyte and lymphocyte subsets. The assessment of functional activity of leukocytes was beyond the scope of the present study. Additionally, the death rate was relatively low as the majority of the participants had mild HF and thus, these study results may not be transferable to HF patients with higher NYHA classes.

## **5.6. Conclusions**

There are significant differences in the numbers of granulocytes and percentage of lymphocytes between patients with HFrEF and HFpEF. High granulocyte counts and low percentage of lymphocytes are independent predictors of worse LVEF and NYHA status in HF.

High monocyte count is a significant independent predictor of death in patients with HFpEF. The study results advance the knowledge of the significant differences in the pattern of leukocytes between HFpEF and HFrEF. This differential effect of different types of leukocytes on LV contractility outcome and may help to better understand the complex pathophysiology of this disorder.

## CHAPTER VI. SUMMARY AND OVERALL CONCLUSIONS

### 6.1. Thesis summary

This thesis provides evidence of significant impairment of both micro- and macrovascular endothelial function in SAs with HF, which accords with similar previous findings from other ethnic groups. In contrast to the measures of endothelial function, parameters of arterial stiffness did not differ between SA subjects with HF and matched control groups.

My thesis work demonstrates for the first time that patients with HF also have significantly increased levels of both MMPs and PMPs, but only MMPs were independently and negatively predictive of LVEF [Chapter III]. In contrast, the work reports for the first time significant abnormalities in monocyte-derived CD34+ and KDR+ EPCs in patients with systolic HF. The numbers of CD34+ monocytes were 2-fold lower in HF subjects than in both control groups with normal LV contractility. Importantly, these abnormalities were evident even despite the similar overall monocyte counts among the groups. Counts of monocyte-derived EPCs did not correlate with parameters of cardiac geometry and function, raising the question of the clinical importance of these cells. However, higher VEGF receptor 1 expression on the Mon2 subsets showed some association with lower LVEF.

My thesis work highlights for the first time the presence of significant ethnic differences in endothelial and blood cellular parameters in patients with systolic HF [Chapter III]. Whites had better FMD than other ethnic groups, but the differences disappeared after the

adjustment for important clinical and demographic co-variables. This analysis suggests that ethnicity-related differences in co-morbidities accompanying HF rather than ethnicity itself may determine the status of macrovascular endothelial dysfunction.

SAs had more severe impairment of microvascular endothelial function compared to other ethnic groups. SA ethnicity was associated with microvascular endothelial dysfunction, even after adjustment for age, presence of hypertension and diabetes, blood pressure and glucose levels. No significant ethnic difference was seen in PWV.

My thesis work shows for the first time significant ethnic difference in monocyte subsets in HF [Chapter III]. The counts and proportion of Mon3, which has been suggested to have reparative potential were significantly higher in HF subjects of AC origin. There were also significant ethnicity-related differences in monocyte expression of SR-AI among HF subjects. These observations accord with better survival of AC subjects with HF, and indicate a possible intrinsic protective/reparative potential in this ethnic group.

In contrast to a recent study showing increased Mon2 levels in European patients with HF compared with controls, there were no significant HF-related changes in this monocyte subset in subjects of SA origin.<sup>425</sup> Nonetheless, my results indicate significant ethnic variations for this subset with white subjects having the highest values, which probably explains the discrepancy.

This work shows for the first time the presence of significant differences in neutrophil counts and lymphocyte percentage in subjects with HFrEF or HFpEF, thus providing

further insight to the possible pathogenic differences between the two conditions [Chapter V]. Of importance, the high monocyte count was a significant independent predictor of mortality in patients with HFpEF, but not in those with HFrEF.

The thesis also advances the methodological approaches to enumeration and characterisation of monocyte subsets and it demonstrates that three human monocyte subsets can be reliably discriminated on the basis of their varying expression of CD14, CD16 and CCR2[Chapter IV].

The thesis work highlights significant differences in the phenotype and functional activity of the monocyte subsets [Chapter IV]. It demonstrates unique characteristics of the CD14<sup>++</sup>CD16<sup>+</sup>CCR2<sup>+</sup> (Mon2) subset featured by attenuated inflammatory properties, high production of the anti-inflammatory cytokine IL-10, and maximal of all monocyte subsets expression of the angiogenic and reparative markers (e.g., Tie2, CXCR4, CD163, VEGF receptors 1 and 2). Of interest this subset was found to be enriched in the bone marrow. Together these observations indicate the presence of a reparative potential of this subset, which accords with previous observations showing an association between higher levels of these cells and better outcome in stroke survivors. The prognostic value of this subset in HF is still to be determined.

Additionally my thesis provides new data on the effects of some biological factors on monocyte subsets and MPAs [Chapter IV]. For example, it was found that there are significant diurnal variations in Mon2 subset with its highest counts seen at 6pm, which paralleled maximal CCR2 expression by the subset, which also peaked at 6pm.

My thesis work demonstrates a prompt increase in monocyte count and the contrasting reduction in MPAs following exercise [Chapter IV]. Although the detailed mechanisms of this phenomenon are not entirely clear, it is likely to reflect ability of the exercise to facilitate preferential migration to tissues of monocytes aggregated with platelets. This is supported by the available data showing the role of platelets in the regulation and acceleration of monocyte homing.<sup>700-701</sup>

This work also reveals a significant post-exercise reduction in CD14 expression by ‘classical’ monocytes, Mon1. Given that CD14 is intimately involved in monocyte antibacterial and inflammatory responses the finding might indicate a potential mechanism linking beneficial/anti-inflammatory effects of exercise and their immunodepressive effects.<sup>26702</sup>

## **6.2. The study limitations**

The study has a number of limitations which need to be considered during the interpretation of the results. The sample size of the ethnic groups is relatively small thus leaving a chance for a bias of the statistical tests, particularly given the necessity of multiple comparisons aiming to provide a more comprehensive picture of endothelial (dys)function and monocyte levels/phenotype. Also the study does not include a control group of healthy subjects of White origin, thus making difficult speculations on whether the observed ethnic differences are purely HF-related or persist from the premorbid state.



Inter-ethnic comparisons are also limited by a single geographic location of the study site, which may thus be biased due to the relatively selective nature of migration processes. Also inter-ethnic cultural diversity (e.g., reflected by nutritional habits) could affect the study results irrespectively of the ethnicity-related genetic background per se. This could also be due to possible variations in health behaviours, for example, reliance of medicine vs. religious believes, compliance with medications.

Admittedly, majority of the patients with HF from SA and AC origins represents the first generation of migrants to the UK with many such subjects derived from less developed regions, with suboptimal maternal nutritional access. These prenatal and early postnatal differences are likely to lead to some degree of epigenetic changes, which is evident from previous data on higher prevalence of cardiovascular risk factors and features of endothelial dysfunction in apparently healthy subjects of SA origin as compared to the White population (discussed in Chapter 2. Literature review). Accordingly the observed ethnicity-associated differences in parameters of endothelial dysfunction and monocyte subsets should be considered as a complex effect of genetic, epigenetic, environmental and behavioural differences attributed to a particular ethnic group.

### **6.3. Overall conclusion**

Systolic HF in SAs is associated with significant abnormalities of the endothelial function and cellular markers of inflammation and repair. A number of significant ethnic differences exist in parameters of endothelial function, characteristics of blood monocyte subsets and EPCs among HF subjects.

The findings further advance knowledge about the role of extracardiac factors in the pathogenesis of HF. This is likely to reflect the multiple roles the endothelium is playing in the regulation of the circulation. Also it is increasingly recognised that a fine balance between detrimental (e.g., excessive inflammation) and reparative factors (e.g., appropriate levels and activity of EPCs and ‘reparative’ monocyte subsets). The study findings provide further evidence of such dysbalance in HF, which may have specific features in HF subjects of different ethnic origin and indicates the presence of significant ethnic differences in the pathogenesis of HF.

#### **6.4. Future research and implication for practice**

Despite significant advances in the management of chronic HF during the last decades this disorder still poses a major threat to public health with a very poor individual outlook. Further developments in this field would require even better understanding of the complex disease pathology (e.g., involving peripheral vascular abnormalities). Future directions in the management of existing HF should perhaps be focused on cardiac repair aiming to reverse unfavourable cardiac remodelling and, ideally, to restore impaired myocardial contractility.

In the thesis study I show prominent abnormalities of microvascular endothelial function in stable HF which are likely to contribute to chronic tissue hypoperfusion, particularly during physical activity, when appropriate vasomotor endothelial response is particularly important.

Accordingly it is tempting to know the impact of impaired microvascular function on physical tolerance (e.g., shortness of breath, and fatigue). This should be tested in a clinical trial where physical tolerance is objectively measured (e.g., by cardio-pulmonary exercise testing).

It is still unknown how the status of microvascular endothelial function affects progression (both from the point of view of symptoms and cardiac deterioration) and outcome (mortality or need for HF-related hospitalization) in patients with HF. The answers to these questions should be obtained in prospective longitudinal studies.

The pathogenic and prognostic roles of microvascular endothelial function should ideally be confirmed by specific interventions aiming at the restoration of the microvascular endothelium. Admittedly pharmaceutical agents strictly specific to the microvascular endothelium are lacking at present. Potential ‘pleiotropic’ benefits of existing medicines (such as statins, or inhibitors of the renin-angiotensin-aldosterone system) or exercise rehabilitation towards the microvascular endothelium could be difficult to separate from other effects of these interventions. However, if prospective clinical trials showed a significant and independent pathogenic and prognostic role of the microvascular endothelial function in HF it could become a target for future endothelium-specific therapies. Obviously such interventions would need to be validated/tested in appropriate experimental models prior to their clinical assessment.

The clear advantage of the laser Doppler-based method of measurement of microvascular endothelial function used in my study is its non-invasiveness and simplicity. It thus may become a surrogate marker of generalized (i.e., not only cutaneous) microvascular dysfunction. The next step in the exploration of such possibility would be direct comparisons of cutaneous laser Doppler-based tests with (semi)invasive measures of microvascular dysfunction in other tissues (e.g., angiography-based methods, or contrastechocardiography with dipyridamole test for assessment of myocardial perfusion).

In my study I observed significant ethnic differences in micro- and macrovascular endothelial function, and in several parameters associated with reparative potential of monocyte subsets and monocyte-derived EPCs. The clinical significance of these findings should be confirmed in prospective clinical trials employing different techniques of cardiac imaging to evaluate possible changes in cardiac geometry, function, and vascularization. These studies should prove or exclude whether the observed ethnicity-related changes in the vascular and monocyte parameters are linked to the ethnicity-related differences in the disease progression and outcome. If the last hypothesis is correct it would be necessary to identify genetic factors and exact molecular mechanisms which drive those ethnicity-related differences.

This thesis study showed a significant increase in the levels of monocyte-derived microparticles in patients with systolic HF where they were significantly associated with poor LV contractility. In the light of the emerging evidence suggesting the role of circulating microparticles as inter-cellular messengers of biological signals (discussed in Chapter II) the pathological and prognostic roles of monocyte-derived microparticles

should be explored in prospective studies. In addition to microparticle enumeration, their phenotypic characterization and assessment of their contents (e.g., proteins, RNA) should be investigated in relation to HF and its progression. The relative simplicity of microparticle enumeration makes them potentially attractive prognostic biomarkers.

The results of my study showed prominent differences between the three monocyte subsets in terms of their phenotype, function, and bone-marrow vs. peripheral blood distribution. However the exact biological role and potential clinical utility of the minor monocyte subsets (i.e., Mon2 and Mon3) are still far from being clear. Different *in vitro* and *in vivo* experiments need be done to address this. For instance, angiogenic and cardiac reparative potential of different monocyte subsets could be explored by their delivery to animal models of ischaemic/myocardial damage. The experiments should be designed to be able to test (i) the ability of individual monocyte subsets to directly differentiate into functional vascular cells or myofibroblasts, (ii) their capacity to augment reparative properties of other cardiac and vascular cells, for example via release of angiogenic and growth factors.

Further research is also needed to establish the mutual developmental relationship between the monocyte subsets and factors regulating their mobilization to the peripheral blood from bone marrow and their homing to tissues. Obtaining data on their *in vivo* functional role of human monocyte subsets is particularly important as there are major differences between human and mouse monocyte subsets and direct extrapolation of animal findings to humans may be inappropriate. The development and utilization of the novel approaches of non-invasive imaging able to track human monocyte subsets *in vivo* would be of great advantage for such analysis.

Apparently the number of potentially interesting future studies related to the thesis findings is not limited to the brief outline above, but the design and details of such analyses should probably represent a continuum built on gradually accumulating evidences, hopefully supporting clinical the utility of my study results.

## APPENDICES

### Appendix 1. Standard Operating Procedure 103 ‘Flow-mediated dilation’

#### SOP 103 Flow Mediated Dilatation (FMD)

Updated by Alena Shantsila November 2009

##### ***Background***

Endothelium-dependent vasodilatation of the brachial artery occurs in response to increased flow. This response can be assessed using ultrasound (1-3). A blood pressure cuff is used to temporally occlude brachial artery. After cuff release, increased blood flow down the brachial artery causes nitric oxide (NO) release from the endothelium and consequent endothelium-dependent brachial artery vasodilatation. The endothelium-independent response can be assessed by administration of a systemic NO donor such as GTN. Abnormality in FMD have been found in patients at risk of atheroma even before evidence of actual atheroma formation, and it is also a predictor of future cardiovascular events in hypertension and coronary artery disease (4). Tremendous interest exists in determining the clinical utility of brachial artery FMD. It has been hypothesised that endothelial function may serve as an integrating index of risk factor burden and genetic susceptibility, and that endothelial dysfunction may serve as a preclinical marker of cardiovascular disease (5). The technique is particularly well suited for study of the earliest stages of atherosclerosis in children and young adults, thus providing maximal opportunity for prevention.

It is critical to point that numerous factors affect flow-mediated vascular reactivity, including surrounding temperature, food, drugs, sympathetic stimuli, period of menstrual cycle in women, among others. Therefore, subjects should fast for at least 8 to 12 h before the study, and they should be studied in a quiet, temperature-controlled room. All vasoactive medications should be withheld for at least four half-lives, if possible. In addition, subjects should not exercise, should not ingest substances that might affect FMD such as caffeine, high-fat foods and vitamin C or use tobacco for at least 4 to 6 h before the study. The investigator should be cognizant of the phase of the subject’s menstrual cycle, as it too may affect FMD (6). Vasomotor endothelial function has well demonstrated diurnal variations. Accordingly all measurements of FMD within particular study have to be performed at uniform time.

FMD assessment in patients with systolic blood pressure above 160 mm Hg has limited informativness a should be avoided. Never attempt to do the test when BP 180 mmHg or above.

##### ***Equipment***

You will need:

Patient (!)  
Vascular scanner free (for around 45 minutes at least)  
Echo jelly (the tubs are refillable)  
ECG monitoring dots  
Manual sphygmomanometer  
GTN spray

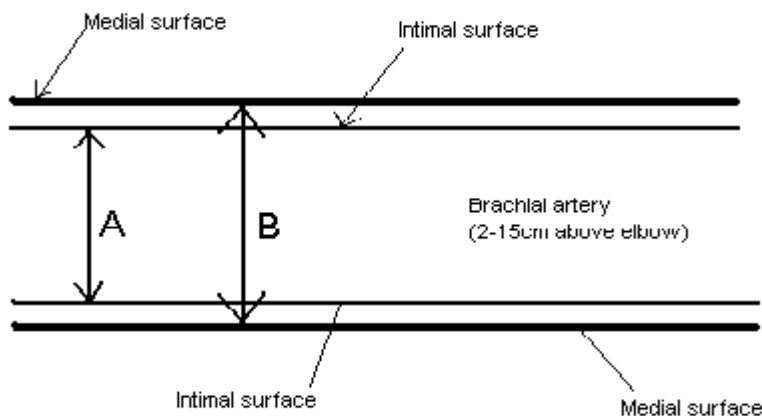
### ***Preparation***

- a. Allow the subject to rest in the room for about 15 minutes before scanning
  - b. Explain details of the procedure and possibility of some discomfort during arm compression
3. Select the probe on echo machine
- a. Turn on echo machine (switch on the front of the machine, towards the left)
  - b. Press 'patient data' on the echo machine console (towards the left) and type necessary patient details (study ID, age, etc.), then press 'close'
  - c. Place the ECG leads on the patient, connect with ECG cable
  - d. Press 'present/transducer' on left side of vertical panel of echo machine console
  - e. Select 'L11-3'; then select 'arterial limb'
  - f. Press 'physio' then 'next' on left-side panel and turn ECG on
4. *Place the Manual sphygmomanometer cuff on the forearm*
5. Label the image (using the 'annotation' button on the left of vertical panel of the console) as 'baseline' using Keyboard under console.
6. The subject is positioned supine with the arm in a comfortable position for imaging the brachial artery. The brachial artery is imaged above the antecubital fossa in the longitudinal plane below the blood pressure cuff. Identify the artery with colour doppler if necessary (button marked 'CFM' on right panel of echo machine) – arterial flow is obviously pulsatile and typically very bright on colour flow (it may be blue or red); once identified as arterial, it is easiest to turn colour flow off again in order to see the arterial wall clearly. A linear segment with clear anterior and posterior intimal interfaces between the lumen and vessel wall is selected for continuous 2D grayscale imaging.
7. Press 'zoom' button, choose desirable visualisation of the artery and press 'zoom' button again.
8. During image acquisition, anatomic landmarks such as veins and fascial planes are noted to help maintain the same image of the artery throughout the study. This makes it much easier to be sure you are measuring the same segment in subsequent measurements. It's also worth finding a vessel that is easy to identify because the later scans are time dependent and you will be under some pressure to find the same vessel quickly.
9. Freeze the image (button to the right of the console) when you have a clear segment seen for several seconds. Scroll backwards and forwards (using the tracker ball) to use the R wave of the ECG cycle so that subsequent readings are taken in the same part of the cycle,



and so that the image quality is optimal. Press 'acquire' to save the image (allows someone else to analyse the image without your measurements being visible); At least 3 cardiac cycles should be saved at approximately 30 sec, 1 min, 2 min, 3 min after blood pressure cuff decompression.

10. Once the image for analysis is chosen, the boundaries for diameter measurements (the lumen-intima or the media-adventitia interfaces) are identified manually with electronic calipers or automatically using edgedetection software. Using 'caliper' button, measure 5 readings from leading edge to leading edge of the vessel. Move the cross cursor to the first point, press the white button on the left of the trackball once you are happy with position, then move to the second area and press the same button again. The machine then displays the distance. Select repeat measurement to take 5 readings. Once you have the readings save the image with the readings visible; take the average of the 5 readings as your measure. The variability of the diameter measurement is greatest when it is determined from a point-to-point measurement of a single frame, and least when there is an average derived from multiple diameter measurements determined along a segment of the vessel. Different people use different places to measure: the important thing is to be consistent between readings and between patients. In the figure, both A and B are acceptable so long as the same method is performed in all patients.



11. Blood flow is estimated by measuring the peak or time-averaging of the pulsed Doppler velocity signal obtained from a midartery sample volume.

12.. Inflate the cuff for at least 20 mm Hg above systolic blood pressure to occlude arterial inflow for 5 min.; warn the patient that it will be uncomfortable! Start timing. Keep an eye on the pressure in the sphygmomanometer: it may drop a little with time and you will need to by pumping more air into the cuff. You can now unfreeze the echo image; delete the label with the 'erase all text' button to the left of the console. Then label the image 'hyperaemia' or similar in preparation for the next image.

13.. At 5 minutes, deflate the cuff rapidly. A midartery pulsed Doppler signal is obtained upon immediate cuff release and no later than 15 s after cuff deflation to assess hyperemic velocity. The ration of blood velocity prior and after the compression serves as a measure of the stimulus (sheer stress) applied at the test. It should be similar for all study groups analysed.

14. Make repeated scans during 3 min. after cuff deflation. It is worthwhile looking for the artery soon (immediately) after cuff deflation so that you can be sure to find the artery within this time, then holding the probe steady until your 3 min. are reached. Try to identify the same segment as you scanned for the first baseline (compare the image you are getting with the image displayed on the mac which you sent to echopac), then freeze the image and repeat the measurement as before, again using the R wave of the ECG cycle.

15. Wait 15 minutes to allow the effect of the FMD to wear off. (You can use this time to gather other information such as history, drug use, or measuring other things such as carotid IMT, delete the label with the 'erase all text' button to the left of the console. Then label the image 'GTN' or similar in preparation for the next image.; make sure you are not doing anything that might upset vasomotor function though (such as blood pressure, venesection). GTN should not be administered to individuals with clinically significant bradycardia or hypotension.

16. Repeat blood flow velocity measurement by the pulsed Doppler..

17. Give 2 puffs of sublingual GTN (warn the patient they may get a mild headache / feel dizzy).

18. Peak vasodilation occurs 3 to 4 min after GTN administration; images should be repeatedly recorded during this time, using synchronisation by the R wave of the ECG cycle.

19. The acquired images are stored in the buffer and need to be saved on the workstation XCelera - press 'end exam' button on the console.

20. Remove sphygmomanometer cuff from the arm, remove the ECG leads from the patient, disconnect from ECG cable. Clean jelly off the patient and thank him/her!

21. Clear up the room: tidy up the examination couch, turn off equipment if not going to be used shortly. Bear in mind that the equipment is expensive and used by a lot of people.

### ***Potential problems***

This technique takes practise – According the current guidelines on FMD assessment it is recommended that at least 100 supervised scans and measurements need to be performed before independent scanning and reading is attempted; 100 scans per year should be performed

to maintain competency. This recommendation is based in part on criteria for ultrasound proficiency established by the Intersocietal Commission for the Accreditation of Vascular Laboratories.

It is worth trying quite hard to get a good image, and be scrupulous in technique in trying to identify the same segment of artery each time. The second scan (FMD / GTN scan as opposed to baseline scans) is a bit pressured as there is a time factor involved. As previously mentioned, try to find the relevant segment of artery early to give you more

time to look. If you don't have time to actually measure the vessel diameter before the next reading is due, you can measure it later from saved images so don't worry.

#### References

1. Celermajer DS, Sorensen KE, Gooch VM, et al. Non-invasive detection of endothelial dysfunction in children and adults at risk of atherosclerosis. *Lancet*. 1992;340:1111-5
2. Chong AY, Blann AD, Lip GY. Assessment of endothelial damage and dysfunction: observations in relation to heart failure. *QJM*. 2003;96:253-67. (Excellent in-house review of FMD as well as other assessments of endothelial dysfunction)
3. Corretti MC, Anderson TJ, Benjamin EJ, et al; International Brachial Artery Reactivity Task Force. Guidelines for the ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery: a report of the International Brachial Artery Reactivity Task Force. *J Am Coll Cardiol*. 2002;39:257-65.
4. Suwaida JA, Hamasaki S, Higano ST, et al. Long-term follow-up of patients with mild coronary artery disease and endothelial dysfunction. *Circulation* 2000;101:948-54.
5. Vogel RA, Corretti MC. Estrogens, progestins, and heart disease: can endothelial function divine the benefit? *Circulation* 1998;97:1223-6
6. Schachinger V, Britten MB, Zeiher AM. Prognostic impact of coronary vasodilator dysfunction on adverse long-term outcome of coronary heart disease. *Circulation* 2000;101:1899-906.

## **Appendix 2. Standard Operating Procedure 107 ‘Measurement of microvascular endothelial function’**

### **Standard Operating Procedure 107 Measurement of microvascular endothelial function**

Written by Will Foster and Andrew Blann August 2005  
Updated by Eduard Shantsila and Andrew Blann December 2008

#### **Health and Safety / COSHH**

The only major hazard in this procedure is the risk of damage to the retina due to the laser. Therefore avoid eye contact. Some subjects may be allergic to Ach & SNP. Some may respond adversely to the electrical current. Obtain consent.

The function of large arteries such as the aorta, brachial and femoral can be assessed by their dilation response to changes in blood flow (i.e., flow mediated dilatation, FMD). This assessment is not possible in the study of small arteries and arterioles, often within vascular beds such as the skin. However, the recent development of perfusion imaging can assess these small arteries. The present SOP is to enable this assessment.

#### **Introduction**

Laser Doppler perfusion imaging is based on four separate technologies

- (a) the Doppler shift phenomenon (change of the wavelength of the moving object proportionally the speed of the object, i.e., blood cells),
- (b) the ability of the Laser imaging system to detect this phenomenon,
- (c) perfusion of skin with pharmacologically active drugs (acetylcholine [Ach] and sodium nitroprusside [SNP]) that will alter small blood vessels close to the surface of the skin, and
- (d) the electrical delivery of these agents into the skin (iontophoresis).

Thus the method allows an evaluation of the perfusion of surface tissues (e.g. skin) in real time scale. Changes in skin blood flow can be determined in response to various stimuli. Vasoactive substances can be used to alter the flow of blood in the skin vessels if delivered by an appropriate means. Iontophoresis is a delivery method that uses the charge of a charged vasoactive substance to allow it to be driven through the skin using an electric current. Sodium nitroprusside is a vasoactive substance with a negative charge that can be delivered through the skin to the microcirculation; once it has passed through the skin it acts as a nitrate donor and therefore acts directly on the vessel's smooth muscle to cause vasodilatation. Thus it is independent of the endothelium in its vasodilatory action. Acetylcholine on the other hand is a positively charged molecule that can similarly be delivered to the subcutaneous tissues by iontophoresis but it acts on the endothelium rather than on the smooth muscle wall, causing endothelial release of nitric oxide. Thus acetylcholine acts in an endothelium-dependent way on the vessel wall to produce

vasodilatation. The vasoactive substances are provided close to the skin in a small perfusion chamber that also acts as an electrode. A second electrode is required to complete the circuit, and this is attached to a nearby section of skin.

### **1. Test solutions**

Quantities of 2% acetylcholine chloride (Ach) and 0.1% sodium nitroprusside (SNP) [both from Sigma-Aldrich] must be prepared in sterile filtered distilled water (Sigma-Aldrich) and appropriate volumes (approximately 1ml aliquots) stored in stored top plastic tubes a fridge at 4°C up to 1 month. These solutions are transferred from the plastic tubes to the iontophoresis chamber with a plastic disposable transfer pipette (stores in preparation room and the laboratory). Ensure the reagents are allowed reach room.

### **2. Patient preparation**

2.1. Use right forearm, in should be clean.

2.2. Ask the patients to put it on a supportive cloth pad on a table with the participant sitting on a chair in a constant room temperature room. Make sure the participant is comfortable and the hand is stable.

2.3. Remove any hair from the forearm gently with a single-use razor.

2.4. Use a Skin Prep (e.g., Skintact) gently to prepare area of the scans, leave the patient to rest for 20 minutes.

### **3. Preparation of the Laser Doppler Flowmeter, Iontophoresis and Data acquisition**

*(Laser Doppler Perfusion and Temperature Monitor DRT4 with Iontophoresis Controller Min1-e, Moor Instruments, Moor Instruments, Axminster, Devon, UK).*

3.1. To switch on the LD, press the black buttons on the main Laser Doppler block, and on Iontophoresis Controller Min1-e, wait for 10 minutes to let the laser warm-up.

3.2. Put two LD chambers connected to the Iontophoresis controller on the front forearm, 15 cm apart using single-use sticky rings provided by Moor Instruments. The first chamber is placed to the upper forearm, the second to the lower forearm.

3.3. Connect LD leads to the chambers: Channel 1 to the top chamber, Channel 2 to the bottom chamber.

3.4. Add Ach solution (~1 ml) to the top chamber using a plastic Pasteur pipette; add SNP solution (~1 ml) to the bottom chamber in a similar way.

3.5. Press top blue square button (RUN) on the LD block, make sure there is a good signal from both channels, press the same button again.

3.6. Now the LD will automatically acquire the baseline skin flow for 1 min, following by delivery of the electric current for another 1 min, and further following by 10 min of recording of post-stimulation signals.

3.7. The chambers can now be removed, and cleaned, a hand washed.

### **4. Data Analysis.**

4.1. Use MoorSoft/DRT4 v. 2.0 software installed on your computer.

4.2. Connect DRT4 to the computer using a grey cable

4.3. Start the software.

4.4. Press 'Download stored data from DRT4' button on the software panel and indicate a folder where the data should be stored.

4.5. Open a file in the scan data using 'File' – 'Open'.

- 4.6. Use 'Edit' – 'Mark' – 'Insert' to put marks at the beginning of the test, beginning of the stimulation, the end of the stimulation, the end of the scan. It is very obvious where to put them as DRT4 places small arrows to point those at the time of acquisition.
- 4.7. Press 'Analyse' – 'New Statistics' and the measurements on the flow before and after the stimulation will be automatically made.
- 4.8. Use 'Analyse' – 'New report' and 'Print' options to obtain a hard copy of your results.
- 4.9. Put results on the mean flow before and after the stimulation into your database for further

## **Appendix 3. Standard Operating Procedure 197 ‘Enumeration of monocytes sub-populations by flow cytometry’**

### STANDARD OPERATING PROCEDURE 197

#### **Enumeration of monocytes sub-populations by flow cytometry**

##### **ES Heart Failure**

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

##### Required pre-training

SOPs on venepuncture and on good clinical practice

SOP 195 – General operation of the flow cytometer

SOP 171 – Operation of the Bayer Advia

##### Contents

Introduction	Page 1
Materials and suppliers	Page 2
Detailed Method	Page 4
Interpretation	Page 7

#### 1. Introduction

Monocytes are large mononuclear cells (MNCs) derived from the bone marrow but on transit to the tissues where they seem likely to become semi-resident macrophages. Traditionally, they have been defined by glass-slide morphology, size, and scatter, but we now have the ability to define monocytes by cell surface molecules, using the FACS. For example, one sub-population bears CD14, another other bears CD16.

Endothelial progenitor cells (EPCs) are of potential importance in the physiological response to endothelial damage. This has led to speculation that the ability to enhance their numbers may allow for a novel approach to modulate various disease states. The definition of these cells has continued to evolve. Currently cited markers include CD34, CD133,

CD309, VEGFR1 and, notably, CD14. Importantly, as these cells mature, the cell surface markers change. In addition, other cells such as monocytes and tissue resident stem cells may cross-differentiate, adding to the complexity of defining this cell population.

This SOP describes enumeration of cells staining for any combination of:

CD34 - widely distributed on stem cells, haematoblasts, progenitor cells etc

VEGFR1. Said to be present specifically on endothelial cells but reported by some to be on monocytes

CD16 – this is an antigen found on the Fc receptors FcγRIIIa and FcγRIIIb. These receptors bind to the Fc portion of IgG antibodies. CD16 is present on natural killer cells, neutrophil polymorphonuclear leukocytes, monocytes and macrophages.

CD14 – a receptor for LPS present on monocytes, macrophages and neutrophils

This SOP demands a monocyte count, itself derived from the full blood count, from the Bayer Advia.

## **2. Materials and Supplier contact details:**

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

BD “FACS Flow” Running solution [Becton Dickinson, Catalogue No. 342003]  
10L containers.

BD “FACS Clean” Cleaning Solution [Becton Dickinson, Catalogue No. 340345]

3 ml BD Falcon tubes [Becton Dickinson, Catalogue No. 352054]

BD Lysing solution [Becton Dickinson Catalogue No. 349202]

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

CD14 -PE conjugated monoclonal antibody - 100 tests [R&D Systems Europe Ltd, Cat No. FAB3832P]

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

CD34 –PerCP conjugated monoclonal antibody - 50 tests [Becton Dickinson, Catalogue No. 345803]

VEGF R1-APC conjugated monoclonal antibody - 100 tests [R&D Systems Europe Ltd Catalogue No. FAB321A]



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

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

Remember to dispose of all material thoughtfully.

### **3. Detailed method**

FIRSTLY

For all WBC analyses a full blood count (Bayer Advia) must be obtained on the same sample of blood that will be used for flow cytometry.

This is essential in order to back-calculate monocyte numbers and thus sub-populations to whole blood

#### **3.1 General Preparation**

##### **3.1.1 Lysing solution.**

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

#### **3.2 Blood sample preparation**

Gently vortex the EDTA or citrate blood sample. Take 0.1 mL of whole blood with yellow tip pipette and add to a 3 mL BD Falcon tube.

Add 10µL each of CD14, CD16, CD34, VEGFR1 fluorochrome labelled antibodies (see 2) with a micropipette. Flush into and out of pipette tip to ensure thoroughly mixed and then gently vortex. Incubate in the dark at room temperature for 20 minutes.

Add 2 ml pre-diluted BD lysing/fixing solution (see 3.1.1) with a clear tip using the 1ml pipette. Incubate for 10 minutes on bench in the dark. Then centrifuge 300g for 5 minutes.

Decant supernatant and add 3 ml of PBS (good quality PBS in the fridge) solution. Vortex gently to resuspend pellet. Then centrifuge once again at 300g for 5 minutes.

Add 0.2 ml of PBS solution and flush into and out of pipette tip, followed by gentle vortex to ensure thoroughly mixed. Store sample in dark at 4°C until ready to be processed (note sample must be processed within three days).

### **3.3 Start up procedure [See SOP 195 on General Operation]**

#### Part 1 – restoring reagents and preparation

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

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

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

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

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

Close the drawer

#### Part 2 - cleaning the machine

Ensure that a 3 ml Falcon tube (labelled 1) approximately 1/3 full of distilled water is positioned over the sample injection port (SIP) – a needle sheath - and that the swing arm is positioned under this tube. Press the prime button on the panel. When the system enters “standby” within 30 seconds then press the “prime” button again. When the standby and low buttons come on again then remove tube 1. We will re-use tube 1 in the shut down procedure.

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

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

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

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

The machine is now ready to run samples.

<b>NB: FACS COMP may need to be run</b>
---

### 3.3 Running blood samples.

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

Click on the ‘Data 1’ folder on the desk top

Click on the ‘Monocyte Protocols’ folder.

Click on the ‘Monocyte Eduard’.

This will open the CellQuest Pro software with the instrument settings.

Click ‘**Connect to Cytometer**’, located under the ‘Acquire’ menu.

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

Click the ‘**Acquire**’ menu once more and click ‘**Show browser**’.

Click directory-‘**Change**’ in order to specify the location folder.

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

Change the custom suffix to the preferred title and number for data and click ‘**OK**’.

Untick the setup box (by clicking on it) in the browser-EPC Acquisition window. Now insert your sample and press “RUN” and “HIGH”.

Each sample is now ready to run. This should be mixed thoroughly and transferred to a 3 mL BD Falcon tube. Open swing arm at bottom right of the cytometer and replace the Falcon tube with the sample to be run. Replace the swing arm under the Falcon tube.

Press the buttons ‘**Run**’ and ‘**High**’ on the control panel of the cytometer.

Click ‘**Acquire**’ on the browser menu. The sample will now run for ~ 2-3 mins. Cell events will be displayed on the screen throughout the process (n.b. the higher the cell density, the more rapidly the cells will be acquired).

Click on ‘**Counters**’ under the ‘**Acquire**’ and observe the events per second which varies from 500 to 5000 depending on various factors. The objective is to acquire 200,000 events for analysis.

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

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

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

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

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

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

### **3.4 Shut-down procedure [See SOP 195 on General Operation]**

In this section we re-use tubes 1 and 2 with distilled water and FACS clean respectively.

Install FACS Clean tube 2 over the SIP needle. Press button ‘**High**’ and ‘**Run**’ on the panel. Leave the support arm out at 90 degrees for approximately 1 minute. This cleans the outer portion of the aspiration sheath. The fluid will be rapidly aspirated, so ensure that the tube doesn’t empty completely.

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

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

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

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

Finally power down the FAC-Scalibur (green button) and Apple Mac.

Clean up !

Note: \* IF THE SYSTEM IS TO BE USED AGAIN ON THE SAME DAY...

LEAVE THE SYSTEM ON STANDBY and then

DEPRESSURISE THE SYSTEM.

#### **4. Interpretation of plots**

For the first ten or so analyses you will need to have all this explained to you by Dr Blann

Collect your plots from the Advia (Figure 1). Obtain the monocyte count ( $0.40 \times 10^3/\mu\text{L} = 0.4 \times 10^6/\mu\text{L}$ ), and the lymphocyte count ( $1.59 \times 10^3/\mu\text{L} = 1.59 \times 10^6/\text{mL}$ ). Therefore Advia defined mononuclear cells (MNCs) =  $1.99 \times 10^6/\text{mL}$ . Hence the monocytes make up  $0.4/1.99 = 20.1\%$ . Similarly the % MNC population in terms of the entire WBCC according to the Advia should be  $1.99/6.11 = 32.57\%$ . Remember these numbers.

Now the FACS plots (Figs 2 & 3, but focussing on Fig 2). The top left initial plots show the FSC/SSC plot (forward and side scatter, all in black). This is needed to gate the presumed monocyte and lymphocyte regions, i.e. = mononuclear cells (MNCs).

Immediately below the figure is a set up data showing the date of the analysis and total WBCC events (e.g.200,000). Below this are the number of gated monocytes (e.g. 12,682) and number of gated lymphocytes (e.g. 45,445). Sum this to give total number of gated MNCs (= 58,127). Note that you can compare the proportion of the Advia defined

monocytes/MNCs (20.1%, from para 1 above) with the same data from the FACS (12,682/58,127 = 21.8% - note the very good agreement, <10%).

### **1. Gating monocyte subpopulations (Figure 2 as a template)**

The central top plot is monocytes by CD14 (horizontal axis) and CD16 (vertical axis), and below it are the numbers, the largest e.g. 12,682 is populated from the FSC/SSC window. Note the three gates:

Gate 1 is CD14 high CD16-ve. The grid analysis gives 9554 events (75.34%).

Gate 2 is CD14 high CD16 +ve. By the same token 1354 events = 10.68%

Gate 3 is CD14 dim CD16 +ve. Ditto 1310 events = 10.33%

You will then need to translate this to meaningful cell biology by converting to cells/ml of whole blood, which is why you need the WBC from the Advia. This section will shortly inform additional subanalyses.

### **2. EPCs from the total MNC population**

Next – top right. This is the total MNC population (i.e. all 58,127 events) classified by VEGFR1 and CD34 staining. Note the quadrant....

UL (MNCs VEGFR1+ve/CD34-ve) is 3679 events from 58127 = 6.33%

UR (MNCs VEGFR1+ve/CD34+ve) 180/58127 = 0.31% = EPCs (?)

LL (MNCs VEGFR1-ve/CD34-ve) is 53996/58127 = 92.89%

LR (MNCs VEGFR1-ve/CD34+ve) is 272/58127 = 0.47%

As before, convert to cells/ml using the MNC and WBC counts from the Advia. N.b. the EPC definition here mimics that of the prostate protocol, but in the latter, they were all CD45-ve – not here, so beware!

### **3. Monocyte subanalyses**

Bottom left gives 9554 CD14+ve/CD16-ve monocytes from gate 1 analysed by VEGFR1 and CD34. Check this by looking at the number of events. Observe the quadrants:

UL = VEGFR1+/CD34- : 2732/9554 = 28.6% of all gate 1 monocyte events

UR = VEGFR1+/CD34+ : 101/9554 = 1.06% of all gate 1 monocyte events

LL = VEGFR1-/CD34- : 6630/9554 = 69.4% of all gate 1 monocyte events

LR = VEGFR1-/CD34+ : 91/9554 = 0.95% of all gate 1 monocyte events

Exactly what these cells are depends on your thoughts of what CD34 and VEGFR1 are found on. Percent of cells is used calculate the absolute of these cells per 1 ml of blood using monocyte numbers from the whole blood sample. Example: The Advia says  $0.4 \times 10^3$  monocytes/ $\mu\text{l}$  = 400,000 cells/mL. As gate 1 = 9554 events from 12,682 monocyte events this is 75.34% so there are  $400,000 \times 75.34\% = 301,360$  CD14+CD16-ve

monocytes/ml. Of these 301,360 cells/ml, 1.06% are additionally VEGFR1+ve/CD34+ve = 319 cells/mL

The middle plot gives the 1354 CD14+ve/CD16+ve monocytes in gate 2 stained by VEGFR1 and by CD34. Observe the quadrants:

UL = VEGFR1+/CD34- :  $715/1354 = 52.81\%$  of all gate 2 monocyte events

UR = VEGFR1+/CD34+ :  $41/1354 = 3.03\%$  of all gate 2 monocyte events

LL = VEGFR1-/CD34- :  $587/1354 = 43.35\%$  of all gate 2 monocyte events

LR = VEGFR1-/CD34+ :  $11/1354 = 0.81\%$  of all gate 2 monocyte events

The bottom right gives analysis of 1310 CD14dim/CD16+ve cells in th3 3<sup>rd</sup> gated region stained by VEGFR1 and by CD34. Observe the quadrants:

UL = VEGFR1+/CD34- :  $52/1310 = 3.97\%$  of all gate 3 monocyte events

UR = VEGFR1+/CD34+ :  $6/1310 = 0.46\%$  of all gate 3 monocyte events

LL = VEGFR1-/CD34- :  $1145/1310 = 87.4\%$  of gate 3 monocyte events

LR = VEGFR1-/CD34+ :  $107/1310 = 8.17\%$  of gate 3 monocyte events

## 5. Interpretation of results

Exactly what these cells are depends on your thoughts of what CD34, VEGFR1, CD14 and CD16 are found on. **A word of warning** Be not 100% confident of the interpretation of your data. Recall that odd CD molecules may be present on cells other than those you presume them to be present upon.

## 6. Validation and quality control

The intra-assay reproducibility of the methods was assessed on six samples of blood, one set of three from a healthy male and second set of three from a woman with a history of renal and ovarian cancer.

Intra-assay coefficients of variation (CV) are derived from the % results of the different subpopulations of monocytes. The initial sorting on monocytes into M1, M2 and M3 give a mean CV of 4.75% (n=6). This contrasts well with the CV of the monocyte count on the Advia of 6.65% (n=2). CVs for the next level of analysis (all monocytes by plus or minus VEGFR1/CD34 grouping gives a mean CV of 6.8% (n=8).

CVs of sub-analyses of the M1, M2 and M3 populations according to VEGFR1/CD34 class were variable. For M1 median (IQ) CV was 8.5% (3.4-20.6), for M2 it was 9.0% (6.2-17.8 and for M3 it was 17.9% (4.1 – 23.4%).

However, this pooling smothers a strong mathematic effect of the raw data. Example, a clutch of 95%-97%-99% gives a CV of 1%, whereas the same variance of 10%-12%-14% gives a CV of 13.6%. Real time examples from this data set are:

Results 50%-96% (n=10) - CV = 2.3%  
Results 10%- 39% (n=6) – CV = 6.7%  
Results 2.2% - 9% (n=9) – CV = 13.7%  
Results <2.2% (n=13) – CV = 17.9%

Thus reproducibility is strongly related to percentages, which may be an argument in favour of analysing raw data, and also possibly an argument for not analysis data where numbers are small as intrinsically CV is likely to be large and the data therefore unreliable.

SOP 197: Enumeration of monocytes sub-populations by flow cytometry

Signed off.....Andrew Blann.....8<sup>th</sup> January 2010.....

Figures not electronic: F1 Advia printout: F2 FACS printout (see ADB)

Raw analyses available from ADB



## **Appendix 4. Standard Operating Procedure 190 ‘Enumeration of microparticles by flow cytometry’.**

### STANDARD OPERATING PROCEDURE 190

#### **Enumeration of Microparticles by Flow Cytometry**

**Eduard Shantsila, Andrew Blann: June 2010**

n.b. Use of the flow cytometry is forbidden without having been officially trained

#### Required pre-training

SOP on venepuncture, good clinical practice

SOP 195 – general operation of the flow cytometer

#### Contents

1. Introduction	Page 1
2. Materials and suppliers	Page 2
3. Detailed Method	Page 4
4. Interpretation and validation	Page 6

#### 1. Introduction

Cellular microparticles (MP) are submicroscopic fragments resulting from the remodelling of plasma membrane in response to numerous conditions, including activation and apoptosis [1]. MPs are generally referred to as 0.1 to 1 µm membrane fragments that often expose the anionic phospholipids phosphatidylserine and membrane antigens representative of their cellular origin. These characteristics discriminate MP from exosomes, which are smaller (<0.1 µm), originate from intracellular multivesicular bodies, and differ in antigenic composition.

MP have been shown to have prothrombotic irrespective of their origin (e.g., from platelets, leucocytes, endothelial cells, red blood cells). However, presence of certain markers (e.g, tissue factor) on MP surface is substantial additional risk of thrombogenesis. Flow cytometry allows identifying and enumerating different types of MP on the basis of

their size, surface antigen presentation and employing count beads (for absolute count of MP number).

This method describes enumeration of:

CD42b (GPIb)+ microparticles for platelet-derived microparticles;

CD144 (VE-cadherin)+ microparticles for endothelial cell-derived microparticles;

CD14+ microparticles for monocyte-derived microparticles

n.b. the SOP may be amended to look at subpopulations of microparticles e.g. those events also bearing tissue factor, CD146 or CD34.

## **2. Materials and Supplier contact details:**

BD “FACS Flow” Running solution [Becton Dickinson Catalogue No. 342003]  
10L containers. Becton Dickinson (UK) Between Towns Road Cowley Oxford  
, Oxfordshire, OX4 3LY Fax: 01865 781578

BD “FACS Clean” Cleaning Solution [Becton Dickinson Catalogue No. 340345]

BD Falcon tubes [Becton Dickinson, Catalogue No. 352054]

BD Lysing solution [Becton Dickinson Catalogue No. 349202]

Sterile Phosphate Buffered Saline solution, 0.5L bottles [????]

CD144-PE conjugated monoclonal antibody - 100 tests [RD Catalogue No.  
FAD9381P](defines EMPs)

CD14-PerCP conjugated monoclonal antibody - 50 tests [RD Catalogue No.  
FAB3832C](defines PMPs)

CD42b-APC conjugated monoclonal antibody - 100 tests [BD Catalogue No.  
551061](defines MMPs)

CytoCount, Count Control Beads – 17 mL [DakoCytomation, Catalogue No. S2366]

Blue pipette tips [Appleton Woods Catalogue No. HTL160] Appleton Woods Ltd  
**Lindon House, Heeley Road Selly Oak, Birmingham B29 6EN Tel 0121 472 7353, Fax  
0121 414 1075. Freefax orderline 0800 387 462**

Yellow pipette tips [Appleton Woods Catalogue No. TG701]

### 3. Detailed method

#### 3.1 General Preparation

Peripheral vein blood sample is to be collected with a 21 gauge needle without applying hemostasis into sodium citrate Vacutainer tubes (BD Diagnostics)

Platelet-free plasma (PFP) has to be prepared by centrifugations for 15 min at 2860g.

Aliquots have to be frozen in 1.5-mL tubes (0.5 mL per tube) and stored at -70°C until use.

#### 3.2 Sample Preparation

PFP has to be unthawed immediately before sample staining.

Prepare 'Mastermix' (MM) of antibodies in the morning of analysis.

Mix 10µL of CD144 and CD14 fluorochrome labelled antibodies and 20µL of CD42b antibody in a BD Falcon tube and dilute 360µL of filtered PBS. This amount of antibody would be sufficient for 20 samples. Total amount of antibodies should be prepared accordingly planned number of samples.

Put 20µL of the MM into BD Falcon tube.

Take 50 µL of thoroughly vortexed PFP with 'wet tip' reverse pipetting technique and add to the tube above without touching antibodies.

Take 25 µL CytoCount, Count Control Beads with 'wet tip' reverse pipetting technique and add to the sample above without touching plasma and antibodies.

Gently vortex the sample. Incubate in the dark at room temperature for 10 minutes.

Ad 100 µL of filtered PBS.

The sample is now ready to be used.

#### 3.3 Start up procedure [See SOP 195 on General Operation]

##### Part 1 – restoring reagents and preparation

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

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

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

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

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

Close the draw

## **Part 2**

Ensure that a 3 ml Falcon tube (labelled 1) approximately 1/3 full of distilled water is positioned over the sample injection port (SIP) – a needle sheath - and that the swing arm is positioned under this tube. Press the prime button on the panel. When the system enters “standby” then press the “prime” button again. Remove tube 1. We will re-use tube 1 in the shut down procedure.

Prepare a second falcon tube (labelled 2) with FACS-clean. This is a smaller box on a shelf at above head height and above the bigger box of sheath flow fluid

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

Return to falcon tube 1 with distilled water. Repeat the above step 6 with this distilled water tube.

The machine is now ready to run samples.

### **3.3 Running Samples.**

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

Click on CellQuest Pro icon on the desk top to open the CellQuest Pro software with the instrument settings.

Click 'Connect to Cytometer', located under the 'Acquisition' menu.

Under the 'Cytometer' menu, click 'Instrument Settings'. Change settings to 'Microparticles' Settings, located within the 'Microparticles' folder. Make sure to click 'SET' prior to clicking 'DONE'.

Click 'Open' to open microparticle acquisition protocol from 'Microparticles' folder. The settings are predefined enumerate events positive for CD144, CD14, CD42b and having size less 1µm but more ~0.3.

Click the 'Acquisition menu' once more and click 'show browser'. Enter the file name and number for data to be stored under and choose an appropriate location to save data to.

Open swing arm at bottom right of the cytometer and replace the Falcon tube with the sample to be run. Replace the swing arm under the Falcon tube.

Press the buttons run and high on the control panel of the cytometer. Click 'Acquire' on the browser menu. The sample will now run for ~ 3 mins. Absolute number of specific microparticle types as,

Number of Cells Counted (e.g. CD42b x CytoCount™ Concentration  
Number of CytoCount™ Beads Counted x 2

CytoCount™ Concentration is to be taken from the reagent pack.

2 – represents a ratio in the volumes of plasma and count beads

Obtain all the data and apply in into the specific spreadsheet you have designed for your project.

### **3.4 Shut-down procedure [See SOP 195 on General Operation]**

In this section we re-use tubes 1 and 2 with distilled water and FACS clean respectively.

Install FACS Clean tube 2 over the SIP needle. Press button high and run on the panel. Leave the support arm out at 90 degrees for approximately 1 minute. This cleans the outer portion of the aspiration sheath. The fluid will be rapidly aspirated, so ensure that the tube doesn't empty completely.

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

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

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

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

Finally power down the FAC-Scalibur (green button) and Apple Mac.

Clean up!

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

#### **4. Interpretation**

Refer to the illustrative figure. It shows three flow cytometer plots.

The left hand plot is the FSC/SSC plot of the sample. The round circle top right are the count beads. The region boxed in the bottom right is for microparticles (MPs).

The middle plot is the plot for platelets microparticles (PMPs). The vertical (Y) axis is the staining with CD42b for platelets. The horizontal (x) axis is not being used for fluorescence but shows the count beads.

Note the cut of lines which define number of PMPs. These have been set by the results of the isotype controls. Hence events greater than about 250 arbitrary units are PMPs

The right hand plot is for monocyte (vertical axis, CD14) and endothelial (horizontal axis, CD144) microparticles (MMPs, EMPs).

Also on the plot are the date, the total number of events (e.g. 364,348), and breakdowns of the different numbers of events in the different quadrants of the plots.

The plots data is the number of PMPs, EMPs and MMPs per microlitre of plasma. These are the numbers you need to add into your spreadsheet.

Typical results are likely to be in the region of.....

4663 PMPs/ $\mu$ L of plasma

2742 EMPs/ $\mu$ L

267 MMPs/  $\mu$ L

Do not be concerned that some numbers fail to add up or match as MP numbers have been back calculated from the concentration of the count beads.

The second illustrative plot is of the isotype controls. Note identical layout but much smaller numbers (of course, ideally these would be zero).

### Validation

Duplicate agreement was obtained for thoroughly vortexed plasma from the same tube.

Intra-assay coefficients of variation were determined on twelve aliquots of plasma from three subjects (i.e. n=4 per assessment). These were analysed in duplicate. Results are shown in table 1. n.b. CDs were obtained from unvortexed plasma placed in the different tubes

Inter-assay coefficients of variation are to be determined.

**Table 1**

#### Duplicate agreement and intra-assay CVs

	Duplicate agreement	Subject A CV	Subject B CV	Subject C CV	Mean CV
<b>PMPs</b>	4.45%	18.9%	12.2%	8.0%	13.0%
<b>EMPs</b>	4.02%	15.0%	3.3%	9.8%	9.4%
<b>MMPs</b>	2.05%	15.2%	10.4%	12.7%	12.8%
<b>Mean</b>	3.5%	16.4%	8.6%	10.2%	<b><u>11.7%</u></b>

STANDARD OPERATING PROCEDURE 190

**Enumeration of Microparticles by Flow Cytometry**

Signed off..... 2010.....

Figures: Illustrative plot attached.

Raw data analyses available from ADB



## **Appendix 5. Standard operating procedure 201 ‘Monocyte subsets, monocyte-platelet aggregates by flow cytometry’**

### STANDARD OPERATING PROCEDURE 201

#### MONOCYTE SUBSETS

##### **Monocyte platelet aggregates by flow cytometry SOP written by Eduard Shantsila and Andrew Blann**

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

#### Required pre-training

SOPs on venepuncture and on good clinical practice

SOP 195 – General operation of the flow cytometer

#### Contents

Introduction	Page 1
Materials and suppliers	Page 2
Detailed Method	Page 3
Interpretation	Page 8
Validation and quality control	Page 9

### **1. Introduction**

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

populations can be further classified by the density of the expression of these markers, for example....

M1 = CD14 strong CD16 negative

M2 = CD14 strong CD16 strong

M3 = CD14 weak CD16 strong

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

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

This SOP describes enumeration of monocyte subsets (dependent on expression of CD14, CD16 and CCR2) and their participation in the formation of MPAs. And of course you will need a platelet count for the project, derived from the full blood count, from the Advia (see SOP 171).

## **2. Materials and Supplier contact details:**

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

BD "FACS Flow" Running solution [Becton Dickinson (BD), Catalogue No. 342003]  
10L containers.

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

BD "FACS Clean" Cleaning Solution [BD Catalogue No. 340345]

BD Lysing solution [BD Catalogue No. 349202]

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

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

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

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

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

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

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

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

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

Remember to dispose of all material thoughtfully.

### 3. Detailed method

#### 3.1 General Preparation

##### 3.1.1 Lysing solution.

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

#### 3.2 Blood sample preparation

Add 12.5µL of Mastermix Absolute Monocyte Count (which includes CD14 2.5 µL, CD16 2.5µL, CD42a 5µL and CCR2 2.5 µL fluorochrome labelled antibodies) with an electronic micropipette. Just place into the tube below a metal grid without touching the pellet.

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

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

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

### 3.3 Start up procedure [See SOP 195 on General Operation]

#### Part 1 – restoring reagents and preparation

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

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

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

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

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

Close the drawer

#### Part 2 - Cleaning the machine

Ensure that a 3 ml Falcon tube (labelled 1) approximately 1/3 full of distilled water is positioned over the sample injection port (SIP) – a needle sheath - and that the swing arm is positioned under this tube. Press the prime button on the panel. When the system enters “standby” within 30 seconds then press the “prime” button again. When the standby and low buttons come on again then remove tube 1. We will re-use tube 1 in the shut down procedure.

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

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

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

Press the '**STANDBY**' and '**LOW**' button on the system.

The machine is now ready to run samples.

<p><b>NB: FACS COMP may need to be run</b></p>
--

### 3.4 Running blood samples.

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

Open CellQuest Pro software

Click 'File' – 'Open'

Click on the 'Monocyte Protocols' folder within 'Data 1' folder.

Click on the 'Monocyte Absolute Count'. This will open study protocol.

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

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

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

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

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

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

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

Open swing arm at bottom right of the cytometer and replace the Falcon tube with the sample to be run. Replace the swing arm under the Falcon tube.

Press the buttons ‘**Run**’ and ‘**High**’ on the control panel of the cytometer.

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

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

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

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

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

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

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

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

### **3.5 Shut-down procedure [See SOP 195 on General Operation]**

In this section we re-use tubes 1 and 2 with distilled water and FACS clean respectively. Install FACS Clean tube 2 over the SIP needle. Press button ‘**High**’ and ‘**Run**’ on the

panel. Leave the support arm out at 90 degrees for approximately 1 minute. This cleans the outer portion of the aspiration sheath. The fluid will be rapidly aspirated, so ensure that the tube doesn't empty completely.

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

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

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

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

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

Note: \* IF THE SYSTEM IS TO BE USED AGAIN ON THE SAME DAY...

LEAVE THE SYSTEM ON STANDBY and then  
DEPRESSURISE THE SYSTEM.

#### **4. Interpretation of plots**

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

##### TOP THREE PLOTS

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

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

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

## CENTRE THREE PLOTS

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

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

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

## LOWER THREE PLOTS (all CD42a versus CCR2)

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

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

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

Other numbers on the sheet (1- 12) refer to mathematical analyses, not to plots, as, follows....

## 5. Interpretation of results (numbers)

This is complicated, so pay attention. There are 12 analyses – the first 4 are raw data:

The total number of events counted and the acquisition date are given top left of the numbers section (i.e. 60,964 on 08-Apr-10).

On the far right is number of count beads (9127) used to quantify events to cells/ $\mu$ L

On the left is some maths from the opening plots showing number of total events collected in this particular analysis and the proportion that are monocytes.

Below this is the maths from Gates 5 and 6 (SSC and CCR2, middle right plot). So there are 667 M2 events and 871 M3 events, giving you relative proportions. This data is used to calculate the absolute count of subsets M2 and M3.

From these analyses numbers 1 – 4 the machine works out for you (given the count bead number in analysis 2 i.e. 9127) the percentage and numbers of monocytes and monocyte subsets, and these are given as numbers 5 – 12 as follows....



Mon is the total number of monocytes per  $\mu\text{L}$ , i.e. 582.95 cells/ $\mu\text{L}$ .  
Mon 1 is the number of M1 monocytes per  $\mu\text{L}$ , i.e. 409.5 cells/ $\mu\text{L}$ .  
Mon 2 is the number of M2 monocytes per  $\mu\text{L}$ , i.e. 98.23 cells/ $\mu\text{L}$ .  
Mon 3 is the number of M3 monocytes per  $\mu\text{L}$ , i.e. 75.22 cells/ $\mu\text{L}$ .

The machine has also worked out the % of each subset immediately below.

Next – for MPAs...

MPA is the total number of MPAs per  $\mu\text{L}$ , i.e. 102.86 cells/ $\mu\text{L}$   
MPA1 is the total number of MPAs in the M1 population, i.e. 71.29 cells/ $\mu\text{L}$   
MPA2 is the total number of MPAs in the M2 population, i.e. 19.28 cells/ $\mu\text{L}$   
MPA3 is the total number of MPAs in the M3 population, i.e. 12.29 cells/ $\mu\text{L}$

From this you can work out the proportions given a calculator. It follows that since you have the platelet count from the Advia, you can also work out how many of the total platelet pool are bound to monocytes. But this is for a separate analysis.

### **Conclusion**

Using this dataset as a template, the numbers that need to go into your spreadsheet are as follows...

Total monocyte count = 582.95 cells/ $\mu\text{L}$

Subsets:        M1 count = 409.50 cells/ $\mu\text{L}$  (70.25%)  
M2 count = 98.23 cells/ $\mu\text{L}$  (16.85%)  
M3 count = 75.22 cells/ $\mu\text{L}$  (12.9%)

Total MPA count = 102.86 cells/ $\mu\text{L}$

Subsets:        MPA1 count = 71.29 cells/ $\mu\text{L}$   
MPA2 count = 19.28 cells/ $\mu\text{L}$   
MPA3 count = 12.29 cells/ $\mu\text{L}$

Once in the spreadsheet, you can easily do the arithmetic for conversion to %

## **6. Validation and quality control (all work done by a single operator)**

**Intraobserver and Interobserver coefficients of variation on the validation work made by Eduard Shantsila are provided in the thesis**

SOP 201: Enumeration of monocytes subsets and  
monocyte platelet aggregates by flow cytometry

Signed off.....Andrew Blann..... 2010.....

Figures not electronic: illustrative plot (see ADB): Raw analyses available from ADB

## **Appendix 6. Standard Operating Procedure 206 ‘Intracellular I-Kappa-Kinase Beta (IKK $\beta$ )’**

### STANDARD OPERATING PROCEDURE 202

#### **Intracellular I-Kappa-Kinase Beta (IKK $\beta$ )**

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

#### Required pre-training

SOPs on venepuncture and on good clinical practice

SOP 193 – General operation of the flow cytometer

SOP 171 – Operation of the Bayer Advia

#### Contents

Introduction	Page 1
Materials and suppliers	Page 3
Detailed Method	Page 4
Interpretation and QC	Page 7
Appendices	Page 10 (flow cytometry printout, signalling figure 1)

#### 1. Introduction

Nuclear factor  $\kappa$ B (NF $\kappa$ B) is a heterodimeric protein composed of different combinations of members of the Rel family of transcription factors which are involved mainly in stress-induced, immune, and inflammatory responses. In addition, these molecules play important roles during the regulation of apoptosis, cell proliferation, development of certain hemopoietic cells, keratinocytes, and lymphoid organ structures (1).

NF $\kappa$ B can be activated by exposure of cells to lipopolysaccharides (LPS) or inflammatory cytokines such as tumour necrosis factor (TNF $\alpha$ ) or interleukin (IL)-1, as well as various

various growth factors, lymphokines, bacterial and viral infection, and by other physiological and non physiological stimuli. The recognition of bacterial and viral products by Toll-like receptors on cells of the innate immune system also results in NF $\kappa$ B induction, leading to the production of proinflammatory cytokines, matrix metalloproteinases, tissue factor, reactive oxidant and nitrogen species and other biologically active substances (1-4).

Inappropriate activation of NF $\kappa$ B has been linked to inflammatory events associated with autoimmune arthritis, septic shock, glomerulonephritis, and **atherosclerosis**. In contrast, complete and persistent inhibition of NF $\kappa$ B has been associated with apoptosis, impaired immune cell development, and delayed cell growth (5). Critically, NF $\kappa$ B represents one of the main transcriptional factors mediating inflammatory responses of monocytes and macrophages. The outstanding technical problem with the measurement of NF $\kappa$ B is that the resting cytosol form and the activated nuclear form are structurally identical and cannot be distinguished by flow cytometry. It can, however, be detected in cell lysate with an electrophoretic mobility shift assay.

I-kappaB molecules are inhibitors of NF $\kappa$ B transcription factors. The I-kappaB kinase (IKK) complex is a phosphorylating enzyme with several subunits. The  $\beta$  subunit is involved in transcriptional activation by phosphorylating I-kappaB, a modification that triggers its ubiquitination and subsequent degradation in proteolysosomes, enabling the passage of (free) NF $\kappa$ B into the nucleus (6). Multiple cytoplasmic and nuclear proteins distinct from the NF $\kappa$ B and I-kappaB proteins are phosphorylated by another catalytic subunit of the IKK complex (IKK $\alpha$ ) which is involved in B lymphocyte maturation. The function of a third IKK subunit (IKK $\gamma$ ) is not fully understood

Therefore levels of the IKK $\beta$  enzyme are a regulator of NF $\kappa$ B in that high levels of the enzyme will lead to the inhibition of I-kappaB and so an increase in the activity of NF $\kappa$ B. This is summarised in Figure 1, final page.

Monoclonal antibodies to IKK $\beta$  have been developed and can be conjugated to fluorochromes. The present SOP exploits this technology in that it is designed to quantify intra-cellular IKK $\beta$  in monocyte subsets, themselves defined by a combination of morphology, CD14 and CD16. However, cells must be permeabilised to allow the conjugated mAbs to enter the cell and thus bind their target

## References:

1. Baldwin AS Jr. The NF-kappa B and I kappa B proteins: new discoveries and insights. *Annu. Rev. Immunol* 1996; 14: 649-83.
2. Pomerantz JL, Baltimore D. Two pathways to NF-KappaB. *Mol Cell* 2002; 10: 693-5.
3. D'Acquisto F, May MJ, Ghosh S. Inhibition of nuclear factor kappa B (NF-B): an emerging theme in anti-inflammatory therapies. *Mol Interv.* 2002;2:22-35.
4. Dixit V, Mak TW. NF-KappaB signaling: Many Roads Lead To Madrid. *Cell* 2002; 111: 615-9.
5. Hinz M, Krappmann D, Eichten A, Heder A, Scheidereit C, Strauss M. NF-KappaB function in growth control: regulation of cyclin D1 expression and G0/G1-to-S-phase transition. *Mol Cell Biol* 1999;19: 2690-8.

6. Schmid JA, Birbach A. IkappaB kinase beta (IKKbeta/IKK2/IKBKB) - a key molecule in signaling to the transcription factor NF-kappaB. Cytokine Growth Fact Rev 2008 ;19:157-65.

### **Legend to figure 1**

Intra-nuclear NFκB binds to the promoter region of various ‘proinflammatory’ genes

Cytoplasmic NFκB may be inhibited by protein I kappa B

However, if I kappa B is phosphorylated it becomes susceptible to degradation and so will not inhibit NFκB

I kappa B can be phosphorylated by the beta subunit of the enzyme I kappa kinase, i.e. IKKβ, which itself needs to be phosphorylated

IKKβ is phosphorylated by TRAF, a downstream product of intracellular second messengers themselves (F) activated by ligand binding of cell surface receptors (G).

### **2. Materials and Supplier contact details:**

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

BD “Cytotfix” Fixation Buffer 100 ml [Becton Dickinson, Catalogue No. 554655]

BD “Cytotfix/Cytoperm” Fixation and Permeabilization Solution 125 ml of 1x solution [Becton Dickinson, Catalogue No. 554722]

BD “Pharm Lyse” Lysing Buffer 100 ml 10x concentrated [Becton Dickinson, Catalogue No. 555899]

BD “Perm/Wash” Buffer 100 ml 10x concentrated [Becton Dickinson, Catalogue No. 554723]

BD “Pharmingen” Stain Buffer (FBS) Dulbecco’s Phosphate-Buffered Saline (DPBS) pH7,4 2% Fetal Bovine Serum 500 ml [Becton Dickinson, Catalogue No. 554656]

BD “FACS Flow” Running solution [Becton Dickinson, Catalogue No. 342003]10L containers

BD “FACS Clean” Cleaning Solution [Becton Dickinson, Catalogue No. 340345]

3 ml BD Falcon tubes [Becton Dickinson, Catalogue No. 352054]

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

Mouse Anti Human CD16 Alexa Fluor 488 conjugated monoclonal antibody 100 tests/1ml [AbD Serotec, Catalogue No. MCA2537A488]

BD Pharmingen PerCP-Cy 5.5 conjugated Mouse Anti Human CD14 monoclonal antibody 0.2mg/ml - 100 tests/ml [Becton Dickinson, Catalogue No. 550787]

IKKbeta-APC conjugated monoclonal antibody 50µg/ml - 100 tests [Becton Dickinson, Catalogue No. 551920]

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

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

Disposable 3 ml Graduated Pasteur Steril 20s (500 pipettes) [Alpha Laboratories, Catalogue No. LW4114]

Remember to dispose of all material thoughtfully.

### 3. Detailed method

#### FIRSTLY

For all WBCC analyses a full blood count (Bayer Advia) must be obtained on the same sample of blood that will be used for flow cytometry.

This is essential in order to back-calculate monocyte numbers and thus sub-populations to whole blood

### **3.1 General Preparation**

#### 3.1.1 Lysing solution.

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

### **3.2 Blood sample preparation**

3.2.1. Place to a falcon tube: 5µl CD16-AF 488, 2.5µl CD14-PerCP-CY5.5 and add 100µl of whole blood sample (collected in an EDTA containing tube). Gently vortex the tube and incubate in a dark box for 15 minutes.

3.2.2. Add 2.0 ml 'PharmLyse' solution, gently vortex and incubate for 10 minutes in the dark box. Spin the tube for 5 minutes at 500g and remove the supernatant by a disposable pipette.

3.2.3. Put 2ml of 'Staining Buffer' in the tube, gently vortex and spin again for 5 minutes at 500g and remove supernatant by disposable pipette.

3.2.4. Add 500µl of 'Fixation and Permeation' solution, gently vortex and incubate the tube for 20 minutes in the dark box. Spin again for 5 minutes at 500g, remove supernatant by a disposable pipette.

3.3.5. Add 2 ml of 'Perm-Wash', gently vortex the sample, wait for a10 minutes and spin for 5 minutes at 500g. Remove the supernatant by a disposable pipette.

3.3.6. Add 10µl of 'IKKbeta-APC' antibody in the sample, gently vortex and incubate 30 minutes in the dark box. Add 2 ml of 'Perm-Wash', spin the tube for 5 minutes at 500g. Remove the supernatant by a disposable pipette.

3.3.7. Add 50µl of 4% PFA/PBS, gently vortex and run sample.

### **3.3 Start up procedure [See SOP 195 on General Operation]**

#### Part 1 – restoring reagents and preparation

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

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

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

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

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

Close the drawer

## Part 2 - cleaning the machine

Ensure that a 3 ml Falcon tube (labelled 1) approximately 1/3 full of distilled water is positioned over the sample injection port (SIP) – a needle sheath - and that the swing arm is positioned under this tube. Press the prime button on the panel. When the system enters “standby” within 30 seconds then press the “prime” button again. When the standby and low buttons come on again then remove tube 1. We will re-use tube 1 in the shut down procedure.

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

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

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

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

The machine is now ready to run samples.

### 3.3 Running blood samples.

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

Click on the ‘Data 1’ folder on the desk top

Click on the ‘Monocyte Protocols’ folder.

Click on the ‘Monocyte Inset IκKB’.

This will open the CellQuest Pro software with the instrument settings.

Click ‘**Connect to Cytometer**’, located under the ‘Acquire’ menu.

Under the ‘Cytometer’ menu, click ‘**Instrument Settings**’. The window appears displaying the compensations and threshold. Change settings by clicking on the **open** icon on the window which displays the folders select ‘Sid’ folder within the ‘Data 1’ folder and click on the ‘Instrument settings’ in this folder. This will update the system settings to the preferred settings for the acquisition. Click ‘**Set**’ on the window and by clicking ‘**Done**’ the window disappears. Make sure to click ‘**Set**’ prior to clicking ‘**Done**’.



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

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

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

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

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

Each sample is now ready to run. This should be mixed thoroughly and transferred to a 3 mL BD Falcon tube. Open swing arm at bottom right of the cytometer and replace the Falcon tube with the sample to be run. Replace the swing arm under the Falcon tube.

Press the buttons '**Run**' and '**High**' on the control panel of the cytometer.

Click '**Acquire**' on the browser menu. The sample will now run for ~ 3-4 mins. Cell events will be displayed on the screen throughout the process (n.b. the higher the cell density, the more rapidly the cells will be acquired).

Click on '**Counters**' under the '**Acquire**' and observe the events per second which varies from 500 to 5000 depending on various factors. The objective is to acquire 400,000 events for analysis of NFkB intracellular signalling.

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

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

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

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

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

Be absolutely sure you have downloaded your results on to paper. Keep this paper safe. Do not assume the computer will keep the results safe, even if you have directed it to do so.

Obtain all the raw data (cell numbers) and apply them into the specific spreadsheet you have designed for your project. The same spreadsheet should have the WBC results from the Advia.

### **3.4 Shut-down procedure [See SOP 195 on General Operation]**

In this section we re-use tubes 1 and 2 with distilled water and FACS clean respectively.

Install FACS Clean tube 2 over the SIP needle. Press button '**High**' and '**Run**' on the panel. Leave the support arm out at 90 degrees for approximately 1 minute. This cleans the outer portion of the aspiration sheath. The fluid will be rapidly aspirated, so ensure that the tube doesn't empty completely.

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

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

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

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

Finally power down the FAC-Scalibur (green button) and Apple Mac.

Clean up !

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

## **4. Interpretation of plots**

Refer to page 10 which shows a typical plot.

The first, FSC vs. SSC plot (top left) is set to collect all monocytes in a sample, even if it results in some contamination with granulocytes and lymphocytes.

The second plot (ungated SSC vs. CD14-PerCP-Cy5.5, top middle) is for the exclusion of granulocytes (large group of events at the middle top part of the plot) from the further analysis.

The top right plot (CD16-AF488 vs. CD14-PerCP-Cy5.5) aims to select monocytes only (subsets, Mon1, Mon2, Mon3). It represents a gate that includes monocytes on the basis of the first plot, excludes granulocytes on the basis of the second plot, and excludes

lymphocytes on the basis of the third plot (see point 4 below). The plot allows the selection of three monocyte subsets: Mon1 (CD14+/CD16- events), Mon2 (CD14+/CD16+ events) and Mon3 (CD14low/CD16+ events).

The left middle plot (events gated from the second plot, CD16-AF488 vs. CD14-PerCP-Cy5.5) is for the exclusion of lymphocytes and natural killers cells (left side of the plot – CD14-negative events).

The three lower range of plots demonstrate the measurement of IKK $\beta$  levels in each monocyte subset. Monocyte subsets Mon1, Mon2 and Mon3 are gated on three different plots with IKK $\beta$  on Y-axis and no fluorochrome in channel FL2. Geometric means on Y-axis of regions M1, M2, and M3 characterise IKK $\beta$  expression in Mon1, Mon2 and Mon3 populations respectively are the key data to be collected.

The units are arbitrary geometric mean fluorescence (GMF).

## **5. Interpretation, validation and quality control**

Collect data on the total white blood cell counts and monocyte count (from the Advia). This is important because it enables a back calculation to the number of M1, M2 and M3 monocytes per ml of venous blood. The implications of differences in levels of IKK $\beta$  are unclear but may relate to levels of NF $\kappa$ B

### Intra-assay reproducibility = 5.2%

This SOP was assessed in 28<sup>th</sup> January 2010, blind by BP, on three vacutainers from two subjects.

#### Subject 1

Levels of IKK $\beta$  expression in M1 was 66.28, 72.47 and 76.24

Mean = 71.67, SD = 4.1, therefore CV = 5.7%

Levels of IKK $\beta$  expression in M2 was 65.6, 74.71 and 73.11

Mean = 71.14, SD = 4.0, therefore CV = 5.6%

Levels of IKK $\beta$  expression in M3 was 60.47, 63.24 and 63.8

Mean = 62.5, SD = 2.3, therefore CV = 2.3%

Mean CV in subject 1 = 4.5%

#### Subject 2

Levels of IKK $\beta$  expression in M1 was 77.63, 69.6 and 70.93

Mean = 72.7, SD = 3.51, therefore CV = 4.8%

Levels of IKK $\beta$  expression in M2 was 80.97, 70.34 and 73.87

Mean = 75.1, SD = 4.4, therefore CV = 5.9%

Levels of IKK $\beta$  expression in M3 was 79.96, 67.49 and 74.44

Mean = 74.0, SD = 5.1, therefore CV = 6.9%

Mean CV in subject 2 = 5.9%

### Inter-assay reproducibility = 10.1%

This was assessed on six samples of VB from the same healthy (?) 55 year old male over a two-month period.

Ikkb in M1 was 59.8, 55.4, 59.4, 71.1, 70.1 and 64.3. Mean = 63.4, SD = 5.8  
So interassay CV = 9.2%

Ikkb in M2 was 63.1, 57.6, 73.7, 74.9, 75.3 and 66.4. Mean = 68.5, SD = 6.6  
So interassay CV = 9.7%

Ikkb in M3 was 65.3, 54.7, 60.6, 70.7, 78.7 and 68.0. Mean = 68.0, SD = 7.8  
So interassay CV = 11.4%

Isotype controls

**Dr Shantsila generated isotype control data with an IgG1-APC conjugate identified a mean expression of IKK $\beta$  in M1 cells of 6.6 units, M2 of 6.4 units and in M3 of 6.5 units.**

SOP 202: I-Kappa-Kinase Beta (IKK $\beta$ ) staining by flow cytometry

Signed off.....

Figures (not electronic): F1 Flow cytometry: F2 Signalling pathway.

Raw analyses available from ADB

## **Appendix 7. Standard Operating Procedure 206 ‘Assessment of monocyte phagocytic activity by Flow Cytometry’**

### STANDARD OPERATING PROCEDURE 206

#### **Assessment of monocyte phagocytic activity by Flow Cytometry**

#### **SOP developed by Eduard Shantsila Winter 2010/2011**

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

#### Required pre-training

SOPs on venepuncture, good clinical practice

SOP 195 – general operation of the flow cytometer

#### Contents

Introduction and brief method	Page 1
Materials and suppliers	Page 2
Detailed Method	Page 2
Data analysis and validation	Page 5

#### 1. Introduction

Monocytes are circulating blood cells participating in innate immunity, inflammatory response and phagocytosis. Monocytes include several subsets that can be discriminated on the basis of surface expression of CD14 (lipopolysaccharide receptor) and CD16 (Fc gamma receptor III). This SOP describes the measurement of the phagocytic activity of:

- CD14+CD16- monocytes (about 85%),
- CD14+CD16+ monocytes (about 5%) and
- CD14<sup>low</sup>CD16+ monocytes (about 10%).

The phagocytic function is evaluated in whole blood by exposing the heparinized blood sample to a conjugate of pHrodo™ *E. coli* BioParticles®. This conjugate is a novel, no-

wash fluorogenic reagent that is only weakly fluorogenic when attached to the outer surface of the phagocyte but is highly fluorescent in the acidic environment of the phagosome upon internalization. This eliminates the wash and quenching steps associated with other phagocytosis assay protocols.

Red blood cells are lysed using the proprietary Lysis Buffer A (Component A) and Buffer B (Component B) followed by centrifugation and washing. The final cell pellet containing white blood cells is resuspended in PBS and is ready for analysis using a flow cytometer. The monocytes subsets are distinguished by gating using forward and scatter properties, CD14 and CD16 expression. Phagocytic activity of monocyte subsets is determined by further gating of individual monocyte subsets and assessment of the amount of phagocytised pHrodo™ *E. coli* BioParticles® particles as the median fluorescent intensity (MFI) in the phycoerythrin (PE) channel which lights up in an acid environment.

#### Brief method

Obtain cells and keep them on ice, bring all reagents to room temperature  
Set up two tubes, add blood to both but only the E coli to one  
Incubate at 37 degree C for 5 mins only  
Add CD14 and CD16 antibodies, back to 37 for 10 minutes  
Lyse red blood cells, wash twice  
Apply to the FACS – voila !

#### **2. Materials and Supplier contact details:**

BD “FACS Flow” Running solution [Becton Dickinson, Catalogue No. 342003]  
10L containers.  
BD “FACS Clean” Cleaning Solution [Becton Dickinson, Catalogue No. 340345]  
3 ml BD Falcon tubes [Becton Dickinson, Catalogue No. 352054]  
Sterile Phosphate Buffered Saline solution, 0.5L bottles [Invitrogen Ltd, Catalogue No 20012-068]

pHrodo™ *E. coli* BioParticles® Phagocytosis Kit for Flow Cytometry [Invitrogen Ltd, Cat No. MP10025]  
CD16-Alexa Fluor 488-conjugated monoclonal antibody [AbD Serotec, Oxford, UK, Cat No. MCA2537A488]  
CD14 -APC conjugated monoclonal antibody [R&D Systems Europe Ltd, Cat No. FAB3832A]

Clear pipette tips [Alpha Laboratories Limited Catalogue No FR1250 1250ul Fastrak Refill NS]  
Yellow pipette tips [Alpha Laboratories Limited Catalogue No FR1200 200ul Fastrak Refill NS]  
Pipettes required: 2-20ul (grey or white), 40-200 ul (yellow); 200-1000ul (purple)

#### 3. Detailed method

### 3.1 General Preparation

#### 3.1.1. Preparing the BioParticles® Solution

3.1.1.1 Add 2.2 mL Buffer B (Component B) to the vial containing the lyophilized product to resuspend the pHrodo™ BioParticles® conjugate. This provides sufficient pHrodo™ BioParticles® conjugate in a 20 µL aliquot for a 20:1 particle-to-phagocyte ratio.

3.1.1.2 Vortex gently for about 1 minute until all the fluorescent particles are homogeneously dispersed.

3.1.1.3 Store the pHrodo™ BioParticles® solution on ice for ~10 minutes prior to use.

#### 3.1.2. Buffers

Bring the Lysis Buffer A (Component A) and Buffer B (Component B) to room temperature before use.

### 3.2 Blood sample preparation

3.2.1. Collect whole blood samples in blood collection tubes containing **heparin**

3.2.2. You can collect and store whole blood samples on ice (preferably) or at 4°C for up to 1 hour prior to using the blood samples in the phagocytosis assay. Do not use any other anticoagulants except heparin for blood collection!

### 3.3. Performing Phagocytosis Assay

3.3.1. To minimize experimental errors and allow proper interpretation of results, use two tubes for each set of experimental samples as outlined in the table below. Each set of control tube and experimental tube is incubated at 37°C.

3.3.2. Mark two tubes: Tube 1 ‘Sample’ and Tube 2 ‘Control’.

3.3.3. Take heparinised blood from ice, put on rotator for 20 sec and add 100 µL of whole blood into Tube 1 and Tube 2.

3.3.4. Add 20 µL of pHrodo™ BioParticles® in Tube 1 and 20 µL of PBS in Tube 2.

3.3.5. Vortex both tube gently and place them into the incubator at 37°C for 5 min.

3.3.6. Take the tubes from the incubator, add CD16 and CD14 antibodies (5 µL each), vortex for 2-3 sec and put back into the incubator for another 10 min. Be fast with this!!! Be precise with timing – it is critical!!!

3.3.7. Take the tubes from the incubator, add 100 µL Lysis Buffer A (Component A) to both tubes, vortex briefly, and incubate at room temperature for 5 minutes.

3.3.8. Add 1 mL Buffer B to the tubes, vortex briefly, and incubate at room temperature for 5 minutes.

3.3.9. Centrifuge the samples at  $500 \times g$  for 5 minutes at room temperature.

3.3.10. Discard the supernatant.

3.3.11. Resuspend the cell pellets with 1 mL PBS.

3.3.12. Repeat wash steps 3.3.11 one more time.

3.3.13. Resuspend the cell pellets in 0.2 mL PBS for flow cytometry analysis. This volume should give a high enough cell count for 400-500,000 events in 6 -7 minutes.

### 3.4. Start up procedure [See SOP 195 on General Operation]

- Turn on FC and after few seconds computer.
- Empty waste (right container) and refill with 360ml distilled water and 40ml bleach (ensure no bleach on gloves)
- Fill machine with left container by FacsFlow to appropriate level (level of indentation)
- Pressurize
- Press LOW/PRIME with the arm closed (distilled water in place)- once PRIME light goes off press once more
- Remove distilled water and place 'top right' on rack
- Insert FacsClean tube (2ml of FacsClean) – opened arm 1 minute, closed 5 minutes - then remove and place 'top left' on rack- set FC on 'high/run'
- Insert a tube with sterile PBS - 1 minute open arm, 5 minutes closed arm

### 3.5. Sample acquisition

- Run FacsComp if you are first user of the flow cytometer during the day
  - Press CellQuest icon
  - 'FILE' – 'Open document' – 'Data 1' – 'Phagocytosis' – 'Phagocytosis'
  - ACQUIRE – 'Connect to cytometer'
  - CYTOMETER – 'Choose instrument settings' – 'Phagocytosis' – 'Set' – 'Done'
  - Change appropriate directory to save flow data
  - Change file name
  - Place Tube 1 into cytometer and press 'Acquire' (press 'Acquire' on top bar, press 'Counters') - once finished, print. Make sure that at least 200 000 events (normally 400000-500000) with at very least 100 events (at least 400 typically) per each monocyte subsets were acquired. Otherwise the is sample is not suitable for analysis.
- Change file name
- Place Tube 2 into cytometer and press 'Acquire' (press 'Acquire' on top bar, press 'Counters')
  - Once finished, print a hard paper copy. When back in ASCOT, show it to Dr Blann or Dr Shantsila for an explanation.

### 3.6 Shut-down procedure [See SOP 195 on General Operation]

In this section we re-use tubes 1 and 2 with distilled water and FACS clean respectively.

Install FACS Clean tube 2 over the SIP needle. Press button '**High**' and '**Run**' on the panel. Leave the support arm out at 90 degrees for approximately 1 minute. This cleans the outer portion of the aspiration sheath. The fluid will be rapidly aspirated, so ensure that the tube doesn't empty completely.

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



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

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

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

Finally power down the FAC-Scalibur (green button) and Apple Mac.

Clean up !

Note: \* IF THE SYSTEM IS TO BE USED AGAIN ON THE SAME DAY...

LEAVE THE SYSTEM ON STANDBY and then

DEPRESSURISE THE SYSTEM.

### **3.6. Data analysis, validation and expected results**

Refer to Illustrative plot 1. There are 12 items of plots and data.....

At the top are (left to right) the forward and side scatter, the side scatter and CD14 plot (defining monocytes), and the CD14/CD16 plot for the three monocyte subsets.

The middle row is the lymphocyte and NK populations, an acquisition record of the proportions of monocyte subsets (and the number of monocytes that are phagocytic), and a plot of the cells staining positive or negative for the E-coli induced fluorochrome.

Below these are the plots of MFI according to monocytes subsets M1, M2 and M3. In M1 and M2 the MFI are 139.49 and 137 arbitrary units respectively. The M3 population clearly stains less powerfully (54,74 units) so presumably is not as involved in phagocytosis.

Illustrative plot 2 is the same sample processed in the absence of the E coli to stimulate the cells. Hence little fluorochrome is activated within the cells.

#### Intra-assay CVs

This was performed by independently processing three blood samples from the same individual. Phagocytic activity of monocyte subsets observed in this healthy volunteer were...

Population	Sample 1	Sample 2	Sample 3	Intra-assay CV
M1	155.38	139.49	147.22	5.4%
M2	155.38	137.00	138.24	7.1%
M3	56.23	54.74	55.73	1.4%

These figures are acceptable.

SOP 206 Phagocytosis Approved 13<sup>th</sup> July 2011

Dr A Blann

Appendices: Illustrative plots 1 and 2

## **Appendix 8. List of the study publications**

*Original manuscripts (available in PUBMED at the time of the thesis submission):*

1. Shantsila E, Bialiuk N, Navitski D, Pyrochkin A, Gill PS, Pyrochkin V, Snezhitskiy V, Lip GY. Blood leukocytes in heart failure with preserved ejection fraction: Impact on prognosis. *Int J Cardiol.* 2012 ;155:337-8. PubMed PMID: 22227252.
2. Shantsila E, Wrigley B, Shantsila A, Tapp LD, Blann AD, Gill PS, Lip GY. Ethnic differences in macrovascular and microvascular function in systolic heart failure. *Circ Heart Fail.* 2011;4:754-62. PubMed PMID: 21914813.
3. Shantsila E, Wrigley B, Tapp L, Apostolakis S, Montoro-Garcia S, Drayson MT, Lip GY. Immunophenotypic characterization of human monocyte subsets: possible implications for cardiovascular disease pathophysiology. *J Thromb Haemost.* 2011;9:1056-66. PubMed PMID: 21342432.

*Literature reviews:*

1. Shantsila E, Lip GY, Gill PS. Systolic heart failure in South Asians. *Int J Clin Pract.* 2011 Dec;65(12):1274-82. PubMed PMID: 22093534.
2. Shantsila E, Kamphuisen PW, Lip GY. Circulating microparticles in cardiovascular disease: implications for atherogenesis and atherothrombosis. *J Thromb Haemost.* 2010;8:2358-68. PubMed PMID: 20695980.

3. Shantsila E, Lip GY. The role of monocytes in thrombotic disorders. Insights from tissue factor, monocyte-platelet aggregates and novel mechanisms. *Thromb Haemost.* 2009;102:916-24. PubMed PMID: 19888530.
  
3. Apostolakis S, Lip GY, Shantsila E. Monocytes in heart failure: relationship to a deteriorating immune overreaction or a desperate attempt for tissue repair? *Cardiovasc Res.* 2010;85:649-60. PubMed PMID:19805399.
  
4. Shantsila E, Lip GY. The endothelium and thrombotic risk in heart failure. *Thromb Haemost.* 2009;102:185-7. PubMed PMID: 19652866.
  
5. Shantsila E, Lip GY. Monocytes in acute coronary syndromes. *ArteriosclerThromb Vasc Biol.* 2009;29:1433-8. PubMed PMID: 19229072.
  
6. Shantsila E, Watson T, Tse HF, Lip GY. New insights on endothelial progenitor cell subpopulations and their angiogenic properties. *J Am Coll Cardiol.* 2008;51:669-71. PubMed PMID: 18261687.
  
7. Shantsila E, Watson T, Lip GY. Endothelial progenitor cells in cardiovascular disorders. *J Am Coll Cardiol.* 2007;49:741-52. PubMed PMID:17306702.

*Published abstracts:*

Shantsila E, Tapp LD, Wrigley BJ, Apostolakis S, Drayson MT, Lip GYH.

Immunophenotypic characterisation of an immature human monocyte subset with angiogenic potential. ATVB 2011 Scientific Sessions, Chicago, Illinois, 28-30 April 2011; Abstract P 653:203

Shantsila E, Gill PS, Lip GYH. Ethnic differences in endothelial function in chronic heart failure. Heart Failure 2011. Congress of the Heart Failure Association of the ESC, Gothenburg, Sweden, 21-24 May 2011;P1099.

Shantsila E, Gill PS, Lip GYH. Ethnic differences in endothelial function in chronic heart failure. Annual Conference of the British Cardiovascular Society, Manchester, UK, 13-15 June 2011. Heart. 2011; 97 Supplement 1: P 102, A59.

## LIST OF REFERENCES

1. Dickstein K, Cohen-Solal A, Filippatos G, McMurray JJ, Ponikowski P, Poole-Wilson PA, Stromberg A, van Veldhuisen DJ, Atar D, Hoes AW, Keren A, Mebazaa A, Nieminen M, Priori SG, Swedberg K, Vahanian A, Camm J, De Caterina R, Dean V, Funck-Brentano C, Hellemans I, Kristensen SD, McGregor K, Sechtem U, Silber S, Tendera M, Widimsky P, Zamorano JL. Esc guidelines for the diagnosis and treatment of acute and chronic heart failure 2008: The task force for the diagnosis and treatment of acute and chronic heart failure 2008 of the european society of cardiology. Developed in collaboration with the heart failure association of the esc (hfa) and endorsed by the european society of intensive care medicine (esicm). *Eur Heart J.* 2008;29:2388-2442
2. Murdoch DR, Love MP, Robb SD, McDonagh TA, Davie AP, Ford I, Capewell S, Morrison CE, McMurray JJ. Importance of heart failure as a cause of death. Changing contribution to overall mortality and coronary heart disease mortality in scotland 1979-1992. *Eur Heart J.* 1998;19:1829-1835
3. Senni M, Tribouilloy CM, Rodeheffer RJ, Jacobsen SJ, Evans JM, Bailey KR, Redfield MM. Congestive heart failure in the community: Trends in incidence and survival in a 10-year period. *Arch Intern Med.* 1999;159:29-34
4. Stewart S, Jenkins A, Buchan S, McGuire A, Capewell S, McMurray JJ. The current cost of heart failure to the national health service in the uk. *Eur J Heart Fail.* 2002;4:361-371
5. Stewart S, MacIntyre K, Hole DJ, Capewell S, McMurray JJ. More 'malignant' than cancer? Five-year survival following a first admission for heart failure. *Eur J Heart Fail.* 2001;3:315-322
6. Cowie MR, Wood DA, Coats AJ, Thompson SG, Suresh V, Poole-Wilson PA, Sutton GC. Survival of patients with a new diagnosis of heart failure: A population based study. *Heart.* 2000;83:505-510
7. Dries DL, Exner DV, Gersh BJ, Cooper HA, Carson PE, Domanski MJ. Racial differences in the outcome of left ventricular dysfunction. *N Engl J Med.* 1999;340:609-616
8. Gill PS, Kai, J., Bhopal, R.S., Wild, S. Health care needs assessment: Black and minority ethnic groups. In: Raftery. J. SA, Mant. J., ed. *Health care needs assessment. The epidemiologically based needs assessment reviews.* Abingdon: Radcliffe Publishing Ltd; 2007.
9. Balarajan R. Ethnicity and variations in mortality from coronary heart disease. *Health Trends.* 1996;28:45-51

10. Balarajan R. Ethnic differences in mortality from ischaemic heart disease and cerebrovascular disease in england and wales. *BMJ*. 1991;302:560-564
11. Srinath Reddy K, Shah B, Varghese C, Ramadoss A. Responding to the threat of chronic diseases in india. *Lancet*. 2005;366:1744-1749
12. Blackledge HM, Newton J, Squire IB. Prognosis for south asian and white patients newly admitted to hospital with heart failure in the united kingdom: Historical cohort study. *BMJ*. 2003;327:526-531
13. Heiat A, Gross CP, Krumholz HM. Representation of the elderly, women, and minorities in heart failure clinical trials. *Arch Intern Med*. 2002;162:1682-1688
14. Carson P, Ziesche S, Johnson G, Cohn JN. Racial differences in response to therapy for heart failure: Analysis of the vasodilator-heart failure trials. Vasodilator-heart failure trial study group. *J Card Fail*. 1999;5:178-187
15. Exner DV, Dries DL, Domanski MJ, Cohn JN. Lesser response to angiotensin-converting-enzyme inhibitor therapy in black as compared with white patients with left ventricular dysfunction. *N Engl J Med*. 2001;344:1351-1357
16. Hunt SA, Abraham WT, Chin MH, Feldman AM, Francis GS, Ganiats TG, Jessup M, Konstam MA, Mancini DM, Michl K, Oates JA, Rahko PS, Silver MA, Stevenson LW, Yancy CW. 2009 focused update incorporated into the acc/aha 2005 guidelines for the diagnosis and management of heart failure in adults: A report of the american college of cardiology foundation/american heart association task force on practice guidelines: Developed in collaboration with the international society for heart and lung transplantation. *Circulation*. 2009;119:e391-479
17. Kubo SH, Rector TS, Bank AJ, Williams RE, Heifetz SM. Endothelium-dependent vasodilation is attenuated in patients with heart failure. *Circulation*. 1991;84:1589-1596
18. Katz SD, Biasucci L, Sabba C, Strom JA, Jondeau G, Galvao M, Solomon S, Nikolic SD, Forman R, LeJemtel TH. Impaired endothelium-mediated vasodilation in the peripheral vasculature of patients with congestive heart failure. *J Am Coll Cardiol*. 1992;19:918-925
19. Bank AJ, Rector TS, Tschumperlin LK, Kraemer MD, Letourneau JG, Kubo SH. Endothelium-dependent vasodilation of peripheral conduit arteries in patients with heart failure. *J Card Fail*. 1994;1:35-43
20. Wisloff U, Stoylen A, Loennechen JP, Bruvold M, Rognum O, Haram PM, Tjonna AE, Helgerud J, Slordahl SA, Lee SJ, Videm V, Bye A, Smith GL, Najjar SM, Ellingsen O, Skjaerpe T. Superior cardiovascular effect of aerobic interval training versus moderate continuous training in heart failure patients: A randomized study. *Circulation*. 2007;115:3086-3094

21. Strey CH, Young JM, Molyneux SL, George PM, Florkowski CM, Scott RS, Frampton CM. Endothelium-ameliorating effects of statin therapy and coenzyme q10 reductions in chronic heart failure. *Atherosclerosis*. 2005;179:201-206
22. Murphy C, Kanaganayagam GS, Jiang B, Chowienczyk PJ, Zbinden R, Saha M, Rahman S, Shah AM, Marber MS, Kearney MT. Vascular dysfunction and reduced circulating endothelial progenitor cells in young healthy uk south asian men. *Arterioscler Thromb Vasc Biol*. 2007;27:936-942
23. Ross R. Atherosclerosis--an inflammatory disease. *N Engl J Med*. 1999;340:115-126
24. Frantz S, Bauersachs J, Ertl G. Post-infarct remodelling: Contribution of wound healing and inflammation. *Cardiovasc Res*. 2009;81:474-481
25. Auffray C, Sieweke MH, Geissmann F. Blood monocytes: Development, heterogeneity, and relationship with dendritic cells. *Annu Rev Immunol*. 2009;27:669-692
26. Serbina NV, Jia T, Hohl TM, Pamer EG. Monocyte-mediated defense against microbial pathogens. *Annu Rev Immunol*. 2008;26:421-452
27. Aldred S, Griffiths HR. Oxidation of protein in human low-density lipoprotein exposed to peroxy radicals facilitates uptake by monocytes; protection by antioxidants in vitro. *Environ Toxicol Pharmacol*. 2004;15:111-117
28. Moore KJ, Freeman MW. Scavenger receptors in atherosclerosis: Beyond lipid uptake. *Arterioscler Thromb Vasc Biol*. 2006;26:1702-1711
29. Makinen PI, Lappalainen JP, Heinonen SE, Leppanen P, Lahtenvuo MT, Aarnio JV, Heikkila J, Turunen MP, Yla-Herttuala S. Silencing of either sr-a or cd36 reduces atherosclerosis in hyperlipidaemic mice and reveals reciprocal upregulation of these receptors. *Cardiovasc Res*. 2010;88:530-538
30. Todt JC, Hu B, Curtis JL. The scavenger receptor sr-a i/ii (cd204) signals via the receptor tyrosine kinase mertk during apoptotic cell uptake by murine macrophages. *J Leukoc Biol*. 2008;84:510-518
31. Kobayashi H, Sakashita N, Okuma T, Terasaki Y, Tsujita K, Suzuki H, Kodama T, Nomori H, Kawasuji M, Takeya M. Class a scavenger receptor (cd204) attenuates hyperoxia-induced lung injury by reducing oxidative stress. *J Pathol*. 2007;212:38-46
32. Chen Y, Wermeling F, Sundqvist J, Jonsson AB, Tryggvason K, Pikkarainen T, Karlsson MC. A regulatory role for macrophage class a scavenger receptors in tlr4-mediated lps responses. *Eur J Immunol*. 2010;40:1451-1460
33. Yu X, Yi H, Guo C, Zuo D, Wang Y, Kim HL, Subjeck JR, Wang XY. Pattern recognition scavenger receptor cd204 attenuates toll-like receptor 4-induced nf- $\kappa$ b



activation by directly inhibiting ubiquitination of tumor necrosis factor (tnf) receptor-associated factor 6. *J Biol Chem*. 2011;286:18795-18806

34. Emura I, Usuda H, Fujita T, Ebe K, Nagai T. Increase of scavenger receptor a-positive monocytes in patients with acute coronary syndromes. *Pathol Int*. 2007;57:502-508

35. Zhang SZ, Jin YP, Qin GM, Wang JH. Association of platelet-monocyte aggregates with platelet activation, systemic inflammation, and myocardial injury in patients with non-ST elevation acute coronary syndromes. *Clin Cardiol*. 2007;30:26-31

36. Hume DA. The mononuclear phagocyte system. *Curr Opin Immunol*. 2006;18:49-53

37. Zhao Y, Glesne D, Huberman E. A human peripheral blood monocyte-derived subset acts as pluripotent stem cells. *Proc Natl Acad Sci U S A*. 2003;100:2426-2431

38. Shantsila E, Watson T, Lip GY. Endothelial progenitor cells in cardiovascular disorders. *J Am Coll Cardiol*. 2007;49:741-752

39. Shantsila E, Watson T, Tse HF, Lip GY. New insights on endothelial progenitor cell subpopulations and their angiogenic properties. *J Am Coll Cardiol*. 2008;51:669-671

40. Shantsila E, Lip GY. Monocyte diversity in myocardial infarction. *J Am Coll Cardiol*. 2009;54:139-142

41. Nahrendorf M, Swirski FK, Aikawa E, Stangenberg L, Wurdinger T, Figueiredo JL, Libby P, Weissleder R, Pittet MJ. The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions. *J Exp Med*. 2007;204:3037-3047

42. Ziegler-Heitbrock L. The cd14+ cd16+ blood monocytes: Their role in infection and inflammation. *J Leukoc Biol*. 2007;81:584-592

43. Tsujioka H, Imanishi T, Ikejima H, Kuroi A, Takarada S, Tanimoto T, Kitabata H, Okochi K, Arita Y, Ishibashi K, Komukai K, Kataiwa H, Nakamura N, Hirata K, Tanaka A, Akasaka T. Impact of heterogeneity of human peripheral blood monocyte subsets on myocardial salvage in patients with primary acute myocardial infarction. *J Am Coll Cardiol*. 2009;54:130-138

44. Urra X, Villamor N, Amaro S, Gomez-Choco M, Obach V, Oleaga L, Planas AM, Chamorro A. Monocyte subtypes predict clinical course and prognosis in human stroke. *J Cereb Blood Flow Metab*. 2009;29:994-1002

45. Moniuszko M, Bodzenta-Lukaszyk A, Kowal K, Lenczewska D, Dabrowska M. Enhanced frequencies of cd14++cd16+, but not cd14+cd16+, peripheral blood monocytes in severe asthmatic patients. *Clin Immunol*. 2009;130:338-346

46. Ziegler-Heitbrock L, Ancuta P, Crowe S, Dalod M, Grau V, Hart DN, Leenen PJ, Liu YJ, MacPherson G, Randolph GJ, Scherberich J, Schmitz J, Shortman K, Sozzani S, Strobl H,

- Zembala M, Austyn JM, Lutz MB. Nomenclature of monocytes and dendritic cells in blood. *Blood*. 2010;116:e74-80
47. Shantsila E, Kamphuisen PW, Lip GY. Circulating microparticles in cardiovascular disease: Implications for atherogenesis and atherothrombosis. *J Thromb Haemost*. 2010
48. Montoro-Garcia S, Shantsila E, Marin F, Blann A, Lip GY. Circulating microparticles: New insights into the biochemical basis of microparticle release and activity. *Basic Res Cardiol*. 2011
49. Shantsila E. Endothelial microparticles: A universal marker of vascular health? *J Hum Hypertens*. 2009;23:359-361
50. Borlaug BA, Paulus WJ. Heart failure with preserved ejection fraction: Pathophysiology, diagnosis, and treatment. *Eur Heart J*. 2011;32:670-679
51. Owan TE, Hodge DO, Herges RM, Jacobsen SJ, Roger VL, Redfield MM. Trends in prevalence and outcome of heart failure with preserved ejection fraction. *N Engl J Med*. 2006;355:251-259
52. Maeder MT, Kaye DM. Heart failure with normal left ventricular ejection fraction. *J Am Coll Cardiol*. 2009;53:905-918
53. Paulus WJ, van Ballegoij JJ. Treatment of heart failure with normal ejection fraction: An inconvenient truth! *J Am Coll Cardiol*. 2010;55:526-537
54. Galasko GI, Senior R, Lahiri A. Ethnic differences in the prevalence and aetiology of left ventricular systolic dysfunction in the community: The harrow heart failure watch. *Heart*. 2005;91:595-600
55. Singh N, Gupta M. Clinical characteristics of south asian patients hospitalized with heart failure. *Ethn Dis*. 2005;15:615-619
56. Ng TP, Niti M. Trends and ethnic differences in hospital admissions and mortality for congestive heart failure in the elderly in singapore, 1991 to 1998. *Heart*. 2003;89:865-870
57. Hughes LO, Raval U, Raftery EB. First myocardial infarction in asian and white men. 1989;298:1345-1350
58. McKeigue PM, Karmi G. Alcohol consumption and alcohol-related problems in afro-caribbeans and south asians in the united kingdom. *Alcohol Alcohol*. 1993;28:1-10
59. Van Driest SL, Ommen SR, Tajik AJ, Gersh BJ, Ackerman MJ. Sarcomeric genotyping in hypertrophic cardiomyopathy. *Mayo Clin Proc*. 2005;80:463-469
60. Dhandapany PS, Sadayappan S, Xue Y, Powell GT, Rani DS, Nallari P, Rai TS, Khullar M, Soares P, Bahl A, Tharkan JM, Vaideeswar P, Rathinavel A, Narasimhan C, Ayapati DR, Ayub Q, Mehdi SQ, Oppenheimer S, Richards MB, Price AL, Patterson N, Reich D, Singh L,

- Tyler-Smith C, Thangaraj K. A common mybpc3 (cardiac myosin binding protein c) variant associated with cardiomyopathies in south asia. *Nat Genet.* 2009;41:187-191
61. Chahal NS, Lim TK, Jain P, Chambers JC, Kooner JS, Senior R. Ethnicity-related differences in left ventricular function, structure and geometry: A population study of uk indian asian and european white subjects. *Heart.* 2010;96:466-471
62. Schofield RS, Wessel TR, Walker TC, Cleeton TS, Hill JA, Aranda JM, Jr. Hyperhomocysteinemia in patients with heart failure referred for cardiac transplantation: Preliminary observations. *Clin Cardiol.* 2003;26:407-410
63. Davis RC, Hobbs FD, Kenkre JE, Roalfe AK, Hare R, Lancashire RJ, Davies MK. Prevalence of left ventricular systolic dysfunction and heart failure in high risk patients: Community based epidemiological study. *BMJ.* 2002;325:1156
64. Alexander M, Grumbach K, Remy L, Rowell R, Massie BM. Congestive heart failure hospitalizations and survival in california: Patterns according to race/ethnicity. *Am Heart J.* 1999;137:919-927
65. Seow SC, Chai P, Lee YP, Chan YH, Kwok BW, Yeo TC, Chia BL. Heart failure mortality in southeast asian patients with left ventricular systolic dysfunction. *J Card Fail.* 2007;13:476-481
66. Davies M, Hobbs F, Davis R, Kenkre J, Roalfe AK, Hare R, Wosornu D, Lancashire RJ. Prevalence of left-ventricular systolic dysfunction and heart failure in the echocardiographic heart of england screening study: A population based study. *Lancet.* 2001;358:439-444
67. Newton JD, Blackledge HM, Squire IB. Ethnicity and variation in prognosis for patients newly hospitalised for heart failure: A matched historical cohort study. *Heart.* 2005;91:1545-1550
68. Joshi PP, Mohanan CJ, Sengupta SP, Salkar RG. Factors precipitating congestive heart failure--role of patient non-compliance. *J Assoc Physicians India.* 1999;47:294-295
69. Sosin MD, Patel JV, Bhatia GS, Hughes EA, Davis RC, Lip GY. Effects of white european, african caribbean and south asian ethnicity on homocysteine levels in patients with systolic heart failure. *Int J Cardiol.* 2008;129:69-75
70. Sosin MD, Bhatia GS, Davis RC, Connolly DL, Lip GY. Heart failure: Treatment and ethnic origin. *Lancet.* 2003;362:919-920
71. Gill PS, Davis R, Davies M, Freemantle N, Lip GY. Rationale and study design of a cross sectional study documenting the prevalence of heart failure amongst the minority ethnic communities in the uk: The e-echoes study (ethnic--echocardiographic heart of england screening study). *BMC Cardiovasc Disord.* 2009;9:47

72. Lip G, Zarifis J, Beevers DG. Acute admissions with heart failure to a district general hospital serving a multiracial population. *Int J Clin Pract.* 1997;51:223-227
73. Liew R, Sulfi S, Ranjadayalan K, Cooper J, Timmis AD. Declining case fatality rates for acute myocardial infarction in south asian and white patients in the past 15 years. *Heart.* 2006;92:1030-1034
74. Lip GY, Khan H, Bhatnagar A, Brahmabhatt N, Crook P, Davies MK. Ethnic differences in patient perceptions of heart failure and treatment: The west birmingham heart failure project. *Heart.* 2004;90:1016-1019
75. Patel JV, Sosin M, Lim HS, Chung I, Panja N, Davis RC, Hughes EA, Lip GY. Raised leptin concentrations among south asian patients with chronic heart failure. *Int J Cardiol.* 2007;122:34-40
76. Jafary FH, Kumar M, Chandna IE. Prognosis of hospitalized new-onset systolic heart failure in indo-asians--a lethal problem. *J Card Fail.* 2007;13:855-860
77. Chong AY, Rajaratnam R, Hussein NR, Lip GY. Heart failure in a multiethnic population in kuala lumpur, malaysia. *Eur J Heart Fail.* 2003;5:569-574
78. Fernandes VR, Cheng S, Cheng YJ, Rosen B, Agarwal S, McClelland RL, Bluemke DA, Lima JA. Racial and ethnic differences in subclinical myocardial function: The multi-ethnic study of atherosclerosis. *Heart.* 2011;97:405-410
79. Agyemang C, Kunst A, Bhopal R, Zaninotto P, Unwin N, Nazroo J, Nicolaou M, Redekop WK, Stronks K. A cross-national comparative study of blood pressure and hypertension between english and dutch south-asian- and african-origin populations: The role of national context. *Am J Hypertens.* 2010;23:639-648
80. Dwivedi G, Beevers DG. Hypertension in ethnic groups: Epidemiological and clinical perspectives. *Expert Rev Cardiovasc Ther.* 2009;7:955-963
81. Lee R, Chan SP, Wong J, Lau D, Ho KT, Ng K. Impact of diabetes mellitus on survival in south east asian patients with congestive heart failure due to left ventricular systolic dysfunction. *Int J Cardiol.* 2010;142:97-100
82. Pannu G, Zaman S, Bhala N, Zaman R. Alcohol use in south asians in the uk. *BMJ.* 2009;339:b4028
83. Niimura H, Patton KK, McKenna WJ, Soultis J, Maron BJ, Seidman JG, Seidman CE. Sarcomere protein gene mutations in hypertrophic cardiomyopathy of the elderly. *Circulation.* 2002;105:446-451
84. Fischbacher CM, Bhopal R, Rutter MK, Unwin NC, Marshall SM, White M, Alberti KG. Microalbuminuria is more frequent in south asian than in european origin populations: A comparative study in newcastle, uk. *Diabet Med.* 2003;20:31-36

85. Cooke GE, Eaton GM, Whitby G, Kennedy RA, Binkley PF, Moeschberger ML, Leier CV. Plasma atherogenic markers in congestive heart failure and posttransplant (heart) patients. *J Am Coll Cardiol.* 2000;36:509-516
86. Joshi PP, Mohanan CJ, Sengupta SP, Salkar RG. Factors precipitating congestive heart failure-role of patient non-compliance. *J Assoc Physicians India.* 1999;47:294-295
87. Barker DJ, Osmond C. Infant mortality, childhood nutrition, and ischaemic heart disease in England and Wales. *Lancet.* 1986;1:1077-1081
88. Ravelli AC, van der Meulen JH, Michels RP, Osmond C, Barker DJ, Hales CN, Bleker OP. Glucose tolerance in adults after prenatal exposure to famine. *Lancet.* 1998;351:173-177
89. Hales CN, Barker DJ. Type 2 (non-insulin-dependent) diabetes mellitus: The thrifty phenotype hypothesis. *Diabetologia.* 1992;35:595-601
90. Osmond C, Barker DJ. Fetal, infant, and childhood growth are predictors of coronary heart disease, diabetes, and hypertension in adult men and women. *Environ Health Perspect.* 2000;108 Suppl 3:545-553
91. Hofman PL, Cutfield WS, Robinson EM, Bergman RN, Menon RK, Sperling MA, Gluckman PD. Insulin resistance in short children with intrauterine growth retardation. *J Clin Endocrinol Metab.* 1997;82:402-406
92. Ong KK, Petry CJ, Emmett PM, Sandhu MS, Kiess W, Hales CN, Ness AR, Dunger DB. Insulin sensitivity and secretion in normal children related to size at birth, postnatal growth, and plasma insulin-like growth factor-I levels. *Diabetologia.* 2004;47:1064-1070
93. Bertram CE, Hanson MA. Animal models and programming of the metabolic syndrome. *Br Med Bull.* 2001;60:103-121
94. Petry CJ, Dorling MW, Pawlak DB, Ozanne SE, Hales CN. Diabetes in old male offspring of rat dams fed a reduced protein diet. *International journal of experimental diabetes research.* 2001;2:139-143
95. Vickers MH, Breier BH, Cutfield WS, Hofman PL, Gluckman PD. Fetal origins of hyperphagia, obesity, and hypertension and postnatal amplification by hypercaloric nutrition. *Am J Physiol Endocrinol Metab.* 2000;279:E83-87
96. Brawley L, Poston L, Hanson MA. Mechanisms underlying the programming of small artery dysfunction: Review of the model using low protein diet in pregnancy in the rat. *Archives of physiology and biochemistry.* 2003;111:23-35
97. Bloomfield FH, Oliver MH, Hawkins P, Holloway AC, Campbell M, Gluckman PD, Harding JE, Challis JR. Periconceptional undernutrition in sheep accelerates maturation of the fetal hypothalamic-pituitary-adrenal axis in late gestation. *Endocrinology.* 2004;145:4278-4285

98. Gluckman PD, Hanson MA, Spencer HG, Bateson P. Environmental influences during development and their later consequences for health and disease: Implications for the interpretation of empirical studies. *Proceedings. Biological sciences / The Royal Society.* 2005;272:671-677
99. Gluckman PD, Hanson MA, Morton SM, Pinal CS. Life-long echoes--a critical analysis of the developmental origins of adult disease model. *Biology of the neonate.* 2005;87:127-139
100. Gluckman PD, Cutfield W, Hofman P, Hanson MA. The fetal, neonatal, and infant environments-the long-term consequences for disease risk. *Early human development.* 2005;81:51-59
101. Barker DJ, Eriksson JG, Forsen T, Osmond C. Fetal origins of adult disease: Strength of effects and biological basis. *Int J Epidemiol.* 2002;31:1235-1239
102. Gibbs WW. The unseen genome: Beyond DNA. *Scientific American.* 2003;289:106-113
103. Heijmans BT, Tobi EW, Stein AD, Putter H, Blauw GJ, Susser ES, Slagboom PE, Lumey LH. Persistent epigenetic differences associated with prenatal exposure to famine in humans. *Proc Natl Acad Sci U S A.* 2008;105:17046-17049
104. Stoger R. The thrifty epigenotype: An acquired and heritable predisposition for obesity and diabetes? *Bioessays.* 2008;30:156-166
105. Evans A, Kalra L. Racial inequity of access to carotid imaging. *Stroke.* 1999;30:2491
106. Blann AD, Lip GY. The endothelium in atherothrombotic disease: Assessment of function, mechanisms and clinical implications. *Blood Coagul Fibrinolysis.* 1998;9:297-306
107. Blann AD, Woywodt A, Bertolini F, Bull TM, Buyon JP, Clancy RM, Haubitz M, Heibel RP, Lip GY, Mancuso P, Sampol J, Solovey A, Dignat-George F. Circulating endothelial cells. Biomarker of vascular disease. *Thromb Haemost.* 2005;93:228-235
108. Nakamura R, Egashira K, Arimura K, Machida Y, Ide T, Tsutsui H, Shimokawa H, Takeshita A. Increased inactivation of nitric oxide is involved in impaired coronary flow reserve in heart failure. *Am J Physiol Heart Circ Physiol.* 2001;281:H2619-2625
109. Arimura K, Egashira K, Nakamura R, Ide T, Tsutsui H, Shimokawa H, Takeshita A. Increased inactivation of nitric oxide is involved in coronary endothelial dysfunction in heart failure. *Am J Physiol Heart Circ Physiol.* 2001;280:H68-75
110. Winyard PG, Spickett CM, Griffiths HR. Analysis of radicals and radical reaction products in cell signalling and biomolecular damage: The long hard road to gold-standard measures. *Biochem Soc Trans.* 2011;39:1217-1220
111. Heymes C, Vanderheyden M, Bronzwaer JG, Shah AM, Paulus WJ. Endomyocardial nitric oxide synthase and left ventricular preload reserve in dilated cardiomyopathy. *Circulation.* 1999;99:3009-3016

112. Calderone A. The therapeutic effect of natriuretic peptides in heart failure; differential regulation of endothelial and inducible nitric oxide synthases. *Heart Fail Rev.* 2003;8:55-70
113. Tada H, Thompson CI, Recchia FA, Loke KE, Ochoa M, Smith CJ, Shesely EG, Kaley G, Hintze TH. Myocardial glucose uptake is regulated by nitric oxide via endothelial nitric oxide synthase in langendorff mouse heart. *Circ Res.* 2000;86:270-274
114. Ruetten H, Dimmeler S, Gehring D, Ihling C, Zeiher AM. Concentric left ventricular remodeling in endothelial nitric oxide synthase knockout mice by chronic pressure overload. *Cardiovasc Res.* 2005;66:444-453
115. Jones SP, Greer JJ, van Haperen R, Duncker DJ, de Crom R, Lefer DJ. Endothelial nitric oxide synthase overexpression attenuates congestive heart failure in mice. *Proc Natl Acad Sci U S A.* 2003;100:4891-4896
116. Wenzel S, Rohde C, Wingerning S, Roth J, Kojda G, Schluter KD. Lack of endothelial nitric oxide synthase-derived nitric oxide formation favors hypertrophy in adult ventricular cardiomyocytes. *Hypertension.* 2007;49:193-200
117. Zhao X, Lu X, Feng Q. Deficiency in endothelial nitric oxide synthase impairs myocardial angiogenesis. *Am J Physiol Heart Circ Physiol.* 2002;283:H2371-2378
118. Drexler H, Kastner S, Strobel A, Studer R, Brodde OE, Hasenfuss G. Expression, activity and functional significance of inducible nitric oxide synthase in the failing human heart. *J Am Coll Cardiol.* 1998;32:955-963
119. Vejstrup NG, Bouloumie A, Boesgaard S, Andersen CB, Nielsen-Kudsk JE, Mortensen SA, Kent JD, Harrison DG, Busse R, Aldershvile J. Inducible nitric oxide synthase (inos) in the human heart: Expression and localization in congestive heart failure. *J Mol Cell Cardiol.* 1998;30:1215-1223
120. Ray R, Shah AM. NADPH oxidase and endothelial cell function. *Clin Sci (Lond).* 2005;109:217-226
121. Westermann D, Riad A, Richter U, Jager S, Savvatis K, Schuchardt M, Bergmann N, Tolle M, Nagorsen D, Gotthardt M, Schultheiss HP, Tschope C. Enhancement of the endothelial nitric oxide synthase attenuates experimental diastolic heart failure. *Basic Res Cardiol.* 2009;104:499-509
122. Chen Y, Hou M, Li Y, Traverse JH, Zhang P, Salvemini D, Fukui T, Bache RJ. Increased superoxide production causes coronary endothelial dysfunction and depressed oxygen consumption in the failing heart. *Am J Physiol Heart Circ Physiol.* 2005;288:H133-141
123. Miller JD, Peotta VA, Chu Y, Weiss RM, Zimmerman K, Brooks RM, Heistad DD. Mnsod protects against cox1-mediated endothelial dysfunction in chronic heart failure. *Am J Physiol Heart Circ Physiol.* 2010;298:H1600-1607

124. Iida S, Chu Y, Francis J, Weiss RM, Gunnett CA, Faraci FM, Heistad DD. Gene transfer of extracellular superoxide dismutase improves endothelial function in rats with heart failure. *Am J Physiol Heart Circ Physiol*. 2005;289:H525-532
125. Landmesser U, Spiekermann S, Dikalov S, Tatge H, Wilke R, Kohler C, Harrison DG, Hornig B, Drexler H. Vascular oxidative stress and endothelial dysfunction in patients with chronic heart failure: Role of xanthine-oxidase and extracellular superoxide dismutase. *Circulation*. 2002;106:3073-3078
126. Kaye DM, Ahlers BA, Autelitano DJ, Chin-Dusting JP. In vivo and in vitro evidence for impaired arginine transport in human heart failure. *Circulation*. 2000;102:2707-2712
127. Feng Q, Lu X, Fortin AJ, Pettersson A, Hedner T, Kline RL, Arnold JM. Elevation of an endogenous inhibitor of nitric oxide synthesis in experimental congestive heart failure. *Cardiovasc Res*. 1998;37:667-675
128. Agnoletti L, Curello S, Bachetti T, Malacarne F, Gaia G, Comini L, Volterrani M, Bonetti P, Parrinello G, Cadei M, Grigolato PG, Ferrari R. Serum from patients with severe heart failure downregulates enos and is proapoptotic: Role of tumor necrosis factor-alpha. *Circulation*. 1999;100:1983-1991
129. Rossig L, Haendeler J, Mallat Z, Hugel B, Freyssinet JM, Tedgui A, Dimmeler S, Zeiher AM. Congestive heart failure induces endothelial cell apoptosis: Protective role of carvedilol. *J Am Coll Cardiol*. 2000;36:2081-2089
130. Seta Y, Shan K, Bozkurt B, Oral H, Mann DL. Basic mechanisms in heart failure: The cytokine hypothesis. *J Card Fail*. 1996;2:243-249
131. Levine B, Kalman J, Mayer L, Fillit HM, Packer M. Elevated circulating levels of tumor necrosis factor in severe chronic heart failure. *N Engl J Med*. 1990;323:236-241
132. Torre-Amione G, Kapadia S, Benedict C, Oral H, Young JB, Mann DL. Proinflammatory cytokine levels in patients with depressed left ventricular ejection fraction: A report from the studies of left ventricular dysfunction (solvd). *J Am Coll Cardiol*. 1996;27:1201-1206
133. Tsutamoto T, Hisanaga T, Wada A, Maeda K, Ohnishi M, Fukai D, Mabuchi N, Sawaki M, Kinoshita M. Interleukin-6 spillover in the peripheral circulation increases with the severity of heart failure, and the high plasma level of interleukin-6 is an important prognostic predictor in patients with congestive heart failure. *J Am Coll Cardiol*. 1998;31:391-398
134. Yin WH, Chen JW, Jen HL, Chiang MC, Huang WP, Feng AN, Young MS, Lin SJ. Independent prognostic value of elevated high-sensitivity c-reactive protein in chronic heart failure. *Am Heart J*. 2004;147:931-938



135. Deswal A, Petersen NJ, Feldman AM, Young JB, White BG, Mann DL. Cytokines and cytokine receptors in advanced heart failure: An analysis of the cytokine database from the vesnarinone trial (vest). *Circulation*. 2001;103:2055-2059
136. Janssen SP, Gayan-Ramirez G, Van den Bergh A, Herijgers P, Maes K, Verbeken E, Decramer M. Interleukin-6 causes myocardial failure and skeletal muscle atrophy in rats. *Circulation*. 2005;111:996-1005
137. Hoare GS, Birks EJ, Bowles C, Marczin N, Yacoub MH. In vitro endothelial cell activation and inflammatory responses in end-stage heart failure. *J Appl Physiol*. 2006;101:1466-1473
138. Suzuki S, Takeishi Y, Niizeki T, Koyama Y, Kitahara T, Sasaki T, Sagara M, Kubota I. Pentraxin 3, a new marker for vascular inflammation, predicts adverse clinical outcomes in patients with heart failure. *Am Heart J*. 2008;155:75-81
139. Mann DL, McMurray JJ, Packer M, Swedberg K, Borer JS, Colucci WS, Djian J, Drexler H, Feldman A, Kober L, Krum H, Liu P, Nieminen M, Tavazzi L, van Veldhuisen DJ, Waldenstrom A, Warren M, Westheim A, Zannad F, Fleming T. Targeted anticytokine therapy in patients with chronic heart failure: Results of the randomized etanercept worldwide evaluation (renewal). *Circulation*. 2004;109:1594-1602
140. Chung ES, Packer M, Lo KH, Fasanmade AA, Willerson JT. Randomized, double-blind, placebo-controlled, pilot trial of infliximab, a chimeric monoclonal antibody to tumor necrosis factor-alpha, in patients with moderate-to-severe heart failure: Results of the anti-tnf therapy against congestive heart failure (attach) trial. *Circulation*. 2003;107:3133-3140
141. Habib F, Dutka D, Crossman D, Oakley CM, Cleland JG. Enhanced basal nitric oxide production in heart failure: Another failed counter-regulatory vasodilator mechanism? *Lancet*. 1994;344:371-373
142. Tentolouris C, Tousoulis D, Antoniadis C, Bosinakou E, Kotsopoulou M, Trikas A, Toutouzas P, Stefanadis C. Endothelial function and proinflammatory cytokines in patients with ischemic heart disease and dilated cardiomyopathy. *Int J Cardiol*. 2004;94:301-305
143. Chong AY, Blann AD, Patel J, Freestone B, Hughes E, Lip GY. Endothelial dysfunction and damage in congestive heart failure: Relation of flow-mediated dilation to circulating endothelial cells, plasma indexes of endothelial damage, and brain natriuretic peptide. *Circulation*. 2004;110:1794-1798
144. Bank AJ, Lee PC, Kubo SH. Endothelial dysfunction in patients with heart failure: Relationship to disease severity. *J Card Fail*. 2000;6:29-36

145. Vittorio TJ, Zolty R, Garg PK, Sarswat N, Tseng CH, Jorde UP, Colombo PC. Interdependence of cardiac and endothelial function in patients with symptomatic chronic heart failure of nonischemic etiology. *Echocardiography*. 2009;26:916-921
146. Bae JH, Bassenge E, Kim MH, Park KR, Kim KY, Synn YC, Schwemmer M. Impact of left ventricular ejection fraction on endothelial function in patients with coronary artery disease. *Clin Cardiol*. 2004;27:333-337
147. Nakamura M, Yoshida H, Arakawa N, Mizunuma Y, Makita S, Hiramori K. Endothelium-dependent vasodilatation is not selectively impaired in patients with chronic heart failure secondary to valvular heart disease and congenital heart disease. *Eur Heart J*. 1996;17:1875-1881
148. Shah A, Gkaliagkousi E, Ritter JM, Ferro A. Endothelial function and arterial compliance are not impaired in subjects with heart failure of non-ischemic origin. *J Card Fail*. 2010;16:114-120
149. Klosinska M, Rudzinski T, Grzelak P, Stefanczyk L, Drozd J, Krzeminska-Pakula M. Endothelium-dependent and -independent vasodilation is more attenuated in ischaemic than in non-ischaemic heart failure. *Eur J Heart Fail*. 2009;11:765-770
150. de Jong RM, Blanksma PK, Cornel JH, Van den Heuvel AF, Siebelink HM, Vaalburg W, van Veldhuisen DJ. Endothelial dysfunction and reduced myocardial perfusion reserve in heart failure secondary to coronary artery disease. *Am J Cardiol*. 2003;91:497-500
151. Canetti M, Akhter MW, Lerman A, Karaalp IS, Zell JA, Singh H, Mehra A, Elkayam U. Evaluation of myocardial blood flow reserve in patients with chronic congestive heart failure due to idiopathic dilated cardiomyopathy. *Am J Cardiol*. 2003;92:1246-1249
152. Mathier MA, Rose GA, Fifer MA, Miyamoto MI, Dinsmore RE, Castano HH, Dec GW, Palacios IF, Semigran MJ. Coronary endothelial dysfunction in patients with acute-onset idiopathic dilated cardiomyopathy. *J Am Coll Cardiol*. 1998;32:216-224
153. Dini FL, Ghiadoni L, Conti U, Stea F, Buralli S, Taddei S, De Tommasi SM. Coronary flow reserve in idiopathic dilated cardiomyopathy: Relation with left ventricular wall stress, natriuretic peptides, and endothelial dysfunction. *J Am Soc Echocardiogr*. 2009;22:354-360
154. Stolen KQ, Kempainen J, Kalliokoski KK, Karanko H, Toikka J, Janatuinen T, Raitakari OT, Airaksinen KE, Nuutila P, Knuuti J. Myocardial perfusion reserve and peripheral endothelial function in patients with idiopathic dilated cardiomyopathy. *Am J Cardiol*. 2004;93:64-68
155. Bitar F, Lerman A, Akhter MW, Hatamizadeh P, Janmohamed M, Khan S, Elkayam U. Variable response of conductance and resistance coronary arteries to endothelial stimulation in

patients with heart failure due to nonischemic dilated cardiomyopathy. *J Cardiovasc Pharmacol Ther.* 2006;11:197-202

156. Elesber AA, Redfield MM, Rihal CS, Prasad A, Lavi S, Lennon R, Mathew V, Lerman LO, Lerman A. Coronary endothelial dysfunction and hyperlipidemia are independently associated with diastolic dysfunction in humans. *Am Heart J.* 2007;153:1081-1087

157. Ma LN, Zhao SP, Gao M, Zhou QC, Fan P. Endothelial dysfunction associated with left ventricular diastolic dysfunction in patients with coronary heart disease. *Int J Cardiol.* 2000;72:275-279

158. Lapu-Bula R, Ofili E. From hypertension to heart failure: Role of nitric oxide-mediated endothelial dysfunction and emerging insights from myocardial contrast echocardiography. *Am J Cardiol.* 2007;99:7D-14D

159. Ferreiro CR, Chagas AC, Carvalho MH, Dantas AP, Scavone C, Souza LC, Buffolo E, da Luz PL. Expression of inducible nitric oxide synthase is increased in patients with heart failure due to ischemic disease. *Braz J Med Biol Res.* 2004;37:1313-1320

160. Colombo PC, Rastogi S, Onat D, Zaca V, Gupta RC, Jorde UP, Sabbah HN. Activation of endothelial cells in conduit veins of dogs with heart failure and veins of normal dogs after vascular stretch by acute volume loading. *J Card Fail.* 2009;15:457-463

161. Dworakowski R, Walker S, Momin A, Desai J, El-Gamel A, Wendler O, Kearney MT, Shah AM. Reduced nicotinamide adenine dinucleotide phosphate oxidase-derived superoxide and vascular endothelial dysfunction in human heart failure. *J Am Coll Cardiol.* 2008;51:1349-1356

162. Rabelo ER, Ruschel K, Moreno H, Jr., Rubira M, Consolim-Colombo FM, Irigoyen MC, Rohde LE. Venous endothelial function in heart failure: Comparison with healthy controls and effect of clinical compensation. *Eur J Heart Fail.* 2008;10:758-764

163. Nightingale AK, Blackman DJ, Ellis GR, Schmitt M, Morris-Thurgood JA, Jones EA, Frenneaux MP. Preservation of venous endothelial function in the forearm venous capacitance bed of patients with chronic heart failure despite arterial endothelial dysfunction. *J Am Coll Cardiol.* 2001;37:1062-1068

164. Funakoshi T, Yamabe H, Yokoyama M. Increased exhaled nitric oxide and impaired oxygen uptake (vo<sub>2</sub>) kinetics during exercise in patients with chronic heart failure. *Jpn Circ J.* 1999;63:255-260

165. Andersson SE, Edvinsson ML, Edvinsson L. Cutaneous vascular reactivity is reduced in aging and in heart failure: Association with inflammation. *Clin Sci (Lond).* 2003;105:699-707

166. Andreassen AK, Gullestad L, Holm T, Simonsen S, Kvernebo K. Endothelium-dependent vasodilation of the skin microcirculation in heart transplant recipients. *Clin Transplant*. 1998;12:324-332
167. Duscha BD, Kraus WE, Keteyian SJ, Sullivan MJ, Green HJ, Schachat FH, Phippen AM, Brawner CA, Blank JM, Annex BH. Capillary density of skeletal muscle: A contributing mechanism for exercise intolerance in class ii-iii chronic heart failure independent of other peripheral alterations. *J Am Coll Cardiol*. 1999;33:1956-1963
168. Kielstein JT, Bode-Boger SM, Klein G, Graf S, Haller H, Fliser D. Endogenous nitric oxide synthase inhibitors and renal perfusion in patients with heart failure. *Eur J Clin Invest*. 2003;33:370-375
169. Naka KK, Tweddel AC, Doshi SN, Goodfellow J, Henderson AH. Flow-mediated changes in pulse wave velocity: A new clinical measure of endothelial function. *Eur Heart J*. 2006;27:302-309
170. Poelzl G, Frick M, Huegel H, Lackner B, Alber HF, Mair J, Herold M, Schwarzacher S, Pachinger O, Weidinger F. Chronic heart failure is associated with vascular remodeling of the brachial artery. *Eur J Heart Fail*. 2005;7:43-48
171. Nakamura M, Sugawara S, Arakawa N, Nagano M, Shizuka T, Shimoda Y, Sakai T, Hiramori K. Reduced vascular compliance is associated with impaired endothelium-dependent dilatation in the brachial artery of patients with congestive heart failure. *J Card Fail*. 2004;10:36-42
172. Heitzer T, Baldus S, von Kodolitsch Y, Rudolph V, Meinertz T. Systemic endothelial dysfunction as an early predictor of adverse outcome in heart failure. *Arterioscler Thromb Vasc Biol*. 2005;25:1174-1179
173. Shechter M, Matetzky S, Arad M, Feinberg MS, Freimark D. Vascular endothelial function predicts mortality risk in patients with advanced ischaemic chronic heart failure. *Eur J Heart Fail*. 2009;11:588-593
174. de Berrazueta JR, Guerra-Ruiz A, Garcia-Unzueta MT, Toca GM, Laso RS, de Adana MS, Martin MA, Cobo M, Llorca J. Endothelial dysfunction, measured by reactive hyperaemia using strain-gauge plethysmography, is an independent predictor of adverse outcome in heart failure. *Eur J Heart Fail*. 2010;12:477-483
175. Katz SD, Hryniewicz K, Hriljac I, Balidemaj K, Dimayuga C, Hudaihed A, Yasskiy A. Vascular endothelial dysfunction and mortality risk in patients with chronic heart failure. *Circulation*. 2005;111:310-314
176. Fischer D, Rossa S, Landmesser U, Spiekermann S, Engberding N, Hornig B, Drexler H. Endothelial dysfunction in patients with chronic heart failure is independently associated with

increased incidence of hospitalization, cardiac transplantation, or death. *Eur Heart J*. 2005;26:65-69

177. Kubrich M, Petrakopoulou P, Kofler S, Nickel T, Kaczmarek I, Meiser BM, Reichart B, von Scheidt W, Weis M. Impact of coronary endothelial dysfunction on adverse long-term outcome after heart transplantation. *Transplantation*. 2008;85:1580-1587

178. Akar JG, Al-Chekakie MO, Fugate T, Moran L, Froloshki B, Varma N, Santucci P, Wilber DJ, Matsumura ME. Endothelial dysfunction in heart failure identifies responders to cardiac resynchronization therapy. *Heart Rhythm*. 2008;5:1229-1235

179. Kistorp C, Chong AY, Gustafsson F, Galatius S, Raymond I, Faber J, Lip GY, Hildebrandt P. Biomarkers of endothelial dysfunction are elevated and related to prognosis in chronic heart failure patients with diabetes but not in those without diabetes. *Eur J Heart Fail*. 2008;10:380-387

180. Chong AY, Freestone B, Patel J, Lim HS, Hughes E, Blann AD, Lip GY. Endothelial activation, dysfunction, and damage in congestive heart failure and the relation to brain natriuretic peptide and outcomes. *Am J Cardiol*. 2006;97:671-675

181. Chong AY, Freestone B, Lim HS, Kistorp C, Gustafsson F, Hildebrandt P, Lip GY. Plasma von willebrand factor and soluble e-selectin levels in stable outpatients with systolic heart failure: The frederiksberg heart failure study. *Int J Cardiol*. 2007;119:80-82

182. Chong AY, Lip GY, Freestone B, Blann AD. Increased circulating endothelial cells in acute heart failure: Comparison with von willebrand factor and soluble e-selectin. *Eur J Heart Fail*. 2006;8:167-172

183. Vila V, Martinez-Sales V, Almenar L, Lazaro IS, Villa P, Reganon E. Inflammation, endothelial dysfunction and angiogenesis markers in chronic heart failure patients. *Int J Cardiol*. 2008;130:276-277

184. Leyva F, Anker SD, Godsland IF, Teixeira M, Hellewell PG, Kox WJ, Poole-Wilson PA, Coats AJ. Uric acid in chronic heart failure: A marker of chronic inflammation. *Eur Heart J*. 1998;19:1814-1822

185. Penna C, Rastaldo R, Mancardi D, Cappello S, Pagliaro P, Westerhof N, Losano G. Effect of endothelins on the cardiovascular system. *J Cardiovasc Med (Hagerstown)*. 2006;7:645-652

186. Good JM, Nihoyannopoulos P, Ghatei MA, Crossman D, Bloom SR, Clark P, Oakley CM, Cleland JG. Elevated plasma endothelin concentrations in heart failure; an effect of angiotensin ii? *Eur Heart J*. 1994;15:1634-1640

187. Ohnishi M, Wada A, Tsutamoto T, Fujii M, Matsumoto T, Yamamoto T, Takayama T, Wang X, Kinoshita M. Endothelin stimulates an endogenous nitric oxide synthase inhibitor,

- asymmetric dimethylarginine, in experimental heart failure. *Clin Sci (Lond)*. 2002;103 Suppl 48:241S-244S
188. Galatius S, Wroblewski H, Sorensen VB, Bie P, Parving HH, Kasstrup J. Endothelin and von willebrand factor as parameters of endothelial function in idiopathic dilated cardiomyopathy: Different stimuli for release before and after heart transplantation? *Am Heart J*. 1999;137:549-554
189. Sakai S, Miyauchi T, Kobayashi M, Yamaguchi I, Goto K, Sugishita Y. Inhibition of myocardial endothelin pathway improves long-term survival in heart failure. *Nature*. 1996;384:353-355
190. Berger R, Stanek B, Hulsmann M, Frey B, Heher S, Pacher R, Neunteufl T. Effects of endothelin a receptor blockade on endothelial function in patients with chronic heart failure. *Circulation*. 2001;103:981-986
191. Shantsila E, Lip GY. The endothelium and thrombotic risk in heart failure. *Thromb Haemost*. 2009;102:185-187
192. Ohlstein EH, Arleth AJ, Storer B, Romanic AM. Carvedilol inhibits endothelin-1 biosynthesis in cultured human coronary artery endothelial cells. *J Mol Cell Cardiol*. 1998;30:167-173
193. Strey CH, Young JM, Lainchbury JH, Frampton CM, Nicholls MG, Richards AM, Scott RS. Short-term statin treatment improves endothelial function and neurohormonal imbalance in normocholesterolaemic patients with non-ischaemic heart failure. *Heart*. 2006;92:1603-1609
194. Erbs S, Beck EB, Linke A, Adams V, Gielen S, Krankel N, Mobius-Winkler S, Hollriegel R, Thiele H, Hambrecht R, Schuler G. High-dose rosuvastatin in chronic heart failure promotes vasculogenesis, corrects endothelial function, and improves cardiac remodeling--results from a randomized, double-blind, and placebo-controlled study. *Int J Cardiol*. 2011;146:56-63
195. Garlichs CD, Zhang H, Mugge A, Daniel WG. Beta-blockers reduce the release and synthesis of endothelin-1 in human endothelial cells. *Eur J Clin Invest*. 1999;29:12-16
196. Mollnau H, Oelze M, August M, Wendt M, Daiber A, Schulz E, Baldus S, Kleschyov AL, Materne A, Wenzel P, Hink U, Nickenig G, Fleming I, Munzel T. Mechanisms of increased vascular superoxide production in an experimental model of idiopathic dilated cardiomyopathy. *Arterioscler Thromb Vasc Biol*. 2005;25:2554-2559
197. Boman K, Jansson JH, Nilsson T, Swedberg K, Cleland JG, Poole-Wilson P. Effects of carvedilol or metoprolol on pai-1, tpa-mass concentration or von willebrand factor in chronic heart failure--a comet substudy. *Thromb Res*. 2010;125:e46-50

198. Hornig B, Arakawa N, Haussmann D, Drexler H. Differential effects of quinaprilat and enalaprilat on endothelial function of conduit arteries in patients with chronic heart failure. *Circulation*. 1998;98:2842-2848
199. Hryniewicz K, Dimayuga C, Hudaihed A, Androne AS, Zheng H, Jankowski K, Katz SD. Inhibition of angiotensin-converting enzyme and phosphodiesterase type 5 improves endothelial function in heart failure. *Clin Sci (Lond)*. 2005;108:331-338
200. Drakos SG, Papamichael CM, Alexopoulos GP, Anastasiou-Nana MI, Stathopoulos JV, Nanas JN. Effects of high doses versus standard doses of enalapril on endothelial cell function in patients with chronic congestive heart failure secondary to idiopathic dilated or ischemic cardiomyopathy. *Am J Cardiol*. 2003;91:885-888
201. Tavli T, Gocer H. Effects of cilazapril on endothelial function and pulmonary hypertension in patients with congestive heart failure. *Jpn Heart J*. 2002;43:667-674
202. Gibbs CR, Blann AD, Watson RD, Lip GY. Abnormalities of hemorheological, endothelial, and platelet function in patients with chronic heart failure in sinus rhythm: Effects of angiotensin-converting enzyme inhibitor and beta-blocker therapy. *Circulation*. 2001;103:1746-1751
203. Poelzl G, Frick M, Lackner B, Huegel H, Alber HF, Mair J, Herold M, Schwarzacher SP, Pachinger O, Weidinger F. Short-term improvement in submaximal exercise capacity by optimized therapy with ace inhibitors and beta blockers in heart failure patients is associated with restoration of peripheral endothelial function. *Int J Cardiol*. 2006;108:48-54
204. Farquharson CA, Struthers AD. Spironolactone increases nitric oxide bioactivity, improves endothelial vasodilator dysfunction, and suppresses vascular angiotensin i/angiotensin ii conversion in patients with chronic heart failure. *Circulation*. 2000;101:594-597
205. Macdonald JE, Kennedy N, Struthers AD. Effects of spironolactone on endothelial function, vascular angiotensin converting enzyme activity, and other prognostic markers in patients with mild heart failure already taking optimal treatment. *Heart*. 2004;90:765-770
206. Abiose AK, Mansoor GA, Barry M, Soucier R, Nair CK, Hager D. Effect of spironolactone on endothelial function in patients with congestive heart failure on conventional medical therapy. *Am J Cardiol*. 2004;93:1564-1566
207. Farquharson CA, Struthers AD. Increasing plasma potassium with amiloride shortens the qt interval and reduces ventricular extrasystoles but does not change endothelial function or heart rate variability in chronic heart failure. *Heart*. 2002;88:475-480
208. Belardinelli R, Solenghi M, Volpe L, Purcaro A. Trimetazidine improves endothelial dysfunction in chronic heart failure: An antioxidant effect. *Eur Heart J*. 2007;28:1102-1108

209. Ito K, Akita H, Kanazawa K, Yamada S, Terashima M, Matsuda Y, Yokoyama M. Comparison of effects of ascorbic acid on endothelium-dependent vasodilation in patients with chronic congestive heart failure secondary to idiopathic dilated cardiomyopathy versus patients with effort angina pectoris secondary to coronary artery disease. *Am J Cardiol.* 1998;82:762-767
210. Hornig B, Arakawa N, Kohler C, Drexler H. Vitamin c improves endothelial function of conduit arteries in patients with chronic heart failure. *Circulation.* 1998;97:363-368
211. Ellis GR, Anderson RA, Chirkov YY, Morris-Thurgood J, Jackson SK, Lewis MJ, Horowitz JD, Frenneaux MP. Acute effects of vitamin c on platelet responsiveness to nitric oxide donors and endothelial function in patients with chronic heart failure. *J Cardiovasc Pharmacol.* 2001;37:564-570
212. Ellis GR, Anderson RA, Lang D, Blackman DJ, Morris RH, Morris-Thurgood J, McDowell IF, Jackson SK, Lewis MJ, Frenneaux MP. Neutrophil superoxide anion--generating capacity, endothelial function and oxidative stress in chronic heart failure: Effects of short- and long-term vitamin c therapy. *J Am Coll Cardiol.* 2000;36:1474-1482
213. Erbs S, Gielen S, Linke A, Mobius-Winkler S, Adams V, Baither Y, Schuler G, Hambrecht R. Improvement of peripheral endothelial dysfunction by acute vitamin c application: Different effects in patients with coronary artery disease, ischemic, and dilated cardiomyopathy. *Am Heart J.* 2003;146:280-285
214. George J, Carr E, Davies J, Belch JJ, Struthers A. High-dose allopurinol improves endothelial function by profoundly reducing vascular oxidative stress and not by lowering uric acid. *Circulation.* 2006;114:2508-2516
215. Doehner W, Schoene N, Rauchhaus M, Leyva-Leon F, Pavitt DV, Reaveley DA, Schuler G, Coats AJ, Anker SD, Hambrecht R. Effects of xanthine oxidase inhibition with allopurinol on endothelial function and peripheral blood flow in hyperuricemic patients with chronic heart failure: Results from 2 placebo-controlled studies. *Circulation.* 2002;105:2619-2624
216. Hambrecht R, Hilbrich L, Erbs S, Gielen S, Fiehn E, Schoene N, Schuler G. Correction of endothelial dysfunction in chronic heart failure: Additional effects of exercise training and oral l-arginine supplementation. *J Am Coll Cardiol.* 2000;35:706-713
217. Hirooka Y, Imaizumi T, Tagawa T, Shiramoto M, Endo T, Ando S, Takeshita A. Effects of l-arginine on impaired acetylcholine-induced and ischemic vasodilation of the forearm in patients with heart failure. *Circulation.* 1994;90:658-668
218. Chin-Dusting JP, Kaye DM, Lefkovits J, Wong J, Bergin P, Jennings GL. Dietary supplementation with l-arginine fails to restore endothelial function in forearm resistance arteries of patients with severe heart failure. *J Am Coll Cardiol.* 1996;27:1207-1213



219. Paul B, Whiting MJ, De Pasquale CG, Mangoni AA. Acute effects of 5-methyltetrahydrofolate on endothelial function and asymmetric dimethylarginine in patients with chronic heart failure. *Nutr Metab Cardiovasc Dis.* 2010;20:341-349
220. Napoli R, Guardasole V, Matarazzo M, Palmieri EA, Oliviero U, Fazio S, Sacca L. Growth hormone corrects vascular dysfunction in patients with chronic heart failure. *J Am Coll Cardiol.* 2002;39:90-95
221. Fichtlscherer S, Rossig L, Breuer S, Vasa M, Dimmeler S, Zeiher AM. Tumor necrosis factor antagonism with etanercept improves systemic endothelial vasoreactivity in patients with advanced heart failure. *Circulation.* 2001;104:3023-3025
222. Fuentes JC, Salmon AA, Silver MA. Acute and chronic oral magnesium supplementation: Effects on endothelial function, exercise capacity, and quality of life in patients with symptomatic heart failure. *Congest Heart Fail.* 2006;12:9-13
223. Patel MB, Kaplan IV, Patni RN, Levy D, Strom JA, Shirani J, LeJemtel TH. Sustained improvement in flow-mediated vasodilation after short-term administration of dobutamine in patients with severe congestive heart failure. *Circulation.* 1999;99:60-64
224. Freimark D, Feinberg MS, Matezky S, Hochberg N, Shechter M. Impact of short-term intermittent intravenous dobutamine therapy on endothelial function in patients with severe chronic heart failure. *Am Heart J.* 2004;148:878-882
225. Schwarz M, Katz SD, Demopoulos L, Hirsch H, Yuen JL, Jondeau G, LeJemtel TH. Enhancement of endothelium-dependent vasodilation by low-dose nitroglycerin in patients with congestive heart failure. *Circulation.* 1994;89:1609-1614
226. Guazzi M, Casali M, Berti F, Rossoni G, Colonna VD, Guazzi MD. Endothelium-mediated modulation of ergoreflex and improvement in exercise ventilation by acute sildenafil in heart failure patients. *Clin Pharmacol Ther.* 2008;83:336-341
227. Ferdinand KC. African american heart failure trial: Role of endothelial dysfunction and heart failure in african americans. *Am J Cardiol.* 2007;99:3D-6D
228. Yamamoto E, Kataoka K, Shintaku H, Yamashita T, Tokutomi Y, Dong YF, Matsuba S, Ichijo H, Ogawa H, Kim-Mitsuyama S. Novel mechanism and role of angiotensin ii induced vascular endothelial injury in hypertensive diastolic heart failure. *Arterioscler Thromb Vasc Biol.* 2007;27:2569-2575
229. Thai H, Wollmuth J, Goldman S, Gaballa M. Angiotensin subtype 1 receptor (at1) blockade improves vasorelaxation in heart failure by up-regulation of endothelial nitric-oxide synthase via activation of the at2 receptor. *J Pharmacol Exp Ther.* 2003;307:1171-1178
230. Varin R, Mulder P, Tamion F, Richard V, Henry JP, Lallemand F, Lerebours G, Thuillez C. Improvement of endothelial function by chronic angiotensin-converting enzyme inhibition in

- heart failure : Role of nitric oxide, prostanoids, oxidant stress, and bradykinin. *Circulation*. 2000;102:351-356
231. Trochu JN, Mital S, Zhang X, Xu X, Ochoa M, Liao JK, Recchia FA, Hintze TH. Preservation of NO production by statins in the treatment of heart failure. *Cardiovasc Res*. 2003;60:250-258
232. Landmesser U, Engberding N, Bahlmann FH, Schaefer A, Wiencke A, Heineke A, Spiekermann S, Hilfiker-Kleiner D, Templin C, Kotlarz D, Mueller M, Fuchs M, Hornig B, Haller H, Drexler H. Statin-induced improvement of endothelial progenitor cell mobilization, myocardial neovascularization, left ventricular function, and survival after experimental myocardial infarction requires endothelial nitric oxide synthase. *Circulation*. 2004;110:1933-1939
233. Greer JJ, Kakkar AK, Elrod JW, Watson LJ, Jones SP, Lefer DJ. Low-dose simvastatin improves survival and ventricular function via eNOS in congestive heart failure. *Am J Physiol Heart Circ Physiol*. 2006;291:H2743-2751
234. Kjekshus J, Apetrei E, Barrios V, Bohm M, Cleland JG, Cornel JH, Dunselman P, Fonseca C, Goudev A, Grande P, Gullestad L, Hjalmarson A, Hradec J, Janosi A, Kamensky G, Komajda M, Korewicki J, Kuusi T, Mach F, Mareev V, McMurray JJ, Ranjith N, Schaufelberger M, Vanhaecke J, van Veldhuisen DJ, Waagstein F, Wedel H, Wikstrand J. Rosuvastatin in older patients with systolic heart failure. *N Engl J Med*. 2007;357:2248-2261
235. Bleske BE, Nicklas JM, Bard RL, Brook RD, Gurbel PA, Bliden KP, Rajagopalan S, Pitt B. Neutral effect on markers of heart failure, inflammation, endothelial activation and function, and vagal tone after high-dose HMG-CoA reductase inhibition in non-diabetic patients with non-ischemic cardiomyopathy and average low-density lipoprotein level. *J Am Coll Cardiol*. 2006;47:338-341
236. Tousoulis D, Antoniadou C, Bosniakou E, Kotsopoulou M, Pitsavos C, Vlachopoulos C, Panagiotakos D, Stefanadis C. Effects of atorvastatin on reactive hyperemia and inflammatory process in patients with congestive heart failure. *Atherosclerosis*. 2005;178:359-363
237. Gounari P, Tousoulis D, Antoniadou C, Kampoli AM, Stougiannos P, Papageorgiou N, Rouliou G, Stefanadi E, Siasos G, Tsioufis C, Stefanadis C. Rosuvastatin but not ezetimibe improves endothelial function in patients with heart failure, by mechanisms independent of lipid lowering. *Int J Cardiol*. 2010;142:87-91
238. Young JM, Strey CH, George PM, Florkowski CM, Sies CW, Frampton CM, Scott RS. Effect of atorvastatin on plasma levels of asymmetric dimethylarginine in patients with non-ischaemic heart failure. *Eur J Heart Fail*. 2008;10:463-466

239. Landmesser U, Bahlmann F, Mueller M, Spiekermann S, Kirchhoff N, Schulz S, Manes C, Fischer D, de Groot K, Fliser D, Fauler G, Marz W, Drexler H. Simvastatin versus ezetimibe: Pleiotropic and lipid-lowering effects on endothelial function in humans. *Circulation*. 2005;111:2356-2363
240. Tousoulis D, Antoniades C, Vassiliadou C, Toutouza M, Pitsavos C, Tentolouris C, Trikas A, Stefanadis C. Effects of combined administration of low dose atorvastatin and vitamin e on inflammatory markers and endothelial function in patients with heart failure. *Eur J Heart Fail*. 2005;7:1126-1132
241. Feng Q, Fortin AJ, Lu X, Arnold JM. Effects of l-arginine on endothelial and cardiac function in rats with heart failure. *Eur J Pharmacol*. 1999;376:37-44
242. Castro PF, Miranda R, Verdejo HE, Greig D, Gabrielli LA, Alcaino H, Chiong M, Bustos C, Garcia L, Mellado R, Vukasovic JL, Godoy I, Lavandero S. Pleiotropic effects of atorvastatin in heart failure: Role in oxidative stress, inflammation, endothelial function, and exercise capacity. *J Heart Lung Transplant*. 2008;27:435-441
243. Rector TS, Bank AJ, Mullen KA, Tschumperlin LK, Sih R, Pillai K, Kubo SH. Randomized, double-blind, placebo-controlled study of supplemental oral l-arginine in patients with heart failure. *Circulation*. 1996;93:2135-2141
244. Rossig L, Hoffmann J, Hugel B, Mallat Z, Haase A, Freyssinet JM, Tedgui A, Aicher A, Zeiher AM, Dimmeler S. Vitamin c inhibits endothelial cell apoptosis in congestive heart failure. *Circulation*. 2001;104:2182-2187
245. Podmore ID, Griffiths HR, Herbert KE, Mistry N, Mistry P, Lunec J. Vitamin c exhibits pro-oxidant properties. *Nature*. 1998;392:559
246. Anlasik T, Sies H, Griffiths HR, Mecocci P, Stahl W, Polidori MC. Dietary habits are major determinants of the plasma antioxidant status in healthy elderly subjects. *Br J Nutr*. 2005;94:639-642
247. Tai MK, Meininger JC, Frazier LQ. A systematic review of exercise interventions in patients with heart failure. *Biol Res Nurs*. 2008;10:156-182
248. Tabet JY, Meurin P, Driss AB, Weber H, Renaud N, Grosdemouge A, Beauvais F, Cohen-Solal A. Benefits of exercise training in chronic heart failure. *Arch Cardiovasc Dis*. 2009;102:721-730
249. Miche E, Herrmann G, Nowak M, Wirtz U, Tietz M, Hurst M, Zoller B, Radzewitz A. Effect of an exercise training program on endothelial dysfunction in diabetic and non-diabetic patients with severe chronic heart failure. *Clin Res Cardiol*. 2006;95 Suppl 1:i117-124

250. Testa M, Ennezat PV, Vikstrom KL, Demopoulos L, Gentilucci M, Loperfido F, Fanelli R, Kitsis RN, Leinwand LA, LeJemtel TH. Modulation of vascular endothelial gene expression by physical training in patients with chronic heart failure. *Ital Heart J.* 2000;1:426-430
251. Parnell MM, Holst DP, Kaye DM. Augmentation of endothelial function following exercise training is associated with increased l-arginine transport in human heart failure. *Clin Sci (Lond).* 2005;109:523-530
252. Hambrecht R, Fiehn E, Weigl C, Gielen S, Hamann C, Kaiser R, Yu J, Adams V, Niebauer J, Schuler G. Regular physical exercise corrects endothelial dysfunction and improves exercise capacity in patients with chronic heart failure. *Circulation.* 1998;98:2709-2715
253. Varin R, Mulder P, Richard V, Tamion F, Devaux C, Henry JP, Lallemand F, Lerebours G, Thuillez C. Exercise improves flow-mediated vasodilatation of skeletal muscle arteries in rats with chronic heart failure. Role of nitric oxide, prostanoids, and oxidant stress. *Circulation.* 1999;99:2951-2957
254. Wang J, Yi GH, Knecht M, Cai BL, Poposkis S, Packer M, Burkhoff D. Physical training alters the pathogenesis of pacing-induced heart failure through endothelium-mediated mechanisms in awake dogs. *Circulation.* 1997;96:2683-2692
255. Gustafsson T, Bodin K, Sylven C, Gordon A, Tyni-Lenne R, Jansson E. Increased expression of vegf following exercise training in patients with heart failure. *Eur J Clin Invest.* 2001;31:362-366
256. Sarto P, Balducci E, Balconi G, Fiordaliso F, Merlo L, Tuzzato G, Pappagallo GL, Frigato N, Zanocco A, Forestieri C, Azzarello G, Mazzucco A, Valenti MT, Alborino F, Noventa D, Vinante O, Pascotto P, Sartore S, Dejana E, Latini R. Effects of exercise training on endothelial progenitor cells in patients with chronic heart failure. *J Card Fail.* 2007;13:701-708
257. Niebauer J, Clark AL, Webb-Peploe KM, Coats AJ. Exercise training in chronic heart failure: Effects on pro-inflammatory markers. *Eur J Heart Fail.* 2005;7:189-193
258. Adamopoulos S, Parissis J, Kroupis C, Georgiadis M, Karatzas D, Karavolias G, Koniavitou K, Coats AJ, Kremastinos DT. Physical training reduces peripheral markers of inflammation in patients with chronic heart failure. *Eur Heart J.* 2001;22:791-797
259. Goto C, Higashi Y, Kimura M, Noma K, Hara K, Nakagawa K, Kawamura M, Chayama K, Yoshizumi M, Nara I. Effect of different intensities of exercise on endothelium-dependent vasodilation in humans: Role of endothelium-dependent nitric oxide and oxidative stress. *Circulation.* 2003;108:530-535
260. McNamara DM, Tam SW, Sabolinski ML, Tobelmann P, Janosko K, Venkitachalam L, Ofili E, Yancy C, Feldman AM, Ghali JK, Taylor AL, Cohn JN, Worcel M. Endothelial nitric

oxide synthase (nos3) polymorphisms in african americans with heart failure: Results from the a-heft trial. *J Card Fail.* 2009;15:191-198

261. Velloso MW, Pereira SB, Gouveia L, Chermont S, Tardin OM, Goncalves R, Camacho V, Contarato Lde F, Quintao M, Oliveira e Alves T, Pessoa LP, Brito Junior A, Ribeiro GS, Mesquita ET. Endothelial nitric oxide synthase glu298asp gene polymorphism in a multi-ethnic population with heart failure and controls. *Nitric Oxide.* 2010;22:220-225

262. Binkley PF, Nunziata E, Liu-Stratton Y, Cooke G. A polymorphism of the endothelial nitric oxide synthase promoter is associated with an increase in autonomic imbalance in patients with congestive heart failure. *Am Heart J.* 2005;149:342-348

263. McNamara DM, Holubkov R, Postava L, Ramani R, Janosko K, Mathier M, MacGowan GA, Murali S, Feldman AM, London B. Effect of the asp298 variant of endothelial nitric oxide synthase on survival for patients with congestive heart failure. *Circulation.* 2003;107:1598-1602

264. Bank AJ, Shammas RA, Mullen K, Chuang PP. Effects of short-term forearm exercise training on resistance vessel endothelial function in normal subjects and patients with heart failure. *J Card Fail.* 1998;4:193-201

265. Kobayashi N, Tsuruya Y, Iwasawa T, Ikeda N, Hashimoto S, Yasu T, Ueba H, Kubo N, Fujii M, Kawakami M, Saito M. Exercise training in patients with chronic heart failure improves endothelial function predominantly in the trained extremities. *Circ J.* 2003;67:505-510

266. Linke A, Schoene N, Gielen S, Hofer J, Erbs S, Schuler G, Hambrecht R. Endothelial dysfunction in patients with chronic heart failure: Systemic effects of lower-limb exercise training. *J Am Coll Cardiol.* 2001;37:392-397

267. Hornig B, Maier V, Drexler H. Physical training improves endothelial function in patients with chronic heart failure. *Circulation.* 1996;93:210-214

268. White RH. The epidemiology of venous thromboembolism. *Circulation.* 2003;107:14-8

269. Dries DL, Rosenberg YD, Waclawiw MA, Domanski MJ. Ejection fraction and risk of thromboembolic events in patients with systolic dysfunction and sinus rhythm: Evidence for gender differences in the studies of left ventricular dysfunction trials. *J Am Coll Cardiol.* 1997;29:1074-1080

270. Pullicino PM, Halperin JL, Thompson JL. Stroke in patients with heart failure and reduced left ventricular ejection fraction. *Neurology.* 2000;54:288-294

271. Siachos T, Vanbakel A, Feldman DS, Uber W, Simpson KN, Pereira NL. Silent strokes in patients with heart failure. *J Card Fail.* 2005;11:485-489

272. Dotsenko O, Kakkar VV. Antithrombotic therapy in patients with chronic heart failure: Rationale, clinical evidence and practical implications. *J Thromb Haemost.* 2007;5:224-231

273. Yusuf S, Pepine CJ, Garces C, Pouleur H, Salem D, Kostis J, Benedict C, Rousseau M, Bourassa M, Pitt B. Effect of enalapril on myocardial infarction and unstable angina in patients with low ejection fractions. *Lancet*. 1992;340:1173-1178
274. Medina P, Navarro S, Estelles A, Espana F. Polymorphisms in the endothelial protein c receptor gene and thrombophilia. *Thromb Haemost*. 2007;98:564-569
275. Toltl LJ, Swystun LL, Pepler L, Liaw PC. Protective effects of activated protein c in sepsis. *Thromb Haemost*. 2008;100:582-592
276. Esmon CT. Thrombomodulin as a model of molecular mechanisms that modulate protease specificity and function at the vessel surface. *FASEB J*. 1995;9:946-955
277. D'Hondt C. Role for heparan sulfate proteoglycan in thrombin-induced calcium transients and nitric oxide production in aortic endothelial cells. *Thromb Haemost*. 2008;100:374-376
278. Kimura C, Oike M. Heparan sulfate proteoglycan is essential to thrombin-induced calcium transients and nitric oxide production in aortic endothelial cells. *Thromb Haemost*. 2008;100:483-488
279. Michiels C. Endothelial cell functions. *J Cell Physiol*. 2003;196:430-443
280. Kato H. Regulation of functions of vascular wall cells by tissue factor pathway inhibitor: Basic and clinical aspects. *Arterioscler Thromb Vasc Biol*. 2002;22:539-548
281. Padro T, Emeis JJ, Steins M, Schmid KW, Kienast J. Quantification of plasminogen activators and their inhibitors in the aortic vessel wall in relation to the presence and severity of atherosclerotic disease. *Arterioscler Thromb Vasc Biol*. 1995;15:893-902
282. Diebold I, Kraicun D, Bonello S, Grolach A. The 'pai-1 paradox' in vascular remodeling. *Thromb Haemost*. 2008;100:984-991
283. Pearson JD, Carleton JS, Gordon JL. Metabolism of adenine nucleotides by ectoenzymes of vascular endothelial and smooth-muscle cells in culture. *Biochem J*. 1980;190:421-429
284. Xin HY, Jiang DJ, Jia SJ, Song K, Wang GP, Li YJ, Chen FP. Regulation by ddah/adma pathway of lipopolysaccharide-induced tissue factor expression in endothelial cells. *Thromb Haemost*. 2007;97:830-838
285. Petersen LC, Albrektsen T, Hjorto GM, Kjalke M, Bjorn SE, Sorensen BB. Factor viia/tissue factor-dependent gene regulation and pro-coagulant activity: Effect of factor viia concentration. *Thromb Haemost*. 2007;98:909-911
286. Chin BS, Conway DS, Chung NA, Blann AD, Gibbs CR, Lip GY. Interleukin-6, tissue factor and von willebrand factor in acute decompensated heart failure: Relationship to treatment and prognosis. *Blood Coagul Fibrinolysis*. 2003;14:515-521

287. Chin BS, Blann AD, Gibbs CR, Chung NA, Conway DG, Lip GY. Prognostic value of interleukin-6, plasma viscosity, fibrinogen, von willebrand factor, tissue factor and vascular endothelial growth factor levels in congestive heart failure. *Eur J Clin Invest.* 2003;33:941-948
288. Bae JS, Rezaie AR. Protease activated receptor 1 (par-1) activation by thrombin is protective in human pulmonary artery endothelial cells if endothelial protein c receptor is occupied by its natural ligand. *Thromb Haemost.* 2008;100:101-109
289. Jafri SM, Ozawa T, Mammen E, Levine TB, Johnson C, Goldstein S. Platelet function, thrombin and fibrinolytic activity in patients with heart failure. *Eur Heart J.* 1993;14:205-212
290. Marcucci R, Gori AM, Giannotti F, Baldi M, Verdiani V, Del Pace S, Nozzoli C, Abbate R. Markers of hypercoagulability and inflammation predict mortality in patients with heart failure. *J Thromb Haemost.* 2006;4:1017-1022
291. Jafri SM, Mammen EF, Masura J, Goldstein S. Effects of warfarin on markers of hypercoagulability in patients with heart failure. *Am Heart J.* 1997;134:27-36
292. Massie BM, Collins JF, Ammon SE, Armstrong PW, Cleland JG, Ezekowitz M, Jafri SM, Krol WF, O'Connor CM, Schulman KA, Teo K, Warren SR. Randomized trial of warfarin, aspirin, and clopidogrel in patients with chronic heart failure: The warfarin and antiplatelet therapy in chronic heart failure (watch) trial. *Circulation.* 2009;119:1616-1624
293. Zimmerman GA, McIntyre TM, Mehra M, Prescott SM. Endothelial cell-associated platelet-activating factor: A novel mechanism for signaling intercellular adhesion. *J Cell Biol.* 1990;110:529-540
294. Chung I, Choudhury A, Lip GY. Platelet activation in acute, decompensated congestive heart failure. *Thromb Res.* 2007;120:709-713
295. Schafer A, Schulz C, Eigenthaler M, Fraccarollo D, Kobsar A, Gawaz M, Ertl G, Walter U, Bauersachs J. Novel role of the membrane-bound chemokine fractalkine in platelet activation and adhesion. *Blood.* 2004;103:407-412
296. Vercauteren M, Remy E, Devaux C, Dautreaux B, Henry JP, Bauer F, Mulder P, Hooft van Huijsduijnen R, Bombrun A, Thuillez C, Richard V. Improvement of peripheral endothelial dysfunction by protein tyrosine phosphatase inhibitors in heart failure. *Circulation.* 2006;114:2498-2507
297. Muldowney JA, 3rd, Painter CA, Sanders-Bush E, Brown NJ, Vaughan DE. Acute tissue-type plasminogen activator release in human microvascular endothelial cells: The roles of galphaq, plc-beta, ip3 and 5,6-epoxyeicosatrienoic acid. *Thromb Haemost.* 2007;97:263-271
298. Bajaj MS, Kuppaswamy MN, Manepalli AN, Bajaj SP. Transcriptional expression of tissue factor pathway inhibitor, thrombomodulin and von willebrand factor in normal human tissues. *Thromb Haemost.* 1999;82:1047-1052

299. Jacquemin M, Neyrinck A, Hermanns MI, Lavend'homme R, Rega F, Saint-Remy JM, Peerlinck K, Van Raemdonck D, Kirkpatrick CJ. Fviii production by human lung microvascular endothelial cells. *Blood*. 2006;108:515-517
300. Han KH, Lim JM, Kim WY, Kim H, Madsen KM, Kim J. Expression of endothelial nitric oxide synthase in developing rat kidney. *Am J Physiol Renal Physiol*. 2005;288:F694-702
301. Balarajan R. Ethnicity and variations in the nation's health. *Health Trends*. 1995;27:114-119
302. Periaswamy R, Gurusamy U, Shewade DG, Cherian A, Swaminathan RP, Dutta TK, Jayaraman B, Chandrasekaran A. Gender specific association of endothelial nitric oxide synthase gene (glu298asp) polymorphism with essential hypertension in a south indian population. *Clin Chim Acta*. 2008;395:134-136
303. Koshy L, Easwer HV, Neetha NV, Natarajan C, Bhattacharya RN, Banerjee M. Role of endothelial nitric oxide synthase gene polymorphisms in predicting aneurysmal subarachnoid hemorrhage in south indian patients. *Dis Markers*. 2008;24:333-339
304. Misra A. C-reactive protein in young individuals: Problems and implications for asian indians. *Nutrition*. 2004;20:478-481
305. Mann DL. Inflammatory mediators and the failing heart: Past, present, and the foreseeable future. *Circ Res*. 2002;91:988-998
306. Torre-Amione G, Kapadia S, Lee J, Durand JB, Bies RD, Young JB, Mann DL. Tumor necrosis factor-alpha and tumor necrosis factor receptors in the failing human heart. *Circulation*. 1996;93:704-711
307. Apostolakis S, Vogiatzi K, Krambovitis E, Spandidos DA. Il-1 cytokines in cardiovascular disease: Diagnostic, prognostic and therapeutic implications. *Cardiovasc Hematol Agents Med Chem*. 2008;6:150-158
308. Armstrong EJ, Morrow DA, Sabatine MS. Inflammatory biomarkers in acute coronary syndromes: Part i: Introduction and cytokines. *Circulation*. 2006;113:e72-75
309. Sun M, Dawood F, Wen WH, Chen M, Dixon I, Kirshenbaum LA, Liu PP. Excessive tumor necrosis factor activation after infarction contributes to susceptibility of myocardial rupture and left ventricular dysfunction. *Circulation*. 2004;110:3221-3228
310. Bozkurt B, Kribbs SB, Clubb FJ, Jr., Michael LH, Didenko VV, Hornsby PJ, Seta Y, Oral H, Spinale FG, Mann DL. Pathophysiologically relevant concentrations of tumor necrosis factor-alpha promote progressive left ventricular dysfunction and remodeling in rats. *Circulation*. 1998;97:1382-1391
311. Dutka DP, Camici PG. Hibernation and congestive heart failure. *Heart Fail Rev*. 2003;8:167-173



312. Nakano M, Knowlton AA, Dibbs Z, Mann DL. Tumor necrosis factor-alpha confers resistance to hypoxic injury in the adult mammalian cardiac myocyte. *Circulation*. 1998;97:1392-1400
313. Lecour S, Smith RM, Woodward B, Opie LH, Rochette L, Sack MN. Identification of a novel role for sphingolipid signaling in tnf alpha and ischemic preconditioning mediated cardioprotection. *J Mol Cell Cardiol*. 2002;34:509-518
314. Maack C, Kartes T, Kilter H, Schafers HJ, Nickenig G, Bohm M, Laufs U. Oxygen free radical release in human failing myocardium is associated with increased activity of rac1-gtpase and represents a target for statin treatment. *Circulation*. 2003;108:1567-1574
315. Bauersachs J, Bouloumie A, Fraccarollo D, Hu K, Busse R, Ertl G. Endothelial dysfunction in chronic myocardial infarction despite increased vascular endothelial nitric oxide synthase and soluble guanylate cyclase expression: Role of enhanced vascular superoxide production. *Circulation*. 1999;100:292-298
316. Blum A. Heart failure--new insights. *Isr Med Assoc J*. 2009;11:105-111
317. Wolf SF, Sieburth D, Sypek J. Interleukin 12: A key modulator of immune function. *Stem Cells*. 1994;12:154-168
318. Swirski FK, Weissleder R, Pittet MJ. Heterogeneous in vivo behavior of monocyte subsets in atherosclerosis. *Arterioscler Thromb Vasc Biol*. 2009;29:1424-1432
319. Roberts CS, Maclean D, Maroko P, Kloner RA. Relation of early mononuclear and polymorphonuclear cell infiltration to late scar thickness after experimentally induced myocardial infarction in the rat. *Basic Res Cardiol*. 1985;80:202-209
320. Maekawa Y, Anzai T, Yoshikawa T, Asakura Y, Takahashi T, Ishikawa S, Mitamura H, Ogawa S. Prognostic significance of peripheral monocytes after reperfused acute myocardial infarction: A possible role for left ventricular remodeling. *J Am Coll Cardiol*. 2002;39:241-246
321. Agema WR, Wouter Jukema J, de Maat MP, Zwinderman AH, Kastelein JJ, Rabelink TJ, van der Wall EE. Pharmacogenetics of the cd14 endotoxin receptor polymorphism and progression of coronary atherosclerosis. *Thromb Haemost*. 2004;91:986-990
322. Hochart H, Jenkins PV, Preston RJ, Smith OP, White B, O'Donnell J. Concentration-dependent roles for heparin in modifying lipopolysaccharide-induced activation of mononuclear cells in whole blood. *Thromb Haemost*. 2008;99:570-575
323. Woollard KJ, Fisch C, Newby R, Griffiths HR. C-reactive protein mediates cd11b expression in monocytes through the non-receptor tyrosine kinase, syk, and calcium mobilization but not through cytosolic peroxides. *Inflamm Res*. 2005;54:485-492

324. Woollard KJ, Loryman CJ, Meredith E, Bevan R, Shaw JA, Lunec J, Griffiths HR. Effects of oral vitamin c on monocyte: Endothelial cell adhesion in healthy subjects. *Biochem Biophys Res Commun.* 2002;294:1161-1168
325. Griffiths HR, Willetts RS, Grant MM, Mistry N, Lunec J, Bevan RJ. In vivo vitamin c supplementation increases phosphoinositol transfer protein expression in peripheral blood mononuclear cells from healthy individuals. *Br J Nutr.* 2009;101:1432-1439
326. Woollard KJ, Phillips DC, Griffiths HR. Direct modulatory effect of c-reactive protein on primary human monocyte adhesion to human endothelial cells. *Clin Exp Immunol.* 2002;130:256-262
327. Arroyo-Espliguero R, Avanzas P, Jeffery S, Kaski JC. Cd14 and toll-like receptor 4: A link between infection and acute coronary events? *Heart.* 2004;90:983-988
328. Satoh M, Shimoda Y, Akatsu T, Ishikawa Y, Minami Y, Nakamura M. Elevated circulating levels of heat shock protein 70 are related to systemic inflammatory reaction through monocyte toll signal in patients with heart failure after acute myocardial infarction. *Eur J Heart Fail.* 2006;8:810-815
329. Choi YH, Lee WH, Lee Y, Kim JK, Lee SY, Park JE. Correlation between monocyte and t-lymphocyte activation markers in patients with acute coronary syndrome. *Jpn Heart J.* 2000;41:605-615
330. Methe H, Kim JO, Kofler S, Weis M, Nabauer M, Koglin J. Expansion of circulating toll-like receptor 4-positive monocytes in patients with acute coronary syndrome. *Circulation.* 2005;111:2654-2661
331. Gokce M, Erdol C, Orem C, Tekelioglu Y, Durmus I, Kasap H. Inflammation and immune system response against unstable angina and its relationship with coronary angiographic findings. *Jpn Heart J.* 2002;43:593-605
332. Lee WH, Lee Y, Jeong JO, Lee SY, Choi YH, Park JE. Activation of cd14 on circulating monocytes in patients with acute coronary syndrome. *Int J Cardiol.* 2001;80:135-142
333. Dunston CR, Griffiths HR. The effect of ageing on macrophage toll-like receptor-mediated responses in the fight against pathogens. *Clin Exp Immunol.* 2010;161:407-416
334. Arroyo-Espliguero R, El-Sharnouby K, Vazquez-Rey E, Kalidas K, Jeffery S, Kaski JC. Cd14 c(-260)t promoter polymorphism and prevalence of acute coronary syndromes. *Int J Cardiol.* 2005;98:307-312
335. Shimada K, Watanabe Y, Mokuno H, Iwama Y, Daida H, Yamaguchi H. Common polymorphism in the promoter of the cd14 monocyte receptor gene is associated with acute myocardial infarction in japanese men. *Am J Cardiol.* 2000;86:682-684, A688

336. Ishikawa Y, Satoh M, Itoh T, Minami Y, Takahashi Y, Akamura M. Local expression of toll-like receptor 4 at the site of ruptured plaques in patients with acute myocardial infarction. *Clin Sci (Lond)*. 2008;115:133-140
337. Satoh M, Shimoda Y, Maesawa C, Akatsu T, Ishikawa Y, Minami Y, Hiramori K, Nakamura M. Activated toll-like receptor 4 in monocytes is associated with heart failure after acute myocardial infarction. *Int J Cardiol*. 2006;109:226-234
338. Hubacek JA, Rothe G, Pit'ha J, Skodova Z, Stanek V, Poledne R, Schmitz G. C(-260)-->t polymorphism in the promoter of the cd14 monocyte receptor gene as a risk factor for myocardial infarction. *Circulation*. 1999;99:3218-3220
339. Heesen M, Blomeke B, Schluter B, Heussen N, Rossaint R, Kunz D. Lack of association between the -260 c-->t promoter polymorphism of the endotoxin receptor cd14 gene and the cd14 density of unstimulated human monocytes and soluble cd14 plasma levels. *Intensive Care Med*. 2001;27:1770-1775
340. Longobardo MT, Cefalu AB, Pezzino F, Noto D, Emmanuele G, Barbagallo CM, Fiore B, Monastero R, Castello A, Molini V, Notarbartolo A, Travali S, Averna MR. The c(-260)>t gene polymorphism in the promoter of the cd14 monocyte receptor gene is not associated with acute myocardial infarction. *Clin Exp Med*. 2003;3:161-165
341. Unkelbach K, Gardemann A, Kostrzewa M, Philipp M, Tillmanns H, Haberbosch W. A new promoter polymorphism in the gene of lipopolysaccharide receptor cd14 is associated with expired myocardial infarction in patients with low atherosclerotic risk profile. *Arterioscler Thromb Vasc Biol*. 1999;19:932-938
342. Koch W, Kastrati A, Mehilli J, von Beckerath N, Schomig A. Cd14 gene -159c/t polymorphism is not associated with coronary artery disease and myocardial infarction. *Am Heart J*. 2002;143:971-976
343. Rechcinski T, Grebowska A, Kurpesa M, Peruga Z, Dziuba M, Krzeminska-Pakula M, Rudnicka W, Chmiela M. Cd14 gene polymorphism 159c/t in a group of patients with coronary artery disease from a population with high morbidity of cardiovascular diseases. *Kardiol Pol*. 2007;65:237-244; discussion 245
344. Carter AM, Grant PJ. Vascular homeostasis, adhesion molecules, and macrovascular disease in non-insulin-dependent diabetes mellitus. *Diabet Med*. 1997;14:423-432
345. Elstad MR, La Pine TR, Cowley FS, McEver RP, McIntyre TM, Prescott SM, Zimmerman GA. P-selectin regulates platelet-activating factor synthesis and phagocytosis by monocytes. *J Immunol*. 1995;155:2109-2122
346. Weyrich AS, McIntyre TM, McEver RP, Prescott SM, Zimmerman GA. Monocyte tethering by p-selectin regulates monocyte chemotactic protein-1 and tumor necrosis factor-

- alpha secretion. Signal integration and nf-kappa b translocation. *J Clin Invest.* 1995;95:2297-2303
347. van der Wal AC, Das PK, Tigges AJ, Becker AE. Adhesion molecules on the endothelium and mononuclear cells in human atherosclerotic lesions. *Am J Pathol.* 1992;141:1427-1433
348. Ma YQ, Plow EF, Geng JG. P-selectin binding to p-selectin glycoprotein ligand-1 induces an intermediate state of alphabeta2 activation and acts cooperatively with extracellular stimuli to support maximal adhesion of human neutrophils. *Blood.* 2004;104:2549-2556
349. Ramos CL, Huo Y, Jung U, Ghosh S, Manka DR, Sarembock IJ, Ley K. Direct demonstration of p-selectin- and vcam-1-dependent mononuclear cell rolling in early atherosclerotic lesions of apolipoprotein e-deficient mice. *Circ Res.* 1999;84:1237-1244
350. Huo Y, Ley K. Adhesion molecules and atherogenesis. *Acta Physiol Scand.* 2001;173:35-43
351. Alon R, Kassner PD, Carr MW, Finger EB, Hemler ME, Springer TA. The integrin vla-4 supports tethering and rolling in flow on vcam-1. *J Cell Biol.* 1995;128:1243-1253
352. Luscinskas FW, Kansas GS, Ding H, Pizcueta P, Schleiffenbaum BE, Tedder TF, Gimbrone MA, Jr. Monocyte rolling, arrest and spreading on il-4-activated vascular endothelium under flow is mediated via sequential action of l-selectin, beta 1-integrins, and beta 2-integrins. *J Cell Biol.* 1994;125:1417-1427
353. Gerszten RE, Lim YC, Ding HT, Snapp K, Kansas G, Dichek DA, Cabanas C, Sanchez-Madrid F, Gimbrone MA, Jr., Rosenzweig A, Luscinskas FW. Adhesion of monocytes to vascular cell adhesion molecule-1-transduced human endothelial cells: Implications for atherogenesis. *Circ Res.* 1998;82:871-878
354. Hillis GS, Dalsey WC, Terregino CA, Daskal I, Nangione A. Altered cd18 leucocyte integrin expression and adhesive function in patients with an acute coronary syndrome. *Heart.* 2001;85:702-704
355. Sardella G, Accapezzato D, Di Roma A, Iacoboni C, Francavilla V, Benedetti G, Musto C, Fedele F, Bruno G, Paroli M. Integrin beta2-chain (cd18) over-expression on cd4+ t cells and monocytes after ischemia/reperfusion in patients undergoing primary percutaneous revascularization. *Int J Immunopathol Pharmacol.* 2004;17:165-170
356. Meisel SR, Shapiro H, Radnay J, Neuman Y, Khaskia AR, Gruener N, Pauzner H, David D. Increased expression of neutrophil and monocyte adhesion molecules lfa-1 and mac-1 and their ligand icam-1 and vla-4 throughout the acute phase of myocardial infarction: Possible

- implications for leukocyte aggregation and microvascular plugging. *J Am Coll Cardiol.* 1998;31:120-125
357. Kawamura A, Miura S, Murayama T, Iwata A, Nishikawa H, Matsuo Y, Tsuchiya Y, Matsuo K, Tsuji E, Saku K. Changes in the expression of leukocyte adhesion molecules throughout the acute phase of myocardial infarction. *J Atheroscler Thromb.* 2004;11:29-34
358. Zhao SP, Li YF. Downregulation of ppargamma expression in peripheral blood monocytes correlated with adhesion molecules in acute coronary syndrome. *Clin Chim Acta.* 2003;336:19-25
359. Trial J, Baughn RE, Wygant JN, McIntyre BW, Birdsall HH, Youker KA, Evans A, Entman ML, Rossen RD. Fibronectin fragments modulate monocyte vla-5 expression and monocyte migration. *J Clin Invest.* 1999;104:419-430
360. Kurosaki K, Ikeda U, Murakami Y, Takahashi M, Shimada K. Inhibition of the renin-angiotensin system: No effect on circulating macrophage colony-stimulating factor levels in acute myocardial infarction. *J Cardiovasc Pharmacol.* 2003;42:37-41
361. Kakio T, Matsumori A, Ono K, Ito H, Matsushima K, Sasayama S. Roles and relationship of macrophages and monocyte chemotactic and activating factor/monocyte chemoattractant protein-1 in the ischemic and reperfused rat heart. *Lab Invest.* 2000;80:1127-1136
362. Ono K, Matsumori A, Furukawa Y, Igata H, Shioi T, Matsushima K, Sasayama S. Prevention of myocardial reperfusion injury in rats by an antibody against monocyte chemotactic and activating factor/monocyte chemoattractant protein-1. *Lab Invest.* 1999;79:195-203
363. Hohensinner PJ, Kaun C, Rychli K, Ben-Tal Cohen E, Kastl SP, Demyanets S, Pfaffenberger S, Speidl WS, Rega G, Ullrich R, Maurer G, Huber K, Wojta J. Monocyte chemoattractant protein (mcp-1) is expressed in human cardiac cells and is differentially regulated by inflammatory mediators and hypoxia. *FEBS Lett.* 2006;580:3532-3538
364. Matsumori A, Furukawa Y, Hashimoto T, Yoshida A, Ono K, Shioi T, Okada M, Iwasaki A, Nishio R, Matsushima K, Sasayama S. Plasma levels of the monocyte chemotactic and activating factor/monocyte chemoattractant protein-1 are elevated in patients with acute myocardial infarction. *J Mol Cell Cardiol.* 1997;29:419-423
365. Morimoto H, Takahashi M, Izawa A, Ise H, Hongo M, Kolattukudy PE, Ikeda U. Cardiac overexpression of monocyte chemoattractant protein-1 in transgenic mice prevents cardiac dysfunction and remodeling after myocardial infarction. *Circ Res.* 2006;99:891-899
366. Nishiyama K, Ogawa H, Yasue H, Soejima H, Misumi K, Takazoe K, Yoshimura M, Kugiyama K, Tsuji I, Kumeda K. Simultaneous elevation of the levels of circulating monocyte

chemoattractant protein-1 and tissue factor in acute coronary syndromes. *Jpn Circ J.* 1998;62:710-712

367. McDermott DH, Yang Q, Kathiresan S, Cupples LA, Massaro JM, Keaney JF, Jr., Larson MG, Vasan RS, Hirschhorn JN, O'Donnell CJ, Murphy PM, Benjamin EJ. Ccl2 polymorphisms are associated with serum monocyte chemoattractant protein-1 levels and myocardial infarction in the framingham heart study. *Circulation.* 2005;112:1113-1120

368. Park HJ, Chang K, Park CS, Jang SW, Ihm SH, Kim PJ, Baek SH, Seung KB, Choi KB. Coronary collaterals: The role of mcp-1 during the early phase of acute myocardial infarction. *Int J Cardiol.* 2008;130:409-413

369. de Lemos JA, Morrow DA, Sabatine MS, Murphy SA, Gibson CM, Antman EM, McCabe CH, Cannon CP, Braunwald E. Association between plasma levels of monocyte chemoattractant protein-1 and long-term clinical outcomes in patients with acute coronary syndromes. *Circulation.* 2003;107:690-695

370. de Lemos JA, Morrow DA, Blazing MA, Jarolim P, Wiviott SD, Sabatine MS, Califf RM, Braunwald E. Serial measurement of monocyte chemoattractant protein-1 after acute coronary syndromes: Results from the a to z trial. *J Am Coll Cardiol.* 2007;50:2117-2124

371. Haim M, Tanne D, Boyko V, Reshef T, Goldbourt U, Battler A, Mekori YA, Behar S. Monocyte chemoattractant protein-1 and recurrent cardiovascular events in patients with stable coronary heart disease. *Clin Cardiol.* 2005;28:31-35

372. Iwai N, Kajimoto K, Kokubo Y, Okayama A, Miyazaki S, Nonogi H, Goto Y, Tomoike H. Assessment of genetic effects of polymorphisms in the mcp-1 gene on serum mcp-1 levels and myocardial infarction in japanese. *Circ J.* 2006;70:805-809

373. Jemaa R, Rojbani H, Kallel A, Ben Ali S, Feki M, Chabrak S, Elasmı M, Taieb SH, Sanhaji H, Souheil O, Mechmeche R, Kaabachi N. Association between the -2518g/a polymorphism in the monocyte chemoattractant protein-1 (mcp-1) gene and myocardial infarction in tunisian patients. *Clin Chim Acta.* 2008;390:122-125

374. Bjarnadottir K, Eiriksdottir G, Aspelund T, Gudnason V. Examination of genetic effects of polymorphisms in the mcp-1 and ccr2 genes on mi in the icelandic population. *Atherosclerosis.* 2006;188:341-346

375. Cermakova Z, Petrıkova J, Arakelyan A, Drabek J, Mrazek F, Lukl J, Petrek M. The mcp-1 -2518 (a to g) single nucleotide polymorphism is not associated with myocardial infarction in the czech population. *Int J Immunogenet.* 2005;32:315-318

376. Hayashidani S, Tsutsui H, Shiomi T, Ikeuchi M, Matsusaka H, Suematsu N, Wen J, Egashira K, Takeshita A. Anti-monocyte chemoattractant protein-1 gene therapy attenuates left

- ventricular remodeling and failure after experimental myocardial infarction. *Circulation*. 2003;108:2134-2140
377. Dewald O, Zymek P, Winkelmann K, Koerting A, Ren G, Abou-Khamis T, Michael LH, Rollins BJ, Entman ML, Frangogiannis NG. Ccl2/monocyte chemoattractant protein-1 regulates inflammatory responses critical to healing myocardial infarcts. *Circ Res*. 2005;96:881-889
378. Schwarz ER, Meven DA, Sulemanjee NZ, Kersting PH, Tussing T, Skobel EC, Hanrath P, Uretsky BF. Monocyte chemoattractant protein 1-induced monocyte infiltration produces angiogenesis but not arteriogenesis in chronically infarcted myocardium. *J Cardiovasc Pharmacol Ther*. 2004;9:279-289
379. Riese U, Brenner S, Docke WD, Prosch S, Reinke P, Oppert M, Volk HD, Platzer C. Catecholamines induce il-10 release in patients suffering from acute myocardial infarction by transactivating its promoter in monocytic but not in t-cells. *Mol Cell Biochem*. 2000;212:45-50
380. van Haelst PL, Tervaert JW, Bijzet J, Balje-Volkers C, May JF, Langeveld B, Gans RO. Circulating monocytes in patients with acute coronary syndromes lack sufficient interleukin-10 production after lipopolysaccharide stimulation. *Clin Exp Immunol*. 2004;138:364-368
381. Poitevin S, Cochery-Nouvellon E, Dupont A, Nguyen P. Monocyte il-10 produced in response to lipopolysaccharide modulates thrombin generation by inhibiting tissue factor expression and release of active tissue factor-bound microparticles. *Thromb Haemost*. 2007;97:598-607
382. Speidl WS, Toller WG, Kaun C, Weiss TW, Pfaffenberger S, Kastl SP, Furnkranz A, Maurer G, Huber K, Metzler H, Wojta J. Catecholamines potentiate lps-induced expression of mmp-1 and mmp-9 in human monocytes and in the human monocytic cell line u937: Possible implications for peri-operative plaque instability. *FASEB J*. 2004;18:603-605
383. Ganne F, Vasse M, Beaudeau JL, Peynet J, Francois A, Mishal Z, Chartier A, Tobelem G, Vannier JP, Soria J, Soria C. Cerivastatin, an inhibitor of hmg-coa reductase, inhibits urokinase/urokinase-receptor expression and mmp-9 secretion by peripheral blood monocytes--a possible protective mechanism against atherothrombosis. *Thromb Haemost*. 2000;84:680-688
384. Schmidt R, Bultmann A, Ungerer M, Joghetaei N, Bulbul O, Thieme S, Chavakis T, Toole BP, Gawaz M, Schomig A, May AE. Extracellular matrix metalloproteinase inducer regulates matrix metalloproteinase activity in cardiovascular cells: Implications in acute myocardial infarction. *Circulation*. 2006;113:834-841
385. Zhao SP, Deng P, Huang HG, Xu ZM, Dai HY, Hong SC, Yang J, Zhou HN. Expression of cox-2 mrna in peripheral blood monocytes from patients with acute myocardial infarction and its significance. *Clin Chem*. 2005;51:2170-2173

386. Askari AT, Brennan ML, Zhou X, Drinko J, Morehead A, Thomas JD, Topol EJ, Hazen SL, Penn MS. Myeloperoxidase and plasminogen activator inhibitor 1 play a central role in ventricular remodeling after myocardial infarction. *J Exp Med.* 2003;197:615-624
387. Kim MP, Zhou M, Wahl LM. Angiotensin ii increases human monocyte matrix metalloproteinase-1 through the at2 receptor and prostaglandin e2: Implications for atherosclerotic plaque rupture. *J Leukoc Biol.* 2005;78:195-201
388. Goldstein JL, Ho YK, Basu SK, Brown MS. Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. *Proc Natl Acad Sci U S A.* 1979;76:333-337
389. Krieger M. The other side of scavenger receptors: Pattern recognition for host defense. *Curr Opin Lipidol.* 1997;8:275-280
390. Silverstein RL, Febbraio M. Cd36 and atherosclerosis. *Curr Opin Lipidol.* 2000;11:483-491
391. Febbraio M, Podrez EA, Smith JD, Hajjar DP, Hazen SL, Hoff HF, Sharma K, Silverstein RL. Targeted disruption of the class b scavenger receptor cd36 protects against atherosclerotic lesion development in mice. *J Clin Invest.* 2000;105:1049-1056
392. Silverstein RL. Inflammation, atherosclerosis, and arterial thrombosis: Role of the scavenger receptor cd36. *Cleve Clin J Med.* 2009;76 Suppl 2:S27-30
393. Fuster V, Moreno PR, Fayad ZA, Corti R, Badimon JJ. Atherothrombosis and high-risk plaque: Part i: Evolving concepts. *J Am Coll Cardiol.* 2005;46:937-954
394. Nakayama M, Kudoh T, Kaikita K, Yoshimura M, Oshima S, Miyamoto Y, Takeya M, Ogawa H. Class a macrophage scavenger receptor gene expression levels in peripheral blood mononuclear cells specifically increase in patients with acute coronary syndrome. *Atherosclerosis.* 2008;198:426-433
395. Dragu R, Huri S, Zuckerman R, Suleiman M, Mutlak D, Agmon Y, Kapeliovich M, Beyar R, Markiewicz W, Hammerman H, Aronson D. Predictive value of white blood cell subtypes for long-term outcome following myocardial infarction. *Atherosclerosis.* 2008;196:405-412
396. Meisel SR, Pauzner H, Shechter M, Zeidan Z, David D. Peripheral monocytosis following acute myocardial infarction: Incidence and its possible role as a bedside marker of the extent of cardiac injury. *Cardiology.* 1998;90:52-57
397. Aliberti G, Pulignano I, Proietta M, De Michele LV, Corvisieri P, Minisola S. Role of monocytes in the early phase of acute myocardial infarction. *Panminerva Med.* 2000;42:179-182



398. Ott I, Neumann FJ, Kenngott S, Gawaz M, Schomig A. Procoagulant inflammatory responses of monocytes after direct balloon angioplasty in acute myocardial infarction. *Am J Cardiol.* 1998;82:938-942
399. Mariani M, Fetiveau R, Rossetti E, Poli A, Poletti F, Vandoni P, D'Urbano M, Cafiero F, Mariani G, Klersy C, De Servi S. Significance of total and differential leucocyte count in patients with acute myocardial infarction treated with primary coronary angioplasty. *Eur Heart J.* 2006;27:2511-2515
400. Hong YJ, Jeong MH, Ahn Y, Yoon NS, Lee SR, Hong SN, Moon JY, Kim KH, Park HW, Kim JH, Cho JG, Park JC, Kang JC. Relationship between peripheral monocytosis and nonrecovery of left ventricular function in patients with left ventricular dysfunction complicated with acute myocardial infarction. *Circ J.* 2007;71:1219-1224
401. Gidron Y, Armon T, Gilutz H, Huleihel M. Psychological factors correlate meaningfully with percent-monocytes among acute coronary syndrome patients. *Brain Behav Immun.* 2003;17:310-315
402. Horne BD, Anderson JL, John JM, Weaver A, Bair TL, Jensen KR, Renlund DG, Muhlestein JB. Which white blood cell subtypes predict increased cardiovascular risk? *J Am Coll Cardiol.* 2005;45:1638-1643
403. Neumann FJ, Zohlnhofer D, Fakhoury L, Ott I, Gawaz M, Schomig A. Effect of glycoprotein iib/iiiA receptor blockade on platelet-leukocyte interaction and surface expression of the leukocyte integrin mac-1 in acute myocardial infarction. *J Am Coll Cardiol.* 1999;34:1420-1426
404. Szabo S, Etzel D, Ehlers R, Walter T, Kazmaier S, Helber U, Hoffmeister HM. Combined thrombolysis with abciximab favourably influences platelet-leukocyte interactions and platelet activation in acute myocardial infarction. *J Thromb Thrombolysis.* 2005;20:155-161
405. Jaster M, Schwimmbeck P, Spencker S, Schultheiss HP, Rauch U. Randomized comparison of platelet-leukocyte aggregates and platelet activation in blood: Heparin-coated coiled wire stent implantation versus balloon angioplasty in acute myocardial infarction. *Thromb Res.* 2003;112:285-289
406. Napoleone E, Di Santo A, Camera M, Tremoli E, Lorenzet R. Angiotensin-converting enzyme inhibitors downregulate tissue factor synthesis in monocytes. *Circ Res.* 2000;86:139-143
407. Soejima H, Ogawa H, Yasue H, Kaikita K, Takazoe K, Nishiyama K, Misumi K, Miyamoto S, Yoshimura M, Kugiyama K, Nakamura S, Tsuji I. Angiotensin-converting enzyme inhibition reduces monocyte chemoattractant protein-1 and tissue factor levels in patients with myocardial infarction. *J Am Coll Cardiol.* 1999;34:983-988

408. Murakami Y, Kurosaki K, Matsui K, Shimada K, Ikeda U. Serum mcp-1 and vegf levels are not affected by inhibition of the renin-angiotensin system in patients with acute myocardial infarction. *Cardiovasc Drugs Ther.* 2003;17:249-255
409. Xiao Z, Theroux P. Clopidogrel inhibits platelet-leukocyte interactions and thrombin receptor agonist peptide-induced platelet activation in patients with an acute coronary syndrome. *J Am Coll Cardiol.* 2004;43:1982-1988
410. Xu ZM, Zhao SP, Li QZ, Nie S, Zhou HN. Atorvastatin reduces plasma mcp-1 in patients with acute coronary syndrome. *Clin Chim Acta.* 2003;338:17-24
411. Deng P, Zhao SP, Dai HY, Guan XS, Huang HG. Atorvastatin reduces the expression of cox-2 mrna in peripheral blood monocytes from patients with acute myocardial infarction and modulates the early inflammatory response. *Clin Chem.* 2006;52:300-303
412. Li CY, Tsai CS, Chueh SH, Hsu PC, Wang JY, Wong CS, Ho ST. Dobutamine inhibits monocyte chemoattractant protein-1 production and chemotaxis in human monocytes. *Anesth Analg.* 2003;97:205-209, table of contents
413. Fraccarollo D, Galuppo P, Schraut S, Kneitz S, van Rooijen N, Ertl G, Bauersachs J. Immediate mineralocorticoid receptor blockade improves myocardial infarct healing by modulation of the inflammatory response. *Hypertension.* 2008;51:905-914
414. Swirski FK, Libby P, Aikawa E, Alcaide P, Luscinskas FW, Weissleder R, Pittet MJ. Ly-6chi monocytes dominate hypercholesterolemia-associated monocytosis and give rise to macrophages in atheromata. *J Clin Invest.* 2007;117:195-205
415. Tacke F, Alvarez D, Kaplan TJ, Jakubzick C, Spanbroek R, Llodra J, Garin A, Liu J, Mack M, van Rooijen N, Lira SA, Habenicht AJ, Randolph GJ. Monocyte subsets differentially employ ccr2, ccr5, and cx3cr1 to accumulate within atherosclerotic plaques. *J Clin Invest.* 2007;117:185-194
416. Veillard NR, Steffens S, Pelli G, Lu B, Kwak BR, Gerard C, Charo IF, Mach F. Differential influence of chemokine receptors ccr2 and cxcr3 in development of atherosclerosis in vivo. *Circulation.* 2005;112:870-878
417. Combadiere C, Potteaux S, Rodero M, Simon T, Pezard A, Esposito B, Merval R, Proudfoot A, Tedgui A, Mallat Z. Combined inhibition of ccl2, cx3cr1, and ccr5 abrogates ly6c(hi) and ly6c(lo) monocytosis and almost abolishes atherosclerosis in hypercholesterolemic mice. *Circulation.* 2008;117:1649-1657
418. Wu H, Gower RM, Wang H, Perrard XY, Ma R, Bullard DC, Burns AR, Paul A, Smith CW, Simon SI, Ballantyne CM. Functional role of cd11c+ monocytes in atherogenesis associated with hypercholesterolemia. *Circulation.* 2009;119:2708-2717

419. Rothe G, Herr AS, Stohr J, Abletshauser C, Weidinger G, Schmitz G. A more mature phenotype of blood mononuclear phagocytes is induced by fluvastatin treatment in hypercholesterolemic patients with coronary heart disease. *Atherosclerosis*. 1999;144:251-261
420. Schlitt A, Heine GH, Blankenberg S, Espinola-Klein C, Dopheide JF, Bickel C, Lackner KJ, Iz M, Meyer J, Darius H, Rupprecht HJ. Cd14+cd16+ monocytes in coronary artery disease and their relationship to serum tnf-alpha levels. *Thromb Haemost*. 2004;92:419-424
421. Mosig S, Rennert K, Krause S, Kzhyshkowska J, Neunubel K, Heller R, Funke H. Different functions of monocyte subsets in familial hypercholesterolemia: Potential function of cd14+ cd16+ monocytes in detoxification of oxidized ldl. *FASEB J*. 2009;23:866-874
422. Auffray C, Fogg D, Garfa M, Elain G, Join-Lambert O, Kayal S, Sarnacki S, Cumano A, Lauvau G, Geissmann F. Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior. *Science*. 2007;317:666-670
423. Soehnlein O, Zernecke A, Weber C. Neutrophils launch monocyte extravasation by release of granule proteins. *Thromb Haemost*. 2009;102:198-205
424. Hristov M, Leyendecker T, Schuhmann C, von Hundelshausen P, Heussen N, Kehmeier E, Krotz F, Sohn HY, Klauss V, Weber C. Circulating monocyte subsets and cardiovascular risk factors in coronary artery disease. *Thromb Haemost*. 2010;104:412-414
425. Barisione C, Garibaldi S, Ghigliotti G, Fabbi P, Altieri P, Casale MC, Spallarossa P, Bertero G, Balbi M, Corsiglia L, Brunelli C. Cd14cd16 monocyte subset levels in heart failure patients. *Dis Markers*. 2010;28:115-124
426. Carmeliet P. Mechanisms of angiogenesis and arteriogenesis. *Nat Med*. 2000;6:389-395
427. Ferrara N. Role of vascular endothelial growth factor in physiologic and pathologic angiogenesis: Therapeutic implications. *Semin Oncol*. 2002;29:10-14
428. Chong AY, Caine GJ, Freestone B, Blann AD, Lip GY. Plasma angiopoietin-1, angiopoietin-2, and angiopoietin receptor tie-2 levels in congestive heart failure. *J Am Coll Cardiol*. 2004;43:423-428
429. Arakawa H, Ikeda U, Hojo Y, Ueno S, Nonaka-Sarukawa M, Yamamoto K, Shimada K. Decreased serum vascular endothelial growth factor concentrations in patients with congestive heart failure. *Heart*. 2003;89:207-208
430. Chin BS, Chung NA, Gibbs CR, Blann AD, Lip GY. Vascular endothelial growth factor and soluble p-selectin in acute and chronic congestive heart failure. *Am J Cardiol*. 2002;90:1258-1260
431. Friehs I, Barillas R, Vasilyev NV, Roy N, McGowan FX, del Nido PJ. Vascular endothelial growth factor prevents apoptosis and preserves contractile function in hypertrophied infant heart. *Circulation*. 2006;114:I290-295

432. Izumiya Y, Shiojima I, Sato K, Sawyer DB, Colucci WS, Walsh K. Vascular endothelial growth factor blockade promotes the transition from compensatory cardiac hypertrophy to failure in response to pressure overload. *Hypertension*. 2006;47:887-893
433. Davis S, Aldrich TH, Jones PF, Acheson A, Compton DL, Jain V, Ryan TE, Bruno J, Radziejewski C, Maisonpierre PC, Yancopoulos GD. Isolation of angiopoietin-1, a ligand for the tie2 receptor, by secretion-trap expression cloning. *Cell*. 1996;87:1161-1169
434. Morisada T, Kubota Y, Urano T, Suda T, Oike Y. Angiopoietins and angiopoietin-like proteins in angiogenesis. *Endothelium*. 2006;13:71-79
435. Hilfiker-Kleiner D, Hilfiker A, Fuchs M, Kaminski K, Schaefer A, Schieffer B, Hillmer A, Schmiedl A, Ding Z, Podewski E, Poli V, Schneider MD, Schulz R, Park JK, Wollert KC, Drexler H. Signal transducer and activator of transcription 3 is required for myocardial capillary growth, control of interstitial matrix deposition, and heart protection from ischemic injury. *Circ Res*. 2004;95:187-195
436. Pearlman JD, Hibberd MG, Chuang ML, Harada K, Lopez JJ, Gladstone SR, Friedman M, Sellke FW, Simons M. Magnetic resonance mapping demonstrates benefits of vegf-induced myocardial angiogenesis. *Nat Med*. 1995;1:1085-1089
437. van Amerongen MJ, Harmsen MC, van Rooijen N, Petersen AH, van Luyn MJ. Macrophage depletion impairs wound healing and increases left ventricular remodeling after myocardial injury in mice. *Am J Pathol*. 2007;170:818-829
438. Zandbergen HR, Sharma UC, Gupta S, Verjans JW, van den Borne S, Pokharel S, van Brakel T, Duijvestijn A, van Rooijen N, Maessen JG, Reutelingsperger C, Pinto YM, Narula J, Hofstra L. Macrophage depletion in hypertensive rats accelerates development of cardiomyopathy. *J Cardiovasc Pharmacol Ther*. 2009;14:68-75
439. Fong GH. Mechanisms of adaptive angiogenesis to tissue hypoxia. *Angiogenesis*. 2008;11:121-140
440. Lambert JM, Lopez EF, Lindsey ML. Macrophage roles following myocardial infarction. *Int J Cardiol*. 2008;130:147-158
441. Manoonkitiwongsa PS, Jackson-Friedman C, McMillan PJ, Schultz RL, Lyden PD. Angiogenesis after stroke is correlated with increased numbers of macrophages: The clean-up hypothesis. *J Cereb Blood Flow Metab*. 2001;21:1223-1231
442. Ogawa K, Suzuki J, Narasaki M, Mori M. Healing of focal injury in the rat liver. *Am J Pathol*. 1985;119:158-167
443. Kobayashi S, Nagaura T, Kimura I, Kimura M. Interferon-gamma-activated macrophages enhance angiogenesis from endothelial cells of rat aorta. *Immunopharmacology*. 1994;27:23-30

444. Sunderkotter C, Steinbrink K, Goebeler M, Bhardwaj R, Sorg C. Macrophages and angiogenesis. *J Leukoc Biol.* 1994;55:410-422
445. Murdoch C, Tazzyman S, Webster S, Lewis CE. Expression of tie-2 by human monocytes and their responses to angiopoietin-2. *J Immunol.* 2007;178:7405-7411
446. Kissel CK, Lehmann R, Assmus B, Aicher A, Honold J, Fischer-Rasokat U, Heeschen C, Spyridopoulos I, Dimmeler S, Zeiher AM. Selective functional exhaustion of hematopoietic progenitor cells in the bone marrow of patients with postinfarction heart failure. *J Am Coll Cardiol.* 2007;49:2341-2349
447. Nonaka-Sarukawa M, Yamamoto K, Aoki H, Nishimura Y, Tomizawa H, Ichida M, Eizawa T, Muroi K, Ikeda U, Shimada K. Circulating endothelial progenitor cells in congestive heart failure. *Int J Cardiol.* 2007;119:344-348
448. Fritzenwanger M, Lorenz F, Jung C, Fabris M, Thude H, Barz D, Figulla HR. Differential number of cd34+, cd133+ and cd34+/cd133+ cells in peripheral blood of patients with congestive heart failure. *Eur J Med Res.* 2009;14:113-117
449. Valgimigli M, Rigolin GM, Fucili A, Porta MD, Soukhomovskaia O, Malagutti P, Bugli AM, Bragotti LZ, Francolini G, Mauro E, Castoldi G, Ferrari R. Cd34+ and endothelial progenitor cells in patients with various degrees of congestive heart failure. *Circulation.* 2004;110:1209-1212
450. Michowitz Y, Goldstein E, Wexler D, Sheps D, Keren G, George J. Circulating endothelial progenitor cells and clinical outcome in patients with congestive heart failure. *Heart.* 2007;93:1046-1050
451. Geft D, Schwartzberg S, Rogowsky O, Finkelstein A, Ablin J, Maysel-Auslender S, Wexler D, Keren G, George J. Circulating apoptotic progenitor cells in patients with congestive heart failure. *PLoS One.* 2008;3:e3238
452. Manginas A, Tsiavou A, Sfyraakis P, Giamouzis G, Tsourelis L, Leontiadis E, Degiannis D, Cokkinos DV, Alivizatos PA. Increased number of circulating progenitor cells after implantation of ventricular assist devices. *J Heart Lung Transplant.* 2009;28:710-717
453. Van Craenenbroeck EM, Beckers PJ, Possemiers NM, Wuyts K, Frederix G, Hoymans VY, Wuyts F, Paelinck BP, Vrints CJ, Conraads VM. Exercise acutely reverses dysfunction of circulating angiogenic cells in chronic heart failure. *Eur Heart J.* 2010;31:1924-1934
454. Dedobbeleer C, Blocklet D, Toungouz M, Lambermont M, Unger P, Degaute JP, Goldman S, Berkenboom G. Myocardial homing and coronary endothelial function after autologous blood cd34+ progenitor cells intracoronary injection in the chronic phase of myocardial infarction. *J Cardiovasc Pharmacol.* 2009;53:480-485

455. Shmilovich H, Ben-Shoshan J, Tal R, Afek A, Barshack I, Maysel-Auslander S, Harats D, Keren G, George J. B-type natriuretic peptide enhances vasculogenesis by promoting number and functional properties of early endothelial progenitor cells. *Tissue Eng Part A*. 2009;15:2741-2749
456. Flores-Ramirez R, Uribe-Longoria A, Rangel-Fuentes MM, Gutierrez-Fajardo P, Salazar-Riojas R, Cervantes-Garcia D, Trevino-Ortiz JH, Benavides-Chereti GJ, Espinosa-Oliveros LP, Limon-Rodriguez RH, Monreal-Puente R, Gonzalez-Trevino JL, Rojas-Martinez A. Intracoronary infusion of cd133+ endothelial progenitor cells improves heart function and quality of life in patients with chronic post-infarct heart insufficiency. *Cardiovasc Revasc Med*. 2010;11:72-78
457. Ingram DA, Mead LE, Tanaka H, Meade V, Fenoglio A, Mortell K, Pollok K, Ferkowicz MJ, Gilley D, Yoder MC. Identification of a novel hierarchy of endothelial progenitor cells using human peripheral and umbilical cord blood. *Blood*. 2004;104:2752-2760
458. Rehman J, Li J, Orschell CM, March KL. Peripheral blood "Endothelial progenitor cells" Are derived from monocyte/macrophages and secrete angiogenic growth factors. *Circulation*. 2003;107:1164-1169
459. Kuwana M, Okazaki Y, Kodama H, Izumi K, Yasuoka H, Ogawa Y, Kawakami Y, Ikeda Y. Human circulating cd14+ monocytes as a source of progenitors that exhibit mesenchymal cell differentiation. *J Leukoc Biol*. 2003;74:833-845
460. Miyamoto T, Ohneda O, Arai F, Iwamoto K, Okada S, Takagi K, Anderson DM, Suda T. Bifurcation of osteoclasts and dendritic cells from common progenitors. *Blood*. 2001;98:2544-2554
461. Crosby JR, Kaminski WE, Schattman G, Martin PJ, Raines EW, Seifert RA, Bowen-Pope DF. Endothelial cells of hematopoietic origin make a significant contribution to adult blood vessel formation. *Circ Res*. 2000;87:728-730
462. Fernandez Pujol B, Lucibello FC, Gehling UM, Lindemann K, Weidner N, Zuzarte ML, Adamkiewicz J, Elsasser HP, Muller R, Havemann K. Endothelial-like cells derived from human cd14 positive monocytes. *Differentiation*. 2000;65:287-300
463. Moldovan NI, Goldschmidt-Clermont PJ, Parker-Thornburg J, Shapiro SD, Kolattukudy PE. Contribution of monocytes/macrophages to compensatory neovascularization: The drilling of metalloelastase-positive tunnels in ischemic myocardium. *Circ Res*. 2000;87:378-384
464. Awad O, Dedkov EI, Jiao C, Bloomer S, Tomanek RJ, Schattman GC. Differential healing activities of cd34+ and cd14+ endothelial cell progenitors. *Arterioscler Thromb Vasc Biol*. 2006;26:758-764

465. Elsheikh E, Uzunel M, He Z, Holgersson J, Nowak G, Sumitran-Holgersson S. Only a specific subset of human peripheral-blood monocytes has endothelial-like functional capacity. *Blood*. 2005;106:2347-2355
466. Dresske B, El Mokhtari NE, Ungefroren H, Ruhnke M, Plate V, Janssen D, Siebert R, Reinecke A, Simon R, Fandrich F. Multipotent cells of monocytic origin improve damaged heart function. *Am J Transplant*. 2006;6:947-958
467. Bruno S, Bussolati B, Scacciatella P, Marra S, Sanavio F, Tarella C, Camussi G. Combined administration of g-csf and gm-csf stimulates monocyte-derived pro-angiogenic cells in patients with acute myocardial infarction. *Cytokine*. 2006;34:56-65
468. Leor J, Rozen L, Zulooff-Shani A, Feinberg MS, Amsalem Y, Barbash IM, Kachel E, Holbova R, Mardor Y, Daniels D, Ocherashvili A, Orenstein A, Danon D. Ex vivo activated human macrophages improve healing, remodeling, and function of the infarcted heart. *Circulation*. 2006;114:I94-100
469. Morimoto H, Takahashi M, Shiba Y, Izawa A, Ise H, Hongo M, Hatake K, Motoyoshi K, Ikeda U. Bone marrow-derived cxcr4+ cells mobilized by macrophage colony-stimulating factor participate in the reduction of infarct area and improvement of cardiac remodeling after myocardial infarction in mice. *Am J Pathol*. 2007;171:755-766
470. Harraz M, Jiao C, Hanlon HD, Hartley RS, Schatteman GC. Cd34- blood-derived human endothelial cell progenitors. *Stem Cells*. 2001;19:304-312
471. Schmeisser A, Garlich CD, Zhang H, Eskafi S, Graffy C, Ludwig J, Strasser RH, Daniel WG. Monocytes coexpress endothelial and macrophagocytic lineage markers and form cord-like structures in matrigel under angiogenic conditions. *Cardiovasc Res*. 2001;49:671-680
472. Kuwana M, Okazaki Y, Kodama H, Satoh T, Kawakami Y, Ikeda Y. Endothelial differentiation potential of human monocyte-derived multipotential cells. *Stem Cells*. 2006;24:2733-2743
473. Vlodavsky I, Fuks Z, Ishai-Michaeli R, Bashkin P, Levi E, Korner G, Bar-Shavit R, Klagsbrun M. Extracellular matrix-resident basic fibroblast growth factor: Implication for the control of angiogenesis. *J Cell Biochem*. 1991;45:167-176
474. Esposito CJ, Popescu WM, Rinder HM, Schwartz JJ, Smith BR, Rinder CS. Increased leukocyte-platelet adhesion in patients with graft occlusion after peripheral vascular surgery. *Thromb Haemost*. 2003;90:1128-1134
475. Kageyama K, Nakajima Y, Shibasaki M, Hashimoto S, Mizobe T. Increased platelet, leukocyte, and endothelial cell activity are associated with increased coagulability in patients after total knee arthroplasty. *J Thromb Haemost*. 2007;5:738-745

476. Bunesco A, Widman J, Lenkei R, Menyes P, Levin K, Egberg N. Increases in circulating levels of monocyte-platelet and neutrophil-platelet complexes following hip arthroplasty. *Clin Sci (Lond)*. 2002;102:279-286
477. Rinder HM, Bonan JL, Rinder CS, Ault KA, Smith BR. Dynamics of leukocyte-platelet adhesion in whole blood. *Blood*. 1991;78:1730-1737
478. Kornberg A, Rahimi-Levene N, Yona R, Mor A, Rachmilewitz EA. Enhanced generation of monocyte tissue factor and increased plasma prothrombin fragment1+2 levels in patients with polycythemia vera: Mechanism of activation of blood coagulation. *Am J Hematol*. 1997;56:5-11
479. Keating FK, Dauerman HL, Whitaker DA, Sobel BE, Schneider DJ. Increased expression of platelet p-selectin and formation of platelet-leukocyte aggregates in blood from patients treated with unfractionated heparin plus eptifibatid compared with bivalirudin. *Thromb Res*. 2006;118:361-369
480. Hayward R, Campbell B, Shin YK, Scalia R, Lefer AM. Recombinant soluble p-selectin glycoprotein ligand-1 protects against myocardial ischemic reperfusion injury in cats. *Cardiovasc Res*. 1999;41:65-76
481. Oberle V, Fischer A, Setzer F, Losche W. Thrombus formation without platelets under inflammatory condition: An in vitro study. *Platelets*. 2007;18:143-149
482. Sarma J, Laan CA, Alam S, Jha A, Fox KA, Dransfield I. Increased platelet binding to circulating monocytes in acute coronary syndromes. *Circulation*. 2002;105:2166-2171
483. Silverstein RL, Asch AS, Nachman RL. Glycoprotein iv mediates thrombospondin-dependent platelet-monocyte and platelet-u937 cell adhesion. *J Clin Invest*. 1989;84:546-552
484. Michelson AD, Barnard MR, Hechtman HB, MacGregor H, Connolly RJ, Loscalzo J, Valeri CR. In vivo tracking of platelets: Circulating degranulated platelets rapidly lose surface p-selectin but continue to circulate and function. *Proc Natl Acad Sci U S A*. 1996;93:11877-11882
485. Poole JC. Phagocytosis of platelets by monocytes in organizing arterial thrombi. An electron microscopical study. *Q J Exp Physiol Cogn Med Sci*. 1966;51:54-59
486. Gawaz MP, Loftus JC, Bajt ML, Frojmovic MM, Plow EF, Ginsberg MH. Ligand bridging mediates integrin alpha iiib beta 3 (platelet gpiib-iiiA) dependent homotypic and heterotypic cell-cell interactions. *J Clin Invest*. 1991;88:1128-1134
487. Simon DI, Ezratty AM, Francis SA, Rennke H, Loscalzo J. Fibrin(ogen) is internalized and degraded by activated human monocytoid cells via mac-1 (cd11b/cd18): A nonplasmin fibrinolytic pathway. *Blood*. 1993;82:2414-2422



488. Kirchhofer D, Riederer MA, Baumgartner HR. Specific accumulation of circulating monocytes and polymorphonuclear leukocytes on platelet thrombi in a vascular injury model. *Blood*. 1997;89:1270-1278
489. Arber N, Berliner S, Pras E, Arber L, Fishelson Z, Kahn Y, Bassat MB, Pinkhas J, Aronson M. Heterotypic leukocyte aggregation in the peripheral blood of patients with leukemia, inflammation and stress. *Nouv Rev Fr Hematol*. 1991;33:251-255
490. Kaplar M, Kappelmayer J, Veszpremi A, Szabo K, Udvardy M. The possible association of in vivo leukocyte-platelet heterophilic aggregate formation and the development of diabetic angiopathy. *Platelets*. 2001;12:419-422
491. Danenberg HD, Kantak N, Grad E, Swaminathan RV, Lotan C, Edelman ER. C-reactive protein promotes monocyte-platelet aggregation: An additional link to the inflammatory-thrombotic intricacy. *Eur J Haematol*. 2007;78:246-252
492. Konieczkowski M, Skrinska VA. Increased synthesis of thromboxane a(2) and expression of procoagulant activity by monocytes in response to arachidonic acid in diabetes mellitus. *Prostaglandins Leukot Essent Fatty Acids*. 2001;65:133-138
493. Goubareva I, Gkaliagkousi E, Shah A, Queen L, Ritter J, Ferro A. Age decreases nitric oxide synthesis and responsiveness in human platelets and increases formation of monocyte-platelet aggregates. *Cardiovasc Res*. 2007;75:793-802
494. Furman MI, Benoit SE, Barnard MR, Valeri CR, Borbone ML, Becker RC, Hechtman HB, Michelson AD. Increased platelet reactivity and circulating monocyte-platelet aggregates in patients with stable coronary artery disease. *J Am Coll Cardiol*. 1998;31:352-358
495. Brambilla M, Camera M, Colnago D, Marenzi G, De Metrio M, Giesen PL, Balduino A, Veglia F, Gertow K, Biglioli P, Tremoli E. Tissue factor in patients with acute coronary syndromes: Expression in platelets, leukocytes, and platelet-leukocyte aggregates. *Arterioscler Thromb Vasc Biol*. 2008;28:947-953
496. Furman MI, Barnard MR, Krueger LA, Fox ML, Shilale EA, Lessard DM, Marchese P, Frelinger AL, 3rd, Goldberg RJ, Michelson AD. Circulating monocyte-platelet aggregates are an early marker of acute myocardial infarction. *J Am Coll Cardiol*. 2001;38:1002-1006
497. Puccetti L, Bruni F, Bova G, Cercignani M, Pompella G, Auteri A, Pasqui AL. Role of platelets in tissue factor expression by monocytes in normal and hypercholesterolemic subjects. In vitro effect of cerivastatin. *Int J Clin Lab Res*. 2000;30:147-156
498. Ichikawa K, Yoshinari M, Iwase M, Wakisaka M, Doi Y, Iino K, Yamamoto M, Fujishima M. Advanced glycosylation end products induced tissue factor expression in human monocyte-like u937 cells and increased tissue factor expression in monocytes from diabetic patients. *Atherosclerosis*. 1998;136:281-287

499. Holschermann H, Terhalle HM, Zakel U, Maus U, Parviz B, Tillmanns H, Haberbosch W. Monocyte tissue factor expression is enhanced in women who smoke and use oral contraceptives. *Thromb Haemost.* 1999;82:1614-1620
500. Kalsch T, Nguyen XD, Elmas E, Grebert N, Suselbeck T, Kluter H, Borggrefe M, Dempfle CE. Coagulation activation and expression of cd40 ligand on platelets upon in vitro lipopolysaccharide-challenge in patients with unstable angina. *Int J Cardiol.* 2006;111:217-223
501. Freeburn JC, Wallace JM, Strain JJ, Sinnamon DG, Craig BM, Johnson D, Gilmore WS. Monocyte tissue factor-like activity in post myocardial infarction patients. *Br J Haematol.* 1998;102:605-608
502. Vieira LM, Dusse LM, Fernandes AP, Martins-Filho OA, de Bastos M, Ferreira MF, Cooper AJ, Lwaleed BA, Carvalho MG. Monocytes and plasma tissue factor levels in normal individuals and patients with deep venous thrombosis of the lower limbs: Potential diagnostic tools? *Thromb Res.* 2007;119:157-165
503. Holschermann H, Haberbosch W, Terhalle HM, Parviz B, Kraus J, Kemkes-Matthes B, Tillmanns H, Kaps M, Stolz E. Increased monocyte tissue factor activity in women following cerebral venous thrombosis. *J Neurol.* 2003;250:631-632
504. Arellano-Rodrigo E, Alvarez-Larran A, Reverter JC, Villamor N, Colomer D, Cervantes F. Increased platelet and leukocyte activation as contributing mechanisms for thrombosis in essential thrombocythemia and correlation with the jak2 mutational status. *Haematologica.* 2006;91:169-175
505. May AE, Neumann FJ, Gawaz M, Ott I, Walter H, Schomig A. Reduction of monocyte-platelet interaction and monocyte activation in patients receiving antiplatelet therapy after coronary stent implantation. *Eur Heart J.* 1997;18:1913-1920
506. Zhao L, Bath PM, May J, Losche W, Heptinstall S. P-selectin, tissue factor and cd40 ligand expression on platelet-leucocyte conjugates in the presence of a gpiib/iiiia antagonist. *Platelets.* 2003;14:473-480
507. Heymans S, Hirsch E, Anker SD, Aukrust P, Balligand JL, Cohen-Tervaert JW, Drexler H, Filippatos G, Felix SB, Gullestad L, Hilfiker-Kleiner D, Janssens S, Latini R, Neubauer G, Paulus WJ, Pieske B, Ponikowski P, Schroen B, Schultheiss HP, Tschope C, Van Bilsen M, Zannad F, McMurray J, Shah AM. Inflammation as a therapeutic target in heart failure? A scientific statement from the translational research committee of the heart failure association of the european society of cardiology. *Eur J Heart Fail.* 2009;11:119-129
508. Falk E. Pathogenesis of atherosclerosis. *J Am Coll Cardiol.* 2006;47:C7-12
509. Davi G, Patrono C. Platelet activation and atherothrombosis. *N Engl J Med.* 2007;357:2482-2494

510. Burnier L, Fontana P, Kwak BR, Angelillo-Scherrer A. Cell-derived microparticles in haemostasis and vascular medicine. *Thromb Haemost.* 2009;101:439-451
511. Dachary-Prigent J, Freyssinet JM, Pasquet JM, Carron JC, Nurden AT. Annexin v as a probe of aminophospholipid exposure and platelet membrane vesiculation: A flow cytometry study showing a role for free sulfhydryl groups. *Blood.* 1993;81:2554-2565
512. Satta N, Toti F, Feugeas O, Bohbot A, Dachary-Prigent J, Eschwege V, Hedman H, Freyssinet JM. Monocyte vesiculation is a possible mechanism for dissemination of membrane-associated procoagulant activities and adhesion molecules after stimulation by lipopolysaccharide. *J Immunol.* 1994;153:3245-3255
513. Combes V, Simon AC, Grau GE, Arnoux D, Camoin L, Sabatier F, Mutin M, Sanmarco M, Sampol J, Dignat-George F. In vitro generation of endothelial microparticles and possible prothrombotic activity in patients with lupus anticoagulant. *J Clin Invest.* 1999;104:93-102
514. Boulanger CM, Amabile N, Tedgui A. Circulating microparticles: A potential prognostic marker for atherosclerotic vascular disease. *Hypertension.* 2006;48:180-186
515. Chironi GN, Boulanger CM, Simon A, Dignat-George F, Freyssinet JM, Tedgui A. Endothelial microparticles in diseases. *Cell Tissue Res.* 2009;335:143-151
516. Jimenez JJ, Jy W, Mauro LM, Soderland C, Horstman LL, Ahn YS. Endothelial cells release phenotypically and quantitatively distinct microparticles in activation and apoptosis. *Thromb Res.* 2003;109:175-180
517. Banfi C, Brioschi M, Wait R, Begum S, Gianazza E, Pirillo A, Mussoni L, Tremoli E. Proteome of endothelial cell-derived procoagulant microparticles. *Proteomics.* 2005;5:4443-4455
518. Feng B, Chen Y, Luo Y, Chen M, Li X, Ni Y. Circulating level of microparticles and their correlation with arterial elasticity and endothelium-dependent dilation in patients with type 2 diabetes mellitus. *Atherosclerosis.* 2010;208:264-269
519. Koga H, Sugiyama S, Kugiyama K, Watanabe K, Fukushima H, Tanaka T, Sakamoto T, Yoshimura M, Jinnouchi H, Ogawa H. Elevated levels of ve-cadherin-positive endothelial microparticles in patients with type 2 diabetes mellitus and coronary artery disease. *J Am Coll Cardiol.* 2005;45:1622-1630
520. Dursun I, Poyrazoglu HM, Gunduz Z, Ulger H, Yykylmaz A, Dusunsel R, Patyroglu T, Gurgoze M. The relationship between circulating endothelial microparticles and arterial stiffness and atherosclerosis in children with chronic kidney disease. *Nephrol Dial Transplant.* 2009;24:2511-2518

521. Bulut D, Maier K, Bulut-Streich N, Borgel J, Hanefeld C, Mugge A. Circulating endothelial microparticles correlate inversely with endothelial function in patients with ischemic left ventricular dysfunction. *J Card Fail.* 2008;14:336-340
522. Werner N, Wassmann S, Ahlers P, Kosiol S, Nickenig G. Circulating cd31+/annexin v+ apoptotic microparticles correlate with coronary endothelial function in patients with coronary artery disease. *Arterioscler Thromb Vasc Biol.* 2006;26:112-116
523. Wang JM, Yang Z, Xu MG, Chen L, Wang Y, Su C, Tao J. Berberine-induced decline in circulating cd31+/cd42- microparticles is associated with improvement of endothelial function in humans. *Eur J Pharmacol.* 2009;614:77-83
524. Bernard S, Loffroy R, Serusclat A, Boussel L, Bonnefoy E, Thevenon C, Rabilloud M, Revel D, Moulin P, Douek P. Increased levels of endothelial microparticles cd144 (ve-cadherin) positives in type 2 diabetic patients with coronary noncalcified plaques evaluated by multidetector computed tomography (mdct). *Atherosclerosis.* 2009;203:429-435
525. Preston RA, Jy W, Jimenez JJ, Mauro LM, Horstman LL, Valle M, Aime G, Ahn YS. Effects of severe hypertension on endothelial and platelet microparticles. *Hypertension.* 2003;41:211-217
526. Wang JM, Huang YJ, Wang Y, Xu MG, Wang LC, Wang SM, Tao J. Increased circulating cd31+/cd42- microparticles are associated with impaired systemic artery elasticity in healthy subjects. *Am J Hypertens.* 2007;20:957-964
527. Wang JM, Su C, Wang Y, Huang YJ, Yang Z, Chen L, Wu F, Xu SY, Tao J. Elevated circulating endothelial microparticles and brachial-ankle pulse wave velocity in well-controlled hypertensive patients. *J Hum Hypertens.* 2009;23:307-315
528. Bakouboula B, Morel O, Faure A, Zobairi F, Jesel L, Trinh A, Zupan M, Canuet M, Grunebaum L, Brunette A, Desprez D, Chabot F, Weitzenblum E, Freyssinet JM, Chaouat A, Toti F. Procoagulant membrane microparticles correlate with the severity of pulmonary arterial hypertension. *Am J Respir Crit Care Med.* 2008;177:536-543
529. Nomura S, Inami N, Shouzu A, Omoto S, Kimura Y, Takahashi N, Tanaka A, Urase F, Maeda Y, Ohtani H, Iwasaka T. The effects of pitavastatin, eicosapentaenoic acid and combined therapy on platelet-derived microparticles and adiponectin in hyperlipidemic, diabetic patients. *Platelets.* 2009;20:16-22
530. Nomura S, Miyazaki Y, Miyake T, Suzuki M, Kawakatsu T, Kido H, Yamaguchi K, Fukuroi T, Kagawa H, Yanabu M. Detection of platelet-derived microparticles in patients with diabetes. *Am J Hematol.* 1993;44:213

531. Omoto S, Nomura S, Shouzu A, Nishikawa M, Fukuhara S, Iwasaka T. Detection of monocyte-derived microparticles in patients with type ii diabetes mellitus. *Diabetologia*. 2002;45:550-555
532. Nomura S, Suzuki M, Katsura K, Xie GL, Miyazaki Y, Miyake T, Kido H, Kagawa H, Fukuhara S. Platelet-derived microparticles may influence the development of atherosclerosis in diabetes mellitus. *Atherosclerosis*. 1995;116:235-240
533. Tan KT, Tayebjee MH, Lim HS, Lip GY. Clinically apparent atherosclerotic disease in diabetes is associated with an increase in platelet microparticle levels. *Diabet Med*. 2005;22:1657-1662
534. Ogata N, Imaizumi M, Nomura S, Shozu A, Arichi M, Matsuoka M, Matsumura M. Increased levels of platelet-derived microparticles in patients with diabetic retinopathy. *Diabetes Res Clin Pract*. 2005;68:193-201
535. Ogata N, Nomura S, Shouzu A, Imaizumi M, Arichi M, Matsumura M. Elevation of monocyte-derived microparticles in patients with diabetic retinopathy. *Diabetes Res Clin Pract*. 2006;73:241-248
536. Goichot B, Grunebaum L, Desprez D, Vinzio S, Meyer L, Schlienger JL, Lessard M, Simon C. Circulating procoagulant microparticles in obesity. *Diabetes Metab*. 2006;32:82-85
537. Casey RG, Joyce M, Roche-Nagle G, Cox D, Bouchier-Hayes DJ. Young male smokers have altered platelets and endothelium that precedes atherosclerosis. *J Surg Res*. 2004;116:227-233
538. Bernal-Mizrachi L, Jy W, Jimenez JJ, Pastor J, Mauro LM, Horstman LL, de Marchena E, Ahn YS. High levels of circulating endothelial microparticles in patients with acute coronary syndromes. *Am Heart J*. 2003;145:962-970
539. van der Zee PM, Biro E, Ko Y, de Winter RJ, Hack CE, Sturk A, Nieuwland R. P-selectin- and cd63-exposing platelet microparticles reflect platelet activation in peripheral arterial disease and myocardial infarction. *Clin Chem*. 2006;52:657-664
540. Craft JA, Marsh NA. Increased generation of platelet-derived microparticles following percutaneous transluminal coronary angioplasty. *Blood Coagul Fibrinolysis*. 2003;14:719-728
541. Zeiger F, Stephan S, Hoheisel G, Pfeiffer D, Ruehlmann C, Kokschi M. P-selectin expression, platelet aggregates, and platelet-derived microparticle formation are increased in peripheral arterial disease. *Blood Coagul Fibrinolysis*. 2000;11:723-728
542. Nomura S, Imamura A, Okuno M, Kamiyama Y, Fujimura Y, Ikeda Y, Fukuhara S. Platelet-derived microparticles in patients with arteriosclerosis obliterans: Enhancement of high shear-induced microparticle generation by cytokines. *Thromb Res*. 2000;98:257-268

543. Chironi GN, Simon A, Boulanger CM, Dignat-George F, Hugel B, Megnien JL, Lefort M, Freyssinet JM, Tedgui A. Circulating microparticles may influence early carotid artery remodeling. *J Hypertens*. 2009
544. Michelsen AE, Brodin E, Brosstad F, Hansen JB. Increased level of platelet microparticles in survivors of myocardial infarction. *Scand J Clin Lab Invest*. 2008;68:386-392
545. Chironi G, Simon A, Hugel B, Del Pino M, Garipey J, Freyssinet JM, Tedgui A. Circulating leukocyte-derived microparticles predict subclinical atherosclerosis burden in asymptomatic subjects. *Arterioscler Thromb Vasc Biol*. 2006;26:2775-2780
546. Huisse MG, Lanoy E, Tcheche D, Feldman LJ, Bezeaud A, Angles-Cano E, Mary-Krause M, de Prost D, Guillin MC, Steg PG. Prothrombotic markers and early spontaneous recanalization in st-segment elevation myocardial infarction. *Thromb Haemost*. 2007;98:420-426
547. Singh N, Gemmell CH, Daly PA, Yeo EL. Elevated platelet-derived microparticle levels during unstable angina. *Can J Cardiol*. 1995;11:1015-1021
548. Matsumoto N, Nomura S, Kamihata H, Kimura Y, Iwasaka T. Association of platelet-derived microparticles with c-c chemokines on vascular complication in patients with acute myocardial infarction. *Clin Appl Thromb Hemost*. 2002;8:279-286
549. Simak J, Gelderman MP, Yu H, Wright V, Baird AE. Circulating endothelial microparticles in acute ischemic stroke: A link to severity, lesion volume and outcome. *J Thromb Haemost*. 2006;4:1296-1302
550. Jung KH, Chu K, Lee ST, Park HK, Bahn JJ, Kim DH, Kim JH, Kim M, Kun Lee S, Roh JK. Circulating endothelial microparticles as a marker of cerebrovascular disease. *Ann Neurol*. 2009;66:191-199
551. Lee YJ, Jy W, Horstman LL, Janania J, Reyes Y, Kelley RE, Ahn YS. Elevated platelet microparticles in transient ischemic attacks, lacunar infarcts, and multiinfarct dementias. *Thromb Res*. 1993;72:295-304
552. Kuriyama N, Nagakane Y, Hosomi A, Ohara T, Kasai T, Harada S, Takeda K, Yamada K, Ozasa K, Tokuda T, Watanabe Y, Mizuno T, Nakagawa M. Evaluation of factors associated with elevated levels of platelet-derived microparticles in the acute phase of cerebral infarction. *Clin Appl Thromb Hemost*. 2010;16:26-32
553. Amabile N, Heiss C, Real WM, Minasi P, McGlothlin D, Rame EJ, Grossman W, De Marco T, Yeghiazarians Y. Circulating endothelial microparticle levels predict hemodynamic severity of pulmonary hypertension. *Am J Respir Crit Care Med*. 2008;177:1268-1275
554. Amabile N, Heiss C, Chang V, Angeli FS, Damon L, Rame EJ, McGlothlin D, Grossman W, De Marco T, Yeghiazarians Y. Increased cd62e(+) endothelial microparticle levels predict

- poor outcome in pulmonary hypertension patients. *J Heart Lung Transplant*. 2009;28:1081-1086
555. Shet AS, Aras O, Gupta K, Hass MJ, Rausch DJ, Saba N, Koopmeiners L, Key NS, Hebbel RP. Sickle blood contains tissue factor-positive microparticles derived from endothelial cells and monocytes. *Blood*. 2003;102:2678-2683
556. Kushak RI, Nestoridi E, Lambert J, Selig MK, Ingelfinger JR, Grabowski EF. Detached endothelial cells and microparticles as sources of tissue factor activity. *Thromb Res*. 2005;116:409-419
557. Jy W, Jimenez JJ, Mauro LM, Horstman LL, Cheng P, Ahn ER, Bidot CJ, Ahn YS. Endothelial microparticles induce formation of platelet aggregates via a von willebrand factor/ristocetin dependent pathway, rendering them resistant to dissociation. *J Thromb Haemost*. 2005;3:1301-1308
558. Brodsky SV, Malinowski K, Golightly M, Jesty J, Goligorsky MS. Plasminogen activator inhibitor-1 promotes formation of endothelial microparticles with procoagulant potential. *Circulation*. 2002;106:2372-2378
559. Nozaki T, Sugiyama S, Koga H, Sugamura K, Ohba K, Matsuzawa Y, Sumida H, Matsui K, Jinnouchi H, Ogawa H. Significance of a multiple biomarkers strategy including endothelial dysfunction to improve risk stratification for cardiovascular events in patients at high risk for coronary heart disease. *J Am Coll Cardiol*. 2009;54:601-608
560. Abrams CS, Ellison N, Budzynski AZ, Shattil SJ. Direct detection of activated platelets and platelet-derived microparticles in humans. *Blood*. 1990;75:128-138
561. Scharf RE, Tomer A, Marzec UM, Teirstein PS, Ruggeri ZM, Harker LA. Activation of platelets in blood perfusing angioplasty-damaged coronary arteries. Flow cytometric detection. *Arterioscler Thromb*. 1992;12:1475-1487
562. Flaumenhaft R, Dilks JR, Richardson J, Alden E, Patel-Hett SR, Battinelli E, Klement GL, Sola-Visner M, Italiano JE, Jr. Megakaryocyte-derived microparticles: Direct visualization and distinction from platelet-derived microparticles. *Blood*. 2009;113:1112-1121
563. Biro E, Akkerman JW, Hoek FJ, Gorter G, Pronk LM, Sturk A, Nieuwland R. The phospholipid composition and cholesterol content of platelet-derived microparticles: A comparison with platelet membrane fractions. *J Thromb Haemost*. 2005;3:2754-2763
564. Tans G, Rosing J, Thomassen MC, Heeb MJ, Zwaal RF, Griffin JH. Comparison of anticoagulant and procoagulant activities of stimulated platelets and platelet-derived microparticles. *Blood*. 1991;77:2641-2648

565. Sinauridze EI, Kireev DA, Popenko NY, Pichugin AV, Panteleev MA, Krymskaya OV, Ataulakhanov FI. Platelet microparticle membranes have 50- to 100-fold higher specific procoagulant activity than activated platelets. *Thromb Haemost.* 2007;97:425-434
566. Ilveskero S, Siljander P, Lassila R. Procoagulant activity on platelets adhered to collagen or plasma clot. *Arterioscler Thromb Vasc Biol.* 2001;21:628-635
567. Ueba T, Haze T, Sugiyama M, Higuchi M, Asayama H, Karitani Y, Nishikawa T, Yamashita K, Nagami S, Nakayama T, Kanatani K, Nomura S. Level, distribution and correlates of platelet-derived microparticles in healthy individuals with special reference to the metabolic syndrome. *Thromb Haemost.* 2008;100:280-285
568. Sossdorf M, Otto GP, Claus RA, Gabriel HH, Losche W. Release of pro-coagulant microparticles after moderate endurance exercise. *Platelets.* 2010
569. Dale GL, Remenyi G, Friese P. Tetraspanin cd9 is required for microparticle release from coated-platelets. *Platelets.* 2009;20:361-366
570. Merten M, Pakala R, Thiagarajan P, Benedict CR. Platelet microparticles promote platelet interaction with subendothelial matrix in a glycoprotein iib/iiiA-dependent mechanism. *Circulation.* 1999;99:2577-2582
571. Siljander P, Carpen O, Lassila R. Platelet-derived microparticles associate with fibrin during thrombosis. *Blood.* 1996;87:4651-4663
572. Ramacciotti E, Hawley AE, Farris DM, Ballard NE, Wroblewski SK, Myers DD, Jr., Henke PK, Wakefield TW. Leukocyte- and platelet-derived microparticles correlate with thrombus weight and tissue factor activity in an experimental mouse model of venous thrombosis. *Thromb Haemost.* 2009;101:748-754
573. Bal L, Ederhy S, Di Angelantonio E, Toti F, Zobairi F, Dufaitre G, Meuleman C, Mallat Z, Boccara F, Tedgui A, Freyssinet JM, Cohen A. Circulating procoagulant microparticles in acute pulmonary embolism: A case-control study. *Int J Cardiol.* 2009
574. Azzam H, Zagloul M. Elevated platelet microparticle levels in valvular atrial fibrillation. *Hematology.* 2009;14:357-360
575. Connor DE, Exner T, Ma DD, Joseph JE. The majority of circulating platelet-derived microparticles fail to bind annexin v, lack phospholipid-dependent procoagulant activity and demonstrate greater expression of glycoprotein iB. *Thromb Haemost.* 2010;103:1044-1052
576. Morel O, Morel N, Freyssinet JM, Toti F. Platelet microparticles and vascular cells interactions: A checkpoint between the haemostatic and thrombotic responses. *Platelets.* 2008;19:9-23
577. Barry OP, Pratico D, Savani RC, FitzGerald GA. Modulation of monocyte-endothelial cell interactions by platelet microparticles. *J Clin Invest.* 1998;102:136-144



578. Gambim MH, do Carmo Ade O, Marti L, Verissimo-Filho S, Lopes LR, Janiszewski M. Platelet-derived exosomes induce endothelial cell apoptosis through peroxynitrite generation: Experimental evidence for a novel mechanism of septic vascular dysfunction. *Crit Care*. 2007;11:R107
579. Jy W, Mao WW, Horstman L, Tao J, Ahn YS. Platelet microparticles bind, activate and aggregate neutrophils in vitro. *Blood Cells Mol Dis*. 1995;21:217-231; discussion 231a
580. George JN, Pickett EB, Saucerman S, McEver RP, Kunicki TJ, Kieffer N, Newman PJ. Platelet surface glycoproteins. Studies on resting and activated platelets and platelet membrane microparticles in normal subjects, and observations in patients during adult respiratory distress syndrome and cardiac surgery. *J Clin Invest*. 1986;78:340-348
581. Barry OP, FitzGerald GA. Mechanisms of cellular activation by platelet microparticles. *Thromb Haemost*. 1999;82:794-800
582. Wencel-Drake JD, Dieter MG, Lam SC. Immunolocalization of beta 1 integrins in platelets and platelet-derived microvesicles. *Blood*. 1993;82:1197-1203
583. Forlow SB, McEver RP, Nollert MU. Leukocyte-leukocyte interactions mediated by platelet microparticles under flow. *Blood*. 2000;95:1317-1323
584. Boing AN, Hau CM, Sturk A, Nieuwland R. Platelet microparticles contain active caspase 3. *Platelets*. 2008;19:96-103
585. Li Y, Lee PY, Sobel ES, Narain S, Satoh M, Segal MS, Reeves WH, Richards HB. Increased expression of fcgammari/cd64 on circulating monocytes parallels ongoing inflammation and nephritis in lupus. *Arthritis Res Ther*. 2009;11:R6
586. Kim KW, Cho ML, Park MK, Yoon CH, Park SH, Lee SH, Kim HY. Increased interleukin-17 production via a phosphoinositide 3-kinase/akt and nuclear factor kappa-dependent pathway in patients with rheumatoid arthritis. *Arthritis Res Ther*. 2005;7:R139-148
587. Martinez MC, Tesse A, Zobairi F, Andriantsitohaina R. Shed membrane microparticles from circulating and vascular cells in regulating vascular function. *Am J Physiol Heart Circ Physiol*. 2005;288:H1004-1009
588. Dean WL, Lee MJ, Cummins TD, Schultz DJ, Powell DW. Proteomic and functional characterisation of platelet microparticle size classes. *Thromb Haemost*. 2009;102:711-718
589. Nomura S, Inami N, Shouzu A, Urase F, Maeda Y. Correlation and association between plasma platelet-, monocyte- and endothelial cell-derived microparticles in hypertensive patients with type 2 diabetes mellitus. *Platelets*. 2009;20:406-414
590. Rauch U, Bonderman D, Bohrmann B, Badimon JJ, Hember J, Riederer MA, Nemerson Y. Transfer of tissue factor from leukocytes to platelets is mediated by cd15 and tissue factor. *Blood*. 2000;96:170-175

591. Aharon A, Katzenell S, Tamari T, Brenner B. Microparticles bearing tissue factor and tissue factor pathway inhibitor in gestational vascular complications. *J Thromb Haemost.* 2009;7:1047-1050
592. Wang JG, Manly D, Kirchhofer D, Pawlinski R, Mackman N. Levels of microparticle tissue factor activity correlate with coagulation activation in endotoxemic mice. *J Thromb Haemost.* 2009;7:1092-1098
593. Aharon A, Tamari T, Brenner B. Monocyte-derived microparticles and exosomes induce procoagulant and apoptotic effects on endothelial cells. *Thromb Haemost.* 2008;100:878-885
594. Mayr FB, Spiel AO, Leitner JM, Firbas C, Jilma-Stohlawetz P, Chang JY, Key NS, Jilma B. Racial differences in endotoxin-induced tissue factor-triggered coagulation. *J Thromb Haemost.* 2009;7:634-640
595. Aras O, Shet A, Bach RR, Hysjulien JL, Slungaard A, Hebbel RP, Escolar G, Jilma B, Key NS. Induction of microparticle- and cell-associated intravascular tissue factor in human endotoxemia. *Blood.* 2004;103:4545-4553
596. Pluskota E, Woody NM, Szpak D, Ballantyne CM, Soloviev DA, Simon DI, Plow EF. Expression, activation, and function of integrin alphabeta2 (mac-1) on neutrophil-derived microparticles. *Blood.* 2008;112:2327-2335
597. Gasser O, Hess C, Miot S, Deon C, Sanchez JC, Schifferli JA. Characterisation and properties of ectosomes released by human polymorphonuclear neutrophils. *Exp Cell Res.* 2003;285:243-257
598. Mesri M, Altieri DC. Endothelial cell activation by leukocyte microparticles. *J Immunol.* 1998;161:4382-4387
599. Scanu A, Molnarfi N, Brandt KJ, Gruaz L, Dayer JM, Burger D. Stimulated t cells generate microparticles, which mimic cellular contact activation of human monocytes: Differential regulation of pro- and anti-inflammatory cytokine production by high-density lipoproteins. *J Leukoc Biol.* 2008;83:921-927
600. Flores-Nascimento MC, Beltrame MP, De Paula EV, Montalvao SL, Pereira FG, Orsi FL, Lorand-Metze I, Annichino-Bizzacchi JM. Microparticles in deep venous thrombosis, antiphospholipid syndrome and factor v leiden. *Platelets.* 2009;20:367-375
601. Bernimoulin M, Waters EK, Foy M, Steele BM, Sullivan M, Falet H, Walsh MT, Barteneva N, Geng JG, Hartwig JH, Maguire PB, Wagner DD. Differential stimulation of monocytic cells results in distinct populations of microparticles. *J Thromb Haemost.* 2009;7:1019-1028

602. Zwicker JI, Liebman HA, Neuberg D, Lacroix R, Bauer KA, Furie BC, Furie B. Tumor-derived tissue factor-bearing microparticles are associated with venous thromboembolic events in malignancy. *Clin Cancer Res.* 2009;15:6830-6840
603. Tesselaar ME, Romijn FP, van der Linden IK, Bertina RM, Osanto S. Microparticle-associated tissue factor activity in cancer patients with and without thrombosis. *J Thromb Haemost.* 2009;7:1421-1423
604. Haubold K, Rink M, Spath B, Friedrich M, Chun FK, Marx G, Amirkhosravi A, Francis JL, Bokemeyer C, Eifrig B, Langer F. Tissue factor procoagulant activity of plasma microparticles is increased in patients with early-stage prostate cancer. *Thromb Haemost.* 2009;101:1147-1155
605. Toth B, Liebhardt S, Steinig K, Ditsch N, Rank A, Bauerfeind I, Spannagl M, Friese K, Reininger AJ. Platelet-derived microparticles and coagulation activation in breast cancer patients. *Thromb Haemost.* 2008;100:663-669
606. Davila M, Amirkhosravi A, Coll E, Desai H, Robles L, Colon J, Baker CH, Francis JL. Tissue factor-bearing microparticles derived from tumor cells: Impact on coagulation activation. *J Thromb Haemost.* 2008;6:1517-1524
607. Leroyer AS, Ebrahimian TG, Cochain C, Recalde A, Blanc-Brude O, Mees B, Vilar J, Tedgui A, Levy BI, Chimini G, Boulanger CM, Silvestre JS. Microparticles from ischemic muscle promotes postnatal vasculogenesis. *Circulation.* 2009;119:2808-2817
608. Heloire F, Weill B, Weber S, Batteux F. Aggregates of endothelial microparticles and platelets circulate in peripheral blood. Variations during stable coronary disease and acute myocardial infarction. *Thromb Res.* 2003;110:173-180
609. Nagy B, Jr., Szuk T, Debreceni IB, Kappelmayer J. Platelet-derived microparticle levels are significantly elevated in patients treated by elective stenting compared to subjects with diagnostic catheterization alone. *Platelets.* 2010;21:147-151
610. Michelsen AE, Noto AT, Brodin E, Mathiesen EB, Brosstad F, Hansen JB. Elevated levels of platelet microparticles in carotid atherosclerosis and during the postprandial state. *Thromb Res.* 2009;123:881-886
611. Mayr M, Grainger D, Mayr U, Leroyer AS, Leseche G, Sidibe A, Herbin O, Yin X, Gomes A, Madhu B, Griffiths JR, Xu Q, Tedgui A, Boulanger CM. Proteomics, metabolomics, and immunomics on microparticles derived from human atherosclerotic plaques. *Circ Cardiovasc Genet.* 2009;2:379-388
612. Leroyer AS, Rautou PE, Silvestre JS, Castier Y, Leseche G, Devue C, Duriez M, Brandes RP, Lutgens E, Tedgui A, Boulanger CM. Cd40 ligand+ microparticles from human

- atherosclerotic plaques stimulate endothelial proliferation and angiogenesis a potential mechanism for intraplaque neovascularization. *J Am Coll Cardiol.* 2008;52:1302-1311
613. Canault M, Leroyer AS, Peiretti F, Leseche G, Tedgui A, Bonardo B, Alessi MC, Boulanger CM, Nalbone G. Microparticles of human atherosclerotic plaques enhance the shedding of the tumor necrosis factor-alpha converting enzyme/adam17 substrates, tumor necrosis factor and tumor necrosis factor receptor-1. *Am J Pathol.* 2007;171:1713-1723
614. Mallat Z, Hugel B, Ohan J, Leseche G, Freyssinet JM, Tedgui A. Shed membrane microparticles with procoagulant potential in human atherosclerotic plaques: A role for apoptosis in plaque thrombogenicity. *Circulation.* 1999;99:348-353
615. Namba M, Tanaka A, Shimada K, Ozeki Y, Uehata S, Sakamoto T, Nishida Y, Nomura S, Yoshikawa J. Circulating platelet-derived microparticles are associated with atherothrombotic events: A marker for vulnerable blood. *Arterioscler Thromb Vasc Biol.* 2007;27:255-256
616. Gawaz M, Neumann FJ, Ott I, Schiessler A, Schomig A. Platelet function in acute myocardial infarction treated with direct angioplasty. *Circulation.* 1996;93:229-237
617. Katopodis JN, Kolodny L, Jy W, Horstman LL, De Marchena EJ, Tao JG, Haynes DH, Ahn YS. Platelet microparticles and calcium homeostasis in acute coronary ischemias. *Am J Hematol.* 1997;54:95-101
618. Bernal-Mizrachi L, Jy W, Fierro C, Macdonough R, Velazques HA, Purow J, Jimenez JJ, Horstman LL, Ferreira A, de Marchena E, Ahn YS. Endothelial microparticles correlate with high-risk angiographic lesions in acute coronary syndromes. *Int J Cardiol.* 2004;97:439-446
619. Morel O, Pereira B, Averous G, Faure A, Jesel L, Germain P, Grunebaum L, Ohlmann P, Freyssinet JM, Bareiss P, Toti F. Increased levels of procoagulant tissue factor-bearing microparticles within the occluded coronary artery of patients with st-segment elevation myocardial infarction: Role of endothelial damage and leukocyte activation. *Atherosclerosis.* 2009;204:636-641
620. Keuren JF, Jie KS, Leers MP. Increased expression of tf on monocytes, but decreased numbers of tf bearing microparticles in blood from patients with acute myocardial infarction. *Eur J Haematol.* 2009;83:387-388
621. Maly M, Hrachovinova I, Tomasov P, Salaj P, Hajek P, Veselka J. Patients with acute coronary syndromes have low tissue factor activity and microparticle count, but normal concentration of tissue factor antigen in platelet free plasma: A pilot study. *Eur J Haematol.* 2009;82:148-153

622. Huisse MG, Ajzenberg N, Feldman L, Guillin MC, Steg PG. Microparticle-linked tissue factor activity and increased thrombin activity play a potential role in fibrinolysis failure in ST-segment elevation myocardial infarction. *Thromb Haemost.* 2009;101:734-740
623. Steppich BA, Braun SL, Stein A, Demetz G, Groha P, Schomig A, von Beckerath N, Kastrati A, Ott I. Plasma TF activity predicts cardiovascular mortality in patients with acute myocardial infarction. *Thromb J.* 2009;7:11
624. Horstman LL, Jy W, Bidot CJ, Nordberg ML, Minagar A, Alexander JS, Kelley RE, Ahn YS. Potential roles of cell-derived microparticles in ischemic brain disease. *Neurol Res.* 2009;31:799-806
625. Geiser T, Sturzenegger M, Genewein U, Haerberli A, Beer JH. Mechanisms of cerebrovascular events as assessed by procoagulant activity, cerebral microemboli, and platelet microparticles in patients with prosthetic heart valves. *Stroke.* 1998;29:1770-1777
626. Lukasik M, Rozalski M, Luzak B, Michalak S, Kozubski W, Watala C. Platelet activation and reactivity in the convalescent phase of ischaemic stroke. *Thromb Haemost.* 2010;103
627. Williams JB, Jauch EC, Lindsell CJ, Campos B. Endothelial microparticle levels are similar in acute ischemic stroke and stroke mimics due to activation and not apoptosis/necrosis. *Acad Emerg Med.* 2007;14:685-690
628. Nozaki T, Sugiyama S, Sugamura K, Ohba K, Matsuzawa Y, Konishi M, Matsubara J, Akiyama E, Sumida H, Matsui K, Jinnouchi H, Ogawa H. Prognostic value of endothelial microparticles in patients with heart failure. *Eur J Heart Fail.* 2010;12:1223-1228
629. Bulut D, Scheeler M, Niedballa LM, Miebach T, Mugge A. Effects of immunoadsorption on endothelial function, circulating endothelial progenitor cells and circulating microparticles in patients with inflammatory dilated cardiomyopathy. *Clin Res Cardiol.* 2011;100:603-610
630. Garcia S, Chirinos J, Jimenez J, Del Carpio Munoz F, Canoniero M, Jy W, Horstman L, Ahn Y. Phenotypic assessment of endothelial microparticles in patients with heart failure and after heart transplantation: Switch from cell activation to apoptosis. *J Heart Lung Transplant.* 2005;24:2184-2189
631. Levy D, Kenchaiah S, Larson MG, Benjamin EJ, Kupka MJ, Ho KK, Murabito JM, Vasan RS. Long-term trends in the incidence of and survival with heart failure. *N Engl J Med.* 2002;347:1397-1402
632. Roger VL, Weston SA, Redfield MM, Hellermann-Homan JP, Killian J, Yawn BP, Jacobsen SJ. Trends in heart failure incidence and survival in a community-based population. *JAMA.* 2004;292:344-350
633. Shantsila E, Lip GY, Gill PS. Systolic heart failure in south asians. *Int J Clin Pract.* 2011;65:1274-1282

634. Blann A, Shantsila E, Shantsila A. Microparticles and arterial disease. *Semin Thromb Hemost.* 2009;35:488-496
635. Shantsila E, Wrigley B, Tapp L, Apostolakis S, Montoro-Garcia S, Drayson MT, Lip GY. Immunophenotypic characterization of human monocyte subsets: Possible implications for cardiovascular disease pathophysiology. *J Thromb Haemost.* 2011;9:1056-1066
636. Shantsila E, Lip GY. The role of monocytes in thrombotic disorders. Insights from tissue factor, monocyte-platelet aggregates and novel mechanisms. *Thromb Haemost.* 2009;102:916-924
637. Shantsila E, Kamphuisen PW, Lip GY. Circulating microparticles in cardiovascular disease: Implications for atherogenesis and atherothrombosis. *J Thromb Haemost.* 2010;8:2358-2368
638. Sieveking DP, Buckle A, Celermajer DS, Ng MK. Strikingly different angiogenic properties of endothelial progenitor cell subpopulations: Insights from a novel human angiogenesis assay. *J Am Coll Cardiol.* 2008;51:660-668
639. Rohde E, Malischnik C, Thaler D, Maierhofer T, Linkesch W, Lanzer G, Guelly C, Strunk D. Blood monocytes mimic endothelial progenitor cells. *Stem Cells.* 2006;24:357-367
640. Rohde E, Bartmann C, Schallmoser K, Reinisch A, Lanzer G, Linkesch W, Guelly C, Strunk D. Immune cells mimic the morphology of endothelial progenitor colonies in vitro. *Stem Cells.* 2007;25:1746-1752
641. Krenning G, van der Strate BW, Schipper M, van Seijen XJ, Fernandes BC, van Luyn MJ, Harmsen MC. Cd34+ cells augment endothelial cell differentiation of cd14+ endothelial progenitor cells in vitro. *J Cell Mol Med.* 2009;13:2521-2533
642. Fujiyama S, Amano K, Uehira K, Yoshida M, Nishiwaki Y, Nozawa Y, Jin D, Takai S, Miyazaki M, Egashira K, Imada T, Iwasaka T, Matsubara H. Bone marrow monocyte lineage cells adhere on injured endothelium in a monocyte chemoattractant protein-1-dependent manner and accelerate reendothelialization as endothelial progenitor cells. *Circ Res.* 2003;93:980-989
643. Corretti MC, Anderson TJ, Benjamin EJ, Celermajer D, Charbonneau F, Creager MA, Deanfield J, Drexler H, Gerhard-Herman M, Herrington D, Vallance P, Vita J, Vogel R. Guidelines for the ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery: A report of the international brachial artery reactivity task force. *J Am Coll Cardiol.* 2002;39:257-265
644. Mohan JS, Lip GY, Blann AD, Bareford D, Marshall JM. Endothelium-dependent and endothelium-independent vasodilatation of the cutaneous circulation in sickle cell disease. *Eur J Clin Invest.* 2011;41:546-551

645. Cracowski JL, Minson CT, Salvat-Melis M, Halliwill JR. Methodological issues in the assessment of skin microvascular endothelial function in humans. *Trends Pharmacol Sci.* 2006;27:503-508
646. Quinones MA, Otto CM, Stoddard M, Waggoner A, Zoghbi WA. Recommendations for quantification of doppler echocardiography: A report from the doppler quantification task force of the nomenclature and standards committee of the american society of echocardiography. *J Am Soc Echocardiogr.* 2002;15:167-184
647. Nagueh SF, Appleton CP, Gillebert TC, Marino PN, Oh JK, Smiseth OA, Waggoner AD, Flachskampf FA, Pellikka PA, Evangelisa A. Recommendations for the evaluation of left ventricular diastolic function by echocardiography. *Eur J Echocardiogr.* 2009;10:165-193
648. Lang RM, Bierig M, Devereux RB, Flachskampf FA, Foster E, Pellikka PA, Picard MH, Roman MJ, Seward J, Shanewise JS, Solomon SD, Spencer KT, Sutton MS, Stewart WJ. Recommendations for chamber quantification: A report from the american society of echocardiography's guidelines and standards committee and the chamber quantification writing group, developed in conjunction with the european association of echocardiography, a branch of the european society of cardiology. *J Am Soc Echocardiogr.* 2005;18:1440-1463
649. Shantsila A, Dwivedi G, Shantsila E, Butt M, Beevers DG, Lip GY. Persistent macrovascular and microvascular dysfunction in patients with malignant hypertension. *Hypertension.* 2011;57:490-496
650. Henry RM, Ferreira I, Kostense PJ, Dekker JM, Nijpels G, Heine RJ, Kamp O, Bouter LM, Stehouwer CD. Type 2 diabetes is associated with impaired endothelium-dependent, flow-mediated dilation, but impaired glucose metabolism is not; the hoorn study. *Atherosclerosis.* 2004;174:49-56
651. Seino Y, Tsukamoto H, Ohki K, Nakamura T, Kashiwagi M, Takano T, Hayakawa H. Abnormal cutaneous vasomotion and reduced cutaneous blood mass remain in congestive heart failure even with normalized cardiovascular hemodynamics. *Am Heart J.* 1993;126:887-895
652. Balmain S, Padmanabhan N, Ferrell WR, Morton JJ, McMurray JJ. Differences in arterial compliance, microvascular function and venous capacitance between patients with heart failure and either preserved or reduced left ventricular systolic function. *Eur J Heart Fail.* 2007;9:865-871
653. Testa M, Yeh M, Lee P, Fanelli R, Loperfido F, Berman JW, LeJemtel TH. Circulating levels of cytokines and their endogenous modulators in patients with mild to severe congestive heart failure due to coronary artery disease or hypertension. *J Am Coll Cardiol.* 1996;28:964-971

654. Wu JR, Lennie TA, De Jong MJ, Frazier SK, Heo S, Chung ML, Moser DK. Medication adherence is a mediator of the relationship between ethnicity and event-free survival in patients with heart failure. *J Card Fail.* 2010;16:142-149
655. Roberts CB, Couper DJ, Chang PP, James SA, Rosamond WD, Heiss G. Influence of life-course socioeconomic position on incident heart failure in blacks and whites: The atherosclerosis risk in communities study. *Am J Epidemiol.* 2010;172:717-727
656. Jolly S, Vittinghoff E, Chattopadhyay A, Bibbins-Domingo K. Higher cardiovascular disease prevalence and mortality among younger blacks compared to whites. *Am J Med.* 2010;123:811-818
657. Markham DW, Dries DL, King LP, Leonard D, Yancy CW, Peshock RM, Willett D, Cooper RS, Drazner MH. Blacks and whites have a similar prevalence of reduced left ventricular ejection fraction in the general population: The dallas heart study (dhs). *Am Heart J.* 2008;155:876-882
658. Krum H, Abraham WT. Heart failure. *Lancet.* 2009;373:941-955
659. Shantsila E, Lip GY. Monocytes in acute coronary syndromes. *Arterioscler Thromb Vasc Biol.* 2009;29:1433-1438
660. Apostolakis S, Lip GY, Shantsila E. Monocytes in heart failure: Relationship to a deteriorating immune overreaction or a desperate attempt for tissue repair? *Cardiovasc Res.* 2010;85:649-660
661. Hendrikx M, Hensen K, Clijsters C, Jongen H, Koninckx R, Bijmens E, Ingels M, Jacobs A, Geukens R, Dendale P, Vijgen J, Dilling D, Steels P, Mees U, Rummens JL. Recovery of regional but not global contractile function by the direct intramyocardial autologous bone marrow transplantation: Results from a randomized controlled clinical trial. *Circulation.* 2006;114:1101-107
662. Jerrard-Dunne P, Evans A, McGovern R, Hajat C, Kalra L, Rudd AG, Wolfe CD, Markus HS. Ethnic differences in markers of thrombophilia: Implications for the investigation of ischemic stroke in multiethnic populations: The south london ethnicity and stroke study. *Stroke.* 2003;34:1821-1826
663. Birns J, Morris R, Jarosz J, Markus H, Kalra L. Ethnic differences in the cerebrovascular impact of hypertension. *Cerebrovasc Dis.* 2008;25:408-416
664. Markus HS, Khan U, Birns J, Evans A, Kalra L, Rudd AG, Wolfe CD, Jerrard-Dunne P. Differences in stroke subtypes between black and white patients with stroke: The south london ethnicity and stroke study. *Circulation.* 2007;116:2157-2164



665. Kalra L, Rambaran C, Chowienczyk P, Goss D, Hambleton I, Ritter J, Shah A, Wilks R, Forrester T. Ethnic differences in arterial responses and inflammatory markers in afro-caribbean and caucasian subjects. *Arterioscler Thromb Vasc Biol.* 2005;25:2362-2367
666. Kalra L, Iveson E, Rambaran C, Sherwood R, Chowienczyk P, Ritter J, Shah A, Forrester T. An international matched cohort study of the contribution of metabolic impairments to subclinical atherosclerosis in united kingdom and jamaican african-caribbeans. *Atherosclerosis.* 2008;199:95-101
667. Kalra L, Iveson E, Rambaran C, Sherwood R, Chowienczyk P, Ritter J, Shah A, Forrester T. Homocysteine, migration and early vascular impairment in people of african descent. *Heart.* 2008;94:1171-1174
668. Khan U, Crossley C, Kalra L, Rudd A, Wolfe CD, Collinson P, Markus HS. Homocysteine and its relationship to stroke subtypes in a uk black population: The south london ethnicity and stroke study. *Stroke.* 2008;39:2943-2949
669. Kalra L, Rambaran C, Iveson E, Chowienczyk PJ, Hambleton I, Ritter JM, Shah A, Wilks R, Forrester T. The role of inheritance and environment in predisposition to vascular disease in people of african descent. *J Am Coll Cardiol.* 2006;47:1126-1133
670. Rambaran C, Chowienczyk P, Ritter J, Shah A, Wilks R, Forrester T, Kalra L. The vascular effects of metabolic impairment clusters in subjects of different ethnicities. *Atherosclerosis.* 2007;192:354-362
671. Watson T, Shantsila E, Karthikeyan VJ, Jessani S, Goon PK, Lip GY. The effects of exercise stress testing, diurnal variation and temporal decline on circulating progenitor cells. *Thromb Haemost.* 2010;103:419-425
672. Yamamoto Y, Gaynor RB. Ikkappa kinases: Key regulators of the nf-kappa pathway. *Trends Biochem Sci.* 2004;29:72-79
673. Hayden MS, Ghosh S. Signaling to nf-kappa. *Genes Dev.* 2004;18:2195-2224
674. Hu Y, Baud V, Delhase M, Zhang P, Deerinck T, Ellisman M, Johnson R, Karin M. Abnormal morphogenesis but intact ikk activation in mice lacking the ikkalph subunit of ikkappa kinase. *Science.* 1999;284:316-320
675. Mercurio F, Zhu H, Murray BW, Shevchenko A, Bennett BL, Li J, Young DB, Barbosa M, Mann M, Manning A, Rao A. Ikk-1 and ikk-2: Cytokine-activated ikkappa kinases essential for nf-kappa activation. *Science.* 1997;278:860-866
676. Ritchie ME. Nuclear factor-kappa is selectively and markedly activated in humans with unstable angina pectoris. *Circulation.* 1998;98:1707-1713
677. Shantsila E, Watson T, Tse HF, Lip GY. Endothelial colony forming units: Are they a reliable marker of endothelial progenitor cell numbers? *Ann Med.* 2007;39:474-479

678. Sunderkotter C, Nikolic T, Dillon MJ, Van Rooijen N, Stehling M, Drevets DA, Leenen PJ. Subpopulations of mouse blood monocytes differ in maturation stage and inflammatory response. *J Immunol.* 2004;172:4410-4417
679. Frankenberger M, Sternsdorf T, Pechumer H, Pforte A, Ziegler-Heitbrock HW. Differential cytokine expression in human blood monocyte subpopulations: A polymerase chain reaction analysis. *Blood.* 1996;87:373-377
680. Cros J, Cagnard N, Woollard K, Patey N, Zhang SY, Senechal B, Puel A, Biswas SK, Moshous D, Picard C, Jais JP, D'Cruz D, Casanova JL, Trouillet C, Geissmann F. Human cd14dim monocytes patrol and sense nucleic acids and viruses via tlr7 and tlr8 receptors. *Immunity.* 2010;33:375-386
681. Rogacev KS, Seiler S, Zawada AM, Reichart B, Herath E, Roth D, Ulrich C, Fliser D, Heine GH. Cd14<sup>++</sup>cd16<sup>+</sup> monocytes and cardiovascular outcome in patients with chronic kidney disease. *Eur Heart J.* 2011;32:84-92
682. De Palma M, Venneri MA, Galli R, Sergi L, Politi LS, Sampaolesi M, Naldini L. Tie2 identifies a hematopoietic lineage of proangiogenic monocytes required for tumor vessel formation and a mesenchymal population of pericyte progenitors. *Cancer Cell.* 2005;8:211-226
683. Venneri MA, De Palma M, Ponzoni M, Pucci F, Scielzo C, Zonari E, Mazzieri R, Doglioni C, Naldini L. Identification of proangiogenic tie2-expressing monocytes (tems) in human peripheral blood and cancer. *Blood.* 2007;109:5276-5285
684. Skrzeczynska-Moncznik J, Bzowska M, Loseke S, Grage-Griebenow E, Zembala M, Pryjma J. Peripheral blood cd14<sup>high</sup> cd16<sup>+</sup> monocytes are main producers of il-10. *Scand J Immunol.* 2008;67:152-159
685. Salonen JT, Nyyssonen K, Salonen R. Body iron stores and the risk of coronary heart disease. *N Engl J Med.* 1994;331:1159; author reply 1160
686. You SA, Wang Q. Ferritin in atherosclerosis. *Clin Chim Acta.* 2005;357:1-16
687. Schaer CA, Schoedon G, Imhof A, Kurrer MO, Schaer DJ. Constitutive endocytosis of cd163 mediates hemoglobin-heme uptake and determines the noninflammatory and protective transcriptional response of macrophages to hemoglobin. *Circ Res.* 2006;99:943-950
688. Kristiansen M, Graversen JH, Jacobsen C, Sonne O, Hoffman HJ, Law SK, Moestrup SK. Identification of the haemoglobin scavenger receptor. *Nature.* 2001;409:198-201
689. Yang S, Zhang L. Glucocorticoids and vascular reactivity. *Curr Vasc Pharmacol.* 2004;2:1-12
690. Chandran DS, Jaryal AK, Jyotsna VP, Deepak KK. Impaired endothelium mediated vascular reactivity in endogenous cushing's syndrome. *Endocr J.* 2011;58:789-799

691. Akaza I, Yoshimoto T, Tsuchiya K, Hirata Y. Endothelial dysfunction associated with hypercortisolism is reversible in Cushing's syndrome. *Endocr J*. 2010;57:245-252
692. Billing AM, Fack F, Turner JD, Muller CP. Cortisol is a potent modulator of lipopolysaccharide-induced interferon signaling in macrophages. *Innate Immun*. 2011;17:302-320
693. Billing AM, Fack F, Renaut J, Olinger CM, Schote AB, Turner JD, Muller CP. Proteomic analysis of the cortisol-mediated stress response in thp-1 monocytes using DIGE technology. *J Mass Spectrom*. 2007;42:1433-1444
694. Kohka H, Iwagaki H, Yoshino T, Kobashi K, Saito S, Isozaki H, Takakura N, Tanaka N. Hydrocortisone sodium succinate suppressed production of interleukin-10 by human peripheral blood mononuclear cells: Clinical significance. *Acta Med Okayama*. 1999;53:55-59
695. Rickard AJ, Young MJ. Corticosteroid receptors, macrophages and cardiovascular disease. *J Mol Endocrinol*. 2009;42:449-459
696. Steer JH, Vuong Q, Joyce DA. Suppression of human monocyte tumour necrosis factor- $\alpha$  release by glucocorticoid therapy: Relationship to systemic monocytopenia and cortisol suppression. *Br J Clin Pharmacol*. 1997;43:383-389
697. Ermetici F, Malavazos AE, Corbetta S, Eller-Vainicher C, Cannavo S, Corsi MM, Ambrosi B. Soluble adhesion molecules levels in patients with Cushing's syndrome before and after cure. *J Endocrinol Invest*. 2008;31:389-392
698. Scheff JD, Calvano SE, Lowry SF, Androulakis IP. Modeling the influence of circadian rhythms on the acute inflammatory response. *Journal of theoretical biology*. 2010;264:1068-1076
699. Lange T, Dimitrov S, Fehm HL, Westermann J, Born J. Shift of monocyte function toward cellular immunity during sleep. *Arch Intern Med*. 2006;166:1695-1700
700. Kuckleburg CJ, Yates CM, Kalia N, Zhao Y, Nash GB, Watson SP, Rainger GE. Endothelial cell-borne platelet bridges selectively recruit monocytes in human and mouse models of vascular inflammation. *Cardiovasc Res*. 2011
701. van Gils JM, da Costa Martins PA, Mol A, Hordijk PL, Zwaginga JJ. Transendothelial migration drives dissociation of platelet-monocyte complexes. *Thromb Haemost*. 2008;100:271-279
702. Oliveira M, Gleeson M. The influence of prolonged cycling on monocyte toll-like receptor 2 and 4 expression in healthy men. *Eur J Appl Physiol*. 2010;109:251-257
703. Simpson RJ, McFarlin BK, McSporran C, Spielmann G, O'Hartaigh B, Guy K. Toll-like receptor expression on classic and pro-inflammatory blood monocytes after acute exercise in humans. *Brain Behav Immun*. 2009;23:232-239

704. Hong S, Mills PJ. Effects of an exercise challenge on mobilization and surface marker expression of monocyte subsets in individuals with normal vs. Elevated blood pressure. *Brain Behav Immun.* 2008;22:590-599
705. Timmerman KL, Flynn MG, Coen PM, Markofski MM, Pence BD. Exercise training-induced lowering of inflammatory (cd14+cd16+) monocytes: A role in the anti-inflammatory influence of exercise? *J Leukoc Biol.* 2008;84:1271-1278
706. Kramer PR, Kramer SF, Guan G. 17 beta-estradiol regulates cytokine release through modulation of cd16 expression in monocytes and monocyte-derived macrophages. *Arthritis Rheum.* 2004;50:1967-1975
707. Huehnergath KV, Mozaffarian D, Sullivan MD, Crane BA, Wilkinson CW, Lawler RL, McDonald GB, Fishbein DP, Levy WC. Usefulness of relative lymphocyte count as an independent predictor of death/urgent transplant in heart failure. *Am J Cardiol.* 2005;95:1492-1495
708. Ommen SR, Hammill SC, Gibbons RJ. The relative lymphocyte count predicts death in patients receiving implantable cardioverter defibrillators. *Pacing Clin Electrophysiol.* 2002;25:1424-1428
709. Ommen SR, Hodge DO, Rodeheffer RJ, McGregor CG, Thomson SP, Gibbons RJ. Predictive power of the relative lymphocyte concentration in patients with advanced heart failure. *Circulation.* 1998;97:19-22
710. Milo-Cotter O, Felker GM, Uriel N, Kaluski E, Edwards C, Rund MM, Weatherley BD, Cotter G. Patterns of leukocyte counts on admissions for acute heart failure-presentation and outcome-results from a community based registry. *Int J Cardiol.* 2011;148:17-22
711. Milo-Cotter O, Teerlink JR, Metra M, Felker GM, Ponikowski P, Voors AA, Edwards C, Weatherley BD, Greenberg B, Filippatos G, Unemori E, Teichman SL, Cotter G. Low lymphocyte ratio as a novel prognostic factor in acute heart failure: Results from the pre-relax-hf study. *Cardiology.* 2010;117:190-196
712. Nunez J, Nunez E, Minana G, Sanchis J, Bodi V, Rumiz E, Palau P, Olivares M, Merlos P, Bonanad C, Mainar L, Llacer A. Effectiveness of the relative lymphocyte count to predict one-year mortality in patients with acute heart failure. *Am J Cardiol.* 2011;107:1034-1039
713. Acanfora D, Gheorghide M, Trojano L, Furgi G, Pasini E, Picone C, Papa A, Iannuzzi GL, Bonow RO, Rengo F. Relative lymphocyte count: A prognostic indicator of mortality in elderly patients with congestive heart failure. *Am Heart J.* 2001;142:167-173
714. Charach G, Grosskopf I, Roth A, Afek A, Wexler D, Sheps D, Weintraub M, Rabinovich A, Keren G, George J. Usefulness of total lymphocyte count as predictor of outcome in patients with chronic heart failure. *Am J Cardiol.* 2011;107:1353-1356

715. Li N, Bian H, Zhang J, Li X, Ji X, Zhang Y. The th17/treg imbalance exists in patients with heart failure with normal ejection fraction and heart failure with reduced ejection fraction. *Clin Chim Acta*. 2010;411:1963-1968
716. Rudolph V, Rudolph TK, Hennings JC, Blankenberg S, Schnabel R, Steven D, Haddad M, Knittel K, Wende S, Wenzel J, Munzel T, Heitzer T, Meinertz T, Hubner C, Baldus S. Activation of polymorphonuclear neutrophils in patients with impaired left ventricular function. *Free Radic Biol Med*. 2007;43:1189-1196
717. Frantz S, Stoerk S, Ok S, Wagner H, Angermann CE, Ertl G, Bauersachs J. Effect of chronic heart failure on nuclear factor kappa b in peripheral leukocytes. *Am J Cardiol*. 2004;94:671-673
718. von Haehling S, Schefold JC, Jankowska E, Doehner W, Springer J, Strohschein K, Genth-Zotz S, Volk HD, Poole-Wilson P, Anker SD. Leukocyte redistribution: Effects of beta blockers in patients with chronic heart failure. *PLoS One*. 2009;4:e6411
719. De Denus S, White M, Tardif JC, Bourassa MG, Racine N, Levesque S, Ducharme A. Temporal increases in subclinical levels of inflammation are associated with adverse clinical outcomes in patients with left ventricular dysfunction. *J Card Fail*. 2006;12:353-359
720. Nagueh SF, Appleton CP, Gillebert TC, Marino PN, Oh JK, Smiseth OA, Waggoner AD, Flachskampf FA, Pellikka PA, Evangelista A. Recommendations for the evaluation of left ventricular diastolic function by echocardiography. *J Am Soc Echocardiogr*. 2009;22:107-133
721. Maeder MT, Thompson BR, Brunner-La Rocca HP, Kaye DM. Hemodynamic basis of exercise limitation in patients with heart failure and normal ejection fraction. *J Am Coll Cardiol*. 2010;56:855-863
722. Wachter R, Schmidt-Schweda S, Westermann D, Post H, Edelmann F, Kasner M, Luers C, Steendijk P, Hasenfuss G, Tschope C, Pieske B. Blunted frequency-dependent upregulation of cardiac output is related to impaired relaxation in diastolic heart failure. *Eur Heart J*. 2009;30:3027-3036
723. Sohn DW, Kim HK, Park JS, Chang HJ, Kim YJ, Zo ZH, Oh BH, Park YB, Choi YS. Hemodynamic effects of tachycardia in patients with relaxation abnormality: Abnormal stroke volume response as an overlooked mechanism of dyspnea associated with tachycardia in diastolic heart failure. *J Am Soc Echocardiogr*. 2007;20:171-176
724. Pamukcu B, Lip GY, Devitt A, Griffiths H, Shantsila E. The role of monocytes in atherosclerotic coronary artery disease. *Ann Med*. 2010;42:394-403