A study of the role of monocyte functional subpopulations in patients with carotid atherosclerosis and coronary artery disease

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Thesis Abstract

This thesis focuses on monocyte subsets and furthers the understanding of monocyte subset relationships to the atherosclerotic disease process, particularly carotid disease (CD) in patients with documented coronary artery disease (CAD). A literature review of monocytes in angiogenesis is presented, revealing a substantial amount of evidence linking monocytes with the angiogenic process. A novel agreeable method of performing carotid contrast ultrasound (CEUS) for the detection of carotid plaque neovascularisation is presented. CEUS is used in those with varying CD and pre-existing CAD to detect the presence of neovascularisation and correlates this with monocyte subset totals determined by flow cytometry/ enzyme-linked immunosorbent assay (ELISA).

Levels of three functional monocyte subsets ((CD14++CD16-CCR2+ (Mon1), CD14++CD16+CCR2+ (Mon2) and CD14+CD16++CCR2- (Mon3)), and expression of their receptors linked to inflammation (toll like receptor (TLR4)), angiogenesis (vascular endothelial growth factor (VEGF), tyrosine kinase receptor (Tie2)), and migration (chemokine receptor (CXCR4)) are compared with severity of CD by division into 4 groups (each with 40 patients); grp.1= CD>50% with pre-existing CAD, grp.2= CD<50% with pre-existing CAD, grp.3=hypercholesterolemia (HC) patients, grp.4= normocholesterolaemic (NC) patients. Mon1 levels were increased with severity of CD, and found to be predictive in those patients with increased carotid intima media thickness, a marker of systemic atherosclerosis. Mon3 known to be increased in stable CAD, was shown to be increased in those with moderate carotid disease but not in the presence of severe disease. All monocyte subsets were shown to demonstrate varying levels of receptor expression mediating the pathological processes occurring at atherosclerotic plaque.

Finally pre and post levels of monocytes where taken on patients after cessation of statin therapy for two weeks. Cessation of statin therapy did not affect monocyte subset counts but caused a reduction in both TLR4 and CXCR4 expression on Mon1. This further advances one's knowledge of statin's complicated characteristics.

This thesis attempts to expand one's understanding of the behavioral heterogeneity of monocytes in atherosclerotic disease and demonstrates CEUS as a feasible research imaging method to address key questions in the field.

Dedication

This Thesis written in 2014 is especially dedicated to my wife Vino, who has stood by me through all and for without whom I would not be where I am today.

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List of abbreviations

CD	- carotid disease
CS	-carotid stenosis
CAD	-coronary artery disease
CUS	-carotid ultrasound
CEUS	-carotid contrast ultrasound
Grp.	-group
ELISA	-enzyme-linked immunosorbent assay
Mon1	-CD14++CD16-CCR2
Mon2	-CD14++CD16+CCR2+
Mon3	-CD14+CD16++CCR2-
VEGF	-vascular endothelial growth factor
Tie	-tyrosine-kinase protein receptor
CXCR	-chemokine receptor
TLR	-toll-like receptor
ICAM	-intracellular adhesion molecule
bFGF	-basic fibroblast growth factor
HIF	-hypoxia inducible factors
MCP	-monocyte chemo-attractant protein

CHAPTER I. INTRODUCTION

1.1. Monocytes and angiogenesis

1.1.1. Introduction

Atherosclerosis is the primary cause for stroke and coronary artery disease in the Western World.(Li and Glass, 2002) It is a chronic inflammatory process characterised by development of lipid rich plaques within the layers of the arterial wall.

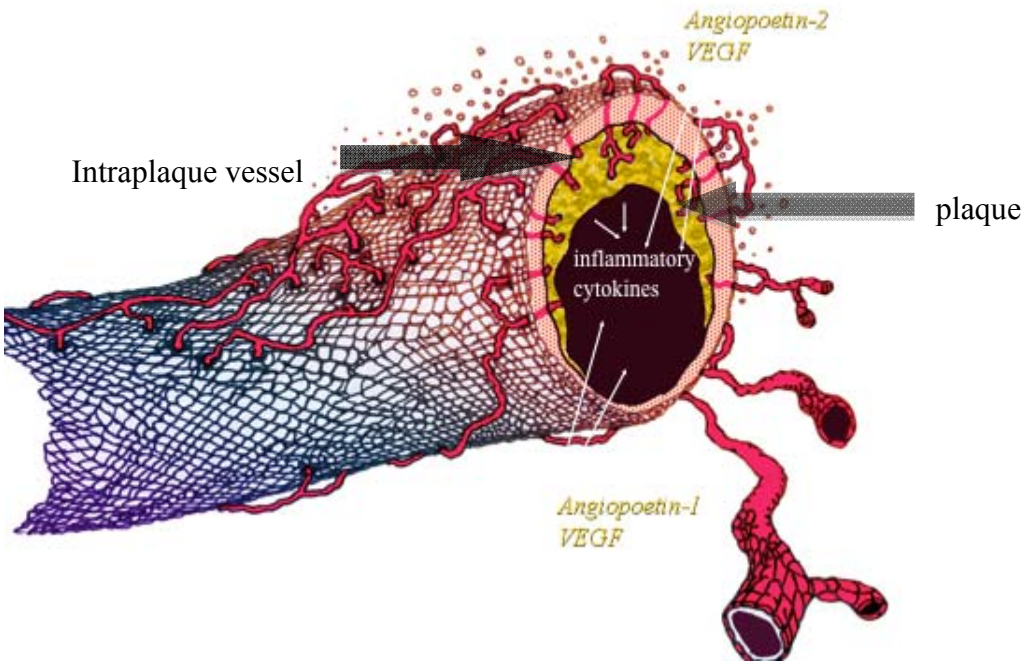


Figure 1: Angiogenesis and inflammation occurring with the arterial wall/vessel.

VEGF, vascular endothelial growth factor

Within this thickened wall is where foam cells, monocyte derived lipid laden macrophages have been recognised.(Glass and Witztum, 2001) The formation of

atherosclerotic plaque is a series of events that is initiated with lipid accumulation (fatty streak) followed by monocyte infiltration and the lipid core formation. Advanced lesions can obstruct arterial lumen, but at any stage atherosclerotic plaque may be complicated by rupture causing a hypoxia/ischemia of the downstream tissues and subsequent vascular complications.

Unhealthy lifestyles, diabetes, obesity, hypertension are still common contributors to the atherogenesis and development of unfavourable events thus prompting identification of new therapeutic targets.(Cannon, 2007) This is particularly true as current treatment modalities such not all patients are suitable for adequate coronary artery bypass grafting or angioplasty.(Zbinden et al., 2005) Of interest, each of the risk factors above triggers numerous pathological pathways involving a number of molecular processes, which include lipid metabolism, coagulation, apoptosis, hypoxia, and the immune response.(Ferrara, 1999, Helisch and Schaper, 2000, Henry et al., 2003)

The body's natural response to ischemia is a reparative mechanism summarised by the term neovascularisation. Neovascularisation includes three processes: angiogenesis, arterogenesis, and vasculogenesis.(Beem, 2008) The formation of new capillary vessels, angiogenesis has been extensively researched and occur in response to a hypoxic environment.(Luque et al., 2009, Bjornheden et al., 1999) Progression and expansion of already existing collateral smooth muscle-type vessels or arterogenesis, is believed to be a mechanism of organ preservation in the presence of vascular occlusion. Vasculogenesis or new vessel growth derived from progenitor/stem cells has been demonstrated in both the adult and embryo.(Silvestre

et al., 2008, Beem, 2008) Understanding these processes of vessel adaptation or formation is fundamental for developing new therapeutic strategies.

Inflammation has been shown to be an essential factor accompanying both the angiogenic and atherogenic pathways.(Silvestre et al., 2008) Monocyte-derived macrophages play a pivotal role in lipid deposition and progression of atherosclerosis, but they are also implicated in the genesis of new vessels.(Li and Glass, 2002) The aim of my first thesis chapter is to present an overview of the available evidence supporting the role of monocytes in angiogenesis.

1.1.2. Search Strategy

I searched the following electronic databases (limiting the search from 1970 up to July 2012): PubMed, Medline, EMBASE and Cochrane Reviews. Given the enormity of this subject area, I have focused on areas of particular relevance to angiogenesis and the role of monocytes in neovascularisation. Multiple formal systematic searches were performed with the aid of a clinical librarian and in accordance with accepted PRISMA guidelines (Appendix 1) to identify relevant publications. The key words used in combinations starting with the stem of monocyte AND angiogenesis then followed by the terms, (again due to the enormity of the subject matter) were: monocyte subsets, neovascularisation, vasculogenesis, angiopoietin, vascular endothelial growth factor (VEGF), Tie2, basic fibroblast growth factor (βFGF), beta adrenergic system, endothelial progenitor cells, hypoxia inducible factors, interleukin, tumour necrosis factor (TnF), vascular adhesion molecule (VCAM), intracellular adhesion molecule (ICAM), monocyte chemoattractant protein (MCP).

The aim of this process was to provide background information and highlight recent advances in the subject matter, for my thesis. Full text articles where available electronically were then obtained from the National Electronic Library for Health (NELH) and from the University of Birmingham Library.

The first search on monocytes and angiogenesis identified 1414 citations. Furthermore with a combination of terms starting with the stem as mentioned above an additional 518 citations were identified. Myself, and other members of our unit, reviewing monocytes and the angiogenic processes, reviewed these citations. (Tapp, 2011, Shantsila, 2007, Wrigley, 2011) All abstracts were read if available, if not, full texts were obtained. Publications focusing on monocytes, their subsets and surface markers were ordered in full text. This totalled 104 articles. Reasons for exclusion of identified literature included: animal studies, articles unavailable, and non-English languages.

The second search, focused on carotid disease and carotid contrast ultrasound. The search terms used in combination were; carotid stenosis, contrast ultrasound, neovascularization. This search revealed, 1346 citations. (Appendix 2) These publication abstracts were all read, and full texts were obtained if the abstract was not available or uncertainty of relevance on reading the abstract. Relevance was agreed with my research supervisors. Reference lists of all articles were searched for further literature. Articles not related to carotid contrast ultrasound were excluded. At the end of this process 22 papers were referenced for this part of the thesis.

In the search of literature on related to the role of statins in monocyte function, terms used in permutation starting with the stem statin were: coronary artery disease, monocyte subsets, and monocytes. Citations not directly related to topic of the search were excluded. This identified 638 citations. Again all abstracts were read, and full articles were obtained when abstracts were not available or the abstracts were unclear. Relevance was agreed with my supervisors. This resulted in 35 papers used for referencing in this section of my thesis.

All articles from all sections once deemed to be relevant were read, classified by subject or publication type and a database compiled using referencing software.

1.1.3. Atherogenesis and plaque neovascularisation

Atherosclerosis is characterised by monocyte adherence to endothelium cell, migration into the arterial wall, and lipid accumulation.(Pietsch et al., 1996) The earliest detectable atherosclerotic change is pathological intimal thickening.(de Groot et al., 2008, Lorenz et al., 2007)

Enlargement of the plaque, results in intraplaque hypoxia that triggers the inflammatory cell infiltration, thus promoting local neovascularisation.(Kwon et al., 1998) Interestingly, although intimal thickening is believed to be an early surrogate marker for atherosclerosis, pathological neovascularisation is implicated in both early and late stages of the disease.(Kwon et al., 1998) For example, in experimental studies on hypercholesterolemia, adventitial neovascularisation in the coronary arteries has been shown to be present even before the actual plaque (protrusion into the lumen) begins to develop.(Kwon et al., 1998) Two instrumental factors

influencing the initiation of intra-arterial neovascularisation appear to be (i) local ischemia, and (ii) either local or systemic inflammatory burden.(Khurana et al., 2005, Doyle and Caplice, 2007, Moulton et al., 2003) Pathological thickening of the intima greater than 100µl increases the distance between the lumen and the inner parts of the vascular wall thus impairing the supply with oxygen and nutrition.(Magnoni et al., 2009) As vascular disease ensures excessive vessel wall thickness, proliferation of the vasa vasorum and intimal neovascularisation is observed. Indeed, the degree of adventitial neovascularisation has recently been demonstrated to be associated with intimal media thickness.(Magnoni et al., 2009)

Evidence of the role of ischemia in the initiation of angiogenesis stems from the demonstration of increased levels of hypoxia inducible factor (HIF-1), which ultimately promotes VEGF production.(Bjornheden et al., 1999, Beem, 2008, Kuwahara et al., 2002) As a potent stimulator of angiogenesis, VEGF is consequently able to create a local proangiogenic environment by mobilising endothelial progenitor cells (EPCs)**.** (Inoue et al., 1998)

Table 1: Angiogenesis inhibitors and stimulators

Stimulators	**Inhibitors**
VEGF	Angiostatin
IL6	TGF-β
TNF	Interferon
bFGF	IL12
PDGF	Anti-thrombin III

bFGF, basic fibroblast growth factor; IL, interleukin; PDGF, platelet derived growth factor; TGF, transforming growth factor; TNF, tumour necrosis factor; VEGF, vascular endothelial growth factor.

Furthermore, aggressive plaque development and accelerated neovascularisation of the vascular wall have been seen following the administration of VEGF in laboratory experiments.(Celletti, 2001)

Hypoxia-independent pathways triggered by an inflammatory stimulus within the vascular wall have also been recognised as modulators of angiogenesis.(Carmeliet, 2003) The density of intra-plaque vessels corresponds to the focal accumulation of inflammatory cells (i.e., monocytes/macrophages) forming a pathological circle: angiogenesis – mobilization of inflammatory cells – angiogenesis.(Moulton et al., 2003) Switching between this inflammatory/angiogenic cascade may be responsible for enhanced plaque progression related to local plaque inflammation and plaque destabilisation. This hypothesis is supported by increased expression of leukocyte adhesion molecules, such as vascular adhesion molecule (VCAM-1) and intracellular adhesion molecule (ICAM-1), on the intimal side of vascular endothelium as opposed to the adventitial side.(O'Brien et al., 1996) Therefore, these observations allude to the notion that the presence of these adhesion molecules on the newly formed vessels is associated with the enhanced accumulation of leukocytes.(O'Brien et al., 1996)

Whilst association of the development and progression of atherosclerosis with macrophages has long been recognised, the function of their blood ancestors, monocytes was less addressed. However, a potential link between monocytes and abnormal plaque angiogenesis has a strong biological justification. Prior to discussing

angiogenesis, an overall understanding of vasculogenesis (new-vessel formation), and its relationship to EPC's needs to be addressed.

1.1.4. Vasculogenesis

Vascular system development in the embryo is termed, vasculogenesis. The first endothelial and haematopoietic cells are derived from a process whereby blood islands are formed from haemangioblasts, otherwise known as mesodermal progenitors in the embryonic yolk sac.(Beem, 2008) These islands of cells differentiate and proliferate form precursors of the vascular wall, angioblasts, which further give origin to endothelial cells.(Beem, 2008, Risau, 1997) Vascular development occurs, as these endothelial cells form the first primitive tubes/vessels. Importantly, it is at this point when both VEGF and basic fibroblast growth factor (bFGF, a critical angiogenic factor), starts to play a role in the process.(Beem, 2008, Tomanek et al., 2001) Activation of bFGF receptors on endothelial cells by bFGF increases the endothelial cell motility, proliferation and proteinase activity.(Beem, 2008, Reiland and Rapraeger, 1993, Klagsbrun, 1992, Mignatti et al., 2007) bFGF can also induce VEGF expression via HIF-1α activation as seen in a study by Shi et al., who showed that HIF-1α induction by bFGF seemed to be an independent pathway triggering VEGF expression in breast cancer.(Hata et al., 1999, Shi et al., 2005) Bovine studies have shown endothelial cell proliferation and capillary formation in the presence of bFGF and VEGF.(Goto et al., 1993)

The identification of EPCs in the adult has led to efforts to understand their contribution to the adult angiogenic processes. However, it must be mentioned that

there is much debate regarding methods of EPC quantification and standardisations. This is due to a significant cell overlap observed and the presence of the progenitors at different stages of maturation.(Schmidt-Lucke et al., 2010, Beem, 2008) Recently Sozer and colleagues have demonstrated that monocytes and EPCs share many characteristics whilst other less differentiated primitive cells produce endothelial cells only in vitro.(Sozer et al., 2008) Further delineation of their phenotype is required.(Beem, 2008)

1.1.5. Angiogenesis

The development of new vasculature, particularly the formation of new capillaries from endothelial cells that "sprout" from existing blood vessels is of importance for a number of pathological and homeostatic processes. It is also fundamental for the embryonic development.(Willems, 2009) Tumour research, the important field for angiogenic studies, has not only suggested a pathological significance of the angiogenic process in cancer but it has led to further efforts to investigate these processes in biological mechanisms of wound healing, ovulation, and tissue repair.(Johnstone and Farley, 2005, Tonnesen et al., 2000)

Naturally healthy tissue requires a supply of nutrients and oxygen. Also the restoration of tissue under ischaemic conditions and tumour growth are dependent on new vessel formation for the supply of nutrients and the removal of degradation products.(Beem, 2008) This understanding of an angiogenic process has led to a vast number of studies on therapeutic approaches to the management of vascular disorders. The primary pathological focus of this evidence has been on understanding the

atherosclerosis-related angiogenesis in plaque formation, and the inhibition of neovascularisation thereby attempting to slow the disease progression.

Interestingly, the main laboratory approach used for understanding of the mechanisms of atherosclerosis-related angiogenesis was based on analysis of ischemic tissues in the presence of pre-existing plaque stenosis as opposed to long-term studies on the development and progression of the disease. For example, a study by McCarthy et al suggested an association between symptomatic carotid disease (plaque) and the presence of intra-plaque neovascularisation.(McCarthy et al., 1999) However this study recruited patients who had pre-existing carotid stenosis and did not study patients who were initially carotid plaque free and developed stenosis over a number of years.(McCarthy et al., 1999) This lack of evidence on early changes and pathological mechanisms has driven the need for non-invasive approaches to detect neovascularisation, such as carotid contrast ultrasonography.

The initiating factor of angiogenesis is a hypoxic environment, often associated with tissue inflammation.(Bosco et al., 2008) The entire pathway is thought to be stimulated by HIF-1α.(Kimura and Esumi, 2003, Bos et al., 2005) In the ischemic environment HIF-1α, escapes degradation due to its transcription being down regulated and its ability to bind other factors (e.g., HIF-1β) activating target genes involved in angiogenesis.(Beem, 2008, Kimura and Esumi, 2003) Interestingly a number of growth factors (e.g., platelet-derived growth factor [PDGF], and bFGF) also share this regulatory hypoxic driven pathway.(Atkinson and Fox, 2004) VEGF, an essential angiogenic modulator has been shown in several in vivo models to induce a strong concomitant angiogenic cascade with HIF-1.(Kuwahara et al., 2002,

Pellizzaro, 2002) VEGF expressed by macrophages and T-lymphocytes stimulates endothelial cells to produce monocyte chemo-attractant protein (MCP-1) hence attracting monocytes and enhancing cell migration by increasing the permeability of the endothelial layer.(Melter et al., 2000, Hong et al., 2005) Dvorak et al. have demonstrated the presence of immature vessels with increased vascular leakage after the addition of VEGF in animal experiments.(Dvorak et al., 1995) Embryologically, the absence of VEGF results in early death due to abnormal blood vessel growth, demonstrating a common link between the physiological and pathological angiogenic pathways.(Carmeliet et al., 1996)

The sprouting of new vessels from pre-existing vasculature is known as angiogenesis.(Ojeifo et al., 1995, le Noble et al., 1998) Angiogenic signals from surrounding cells lead to vasodilatation and an increase in vascular permeability.(Beem, 2008, Kimura and Esumi, 2003, Pamukcu et al., 2010, Wu et al., 2009) Digestive enzymes such as collagenase and matrix metalloproteinases (MMPs) partially destroy the basement membrane.(Beem, 2008, Folkman and Klagsbrun, 1987, Sengupta and MacDonald, 2007) The plasma proteins then form a fibrin rich matrix, with a lumen forming in the proliferating capillary when the activated endothelial cells migrate towards the site.(Ojeifo et al., 1995) Ultimately, the newly formed capillaries become part of the existing circulation in a process in which shear stress is a critical factor.(Marumo et al., 1999)

Although inflammation plays a significant role in angiogenesis, multiple other processes are implicated in development of new vessels, including cell-to-extracellular matrix interactions, vascular wall maturation and basal lamina

modifications.(Pamukcu et al., 2010, Hoefer et al., 2004) The behaviour of endothelial cells is significantly influenced by inflammatory leukocytes able to release the number of pro-angiogenic factors, such as VEGF, hepatocyte growth factor (HGF), tumour necrosis factor-α (TNFα), and interleukin (IL)-8.(Hoeben et al., 2004, Ding et al., 2004, Ding et al., 2003, Hoefer et al., 2002, Li et al., 2003)

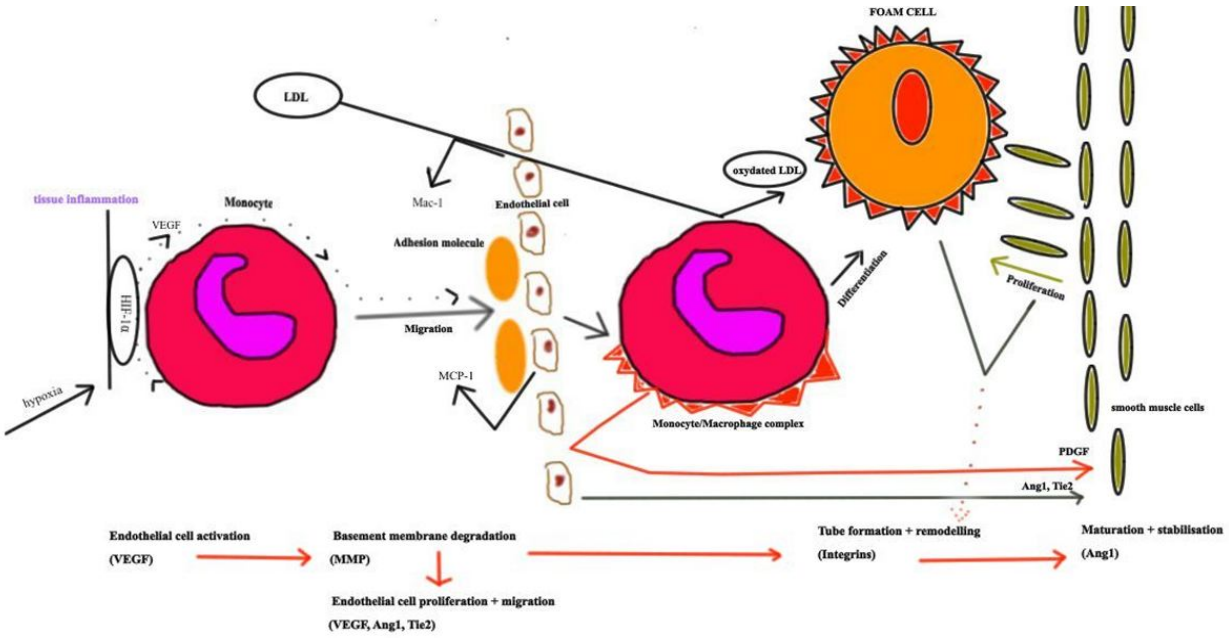
In areas of atherogenic lesions chronic infection, cigarette smoking, free radicals, hypertension, and diabetes have all been implicated as causes in the activation of endothelial cells.(Marumo et al., 1999, Ross, 1999, Osterud and Bjorklid, 2003) The increased shear stress acting through both membrane structures and cell junction molecules, stimulate quiescent endothelial cells lining the vascular wall.(Resnick et al., 2003) This intracellular signalling triggers the expression of genes like MCP-1 mRNA, involved in induction of transcription factors responsible for shear stress-mediated effects.(Ojeifo et al., 1995, Pamukcu et al., 2010, Chien et al., 1998) This sequence of events in the presence of hypercholesterolemia triggers expression of adhesion molecules, particularly P-selectin, E-selectin, VCAM-1, ICAM-1, as well as MCP-1 release and activation of genes responsible for the expression of chemokine receptor 2 ((CCR2) (MCP-1 receptor).(Pamukcu et al., 2010, Wu et al., 2009, Sadhu et al., 2007, Ohno et al., 1995)

1.1.6. Monocytes in angiogenesis

Oxygen-deprived intima of the arterial wall recruits circulating monocytes via specific integrin receptors (Mac-1) that interact with the endothelial adhesion molecules.(Hoefer et al., 2004, Schuler et al., 2003, van Weel et al., 2007)

Figure 4: Monocytes role in angiogenesis.

HIF, hypoxia induced factor, VEGF, vascular endothelial growth factor, MMP, Matrix metalloproteinase, Ang, Angiopoietin, Tie, Tyrosine Kinase, PDGF, Platelet derived growth factor, MCP, Monocyte chemotactic protein, MAC, Macrophage antigen, LDL, Low density lipoprotein.



Hajjar et al. have shown that this binding predominantly occurs at the tight junctions of the endothelial cells and allows monocyte entry into the sub-endothelial space.(Hajjar and Nicholson, 1995) VEGF, expressed by macrophages activates the production of MCP-1 by the endothelial cells and an increase in the permeability of the endothelial layer. (Marumo et al., 1999)

Table 2: Examples of animal and human studies implicating monocytes in angiogenesis.

Study	Model	Mediating factor	Study design	Study finding
(Capoccia et al., 2008)	Mouse	MCP-1	Direct injection of bone marrow cells into blood of surgically induced hind limb ischaemia. Adductor hind leg muscle was then subjected to flow cytometry, ELISA, and immunofluorescence. Bone marrow cells were also harvested and transplanted into wild type mice.	Inflammatory subset of monocytes was selectively recruited to the site of insult in parallel with increased MCP-1 amounts. Two waves of monocyte proliferation were demonstrated in the presence of angiogenesis and inflammation.
(Arras et al., 1998)	Rabbit	TNF, bFGF	Femoral artery occlusion of rabbit hind limb for 3 and 7 days, with randomly given lipopolysaccharide. Further control animals were tested at 21 days for comparison. Carotid artery catheters were also placed for proliferation analysis.	After day 7 of induced hypoxia, maximal macrophage proliferation was present being associated with higher TNF and bFGF levels. Monocytes/macrophage activation played important role in angiogenesis and vessel growth in the presence of hypoxia.
(Hong et al., 2005)	Rat, Chick	MCP-1, VEGF	Thoracic and abdominal aortas were obtained from 5-week-old rats. VEGF was analysed by mRNA expression using PCR. MCP-1 was analysed in vivo using chick chorioallantoic membrane.	Monocytes were implicated in angiogenesis in MCP-1-mediated manner and related to HIF and VEGF-A up-regulation.
(Cursiefen C et al., 2004)	Mouse	VEGF	Mouse model of suture induced inflammatory corneal neovascularisation. Immunochemistry and morphometry were used to analyse angiogenesis in the cornea.	VEGF mediates the recruitment of monocytes/macrophages resulting in the initiation of neovascularisation in the presence of inflammation but also amplifies the pathological process of both angiogenesis.
(Celletti et al., 2001)	Mouse, Rabbit	VEGF	Cholesterol fed mice were administered intra-peritoneal VEGF and albumin. Combination of FACS analysis and histological section were used for blood and plaque progression analysis respectively.	VEGF promoted angiogenesis but also played a role in plaque progression associated with monocyte/macrophage accumulation.

			Plaque formation was also analysed in the same manner in rabbit to eliminate cross species-specific effect.	
(Murdoch et al., 2008)	Human	Tie-2, Ang 2	Flow cytometry and chemotaxis micro-chamber technique in healthy donors.	Ang-2 recruited Tie2+ monocytes to both tumours and sites of inflammation and enhanced expression of proangiogenic cytokines.
(Eubank TD et al., 2003)	Human	M-CSF, VEGF	Isolated human monocytes were stimulated with M-CSF. ELISA was used for VEGF analysis.	M-CSF enhanced production of VEGF and angiogenesis by human monocytes.
(Venneri et al., 2007)	Human	Tie-2	Healthy blood donors and surgically resected tumour tissue. Analysis performed using flow cytometry, western-blot analysis, immunohistochemistry and migration assays.	Tie2+ monocytes were associated with angiogenesis.

MCP- monocyte chemotactic protein, VEGF- vascular endothelial growth factor, EC- endothelial cell, HIF- hypoxia inducible factor, bFGF- basic fibroblast factor, PCR-polymerase chain reaction, Ang-2- Angiopoietin, Tie2 – Tyrosine Kinase, M-CSF- macrophage colony-stimulating factor.

The chronic low-grade inflammation inside the vascular wall has been shown to be associated with monocyte infiltration. The monocyte maturation to macrophages is accompanied by the production of cytokines and growth factors.(Hoefer et al., 2004, Hoefer et al., 2002, Arras et al., 1998)

Plaque monocytes/macrophages interact with collagen and proteoglycans in the extracellular matrix by expressing proteases like urokinase plasminogen activator (u-PA).(Menashi et al., 1993) uPA activates plasmin, which in turn degrades the extracellular matrix.(Menashi et al., 1993) The monocytes produce PDGF, which induces mitotic activity of the endothelial cells and vascular smooth muscle cells.(Hoppenreijs, 1994, Polverini et al., 1977) Activated plaque monocytes/macrophages ingest the oxidised lipids and become lipid-laden ‘foam’ cells. It is believed that ‘foam’ cells promote vascular remodelling by stimulation of smooth muscle cell migration and a subsequent shift in endothelial function.(Kruth, 2001)

Whilst there is a distinct relationship between monocytes and angiogenesis in the atherosclerotic lesions, controversy surrounds the origin of the native endothelial cells as well as the role of specific subtypes of monocyte populations such as CD14+/VEGFR-2+ monocytes.(Elsheikh et al., 2005) Animal studies have demonstrated that although endothelial cells play a role in the initiation atherosclerotic process they themselves may be bone marrow-derived as in tumour-associated blood vessels.(Nolan et al., 2007) Once monocytes have infiltrated the tissue layers a proportion of them will differentiate into dendritic cells triggering the activation of antigen specific T lymphocytes associated with creation of the local inflammatory environment.(Randolph et al., 1998)

Large proportion of circulating EPCs was found to be of monocytic origin.(Shantsila et al., 2007) Human monocytes include a population of cells able to obtain endothelial cell phenotype in culture.(Fernandez Pujol et al., 2000) Cultures of so-called 'early' EPCs are mainly comprised of monocytes and T-cells and their formation is strictly dependent upon monocytes presence.(Shantsila and Lip, 2009) Additionally, monocytes constitute the dominant population among circulating cells expressing type 2 receptors for VEGF (VEGFR2).(Romagnani et al., 2005) Cells bearing CD14 (a monocyte marker) are capable of improving re-endothelialisation after carotid balloon injury in animals and this process depends upon the levels of a major factor stimulating monocyte mobilisation, MCP-1.(Fujiyama et al., 2003) Elsheikh et al have reported that transplantation of CD14+/VEGFR2+ cells into balloon-injured femoral arteries of nude mice significantly contributed to their efficient re-endothelialisation.(Elsheikh et al., 2005) These data support the possible involvement of monocytes in hypoxia-induced VEGF-mediated formation of vasa vasorum.

1.1.7. Angiopoietins

Homeostasis of the vascular system is supported by secreted glycoproteins called angiopoietins (Ang).(Barton et al., 2005) These angiopoietins function as growth factors to aid angiogenesis. However, Metheny-Barlow et al. have shown that his may not necessarily be the case for angiopoietin 1 (Ang1), which, under specific conditions, may act as an inhibitor of the angiogenic process.(Metheny-Barlow and Li, 2003)

There are 4 main ligands in the angiopoietin group (Ang1, Ang2, Ang3, Ang4).(Thomas and Augustin, 2009) Ang1 and Ang2 have been well studied and have a strong affinity to tyrosine kinase receptors.(Lemieux et al., 2005) Both Ang1 and Ang2 can be found in high concentration in tumours, particularly angiosarcoma suggesting their role in both tumour angiogenesis and progression.(Amo et al., 2004, Brown et al., 2000) Shim et al. have demonstrated differences between Ang1 and Ang2 in their response to hypoxia. Ang2 was up regulated in the presence of ischemic tissue whereas Ang1 was mostly associated with malignancy. However, both are implicated in the angiogenic processes.(Shim et al., 2007) The family of receptors, which primarily maintains Ang influence and ability to be expressed in endothelial cells, is a tyrosine kinase, Tie2. Tie2 is involved in the stabilization of mature blood vessels, promoting the interaction between endothelial cells and supporting periendothelial cells. (Thurston, 2003)

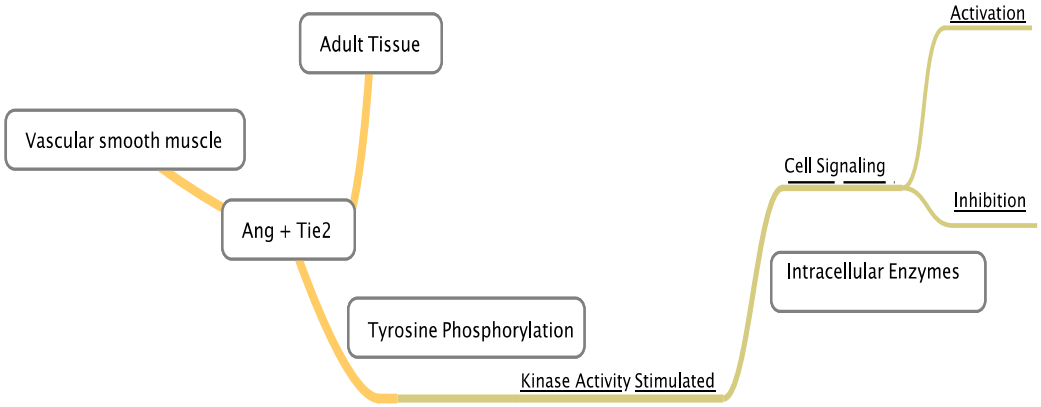


Figure 3: Interaction of tyrosine kinase receptor type 2 (Tie2) with vascular smooth muscle.

Ang, angiopoietin.

Animal studies have shown that absence of either Ang1 or Tie2 results in incomplete vascular development and death.(Suri et al., 1996, Sato et al., 1995) Interestingly, the interaction between Tie1 and Tie2 remains primarily unclear but it is known that none of the Ang family members directly binds Tie1, yet Tie2 inhibits Tie1-mediated regulatory control of endothelial cell function.(Saharinen et al., 2005)

Hauer et al. have demonstrated an overall reduction of experimental atheroma after Tie2 inhibition.(Hauer et al., 2009) This again reveals a significant therapeutic potential of this pathway. More recently, Wu et al. have shown a relationship between both Ang2 and VEGF in genetically modified mouse studies.(Wu and Liu, 2010) Although, the angiogenic effects was greater in the lymphatic tissue rather than in blood vessels, the studies raised interest in the development in anti-angiogenic therapies.(Wu and Liu, 2010) Recently, Saharinen et al. have suggested that the two systems (i.e., mediated by VEGF and Ang) played different roles in blood and lymphatic vessel growth.(Saharinen et al., 2010)

Another study demonstrated not only a link between both VEGF and Ang2 but also a clear difference in how they regulated the angiogenic pathways.(Fujiyama et al., 2001) Indeed, Ang1 has showed an inhibitory role against the actions of Tie2 in blood vessel maturation whilst Ang2 expression counteracted this Ang1 effect, thus promoting vascular stabilisation.(Hawighorst Skobe, 2002) Once again this antagonist relationship has sparked interest from both a scientific and therapeutic point of view.

1.1.8. Vascular endothelial growth factor

MCP-1, although known primarily to play a role in inflammation has been shown to be a chemokine with angiogenic properties.(Hong et al., 2005) Hong et al demonstrated that the MCP-1-mediated angiogenic cascade is maintained and modulated by VEGF.(Hong et al., 2005) Further evidence to this relationship and the monocyte role in angiogenesis has been shown by in vitro treatment of human monocytes with VEGF obtained from tumour cells, resulting in both monocyte activation and migration.(Barleon et al., 1996)

VEGF is a pro-angiogenic growth factor primarily involved in the initiation of new capillary formation.(Risau, 1997) VEGF is involved in embryonic angiogenesis but it is as well a potent signalling protein, which stimulates vasculogenesis and angiogenesis in the presence of injury, exercise, and formation of collaterals.(Prior et al., 2004, Miquerol et al., 2000) There are 4 well-known VEGF derivatives plus one placental growth factor.

Table 3: Family of VEGF and their functions.

Type	Function
VEGF A	Angiogenesis
	Chemotactic
	Vasodilatation
VEGF B	Embryonic
VEGF C	Lymphangiogenic
VEGF D	Lung
	Lymphatic's
PlGF	Vasculogenesis

PlGF, placental growth factor; VEGF, vascular endothelial growth factor.

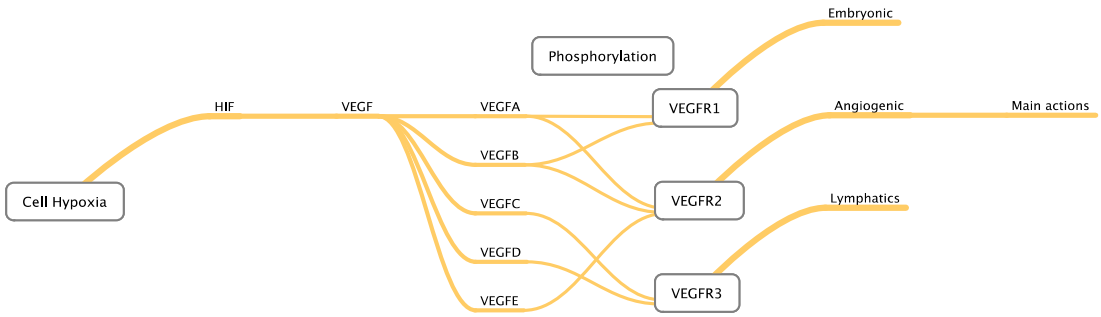


Figure 4: Family of VEGFs and their functions.

HIF, hypoxia induced factor, VEGF, vascular endothelial growth factor, VEGFR, vascular endothelial growth factor receptor

Interestingly, excessive VEGF expression has been linked to the progression of malignancy, and retinal eye disease.(Guo et al., 2010) VEGF main action however, is mediated by binding of tyrosine-kinase receptors.(Shibuya et al., 1990)

VEGFR2 are essential for endothelial cell survival.(Kabrun et al., 1997) Absence of VEGFR2 is incompatible with development of endothelial and haematopoietic cells in

animals.(Matthews et al., 1991) In contrast VEGFR1 is not obligatory for endothelial cell differentiation but it is required for embryonic development.(Fong et al., 1995) Interestingly, a possible antagonistic relationship between VEGFR1 and VEGFR2 has highlighted the intricate relationship between promoting and maintaining vascular development in ischemia, cancer, and other pathological processes.(Yancopoulos et al., 2000) Unfortunately, despite the primary role of VEGFR2 in both vasculogenesis and angiogenesis, the molecular mechanisms controlling its genetic expression are still at an early stage of recognition, representing justification for renewed focus in the critical process of protein modulation in a therapeutic respect.(Guo et al., 2010) This is especially true since it has been demonstrated that tumour genesis itself involves specific angiogenic factors based on tumour type.(Hayes et al., 2007)

1.1.9. Monocyte heterogeneity

Monocyte subsets in particular are believed to play a differential role in intra-plaque angiogenesis and tissue repair.(Pamukcu et al., 2010) The subsets differ in phenotype, granulation, size, morphology and genetic make up.(Yona and Jung, 2010) Over the last thirty years, human monocyte subsets were distinguished based on their surface CD14/CD16 expression as ‘classical’ CD14++CD16– cells and less frequent ‘non-classical’ CD14+CD16++ blood monocytes.(Grage-Griebenow et al., 2001) However this diversity amongst monocyte subsets is not unique to humans, and has been shown in animals.(Chamorro et al., 2005, Ahuja et al., 1995)

A third subset can be distinguished by surface expression of CCR2**.** (Shantsila et al., 2011)

Table 4: Monocytes subsets and their functions.

Monocyte subset	Expression	Primary Function
Mon1 (classical)	CD14++CD16–CCR2+	Phagocytosis Cytokine production
Mon2 (intermediate)	CD14++CD16+CCR2+	Angiogenesis
Mon3 (non-classical)	CD14+CD16++CCR2–	Collagen deposition, Anti-inflammatory effects

Interestingly, this CD14++CD16+CCR2+ monocytes phenotypically resemble the previously reported pro-angiogenic monocytes.(Shantsila et al., 2011) For example, De Palma et al., and Venneri et al. have demonstrated distinct pro-angiogenic properties of Tie2-expressing monocytes.(De Palma et al., 2005, Venneri et al., 2007) This conclusion lends to an earlier study by Lu et al., on bone marrow derived vascular progenitors, which demonstrated blood vessel formation to be an angiogenic process (from pre-existing vessels) but also having a vasculogenic component. In other words, growth factors, cytokines and other key proangiogenic contributions derive not only from local tissues but also from bone marrow.(Lu et al., 2008)

The identification of each subset thus allows further research into their respective physiological functions. Ziegler-Heitbrock et al. have shown that CD14+CD16+ monocytes have some features common with mature tissue macrophages.(Ziegler-Heitbrock et al., 1993) However, animal-based studies on monocyte subsets are controversial due to substantial differences between human and murine monocyte subsets.(Qu et al., 2004, Auffray et al., 2009) Nahrendorf and colleagues compared monocytes in a model of mouse myocardial infarction and suggested that specific

signalling may depend on site and type of hypoxia/ischemia insult and time of recovery from this injury.(Nahrendorf et al., 2007)

Although three monocyte subsets are now recognised, the majority of published studies only refer to two monocyte subpopulations (i.e., CD14+CD16- and CD14+CD16+ monocytes) without further subdivision of the CD16+ cells, and thus careful interpretation of such data is required. The CD16+ monocytes are infrequent (less than 15% in healthy humans), but their proportions are increased in patients with stenotic CAD, and myocardial infarction, being related to the up-regulation of inflammatory cytokines.(Schlitt et al., 2004, A.M Gotto Jr et al., 1990, Grage-Griebenow et al., 2001) These data might indicate a possible role of CD16+ monocytes for the advanced inflammation-mediated arterogenesis/intraplaque angiogenesis. However, the relation of monocyte populations to systemic atherosclerosis (IMT, adventitial vasa vasorum) and high-risk indices for plaque destabilisation is clearly understudied. Subset specificity may also be dependent on expression of multiple receptors and MCP-1-mediated signalling.(Capoccia et al., 2008) Indeed, angiogenesis and monocyte subset involvement is a multiple stepwise process, which consists of two areas of recruitment, local and bone marrow, which are specific to the stimulating environment.(Capoccia et al., 2008) Further studies are being performed to delineate the specific functions of each of the monocyte subsets, but their specific roles in plaque progression, stability and rupture remains insufficiently understood at present.(Mehta NN, 2012)

1.1.10. Plaque instability and rupture

The volatile nature of an atheromatous plaque is responsible for approximately 60% of symptomatic carotid artery disease and about 75% of acute coronary events. (Burke

et al., 1997, Redgrave et al., 2006) Neovascularisation recently has been implicated as a possible contributor to the process by which an asymptomatic fibro-atheromatous plaque becomes a lesion vulnerable to rupture, although the precise mechanism of how this occurs remains unclear.(Kolodgie et al., 2003, Takaya et al., 2005, Milei et al., 1998) As the plaque progresses, the adventitial vasa vasorum is the site of initiation of intra-plaque vessels formation. (Kumamoto et al., 1995) Evidence has shown the presence of neo-vessels within the plaque has been associated to its rupture. (Kumamoto et al., 1995) In the comparison of stable plaques to those in both vulnerable and ruptured plaques, there is a 2 to 4 fold increase in the number of vasa vasorum, respectively. (Virmani, 2005) Although plaque deposits themselves may be localised and unique, the changes found in the arterial wall vascularisation are known to be systemically widespread, lending to the notion of atherosclerosis being a pan-arterial disease.(Fleiner et al., 2004) However the factors which trigger the change, from a nonthreatening to unstable plaque remains poorly understood. The synthesis of pro-inflammatory molecules such as IL-6 and tumour necrosis factor-α, mediated by stimulation of TLR4 are upgraded by activated monocytes. (Satoh et al., 2006, Shantsila and Lip, 2009) The interaction between EPC and white blood cells results in an inflammatory cascade resulting from the interaction amongst CD14, a monocyte endotoxin receptor, acting together with a co-receptor, TLR leading to in monocytic activation. (Agema WR, 2004, Lauener RP, 1990) This monocyte activation subsequently enhances the affinity of monocyte ligands to adhesion molecules thus promoting monocyte–endothelium adhesion. (Lauener RP, 1990) Evidence for this has been demonstrated by the presence of micro-vessels within lipid-rich plaques strongly expressing adhesion molecules (ICAM-1, VCAM-1) thereby facilitating trans-endothelial migration of inflammatory cells (i.e. monocytes) into the plaque microenvironment. (O'Brien et al., 1996, van der Wal et al., 1992) This evidence

implicates the potential involvement of monocytes and there role in plaque neovascularisation and plaque rupture.

1.1.11. Monocytes in Atherosclerosis Progression

Uncontrolled lipid accumulation followed by rapid monocyte infiltration and phagocytosis of LDL mediated by scavenger receptors subsequently results in macrophage apoptosis. This understandably increases the atherosclerotic plaque core with ensuing necrotic tissue, collagen deposition and migration of smooth muscle.(Pamukcu et al., 2010) This pattern of monocyte/macrophage deposition and removal although protective by nature is only mediated by the inflammatory reaction it manifests itself. This unbalanced inflammation with excessive cytokine release from monocytes and enhanced monocyte expression of the TLRs promotes both angiogenesis and plaque growth and destabilisation.(Satta N, 1994, Dachary-Prigent J, 1993, Burnier L, 2009) Administration of statins to subjects with hypercholesterolaemia inhibited the expression of monocyte pro-inflammatory cytokines (TNF and IL1β) and the treatment has a well-documented capacity to reduce risk of unfavourable event in patients with stable coronary heart disease. (Ferro et al., 2000) Moreover, the long-term treatment with statins can prevent progression or even lead to regression of the atheroma, although the relative magnitude of lipid-independent pleiotropic effects of the statins in the overall benefits of the drugs remain unclear.(MAAS, 1994)

1.1.12. Adrenergic system and angiogenesis

The adrenergic system have been shown to be implicated in regulation of expression of pro-angiogenic factors and angiogenesis.(Ristori C, 2011) For instance, high norepinephrine levels are linked to increased VEGF expression.(Guo K, 2009, Yang EV, 2009) Although the mechanisms of norepinephrine-mediated VEGF up regulation in atherosclerosis remain unclear, a post transcriptional mechanism has been revealed by which norepinephrine induced HIF α-1 production modulated VEGF expression in cancer cells.(Park SY, 2011) Interestingly this recent study draws attention to an older study that demonstrated norepinephrine's effect on VEGF was mediated by a paracrine mechanism.(Weil J, 2003)

In the absence of ischaemia or even exercise, the alpha adrenergic (α_1A) system has been demonstrated to increase capillary blood flow via an increase in capillarity of skeletal muscles.(Dawson JM, 1989) In 2008, Ciccarelli demonstrated α_1A to inhibit angiogenesis by interfering with EC proliferation and responsiveness to VEGF.(Ciccarelli M, 2008, Carmeliet P, 2000) Although expressed in many tissues, the primary role of beta-adrenergic system (β_2A) receptors overall remains unclear. (Iaccarino G, 2005) In 2008, Leosco and colleagues demonstrated the promotion of angiogenesis with exercise resulting in improved β_2A signalling. (Leosco D, 2008) This promotion of angiogenesis is also evidenced by the use of propranolol, a potent adrenergic blocking agent in the treatment haematopoietic malignancies whereby its anti-inflammatory and anti angiogenic properties have been demonstrated to reduce VEGF production and matrix metalloproteinase-2 (MMP-2) activity.(Hajighasemi F, 2009) In the presence of ischaemia, Iaccarino et al., observed adrenergic down regulation of β_2A with enhancement and preservation of capillaries along with the promotion of endothelial cell proliferation suggesting a vital role in regulation of

angiogenesis by the β_2A system. (Iaccarino G, 2005) The augmentation of EC, shown to be demonstrated after a single episode of exercise, clearly has demonstrated a link between ischaemia and angiogenesis.(Rehman J, 2004) Most recently emphasis has been placed on identification of specific β_2A receptor subtypes on lipopolysaccharide laden monocytes implicated in creation of the pro-inflammatory state, mediated by cytokine modulation and cyclic adenosine monophosphate (cAMP) dependent mechanisms.(Grisanti LA, 2010) Galasso et al. most recently, have shown a relationship between EPC cells and β_2A receptors, going as far to demonstrate EPC's ability to express β_2A receptors, which in turn promotes EPC migration, and proliferation thereby augmenting angiogenesis. (Galasso G, 2013)

1.1.13. Conclusion

Atherosclerosis and its angiogenic component is an obvious feature of vascular disease. The regeneration of vascular beds with capillary sprouting relies on angiogenesis. Atherosclerosis has an inflammatory component, and MCP-1 plays a role in recruiting monocytes to the site of insult. Monocytes, important members of the innate immune system have been shown to play an intricate role in angiogenesis. Neovascularisation is a major contributor to plaque progression. Much more research is required to establish the exact mechanisms underlying the tightly regulated angiogenic processes. One approach would be to continue to investigate the potential presence of various subtypes of monocytes and their specific receptors and roles in cardiovascular disease. This hopefully would be useful for the development of new therapeutic targets for prevention of progression of atherosclerotic disease and its complications.

1.2. Carotid Ultrasound

Stroke is the leading cause of adult disability and the second leading cause of death in both Europe and the United States. (Feigin, 2005) In the United Kingdom, it is estimated that the direct cost of stroke to the National Health Service is 2.8 billion pounds. (House of Commons, 2006) Leary et al. reported that in 1998 in the United States, 11 million people had experienced a neurovascular event at some point during their lifetime and, of those, only 770,000 were symptomatic. (Leary and Saver, 2003) They concluded that there were a substantial number of affected patients that were either unreported or even silently affected. It is estimated that 20-25% of strokes originate from the carotid artery and that carotid imaging should be part of any clinical stroke evaluation. (Weinberg, 2010, Gleason et al., 2001)

Although angiography has remained the gold standard of imaging for any blood vessel, it tends only to be used when non-invasive methods require further clarification. (Weinberg, 2010) Carotid Doppler ultrasound (CUS) remains the primary method of detecting CD. (Wardlaw and Lewis, 2005) A number of clinical studies have shown that this non-invasive, inexpensive and reproducible modality, using various post-processing algorithms, can accurately measure percentage stenosis using peak systolic velocity.(Alexandrov et al., 1997) The North American Symptomatic Carotid Endarterectomy Trial (NACSET), and the European Carotid Surgery Trial (ECST), both differed on how to calculate the degree of CD. Both trials calculated the lumen diameter at the point of maximum of disease but NACSET divided this measurement by the distal ICA lumen (normal segment) whereas ECST used an estimated normal lumen diameter at the site of maximum disease. The NASCET method produced lower measurements of disease then that of ECST, significantly affecting overall outcomes lending to the continuing disagreement.

(Rothwell et al., 2003) For the purpose of this thesis, the ECST method was used, as it remains widely adopted by the UK. (Grant et al., 2003)

NACSET, the Asymptomatic Carotid Artery Stenosis Trial (ACAST) and the Asymptomatic Carotid Surgery Trial (ACST), in particular, have all established guidelines for the use of CUS alone as the primary tool for determining the need for surgical intervention. (Barnett et al., 1998, Chambers and Donnan, 2005, Study et al., 1995, Rothwell and Goldstein, 2004, Moneta et al., 1993) These large studies have shown benefit from surgical intervention (carotid endarterectomy, (CEA)) in patients with greater that 70% stenosis, but significant controversy surrounds the management of asymptomatic disease. (Barnett et al., 1998, Stansby et al., 2011) Nicolaides et al. specifically investigated the asymptomatic internal carotid artery stenosis and demonstrated a stroke risk of approximately 1% per year when the stenosis was greater than 70%.(Nicolaides et al., 2010) This is in direct contrast to symptomatic disease, which the NASCET trial showed the five-year stroke risk in patients with 60-99% stenosis to be 16.2%, while in those with less then 60% the risk was reduced to 8%. (Barnett et al., 1998) Most recently, Hirt investigated the progression of asymptomatic carotid disease with >50% stenosis and concluded that, over five years, the rapid progression of stenosis poses a significant stroke risk. (Hirt, 2011) In asymptomatic patients, the risk of stroke has been shown to be significantly increased in those with >90% CD, hence asymptomatic disease may not be benign, simply because most cerebrovascular events happen in patients who are not symptomatic. (Rothwell et al., 2004) These results continue to fuel scientific and clinical interest in the identification of plaque characteristics using imaging techniques, particularly CUS, to predict stroke risk, as well as to develop therapeutic strategies to prevent or inhibit disease progression.

Parasekevas et al. showed that the presence of carotid disease is a marker of widespread atherosclerotic disease. (Paraskevas et al., 2007) Pathological intimal media thickening (IMT) is recognised as the earliest atherosclerotic change and is primarily characterised by surface smooth muscle cells overlying relatively acellular lipid rich pools. (Kumamoto et al., 1995) IMT measurement remains objective, as IMT is not a defined morphological structure but an observation on ultrasound for which no clear anatomical structure is identified. (Coli et al., 2008) The body's natural response to ischaemia is a repairing mechanism summarised by the term neovascularisation. This process is thought to originate in the adventitia. (Kumamoto et al., 1995) Histological studies have demonstrated that the development of an unstable plaque is related to plaque inflammation and neovascularisation. (Giannoni et al., 2009) The presence of plaque neovascularisation has been shown to be an acute predictor of unstable atherosclerotic lesions in cardiovascular and cerebrovascular patients. (Vicenzini et al., 2009) Understanding these processes of vessel adaptation or formation is fundamental to developing new therapeutic strategies or imaging techniques.

Carotid contrast ultrasound (CEUS) imaging is an evolving technique, which focuses directly on the adventitial vasa vasorum (VV). These microvessels that supply vascular nutrition were first studied in the early 1980s in association with coronary disease, and have more recently been implicated in the progression of atherosclerotic plaques.(Barger et al., 1984, Magnoni et al., 2009) Atherosclerotic intraplaque neovascularisation is associated with high-risk plaque destabilisation. (Vicenzini et al., 2009) Under-nutrition and resulting ischemia of the growing atherosclerotic lesions triggers growth of new intra-wall/intra-plaque microvessels from adventitia, often being also associated with inflammatory processes. (Giannoni and Vicenzini, 2009, Kumamoto et al., 1995)

The use of contrast microbubble agents in the delineation of carotid plaque vascularisation was first described by Feinstein. (Feinstein, 2006) Huang et al. demonstrated a clear correlation with contrast ultrasound and plaque morphology. (Huang et al., 2007) Since then, a number of studies have further enhanced the rationale for improving the technique but, to the knowledge of the authors, an agreeable validated methodology has not been developed to date. These studies are all limited by their varying methods of dosage/rate of contrast, equipment used, and assessment of neovascularisation.

Quantification of plaque neovascularisation may improve identification of high risk lesions limited clinical tools are currently available. Ultrasound contrast agents remain within the vascular space, and therefore can be detected by their reflective resonation to highlight the presence of vascular disease.(Clevert et al., 2011) More recently, carotid contrast imaging, an evolving technique, has been used to focus on the plaque and adventitial vasa vasorum.(Clevert et al., 2011) However, reproducibility of the method is poorly established.(Hoogi et al., 2011, Papaioannou et al., 2009, Coli et al., 2008, Huang et al., 2007, Giannoni et al., 2009, Magnoni et al., 2009)

Therefore, one of the aims of my thesis was to develop a method for the accurate and agreeable detection of carotid plaque vasculature using CEUS.

Table 5: Comparison of carotid contrast studies.

Study	n	U/S machine	Probe	Contrast Agent	Injection of contrast	Time to scan	Duration of Scan	♯ of reviewers	Variability	Assessment of neovascularisation
(Shah et al., 2007)	17	GE Vivid 7 & ATL HDI 5000	GE: 7L ATL: 7-4 L	Optison	Bolus 0.5-1.0ml + Saline 2-3ml	15-30sec	NS	3	NS	o= no neovascularisation within plaque 1= limited appearance of neovascularisation within plaque 2= moderate neovascularisation within plaque 3= pulsating, arterial vessel within plaque
(Papaioannou et al., 2009)	1	GE Vivid 7	10L	Sonovue	Bolus 2.5cc + 2.5cc Saline	during injection	90sec	NS	NS	ROI enhancement
(Coli et al., 2008)	32	GE Vivid 7	7L	Optison	Bolus 2ml + repeat (dilution 3ml contrast + 7ml Saline) Max 2 bolus	NS	2 min after each bolus	2	NS	1= no bubbles within plaque or bubbles confined to plaque adventitial side 2= bubbles reaching plaque core and/or extensive contrast agent enhancement throughout plaque
(Huang et al., 2007)	63	Acuson Sequoia 512 scanner	15L	Sonovue	2.4ml bolus Over 3	NS	NS	NS	NS	Time intensity curve analysis

		(Siemens)			sec					
(Giannoni et al., 2009)	77	Acuson Sequoiq 512 (Siemens)	15L	Sonovue	2.5ml bolus followed by two other 1.25 bolus each followed by Saline	NS	NS	NS	NS	type 1= rare, discrete contrast enhancement with microvessels large diameter 5-60μm type II – diffuse contrast enhancement, with microvessels of small diameter, 20-30 m, with VEGF staining.
(Vicenzini et al., 2007)	23	Acuson Sequoiq 512 (Siemens)	15L	Sonovue	Bolus 2.5ml + 10ml saline flush	NS	NS	NS	NS	Observation only
(Magnoni et al., 2009)	25	GE Vivid 7	7L	Optison	Bolus 2ml + repeated Max=6ml	30 seconds	2 min	2	95% limits of the agreements varied between .131 and 0.134	Blood flow imaging modality with background subtraction (periadventitial study)

n, number of patients; U/S, ultrasound; Probe, MHZ-Linear Array; NS, not stated; ♯, number.

CHAPTER II. METHODS

2.1. Patient selection

I recruited 160 patients divided into 4 groups: (i) 40 patients with carotid disease (CD) ≥50% and documented CAD (grp.1 CD. >50), (ii) 40 patients with carotid disease <50% and documented CAD (grp.2 CD<50), (iii) 40 asymptomatic control subjects with hypercholesterolaemia (grp.3 HC) and (iv) 40 asymptomatic control subjects without hypercholesterolaemia (grp.4 NC). The number of patients recruited was calculated based on my hypothesis that my primary cellular marker, $CD14^{low}/CD16^{+}$ monocytes, will be raised by 0.3 standard deviation (i.e. from 50 cells/μl in controls to 55.9 cells/μl in patients with CAD without carotid stenosis and further to 61.8 cells/μl in CAD patients with carotid stenosis ≥50%.

The participants were recruited from Sandwell and West Birmingham Hospital NHS Trust during the period May 2010 and May 2011. My study was approved by the local research ethics committee and all participants gave written informed consent.

I assessed CD and intraplaque neovascularisation using CEUS (see below). I confirmed the presence of stable CAD by history and/or previous elective coronary angiography, with no acute hospital admission for ≥3 months. Grp.3 HCs were recruited from our outpatient lipid clinic and had total cholesterol level above 5.0 mmol/L but without clinical evidence of symptomatic arthrosclerosis. I identified and recruited grp. 4 NC from hospital staff, relatives of patients, and local general practices and were healthy with no clinical evidence of symptomatic arthrosclerosis and cholesterol levels below 5.0 mmol/L. I excluded from all groups any patient with

the following criteria; any neoplastic, inflammatory diseases, and significant kidney disease.

All subjects were invited to attend our research clinic in the morning, after abstaining from smoking from midnight of the preceding day. Following a 20-minute supine rest, a 20ml blood sample was taken for flow cytometric studies and ELISA (below) and carotid ultrasound imaging was performed (below).

2.2. Flow cytometry

I performed flow cytometric analysis using the BD FACS Calibur flow cytometer (Becton Dickinson, Oxford, UK [BD]) as published previously by my supervisor.(Shantsila et al., 2011) The technique is robust and highly reproducible.

2.2.1. Absolute count of monocyte subsets

Mouse anti-human monoclonal fluorochrome-conjugated antibodies anti-CD16-Alexa Fluor 488 (clone DJ130c, AbD Serotec, Oxford, UK), anti-CD14-PE (clone MϕP9, BD), anti-CD42a-PerCP (clone Beb1, BD) and anti-CCR2-APC (clone 48607, R&D) were mixed with 50µl of fresh EDTA anticoagulated whole blood in BD TruCount tubes (BD) containing a strictly defined number of fluorescent count beads.(Shantsila et al., 2011, Shantsila et al., 2012, Tapp et al., 2012, Wrigley et al., 2013) 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, side scatter properties versus CD14 expression to exclude granulocytes, and un-gated CD14 versus CD16 expression to

exclude natural killer lymphocytes. Monocyte subsets were defined as Mon1, Mon2, and Mon3. Monocyte platelet aggregates (MPAs) were defined as events positive to both monocyte markers (as above) and the platelet marker CD42a (glycoprotein IX). Absolute counts of monocyte subsets (cells/µl) were calculated count beads according to the manufacturer recommendations.

2.2.2. Expression of surface antigens on monocyte subsets

For my analysis of surface antigens, 100µl of whole blood 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 phosphate buffered saline followed by immediate flow cytometric analysis. Anti-CD16-Alexa Fluor 488 (clone DJ130c, AbD Serotec, Oxford, UK) and anti-CD14-PerCP-Cy5.5 (clone M5E2, BD) were used for definition of monocyte subsets into Mon1, Mon2, and Mon3. PE-conjugated antibodies were used against TLR4 (clone 285219, R&D), CXCR4 (clone 12G5, R&D). APC-conjugated antibodies were used against IL6 receptor (clone 17506, R&D), integrin α4/CD49d, VCAM-1 (clone 7.2R, R&D), VEGF (clone 89106, R&D), Tie2 (clone 83715, R&D).

Table 6:Variability

Flow Cytometry:			
	Protocol Validation	Monocytes	4.6%
	Inflammatory markers:	ILR6	5.6%
	Angiogenic markers:	VEGFR	5.7%
	Angiogenic markers:	TIE2	2.3%

ILR6, interleukin-6 receptor, VEGFR, vascular endothelial growth factor receptor, TIE2, tyrosine kinase receptor

2.3. Carotid Ultrasound

I used a Philips Compact Xtreme CX50 (Bothel, WA, USA) portable ultrasound system with an L12-3 (3-12 MHz frequency) probe was for imaging of the carotid arteries. All images I captured digitally were transferred to a Philips multi-modality image management system, Xcelera with QLAB 7.1 software for analysis and assessment. Each patient underwent CUS followed by contrast in a single session. Images were acquired with the participant's head rotated 45° away from the side of study. A magnified, grey-scale B-mode images of the carotid artery were obtained bilaterally at 3 levels: distal common carotid artery (CCA), carotid bifurcation and the proximal internal carotid artery (ICA) in three different longitudinal planes (anterior oblique, lateral and posterior oblique) and also in the transverse projection. (Grant et al., 2003) I captured a single static image for the CCA, and 2 images, each at different angles, were captured for the carotid bifurcation and the ICA. All still images were captured at end-diastole. A 2 to 4 sec real-time cine video of the CCA and carotid bifurcation was recorded, capturing at least 3 complete cardiac cycles.

IMT and the degree of CD (≥ 50% or less 50%) were determined according to the current guidelines. (Wardlaw and Lewis, 2005) The inter- and intra- observer coefficient of variation in our laboratory was <5%.

2.4. Contrast carotid ultrasound

The Philips CX50 carotid contrast ultrasound pre-sets I used were as follows: frequency: 26Hz, dynamic range: 66, Edge 3, Map G, Ave. 2, Depth 3 cm.

I performed CEUS using Sonovue (Bracco, Milan, Italy) echo contrast determined from my previously published validated protocol. (Jaipersad AS, 2013a)

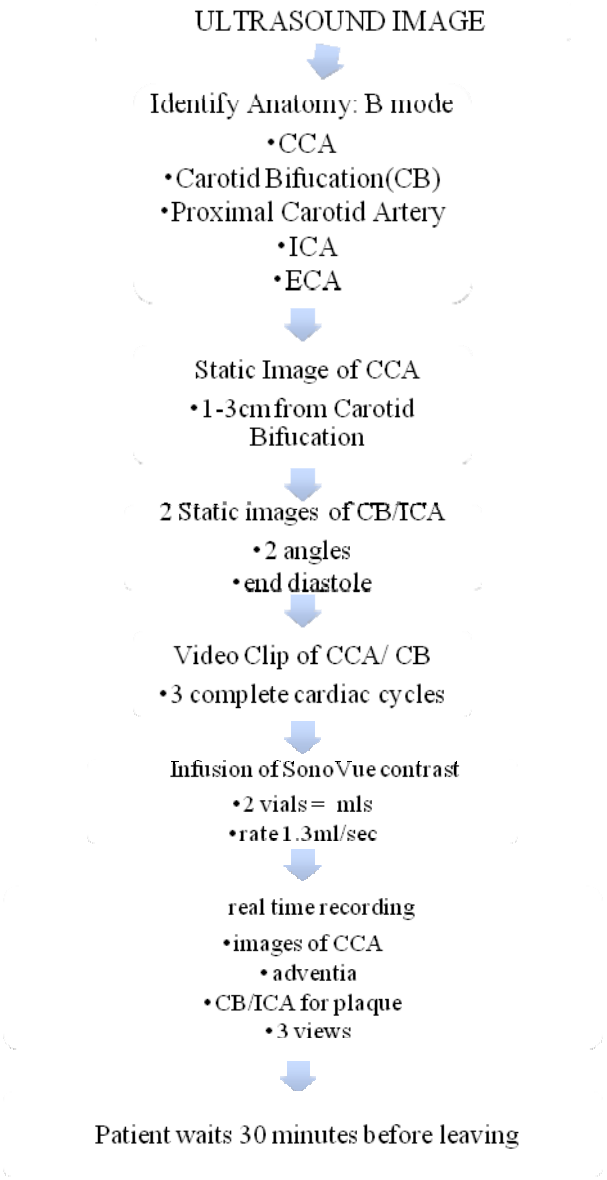


Figure 5: Algorithm used for carotid contrast ultrasound.
CCA, common carotid artery, ICA, internal carotid artery, ECA, external carotid artery

In my protocol, SonoVue (Bracco Imaging S.p.A., Milan, Italy) contrast agent (sulpher hexafluoride microbubbles) was injected using a Vuejet (BR-INF100) infusion pump (Bracco Imaging S.p.A., Milan, Italy). SonoVue has a half-life of 6 min and is cleared by the respiratory system.(Clevert et al., 2011) Documented overall reporting rate of serious adverse effects like sensory motor paresis or chest pain with ST-segment elevation on ECG are less than 0.01%.(Piscaglia and Bolondi, 2006) An infusion pump was used to achieve standardized results, by improving image quality and minimization of artifacts.(Boyajian R.A., 2000, Albrecht et al., 1998) The contrast was infused via an antecubital vein (20 Gauge Venflon) with rate of 1.3 ml/s with 6 ml of SonoVue typically used.

A linear array probe, with a mechanical index 0.08-0.10, was used to achieve the best possible visualization of plaque morphology and vascularization, starting 30 sec after beginning the infusion. During the contrast-enhanced ultrasound imaging I gave special attention to the previously identified lesions. All patients tolerated the exam well with no side effects. High quality images were available for all patients. After 30 min the patients were deemed fit to go home.

Neovascularisation was measured by 2 independent observers of which one was myself, to test inter-observer variability, and repeated by myself to test intra-observer variability on 3 separate occasions (initial review, at week 2, and at 3 months). Plaque neovascularisation was categorised as follows: Grade 0 - no contrast within the plaque; Grade 1 - bubbles confined to the plaque adventitial side; or Grade 2 - bubbles reaching the plaque core and/or contrast agent enhancement throughout the plaque**.**

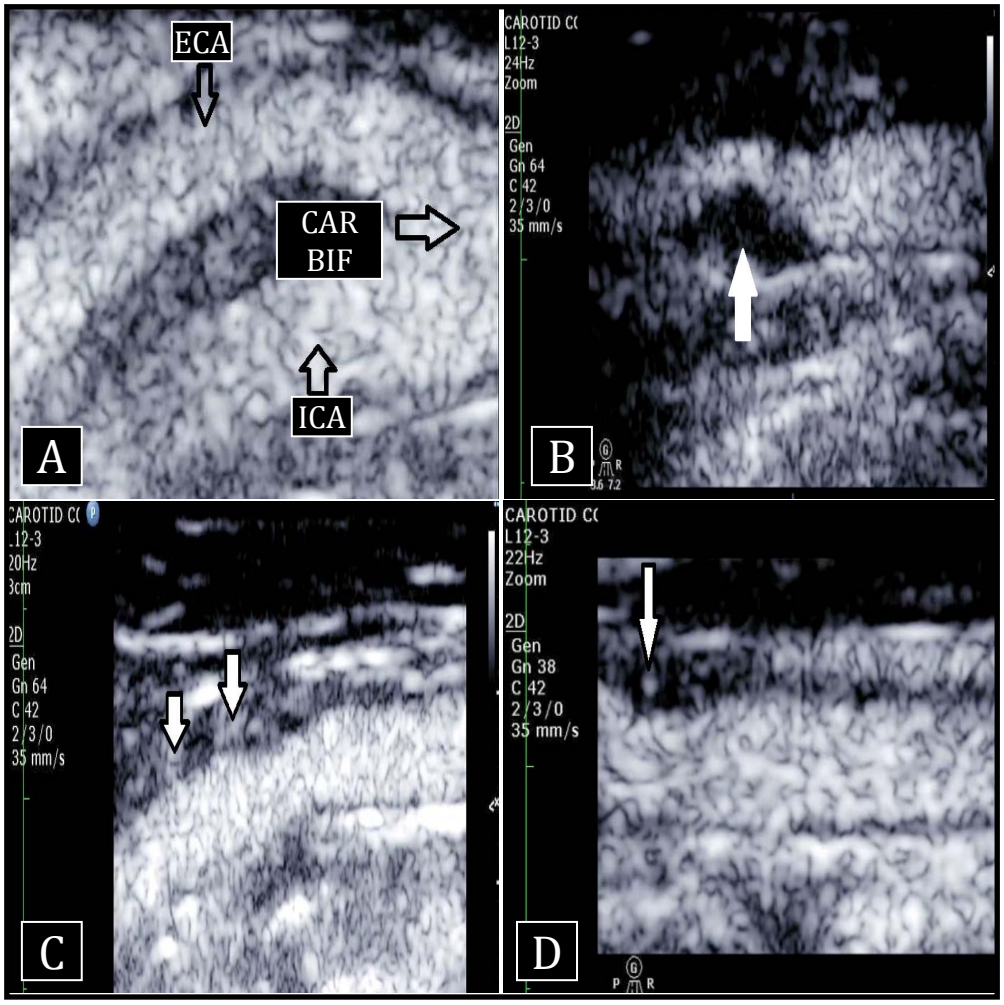


Figure 6. Contrast-enhanced Carotid Ultrasound.
A: Carotid bifurcation with contrast agent. B: Grade 0 ICA lesion (no contrast within the plaque); C: Grade 1 ICA lesion (bubbles confined to the plaque adventitial side); D: Grade 2 ICA lesion (bubbles reaching the plaque core and/or contrast agent enhancement throughout the plaque). CAR BIF, carotid bifurcation, ECA, external carotid artery, ICA, internal carotid artery

The inter- and intra- observer coefficient of variation in our laboratory was <5%. (Jaipersad AS, 2013a)

2.5. Enzyme-linked immunosorbent assay (ELISAs)

Following centrifugation of peripheral venous blood the plasma samples I obtained were stored at –70^{o}C for batched analysis. I measured Tie 2, angiopoietin and VEGF

in citrated plasma by commercial ELISAs technique (R&D Systems, Abingdon, UK). Intra- and inter-assay coefficients of variation were <5% and <10% respectively. My lower limits of detection for Tie2 and angiopoietin were 0.16 ng/mL and 31.2 pg/ml for VEGF.

2.6. Power calculation

Based on my units previous work (Shantsila et al., 2011), the calculated minimum number of participants required to achieve 80% power to detect a difference of 0.5 standard deviations in mean monocyte count between the study groups was n=35 for the cross-sectional study

2.7. Statistical analysis

I expressed normal data as mean [standard deviation, SD], and non-normal data as median [interquartile range, IQR]. The between group comparisons were done using ANOVA with Tukey's post-hoc test (normal data) or Kruskal-Wallis text with Dunn's post-hoc test (non-normal data). The predictive value of the study parameters for CD (assessed as maximal stenosis percentage detected from both sides), and from IMT (assessed as average IMT from the right and left sides), were established using linear regression analysis. My analyses were done across the whole study population (n=160).

Predictive value for intraplaque neovascularisation were done using multinomial regression analysis among participants with detectable atherosclerotic plaques and

good quality contrast study (n=128). The choice of variables for multivariate regression analyses was based on their significance on univariate analysis and relevance. Two-tailed p values were used. Correlation analyses were performed using Spearman test. The differences/effects were considered significant with p value less 0.05, except for comparisons of surface monocyte markers where p value less 0.01 was considered significant to account for multiple comparisons.

In chapter 5, my values of the study parameters before and after the statin withdrawal were compared using paired T-test (normal data) or Wilcoxon text (non-normal data). Correlation analysis was done using Pearson test (normal data) or Spearman test (non-normal data). I hypothesized an intervention effect of 4 units (standard deviation 10 units) in a test statistic with a normal distribution. To achieve this at $2p<0.05$ and 1-beta = 0.8, 52 paired samples are required. Therefore for additional confidence in view of multiple analyses, I recruited in excess of this number.

I performed statistical analysis with SPSS version20 software (IBM) and produced graphs with GraphPad Prism 4.0 software (La Jolla, CA, USA), and calculated the power calculation on Minitab 16 (Minitab Inc., Philadelphia, USA).

CHAPTER III. EVALUATION OF CAROTID PLAQUE NEOVASCULARISATION USING CONTRAST ULTRASOUND

3.1. Abstract

CEUS is increasingly used to improve visualisation of carotid arteries. However, its reproducibility and utility for clinical research are not well established. The aim of the present study was to assess agreeability of detection of carotid artery wall neovascularisation using CEUS. Complete sequenced CEUS images from 10 individuals were analysed for the presence of carotid arterial wall neovascularisation. The images were acquired using Philips CompactXtreme CX50 ultrasound unit with an L12-3 probe and Bracco SonoVue contrast agent. The carotid wall neovascularisation was graded by 2 independent observers with inter-/intra-observer agreement (κ) calculated. Inter-observer κ values for intraplaque neovascularisation (mean [95% confidence interval]) was 0.67 [0.40-0.94] for the left side. Intra-observer κ values for intraplaque neovascularisation was 0.65 [0.38-0.92]. No study-related complications were observed. The CEUS method although semi-quantitative shows moderate-to-strong intra- and inter-agreement for the results and can be used for clinical research purpose.

3.2. Aim

Atherosclerotic intraplaque neovascularisation is associated with high-risk plaque destabilisation. (Vicenzini et al., 2009) Under-nutrition and resulting ischemia of the growing atherosclerotic lesions triggers growth of new intra-wall/intra-plaque

microvessels from adventitia, often being also associated with inflammatory processes. (Giannoni and Vicenzini, 2009, Kumamoto et al., 1995)

Quantification of plaque neovascularisation may improve identification of high risk lesions limited clinical tools are currently available. Ultrasound contrast agents remain within the vascular space, and therefore can be detected by their reflective resonation to highlight the presence of vascular disease.(Clevert et al., 2011) More recently, carotid contrast imaging, an evolving technique, has been used to focus on the plaque and adventitial vasa vasorum.(Clevert et al., 2011) However, reproducibility of the method is poorly established.(Hoogi et al., 2011, Papaioannou et al., 2009, Coli et al., 2008, Huang et al., 2007, Giannoni et al., 2009, Magnoni et al., 2009) The aim of this chapter of my thesis was to evaluate agreeability of detection of carotid plaque vasculature using carotid contrast ultrasound.

3.3. Methods

I performed this validation study on 10 complete sequenced ultrasound images selected randomly from a larger pool of 160 patients by myself, and I individually analysed each for the presence of carotid arterial wall neovascularisation. Neovascularisation was assessed by 2 observers of which one was myself and another independent operator, to test inter-observer variability, and repeated by myself to establish the test intra-observer variability on 3 separate occasions (initial review, at week 2, and at 3 months). The patients personal details were removed from the scans to avoid possible bias during their assessment.

CEUS was performed as discussed in the main methodology section.

I calculated using SPSS version 20 (IBM) the agreement between the 2 reviewers or between different time-points (kappa, κ). (Cohen, 1968) Landis and Koch graded κ values as follows: κ <0 - no conformity; κ = 0-0.20 - slight agreement; κ = 0.2-0.40 - fair agreement; κ = 0.41-0.60 - moderate agreement; κ = 0.61-0.80 - substantial agreement; and κ = 0.81-1 - almost perfect agreement. (Landis and Koch, 1977)

3.4. Results

Ten patients were studied (mean [standard deviation] age 66 [13] years, 50% male). Six patients had history of hypertension, 7 had stable coronary artery disease, 3 were current smokers, and 9 received statins. Two patients had no CD, 4 had unilateral CD, and 2 subjects had >50% CD. Inter-observer κ values for intraplaque neovascularisation (mean [95% CI]) was 0.67 [0.40-0.94] for the left side. Intra-observer κ values for intraplaque neovascularisation (mean [95% CI]) was 0.65 [0.38-0.92] for measurements taken from both (i.e. right and left) sides.

3.5. Discussion

There is a growing awareness that a number of molecular processes such as inflammation, lipid accumulation, angiogenesis and thrombosis may occur at sites of plaque development and that these molecular processes are highly related to the physiological progression to rupture. (Nighoghossian et al., 2005) Although the precise method of remodeling of an asymptomatic plaque into a vulnerable plaque

remains unclear, neovascularisation has been implicated as an important contributor to plaque rupture. (Kolodgie et al., 2003, Takaya et al., 2005) Compared with stable patients, those with vulnerable coronary plaques had a 2-fold increase in their vasa vasorum density increasing further to 4-fold in ruptured plaques. (Virmani, 2005) These changes in the arterial wall are not specific to any particular arterial bed but occur throughout the arterial vasculature, reflecting a systemic nature atherosclerosis. (Fleiner et al., 2004, Nazemi et al., 1990, Wakhloo et al., 2004)

In the current study, I was able to demonstrate, using a standardised technique with the use of SonoVue contrast agent, the presence of contrast agent within the atherosclerotic plaque. The study results confirm the potential of contrast-enhanced carotid ultrasonography for assessment of intra-plaque neovascularisation. (Xiong et al., 2009) The inter- and intra-observer variability for detecting the presence of carotid plaque neovascularisation can be acceptable for research purposes. Clinical utility of the approach needs to be assessed in prospective studies.

3.6. Conclusion

The application of this method can potentially be adapted, following further analysis, for the development of quantitative measurements; this application is currently under review. The clinical implications of carotid contrast ultrasound may be at the forefront of further enhancing plaque knowledge, predicting stroke risk and helping to develop targeted therapeutic strategies on plaque remodeling and stabilisation. The method I propose here for detecting neovascularisation is novel and accurate and although it remains a subjective method, my study has demonstrated that it is agreeable.

CHAPTER IV. EXPRESSION OF MONOCYTE SUBSETS AND ANGIOGENIC MARKERS IN RELATION TO CAROTID PLAQUE NEOVASCULARISATION IN PATIENTS WITH PRE-EXISTING CORONARY ARTERY DISEASE AND CAROTID DISEASE

4.1. Abstract

Aim: To characterise blood monocyte subsets in patients with different degrees of carotid atherosclerosis and pathological carotid plaque neovascularisation.

Methods: Assessment of carotid plaque neovascularisation using contrast ultrasonography and flow cytometric quantification of monocyte subsets and their receptors involved in inflammation, angiogenesis and tissue repair was done in 40 patients with carotid disease (CD) ≥50% and documented CAD (grp.1 CD>50), 40 patients with CD <50% and documented CAD (grp.2 CD<50), 40 hypercholesterolaemic controls (grp.3 HC group) and 40 normocholesterolaemic controls (grp.4 NC).

Results: Grp.1 CD>50 and grp.2 CD<50 groups had increased counts of Mon1 ("Classical" CD14++CD16-CCR2+ cells) compared to grp.3 HCs (p=0.03, and p=0.009). Mon3 (CD14+CD16++CCR2- cells) were only increased in grp.2 CD<50 compared with grp.3 HCs (p<0.01). Both grp.1 CD>50 and grp.2 CD<50 groups showed increased expression of proinflammatory interleukin-6 receptor on Mon1 and Mon2 (CD14++CD16+CCR2+ cells); TLR4, proangiogenic Tie2 on all subsets (p<0.01 for all). In multivariate regression analysis only high Mon1 count was a significant predictor of carotid disease (p=0.04) and intima-media thickness (p=0.02).

In multivariate regression analysis only Mon1 subset was significantly associated with severe, Grade 2 neovascularisation (p=0.034).

Conclusion: Classical monocytes (Mon1) represent the only monocyte subset predictive of the severity of carotid and systemic atherosclerosis, such as carotid IMT, degree carotid disease and presence of carotid intraplaque neovascularisation.

4.2. Introduction

The changes in arterial wall vascularisation are uniformly present in different arterial beds (in contrast to often selected location of the plaques themselves) and support the concept of symptomatic atherosclerosis as a panarterial disease.(Fleiner et al., 2004) However, the factors/ mechanisms triggering the switch from relatively benign systemic atherosclerosis (i.e. intima-media thickening, increased density of adventitial vasa vasorum) to rapidly progressive stenotic or associated with intraplaque neovascularisation are poorly understood.

A potential link between monocytes and abnormal plaque angiogenesis has a strong biological justification. Monocytes activity is directly associated with the inflammatory status. Activated monocytes promote the synthesis of proinflammatory molecules, such as interleukin-6 (IL-6) and tumour necrosis factor-α, mediated by stimulation of Toll-like receptors (TLR4).(Satoh et al., 2006, Shantsila and Lip, 2009) Monocyte activation enhances the affinity of monocyte ligands to adhesion molecules, thus promoting monocyte–endothelium adhesion.(Lauener et al., 1990) In support of this argument, microvessels within lipid-rich plaques strongly express adhesion molecules, such as vascular cell adhesion molecule (VCAM), with

monocytes expressing receptors to the molecule.(O'Brien et al., 1996, van der Wal AC, 1992) Their interaction may facilitate transendothelial migration of inflammatory cells (i.e. monocytes) into the plaque microenvironment. Also, monocytes per se have proangiogenic properties.(Jaipersad AS, 2014) Additionally, monocytes have been found to constitute the dominant population among circulating cells expressing type2 receptor for vascular endothelial growth factor (VEGF), Tie2, CXCR4 – which are receptors implicated in angiogenesis and tissue remodelling.(Romagnani et al., 2005) Whilst a role for macrophages in the development and progression of atherosclerosis has long been recognised, the role of their blood-borne ancestors – monocytes, represented by different functional subsets – has scarcely been addressed.(Schmitz and Grandl, 2008)

In my study I firstly aimed to assess different monocyte subsets and their expression of receptors involved in inflammation, angiogenesis/tissue remodelling in patients with different degrees of carotid atherosclerosis with evidence of a systemic atherosclerotic process (i.e., concomitant coronary artery disease (CAD)), who were compared to hypercholesterolaemic and normocholesterolaemic controls. Second, I aimed to establish a relation between the monocyte subsets and carotid plaque neovascularisation, by using a novel method of contrast carotid ultrasonography as previously published by our unit. (Jaipersad AS, 2013a)

4.3. Methods

I performed both flow cytometry and CUS as discussed in the main methodology section.

4.4. Results

4.4.1. Monocyte subsets and carotid atherosclerosis

The study groups were matched for age and gender, but, as expected, there were significant differences between the groups in clinical characteristics.

Table 7. Demographic, clinical and carotid artery characteristics in the study groups

	Grp. 1 CD>50 (n=40)	Grp.2 CD<50 (n=40)	Grp. 3 HC (n=40)	Grp. 4 NC (n=40)	p value
Age, years	70 [9]	69 [9]	67 [7]	67 [6]	0.22
Gender (male), n [%]	24 [60]	27 [68]	17 [43]	17 [43]	0.053
Systolic BP, mmHg	139 [20]	137 [21]	148 [19]	143 [23]	0.08
BMI, kg/m2	26 [5]	28 [7]	29 [6]	27 [5]	0.12
Cholesterol, mmol/L	4.0 [0.6]†‡	3.9 [0.6]†‡	6.7 [1.1]‡	3.3 [0.9]	<0.001
eGFR, mL/min/1.67	71 [9]	74 [11]	72 [10]	73 [10]	0.59
Hypertension, n [%]	28 [70]†‡	23 [58]‡	17 [43]‡	0	<0.001
Diabetes, n [%]	10 [25]†‡	7 [18]†‡	1 [3]	0	0.001
Current smokers, n [%]	16 [40]†‡	12 [30]‡	6 [15]	7 [18]	0.022
Aspirin, n [%]	37 [93]†‡	36 [90]†‡	6 [15]‡	0	<0.001
ACEI/ARB, n [%]	23 [58]†‡	17 [43]†‡	4 [10]‡	0	<0.001
CCB, n [%]	20 [50]†‡	9 [23]‡	10 [25]‡	0	<0.001
Beta-blocker, n [%]	15 [38]*†‡	21 [53]†‡	4 [10]‡	0	<0.001

Statin, n [%]	40 [100]†‡	40 [100]†‡	0	0	<0.001
Intima-media thickness					
Right CCA, mm	0.75 [0.2]	0.74 [0.1]	0.68 [0.1]	0.70 [0.1]	0.07
Left CCA, mm	0.78 [0.2]	0.81 [0.2]	0.74 [0.1]	0.76 [0.1]	0.15
Carotid Disease					
Right CCA, n [%]					
<20%	6 [15]	13 [33]	32 [80]	31 [78]	<0.001
20-49%	8 [20]	27 [68]	8 [20]	9 [23]	
>50%	26 [65]	10 [25]	0	0	
Left CCA, n [%]					
<20%	7 [18]	7 [18]	25 [63]	30 [75]	<0.001
20-49%	4 [10]	33 [83]	15 [38]	10 [25]	
>50%	29 [73]	0	0	0	
Plaque neovascularisation					
Right CCA, n [%]					
Grade 1	17 [43]	14 [35]	2 [5]	2 [5]	<0.001
Grade 2	4 [10]	4 [10]	5 [13]	1 [3]	
Left CCA, n [%]					
Grade 1	11 [28]	13 [33]	9 [23]	7 [18]	0.005
Grade 2	7 [18]	4 [10]	8 [20]	3 [8]	

*p<0.05 vs. grp2. CD<50 group, †p<0.05 vs. grp.3 HCs, ‡p<0.05 vs. grp.4 NCs. Continuous data are presented as mean [standard deviation] for normal data or median [interquartile range] for non-normal data. ACEI/ARB, angiotensin converting enzyme inhibitor/angiotensin receptor blocker; BMI, body mass index; BP, blood pressure; CCA, common carotid artery; CCB, calcium channel blocker; CD>50, carotid disease ≥50 and CAD; CD>50, carotid disease <50 and CAD; eGFR, estimated glomerular filtration rate; HC, hypercholesterolaemic controls; NC, normocholesterolaemic controls.

A proportion of control subjects had non-significant asymptomatic carotid atherosclerotic lesions (i.e., <50%) reflecting the natural pattern of high prevalence of subclinical stable atherosclerotic plaques in older populations. The grp.1 CD>50 and grp.2 CD<50 groups were well matched for majority of demographic and clinical characteristics. There was no significant difference in IMT between the study groups. All patients from grp.1 CD>50 and grp. 2 CD<50 groups received statins, whilst control groups were free of statins.

Grp. 1 CD>50 group had increased counts of Mon1 compared to grp.3 HCs ($p=0.03$) and MPAs lower than in grp.2 CD<50 group ($p<0.001$). This group also had increased expression of CD14 on Mon2 ($p=0.001$ vs. grp.3 HC); IL6R on Mon1 and Mon2 ($p<0.001$ vs. grp.4 NC); TLR4 on Mon1 and Mon3 vs. grp.2 CD<50 ($p<0.01$) and TLR4 on all subsets vs. grp.3 HC and grp.4 NC ($p<0.001$); VEGF receptor 2 on Mon2 ($p<0.001$ vs.grp.4 NC) and on Mon3 ($p<0.01$ vs. grp.3 HC and $p<0.001$ vs. grp.4 NC); CXCR4 on all subsets ($p<0.001$ vs. grp.4 NC for all 3 subsets); Tie2 on Mon1 ($p<0.001$ vs. grp.4 NC and grp.3 HC), Mon2 ($p<0.001$ vs. grp.4 NC), and Mon3 ($p<0.01$ vs. grp.4 NC). Grp.1 CD>50 group had decreased CD16 expression on all subsets ($p\leq0.001$ vs. all other groups).

Similarly to grp.1 CD>50, grp.2 CD<50 group had increased counts of Mon1 compared to grp.3 HCs ($p=0.009$).

Table 8. Monocyte subsets in the study groups

	Grp.1 CS>50 (n=40)	Grp.2 CS<50 (n=40)	Grp.3 HC (n=40)	Grp.4 NC (n=40)	p value
Counts of monocyte subsets and MPAs					
Mon1, per µL	472 [153]‡	484 [148]‡	420 [121]	385 [130]	0.005
Mon2, per µL	36 [27-67]	35 [27-58]	32 [20-60]	25 [18-50]	0.13
Mon3, per µL	56 [42-78]	62 [46-77]‡	49 [38-73]	44 [34-54]	0.008
MPA, per µL	79 [60-104]*	142 [91-162]‡	93 [77-129]‡	61 [39-100]	<0.001
Monocyte surface markers					
CCR2 (Mon1)	157 [46]	160 [32]	154 [31]	141 [38]	0.093
CCR2 (Mon2)	130 [42]	116 [22]	117 [25]	112 [26]	0.049
CCR2 (Mon3)	15 [2]	16 [2]‡	15 [1]	15 [3]	0.002
CD14 (Mon1)	1351 [286]	1373 [248]	1291 [336]	1434 [308]	0.192
CD14 (Mon2)	1435 [361]†	1349 [327]	1129 [312]	1308 [388]	0.001
CD14 (Mon3)	154 [53]	160 [34]	150 [39]	163 [45]	0.52
CD16 (Mon1)	8.4 [2.1]*†‡	11.5 [1.8]	11.7 [1.8]	12.3 [2.1]	<0.001
CD16 (Mon2)	45 [12]*†‡	66 [11]	67 [8]	66 [10]	<0.001
CD16 (Mon3)	123 [50]*†‡	175 [59]	187 [57]	182 [64]	<0.001
IL6R (Mon1)	75 [15]‡	78 [15]‡	67 [23]‡	53 [22]	<0.001
IL6R (Mon2)	66 [12]‡	69 [12]‡	59 [20]‡	44 [21]	<0.001
IL6R (Mon3)	34 [8]	40 [11]	42 [13]	34 [13]	0.003
TLR4 (Mon1)	5.7 [4.5-7.1] *†‡	3.7 [2.4-4.7]†	1.6 [1.5-1.8]	1.7 [1.5-4.7]	<0.001
TLR4 (Mon2)	8.7 [5.9-14]†‡	5.3 [2.4-8.7]†‡	1.3 [1.3-1.5]	1.3 [1.2-6.7]	<0.001
TLR4 (Mon3)	3.4 [2.6-4.1]*†‡	1.9 [1.5-2.9]‡	1.3 [1.2-1.4]	1.2 [1.1-2.4]	<0.001

CD49d (Mon1)	10 [7.7-13]*	17 [12-22]†‡	11 [5.4-18]‡	2.9 [2.3-10]	<0.001
CD49d (Mon2)	18 [14-23]*	37 [25-44]†‡	26 [8.6-33]	6.5 [3.6-21]	<0.001
CD49d (Mon3)	28 [26-37]*	53 [32-69]‡	36 [19-49]	25 [15-31]	<0.001
VEGF receptor 2 (Mon1)	6.6 [5.4-8.1]	6.0 [4.9-7.1]	5.8 [4.7-7.0]	5.4 [4.0-7.9]	0.087
VEGF receptor 2 (Mon2)	12 [10-18]‡	10 [8.3-14]	10 [6.9-13]	8.4 [5.6-12]	0.001
VEGF receptor 2 (Mon3)	3.7 [2.8-4.8]†‡	2.9 [2.2-3.5]†	2.5 [1.8-3.9]	1.8 [1.4-2.6]	<0.001
CXCR4 (Mon1)	14 [12-16]‡	12 [11-15]	13 [12-16]‡	11 [8.7-13]	<0.001
CXCR4 (Mon2)	19 [16-24]‡	18 [14-25]	18 [15-26]‡	14 [10-20]	0.001
CXCR4 (Mon3)	6.7 [5.4-8.7]‡	5.1 [4.0-7.3]‡	5.6 [4.0-7.7]‡	3.2 [2.4-5.4]	<0.001
Tie2 (Mon1)	6.1 [5.0-7.6]†‡	5.0 [4.3-6.4]‡	4.3 [2.5-5.2]	1.9 [1.6-4.9]	<0.001
Tie2 (Mon2)	10 [7.9-15]‡	11 [7.6-15]‡	7.6 [4.4-12]	3.6 [2.2-10]	<0.001
Tie2 (Mon3)	8.2 [6.0-10]‡	8.0 [6.8-10]‡	7.1 [5.6-8.5]	5.7 [4.6-8.3]	0.001

*p<0.05 vs. grp.2 CD<50 group (p<0.01 for the surface markers), †p<0.05 vs. grp.3 HCs (p<0.01 for the surface markers), ‡p<0.05 vs. grp.4 NCs (p<0.01 for the surface markers).

ANOVA with Tukey's post-hoc test (normal data) or Kruskal-Wallis text with Dunn's post-hoc test (non-normal data). Data are presented as mean [standard deviation] for normal data or median [interquartile range] for non-normal data. CD>50, carotid disease ≥50% and CAD; CD>50, carotid disease <50% and CAD; IL6R, interleukin 6 receptor; Mon1, CD14++CD16-CCR2+ monocytes; Mon2, CD14++CD16+CCR2+ monocytes; Mon3, CD14+CD16++CCR2- monocytes; TLR4, toll-like receptor 4; VEGF, vascular endothelial growth factor; HC, hypercholesterolaemic controls; NC, normocholesterolaemic controls.

But Mon3 were also increased in grp.2 CD<50 compared with grp.3 HCs (p<0.01). MPA were higher in grp.2 CD<50 than in both grp.1 CD>50 (p<0.001) and grp.4 NCs (p<0.001). The grp.2 CD<50 group showed increased expression of CCR2 on Mon3 (p=0.002 vs. grp.4 NC); IL6R on Mon1 and Mon2 (p<0.001 vs. grp.4 NC); TLR4 on

Mon1 (p<0.001 vs. grp.3 HC), Mon2 (p<0.001 vs. grp.3 HCs, p<0.01 vs. grp.4 NCs), and Mon3 (p<0.001 vs. grp.4 NC); VEGF receptor 2 on Mon3 (p<0.01 vs. grp.3 HC); CXCR4 on Mon3 (p<0.01 vs. grp.3 HC); Tie2 on all subsets vs. grp.4 NC (p<0.001 for Mon1 and Mon2, p<0.01 for Mon3). Patients from the grp.2 CD<50 had the highest of all groups expression of CD49d on all monocyte subsets (p<0.01 for all). In contrast to grp.1 CD>50, monocyte CD16 expression was not changed in grp.2 CD<50 patients.

In univariate regression analysis, higher Mon1 counts were predictive of degree of maximal CD (p<0.001), whilst there was modest predictive value for Mon3 (p=0.03) and a non-significant trend for Mon2 (p=0.06).

Table 9: Linear regression analysis for predictors of carotid stenosis and intima-media thickness.

Predictor	Stenosis*			Intima-media thickness†		
(n=160)	B [SE]	β	p	B [SE]	β	p
Univariate analysis						
Age	0.6 [0.2]	0.21	0.01	0.01 [0.00]	0.36	<0.001
Male gender	12 [3]	0.26	<0.001	0.05 [0.02]	0.19	0.02
Systolic BP	-0.1 [0.1]	-0.09	0.23	0.00 [0.00]	0.00	0.96
BMI	-0.6 [0.3]	-0.16	0.04	0.00 [0.00]	-0.03	0.70
CAD	32 [2]	0.74	0.000	0.05 [0.02]	0.21	0.01
Hypertension	14 [3]	0.32	0.000	0.02 [0.02]	0.09	0.28
Diabetes	23 [5]	0.33	0.000	0.05 [0.03]	0.13	0.11
Smoking	13 [4]	0.26	0.001	0.04 [0.02]	0.14	0.08

Aspirin	28 [3]	0.65	0.000	0.06 [0.02]	0.23	0.01
ACEI/ARB	21 [3]	0.44	0.000	0.05 [0.02]	0.17	0.03
Statin	32 [2]	0.74	0.000	-0.01 [0.01]	-0.04	0.62
Cholesterol	-2.8 [1.1]	-0.20	0.01	-0.01 [0.01]	-0.13	0.10
eGFR	-0.20 [0.17]	-0.09	0.24	-0.001 [0.001]	-0.07	0.40
Mon1	0.04 [0.01]	0.26	<0.001	0.000 [0.000]	0.16	0.05
Mon2	0.11 [0.06]	0.15	0.060	0.001 [0.000]	0.17	0.03
Mon3	0.15 [0.07]	0.17	0.030	0.001 [0.000]	0.13	0.11
MPA	0.01 [0.03]	0.03	0.68	0.000 [0.000]	0.10	0.24
Multivariate analysis*						
Mon1	0.02 [0.01]	0.15	0.04	0.03 [0.01]	0.19	0.02
Mon2	0.05 [0.05]	0.06	0.36	0.06 [0.06]	0.08	0.27
Mon3	-0.01 [0.06]	-0.02	0.82	0.06 [0.07]	0.06	0.41
MPA	-0.02 [0.03]	-0.04	0.53	0.00 [0.03]	-0.01	0.92

*Adjusted for age, sex, hypertension, diabetes, smoking, cholesterol level for multivariate analysis.

†For multivariate analysis, adjusted for age, gender, smoking for multivariate analysis

ACEI/ARB, angiotensin converting enzyme inhibitor/angiotensin receptor blocker; B, coefficient of regression; BMI, body mass index; BP, blood pressure; CAD, coronary artery disease; eGFR, estimated glomerular filtration rate; IMT, intima-media thickness, Mon1, CD14++CD16-CCR2+ monocytes; Mon2, CD14++CD16+CCR2+ monocytes; Mon3, CD14+CD16++CCR2- monocytes; MPA, monocyte-platelet aggregates; SE, standard error.

In multivariate regression analysis only high Mon1 counts remained a significant predictor of CD (p=0.04). Mon1 was a significant predictor of IMT on multivariate analysis (p=0.02).

4.4.2. Monocyte subsets and plaque neovascularisation

From the whole study population, 128 subjects had atherosclerotic plaques suitable for assessment of intraplaque neovascularisation.

Table 10: Demographic and clinical characteristics of patients with different grades of plaque neovascularisation

	Grade 0 (n=42)	Grade 1 (n=54)	Grade 2 (n=32)	p value
Age, years	68 [8]	71 [8]	68 [8]	0.18
Gender (male), n [%]	24 [57]	32 [59]	23 [72]	0.39
Systolic BP, mmHg	141 [19]	146 [21]	136 [24]	0.13
BMI, kg/m2	29 [7]*	26 [5]	27 [5]	0.036
Cholesterol, mmol/L	4.4 [1.6]	4.3 [1.3]	4.9 [1.8]	0.21
eGFR, mL/min/1.67	75 [10]*	71 [10]	69 [8]	0.030
CAD, n [%]	22 [52]	37 [69]	19 [59]	0.27
Hypertension, n [%]	18 [43]	25 [46]	18 [56]	0.50
Diabetes, n [%]	6 [14]	5 [9]	6 [19]	0.44
Current smokers, n [%]	10 [24]	19 [35]	8 [25]	0.41
Aspirin, n [%]	19 [45]	36 [67]	20 [63]	0.074
ACEI/ARB, n [%]	11 [26]	20 [37]	12 [38]	0.46
Statins, n [%]	22 [52]	37 [69]	19 [59]	0.27

Grade 0 = no detectable intraplaque neovascularisation

Grade 1 = neovascularisation (mild)

Grade 2 = neovascularisation (severe)

P<0.05 vs. Grade 2. Continuous data are presented as mean [standard deviation] for normal data or median [interquartile range] for non-normal data. ACEI/ARB, angiotensin converting enzyme inhibitor/angiotensin receptor blocker; BMI, body mass index; BP, blood pressure; CCA, common carotid artery; eGFR, estimated glomerular filtration rate.

The Grade 0 group has slightly higher body mass index (BMI) and estimated glomerular filtration rate (eGFR) then Grade 2 group (p=0.03 for both). Patient with Grade 2 neovascularisation had higher Mon1 count compared to those with Grade 0 (p=0.048 for ANOVA and p=0.06 for post-hoc analysis) on multivariate analysis.

Table 11: Monocyte subsets and plasma markers of angiogenesis in patients with different grades of plaque neovascularisation

	Grade 0 (n=42)	Grade 1 (n=54)	Grade 2 (n=32)	p
Counts of monocyte subsets and MPAs				
Mon1, per μL	406 [124]*	464 [150]	482 [149]	0.048
Mon2, per μL	42 [23-59]	32 [23-60]	34 [29-54]	0.71
Mon3, per μL	50 [36-71]	57 [40-77]	48 [35-74]	0.44
MPA, per μL	82 [60-126]	94 [57-142]	99 [77-137]	0.33
Monocyte surface markers				
CCR2 (Mon1)	155 [37]	149 [34]	165 [37]	0.14
CCR2 (Mon2)	122 [30]	114 [30]	127 [35]	0.13
CCR2 (Mon3)	16 [2]	15 [2]	16 [2]	0.67
CD14 (Mon1)	1398 [344]	1353 [292]	1334 [276]	0.65
CD14 (Mon2)	1343 [357]	1321 [365]	1321 [366]	0.95

CD14 (Mon3)	156 [38]	154 [38]	161 [50]	0.73
CD16 (Mon1)	11 [3]	10 [2]	10 [2]	0.02
CD16 (Mon2)	61 [12]	58 [15]	61 [16]	0.59
CD16 (Mon3)	178 [64]	150 [60]	161 [56]	0.09
IL6R (Mon1)	67 [23]	69 [22]	74 [17]	0.36
IL6R (Mon2)	59 [21]	61 [20]	65 [15]	0.37
IL6R (Mon3)	39 [15]	35 [10]	37 [9]	0.19
TLR4 (Mon1)	2.9 [1.6-5.1]	4.5 [1.8-5.9]	3.3 [1.6-5.6]	0.05
TLR4 (Mon2)	3.6 [1.3-7.8]	6.2 [1.6-12]	3.3 [1.3-10]	0.07
TLR4 (Mon3)	1.7 [1.3-3.0]	2.6 [1.4-3.9]	1.8 [1.3-3.2]	0.06
CD49d (Mon1)	10 [6.1-18]	11 [7.1-18]	11 [8.1-17]	0.74
CD49d (Mon2)	21 [12-39]	24 [15-35]	24 [14-32]	0.67
CD49d (Mon3)	29 [22-47]	34 [25-52]	37 [24-52]	0.41
VEGF receptor 2 (Mon1)	6.5 [5.0-7.5]	5.5 [4.2-7.2]	6.1 [5.2-6.9]	0.13
VEGF receptor 2 (Mon2)	10.4 [8.3-15]	10 [7.4-15]	10.5 [7.9-15]	0.80
VEGF receptor 2 (Mon3)	2.9 [2.1-4.1]	2.9 [2.1-3.8]	3.0 [2.3-4.0]	0.93
CXCR4 (Mon1)	12 [11-15]	13 [11-15]	13 [11-16]	0.47
CXCR4 (Mon2)	17 [13-23]	19 [16-26]	18 [15-23]	0.16
CXCR4 (Mon3)	4.7 [3.8-6.3]	5.7 [4.4-8.5]	6.4 [3.9-7.6]	0.12
Tie2 (Mon1)	4.8 [3.8-6.4]	5.0 [3.9-7.1]	5.0 [4.2-6.1]	0.70
Tie2 (Mon2)	8.3 [5.7-14]	10 [7.0-15]	10 [6.8-12]	0.45
Tie2 (Mon3)	7.3 [5.7-8.7]	7.4 [6.0-8.8]	8.4 [6.0-11]	0.28
Plasma markers of angiogenesis				
Tie 2, ng/mL	139 [106-186]	128 [103-165]	126 [104-163]	0.84
Angiopoietin 2, ng/mL	2.5 [1.6-3.9]	2.5 [1.6-4.3]	2.2 [1.2-3.9]	0.92

VEGF, pg/mLs	5 [0-93]	20 [0-115]	30 [0-225]	0.62

*p=0.06 vs. Grade 2. ANOVA with Tukey's post-hoc test (normal data) or Kruskal-Wallis text with (non-normal data). Data are presented as mean [standard deviation] for normal data or median [interquartile range] for non-normal data. IL6R, interleukin 6 receptor; Mon1, CD14++CD16-CCR2+ monocytes; Mon2, CD14++CD16+CCR2+ monocytes; Mon3, CD14+CD16++CCR2- monocytes; MPA, monocyte-platelet aggregates; TLR4, toll-like receptor 4; VEGF, vascular endothelial growth factor.

There were no differences in levels of other monocyte subsets and MPA, nor in expression the studied monocyte surface markers at the $p<0.01$ significance threshold. No differences between groups were seen in levels of plasma markers of angiogenesis, with no correlation found between their levels and density of the correspondent receptors on monocytes.

In univariate regression analysis, predictors of Grade 1 neovascularisation were low BMI (p=0.02), smoking (p=0.02), degree of CD (p=0.02) and lower eGFR (p=0.05 for a trend).

Table 12: Multinomial regression analysis for predictors of plaque angiogenesis

n=128	Grade 1 vs. Grade 0 OR [95% CI]	p	Grade 2 vs. Grade 0 OR [95% CI]	p
Univariate analysis				
Age	1.04 [0.99-1.10]	0.11	1.00 [0.94-1.06]	1.00
Gender	1.09 [0.48-2.47]	0.83	1.92 [0.72-5.12]	0.19
BMI	0.91 [0.84-0.98]	0.02	0.94 [0.87-1.02]	0.15
CAD	0.51 [0.22-1.16]	0.11	0.75 [0.30-1.91]	0.55

Hypertension	0.87 [0.39-1.96]	0.74	0.58 [0.23-1.48]	0.25
Diabetes	1.63 [0.46-5.77]	0.45	0.72 [0.21-2.49]	0.61
Smoking	3.25 [1.21-8.74]	0.02	2.20 [0.95-5.05]	0.06
Aspirin	0.39 [0.17-0.90]	0.03	0.50 [0.19-1.27]	0.14
ACEI/ARB	0.60 [0.25-1.46]	0.26	0.59 [0.22-1.60]	0.30
Carotid stenosis	1.03 [1.01-1.05]	0.02	1.03 [1.00-1.06]	0.03
Cholesterol	0.94 [0.72-1.24]	0.68	1.20 [0.90-1.60]	0.22
eGFR	0.96 [0.92-1.00]	0.05	0.94 [0.89-0.99]	0.02
Mon1	1.00 [1.00-1.01]	0.046	1.00 [1.00-1.01]	0.023
Mon2	1.00 [0.98-1.01]	0.83	1.00 [0.99-1.02]	0.72
Mon3	1.01 [0.99-1.03]	0.23	1.01 [0.99-1.03]	0.45
MPA	1.00 [0.99-1.01]	0.71	1.00 [1.00-1.01]	0.35
Multivariate analysis				
Mon1*	1.03 [0.99-1.06]	0.11	1.04 [1.01-1.8]	0.022
Mon1†	1.03 [0.99-1.06]	0.13	1.04 [1.00-1.08]	0.034

* Adjusted for age, BMI, eGFR, aspirin, statins

† Adjusted for age, BMI, eGFR, carotid artery disease, aspirin, statins

ACEI/ARB, angiotensin converting enzyme inhibitor/angiotensin receptor blocker; BMI, body mass index; CAD, coronary artery disease; eGFR, estimated glomerular filtration rate; Mon1, CD14++CD16-CCR2+ monocytes; Mon2, CD14++CD16+CCR2+ monocytes; Mon3, CD14+CD16++CCR2- monocytes; MPA, monocyte-platelet aggregates.

Predictors of Grade 2 neovascularisation were degree of CD (p=0.03), reduced eGFR (p=0.02), with a trend seen for smoking (p=0.06). High Mon1 count was significantly associated with both Grade 1 (p=0.046) and Grade 2 (p=0.023) neovascularisation. On multivariate analysis, Mon1 remained significantly associated with Grade 2

neovascularisation after adjustment for age, BMI, eGFR, CD, aspirin and statin use (p=0.034).

4.5. Discussion

My study demonstrates for the first time that counts of 'classical' monocytes (Mon1) is the only monocyte subset associated with severity of carotid atherosclerosis independently of other risk factors. This subset was also the only monocyte subset which counts were predictive of IMT, a marker of carotid and generalised atherosclerosis.(Bots and Grobbee, 2002, Nambi et al., 2012)

Despite some controversy in respect to the link between the total monocyte count and carotid atherogenesis, overall, the published data support the role of monocytosis in carotid atherogenesis.(Johnsen et al., 2005, Huang et al., 1996) In 2011, Hoogi et al, was able to demonstrate a clear correlation between the findings of neovascularisation on carotid contrast ultrasound and histological analysis. (Hoogi et al., 2011) In a prospective study of subjects free from carotid disease during a 10-year follow up, total monocyte count was significantly predictive of future plaque development.(Johnsen et al., 2005) Until now it has been unclear which of the monocyte subsets were principally implicated in the process.

Of interest Mon3 monocytes (i.e., 'non-classical' monocytes) which were previously shown to be increased in stable CAD were only significantly increased in patients with moderate, but not with severe, carotid stenosis in the present study.(Schlitt et al., 2004) The exact reasons of this difference I cannot fully explained based on design of

the present study but it could be due to predominant role of the subset in the initial development and growth of atherosclerotic plaques. I also observed a similar phenomenon in relation to expression of CD49d by all monocyte subsets (i.e.. a receptor that mediates recruitment of monocytes to areas of vascular inflammation and atherogenesis)(O'Brien et al., 1996) and levels of MPAs (another phenomenon contributing to monocyte migration to the vascular tissues).(van Gils et al., 2008)

Not only numbers of monocytes but also their functional status could play a role in atherosclerotic plaque formation. My present study supports this hypothesis by demonstrating significant upregulation of different monocyte receptors mediating inflammatory and angiogenic processes in the patients with carotid atherosclerosis. These changes were not confined to a single monocyte subset but were present variably across all 3 monocyte subpopulations, possibly reflecting complex implication and functions of different subsets in atherogenesis. Of note, some features on excessive proinflammatory activation, such as increased expression of IL6R on Mon2 was only seen in grp.1 CD>50 patients suggesting higher degree on monocyte proatherogenic changes in subjects prone to more severe coronary atherosclerosis. Interestingly, patients with the severe carotid stenosis had highly significant reduction in CD16 expression by all monocytes. The presence of this phenomenon does not have a straightforward explanation and farther investigation of related changes in monocyte functionality may be of interest.

My present study is the first to evaluate the role of monocytes and their subsets in carotid plaque neovascularisation and to show a significant and independent association of Mon1 subset in development of plaque neovascularisation. Two major factors influencing intravascular neovascularisation are local ischaemia and

inflammatory burden (either local or systemic). Diffusion of oxygen and other nutrients is limited to 100μm from the lumen of the blood vessel, which in normal arteries is adequate to nourish the inner media and intimal layers. As vessel wall thickness increases in the setting of vascular disease, proliferation of the vasa vasorum and intimal neovascularization is observed and the degree of adventitial neovascularization is associated with carotid IMT. (Magnoni et al., 2009) The role of local ischemia in angiogenesis is supported by evidence of a rabbit model of high local levels of hypoxia inducible factor-1, (Bjornheden et al., 1999) which is a recognised promoter of production of vascular endothelial growth factor (VEGF). (Kuwahara et al., 2002) Consequently, VEGF (a potent stimulator of angiogenesis) is able orchestrate a local proangiogenic environment and to mobilise endothelial progenitors. Indeed, VEGF is abundantly expressed within atherosclerotic lesions. (Inoue et al., 1998) Lack of significant systemic plasma VEGF levels changes could be due to the relatively small area of localised carotid plaque angiogenesis.

Hypoxia-independent pathways of angiogenesis within the vessel wall have been identified and appear to depend on an inflammatory stimulus.(Carmeliet, 2003) The density of intraplaque vessels corresponds to the focal accumulation of inflammatory cells (such as monocytes/macrophages) forming a vicious circle: *enhanced angiogenesis – enhanced mobilization of inflammatory cells – enhanced angiogenesis*.(Moulton et al., 2003) The present study provides the first clinical evidence of pathological carotid intraplaque angiogenesis to 'classical' Mon1 subset with potent proinflammatory and phagocytic properties. (Shantsila et al., 2011, Ziegler-Heitbrock et al., 2010)

4.6. Limitations/Future Studies

My study is limited by its observational nature and clinical significance of the finding for adverse outcome is still to be established. Characteristics and counts of blood monocytes were used as surrogates of monocyte activity i.e.: immunity, angiogenesis, reparative processes and atherogenesis (Wrigley BJ, 2011, Shantsila et al., 2011), which may differ for their distribution within atherosclerotic plaques. My data are obtained from relatively small study population and require further confirmation from larger prospective analyses.

Another limitation of my study is the use of statin by my patients and the pleomorphic properties that each of the various statins have on both the atherosclerotic and angiogenic pathways. This topic is currently part of my subsequent analysis. (Jaipersad AS, 2013b)

My thesis data suggest that plaque neovascularisation is a process occurring both in early and late plaque development indicating that monocyte markers may be associated with specific carotid plaque characteristics. The process of an asymptomatic plaque progression to a vulnerable plaque remains unclear. Therefore carotid plaques could be separated based on their complications (i.e.; ulcerative, haemorrhagic) so that an association between monocyte parameters and carotid plaque complications.

My study was able to demonstrate the classical monocyte (Mon1) to be predictive of IMT and associated with severity of carotid atherosclerosis. However, it also demonstrated that the non-classical monocytes (Mon3) were significantly raised in the presence of moderate but not severe carotid disease. The significance of these

findings could be confirmed in larger scale studies with longer follow up looking at the development of carotid plaques to establish associations of the levels of individual monocyte subsets. This type of study would help highlight interactions of monocyte subsets at various stages of angiogenesis and potentially confirm that specific subpopulations are more critical for the plaque rupture acting on the plaque neovascularization.

The specific role of the various monocyte subsets in atherosclerotic process still remains uncertain. My thesis provides further evidence for a variation in monocyte subset presence in those patients with carotid plaque disease and pre-existing CAD. However, further studies directed at isolating specific subset types and specifically tracing their presence within the carotid plaques, would be of significant value. The ability to inhibit or artificially introduce a monocyte subset i.e. Mon1, and observe the effect on neovascularisation within carotid plaque could help better understand the atherosclerotic process.

4.7. Conclusion

Classical monocytes (Mon1) represent the only monocyte subset predictive of the severity of carotid and systemic atherosclerosis, such as carotid IMT, degree CD and presence of carotid intraplaque neovascularisation. All monocyte subsets showed varying changes in expression of receptors mediating inflammatory, angiogenic and remodelling processes in patients with carotid atherosclerosis. These observations may have important implications for the pathogenesis of carotid plaque and intraplaque angiogenesis.

CHAPTER V: THE EFFECT OF STATIN THERAPY WITHDRAWAL ON MONOCYTE SUBSETS

5.1. Abstract

Background: Three functionally distinct monocyte subsets have been identified. Statins are of undoubted effect in atherosclerosis, and have numerous pleiotropic effects that contribute to their clinical success, but the effect of these drugs on monocytes subsets is unclear. I hypothesised a beneficial effect of statins on key receptor expression by monocyte subsets.

Material and methods: Effects of temporal (2 weeks) cessation of statin therapy by 66 patients with stable coronary artery disease on monocyte subsets (CD14++CD16-CCR2+ (Mon1), CD14++CD16+CCR2+ (Mon2) and CD14+CD16++CCR2-(Mon3)]), their aggregates with platelets, and their expression of a number of receptors involved in inflammation (IL-6 receptor), adhesion (vascular cell adhesion molecule [VCAM]), angiogenesis (vascular endothelial growth factor [VEGF]) and repair were assessed by flow cytometry.

Results: Statin cessation did not lead to any significant changes in absolute numbers of monocyte subsets or the degree of their aggregation with platelets. All monocyte subsets showed significant downregulation of expression of vascular endothelial factor receptor 2, Tie2, and Toll-like receptor-4 (TLR4) (all changes $p<0.01$). Expression of CXCR4 was only reduced in Mon1 cells ($p=0.013$). There was no significant change in the expression of CD14, CD16, CCR4, IL6 receptor and VCAM (all p=NS).

Conclusions: Statin withdrawal does not affect counts of any of monocyte subsets, but leads to downregulation of expression of TLR4 and receptors related to angiogenesis

on all subsets, as well as a decrease in density of CXCR4 expression on ‘classical’ Mon1. These data provide further support of pleiotropic effects of statins and their effects monocyte pro-angiogenic and pro-reparative characteristics.

5.1. Introduction

Monocyte-derived macrophages play a considerable role in pathogenesis of atherosclerosis.(Napoli et al., 1997, Pamukcu et al., 2010) They scavenge and accumulate lipids thus contributing to the formation the lipid-laden core of the plaque, and release numerous proatherogenic cytokines (such as interleukin-6) and other biologically active molecules. Of note, the changes in monocyte phenotype and function in atherosclerosis occur before their migration to the tissues whilst still in circulation.(Cushing and Fogelman, 1992) Activation of circulating monocytes is well documented in both CAD and its complications, such as myocardial infarction.(Jugdutt, 2002, Maekawa et al., 2002) A high monocyte count is a strong predictor of CAD development as well as poor outcome in patients with myocardial infarction. More recently, potentially important differences have been found between different functional monocyte subsets in the relation to atherogenesis and plaque destabilisation.(Wu et al., 2009, Tsujioka et al., 2009)

Statins have dramatically changed and prognosis of patients with different forms of atherosclerosis and have become a paradigm their treatment.(MAAS, 1994) Although the main effect of statins is related to inhibition of cholesterol production, numerous pleiotropic effects have also been reported.(Koh, 2000, Crisby et al., 2001) For example, statins' capacity to ameliorate low-grade inflammation, common in atherosclerosis, helps to improve prognosis in CAD irrespectively of their cholesterol lowering action.(Ridker et al., 2005) Better understanding of pleiotropic effects of the stains could help to identify novel therapeutic targets.

Given the intimate involvement of monocytes in atherogenesis it is natural to explore direct effects of statins on monocyte numbers, subsets and phenotypes.(Ferro et al., 2000, Han et al., 2005) In doing this the differences between individual monocyte subsets need to be considered. The most straightforward way of testing this is to evaluate monocyte changes after initiations of statins. However, this approach has several limitations. Majority of patients diagnosed with CAD simultaneously start several treatments potentially affecting monocytes (e.g., angiotensin converting enzyme [ACE] inhibitors, aspirin). Also, even if deemed ethical, temporal delay in initiation of those medications would only allow assessment short-term effects of statins. However, it is known that short-term pleiotropic effects of the statins may be profoundly different from the long-term effects, as this is the case with endothelial progenitor cells.(Vasa et al., 2001, Hristov et al., 2007)

In this chapter of my thesis, I tested the hypothesis that a beneficial effect of statins on monocyte subsets would be seen, as defined by the expression of key surface receptors, using a model of the two-week cessation of this drug.

5.3 Methods

5.3.1. Study population

I recruited 66 patients with known treated CAD and carotid atherosclerosis confirmed by ultrasonography and who attended the Sandwell and West Birmingham Hospitals NHS Trust between November 2009 and November 2012 and met the study criteria. Exclusion criteria comprised any hospital admissions within preceding 3 months, infectious and inflammatory disorders, cancer, significant valvular heart disease, atrial fibrillation, renal failure [creatinine levels > 200 μg/mL], immune-modulating drugs and hormone replacement therapy.

The study participants were invited to attend the research clinic in the morning, after abstaining from smoking from midnight of the preceding day. Following a 20-minute supine rest, a 20 ml blood sample was taken for laboratory analyses. After the baseline analysis patients stopped their statin therapy till the second blood sampling 14 days later, and then resumed. The study was performed in accordance with the Helsinki declaration and was approved by the Coventry Research Ethics Committee. All participants provided written informed consent.

5.3.2. Flow cytometry

I performed flow cytometry analysis as discussed previously in the main methodology section.

5.3.3 Statistical analysis

Statistical analysis was performed as I presented in the main methodology section.

5.4. Results

5.4.1. Study population

Sixty-six patients completed the study (aged 70 [8.4] years, 42 [64%] male, body mass index 27 [5.1] kg/m^2, systolic blood pressure 138 [20]/70 [12] mm Hg, estimated glomerular filtration rate 71 [10] ml/min/1.73 m^2, and total cholesterol 3.9 [0.6] mmol/L). Medical records showed previous myocardial infarction in 18 [27%], hypertension in 42 [64%], and diabetes in 13 [20%] patients. Current smokers were 23 [35%] patients. Their medications included aspirin in 60 [91%], clopidogrel in 13 [20%], beta-blockers in 32 [49%], ACE inhibitors or angiotensin receptor blockers in 40 [61%] patients. The medications remained the same during the period of the study. All patients received long-term treatment with statins before their withdrawal, mostly simvastatin (42 [64%] patients) and atorvastatin (16 [24%]).

5.4.2. Monocyte parameters

The statin cessation did not lead to any significant changes in levels of monocyte subsets or degree of their aggregation with platelets.

Table 13: Monocyte parameters before and after statin withdrawal

	Mon1			Mon2			Mon3		
	On statin	After statin	p value*	On statin	After statin	p value*	On statin	After statin	p value*
Count, per µL	475 [150]	461 [165]	0.30	36 [29-59]	39 [26-54]	0.40	58 [46-77]	56 [38-76]	0.054
Monocyte, %	81 [76-86]	81 [75-86]	0.96	7 [5-10]	7 [5-11]	0.82	11 [8-14]	11 [7-14]	0.88
MPA, per µL	77 [51-116]	79 [45-125]	0.74	11 [7-16]	11 [7-16]	0.30	8 [6-11]	7 [5-11]	0.47
MPA, %	16 [13-21]	17 [14-22]	0.32	25 [18-40]	28 [19-38]	0.26	13 [11-16]	14 [12-16]	0.29
CD14, MFI	1350 [262]	1296 [274]	0.10	1378 [348]	1309 [337]	0.11	155 [45]	148 [43]	0.08
CD16, MFI	10 [3]	10 [3]	0.32	55 [16]	56 [16]	0.32	148 [64]	153 [54]	0.38
CCR2, MFI	161 [40]	163 [36]	0.62	123 [35]	126 [31]	0.47	16 [2]	16 [2]	0.95
Toll-like receptor 4, MFI	4.7 [3.6-6.6]	4.0 [3.3-5.0]	<0.001	7.5 [4.2-13]	6.1 [4.0-8.8]	0.002	3.0 [1.9-4.0]	2.4 [1.9-3.3]	0.006
IL-6 receptor, MFI	75 [14]	75 [17]	0.96	67 [12]	68 [15]	0.48	36 [9]	38 [11]	0.08
VCAM-1 receptor, MFI	12 [9-16]	11 [9-16]	0.85	23 [16-37]	26 [19-34]	0.31	37 [26-54]	37 [31-55]	0.15

VEGF receptor 2, MFI	6.3 [5.2-7.3]	5.5 [4.4-6.5]	<0.001	12 [8.8-17]	9.6 [7.8-12]	<0.001	3.2 [2.6-4.1]	2.8 [2.4-3.6]	<0.001
CXCR4, MFI	13 [11-16]	12 [11-14]	0.013	19 [16-24]	19 [15-21]	0.28	6.0 [4.8-8.3]	5.9 [4.7-7.2]	0.07
Tie2, MFI	5.7 [5-7]	4.9 [4-6.0]	<0.001	10.7 [8-16]	8.5 [7-11]	<0.001	8.0 [7-10]	7.2 [6-8]	<0.001

*Paired T-test or Wilcoxon test. Data are presented as mean [standard deviation] or median [interquartile range]. IL, interleukin; MFI, median fluorescence intensity; Mon1, CD14++CD16-CCR2+; Mon2, CD14++CD16+CCR2+; Mon3, CD14+CD16++CCR2-; MPA, monocyte-platelet aggregates; VEGF, vascular endothelial growth factor; VCAM-1 receptor, vascular cell adhesion molecule-1.

Following statin withdrawal all monocyte subsets showed significant down-regulation of expression of VEGF receptor 2, Tie2, and TLR4.

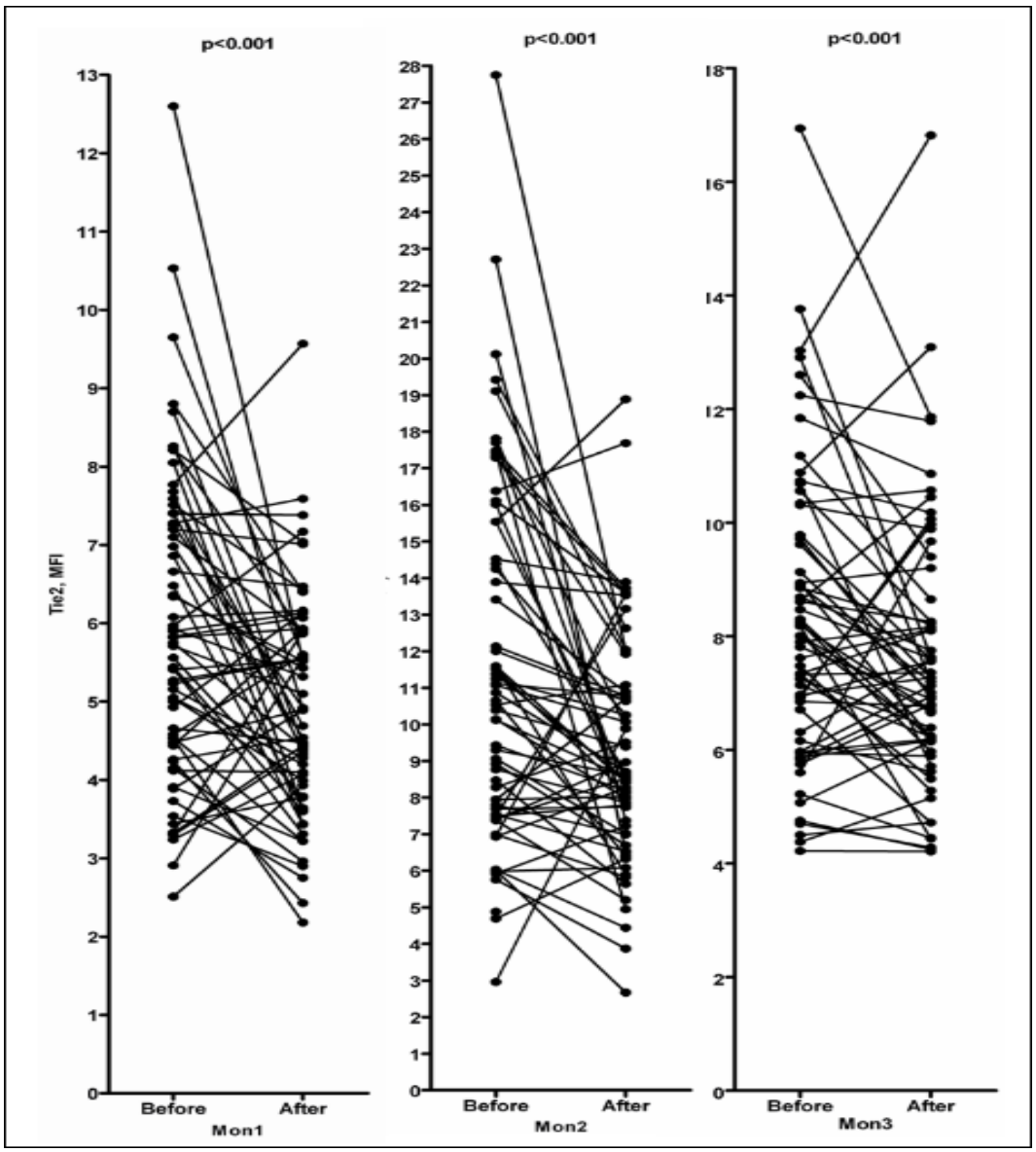


Figure 7. Effects of statin cessation on monocyte Tie2 expression

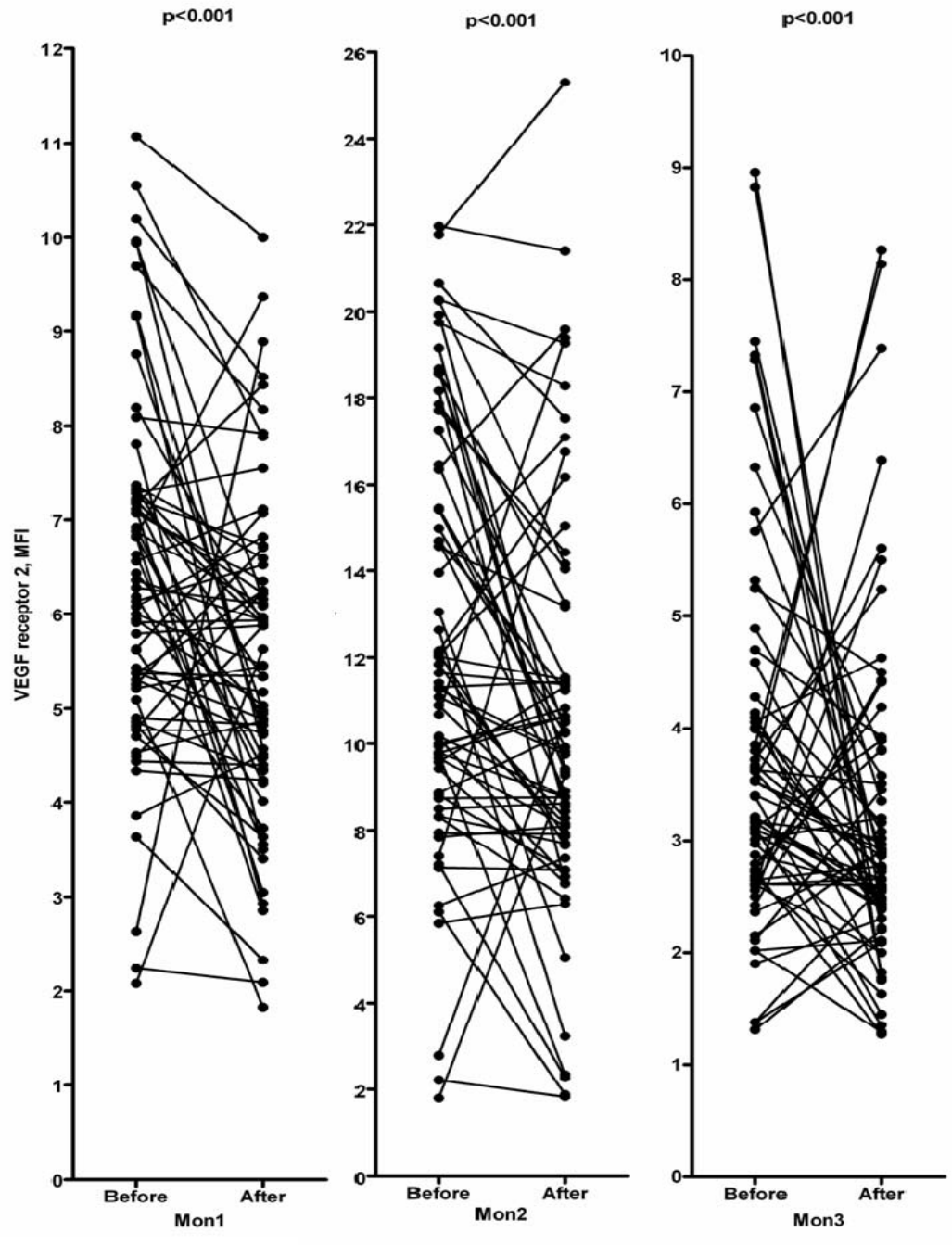


Figure 8. Effects of statin cessation on monocyte vascular endothelial growth factor receptor 2 expression

Expression of CXCR4 was only reduced on Mon1. There was no significant change in monocyte expression of CD14, CD16, CCR4, and receptors to IL6 and VCAM-1.

5.4.3. Plasma parameters

The statin withdrawal resulted in significant reduction of plasma Tie2 levels, but did not change in VEGF or angiopoietin-2.

Table 14: Plasma angiogenic factors before and after statin cessation

	On statin	After statin	p value*
Tie 2, ng/mL	126 [101-163]	113 [93-143]	0.008
Angiopoietin 2, ng/mL	2.4 [1.6-4.7]	2.6 [1.8-4.9]	0.11
VEGF, pg/mLs	20 [0-95]	10 [0-103]	0.60

*Wilcoxon test. Data are presented as median [interquartile range]. VEGF, vascular endothelial growth factor

There was no correlation between monocyte Tie2 density and plasma Tie2 and angiopoietin-2 levels, and between monocyte VEGF receptor 2 expression and plasma VEGF levels.

Table 15. Correlation analysis of plasma angiogenic factors and monocyte receptors

	Mon1		Mon2		Mon3	
	r	p value	r	p value	r	p value
	Monocyte Tie2 expression					
Plasma Tie2	0.07	0.59	0.11	0.40	0.04	0.77
Plasma	0.00	0.99	0.00	0.98	-0.12	0.32

angiopoietin 2						
	Monocyte VEGF receptor 2 expression					
Plasma VEGF	0.03	0.82	0.03	0.84	-0.10	0.44

VEGF, vascular endothelial growth factor. Mon1, CD14++CD16-CCR2+; Mon2, CD14++CD16+CCR2+; Mon3, CD14+CD16++CCR2-.

5.5. Discussion

The main finding of my study is that statin cessation led to significant down-regulation of receptors involved in angiogenesis on all monocyte subsets. Statin cessation was also related to reduction of CXCR4 expression on 'classical' Mon1.

Statins are known to modulate angiogenesis with most (but not all) reports indicating their pro-angiogenic and pro-reparative properties.(Pourati et al., 2003, Asai et al., 2012, Zhang et al., 2012, Zaitone and Abo-Gresha, 2012, Koutouzis et al., 2007) Our group has previously shown that high-dose atorvastatin increased levels of circulating progenitor cells and angiopoietin-2, but reduced plasma VEGF concentrations.(Jaumdally et al., 2011, Jaumdally et al., 2010) The pro-angiogenic and pro-reparative effects of statins appear to be drug and dose dependent and involve numerous pathways.(Camnitz et al., 2012) My findings indicate that overall statin treatment is associated with enhancement of monocyte expression of receptors involved in these processes. Whilst the effects of statin withdrawal on monocyte VEGF receptor 2 and Tie2 were uniform over all monocyte subsets, CXCR4 was only decreased on Mon1, with a similar trend for Mon3. No effect was found in relation to CXCR4 expression on Mon2, subsets featured by the highest of all subsets expression

of the receptor. This could reflect a differential role of monocyte receptors in complex CXCR4-mediated inflammatory and reparative effects.(Liehn et al., 2011) However the absolute change in the receptor density was small and the results need to be interpreted cautiously.

Although, my overall data suggest that statins reduce monocyte TLR4 expression, there is some controversy in this regard. Most of the data come from in-vitro experiments or studies on healthy normolipidemic volunteers. For instance, 4-week administration of 20 mg daily of atorvastatin reduced TLR4 expression in only 12 healthy subjects with similar effects also seen in vitro.(Methe et al., 2005) However in a double-blind, placebo-controlled study on healthy volunteers high-dose simvastatin did not influence monocyte surface TLR4 density, but diminished its up-regulation in response to endotoxemia.(Niessner et al., 2006) Statins were unable to control TLR4 over-activation in patients with chronic idiopathic urticaria.(Azor et al., 2011) In a study of 140 patients undergoing carotid endarterectomy with half of patients not receiving statins, immunohistochemically assessed macrophage density of TLR was higher among statin naïve subjects.(Katsargyris et al., 2011) However it is not clear whether the findings could be affected by clinical factors on which a decision was made about the statin treatment. Admittedly statins differ in relation to their capacity to reduce monocyte TLR4 levels, with such properties seen for atorvastatin, but not rosuvastatin.(Satoh et al., 2012) Finally the response of monocyte TLR4 to statins is affected by genetic polymorphisms.(Boekholdt et al., 2003) Of interest, given the recently demonstrated involvement of the TLR4 in pro-angiogenic pathways the down-regulation in the receptor after the statin cessation seems to accord with other changes in receptors related to angiogenesis.(Grote et al., 2010) Although reasoning

for this part of my findings remains unclear, the effects of statins may still be more complex than originally thought.

5.6. Limitations/Future Studies

The study population is relatively small, which could result in omission of some subtle changes in monocyte parameters. The study was performed over the interval of two weeks and does not provide information on shorter-term or longer-term effects of statin withdrawal. The study does not analyse functional status on monocytes and does not provide mechanistic insight into the findings.

The results of this thesis demonstrate the common notion that statin therapy and its pleiotropic effects are generally very complex. Evidently a study of similar design is required on a larger scale with a focus mainly on statins to further emphasise the results shown in this study. The finding that statin cessation led to a significant down regulation of receptors on all monocyte subsets involved in angiogenesis may have significant implications on those patients whose statin medication is discontinued for clinical and non clinical reasons.

To provide complimentary knowledge to the findings in my study, the effects of different statin treatments on modulation of monocyte-associated plaque progression and neovascularisation could be investigated. The implications here on monocytes and plaque morphology would have immense therapeutic applications.

5.7. Conclusion

In conclusion, my study results suggest that statin withdrawal does not affect counts of any of monocyte subsets. However, it leads to downregulation of expression of TLR4 and receptors related to angiogenesis on all subsets, and decrease in density of CXCR4 expression on 'classical' Mon1. These data provide further support of pleiotropic effects of statins and their effects monocyte proangiogenic and proreparative characteristics.

CHAPTER VI. SUMMARY AND OVERALL CONCLUSIONS

6.1. Thesis summary

My thesis begins with a review of literature on neovascularisation in plaque growth, vasculogenesis, angiogenesis, and then addresses the role of monocytes and their subpopulations role in mediating angiogenic signalling. The implication of these processes in the presence of atherosclerosis is discussed in section 1.1. The importance of monocytes and their role in relation to coronary heart disease and carotid plaque disease is emphasised. However, significant gaps remain in understanding of the roles of individual monocyte subsets and their functional characteristics in systemic and carotid plaque atherogenesis.

The potential of carotid ultrasound and the evolution of CEUS in characterisation of carotid atherosclerosis are discussed in section 2.3 and chapter 3. There is growing evidence supporting the use of CEUS in the use carotid plaque analysis. In Chapter 3, CEUS is used to demonstrate the presence of neovascularisation within the carotid plaques. There is an increasing use of contrast ultrasound in experimental research and this method is now being applied to the clinical setting. However, the technique is still to be validated and standardised for robust use in clinical research. As part of my thesis, I present a chapter devoted to the standardisation and assessment of agreeability of the method of CEUS for detection of carotid wall neovascularisation.

In Chapter 4, patients with pre-existing CAD and CD were recruited to demonstrate differences in monocyte subsets and their expression of inflammatory and angiogenic

receptors in relation to CEUS detected carotid plaque neovascularisation. My study showed for the first time, that classical monocytes (Mon1) were associated with more advanced CD, independent of other risk factors and the same subsets were also linked to increased carotid IMT. I also found that non-classical Mon3 subset, known in the literature to have reparative potential were increased in stable CAD and moderate CD (<50%) but not severe CD (>50%). There was a significant increase in expression of monocyte receptors involved in their migration to tissues (i.e., CD49d), and levels monocyte-platelet interactions also known facilitate monocyte trans-endothelial migration. Interestingly, patients with CD>50% had a significant reduction of CD16 expression by all monocytes, a trend not easily explained in this study but an indication for further monocyte functional studies.

Chapter 5 highlights and contributes to the accumulating evidence on the pleomorphic effects of statins. My thesis demonstrates an enhanced monocyte effect in angiogenesis on those taking statins, demonstrated by increased expression pro-angiogenic markers. The main focus of this chapter however, emphasised the effect of statin withdrawal (2 weeks) on monocyte subsets and their angiogenic markers. Overall, there was no reduction in monocyte counts after statin withdrawal but controversially a significant reduction in monocyte expression of TLR4 on all subsets and a decrease in CXCR4 on Mon1 was observed. My analysis suggests a complex role of statin therapy and modulation of monocyte-mediated inflammation, angiogenesis and repair.

6.2. Future research

Despite advances in the management of atherosclerosis, progressively aging population results in significant number of patients suffering the consequences of atherosclerotic disease. A considerable amount of research is being conducted to prevent or slow the progression of the carotid artery disease. Due to the complex nature of the disease pathology and the intricate pathological processes occurring within the arterial walls, further developments in this field would require even better understanding of the mechanisms and pathways involved. My thesis has provided insight into: 1) the presence of pathological neovascularisation within the atherosclerotic carotid plaque; 2) the relationship between the presence of neovascularisation and monocyte subsets and their angiogenic receptor expression; 3) the relationship of monocyte characteristics to cessation of statin therapy. Several questions still however remain unanswered. The following is a framework of studies, which could be performed to address these notions.

6.2.1 Carotid Contrast Ultrasound

1. In this thesis study, I have shown a agreeable method of performing CEUS. However further research into methods aiming to delineate and quantify plaque features would allow for standardisation of technique across multiple research centres and potentially for its clinical utilisation in the future. Also, MRI and CT modalities are increasingly used to assess CD and these techniques with their high specificities and sensitivities would need to be assessed against CEUS outcomes.

2. Histological characteristics of high risk and ruptured plaques are well documented, but methods to visualise these plaques in vivo remain limited. To test this, other aspects of plaque morphology i.e.; ulceration, plaque volume could be measured in the presence of new onset neovascularisation in asymptomatic individuals undergoing long term follow up with the CEUS method and to demonstrate the evolution of plaque progression and its changes when complications develop. This could perhaps result in a model for prediction of atherosclerotic clinical events and shed insight into how these varying plaque characteristics interact to cause instability.

3. My thesis presents for the first time, a novel agreeable method of using CEUS to detect the presence of neovascularisation within existing carotid plaque. Further studies would be required using both histological and novel advanced imaging methods, like magnetic resonance imaging (MRI) to test CEUS sensitivity and specificity at detecting of the presence of neovascularisation. Confirmation of CEUS accuracy would allow for its widespread use as a method of detecting neovascularisation, and possibly extending its application to directed therapeutic interventions.

6.2.2. Monocyte subsets

1. My thesis data suggest that plaque neovascularisation is a process occurring both in early and late plaque development indicating that monocyte markers may be associated with specific carotid plaque characteristics. The process of

an asymptomatic plaque progression to a vulnerable plaque remains unclear. Therefore carotid plaques could be separated based on their complications (i.e.; ulcerative, haemorrhagic) so that an association between monocyte parameters and carotid plaque complications.

2. My study was able to demonstrate the classical monocyte (Mon1) to be predictive of IMT and associated with severity of carotid atherosclerosis. However, it also demonstrated that the non-classical monocytes (Mon3) were significantly raised in the presence of moderate but not severe carotid disease. The significance of these findings could be confirmed in larger scale studies with longer follow up looking at the development of carotid plaques to establish associations of the levels of individual monocyte subsets. This type of study would help highlight interactions of monocyte subsets at various stages of angiogenesis and potentially confirm that specific subpopulations are more critical for the plaque rupture acting on the plaque neovascularization.

3. The specific role of the various monocyte subsets in atherosclerotic process still remains uncertain. My thesis provides further evidence for a variation in monocyte subset presence in those patients with carotid plaque disease and pre-existing CAD. However, further studies directed at isolating specific subset types and specifically tracing their presence within the carotid plaques, would be of significant value. The ability to inhibit or artificially introduce a monocyte subset i.e. Mon1, and observe the effect on neovascularisation within carotid plaque could help better understand the atherosclerotic process.

6.2.3 Statins

1. The results of this thesis demonstrate the common notion that statin therapy and its pleiotropic effects are generally are very complex. Evidently a study of similar design is required on a larger scale with a focus mainly on statins to further emphasise the results shown in this study. The finding that statin cessation led to a significant down regulation of receptors on all monocyte subsets involved in angiogenesis has profound implications on those patients whose statin medication is discontinued for clinical and non clinical reasons.
2. To provide complimentary knowledge to the findings in my study, the effects of different statin treatments on modulation of monocyte-associated plaque progression and neovascularisation could be investigated. The implications here on monocytes and plaque morphology would have immense therapeutic applications.

Further research is also needed to establish the role of genetic polymorphism of monocyte subset receptors in the development of plaque progression to symptomatic disease. This will help to understand roles of particular monocyte subsets in the development of atherosclerotic disease and plaque instability. Also, future studies are needed on the relationship of familial hyperlipidaemia and monocyte subset levels in the development of atherosclerotic disease whilst taking into account for monocyte differences in respect to the receptor expression in familial hyperlipidaemia.

6.3. Overall conclusion

New vessel formation within atherosclerotic plaques plays a significant role in the clinical presentation of CAD and cerebrovascular events. Blood monocytes play a role in both ischaemia and inflammation, two processes implicated in angiogenesis. The use of CEUS in my study demonstrates a feasible method to assess carotid plaque for the presence of neovascularisation and standardises how CEUS should be performed for research purposes. My analysis further advances one's understanding of the role of monocyte subsets and their potential individual characteristics in patients with pre-existing CAD and CD. In particular it has produced evidence of the role of Mon1 as a predictor of severity of CD. My thesis also provides further insight into the pathological process occurring in the atherosclerotic plaque by demonstrating varying degrees of expression of monocyte receptors mediating angiogenesis, and inflammation from all monocyte subsets.

Statin usage in clinical practise remains the mainstay of prevention against the atherosclerotic process. My study begins to explore the relationship between statins and carotid plaque in the presence of pre-existing CAD whose prescribed statins were stopped. My study findings demonstrate that although statin withdrawal for 2 weeks does not affect monocyte subsets, it down-regulates expression of receptors involved in: angiogenesis (Tie2), and inflammation (TLR4), on all monocyte subsets. Interestingly, reparative receptor expression (CXCR4) was only decreased on Mon1. My findings not only lends to the current understanding of affects of statin therapy in high-risk atherosclerotic patients but also contributes to the complexity of statin effects on the atherosclerotic processes occurring at the molecular level.

These observations presented in my thesis on monocytes and subpopulations in relations to neovascularisation and statin therapy usage, offer hope for the better understanding of the intricate mechanisms occurring during the atherosclerotic process in the presence of CAD and CD. But the limitations of the experimental models involved need careful consideration before extrapolating to the clinical scenario.

Appendix 1: PRISMA Checklist

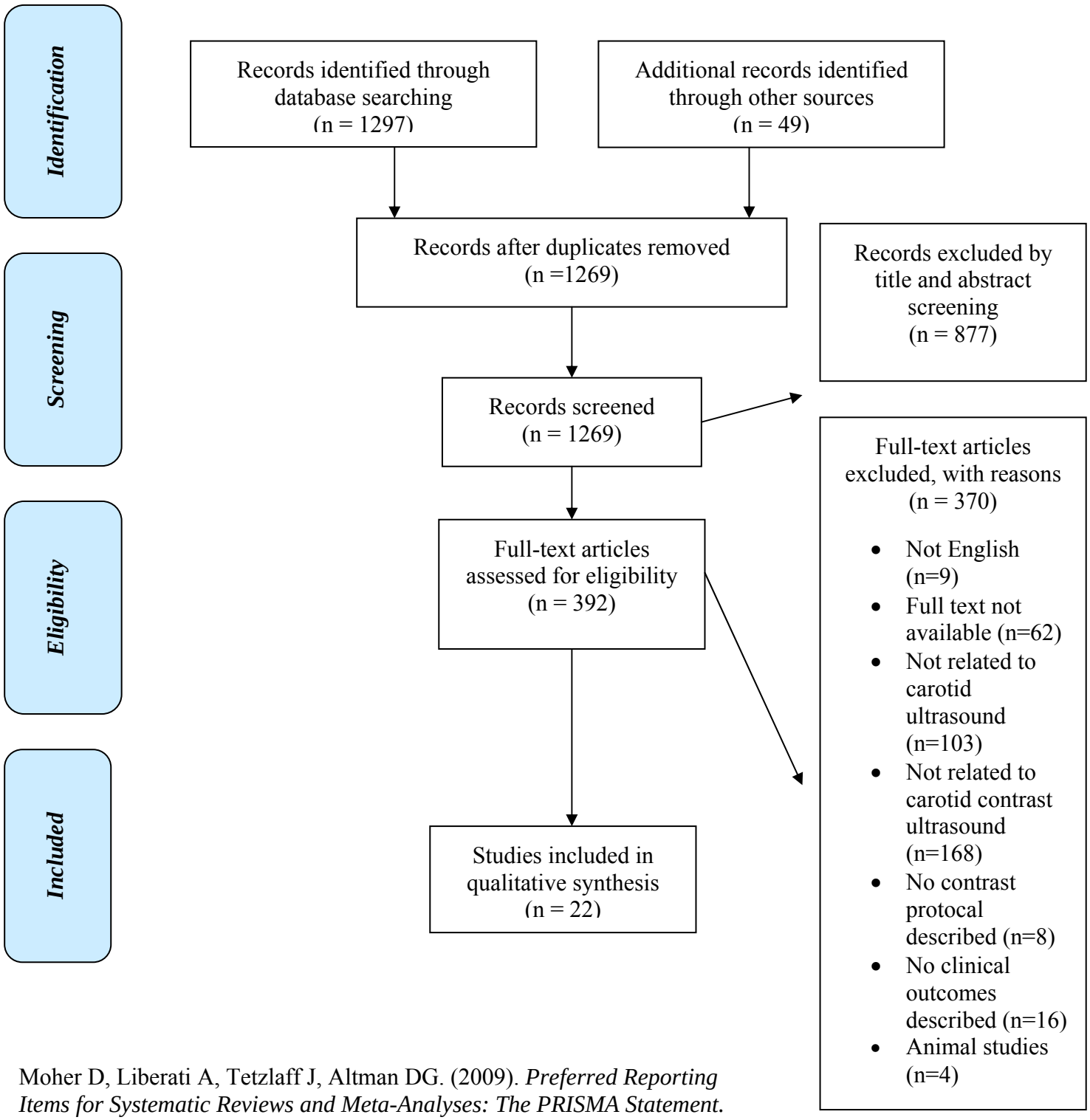
PRISMA 2009 Checklist

Section/topic		Checklist item
METHODS		
Eligibility criteria		Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.
Information sources		Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.
Search		Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.
Study selection		State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).
Data collection process		Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.
Data items		List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.
RESULTS		
Study selection		Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.

Moher D, Liberati A, Tetzlaff J, Altman DG. (2009). *Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement.* . Available: http://www.prisma-statement.org/2.1.2%20-%20PRISMA%202009%20Checklist.pdf. Last accessed 13th August 2011.

Appendix 2: PRISMA Flow Diagram

PRISMA 2009 Flow Diagram



Moher D, Liberati A, Tetzlaff J, Altman DG. (2009). *Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement.* . Available: http://www.prisma-statement.org/2.1.4%20-%20PRISMA%202009%20Diagram.pdf. Last accessed 13th August 2011.

Appendix 3: SOP Carotid Ultrasound

STANDARD OPERATING PROCEDURE CUPNASA

Carotid Contrast Ultrasound protocol

Written by A. Jaipersad, A. Shantsila, E. Shantsila

N.B. Use of the Phillips GX50 is forbidden

Without having been officially trained

Required pre-training

1. SOPs on venepuncture and on good clinical practice

Contents

Introduction
Brief Description of method
Standard and contrast-enhanced carotid ultrasound imaging

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Intima Media Thickness
Parameters to analyse
Plaque morphology analysis
Stenosis
Contrast image analysis
References

Introduction

Vasa Vasorum (VV) is known to be prominent in areas of atherosclerosis and contribute to vascular wall nutrition.[1] Their development not only contributes to structural properties of the arterial wall but they have been implicated in various pathological processes; inflammation, hemorrhage and plaque rupture.[2]
Imaging strategies for VV are currently limited. B mode ultrasonography is the gold standard in measuring plaque structure but lacks detail when looking at neovascularization. Contrast Ultrasound (CU) has now been for sometime utilized for use in the cardiology setting. Contrast enhanced ultrasound for carotid plaque enhancement although first documented in the literature over 10 years ago, recently has emerged as a method that is easy, feasible and reproducible. Currently however, the analysis remains less quantitative and more subjective. The aim of the SOP is to document the methodology in obtaining CEUS images.

Brief Description of method

Magnified, gray scale and contrast images of the carotid artery at three levels on

bilaterally of each participant are to be obtained. The segments to be imaged are the distal common carotid artery, the carotid bifurcation, and the proximal internal carotid artery. Images should be acquired with the participant's head rotated 45 degrees away from the side of study. A single image ought to be captured at the common carotid level of the artery and two images each from a different angle must be captured of both the carotid bifurcation and the internal carotid artery. Three ml of Sonovue contrast are to be delivered via an infusion pump at a rate 1.3ml/sec. A video stream of the common carotid artery will be recorded and will be accompanied by a 2 to 4 second real-time cine loop demonstrating at least three complete cardiac cycles. In addition, the common carotid, the carotid bifurcation and internal carotid segments will be captured as a single or static image. All static images should be captured at end-diastole. Captured images should visualize the intima-media complex of the far wall of each segment studied over a 1-cm length of the artery.

Standard and contrast-enhanced carotid ultrasound imaging.

First, the carotid bifurcation is imaged bilaterally by B-mode ultrasound, color Doppler, and pulsed-wave Doppler, and the examination is digitally stored for later review. Special care is to be taken to image and record all distinct plaques seen at each side.
The patient is then submitted to contrast-enhanced ultrasound imaging, with special attention to the previously identified lesions. The preset real-time, contrast-specific imaging modality is switched on and image settings adjusted to maximize contrast signal visualization.

The contrast ultrasound investigation is performed with an infusion in an antecubital vein (20 Gauge Venflon) of Sonovue 6mL (Bracco), at a rate of 1.3ml/sec. The linear array probe with a mechanical index 0.08 to 0.10, with contrast pulse sequencing continuous real-time recording is used to achieve the best visualization of the plaque morphology and vascularization. Good images can be obtained for about 3 minutes. For a single examination, no more than 2 vials of Sonovue are required. The studies are to be digitally stored for later analysis by pressing end exam button. The patients are to be observed for 30 min before returning to their wards/home.

Carotid assessment in patients with systolic blood pressure above 160 mm Hg has limited information and should be avoided. Never attempt to do the test when BP 180 mmHg or above as documented by Sonovue.

Contrast Preparation:

Required:

1. BRACCO VUEJECT (BR-INF100) Infusion Pump with wired screen remote.
2. Kit for Balancing pump through Echo- Contrast
   Consists of: -
   - 20ml Syringe
   - Intra venous Cannula 20G
   - Infusion Line
3. 2X BRACCO Sonovue: sulpher hexafluoride microbubbles 8 microlitres/ml

powder and solvent for dispersion.

Consists of: - Vial with 25mg lyophilized powder
- 1 pre-filled syringe containing 0.9% sodium chloride with separate piston.
- 1 spike transfer system.

Preparation of Contrast:

1. Switch on infusion pump by pressing ON/OFF button on machine. System will run through pre-checks. Once completed, screen will request to insert syringe and close cover.

NOTE: This process takes time. Perform prior to preparing contrast material.

2. Open all material as listed above.
3. Remove cap from power vial and connect to spike system. An audible click can be heard.
4. Connect piston to pre-filled syringe and connect to spike system with twist motion.
5. Reconstitute power with sodium chloride by depressing piston.
6. Disconnection pre-filled syringe and connect 20ml syringe. Fill 20ml syringe and depress back into powder vial multiple times to remove all traces of powder residue in vial.
7. Remove filled 20ml syringe and connect to line. Flush line to remove air.
8. Place filled 20ml syringe and prepped line into infusion machine, being careful to feed line into machine.
9. Close cover.
10. Infusion System will then run through a checking cycle which if correct will cycle the inserted syringe. A countdown timer will appear on the remote.
11. The contrast is now prepared and ready to be used.

Note: We do not connect the line to the patient until all B-mode images are taken (steps 5-13 as listed below).

Procedure

Participant Preparation:

The carotid ultrasound procedures should be performed with the subject in the supine position with a 20G intra venous cannula placed. The subject is made comfortable in a position that allows head rotation to either side. The imager stands or is seated at the end of the exam table near the participant's head. The top of the head should be about three inches from the end of the exam table and the head is to be rotated 45 degrees away from the side being scanned.

a. Allow the subject to rest in the room for about 20 minutes before scanning
b. Helps to remove patient jewellery and protect clothing with paper provided.
c. Explain details of the procedure and possibility of allergic reaction.

Turn machine ON by pressing *ON/OFF* button.

Note: - Turning U/S on and will automatically load Carotid contrast Protocol. (Also available by pressing **PROTOCOL** on machine) which starts in B-mode and must be switched to **Contrast on mode** by the button at the top of the machine (shown on screen).

Machine Set-up, Probe Frequency and Magnification

Pre-sets have been defined for the Philips GX50 device. The settings will be set at the start of the study by the Phillips engineer and should not be altered. Each machine will have only one probe. The probe that is used for the scan is L12-3. The probe frequency is set to 3 to 12 MHz for scanning the common carotid artery and the internal carotid artery.

Philips GX50 Carotid Contrast Ultrasound Pre-sets
Frequency: 26Hz
66 Dyn Range
Res
Edge 3
Map G
Ave. 2
Depth 3cm

- Pressing **ACQUIRE** will save the image to the active memory and show on the right of the screen the image captured.
- Automatic return to B-Mode, if not press **2D** button to return.
- Only pressing **END EXAM** will save to the hard drive.

Participant information should be entered on the ultrasound machine's demographic information screen to identify the scan as a Vascular Contrast scan.

To enter subject information to GX50 demographic information screen:

Press ***PATIENT*** on the machine.

The participant ID number is entered in the MRN section.
Hit "Return tab" until you reach the Last Name field, repeat to enter forename, date of birth, sex and activate pull down menu for scan type: "Vascular".
Hit the OK to begin scanning.

1. Position patient on bench lying down with head up and neck stretched exposing area to scan (as above).
2. Place Stickers for ECG as labelled on Leads (Red [Right} Yellow [Left] Green [Front] diagram on leads.
3. Pressing **PHYSIO** button on U/S will toggle between ECG leads.
4. Turn room Lights off. Scanning is now ready to begin.

Anatomical Sites of Interest

The extra cranial carotid arteries are the largest arteries in the neck. The right

common carotid artery originates from the innominate artery on the right and the left common carotid artery originates directly from the aortic arch. Each common carotid artery ascends in the neck lateral to and posterior to the trachea. At the approximate level of thyroid cartilage, slightly below the angle of the mandible, the common carotid artery bifurcates into the external and internal carotid arteries

According to standard protocol the carotid ultrasound image is captured in static grey-scale and real-time cine loops from the common carotid, bifurcation and internal carotid on both the right and the left side.

1. Distal common carotid: the 1 cm segment of the common carotid artery proximal to the origin of the carotid bifurcation, where the near and far walls of the artery are parallel to one another. The common carotid artery dilates just before bifurcating into the internal and external carotid arteries.

2. Carotid bifurcation: for study purposes this arterial segment is defined as the flow divider extending into the proximal internal carotid artery. It ends when the walls of the artery lose their curvature and become parallel.

3. Internal carotid artery: anatomically, the tip of the bifurcation defines the proximal end. The vessel then ascends in the neck and enters the base of the skull. The internal carotid artery has no branches in the neck. For the purposes of this protocol, the study will be limited to the initial 10 mm of the internal carotid artery distal to the bifurcation.

4. External carotid artery: originates at the carotid bifurcation and is more superficial and nearer the mid- line than the internal carotid. The external carotid artery lies anterior and slightly medially to the internal carotid artery in 90% of individuals. The external carotid artery is usually smaller than the internal and it has branches that supply the neck and face, the first branch is usually identified as the superior thyroid artery. No images of the external are recorded.

2D Procedure:

5. Put contact gel onto probe and starting with Right side, identify Common Carotid artery. Press **TEXT** button to label scan image (drop down menu) with appropriate side.
6. Use **GAIN** button to improve image.
7. Press **Freeze** to still the image and adjust using trackball in accordance with ECG (the peak R wave). Press **ACQUIRE** once satisfactory image is obtained.
8. Press **Zoom** button and adjust box size and position using **trackball**. Use right button to toggle between.
9. Press **M-mode** button and position cursor with trackball. Press **M-mode** again to activate (which will display two images; left M-mode and right B- mode) and **Freeze** followed by **ACQUIRE** to save image. Automatic return to B-Mode, if not press **2D** button to return.
10. Press **PW** (Pulse Wave) button to activate doppler and position indicator using trackball and press **PW** to activate and acquire pressure readings. (If does not

work press **HQ** button to activate. Confirm angle is 60 degrees. Press **Acquire** to save. Automatic return to B-Mode, if not press **2D** button to return.

11. To measure: press **Calliper** button i.e. small lesions and use **trackball** to adjust. **ACQUIRE** to save. Automatic return to B-Mode, if not press **2D** button to return.
12. Pressing **CALC** button will produce a drop down menu: to calculate percent stenosis based on true diameter (lumen to lumen) and residual diameter measurements. Carotid image view must be in cross section. Choose CCA or ICA as required. Adjust using **trackball**. Press **Acquire** to save. Automatic return to B-Mode, if not press **2D** button to return.
13. Repeat steps 5-8, 10-12 to acquire images of Carotid Bulb, Plaque if present and Internal Carotid Artery.
14. Repeat steps 5-12 and 13 for Left side.

Contrast Procedure:

15. Press **PURGE** on Infusion machine to prep infusion line.
    ALL infusion adjustments can be now made using wired remote.
16. Press **START** and adjust rate of infusion (1.3ml/s) by pressing **ARROW** buttons. This will allow 3 minutes of scanning to be performed with a 1 minute remaining audible warning and automatic shut off. Pressing **OFF** on remote or infusion machine will shut off, as well.
17. On GX50 press **Contrast ON** button (identified at the bottom of the screen) to switch on contrast identification mode.
18. Begin scanning starting with Patient Right Side.
19. Repeat steps: 5-8 and 13 to acquire detail images.
20. Repeat steps 5-8 for Left side.
21. Once finished, press **END EXAM**. This will save patient data with images on hard drive into folder.
22. The patient is observed for 5 minutes with ECG leads in place while infusion pump is disconnected. Then ECG leads are removed with pads.

NOTE: Blood pressure is taken if patient feels ill effects and ECG leads remain on for 15 minutes.

23. The intravenous cannula is removed and pressure applied to site with the patient now sitting up and reviewing scan images with imager.
24. The patient is observed for 15minutes after procedure.
25. Pressing **OFF** on the machine will switch off safely.

Analysis of Images:

Using Xcelera and Qlab version 7.1 by Philips Healthcare.

1. Connect GX50 to Ethernet cable of Xcelera computer.
2. Switch GX50 on. Machine will warm up.
3. Press Review button. This will bring up a list of folders on hard drive. If not, using trackball at top of screen click on export icon. This will change screen.
4. Select folder to export and select export at bottom of screen. This will take sometime to complete. No message will be given to tell when complete but next to study will be a red export icon.

NOTE: On the right side of screen (TOP) will be a menu to toggle between Xcelera export and DVD export. Check to make sure Xcelera is chosen.

5. Main computer can now be used to analyse study.

6. With Xcelera open as per standard. Enter MRN details as entered earlier on GX50 to identify folder and load images.
7. Toggle images with mouse.
8. Once image is selected, right click mouse button to open Q-lab for Intima Media Thickness (IMT) or double click to open Dicomm viewer to measure parameters as below.

Note: At top to viewer, make sure carotid protocol is selected from drop down menu. This allows measurements to be performed.

Intima Media Thickness

Measured in Qlab as an automated procedure by clicking IMT icon listed on left. If automation unclear adjustment can be made using the icons listed on the left of the screen, taking care to keep the 10mm distance required and criteria as well established in the literature. Anterior wall IMT is measured during contrast phase.

Parameters to analyse

Lesion

Side	Success %	Image time	IMT (mm)	True Diameter (cm)	Residual Diameter (cm)	% Stenosis
Right			**A:**			
			P:			
Left			**A:**			
			P:			

Plaque morphology analysis:

Each visible plaque is to be classified in terms of echogenicity at standard imaging, according to well-established criteria:
Class I: uniformly echolucent
Class II: predominantly echolucent
Class III: predominantly echogenic
Class IV: uniformly echogenic
Class V: extensive calcification with acoustic shadowing

Stenosis

The degree of stenosis is to be measured according to European Carotid Surgery Trial (ECST) criteria and according to blood flow velocities. This parameter was acquired at time of scanning. Ratio can be calculated using Xcelera measurement tool as listed in carotid protocol.

Side	PSV (cm/s) CCA	ICA	EDV (cm/s) CCA	ICA	ICA/CCA Ratio
Right					
Left					

Contrast image analysis:

In contrast-enhanced images, all the plaques appear dark and hypoechoic while the adventitia still appears as a bright echogenic line. Moving bright spots within the plaque or on its adventitial side are considered to represent the contrast agent's bubble signal coming from plaque neovascularization. Fixed echogenic spots are considered to be strong tissue acoustic reflectors.
Plaque neo- vascularization should categorized as follows:

Grade 0: No contrast within Plaque
Grade 1: No bubbles within the plaque or bubbles confined to plaque adventitial side and/or shoulder;
Grade 2: bubbles reaching plaque core and/or extensive contrast-agent enhancement throughout the plaque.

Carotid Contrast Ultrasound SOP References:

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Appendix 4: Carotid Contrast Ultrasound Data Sheet

CUPNASA Study: Data analysis
Anthony Jaipersad

plasma code:
Scan code:
Flow code:

Patient ID:
Date of Analysis:
Date of Scan:

Lesion

Side	**Success %**	**Image time**	**IMT (mm)**	**True Diameter (cm)**	**Residual Diameter (cm)**	**% Stenosis**
Right			**P:**			
Left			**P:**			

Plaque morphology: **Right:** **Left:**
0. No lesion I. Uniformly Echolucent II. Predominantly Echolucent III. Predominantly Echogenic IV. Uniformly Echogenic V. Extensive Calcification/Shadowing

AVV: **Right:** **Left:**
Grade 0: no contrast within plaque
Grade 1: microbubbles within plaque or bubbles confined to adventitial side
Grade 2: clear visible microbubbles reaching plaque core or enhanced plaque.
Grade 5: no comment

Image Quality: **Right:** **Left:**
Excellent/good/Fair

Comments:

Signature of Reviewer:
Printed Name:

Appendix 5: Flow Cytometry Protocol

Flow Cytometry Protocol

Sign in- FC logbook and laboratory logbook
Pipettes required – 2-20ul (grey)
5-40ul (red)
40-200 ul (yellow)
200-1000ul (purple)
10-100ul and 100-1000ul digital

1. Sign laboratory logbook
2. Centrifuge 4 blood tubes (2 x citrate, 1 x serum, 1 x EDTA)- 2860RPM for 15 mins
3. Label tubes 1-3 and additional tube for absolute count (AC)
4. Place Antibodies from 'master mix'
   Tube 1 22.5ul (red)
   2 22.5ul (red)
   3 22.5ul (red)
   AC 12.5ul (grey)- place on opposite side to count beads

5. Add blood to tubes

   AC
   Take 10-100ul digital pipette
   Set to 2 x 50ul
   Withdraw blood then expel by pressing reset, take up blood once again
   Wipe pipette tip to remove excess blood
   Eject **50ul** into AC tube
   Replace tube top

   Tubes 1-3
   Take 100-1000ul digital pipette
   Set to 10 x 100ul
   Withdraw blood then expel by pressing reset, take up blood once again
   Wipe pipette tip to remove excess blood
   Eject **100ul** into tubes 1-3 (1 press)

   Replace rest of blood into EDTA blood tube

6. Vortex all samples (level 3)
7. Place samples in dark for 15 minutes (taken from when last sample prepared)
8. Prepare Flow Cytometer

Turn on FC and after few seconds computer.
Empty waste and refill with 360ml distilled water and 40ml bleach (ensure no bleach on gloves)
Fill machine with Facs flow 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- open 1 minute, closed 5 minutes- then remove and place 'top left' on rack- set FC on 'high/run'
Insert PBS- 1 minute open, 5 minutes closed

9. Use time to prepare ELISA samples for storage
10. **Lysing**
    Purple pipette (200-1000ul)
    2 x 1000ul i.e.. 2mls Facslyse tubes 1-3
    450ul AC tube
10. Place tops on tubes and vortex
11. Place AC in dark for 15 minutes- after which sample ready to be processed
    Place tubes 1-3 in centrifuge- leave for 10 minutes before spinning for 5 minutes (300RPM)
12. Use time to finish ELISA preparation if needed

Prepare Computer
Calibration- 1000ul (purple pipette) of Facs flow- tubes A &B
Place 1 drop of Blue top and white top into A and B, Green, red, pink top into B only
Calibration- open Facscomp icon- enter initials, accept, run
Vortex tube A and B- insert A followed by B

13. Remove tubes from centrifuge- Decant 1-3
14. Add PBS- Large pipette with PBS nozzle
    3mls into tubes 1-3
15. Replace tops and vortex
16. Place tubes 1-3 in centrifuge- 300RPM for 5 minutes
17. For AC- purple pipette (200-1000)
    Add PBS 750ul x 2
    Vortex

Finish computer preparation
Open CellQuest icon
Close box, don't save
FILE- open document, data 1, Mon protocols, Mon no wash
ACQUIRE- connect to cytometer
CYTOMETER- choose instrument settings, Mon no wash, Set, Done
Drag small symbol up and 'unclick' setup
Change directory- e.g. NST-SP 241109
Change file name- e.g. MonAC NST1-1 SP

18. Place AC into cytometer and press acquire (press acquire on top bar, press counters)- once finished, print
19. Remove tubes 1-3 from centrifuge
20. Decant all tubes
21. Re-suspend with yellow pipette- 100ul PBS

Before running tubes 1-3- Empty FC waste and add more facsflow
To run tubes 1-3
On computer- Close box, don't save
FILE- open document, choose Mon wash Luke Ben
CYTOMETER- Install settings open; choose Luke Ben, set, done
WINDOWS- show browser, click Monocyte Tony- clear panel, load tubes from Mon Tony
Change directory and file name as before
22. Vortex and run tubes 1-3, changing file on each sample (1, 2, 3 etc.)- print after each
23. At end- Facsclean open 1 min, closed 5 mins, during which time clean up!
24. Distilled water open 1 min, closed 5 mins
25. Press standby and low
26. Depressurize- shutdown computer
27. After 5 minutes- close flow cytometer by green button

Appendix 6: SOP 198 Monocytes

STANDARD OPERATING PROCEDURE 198

Absolute count of monocyte subsets and assessment of surface marker expression on them by Flow Cytometry
SOP written by Dr Eduard Shantsila & Dr A. Blann

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

Required pre-training

4. SOPs on venepuncture and on good clinical practice
5. SOP 171 – Operation of the Bayer Advia
6. SOP 193 – General operation of the flow cytometer

Contents

Introduction Page 1

Materials and suppliers Page 2

Detailed Method Page 4

1. Introduction

Monocytes are circulating blood cells that participate in innate immunity and inflammatory response as well as in other processes such as angiogenesis, formation of tissue macrophages and dendritic cells, etc. Several monocyte subsets can be discriminated, such as on the basis of surface expression of:

- CD14 (lipopolysaccharide receptor)
- CD16 (Fcγ receptor III), and
- CCR2 (the receptor for monocyte chemoattractant protein [MCP-1]).

This SOP describes enumeration and characterisation monocyte subsets of:

- CD14+CD16- monocytes (about 85%),
- CD14+CD16+ monocytes (about 5%) and
- CD14lowCD16+ monocytes (about 10%): and all by expression of CCR2

However, multiple analyses are possible because the FC and its lasers can accommodate four different fluorochromes:

- FL1 is set up for AF488

- FL2 is set up for PE
- FL3 is set up for Per-CP-Cy5.5
- FL4 is set up for APC

This means that other cell surface and intracellular molecules can be probed, assuming a relevant antibody conjugated to a useable fluorochrome can be sourced. It follows that various sub-protocols can be derived. Therefore this SOP comes in three stages:

(a) An absolute white blood cell count that gives a monocyte count is necessary to ensure results are fed back mathematically so the number of each type of cell per ml of venous blood can be determined.

(b) The assessment of surface expression of various receptors i.e. CD14 and CD16 on the surface of monocyte subsets – performed with 'lyse-and-wash' protocol. This part of protocol can be done without CCR2 so that two of four channels are available of FACSCalibur flow cytometer (i.e., PE- and APC-channels) can be used for evaluation of other parameters. This section will be generic for all subsequent sub-protocols.

(c) The profile of these sub-protocol parameters may vary depending on the aim of a sub-project and may include receptors mediating different but specific functions of monocytes. These include potential roles in….

- Platelet marker (CD42a) to detect monocyte –platelet aggregates (MPAs). **This sub-SOP was part validated for intra-assay CV on 14th January 2010**

- Intracellular I-kappa-kinase-β (IKKβ). **This sub-SOP was part validated for intra-assay CV and isotype control on 11th February SOP 20**

- Angiogenesis (as can be marked by molecules such as VEGF receptors 1 and 2, CXCR4 [stromal cell-derived factor-1], CD34)

- Inflammatory responses (interleukin-6 receptor, Toll-like receptor 4)

- Differentiation and migration (receptors to adhesion molecules ICAM-1 and VCAM-1), i.e. integrin β2/CD18, integrin α4/CD49d

- Scavenger receptors (CD204, CD163)

It follows from the above that considerable thought must be directed towards these complex assays, especially as different antibodies may be conjugated to the same fluorochrome.

2. Materials and Supplier contact details:

2.1 General reagents and disposables

1) BD “FACS Flow” Running solution [Becton Dickinson, Catalogue No. 342003] 10L containers.
2) BD “FACS Clean” Cleaning Solution [Becton Dickinson, Catalogue No. 340345]
3) 3 ml BD Falcon tubes [Becton Dickinson, Catalogue No. 352054]
4) BD Lysing solution [Becton Dickinson Catalogue No. 349202]
5) Sterile Phosphate Buffered Saline solution, 0.5L bottles [Invitrogen Ltd, Catalogue No 20012-068]
6) BD TruCount tubes [Becton Dickinson Catalogue No. 340334]
7) Clear pipette tips [Alpha Laboratories Limited Catalogue No FR1250 1250ul Fastrak Refill NS]
8) Yellow pipette tips [[Alpha Laboratories Limited Catalogue No FR1200 200ul Fastrak Refill NS]
9) Pipettes required 2-20ul (grey top) 5-40ul (red top) 40-200 ul (yellow top) 200-1000ul (purple top) 10-100ul and 100-1000ul digital

2.2 Antibodies - Generic

10) CD14-PE conjugated monoclonal antibody [R&D Systems Europe Ltd, Cat No. FAB3832P] – this is for the absolute counts (AC) of cells only – not the mastermixes (see below)
11) CD14-PerCP-Cy5.5 conjugated monoclonal antibody [Becton Dickinson Catalogue No. 550787] – for mastermixes (see below)
12) CD16-Alexa Fluor 488-conjugated monoclonal antibody [AbD Serotec, Oxford, UK, Cat No. MCA2537A488]
13) CCR2-APC conjugated monoclonal antibody [R&D Systems Europe Ltd, Cat No. FAB151A]

2.3 Antibodies – Sub-protocols: for example:

14) CD42a-PerCP conjugated monoclonal antibody [Becton Dickinson, Catalogue No. 340537]
15) IKKbeta-APC conjugated monoclonal antibody 50µg/ml - 100 tests [Becton Dickinson, Catalogue No. 551920]
16) VEGFR1-APC conjugated monoclonal antibody (R&D Systems Europe Ltd Catalogue No. FAB321A)
17) VEGFR2 (KDR, CD309)-APC conjugated monoclonal antibody [R&D Systems Europe Ltd Catalogue No. FAB321A]
18) CD34-PE conjugated monoclonal antibody (Becton Dickinson, Catalogue No. 555822)
19) TLR4 (CD284)-PE conjugated monoclonal antibody [R&D Systems Europe Ltd Catalogue No. FAB14781P]
20) IL-6 receptor α-APC conjugated monoclonal antibody [R&D Systems Europe Ltd Catalogue No. FAB227A]
21) Mac-1-PE conjugated monoclonal antibody (CD11/CD18, receptor for ICAM-1) [R&D Systems Europe Ltd Catalogue No. FAB1730P]

22) Integrin α4/CD49 complex-APC conjugated monoclonal antibody [R&D Systems Europe Ltd Catalogue No. FAB1354A]
23) CD163-APC conjugated monoclonal antibody [R&D Systems Europe Ltd Catalogue No. FAB1607A]
24) CXCR4-PE conjugated monoclonal antibody (CD184) [R&D Systems Europe Ltd Catalogue No. FAB170P]

3.1 General Preparation

3.1.1 Lysing solution (kept on the shelf in flow cytometry room at room temperature).

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). When this is made up the operator must ensure that date is put on the bottle.

3.1.2. Mastermixes (MMs)

These are made up to define the particular subject of the sub-protocol. There are separate tubes for different sub-projects. All MMs are composed to 100 ul of blood, 5 ul CD16-AF488 and 2.5 ul CD14-PerCP-Cy5.5. Sub-MMs for individual sub-protocols then have other antibodies in addition. For example…..

MM1: IL6-R-APC and TLR4-PE

3.1.3. Time from blood sample collection by aseptic venepuncture (see appropriate guidelines) to beginning of sample preparation should be less 30 min, and must not be more than 60 min.

3.2 Blood sample preparation

3.2.1. Place EDTA blood sample on rotator.
3.2.2. Label tubes 1-5 and additional tube for absolute count (AC)
3.2.3. Place Antibodies from 'mastermixes':

Tube 1 - 22.5ul (red top)
Tube 2 - 22.5ul (red top)
Tube 3 - 22.5ul (red top)
Tube 4 - 27.5ul (red top)
Tube 5 - 22.5ul (red top)

AC tube - 12.5ul (grey top) - place on opposite side to count beads. Do not touch the pellet!!!! If this happened – change the tube

.

3.2.4. Add blood to tubes

a) Absolute count tube:

- Take 10-100ul digital pipette
- Set to 2 x 50ul

- Withdraw blood then expel by pressing 'RESET', take up blood once again
- Wipe pipette tip to remove excess blood
- Eject 50ul into AC tube – keep the tip well above the metal retainer. Do not touch pellet!!!
- Replace tube top

b) MM Tubes 1-5

- Take 100-1000ul digital pipette
- Set to 10 x 100ul
- Withdraw blood then expel by pressing reset, take up blood once again
- Wipe pipette tip to remove excess blood
- Eject 100ul into tubes 1-5 (1 press)
- Replace rest of blood into EDTA blood tube (keep it in case you need to repeat the procedure)

3.2.5. Vortex all samples (speed 3 for 3 sec) [what speed and duration?]
3.2.6. Place samples in dark for 15 minutes (taken from when last sample prepared)

3.3 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

3.4. Further sample preparation

3.4.1. Red blood cell lysing
- Use purple pipette (200-1000ul)
- Put 2mls of Facslyse in Tubes 1-5
- Put 450ul in AC tube
- Place tops on tubes and vortex (speed 3 for 3 sec)
- Place AC tube in dark for 15 minutes- after which add 1.5 mls of PBS, vortex - sample ready to run
- Place tubes 1-5 in centrifuge- leave for 10 minutes before spinning for 5 minutes (300RPM)
- Remove tubes 1-5 from centrifuge, decant, add 3 mls of PBS, replace tops, vortex, spin again - 300RPM for 5 minutes
- Decant supernatant, resuspend in 100 ul of PBS – sample ready to run

3.5. Sample acquisition

3.5.1. Absolute count
- Run FacsComp if you are first user of the flow cytometer during the day
- Press CellQuest icon
- 'FILE' – 'Open document' – 'Data 1' – 'Mon protocols' – 'Mon no wash'
- ACQUIRE – 'Connect to cytometer'
- CYTOMETER – 'Choose instrument settings' – 'Mon no wash' – 'Set' – 'Done'
- Change appropriate directory to save flow data
- Change file name
- Place AC into cytometer and press 'Acquire' (press 'Acquire' on top bar, press 'Counters') - once finished, print

3.5.2. Expression of surface markers
- 'FILE' – 'Open document' - choose – 'Mon wash Luke/Ben'
- CYTOMETER – 'Instrument settings' – 'Open' - choose 'Mon wash Luke/Ben' – 'Set' – 'Done'
- 'WINDOWS' – 'Show browser'
- Change directory and file name as before
- Vortex and run tubes 1-5, changing file on each sample (1, 2, 3 etc.)- print after each

Absolute counts of monocyte subsets are performed automatically by flow cytometry software and calculated as number of count beads in tube x number of monocytes collected/(number of collected count beads x volume of blood, µl).

For example: typical number of count beads in a tube – 50000
Number of CD14+CD16- monocytes collected – 5000
Number of collected count beads – 10000
Volume of blood to be used – 50 µl.
Absolute count of CD14+CD16- monocytes will be: 50000 x 5000/(10000 x 50) = 500 cells/µl

3.6. Shut-down procedure [See SOP 195 on General Operation]

1. In this section we re-use tubes 1 and 2 with distilled water and FACS clean respectively.

2. 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.

3. 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.

4. 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'.**

5. 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.

6. 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.

7. Finally power down the FAC-Calibur (green button) and Apple Mac.

8. Clean.

9. 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 the results

Part 1 of the analysis is the number is the number of monocytes in classes 1, 2 and 3, as defined by CD14, C16 and CCR2 and is generic to all sub-protocols. This data will be in all operators spreadsheets. Separation of monocytes from other blood cells and debris is based of three gates (see Appendix 1). Gate 1 employs region 'Mon' to include all monocytic cells on the basis of FSC/SSC characteristics. Gate 2 employs region 'Monos' to exclude granulocytes from analysis. Gate 3 employs region 'NK Lymph' to exclude them from analysis. Monocyte subsets are defined as:

Mon1 – CD14+CD16-CCR2+ cells
Mon2 – CD14+CD16+CCR2+ cells and
Mon3 – CD14lowCD16+CCR2+.

Absolute counts of monocyte subsets are performed automatically by flow cytometry software and calculated as number of count beads in tube x number of monocytes collected/(number of collected count beads x volume of blood, μl).

The protocol employs a 'single platform' approach (i.e., full blood count is not needed for obtaining absolute count). The number of count beads in the pellet has been included in protocol formulas (by Dr. Eduard Shantsila). Absolute count of monocyte subsets and monocyte-platelet aggregates (per μl of blood) is performed automatically and represented on both screen of flow cytometer and printout. See statistics of monocyte count and immunophenotypic characteristics obtained from healthy volunteers in table 1.

The percentage of cells expressing PE-conjugated markers (MAC-1, TLR4, CXCR4, VEGF receptor 1, CD34 is estimated as proportion of cells positive to these markers (upper right plus lower right quadrants on correspondent plots).

The percentage of cells expressing APC-conjugated markers (Integrin α4/CD49 complex, CD163, IL6-receptor, KDR, CD204) is estimated as proportion of cells

positive to these markers (upper left and upper right quadrants on correspondent plots).

SOP 198 Monocyte Table

Comparative characteristics of monocyte subsets – expected results

Parameter	**Mon1**	**Mon2**	**Mon3**
Absolute count, cell /μl	360.1±87.0	24.5±14.8	38.9±12.7
Mon, %*	84.6±5.77	5.90±3.40	9.51±3.74
MPA, cell /μl	37.0±10.9	3.68±2.45	4.59±1.69
MPA, %**	10.4±2.30	16.0±5.65	11.95±2.56
CD204, %***	24.5±14.9	56.9±16.7	61.1±14.29
CD163, %***	98.6±1.27	99.7±0.32	70.3±13.7
Integrin β2/CD18, %***	100 (100-100)	100 (100-100)	99.9 (99.8-100)
Integrin α4/CD49d, %***	74.7 (59.8-82.2)	94.7 (91.1-96.7)	99.1 (98.2-99.4)
CXCR4, %***	91.0 (79.3-96.2)	95.6 (92.0-97.9)	51 (42.9-58.9)
VEGF receptor 1, %***	18.2 (14.5-52.0)	69.6 (62.3-85.3)	28.3 (20.75-33.23)
KDR, %***	5.00 (3.38-7.23)	8.25 (5.65-10.9)	2.80 (2.23-4.58)
CD34, %***	0.45 (0.2-1.0)	1.45 (0.83-3.3)	2.40 (1.8-3.7)
TLR4, %***	18.4 (14.3-32.2)	63.2 (46.4-75.2)	21.4 (18.4-29.9)
IL6 receptor, %***	100 (99.9-100)	100 (99.9-100)	96.3 (93.5-97.9)

Data is mean (SD) or median (IQR). Mon1 – CD14+CD16-CCR2+ monocytes, Mon2 – CD14+CD16+CCR2+ monocytes, CD14+CD16+CCR2- monocytes, MPA – monocyte-platelet aggregates, VEGF – vascular endothelium growth factor, TLR4 – Toll-like receptor-4, IL6 – interleukin 6 * Per cent of total monocyte count, **Per cent of MPA compared to the whole monocyte subset, ***Per cent of positive cells within a monocyte subset.

Appendix 7: SOP 66 VEGF

STANDARD OPERATING PROCEDURE 66

Vascular Endothelial Growth Factor (VEGF)

ELISA developed by Funmi Belgore and Andrew Blann, 1997 and 1998. This SOP was updated by Balu Balakrishnan August 2007

Introduction Page 1
Materials Page 2
Method Page 6

1. INTRODUCTION

Synopsis:

Vascular endothelial growth factor (VEGF) is a multi-functional peptide capable of inducing angiogenesis and may have effects on endothelial integrity. It has been implicated in neovascularisation in adult pathophysiology. This ELISA uses commercial antibody and will require about 4-6 hours to complete.

Brief Method:

1. Coat microtitre plate with 112 µl of primary antisera l in 20 ml PBS buffer for 2 plates overnight in the fridge.

2. Wash, block with 100 µl /well of 5% Marvel (1g in 20mls PBS-T for 2 plates) for 1 hour at RT.

3. Wash, add 100 µl of neat **plasma**, or neat tissue culture fluid, and recombinant standards for 2 hours at RT. Standards are double diluted down the plate. Use fresh tips for each sample.

4. Wash, add 112 µl of biotinylated anti-human VEGF antibody in 20 ml PBS tween for 90 minutes at RT.

5. Wash, add Streptavidin (100µl/well) for 45 minutes at RT.

6. Wash, add 100 µl substrate (Solutions A and B) .Blue colour develops well with in 20 to 30 minutes

7. Stop with 50 µl/well acid. Colour goes yellow. Read at 450 nm.

Expected values:

Data usually not normally distributed. Controls generally have median values of about 30-50 pg/ml (but IQR may be over 200pg/ml) Patients' median values generally 100 to over 200 pg/ml. Again, wide IQRs.

2. MATERIALS

2.1 Coating the plate with 1% AB

The plates are coated with 20ml of 1%AB.To make up the buffer use 20ml of PBS (1X), liquid 20012-068 from Life technologies and add 112 µl of 1%AB from the stock solution.

2.2 Primary Antiserum

2.2.1 The primary antisera is goat anti-human VEGF antibody.

Supplier, storage and location

From R&D systems, Tel: 01235 529 449, Fax: 01235 533 420.
Catalog Number: DY293B Duoset kit

Reconstitute vial with 1ml PBS and aliquot out to 112µl aliquots and store in the –70 freezer until use.

Add 20mls of pbs to give 20µg/20mls or 1µg/ml, i.e. the working concentration (enough for 2 plate)

2.3 Microtitre plates

2.3.1 Flat-bottomed 96 well microtitre plates (Immunlon 2)

Supplier, storage and location

Thermo Life Sciences, Basingstoke. Telephone 01256 817 282.

2.4 Yellow and Blue Tips

.
2.4.1 Yellow and blue tips

Supplier, storage and location

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, Website www.appletonwoods.co.uk.

Our account number DUD 001, we have various discounts for various goods.

Tips stored at RT in plastic bags/boxes under the desk in the main lab.

2.5 Wash Buffer

To make this up, you need

2.5.1 Phosphate buffer saline tablets. Sigma catalogue number P 4417. Buy in lots of 50 or
100 tablets. Kept on the shelves on the right hand side.

2.5.2 Tween 20. Sigma Catalogue number P 1379. Order in 100 ml lots. Also kept on the
shelves on the far right hand side.

2.5.3 Distilled water.

Supplier

Sigma – Aldrich, 0800 717181. Our account number is 782274. They will ask for your name (use mine) and order number (use your initials).

Method/storage/supplier

To one litre of water, add 5 tablets and 0.5 ml Tween (use a blue tip and micropipette). Place on rotamixer with stir bar, wait until tablets have dissolved. We generally make up several litres at a time. Stored at room temperature on the rotamixer.

n.b. Generally, make up 2, 3 or more litres in one batch.

Biohazard/COSHH

(n.b. this is not an official assessment!) nil unless you eat/drink a few kilos/litres. Wash off spills with water. Otherwise maintain good laboratory practice.

2.6 Secondary Antiserum

2.6.1 The secondary antisera is biotinylated goat anti-human VEGF polyclonal antiserum (conjugated to biotin).

Supplier, storage and location

From R&D systems, Tel: 01235 529 449, Fax: 01235 533 420.
Catalog Number: DY293B Duoset kit

Reconstitute the vial with 1ml of PBS-0.1%BSA (gives stock of 18µg/ml of stock) and aliquot into 112µl aliquots and store in the –70 freezer until use.

Add 20mls of PBS-T to give 1µg /10mls or 500ng/ml (enough for 1 plate).

2.7 Steptavidin-HRP conjugate

Streptavidin binds to biotin, thus amplifying the signal. HRP = horse radish peroxidase. Obtain from R&D Systems, Abingdon, as for the antibodies and rVEGF standard.

From R&D systems, Tel: 01235 529 449, Fax: 01235 533 420
Catalogue number: DY998

The order consists of five small glass vials, each with 1 ml fluid. Store in the 4^{o} Fridge. Do not freeze. However: Extravidin peroxidase can also be obtained from Sigma: Catalogue number: E 2886. Add 10µl of extravidin peroxidase to 10mls of PBS-T for 1 plate.

2.8 Substrate

This is/are Solutions A and B from R&D Systems.

Biohazard/COSHH

(n.b. this is not an official assessment!) nil unless you eat/drink a few kilos/litres. Wash off spills with water. Otherwise maintain good laboratory practice.

2.9 Standards

Supplier/storage
From R&D systems, Tel: 01235 529 449, Fax: 01235 533 420.
Catalog Number: DY293B Duoset kit

The stock solution of rVEGF standard was prepared by adding 500ul of Pbs Buffer .Then aliquoted out into vials containing 33ul each so as to attain a top standard of 4 ng when made up to one ml with Pbs.

2.10 Stop Solution

1 mol/L hydrochloric acid. No need to dilute.

Supplier/ Storage

Sigma, Catalogue number 920-1. Order in lots of 1 gallon (= 3.8 L).

Biohazard/COSHH
(n.b. this is not an official assessment!) **Considerable**. Do not get on skin/clothes. Wash off spills with water. Otherwise maintain good laboratory practice.

3. METHOD

Step 1 Coating microtitre plates

Using a micropipette and a yellow tip, transfer the 112 µL of primary antibody aliquots (2.2) to approximately 20 mls Pbs buffer (2.1) and, using an eight-channel micropipette with yellow tips, apply 100 µL to each well of the micro titre plate (2.3).

Place in lunch box with lid and label. Incubate in the fridge overnight or two hours at room temperature.

Step 2 Wash and block

Using the eight-channel washing manifold (Sigma), wash out the unbound antiserum with three lots of >250 µL PBS/tween (2.5). Blot out on tissue paper between each step.

Using the 8-channel pipette, add 100 µl of 5% Marvel (2.9) into each well and incubate at RT for 1 hours.

Step 3. Samples of plasma, serum or tissue culture fluid AND standards (need to made up fresh each time

Wash again as above.

Thaw plasma/serum samples in warm water in the sink, but never for long, just to take the chill off. Even from –70^{o}C, small aliquots are quick to thaw.

100µl of neat plasma/culture medium added to each well. CHANGE TIPS EACH SAMPLE

The Standard Curve [correct at 6.2.2007]

The standard consisting of a small amount of rVEGF has been pre-aliquoted and stored in the top compartment of the -70^{o}C freezer in the box labelled VEGF.

Stock
Current top batch is 33 ul of fluid with 4 ng of protein per vial. Therefore take one of these and make it up to one ml for 4 ng\ml working concentration.

Working
Place top standard of 200 ul in wells of columns 11 and 12 of row A in two plates. This will be 4 ng/mL. Use the leftover 100 ul in the wells for double dilution on the plates.

Take
Of this leftover, take 100 ul in the wells below i.e. 11B and 12 B mix well continue the same all the way to the bottom of the plate. Double dilute the standards to the next but last wells that is 11g and 12 g maintain 11h and 12h as blanks (free of VEGF).

Replace the plates in plates in the lunch box as before and incubate for 2 hours at RT.

Discard all left over eppendorf vials.

Step 5 – Wash and Secondary (Detection) Antibody

Wash as above.

The secondary Ab should have been aliquoted and stored in the –70 (2.6). Transfer the secondary to 20mls of PBS-T (make sure it is well mixed) and add 100µl into each well with 8-channel pipette. Incubate at RT for ninety minutes.

Step 6 – Wash and Conjugate

Wash again as above.

Using a micropipette and a yellow tip, add 100µl of Steptavidin-HRP to approximately 20mls of PBS-T (2.10) and, using an eight-channel micropipette with yellow tips, apply 100 µL to each well. Place back in the lunch box with lid and label and incubate on the bench for 30 minutes.

n.b. discard the tips immediately: a mere 1 molecule of contaminating enzyme will work on your substrate.

Step 7 – Wash Again

As 3.2, but be very thorough at this stage. Being slack here means the blanks will be positive.

Step 8 – Colour Development

This is R&D Systems solutions A and B. Get them out of the 'fridge in plenty of time to warm up e.g. on the bench on in warm water. Mix them before use

Add 100 µL substrate in a controlled manner, and then the acid at the same speed in the same direction (e.g.. both right to left). It may be that you have to stop the reaction at the start of the first plate as soon as you have finished adding substrate to the end of the second plate.

Exactly when to stop the reaction is a learned skill. Generally, when you can differentiate a gradient between all the standards and the blanks are still blank.

Wait till the standards develop .The observer must be able to differentiate between the last two standards.

n.b. if the reaction is slow the most likely reason is that the substrate buffer is out of pH

Step 9 – Reading and Calculation

9.1 Put the ELISA reader on to warm up (see separate instructions on the wall above the
machine). Ensure the wavelength is 450 nm.

9.2 Construct a standard curve using 3 log graph paper from the standards and read off your pg/ml from the optical densities.

Step 10 – Disposal: See GLP document

All tips and leftover whole plasma to go in sharps bin. Microtitre plates to be emptied down a sink, then washer out with tap water, then put in yellow bins.

SOP SIGNED OFF BY ANDREW BLANN (Date)

MASTER PAPER COPY KEPT BY RUBY STONE.

SOP 66 VEGF REFERENCES

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Appendix 8: SOP 19 Tie 2 ELISA

STANDARD OPERATING PROCEDURE 19

Tie-2 ELISA

Written by Balu Balakrishnan 2013

1. INTRODUCTION

Synopsis:

Vascular endothelial growth factor (VEGF) is a multi-functional peptide capable of inducing angiogenesis and may have effects on endothelial integrity. It has been implicated in neovascularisation in adult pathophysiology. This ELISA uses commercial antibody and will require about 4-6 hours to complete.

Brief Method:

8. Coat microtitre plate with 112 µl of primary antisera l in 20 ml PBS buffer for 2 plates overnight in the fridge.

9. Wash, block with 300 µl per well of reagent diluent for 1 hour at RT.

10. Wash, add 10 µl of neat plasma to 90 µl of reagent diluent, or neat tissue culture fluid, and recombinant standards for 2 hours at RT. Standards are double diluted down the plate. Use fresh tips for each sample.

11. Wash, add 112 µl of Detection Antibody in 20 ml of reagent diluent for 2 hours at RT

12. Wash, add Streptavidin (100µl/well) for 20 minutes at RT.

13. Wash, add 100 µl substrate (Solutions A and B) .Blue colour develops well with in 20 to 30 minutes

14. Stop with 50 µl/well acid. Colour goes yellow. Read at 450 nm.

Range:156 - 10,000 pg/mL

2. MATERIALS

2.1 Coating the plate with 1% AB

The plates are coated with 20ml of 1%AB.To make up the buffer use 20ml of PBS (1X), liquid 20012-068 from Life technologies and add 112 µl of 1%AB from the stock solution.

2.2 Primary Antiserum

The mouse anti-human Tie-2 antibody.

Supplier, storage and location

From R&D systems, Tel: 01235 529 449, Fax: 01235 533 420.
Catalog Number: DY5159 Duoset kit

Reconstitute vial with 1ml PBS and aliquot out to 112µl aliquots and store in the –70 freezer until use.

Add 20ml of PBS (1X), liquid 20012-068 from Life technologies for the working concentration enough for 2 plate.

2.3 Microtitre plates

2.3.1 Flat-bottomed 96 well microtitre plates .

Supplier, storage and location

Thermo Life Sciences, Basingstoke. Telephone 01256 817 282.

2.4 Yellow and Blue Tips

.
Yellow and clear tips

Supplier, storage and location
Alpha Laboratories Limited
Tel – 02380483000

2.5 Wash Buffer

To make this up, you need

Phosphate buffer saline tablets. Sigma catalogue number P 4417. Buy in lots of 50 or 100 tablets. Kept on the shelves on the right hand side.

Tween 20. Sigma Catalogue number P 1379. Order in 100 ml lots. Also kept on the shelves on the far right hand side.
Distilled water.

Supplier

Sigma – Aldrich, 0800 717181.

Method/storage/supplier

To one litre of water, add 5 tablets and 0.5 ml Tween (use a blue tip and micropipette). Place on rota mixer with stir bar, wait until tablets have dissolved. We

generally make up several litres at a time. Stored at room temperature on the rotamixer.

n.b. Generally, make up 2, 3 or more litres in one batch.

Biohazard/COSHH

(n.b. this is not an official assessment!) nil unless you eat/drink a few kilos/litres. Wash off spills with water. Otherwise maintain good laboratory practice.

2.6 Secondary Antiserum

2.6.1 The secondary antisera is biotinylated mouse anti-human Tie-2 polyclonal antiserum (conjugated to biotin).

Supplier, storage and location

From R&D systems, Tel: 01235 529 449, Fax: 01235 533 420.
Catalog Number: DY5159 Duoset kit

Reconstitute the vial with 1ml of PBS-0.1%BSA and aliquot into 112µl aliquots and store in the –70 freezer until use.

Add 20mls of PBS-T to give 1µg /10mls or 500ng/ml (enough for 1 plate).

2.7 Steptavidin-HRP conjugate

Streptavidin binds to biotin, thus amplifying the signal. HRP = horse radish peroxidase. Obtain from R&D Systems, Abingdon, as for the antibodies and Tie-2 standard.

From R&D systems, Tel: 01235 529 449, Fax: 01235 533 420
Catalogue number: DY998

2.8 Substrate

This is/are Solutions A and B from R&D Systems.

Biohazard/COSHH

(n.b. this is not an official assessment!) nil unless you eat/drink a few kilos/litres. Wash off spills with water. Otherwise maintain good laboratory practice.

2.9 Standards

Supplier/storage

From R&D systems, Tel: 01235 529 449, Fax: 01235 533 420.

Catalog Number: DY5159 Duoset kit

The stock solution of Tie-2 standard was prepared by adding 500ul of Pbs Buffer. Then aliquoted out into vials containing 43.5ul each so as to attain a top standard of 10 ng when made up to one ml with Pbs.

2.10 Stop Solution

1 mol/L hydrochloric acid. No need to dilute.

Supplier/ Storage

Sigma, Catalogue number 920-1. Order in lots of 1 gallon (= 3.8 L).

Biohazard/COSHH
(n.b. this is not an official assessment!) **Considerable**. Do not get on skin/clothes. Wash off spills with water. Otherwise maintain good laboratory practice.

3. METHOD

Step 1 Coating microtitre plates

Using a micropipette and a yellow tip, transfer the 112 µL of primary antibody aliquots to approximately 20 mls Pbs buffer and, using an eight-channel micropipette with yellow tips, apply 100 µL to each well of the micro titre plate. Place in a box with lid and label. Incubate in the fridge overnight or two hours at room temperature.

Step 2 Wash and block

Using the eight-channel washing manifold (Sigma), wash out the unbound antiserum with three lots of >250 µL PBS/tween . Blot out on tissue paper between each step.

Using the 8-channel pipette, add 300 µl of Reagent diluent into each well and incubate at RT for 1 hours.

Step 3. Samples of plasma, serum or tissue culture fluid AND standards (need to be made up fresh each time)

Wash again as above.

Thaw plasma/serum samples in warm water in the sink, but never for long, just to take the chill off. Even from –70^{o}C, small aliquots are quick to thaw.

10µl of neat plasma to 90µl of Reagent diluent added to each well. CHANGE TIPS EACH SAMPLE

The Standard Curve

Stock

Current top Std is 43.5 ul of fluid with 10ng of protein per vial. Therefore take one of these and make it up to one ml for 10 ng\ml working concentration.

Working
Place top standard of 200 ul in wells of columns 11 and 12 of row A in two plates. This will be 10 ng/mL. Use the leftover 100 ul in the wells for double dilution on the plates.

Take
Of this leftover, take 100 ul in the wells below i.e. 11B and 12 B mix well continue the same all the way to the bottom of the plate. Double dilute the standards to the next but last wells that are 11g and 12 g maintain 11h and 12h as blanks (free of VEGF).

Replace the plates in plates in the box as before and incubate for 2 hours at RT.

Discard all left over eppendorf vials.

Step 5 – Wash and Secondary (Detection) Antibody

Wash as above.

The secondary Ab should have been aliquoted and stored in the –70 or in the duoset box. Transfer the secondary to 20mls of PBS-T (make sure it is well mixed) and add 100µl into each well with 8-channel pipette. Incubate at RT for 2 hours.

Step 6 – Wash and Conjugate

Wash again as above.

Using a micropipette and a yellow tip, add 100µl of Steptavidin-HRP to approximately 20mls of PBS and, using an eight-channel micropipette with yellow tips, apply 100 µL to each well. Place back in the box with lid and label and incubate on the bench for 30 minutes.

n.b. discard the tips immediately: a mere 1 molecule of contaminating enzyme will work on your substrate.

Step 7 – Wash Again

As 3.2, but be very thorough at this stage. Being slack here means the blanks will be positive.

Step 8 – Colour Development

This is R&D Systems solutions A and B. Get them out of the ‘fridge in plenty of time to warm up e.g. on the bench on in warm water. Mix them before use

Add 100 µL substrate in a controlled manner, and then the acid at the same speed in the same direction (e.g.. both right to left). It may be that you have to stop the

reaction at the start of the first plate as soon as you have finished adding substrate to the end of the second plate.

Exactly when to stop the reaction is a learned skill. Generally, when you can differentiate a gradient between all the standards and the blanks are still blank.

Wait till the standards develop .The observer must be able to differentiate between the last two standards.

n.b. if the reaction is slow the most likely reason is that the substrate buffer is out of pH

Step 9 – Reading and Calculation

9.3 Put the ELISA reader on to warm up (see separate instructions on the wall above the
machine). Ensure the wavelength is 450 nm.

9.4 Construct a standard curve using 3 log graph paper from the standards and read off your pg/ml from the optical densities.

Step 10 – Disposal: See GLP document

All tips and leftover whole plasma to go in sharps bin. Microtitre plates to be emptied down a sink, then washer out with tap water, then put in yellow bins.

SOP SIGNED OFF BY ANDREW BLANN (Date)

Appendix 9: Published Papers

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