Waterford Institute *of* Technology

Cell-derived Microparticles as Part of Multimarker Strategies to Improve Vascular Risk Prediction

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Presentations and Publications

1. Abstracts of Chapters 4 and 5 have been presented orally at the following conferences/meetings;

a. 2nd Annual Joint Irish Association of Vascular Surgeons/Northern Ireland Vascular Society Conference 2012. Title: Predicting carotid artery disease and plaque vulnerability from cell-derived microparticles: The Carotid Artery Biomarker Study

b. 8th Annual meeting of the Irish Cytometry Society 2012. Title: Predicting carotid artery disease and plaque vulnerability from cell-derived microparticles: The Carotid Artery Biomarker Study

c. The Society of Academic & Research Surgery Meeting 2013. Title: Predicting carotid artery disease and plaque vulnerability from cell-derived microparticles: The Carotid Artery Biomarker Study

d. 3rd Annual Joint Irish Association of Vascular Surgeons/Northern Ireland Vascular Society Conference 2013. Title: Prognostic potential of cell-derived microparticles and soluble vascular biomarkers in carotid arterial disease

e. European Society of Vascular Surgery Spring Meeting 2013. Title: Novel vascular biomarkers of carotid arterial disease: The Carotid Artery Biomarker Study

2. Abstracts of chapters 4 and 5 have also been published in the British Journal of Surgery Online. Title: Predicting carotid artery disease and plaque vulnerability from cell-derived microparticles: The Carotid Artery Biomarker Study

3. Chapter 6 was presented orally at the American College of Sports Medicine Annual Meeting 2013. Title: Influence of a 24 week low carbohydrate diet on endothelial and inflammatory biomarkers in overweight women

4. Other achievements based on work in this thesis include;

a. Chapters 4 and 5, WIT Research Day 2013, Best Oral Presentation. Title: Cell-derived microparticles as diagnostic biomarkers of carotid artery disease

b. Chapters 5 and 5, WIT Research Day 2013. Poster titled: Cell-derived microparticles as diagnostic biomarkers of carotid artery disease

c. Chapter 6, WIT Research Day 2012. Poster titled: Effects of a 24-week low carbohydrate diet on vascular health biomarkers

Declaration

Waterford Institute *of* Technology

This thesis is a presentation of my original research work. Wherever contributions of others are involved, every effort is made to indicate this clearly, with due reference to the literature, and acknowledgement of collaborative research and discussions.

The work was done under the guidance of Dr. Michael Harrison and Dr. Orla O'Donovan at Waterford Institute *of* Technology and Professor Keith S. Cross at Waterford Regional Hospital.

Signature: ..... Date: .....

Antony Lubonga Wekesa B.Sc. (Hons)

Abstract

Author: Antony Lubonga Wekesa

Title: Cell-derived Microparticles as Part of Multimarker Strategies to Improve Vascular Risk Prediction

Candidate biomarkers should possess some of the following characteristics: identify individuals with disease, distinguish disease stage and monitor changes in health with treatment. There is increasing interest in the potential of cell-derived microparticles to act as sensitive biomarkers of vascular health status. Cell-derived microparticles (MP) are small (0.1-1.0 μm) plasma membrane derived vesicles shed into circulating blood by most cells, including platelets and endothelial cells. They may act not only as biomarkers but also mediators of vascular disease transferring biological agents that mediate inflammation, coagulation and vascular remodelling.

In a series of studies, we have demonstrated that;

1. A number of small methodological studies undertaken suggest that flow cytometry can enumerate MP reproducibly but that variability can occur during sample processing through the double centrifugation protocol. Day to day biological variation also exists.
2. Annexin V+ MP (mainly of platelet origin) were higher in carotid artery disease cases compared to age-matched controls and added to predictive ability in a multivariable model.
3. CD31+CD41- endothelial MP (EMP) were higher in carotid artery patients with unstable plaques, classified post-surgery by immunohistochemistry. CD31+CD41- EMP, but not any subset of platelet MP, added to predictive ability in multivariable models to predict unstable plaques.
3. CD31+CD41- EMP in addition to other soluble markers were lower following a low carbohydrate diet that involved moderate reductions in body weight and waist circumference, with the effect size (Cohen's d) greatest for CD31+CD41- EMP.
4. Other cellular vascular biomarkers were investigated, specifically endothelial progenitor cells and platelet monocyte aggregates but were not useful as biomarkers. A number of protein vascular biomarkers showed potential both in predicting disease and monitoring changes in health status, specifically the acute phase protein serum amyloid A.

Despite the methodological difficulties that exist when enumerating MP, various subsets have shown potential as stand-alone biomarkers and in multivariable models. Future studies are justified including studies to examine the ability of MP to predict asymptomatic carotid artery disease and prospective studies to determine the prognostic ability.

Acknowledgment

I am deeply indebted to Dr. Michael Harrison, Dr. Orla O'Donovan and Professor Keith S. Cross, for introducing me to this important and interesting field of vascular biomarkers. Their constant encouragement, helpful comments and supervision enabled me to accomplish my research objectives.

I wish to express my sincere appreciation to all the participants for giving up their valuable time to take part in this research and making it what it is.

My deep appreciation goes to staff of the Departments of Vascular Surgery and Histology (Waterford Regional Hospital), notably Mr. Joseph Dowdall, Dr. Rob Landers, Lorraine Byrne, Dr. Marianne Doyle and Dr. Michelle Griffin, for helping with participant recruitment, vascular imaging and analysis of carotid plaques.

I wish to thank Dr. Lorna Doyle and members of the Biomedical Research Cluster for their assistance, support and constructive criticism.

My profound gratitude to my colleagues, Dr. John Paul Phelan, Doreen FitzMaurice, Mr. Mark Ross and Mr. Bruce Wardrop, and the wonderful teams at the Pharmaceutical and Molecular Biotechnology Research Centre and Research Support Unit, for their assistance and support, and with whom I exchanged excellent ideas during the period of my research work.

To all I say thank you and God bless.

Finally I wish to acknowledge the Technological Sector Research Funding Program for funding this research.

Dedication

This work is dedicated to:

My mom, Ruth, who brought me the world of love

My dad, Julius (RIP), who brought me the world of words

My siblings, Martin, Evelyn, Harry, Millicent, Metrine, Gertrude and Irene, for bringing me the world

My uncles Simon, John, Jotham and Nicholas for the encouragement and support

To all I say,

Thank you and God bless.

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List of Abbreviations

1.	AA	Arachidonic acid
2.	ACVD	Atherosclerotic cardiovascular disease
3.	APOA1	Apolipoprotein A1
4.	APOB	Apolipoproteins B
5.	AUC	Area under the curve
6.	bFGF	Basic fibroblast growth factor
7.	BMI	Aody mass index
8.	CAS	Aarotid angioplasty with stenting
9.	CEA	Carotid endarterectomy
10.	CFU	Colony forming units
11.	CPC	Circulating progenitor cells
12.	CRP	C-reactive protein
13.	CT	Computed tomography
14.	EC	Endothelial cells
15.	ECM	Extracellular matrix
16.	EDTA	Ethylenediaminetetraacetic acid
17.	EMP	endothelial microparticles
18.	EPC	Endothelial progenitor cells
19.	EPCR	Endothelial protein C receptor
20.	Flt-1	Fms-like tyrosine kinase 1
21.	FXa	Coagulation factor Xa
22.	G-CSF	Granulocyte colony stimulating factor
23.	GM-CSF	Granulocyte-macrophage colony stimulating factor
24.	HDL-cholesterol	High density lipoprotein cholesterol
25.	hsCRP	High sensitivity assays
26.	IGF-1	Insulin-like growth factor-1
27.	IL-6	Interleukin 6
28.	IL-8	Interleukin 8
29.	ISHAGE	International Society of Hematotherapy and Graft Engineering
30.	LDL-cholesterol	low density lipoprotein cholesterol
31.	LECA	Left external carotid artery
32.	LICA	Left internal carotid artery
33.	Low CHO	Low carbohydrate
34.	MCP-1	Monocyte chemoattractant protein 1
35.	mKitL	Membrane-bound Kit ligand
36.	MMP	Matrix metalloproteinases
37.	MP	cell-derived microparticles
38.	MP	Cell-derived microparticles
39.	MRI	Magnetic resonance imaging
40.	NEFA	Non-esterified fatty acid
41.	OEC	Outgrowth endothelial cell
42.	PE	Phosphatidylethanolamine
43.	PET	Positron emission tomography
44.	PLA	Platelet leukocyte aggregates
45.	PMA	Platelet monocyte aggregates

46. PMP	Platelet microparticles
47. PPACK	D-Phenylalanyl-L-prolyl-L-arginine chloromethyl ketone
48. PS	Phosphatidylserine
49. PSGL-1	P-selectin glycoprotein ligand-1
50. RBC	Red blood cells
51. ROC	Receiver operator curve
52. SAA	Serum amyloid A
53. SDF-1	Stromal-derived factor-1
54. sE-selectin	Soluble E-selectin
55. sFlt-1	Soluble fms-like tyrosine kinase-1
56. sICAM-1	Soluble intercellular adhesion molecule 1
57. sKitL	Soluble Kit ligand
58. SM	Sphingomyelin
59. SMC	Smooth muscle cells
60. sP-selectin	Soluble P-selectin
61. sVCAM-1	Soluble vascular adhesion molecule 1
62. TF	Tissue factor
63. TFPI	Tissue factor pathway inhibitor
64. TGFβ1	Transforming growth factor beta 1
65. TIA	Transient ischemic attacks
66. Tie-2	Tyrosine-protein kinase receptor
67. TIMPs	Tissue inhibitors of metalloproteinases
68. TM	Thrombomodulin
69. TXA_2	Thromboxane A_2
70. VCAM-1	Vascular adhesion molecule 1
71. VEGF	Vascular endothelial growth factor
72. VEGFR-2	Vascular endothelial growth factor receptor
73. vWF	Von Willebrand facto

Chapter 1

Literature Review

1. Literature review

1.1 Anatomy of the Arterial Wall

The structure of the carotid artery is composed of three layers: the tunica intima, tunica media, and tunica adventitia (figure 1.1).

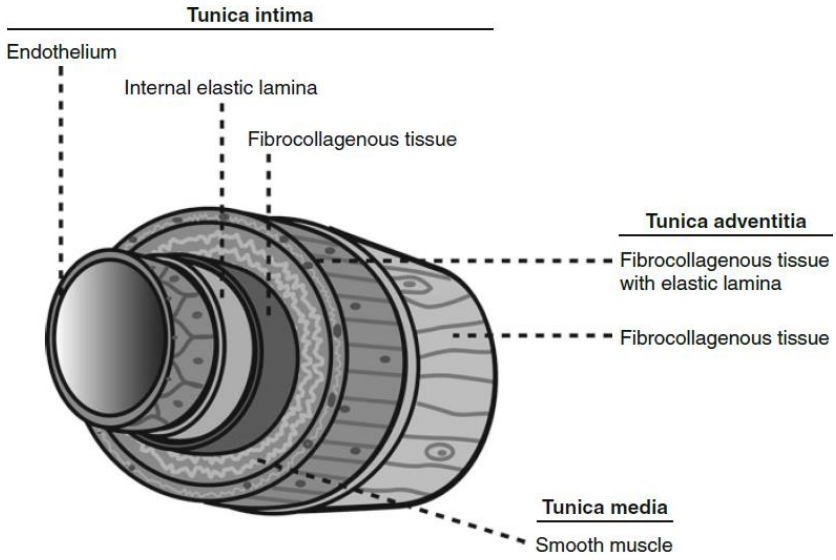


Figure 1.1: The mural structure of the carotid artery
The carotid artery is composed of three layers: the tunica intima, tunica media, and tunica adventitia
Hall and Bassiouny 2012

Each layer plays a specific and essential role in the overall function of the artery. The *tunica intima* is the inner layer composed of a monolayer of endothelial cells (Abate et al., 1993), elastic lamina and fibrocollagenous tissue. The EC are directly adjacent to blood flow and have surface receptors that interact with blood proteins and molecules to regulate vascular permeability as well as playing a key role in platelet aggregation and resistance to thrombosis. The *tunica media* is the middle layer composed of an inner circumferential layer and an outer longitudinal layer of smooth muscle cells (SMC) surrounded by a matrix of elastin, collagen, and proteoglycans (Clark and Glagov, 1985). It provides structure and maintains vascular tone.

Surrounding the *tunica media* is the *tunica adventitia* composed of an external elastic lamina and the fibrocollagenous tissue. These two layers are remarkably strong and composed mostly of collagen and autonomic nerve fibers that extend into *tunica media*.

1.2 Atherosclerosis

The term atherosclerosis comes from the Greek words *athero*, meaning gruel, and *sclerosis*, meaning hardening. Atherosclerosis was initially thought to be a bland proliferative process caused by accumulation of lipids within the artery (Ross and Glomset, 1976). However, it is now regarded as a complex multifactorial disease that develops in the arterial wall as a result of physical or metabolic injury disrupting endothelial integrity. The injurious stimuli include oxidative stress, shear stress and hyperlipidaemia (Ross, 1999). The disease results in excessive inflammatory and fibro-proliferative reactions within the arterial wall (figure 1.2) (Hansson et al., 2006, Mallika et al., 2007, Libby et al., 2009).

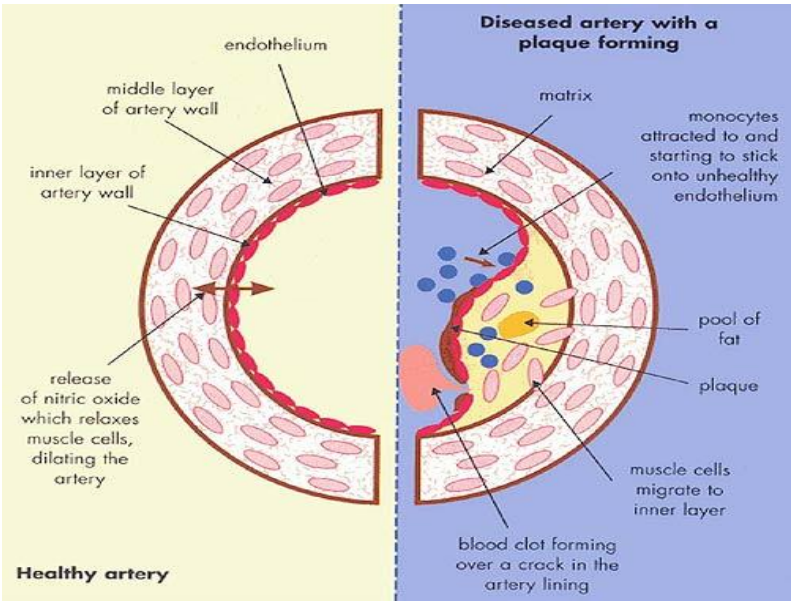


Figure 1.2: Contrast between a healthy and an atherosclerotic artery
The figure depicts the structural differences between a healthy and an atherosclerotic artery.
Retrieved from http://www.drugdevelopment-technology.com/projects/etc/etc2.html
Accessed 20th September 2012

Disruption of the endothelium alters the expression of cellular adhesion molecules and other surface receptors resulting in altered interaction between blood cells and endothelial cells (Hansson et al., 2006). The endothelial cells (EC) increase expression and secretion of chemokines and growth factors causing chemoattraction and adherence of leukocytes (monocyte/macrophages and T-cells) on to the endothelium. The leukocytes then adhere firmly onto the endothelium and transmigrate into the sub endothelial layer (figure 1.3) (Arici and Walls, 2001).

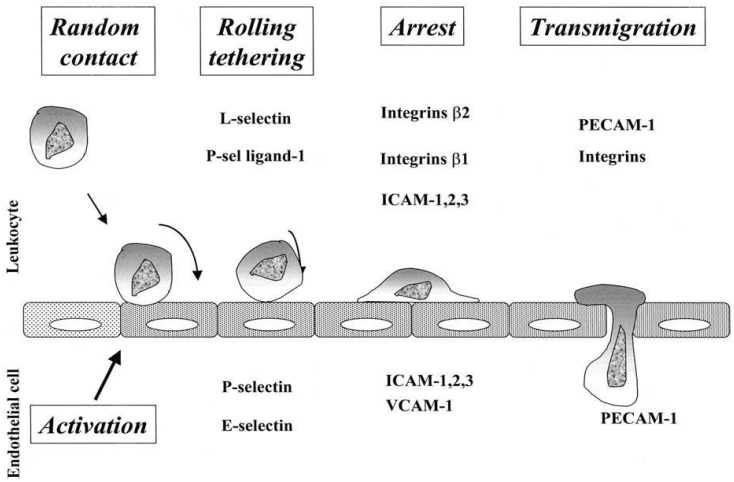


Figure 1.3: Interactions between leukocytes and endothelium
Mechanisms involved in the interaction between blood cells and endothelial cells is mediated by cellular adhesion molecules that mediate rolling, tethering, arrest and transmigration of leukocytes into the endothelium
(Brevetti et al., 2006)

Inside the sub endothelial layer leukocytes accumulate modified lipids becoming large foam cells. The foam cells and T-cells aggregate forming fatty streaks within the sub endothelial layer (Arici and Walls, 2001). With continued inflammation and proliferation smooth muscle cells (SMC), the fatty streaks progress to an intermediate lesion and ultimately to a fibrous plaque. A number of factors have been identified that contribute to the progression, form an early lesion to either a pathologic or symptomatic plaque. These factors include hemodynamic, metabolic, environmental, and genetic risk factors that perpetuate the inflammatory and fibro-proliferative processes (Hall and Bassiouny, 2012).

1.2.1 Interaction of platelets, endothelial cells and leukocytes in atherosclerosis

Interactions between platelets, EC and leukocytes occur when the endothelium is vascular damaged (figure 1.4). Injured EC down regulate antiplatelet prostaglandins and express molecules such as fibronectin, soluble intercellular adhesion molecule 1

(sICAM-1), endothelial P-selectin, E-selectin, integrin $\alpha_v\beta_3$, and Von Willebrand factor (vWF) which promote platelet adhesion and degranulation (Bombeli et al., 1998, Frenette et al., 1998). The activated platelets then interact with leukocytes via engagement of P-selectin expressed on platelets with P-selectin glycoprotein ligand-1 (PSGL-1) expressed on leukocytes forming platelet leukocyte aggregates (Michelson et al., 2001, Michelson et al., 1996, Huo et al., 2003). The expression of adhesion molecules initiates a cascade of atherogenic events. The interaction of platelets and leukocytes causes surface expression of procoagulant molecules such as tissue factor (TF) that bind coagulation factor Xa (FXa) and fibrinogen (Barnard et al., 2005). In addition to expression of procoagulant molecules, the platelet leukocyte interaction causes platelets to donate cholesterol to monocytes. This suggests that platelet adhesion may contribute to lipid macrophage differentiation (Mendelsohn and Loscalzo, 1988). After formation the aggregates localize to the injured endothelium (Huo et al., 2003), which may contribute to atherogenesis and plaque formation (Gawaz et al., 2005).

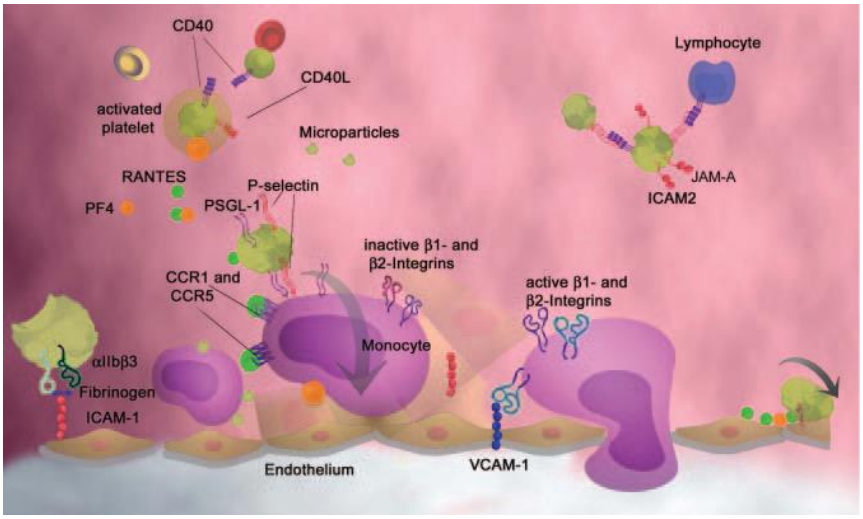


Figure 1.4: Interactions of platelets with different leukocyte subtypes that regulate vascular inflammation.
A multitude of molecular mechanisms and platelet derived components mediate and regulate platelet-induced leukocyte infiltration including chemokine deposition and mononuclear and endothelial cell activation
(von Hundelshausen and Weber, 2007).

1.2.2 Vascular endothelium in atherosclerosis

The vascular endothelium is strategically located between the blood and underlying arterial tissues and performs a variety of important physiological functions. It i) acts as a barrier between flowing blood and vascular wall, ii) regulates vascular growth, platelet function and coagulation, iii) modulates vascular tone, calibre and blood flow, iv) responds to and regulates numerous signal transduction, humoral, neural and mechanical stimuli, v) synthesizes and releases vasoactive substances (e.g. nitric acid), vi) regulates cholesterol and lipid homeostasis and vi) regulates immunity and inflammation (Mehta and Malik, 2006, Simionescu and Antohe, 2006, Sima et al., 2009a). Pathological conditions such as hyperlipidaemia may cause EC damage thereby altering the endothelial function. This leads to atherogenesis, progression and eventually plaque formation (Sima et al., 2009b).

1.2.3 Formation of atheromatous plaques

Following disruption of the endothelium, fatty streaks start forming within the human arterial walls. The fatty streaks develop to intermediate lesions and eventually to fibrous or thrombus plaques (figure 1.5).

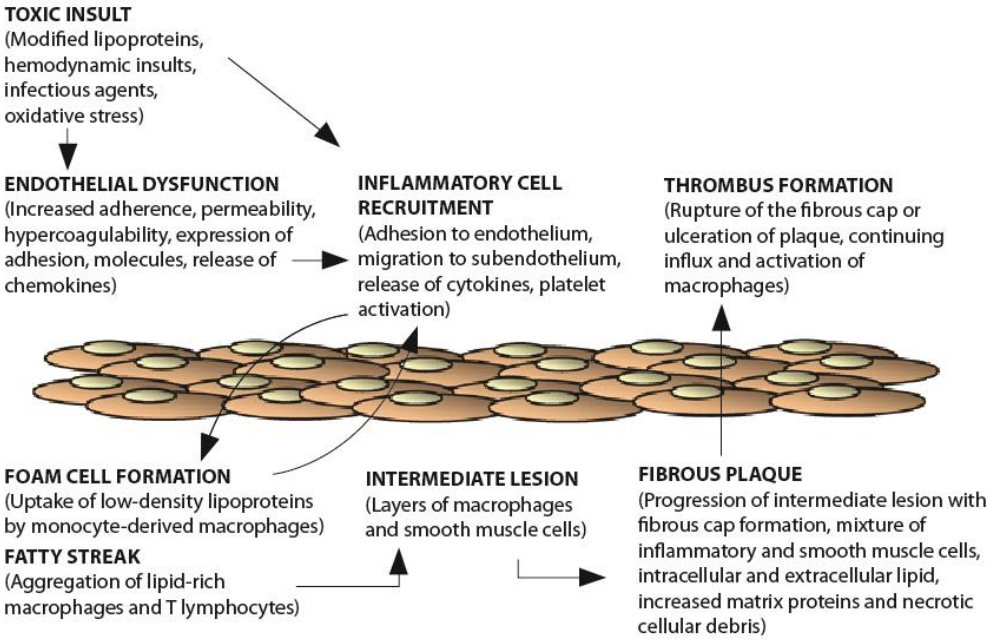


Figure 1.5: Development of atherosclerotic plaques according to "response-to-injury" hypothesis.
Toxic insult causes endothelium dysfunction which leads to recruitment of inflammatory cells into the endothelium. The inflammatory cells form foam cells, fatty streaks which develop to intermediate lesions and eventually to fibrous or thrombus plaques
(Arici and Walls, 2001)

The factors determining this progression include plaque composition, physical and biochemical stresses (Hall and Bassiouny, 2012). Formation of atheromatous plaques is as a result of complex cellular interactions between SMC and EC. These interactions include accumulation of extracellular lipids, macrophage and foam cell formation, neovascularisation and SMC structural adjustment. Covering the plaque is a fibrous cap consisting of connective tissues arising from SMC and extracellular matrix

(ECM) (Stary, 2000, Stary et al., 1995, Virmani et al., 2000). Formation of the fibrous cap is mediated by cytokines and growth factors produced by T-lymphocytes and activated macrophages. They stimulate proliferation of SMC and production of ECM (Halvorsen et al., 2008). The fibrous cap provides structural integrity and encloses the atheroma.

During early atherogenesis, the atheroma contains fatty streaks of sub endothelial foam cell aggregates and SMC proliferates. Advanced atheroma is predominantly composed of foamy macrophages and small amounts of T-lymphocytes, SMC, mast cells and platelets. Recruitment of these cells is a continuous process, from onset of atherosclerosis to associated plaque complications. The level of SMC proliferation determines the type of fibrous cap (table 1.1). Increased influx and proliferation produces thick stable caps whereas reduced influx and proliferation produces thin unstable caps. The strength of the fibrous cap depends on a dynamic balance between collagen synthesis and its degradation by proteolytic enzymes such as matrix metalloproteinases (MMPs) secreted by leukocytes within the intima (Welgus et al., 1990, Galis et al., 1994). When an equal balance is maintained between synthesis and degradation, plaques are stable and likewise when there is a shift towards increased synthesis and reduced breakdown. Reduced collagen synthesis and increased degradation leads to unstable plaques (Libby, 2008, Halvorsen et al., 2008).

Unstable plaques are also known as vulnerable plaques because they reflect a greater cap weakening and rupture. They contain a larger lipid pool representing a more active atherosclerotic process with significant amounts of T-lymphocytes and activated macrophages. They secrete TFs and proteolytic enzymes which mediate and

degrade collagen. The TFs prevent synthesis of interstitial collagen inhibiting proliferation; promoting apoptosis of SMC and atheroma cells. The enzymes break down the ECM components of the fibrous cap. These processes serve to weaken the cap and thus increase the risk of rupture (Halvorsen et al., 2008, Arroyo and Lee, 1999) resulting in thromboembolic events and cerebrovascular ischemia (Naghavi et al., 2003).

Table 1.1: Characteristics of stable and unstable plaques

	Stable	**Unstable**
Fibrous cap	Solid, thick, dense	Thin or virtual absent
Extracellular lipid content	Small or no lipid pool	Large lipid pool
Influx and proliferation of SMC	Decreased	Increased
Effect on arterial lumen diameter	Narrows	Narrows then becomes compensated as plaque grows
MMP expression & activity	Reduced	Increased
Inflammatory cytokine expression & activity	Reduced	Increased
Apoptotic cell death	Reduced	Increased
Necrotic core	Reduced	Prominent
Plaque disruption	Not prone	Prone
Inflammatory cells	Small burden of inflammatory cells, T-lymphocytes and activated macrophages	Greater burden of inflammatory cells, T-lymphocytes and activated macrophages
Inflammatory cells	Small burden of inflammatory cells, T-lymphocytes and activated macrophages	Greater burden of inflammatory cells, T-lymphocytes and activated macrophages

SMC = smooth muscle cells; MMP = matrix metalloproteinase

1.3 Carotid Artery Disease

The right and left common carotid arteries supply oxygenated blood to the brain, eyes, throat, neck glands, tongue, face, mouth and ears. The right common carotid artery branches from the brachiocephalic artery whereas the left common carotid artery branches from the aorta. The common carotids branch into internal and external carotid arteries (figure 1.6).

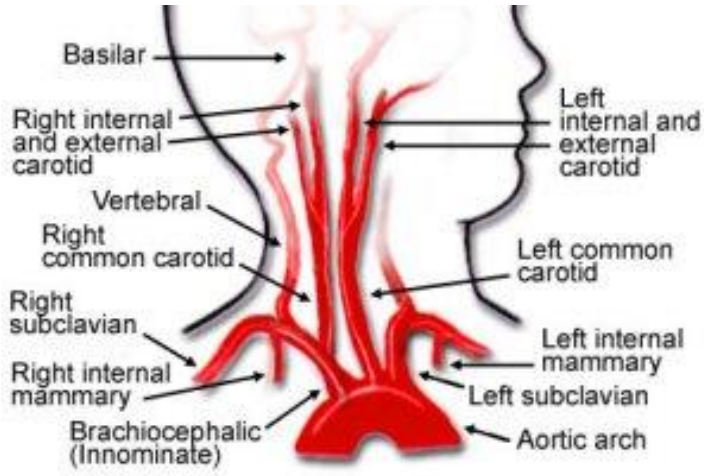


Figure 1.6: The carotid and related arteries.
The right and left common arteries originate from the brachiocephalic and aorta respectively. They branch into branch into internal and external carotid arteries supplying oxygenated blood to the brain, eyes, throat, neck glands, tongue, face, mouth and ears.
Retrieved from
http://www.texasheartinstitute.org/HIC/Topics/Cond/CarotidArteryDisease.cfm
Accessed 12th June 2010

Carotid artery disease is a disease in which plaque builds up in the carotid arteries. It is a common manifestation of generalized atherosclerosis and is closely associated with an increased risk of stroke and myocardial infarction (Hollander et al., 2002). The disease remains one of the leading causes of cerebral ischemic events (Kadoglou et al., 2008, O'Leary et al., 1999). At least 20 % of cerebral ischemic strokes

are attributed to carotid bifurcation disease (Chaturvedi et al., 2005, Donnan et al., 2008, Timsit et al., 1992). Hyperlipidemia, hypertension, hyperglycemia, hyperhomocysteinemia, smoking, male gender and family history of atherosclerosis are some of the risk factors implicated in its pathogenesis.

Carotid artery disease usually develops where the common carotid arteries split into internal and external carotid arteries (figure 1.7A). The plaque encroaches on the lumen of the internal carotid artery extending in to the common carotid artery. As the plaque develops, an hourglass configuration to the stenosis forms within the lumen (figure 1.7C) (Wilterdink and Easton, 1992).

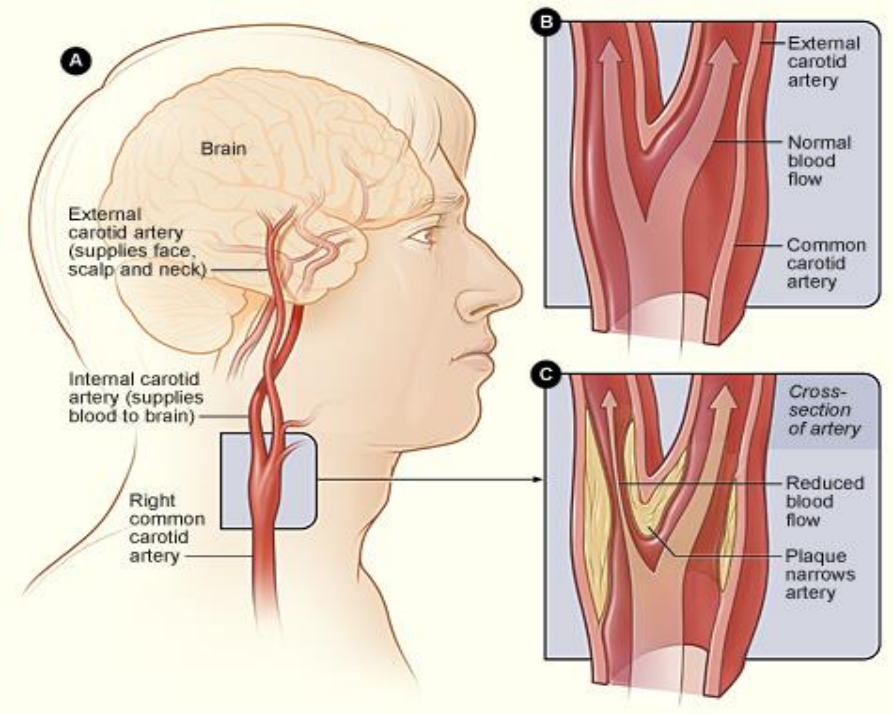


Figure 1.7: Right common carotid artery
A: bifurcation of right common carotid artery into external and internal common carotid arteries. B: cross section of a normal common carotid artery. C: plaque-encroached common carotid artery.
(Wilterdink and Easton, 1992)

The fact that bifurcation of the common carotid arteries is a region of flow division and low shear stress (figure 1.7B) suggests that fluid dynamics and vessel geometry play a key role in the inception of atherosclerotic plaque (figure 1.8) (Friedman et al., 1981, Ku et al., 1985, Zarins et al., 1983).

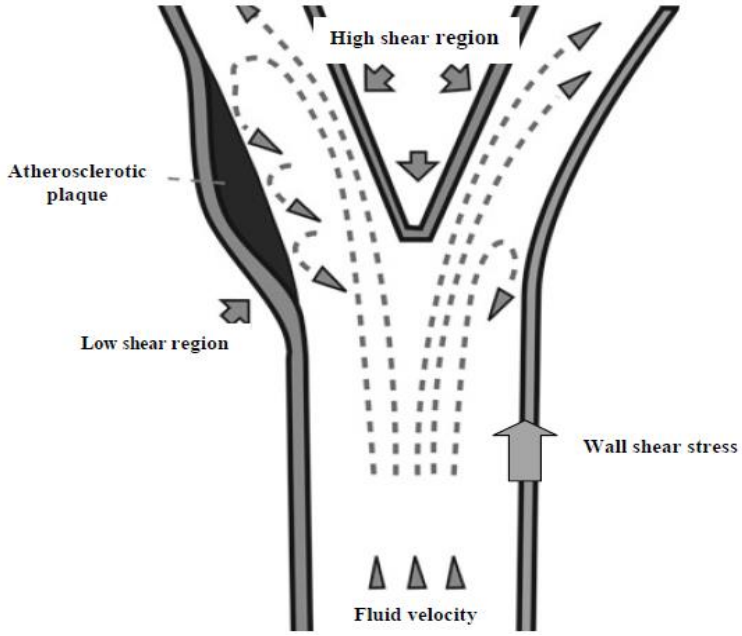


Figure 1.8: Wall shear stress with boundary layer separation
Blood flow near the centre of the artery is laminar. Blood flow near the intima, referred to as the boundary layer, is slower and has more disturbed currents. This is referred to as boundary layer separation. The areas of lower shear force (< 4 dyn/cm^2) have been found to induce endothelial injury and are, as demonstrated at the carotid bifurcation, typically found on the outer walls at arterial branch points
(Adapted from Hall and Bassiouny 2012)

Advanced cases of stenosis are characterized by a reduction in vessel diameter (stenosis). Stenosis is caused by accumulation of plaque in the lumen pushing the plaque into the lumen as it grows. If the plaque ruptures, thrombus can become superimposed on the atheroma further increasing stenosis. Rupture of the plaque also releases thrombus into circulation which may embolise. Stenosis with inadequate collateral compensation and embolism of the thrombotic material may manifest as transient ischemic attacks (TIA) or stroke. Some of the symptoms of TIA or

stroke include blurred vision, confusion, loss of memory, loss of sensation, problems with speech and language, vision loss and weakness in one part of the body (Kistler et al., 1984).

The type and location of plaque determines whether a patient experiences warning symptoms or not. TIA is often the first symptom of carotid atherosclerosis. In TIA, these warning symptoms resolve on their own within minutes of onset, leaving no residual effects. In contrast, strokes which may result in permanent damage.

1.3.1 Evaluating carotid artery disease

Carotid artery disease can be evaluated by several methods. Blood tests to determine cholesterol, triglycerides and glucose levels and a physical examination involving auscultation of the neck for bruits are routine undertaken. The carotid arteries may also be imaged to examine the internal morphology and blood flow patterns. Imaging provides information on the degree of stenosis and plaque parameters such as total plaque area, number of plaques and plaque structural morphology. In addition, carotid imaging allows for early detection, clinical staging, surgical road mapping, and postoperative therapeutic surveillance. Four of the most common methods of imaging the carotid artery are angiography, computed tomography angiography, magnetic resonance angiography and duplex ultrasonography.

1.3.1.1 Duplex ultrasonography

This method incorporates two elements, grayscale or B-mode ultrasound to visualize the structure or architecture of the arterial wall, and colour Doppler ultrasound to visualize the flow or movement of blood within the artery. It examines the carotid plaque and measures flow velocity/degree of stenosis (figure 1.9) on the basis of the waveform and spectral analysis of the common carotid artery and its major branches, especially the internal carotid artery.

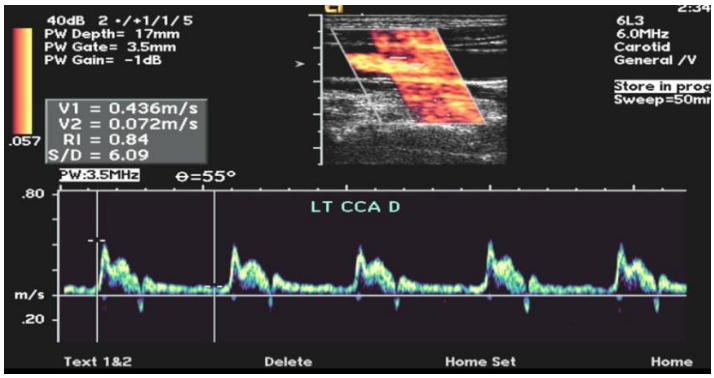


Figure 1.9: Duplex carotid sonography image
The image depicts carotid artery stenosis of the common carotid artery (upper right image) and the waveform and spectral analysis of the same common carotid artery (bottom image)
Retrieved from http://emedicine.medscape.com/article/417524-overview
Accessed 9th December 2012

1.3.1.2 Computed tomography

This method uses x-rays to make detailed pictures of the arterial structure providing an accurate means of assessing stenosis and carotid plaque. It generates a three-dimensional image (figure 1.10) of the inside of the artery from a large series of two-dimensional X-ray images taken around a single axis of rotation. In addition to estimating the degree of stenosis, computed tomography can depict carotid ulcers and thrombosis, and detect carotid plaques.

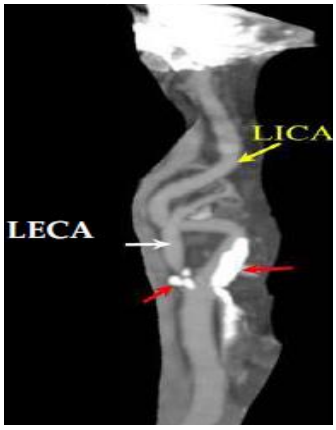


Figure 1.10: Computed tomography angiography image
A high-grade stenosis of the origin of the LECA (white arrow) and dense calcifications (red arrows) near the origins of both the LECA and the carotid bulb. The LICA is not significantly stenotic (yellow arrow). LECA = left external carotid artery, LICA = left internal carotid artery.
Retrieved from http://emedicine.medscape.com/article/417524-overview
Accessed 9th December 2012

1.3.1.3 Magnetic resonance angiography

This method uses powerful magnets and radio waves for detecting structural abnormalities of the carotid artery (figure 1.11). It evaluates the artery for stenosis or aneurysms (vessel wall dilatations, at risk of rupture).

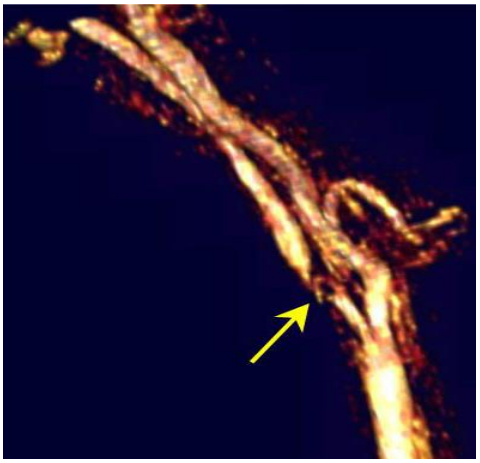


Figure 1.11: Carotid magnetic resonance angiogram image
Image demonstrating very high-grade stenosis (yellow arrow) in the proximal internal carotid artery in a patient with a recent onset of stroke
Retrieved from http://emedicine.medscape.com/article/417524-overview. Accessed 9th December 2012.

3.2 Treatment options for carotid artery disease

Carotid artery disease can be treated using non-invasive and invasive treatments. Non-invasive options include use of blood thinner medication - aspirin, clopidogrel (Plavix) and warfarin (Coumadin), HMG CoA reductase inhibitors - altoprev (lovastatin), crestor (rosuvastatin), lescol (fluvastatin) and lipitor (atorvastatin), and anti-hypertensive medication - acetazolamide (diamox), bisoprolol (zebeta) and amlodipine (norvasc). Medications are normally taken in combination with a reduced dietary intake of saturated fat and salt, smoking cessation and increased levels of physical activity. Carotid endarterectomy (CEA) and carotid angioplasty with stenting (CAS) are the most commonly used invasive procedure to treat carotid artery disease. CEA involves surgical removal of the plaque from the artery whereas CAS involves repairing the blockage in the carotid artery by inserting a balloon-tipped catheter and/or stent.

1.4 Biomarkers

A biomarker is defined as as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention” (NIH, 2001). In general, biomarkers are cells, molecules, hormones, enzymes, or other proteins that can be measured in the tissues and body fluids such as blood and urine. These systemic measurements may provide vital information reflecting health status, disease risk and disease progression (Paramo et al., 2007).

1.4.1 Measures of biomarker test performance

For any biomarker to be utilised clinically, it should have the ability to identify high-risk patients, be accurate and reliable, provide good sensitivity, specificity and predictive value and add predictive value to traditional biomarkers (Paramo et al., 2007). Sensitivity is the ability of a test to detect disease, when it is truly present, specificity is the ability of a test to exclude the disease in patients who do not have disease whereas predictive value is the probability that the test will give the correct diagnosis. These and other commonly used test indicators in diagnostic research are summarised in table 1.2.

Table 1.2: Commonly used test indicators in diagnostic research

Test indicator	**Formula**	**Definition**
Sensitivity (true positive rate, TPR)	TP/(TP + FN)	Proportion positive test results among diseased
Specificity (true negative rate, TNR)	TN/(TN + FP)	Proportion negative test results among the "healthy"
Positive predictive value (PPV)	TP/(TP + FP)	Proportion diseased among subjects with a positive test result
Negative predictive value (NPV)	TN/(TN + FN)	Proportion non diseased among subjects with a negative test result
Likelihood ratio of a positive test result (LR+)	sensitivity/(1-specificity)	Ratio of a positive test result among diseased to the same result in the "healthy"
Likelihood ratio of a negative test result (LR-)	(1-sensitivity)/specificity	Ratio of a negative test result among diseased to the same result in the "healthy"
Accuracy	(TP + TN)/(TP + TN + FP + FN)	Proportion correctly identified subjects
Youden's index	sensitivity +specificity-1	

TP=true positives, FP =false positives, FN=false negatives, and TN=true negatives.
(Glas et al., 2003)

1.4.2 Vascular biomarkers

Despite great progress in the treatment of vascular diseases, they remain the leading cause of death in developed countries (Gerszten and Wang, 2008). Current medical knowledge is unable to effectively predict those at risk of vascular diseases (Martin-Ventura et al., 2009). Risk stratification is normally based on the measurement of biomarkers and non-invasive evaluation of vascular structure or function. These parameters have proven to be efficient tools of assessing vascular structure or function (Ambrose and Srikanth, 2010) but lack the appropriate criteria of sensitivity, specificity, cost-effectiveness and intra-observer reproducibility (Hlatky et al., 2009). In addition, many serum biomarkers in cardiovascular disease have been studied in coronary artery disease and very few in patients with carotid artery disease. Consequently new biomarkers are needed to supplement the information obtained from traditional biomarkers (Gerszten and Wang, 2008) and improve predictive capabilities (Tardif et al., 2006). This has led to increased quest for new candidates.

Atherosclerotic cardiovascular disease (ACVD) is a multifactorial disease that involves significant alteration in the structure and function of the vascular endothelium. Atherogenic mediators reflecting alterations of the main regulatory functions of the endothelium can be evaluated as biomarkers. They include circulating platelets, EC, cellular aggregates and circulating progenitor cells (CPC) involved in either endothelial damage or regeneration. Platelet microparticles (PMP), endothelial microparticles (EMP), platelet monocyte aggregates (PMA) and CPC are emerging cellular biomarkers that can be used to non-invasively evaluate vascular integrity and

risk. As such, they may be considered as integrative biomarkers of vascular competence (Sabatier et al., 2009). These novel biomarkers might add to the predictive value of the traditional risk markers such as the atherogenic lipoprotein phenotype, inflammatory mediators and glycemic parameters to improve assessment of vascular diseases such as carotid artery disease (Koenig, 2010).

1.4.3 Carotid artery disease biomarkers

Evaluation of carotid artery disease using vascular imaging techniques allows investigators to collect information on vascular anatomy, carotid stenosis and atherosclerotic plaque morphology. The value of various imaging techniques are summarised in table 1.3.

These vascular imaging techniques provide useful diagnostic information. Nonetheless, no imaging modality at present has been shown to improve the identification of patients at highest risk of neurologic events beyond the severity of stenosis (Sharma et al., 2009). At present, it is impossible to assess whether a carotid plaque will become symptomatic or to predict when symptoms will occur (Hermus et al., 2010).

Advances in cellular and molecular pathophysiology and the demand to accurately identify carotid plaques which confer excess risk of neurologic events and choose the optimal interventional strategy have stimulated great interest in the development of novel diagnostic markers (Avgerinos et al., 2011). The necessity to expand and improve the diagnostic field is also supported by the fact that >50 % of patients with cardiovascular disease lack any of the conventional risk factors (cigarette

smoking, diabetes, hyperlipidaemia, and hypertension) implying that other factors play a significant role in the development of the disease (Futterman and Lemberg, 1998). In the recent past, serum, plasma and cellular biomarkers have emerged as potential predictors of carotid artery disease natural history, interventional outcome, perioperative risks and as therapeutic targets individually or in combination (Avgerinos et al., 2011, Hermus et al., 2010).

Table 1.3: Information collected vascular imaging techniques of carotid artery disease

Vascular imaging technique	**Information collected**
Ultrasound	Degree of stenosis Flow velocity Grading of echolucency/echogenicity Surface irregularity Grayscale median
CT or MRI	Degree of stenosis Thin fibrous cap Lipid core Carotid ulceration Intraplaque haemorrhage, Thrombosis
Angiography	Degree of stenosis Calcification Fibromuscular hyperplasia Carotid kinking or folding Focal thrombus formation Intimal dissection
Intravascular ultrasound and PET	Plaque vulnerability

CT; computed tomography, MRI; magnetic resonance imaging, PET; positron emission tomography.
Avgerinos et al., 2011, Liapis et al., 2009, Hermus et al., 2010b

1.4.3.1 The multiple biomarker strategy

As our understanding of the biology of atherothrombosis improves (Ramsey et al., 2010, Libby et al., 2010), several candidate vascular biomarkers reflecting inflammation, apoptosis, proteolysis, thrombosis and angiogenesis are being evaluated

as potential clinical tools for improving risk prediction (Koenig and Khuseyinova, 2007). There have been several recent attempts to identify a panel of vascular biomarkers with a high predictive value. Several cellular and protein molecules continue to be evaluated both as single markers, and in multi-marker approaches. These biomarkers represent various cellular derivatives relevant to the pathophysiology of atherothrombosis such as markers of the inflammatory response and include C-reactive protein (CRP) and serum amyloid A (SAA), lipid-related parameters and proteolytic markers. In addition, there are biomarkers representing circulating cells such as markers of angiogenesis (CPC) and activation (PMA); and cell-derived vesicles such as markers of apoptosis or activation (PMP and EMP). Besides providing information about cellular activation, endothelial damage and regeneration, these cellular biomarkers may aid in improved cardiovascular risk prediction and outcome. Consequently, multimarker approaches may be the appropriate way to define the most suitable panels of cardiovascular biomarkers for risk assessment. The use of multiple biomarkers (table 1.4) has recently been shown to improve risk stratification for cardiovascular events in high risk individuals (Nozaki et al., 2009, Pelisek et al., 2009, Wang et al., 2006, Zethelius et al., 2008, Ikonomidis et al., 2008, Georgescu et al., 2012, Burger and Touyz, 2012).

Table 1.4: Studies of cardiovascular disease that have employed a multimarker approach in identifying or classifying disease

Reference	Vascular markers	Cardiovascular condition	Results
(Nozaki et al., 2009)	EMP BNP hsCRP	Coronary heart disease	The C-statistic increased when each biomarker or combinations of biomarkers were combined with the Framingham risk model
(Pelisek et al., 2009)	MMP 1 MMP 7 TIMP 1 IL-8	Carotid artery disease	The combination of MMP-1, MMP-7, TIMP-1 and IL-8 gave the highest positive and negative predictive values
(Heider et al., 2009)	MMP 7 MMP 8 MMP 9	Carotid artery disease	The combination of more than one biomarker increased the positive predictive value for neurological symptoms

BNP; brain natriuretic peptide, EMP; endothelial microparticles, hsCRP; high sensitivity C-reactive protein, IL-8; interleukin 8, MMP; matrix metalloproteinases, TIMP 1; Tissue inhibitor of metalloproteinases-1

1.4.4 Protein vascular biomarkers

Circulating molecules such as total cholesterol, low density lipoprotein cholesterol (LDL-cholesterol), high density lipoprotein cholesterol (HDL-cholesterol), non-esterified fatty acid (NEFA), triglycerides, apolipoprotein A1 (APOA1) and apolipoproteins B (APOB) levels have for a long time been used to assess cardiovascular risk. However, a considerable number of at risk patients cannot be identified on the basis of these risk factors alone (Khot et al., 2003). There is need to identify novel circulating/soluble vascular biomarkers to identify at risk individuals that cannot be identified using these traditional risk factors. There is growing interest in novel protein biomarkers such as inflammatory, proteolytic and angiogenic proteins. These protein biomarkers are being investigated to determine their potential to assess cardiovascular risk and predict cardiovascular diseases. Several studies have compared these protein biomarkers between atherosclerotic patients and controls (tables 1.5 and 1.6). Such protein biomarkers include;

i. Inflammatory proteins, C-reactive protein and serum amyloid A
ii. Matrix metalloproteinases
iii. Soluble cellular adhesion molecules
iv. Soluble thrombomodulin
v. Growth factors, vascular endothelial growth factor and basic fibroblast growth factor and their receptors, soluble fms-like tyrosine kinase 1 and tyrosine kinase-2

1.4.4.1 Inflammatory proteins, C-reactive protein and Serum Amyloid A

C-reactive protein is a pentameric protein primarily synthesised by hepatocytes. It is an acute-phase response protein that can increase up to a 10,000-fold due to de novo hepatic synthesis during an infection, sepsis, tissue necrosis, trauma, cancer or various inflammatory diseases (Hirschfield and Pepys, 2003). The hepatic synthesis of CRP is under the control of a cascade of cytokines, including interleukins-1 (IL-1), -6 (IL-6), and tumour necrosis factor-α (TNF-α), originating at the site of pathology (Hirschfield and Pepys, 2003, Gabay and Kushner, 1999). Conventional CRP assays are used to measure high CRP levels expressed in acute infection or inflammation (~10-20 mg/L). However, high sensitivity assays (hsCRP) are used to measure lower CRP levels (0.3 mg/L) implicated in increased cardiovascular risk (de Ferranti and Rifai, 2007).

Increasing evidence suggests that CRP may serve as a proatherogenic factor. Previous studies have shown elevated levels of CRP in patients with atherosclerosis compared to healthy controls (Zeller et al., 2005, Zhou et al., 2009, Rerkasem et al., 2002, Johnson et al., 2004), and indeed higher levels have been reported in individuals with more advanced atherosclerotic disease (Momiyama et al., 2009, Liuzzo et al., 1994, Alvarez Garcia et al., 2003).

Serum Amyloid A is another acute phase protein produced by the liver in response to both acute and chronic inflammatory stimuli (Malle et al., 1993). Release of SAA is stimulated by immune system modulators such as interleukin-1, interleukin-6 and tumour necrosis factor. Although the function of SAA is unclear, evidence suggests

that it is involved in atherosclerosis (Badolato et al., 1994, Meek et al., 1994, Liao et al., 1994).

1.4.4.2 Matrix metalloproteinases

Matrix metalloproteinases belong to a family of multidomain zinc-dependent endopeptidases that promote degradation of all protein and proteoglycan-core-protein components of the extracellular matrix (Galis and Khatri, 2002). Their activity is regulated mainly by tissue inhibitors of metalloproteinases (TIMPs). MMPs are widely expressed in ells involved in atherosclerosis including monocytes/macrophages, EC and SMC (Jones et al., 2003). Although undetectable in physiological circumstances, they are prominently expressed during biological processes such as angiogenesis (Vu et al., 1998, Lozonschi et al., 1999, Guedez et al., 2001, Hajitou et al., 2001, Chan et al., 1998), and vascular remodelling (Lijnen et al., 1999).

Matrix metalloproteinases increase migration and proliferation of vascular SMC surrounding atherosclerotic plaques (Ravn and Falk, 1999). They also promote plaque instability by degrading the ECM in the fibrous cap (Ardans et al., 2001, Noji et al., 2001, Loftus et al., 2000). These suggest that MMP are proatherogenic. Indeed, elevated levels of MMP have been found in patients with unstable atherosclerosis (Sapienza et al., 2005) and advanced atherosclerotic disease (Momiyama et al., 2009).

1.4.4.3 Soluble cellular adhesion molecules

Cellular adhesion molecules are molecules expressed on cells including endothelial cells, leukocytes and platelets that mediate the adhesion of the cell to

other cells or to the ECM. They are composed of three superfamilies: selectins, immunoglobulins and integrin's. The adhesion molecules ICAM-1, ICAM-3 or CD50, and vascular adhesion molecule 1 (VCAM-1) are members of the large immunoglobulin superfamily whereas endothelial-leukocyte adhesion molecule-1 (E-selectin or CD62E) and platelet activation-dependent granule to external membrane protein (P-selectin or CD62P) are members of the selectin superfamily. Adhesion molecules are expressed by vascular cells including leukocytes (ICAM-1, ICAM-3), EC (ICAM-1, VCAM-1, E-selectin, P-selectin), macrophages (VCAM-1) and platelets (P-selectin) (Albelda et al., 1994, Emara et al., 2012, Simmons et al., 1992).

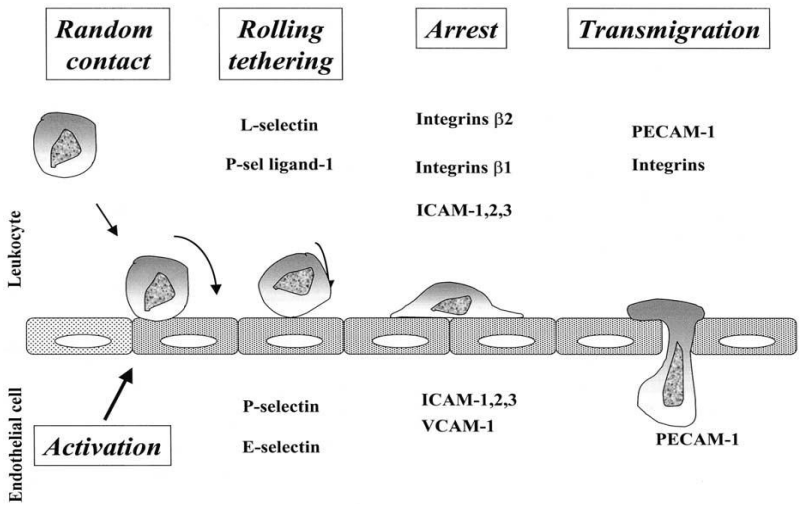


Figure 1.12: Role of adhesion molecules in atherogenesis
P-selectin and E-selectin mediate rolling and tethering of leukocytes on the endothelial wall, intercellular adhesion molecules 1 & 3 and vascular adhesion molecule 1 induce firm adhesion of the leukocytes on to endothelial surface whereas platelet endothelial cell adhesion molecule 1 mediate transmigration into the arterial wall
(Brevetti et al., 2006)

Cellular adhesion molecules have a critical role in atherogenesis as they mediate the interaction of leukocytes with endothelial cells, resulting ultimately in the transmigration of inflammatory cells into the arterial intima (figure 1.12). The selectins (P-selectin and E-selectin) mediate rolling and tethering of leukocytes on the

endothelial wall whereas ICAM-1, ICAM-3 and VCAM-1 induce firm adhesion of the leukocytes on to endothelial surface.

Elevated soluble forms of adhesion molecules are detectable in plasma (Blankenberg et al., 2003) of patients with carotid and coronary atherosclerosis (Hwang et al., 1997, Shyu et al., 1997, Mocco et al., 2001, Debing et al., 2008, Guray et al., 2004, Lu et al., 2010, Frijns et al., 1997).

1.4.4.4 Soluble thrombomodulin

Thrombomodulin (TM) is a cell surface-expressed glycoprotein which is predominantly synthesized and expressed by EC.

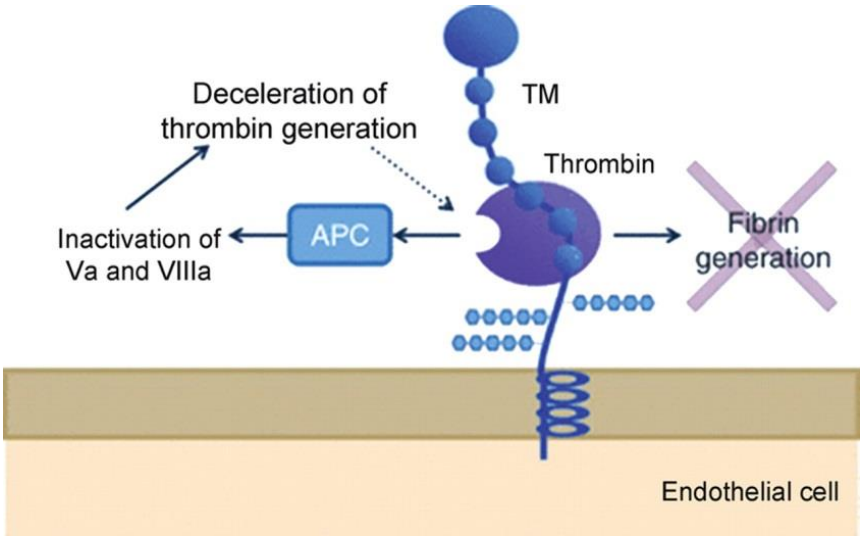


Figure 1.13: The role of thrombomodulin in blood coagulation
Thrombin attaches to thrombomodulin forming a thrombin-thrombomodulin complex which inhibits fibrin generation. Thrombomodulin also mediates protein S activation which inactivates factors Va and VIIIa decelerating thrombin generation.
Retrieved from www.neurology.org
Accessed 09th August 2013

It is an important anticoagulant protein which acts as a thrombin receptor on EC and a critical cofactor for thrombin-mediated activation of protein C. It reduces

blood coagulation by converting thrombin from a procoagulant to an anticoagulant enzyme. Thrombin attaches to TM forming a thrombin-TM complex which inhibits fibrin formation, platelet activation (Esmon et al., 1982) and thrombin mediated protein S activation (Mitchell et al., 1986) (figure 1.13). Thus, the thrombin-TM pathway mediates a major physiological antithrombotic mechanism in EC making TM an important regulator involved in maintaining the fluidity of circulating blood (Tohda et al., 1998).

Thrombomodulin is usually attached to EC as membrane bound TM. However, it also exists in plasma as soluble thrombomodulin (sTM) (Ishii and Majerus, 1985), likely produced by proteolytic cleavage from cellular TM after EC damage (Ishii et al., 1991, Ishii et al., 1990). There is contradicting evidence of the role of TM in atherosclerosis. Only a few studies suggest that increased TM expression inhibits atherosclerosis (Salomaa et al., 1999, Wu et al., 2003). However, majority of the previous studies suggest that increased levels of circulating TM promote atherosclerosis (Blann et al., 1997a, Blann et al., 1996, Seigneur et al., 1993).

1.4.4.5 Growth factors, vascular endothelial growth factor and basic fibroblast growth factor

Vascular endothelial growth factor (VEGF) is a glycoprotein secreted in the vascular wall by EC and SMC. As a sub-family of growth factors VEGF are important signalling proteins involved in both vasculogenesis and angiogenesis (Fong et al., 1995, Shalaby et al., 1995). VEGF interacts with the endothelium via a high affinity membrane-spanning receptor, Flt-1, promoting monocyte activation and migration,

and vascular SMC migration (Barleon et al., 1996, Clauss et al., 1990, Grosskreutz et al., 1999), and endothelial progenitor cells (EPC) mobilisation from bone marrow (Lyden et al., 2001, Hattori et al., 2001). Members of the VEGF sub-family include VEGF A, B, C and D, and placenta growth factor (PlGF).

There is conflicting evidence regarding the role of VEGF in atherosclerosis. VEGF may promote atherogenesis by inducing neointimal angiogenesis and intimal hyperplasia, and recruiting/activating monocytes (Yonemitsu et al., 1996, Lazarous et al., 1996, Inoue et al., 1998, Zhao et al., 2002). Elevated levels of VEGF have been found in atherosclerotic patients compared to healthy controls (Blann et al., 2001, Blann et al., 2002, Hojo et al., 2000, Lee et al., 2001). VEGF may also be anti-atherogenic. It is released following vascular injury, accelerating re-endothelialisation of the injured vessels thereby maintaining endothelial integrity (Van Belle et al., 1997, Asahara et al., 1995).

Basic fibroblast growth factor (bFGF) is a pluripotent angiogenic growth factor (Miao et al., 1996, Givol and Yayon, 1992) that exerts a number of trophic effects on vascular target cells such as SMC and EC (Ferrara et al., 1992, Klagsbrun, 1989). It can stimulate processes that are characteristic of angiogenesis including EC migration, invasion, and production of plasminogen activator (Montesano et al., 1986). Basic FGF has the potential to accelerate atherosclerosis by promoting SMC proliferation or stimulating angiogenesis following vascular injury (Edelman et al., 1992, Unger et al., 1994, Lindner et al., 1991, Nabel et al., 1993).

1.4.4.6 Soluble fms-like tyrosine kinase 1 and Tyrosine kinase-2

Fms-like tyrosine kinase 1 (Flt-1) and tyrosine kinase-2 (Tie-2) are endothelial receptors for the angiogenic growth factors, Flt-1 for the VEGF family (Shibuya, 2006) and Tie-2 for angiopoietin 1 and 2 (Teichert-Kuliszewska et al., 2001), respectively. Flt-1 is highly expressed in vascular EC whereas Tie-2 is expressed almost exclusively on the endothelium of actively growing blood vessels in both the embryonic and the adult vasculature (Dumont et al., 1992, Wong et al., 1997). Both receptors have a role in angiogenesis. Flt-1 is involved in the recruitment of haematopoietic precursors, release of growth factors from the liver and in the mediation of monocyte and macrophage migration (Olsson et al., 2006, Kowanetz and Ferrara, 2006). Tie-2, interacts with its ligands promoting angiogenesis by regulating EC survival and migration (Sato et al., 1995) and blood vessel maturation and maintenance (Schlaeger et al., 1997, Motoike et al., 2000, Dumont et al., 1994). Reduced plasma levels of Flt-1 have been reported in patients with peripheral arterial disease compared with the age- and sex-matched healthy controls (Blann et al., 2002).

Table 1.5: Comparison of circulating vascular biomarkers between atherosclerotic patients and controls

Reference	Condition	Cases	Healthy controls	Vascular markers	Findings
(Debing et al., 2008)	Carotid artery disease	180	180	hsCRP sVCAM-1 sICAM-1 HDL-C Glucose	Higher hsCRP, sVCAM-1, sICAM-1, TG, glucose in cases than controls Lower TC and HDL-C in cases than controls
(Rerkasem et al., 2002)	Carotid artery disease	137	12	hsCRP	Higher hsCRP in cases than controls
(Shyu et al., 1997)	Acute ischaemic stroke	51	25	sICAM-1	Higher sICAM-1 in cases than controls
(Taurino et al., 2008)	Carotid artery disease	15	8	MMP-9	Higher MMP-9, MMP-2 in cases than controls
(Sapienza et al., 2005)	Carotid artery disease	53	15	MMP-1 MMP-2 MMP-3 MMP-9	Higher MMP-1, MMP-2, MMP-3, MMP-9 in cases than controls
(Mocco et al., 2001)	Carotid artery disease	54	5	sICAM-1	Higher sICAM-1 in cases than controls
(Frijns et al., 1997)	Carotid artery disease	62	34	sP-selectin sE-selectin	Higher sP-selectin, sE-selectin in cases than controls
(Blann et al., 1997b)	Carotid artery disease	55	55	sP-selectin TM	Highers sP-selectin in cases than controls
(Zeller et al., 2005)	Cerebrovascular Disease	58	58	CRP	Higher CRP in cases than controls

Table 1.5 continued

Reference	Condition	Cases	Healthy controls	Vascular markers	Findings
(Zhou et al., 2009)	Cerebrovascular disease	136	67	hsCRP Glucose TG TC HDL-C LDL-C	Higher hsCRP, glucose, TG, TC, LDL-C in cases than controls Lower HDL-C in cases than controls
(Hwang et al., 1997)	CHD Carotid artery diseases	476	316	sICAM-1	Higher levels of sICAM-1 in cases
(Momiyama et al., 2009)	Coronary artery disease	220	45	hsCRP MMP-8 TC HDL-C	Higher hsCRP and MMP-8 in cases than controls Lower TC and HDL-C in cases than controls
(Fyfe et al., 1997)	Coronary artery disease	23	12	SAA	Higher SAA in cases than controls
(Noji et al., 2001)	Coronary atherosclerosis	53	133	MMP-9 MMP-2 TC TG HDL-C LDL-C	Higher MMP-9, TC, TG, LDL-C in cases than controls Lower MMP-2, MMP-3 and HDL-C in cases than controls
(Guray et al., 2004)	Coronary atherosclerosis	122	29	sVCAM-1 sICAM-1 sE-selectin sP-selectin	Higher sVCAM-1, sICAM-1, sE-selectin, sP-selectin in cases than controls

Table 1.5 continued

Reference	Condition	Cases	Healthy controls	Vascular markers	Findings
(Lu et al., 2010)	Coronary atherosclerosis	128	31	sVCAM-1 sICAM-1 sE-selectin sP-selectin	Higher sVCAM-1, sICAM-1, E-selectin, sP-selectin in cases than controls
(Jousilahti et al., 2001)	CHD	196	1123	CRP SAA	Higher CRP, SAA in cases than controls
(Blann et al., 1996)	IHD	116	116	sE-selectin TM	Higher sE-selectin in cases than controls
(Tayebjee et al., 2005)	PAD	79	42	MMP-9 TC HDL-C	Higher MMP-9 in cases than controls Lower TC and HDL-C in cases than controls
(Blann et al., 2002)	Coronary artery disease PAD	140	70	VEGF sFlt	Higher VEGF in cases than controls Lower sFlt-1 in cases than controls
(Seko et al., 1997)	AMI	19	19	VEGF	Higher VEGF in cases than controls

AMI; acute myocardial infarction, CAD; coronary artery disease, CHD; coronary heart disease, CLI; critical limb ischaemia, HDL-C; HDL cholesterol, hsCRP; high sentivity C-reactive protein, IC; intermittent claudication, IHD; Ischaemic heart disease,LDL-C; LDL cholesterol, MMP; matrix metalloproteinase, SAA; serum amyloid A, sE-selectin; soluble E-selectin, sICAM; soluble intracellular adhesion molecule, sVCAM; soluble vascular adhesion molecule, sFlt; soluble fms-tyrosine kinase, TC; Total cholesterol, TG; triglycerides, TM; thrombomodulin, UAP: unstable angina pectoris, SAP: stable angina pectoris, PAD; peripheral arterial disease, VEGF; vascular endothelial growth factor, ApolipoproteinA-I, Apolipoprotein B-100, MVD; multiple vessel disease, SVD; single vessel disease, NV: normal vessel

Table 1.6: Comparison of circulating vascular biomarkers between unstable and stable carotid artery disease

Reference	Condition	Stable disease	Unstable disease	Vascular markers	Findings
(Alvarez Garcia et al., 2003)	Carotid artery disease	29	33	hsCRP	Higher hsCRP in patients with unstable plaques compared with patients with stable plaques
(Pelisek et al., 2009)	Carotid artery disease	37	64	MMP-1 MMP-7	Higher MMP-1, MMP-7 in patients with unstable plaques compared with patients with stable plaques
(Sapienza et al., 2005)	Carotid artery disease	24	29	MMP-1 MMP-2 MMP-3 MMP-9	Higher MMP-1, MMP-2, MMP-3, MMP-9 in patients with unstable plaques compared with patients with stable plaques
(Alvarez Garcia et al., 2004)	Carotid artery stenosis	13	27	MMP-2 MMP-9	Higher MMP-9 in patients with unstable plaques compared with patients with stable plaques

hsCRP; high sentivity C-reactive protein, MMP; matrix metalloproteinase

1.4.5 Cellular vascular biomarkers

Various pathophysiological processes such as inflammation, lipid accumulation, apoptosis, proteolysis, thrombosis, and angiogenesis have been associated with atherothrombosis and accompanying secondary clinical events. In response to these processes, blood and vascular cells may release molecules into the bloodstream. These molecules may be investigated as circulating biomarkers reflecting the atherosclerotic processes. However, despite the irreplaceable utility of biomarkers such as lipid phenotype and CRP there is still a need to identify and implement novel biomarkers of vascular health. The traditional biomarkers provide an incomplete picture of the multifactorial nature of ACVD. In addition, traditional biomarkers have a low predictive value and limited accuracy in detecting the presence and severity of vascular diseases such as atherosclerosis (Packard and Libby, 2008).

There is increasing evidence to indicate that a number of vascular diseases may be caused by increased endothelial damage and loss of endothelial repair. A number of cellular and cell-derived biomolecules such as PMA, MP and CPC have a role in the onset, progression, and regression of atherosclerosis. MP and PMA are potential pathogenic vectors able to accelerate endothelial damage and promote disease progression whereas CPC mediate endothelial repair and regeneration. Focus is now shifting towards multimarker strategies combining MP and CPC as integrative markers of vascular risk and health. In addition, the MP and CPC may be used as non-invasive markers to define the 'vascular competence' of patients thereby offering new perspective to assess vascular damage. Indeed, a change in the ratio of MP to CPC may

reflect an imbalance between endothelial damage and repair that could be useful to identify patients with damaged vasculature (Pirro *et al*., 2006, Sabatier *et al*., 2009).

The diagnostic role of MP, CPC and PMA has not been fully identified. However, MP, CPC and PMA may be evaluated as vascular biomarkers that would add to the predictive value of other vascular biomarkers and thus improve assessment of vascular diseases such as carotid artery disease.

1.4.5.1 Cell-derived microparticles

Cell-derived microparticles were first described by Chargaff and West in 1946 as a perceptible factor in platelet free plasma with the potential to generate thrombin (Chargaff and West, 1946). In 1967, Peter Wolf described them as ‘‘platelet dust’’, a fraction containing mainly lipid-rich particles separated by ultracentrifugation from fresh plasma (Wolf, 1967). Microparticles are 0.1-1 µm in diameter (Thery *et al*., 2009) or ~0.1-4 µm in blood plasma (Gyorgy *et al*., 2011), but the lower cut-off remains to be established (Yuana *et al*., 2011). These plasma membrane derived vesicles are shed into circulating blood by cells such as platelets, red blood cells (RBC), leukocytes, and EC (Burnier *et al*., 2009). The majority of the MP in circulation in healthy subjects originate from platelets (George *et al*., 1982, Berckmans *et al*., 2001) making up to 70-90 % of MP in circulation (Horstman and Ahn, 1999), followed by RBC-derived MP (Lynch and Ludlam, 2007, Piccin *et al*., 2007). Lower numbers originate from EC, monocytes and lymphocytes. Most MP studies have predominantly characterized MP derived from RBC, platelets and EC.

Microparticles can be released into circulation upon cellular activation, cell injury or following cell activation-independent processes, including senescence and apoptosis (Beaudoin and Grondin, 1991, Perez-Pujol *et al*., 2007). The rate of steady state release of budding/shedding MP (Cocucci *et al*., 2009) is generally low (except for tumours that release them constitutively) (Smalley *et al*., 2008). MP constitutes a heterogeneous population occurring in different bodily fluids such as blood and synovial fluid. They also differ in terms of the cells of origin, numbers, size, antigenic composition and functional properties (Fourcade *et al*., 1995, Diamant *et al*., 2004). Previous studies have reported varying normal reference ranges of MP in the peripheral blood of healthy subjects, 0.51×10^5 to 2.82×10^5 microparticles/mL (Grant *et al*., 2011) and 2×10^5 to 2×10^5 microparticles/mL (Ansa-Addo *et al*., 2010). This translates to 5 - 50 μg/mL. Since 1946 when they were first described, MP have been extensively studied and described. Several recent review articles have summarized their basic characteristics, molecular and functional aspects (Thery *et al*., 2009, Cocucci *et al*., 2009, Leroyer *et al*., 2010, Dignat-George and Boulanger, 2011, Piccin *et al*., 2007, Little *et al*., 2010, Amabile *et al*., 2010).

Microparticle Composition

The composition (figure 1.14) of MP depends on the parent cell and the cellular mechanisms triggering their formation. An MP consists of a phospholipid bilayer containing embedded proteins. The composition and the distribution of phospholipids within the bilayer is highly specific: negatively charged phospholipids phosphatidylserine (PS) and phosphatidylethanolamine (PE) are located on the external membrane layer, while the neutral phospholipids, phosphatidylcholine

(Eagleton *et al*.) and sphingomyelin (SM) are found on the inner side of the cell membrane (Diamant *et al*., 2004). The phospholipid content of MP is reported to be around 59 % PC, 20.6 % SM, 9.4 % PE and 1.05 % PS (Weerheim *et al*., 2002). This latter phospholipid, PS is significantly important in MP analysis despite its low content on MP when compared to other phospholipids. PS is crucially involved in the procoagulant activity of MP, as the tenase and prothrombinase complexes assembles on PS (Sinauridze *et al*., 2007). The protein component of the MP originates partly from the parent cell occurring as cell-specific antigens or activation markers on the outer surface of the MP (Zwaal and Schroit, 1997). Microparticles also carry MMP 2 and 9, which play a role in basement cell matrix degradation (Taraboletti *et al*., 2002).

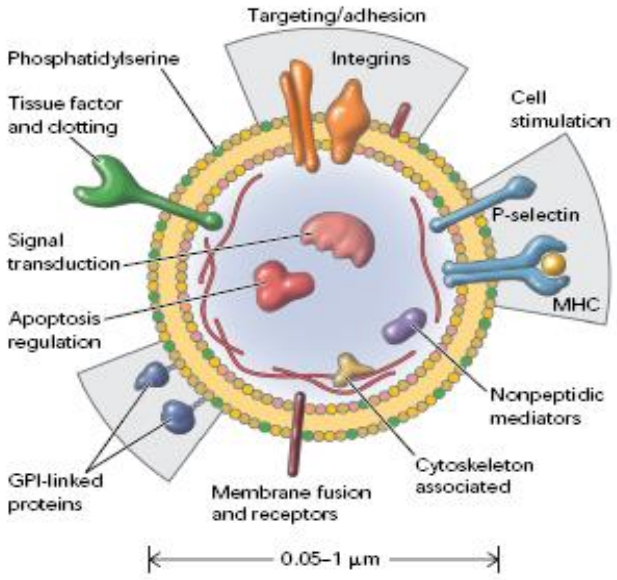


Figure 1.14: Composition of cell-derived microparticles
A microparticle consists of a phospholipid bilayer composed of phosphatidylserine, phosphatidylethanolamine, phosphatidylcholine and sphingomyelin. Within the bilayer are embedded proteins such as P-selectin.
(Hugel et al., 2005)

Microparticle Formation

In steady state, the plasma membrane is asymmetric with respect to the composition and the distribution of phospholipids in its inner and outer layers (Piccin et al., 2007). This asymmetry is maintained by five enzymes namely; gelsoli, aminophospholipid translocase (flippase), floppase, scramblase and calpain (Connor *et al*., 1992). PC and SM are primarily located in the outer membrane, while PS and PE are present within the inner membrane (Diamant et al., 2004).

Although phospholipid asymmetry is essential, a loss of asymmetry may arise during cell activation, apoptosis, senescence, injury or necrosis. Loss of this asymmetry results in general redistribution of phospholipids, leading to cell membrane restructuration, PS externalization, and MP generation. The enzymes involved in maintaining the cell membrane phospholipids asymmetry are also involved in MP generation.

MP generation (figure 1.15) involves the release of intracellular calcium (Ca^{2+}) from the ER. This increases the concentration of Ca^{2+} in the cytosol, changing the dynamic asymmetric steady state maintained by these enzymes. These cellular events contribute to compromise the phospholipid asymmetry of the cell thus releasing MP into the bloodstream (Daleke, 2003).

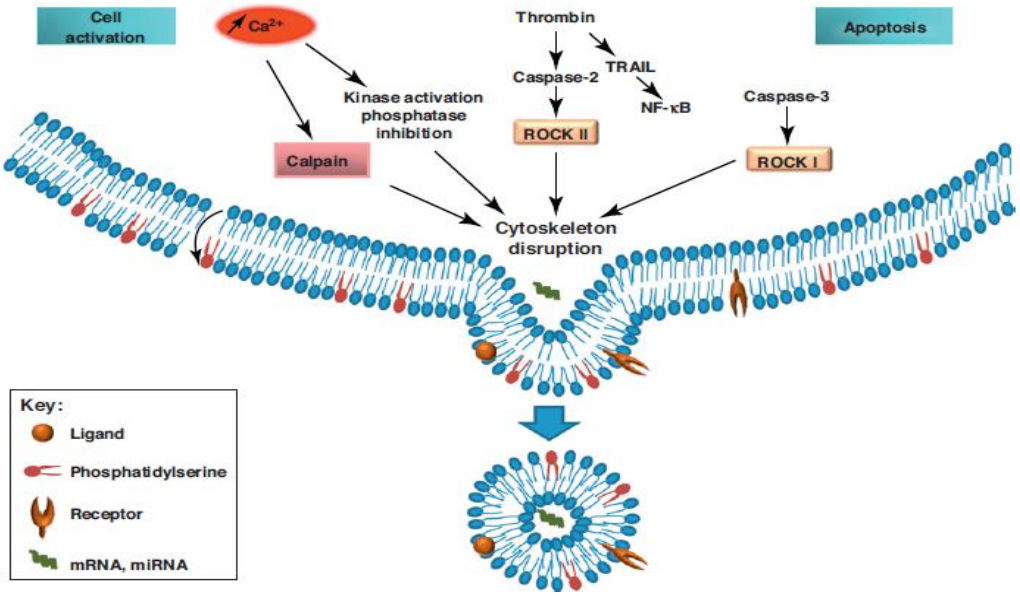


Figure 1.15: Basic schematic representation of the different steps leading to formation of cell-derived microparticles (MP)
During cell activation, an increase in intracellular calcium induces externalization of PS. Calcium increase is also associated with calpain activation, and this is required for cytoskeleton disruption and MP formation. Under apoptosis induction, cleavage of caspases induces ROCK activation, leading to cytoskeleton alteration and blebbing. ROCK, Rho kinase; TRAIL, TNF-related apoptosis-inducing ligand, PS; phosphotidylserine TRENDS in Pharmacological Sciences

The enzyme scramblase allows phospholipids to move across the plasma membrane (Zwaal *et al*., 1993). Activation of scramblase and inhibition of translocase because of high cytoplasmic Ca^{2+} concentration initiates the loss of phospholipid asymmetry and exposure of PS on the outer plasma membrane. In summary, an MP is released following activation, cytoskeleton disruption, general phospholipid redistribution and PS externalization.

Microparticle Functions

Microparticles contribute to a variety of physiological and pathological processes. They readily circulate in the vasculature due to their small size, easily eliciting both local and long range processes (Ardoin *et al*., 2007).

1. Microparticles in intercellular communication

Microparticles are efficient vectors that exchange biological information between cells. As vectors, MP carry surface antigens, cytoplasmic proteins, or nucleic acids from their parent cells to local or distal cells. When released into the bloodstream they can act as messengers delivering a variety of cargos, such as cell surface receptors, proinflammatory cytokines, signalling molecules and mRNA to target cells. These capabilities confer on MP a major role in intercellular communication by binding to their targets via surface receptors or ligands and via lipid membrane fusion, thus facilitating cell-cell interactions (figure 1.16).

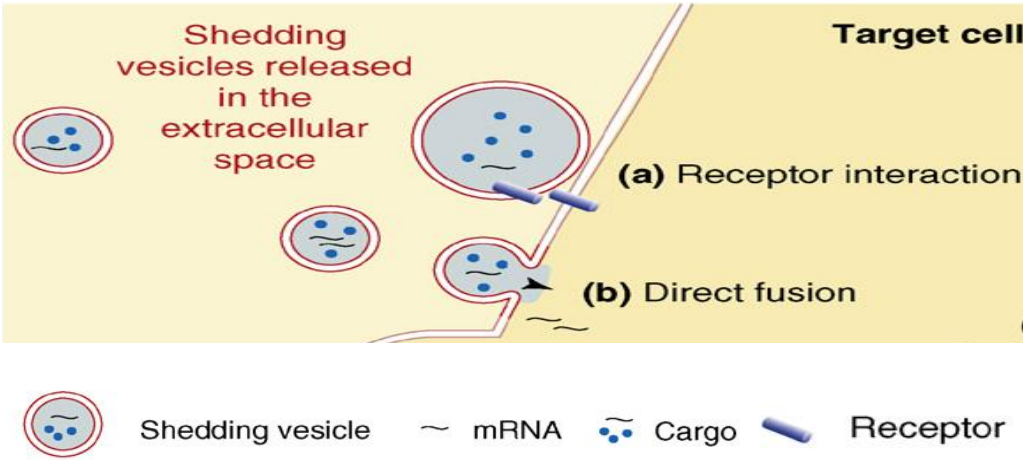


Figure 1.16: Interactions of shedding cell-derived microparticles/vesicles (MP) with their target cells
The shedding MP, released by a parent cell to the extracellular space can (a) bind via specific receptors (blue cylinders) to the surface of the target cell or (b) fuse with the target cell plasma membrane discharging their cargo into the cytosol.
(Cocucci et al., 2009)

Microparticles are therefore important diffusible vectors in the transcellular exchange of biological information (Mesri and Altieri, 1999, Tabibzadeh *et al*., 1994, Baj-Krzyworzeka *et al*., 2002, Greco *et al*., 2001). They are involved in transport through intercellular interaction, and delivery by transfer of surface molecules such as receptors, membrane bioactive molecules (Berckmans *et al*., 2002) and cytoplasmic

proteins to other cells (Fritzsching *et al*., 2002, Barry *et al*., 1998) or to other MP (Barry and FitzGerald, 1999). There is growing consensus that MP act as messengers of inflammatory response, coagulation and vascular remodelling and angiogenesis.

2. Microparticles in inflammatory responses

Microparticles have a both proinflammatory and anti-inflammatory effect on EC and the vascular wall. Results from *in vitro* studies have found that MP stimulate the release of proinflammatory endothelial cytokines such as interleukin 6 (IL-6) and monocyte chemoattractant protein 1 (MCP-1) and induce expression of endothelial cytoadhesins such as ICAM (Pompilio *et al*.), VCAM and E-selectin (Barry *et al*., 1997, Mause *et al*., 2005, Jy *et al*., 1995, MacKenzie *et al*., 2001, Mesri and Altieri, 1999). In addition to the proinflammatory role, MP play an anti-inflammatory role as demonstrated by *in vitro* studies (Gasser and Schifferli, 2004, Dalli *et al*., 2008, Koppler *et al*., 2006). In these *in-vitro* studies MP stimulated the release of anti-inflammatory factors such as transforming growth factor beta 1 (TGFβ1) and interleukin-10 from macrophages resulting in a reduction in circulating levels of tumour necrosis factor alpha (TNFα) and IL-8.

3. Microparticles in coagulation

Microparticles promote coagulant (figure 1.17) through exposure of PS and TF on the outer membrane. Exposure of PS and TF increases their ability to interact with clotting factors. PS exposure promotes the assembly of clotting enzymes and provides a catalytic surface for TF activity. Interaction between clotting factors, PS and TF leads to the formation of tenase and prothrombinase complexes that are essential in

platelet-plasma membrane vesiculation. Both complexes play a central role in coagulation enabling the entire coagulation system to proceed on the restricted surface of platelets, rather than in the fluid phase (Simoncini et al., 2009, Del Conde et al., 2005, Andrews and Berndt, 2004)

Microparticles are also involved in anticoagulant pathways (figure 1.17) through the expression of thrombomodulin, TF pathway inhibitor (TFPI), endothelial protein C receptor (EPCR) or protein S on the MP surface. Thrombomodulin binds to TF forming a complex which has no procoagulant effects. TFPI down-regulates activation of the TF-factor VIIa complex whereas EPCR binds protein C, which together with its cofactor (protein S) proteolytically inactivates factor Va and VIIIa (Satta *et al*., 1997, Perez-Casal *et al*., 2005). In addition, the negatively charged PS, exposed on MP may promote assembly of protein C anticoagulant enzyme complex (Morel et al., 2009).

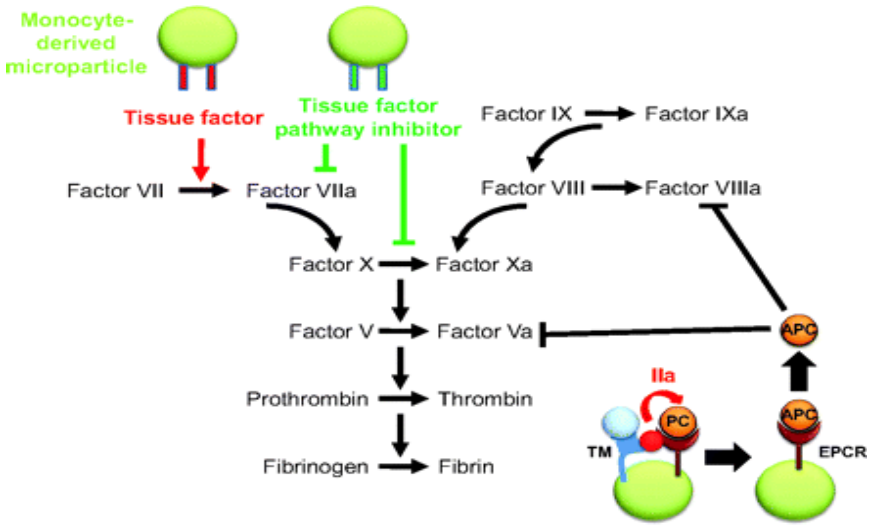


Figure 1.17: Microparticles and coagulation
Tissue factor expressed on microparticles promotes coagulation whereas tissue factor inhibitor inhibits coagulation
(Angelillo-Scherrer, 2012)

4. Microparticles in vascular remodelling

Microparticles interact with the ECM where they localize and activate MMP-2 to modify the surrounding matrix molecules. They bind and activate both endogenous and exogenous proMMP-2 to active MMP-2 (Lozito and Tuan, 2012). MP act as a cellular source of arachidonic acid (AA) and thromboxane A2 (TXA_2) which might have an effect on vascular smooth muscles. AA is present in phospholipids especially, PE and PC that make up the MP membrane (Pfister, 2004).

5. Microparticles in angiogenesis

Endothelial MP promote angiogenesis by firstly activating surface expressed plasminogen into plasmin thus supporting plasmin generation and dissemination. In turn plasmin regulates EPC mediated tube formation (Lacroix *et al*., 2007), Secondly MP promote angiogenesis by increasing EPC number and differentiation (Hristov *et al*., 2004). MP also mediate different stages of angiogenesis and induce endothelial migration and tube formation by delivering bioactive lipids such as AA (Barry *et al*., 1997, Barry *et al*., 1999) and sphingosine-1-phosphate (S1P) (Alvarez *et al*., 2007, Sano *et al*., 2002) released by platelets. These lipids modulate platelet, monocyte and EC function.

6. Microparticles in cell survival and apoptosis

The release of MP can protect cells against the consequences of external stimuli or stress. EMP carrying the lytic complement C5b-9 complex are protected from complement-induced lysis (Sims and Wiedmer, 1995). Compared to parent cells, MP contain increased concentrations of chemotherapeutics, oxidized phospholipids or

caspases 3 suggesting a protective mechanism to prevent accumulation of these toxic materials to dangerously high levels (Nomura *et al*., 2008, Abid Hussein *et al*., 2005, Freyssinet and Toti, 2010a).

Characterization of cell-derived microparticles

In defining or characterizing MP, three properties are recognized; i) their size, ii) the expression of specific antigens of cells from which they derive and iii) the ability to bind annexin V (which reflects membrane flipping).

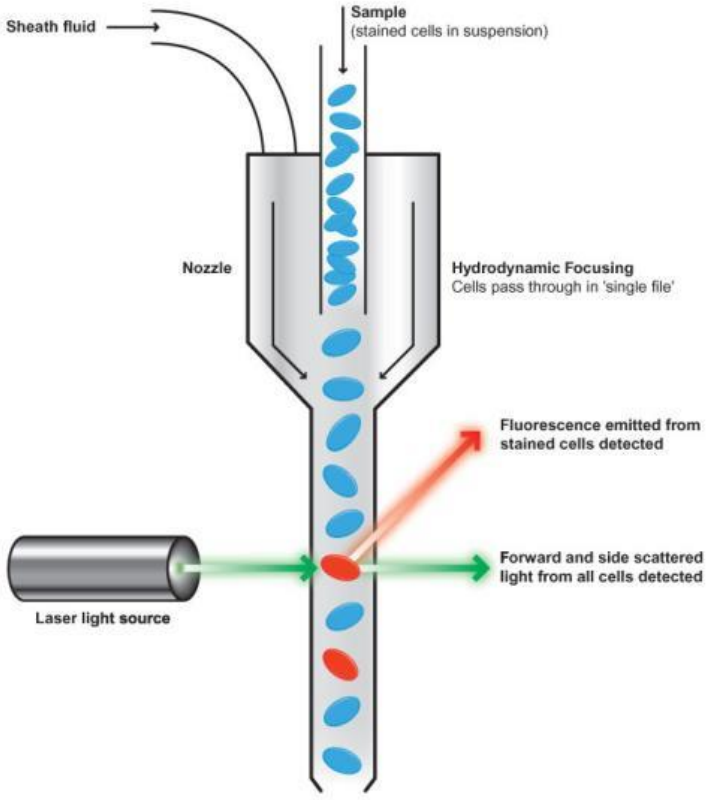


Figure 1.18: The principles of flow cytometry
Retrieved from
http://www.abcam.com/ps/CMS/Images/Flow-Cytometry-Diagram2.jpg
Accessed 09th August 2013

Flow cytometry (figure 1.18) is the most extensively used technique for enumeration and characterization of MP (Lacroix *et al*., 2010). It detects MP in blood

or blood fractions and from other body fluids such as synovial fluids (Horstman and Ahn, 1999, Berckmans *et al*., 2002). The procedure utilizes labelled antibodies against cell-specific markers in quantifying and establishing cellular origin of MP (Diamant *et al*., 2004). Cells and particles are suspended in a stream of fluid and analysed as they pass by a flow cell interacting with a laser beam. The cells and particles scatter light and emit fluorescence that is detected by the instrument allowing for simultaneous multiparametric analysis of their physical and multiple antigen characteristics (Lacroix *et al*., 2010).

Despite flow cytometry being widely used in research, details regarding the optimisation of pre-analytical variables such as sampling conditions, processing and storage of blood samples are sparse. In addition, there are no clear understanding on how type of flow cytometer, settings of the flow cytometer, use of counting beads, sources and concentrations of antibodies, effects MP counts. Previous studies have shown that pre-analytical and analytical issues related to plasma preparation, microparticle preparation and analysis are critical in microparticle detection and enumeration (Yuana et al., 2011). Especially the presence of residual platelets, anticoagulant used in blood collection tubes, storage of the blood/plasma, freezing/thawing of the plasma and centrifugation can influence MP counts.

The type of anticoagulant used in blood collection tubes influences MP counts. Shah *et al.,* (2008) found that blood samples collected in tubes containing heparin and D-Phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK) anticoagulants had elevated platelet- and erythrocyte-derived MP compared to samples anticoagulated with acid-citrate-dextrose or sodium citrate (Shah *et al*., 2008). Connor *et al.,* (2009)

reported that the number of annexin V+ MP was increased 60 min following blood collection in sodium citrate-anticoagulated samples compared to ethylenediaminetetraacetic acid (EDTA) anticoagulated samples (Connor *et al*., 2009). Conversely, 3 h after blood collection PMP counts increased two-fold in plasma from citrated blood but were relatively stable in plasma isolated from citrate-theophylline-adenosine-dipyridamole blood (Kim *et al*., 2002).

The centrifugation protocol can also affect microparticle counts. Double centrifugation of MP prior to freezing decreases the number of annexin V+ MPs (Ayers *et al*., 2011) whereas high speed centrifugation leads to a significant loss of CD31+ CD42b- endothelial MPs (van Ierssel *et al*., 2010). Dey-Hazra and co-workers found 10- to 15-fold higher absolute and platelet-derived microparticles using initial lower centrifugation speeds at 1500 × g compared with protocols using centrifugation speeds at 5000 × g (Dey-Hazra *et al*., 2011).

Storage at low temperatures has been found to significantly reduce annexin V+ and TF+ microparticles (Shah *et al*., 2008). Long-term storage of MP samples at -80°C decreased MP levels (Ayers *et al*., 2011). Freezing MP samples at -80°C increased CD31+CD42b- and CD62E+ EMP counts, and lowered CD144+ EMP (van Ierssel *et al*., 2010). In addition, storing samples for MP detection at -80°C decreased microparticle levels at day 28, 42, and 56 compared with a fresh sample (Dey-Hazra *et al*., 2011).

Other variables that have been investigated include, freeze thaw and washing of MP samples, and filtration of buffers. Ayers *et al* (2011) found that a single freeze thaw cycle of samples led to an increase in annexin V+ MP and PMP. However,

washing samples resulted in decreased annexin V+ MP and PMP (Ayers *et al*., 2011). PMP levels were significantly reduced when samples were thawed on ice compared with 37^{o}C and room temperature whereas MP of endothelial origin appeared to be less influenced by the thawing procedure. Compared to annexin V+ there is a larger decrease in CD41+ PMP when placed on ice (Trummer *et al*., 2009). The amount of background noise was reduced significantly following filtration of buffer with a 0.2 µm filter (Dey-Hazra *et al*., 2011).

The size of the MP is another limitation when enumerating circulating levels of MP. Because of their small size MP have a low degree of expression of specific surface markers. Many of the MP are apoptotic in nature and therefore tend to cause a high degree of non-specific binding (Freyssinet and Toti, 2010b). In addition, no uniform definition of MP exists making it difficult to classify MP subtypes from the same parental cells (Shah *et al*., 2008).

Microparticle analysis is also affected by protein complexes such as insoluble immune complexes. These affect MP purification by differential centrifugation and analysis by flow cytometry. This is especially common with samples obtained from disease states such as autoimmune diseases, hematologic disorders, infections and cancer in which immune complex formation is common. Protein complexes may interfere with MP detection and quantification due to their overlap in biophysical properties such as size, light scattering and sedimentation (Gyorgy *et al*., 2011).

In summary, current methods used to detect MP and accurately measure MP using flow cytometry are hampered by a lack of standardization. Standardization is

essential for successful development of MP technologies. The development of standardized MP technologies would allow for direct comparison of results between studies and lead to a greater understanding of MP in health and disease. Although the standardization of pre-analytical and analytical variables for enumeration of MP remains a significant challenge newer approaches are being investigated. An international collaboration is working on standardization of flow cytometric detection and quantitation of MP (Lacroix *et al*., 2010). Issues under investigation are sizing, probing and counting. The use of size-calibrated beads offers new options for standardized counting and enables calculation of absolute number of MP (Lacroix *et al*., 2010, Tramontano *et al*., 2010). Newer instruments able to detect and quantitate smaller MP (extending the limit to 0.1 μm) are now available. Researchers are using new fluorescent dyes to improve MP detection and quantitation. The combination of multicolour antibodies such as (CD31+CD41- , CD31+ CD42b-, CD105+ CD45-) may improve the specificity and monochrome composite markers (CD144+ CD105+, CD146+ CD105) to improve the sensitivity (Lacroix *et al*., 2010).

Besides flow cytometry, other MP assays have been developed including, single particle assays and bulk assays (table 7). Single particle assays include atomic force microscopy (Yuana *et al*., 2010) and high-sensitivity microscopy (Reininger *et al*., 2006). These two procedures can be used for accurate determination of MP size and shape, but cannot be used for routine analysis of clinical samples (Yuana *et al*., 2010). Bulk assays include immunoassays, functional assays and hybrid assays that detect antigens expressed on MP (Mallat *et al*., 2000), PS/TF dependent procoagulant activity (Exner *et al*., 2003, Camoin-Jau *et al*., 2009) and prothrombinase activity (Mallat *et al*.,

2000, Hugel *et al*., 1999, Jy *et al*., 2004) respectively. However, bulk assays do not provide size information or single particle counts (Lacroix *et al*., 2010). Other available MP analysis techniques include dynamic light scattering (Lawrie *et al*., 2009, Maurer-Spurej *et al*., 2006), high performance liquid chromatography (Weerheim *et al*., 2002), capillary electrophoresis (Xiong *et al*., 2003) and mass spectrometry (Jin *et al*., 2005, Miguet *et al*., 2006). Flow cytometry has a major advantage over the other techniques in that each MP is interrogated individually, and allows for the identification and quantification of MP subpopulations based on antigen expression (table 1.7).

Table 1.7: Advantages and limitations of techniques in microparticle measurement

Technique	Quantification (Bulk quantitation)	Enumeration (single particle counting)	Origin	Specificity	Sizing	Cost/complexity of instrumentation	Practicability
FCM	++	+++	+++	++	+	-	++
Immunoassays	+++	-	+	+	-	+	+++
Functional assays	+++	-	+	+	-	+	+++
DLS	++	+	-	-	++	+	+
AFM	-	++	+	++	+++	-	-
Electron microscopy	-	+	+	+++	++	-	-
RICM	-	+	+	+	++	-	-

FCM, flow cytometry; DLS, dynamic light scattering; AFM, atomic force microscopy; RICM, reflection interference contrast microscopy

Endothelial microparticles

Endothelial MP were first described in patients with lupus anticoagulant presenting thrombotic complications. They were defined as small vesicular structures with a heterogeneous diameter (0.1-1 μm) resulting from the remodelling of membrane phospholipids and expressing PS and antigens representative of EC (Combes *et al*., 1999). Although the precise mechanisms leading to *in vivo* MP generation by EC remain unclear EC may shed membranous vesicles termed endothelial microparticles (EMP) upon activation or apoptosis (Jimenez et al., 2003). EMP represent a relatively small (5-15%) but very important subset of all circulating MP (Hussein *et al*., 2003, Combes *et al*., 1999, Preston *et al*., 2003, Martinez *et al*., 2005).

The stimulus that triggers the release of EMP determines their protein composition. Proteins present on EMP mostly originate from the plasma membrane, cystolic fraction, cytoskeleton or mitochondria of the parent cell (Peterson *et al*., 2008). The quantity and phenotype of EMP released reflects the states of the parent cell. EMP express distinctive antigens depending on whether they are released following activation or apoptosis (table 1.8). *In vitro* studies have found that EMP released following apoptosis express constitutive antigens (for example CD31, CD105, CD144, CD146) and PS (Chironi *et al*., 2009). EMP released upon activation of EC express increased levels of inducible antigens (for example CD62E, CD54, CD106, TF) (Jimenez et al., 2003). In addition, apoptotic EC shed EMP with procoagulant activity (Casciola-Rosen *et al*., 1996).

Table 1.8: Examples of endothelial cell associated markers

Endothelial marker	Action	Cell of origin	Expression	Counter-receptor	Target cells
CD62E (E-selectin)	Rolling	Activated EC	Activation	L-selectin, β2 integrins	WBC
CD106 (VCAM-1)	Adhesion	EC	Activation	VLA-4	Monocytes, lymphocytes
CD54 (ICAM-1)	Leukocyte transmigration	EC, macrophages, lymphocytes	Activation	LFA-1	leukocytes
CD105 (Endoglin)	Angiogenesis	EC, pre-b lymphocytes	Constitutive	CD105	EC
CD31 (PECAM-1)	Adhesion	EC, leukocytes	Constitutive	PECAM-1	EC, leukocytes
CD144 (VE-cadherin)	Adhesion	EC	Constitutive	CD144	EC
CD146 (MCAM)	Monocyte transmigration, angiogenesis	EC	Constitutive	Unknown	EC, monocytes

EC, endothelial cells; ICAM-1' Intercellular Adhesion Molecule 1; VCAM-1, Vascular Adhesion Molecule ; PECAM-1, Platelet endothelial cell adhesion molecule 1; MCAM, melanoma cell adhesion molecule; LFA-1, Lymphocyte function-associated antigen ; VLA-4, late activation antigen-4.

Tramontano et al., 2010

Endothelial MP carry many markers of the parent cells (figure 1.19), the analysis of which can provide useful information on EC status, for example, CD54+, CD62E+ and CD106+ EMP predominantly reflect inflammatory EC activation, while TF+ EMP reflect prothrombotic changes of EC and annexin V+ EMP characterize endothelial apoptosis (Jimenez et al., 2003).

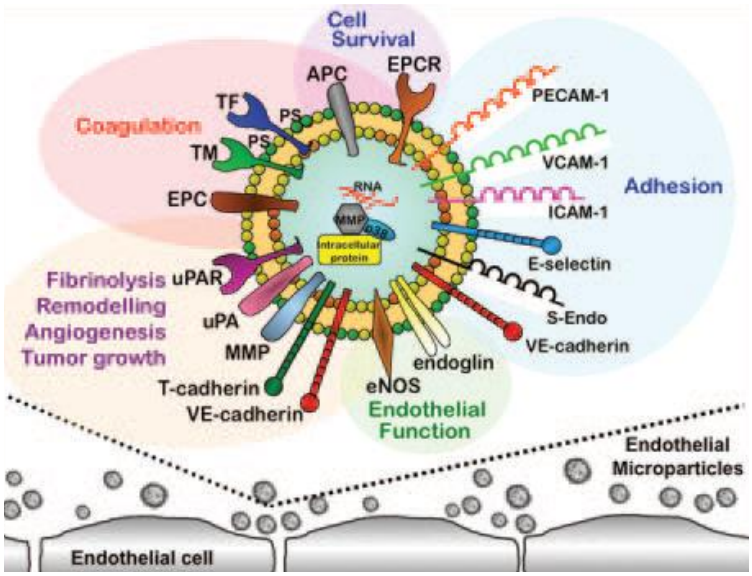


Figure 1.19: Schematic representation of the panel of molecules conveyed by endothelial microparticles and the associated biological effects.
EPCR, endothelial protein C receptor; PECAM-1, platelet endothelial cell adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; ICAM-1, intercellular cell adhesion molecule-1; E-selectin, endothelial selectin; S-Endo, CD146/melanoma cell adhesion molecule; VE-cadherin, vascular endothelial cadherin; eNOS, endothelial NO synthase; MMP, matrix metalloproteases; uPA, urokinase plasminogen activator; uPAR, urokinase plasminogen activator receptor; EPC, endothelial protein C; TM, thrombomodulin.
Dignat-George and Boulanger, 2011

Unfortunately, most of these markers lack exclusive endothelial expression and are expressed on other cell types such as CD105 (activated monocytes/macrophages and bone marrow cell subsets), CD31 (activated platelets, PMP and leukocyte subsets), CD146 (pericytes, tumour cells and activated T-cells), CD54 (leukocytes and αv

integrin) whereas CD51 (monocytes/macrophages and platelets) (Dignat-George and Boulanger, 2011). Only CD62E and CD144 are specific for EMP (Koga *et al*., 2005).

Endothelial microparticles in atherogenesis and atherothrombosis

Endothelial damage may lead to EC activation, EC apoptosis and inflammatory activation which may cause EMP production. The EMP may mobilise inflammatory leukocytes attenuating the inflammatory process. EMP may also activate platelets and promote angiogenesis. These processes drive the atherogenic process leading to plaque progression, prothrombotic activation, plaque destabilisation and atherothrombotic events (Shantsila et al., 2010).

Endothelial microparticles as vascular biomarkers

Identification of EMP in the circulation has raised considerable interest as non-invasive markers of vascular damage. Given that EMP are released under conditions of cell stress/damage, it is not surprising that plasma levels of EMP are increased in most cardiovascular diseases. There is strong evidence to suggest that MP of endothelial origin may be reflective of endothelial damage and may in fact contribute to endothelial damage (Chironi *et al*., 2009, Leroyer *et al*., 2010, Dignat-George and Boulanger, 2011, Boulanger *et al*., 2008). EMP are thought to be directly indicative of endothelial cell stress/damage, and may also reflect endothelial inflammation, increased coagulation, and vascular tone (Leroyer *et al*., 2010).

EMP are emerging as putative biomarkers reflect endothelial injury. They are associated with a number of established cardiovascular risk factors, are indicative of a

poor clinical outcome in atherothrombotic disorders and correlate with established parameters of endothelial damage (Sabatier *et al*., 2009). They have been found to be an independent predictor of future cardiovascular events in patients at high risk for coronary heart disease (Nozaki *et al*., 2009, Georgescu *et al*., 2012). There is growing interest in multimarker strategies combining EMP with EPC levels as integrative markers of vascular health (Nozaki *et al*., 2009, Georgescu *et al*., 2012).

Studies that have compared levels of endothelial microparticles between atherosclerotic patients and controls

Several case control studies have shown increased EMP levels in patients with atherosclerotic disease compared to control subjects (table 1.9). Bernal-Mizrachi *et al.,* (2003) evaluated two species of EMP (CD31 and CD51) in coronary artery patients and found significantly higher EMP in patients than in healthy volunteers (control subjects) with no history of vascular occlusive disorders (Bernal-Mizrachi *et al*., 2003). Similarly, a study by Mallat *et al., (2000)* found significantly elevated levels of circulating procoagulant EMPs in patients with coronary artery disease than in patients with angiographic documentation of absence of coronary artery disease (Mallat *et al*., 2000). EMP have also been evaluated in patients with cerebrovascular disease. Simak *et al., (2006)* found significantly higher PS positive EMP counts in acute ischemic stroke patients relative to the age-matched control subjects (Simak *et al*., 2006) whereas Jung *et al., (2009)* found higher CD62E+, CD31+ CD42b− and CD31+ annexin V+ EMP in patients with acute stroke compared to patients with vascular risk factors but no stroke events (Jung *et al*., 2009). These findings indicate that EMP may contribute to atherosclerotic diseases and may be useful for assessing damage to the endothelium.

Endothelial MP phenotypic profiles reflect distinct phenotypes of atherosclerosis and may be associated with different disease severities. EMP levels have been found to be higher in acute coronary syndrome patients compared to patients with stable angina. Higher CD31+CD42- EMP were found in patients with severe stenosis compared to milder stenosis. The same study also found 2.5-fold greater EMP levels in patients with higher risk lesions compared to low risk lesions (Bernal-Mizrachi et al., 2004). Similarly, Mallat *et al.,* (2000) found significantly higher levels of procoagulant EMP in patients with acute coronary syndrome than stable angina (Mallat *et al*., 2000). There was a significant relation between circulating EMPs and severity of acute ischemic stroke. Simak *et al*., (2006) found significantly higher endoglin+, PS+ and ICAM+ EMP in patients with moderate-severe stroke compared to mild stroke patients (Simak *et al*., 2006). These data suggests EMP may contribute to the progression of atherosclerosis from a stable state to an unstable state. Besides indicating EC injury in atherosclerosis, EMP may be promising markers of assessing the severity and/or stage of atherosclerosis.

Table 1.9: Summary of studies that have compared cell-derived microparticles in atherosclerotic vascular disease patients and controls

Reference	Disease	Cases	Controls	EMP markers	Findings
(Bernal-Mizrachi *et al*., 2003)	Coronary artery disease	38 MI 26 UA 20 SA	42	CD31+CD42- CD51	CD31+ EMP higher in cases compared to controls (7500 vs. 800 counts/µL) CD31+ EMP higher in ACS than SA CD51+ EMP higher in cases compared to controls (1800 vs. 600 counts/µL) PMP higher in cases compared to controls PMP higher in ACS patients than SA patients and controls PMP higher in MI patients than UA patients
(Mallat *et al*., 2000)	Coronary artery disease	12 SA 27 ACS	12	CD146+ CD31+	EMP higher in cases compared to SA and controls (22.2±2.7 vs. 10.1±1.6 vs. 9.9±1.6 nmol/L PS equivalent) EMP higher in ACS patients compared to SA patients (22.2±2.7 vs. 10.1±1.6 nmol/L PS equivalent)
(Bernal-Mizrachi et al., 2004)	Coronary artery disease	15 MI 20 UA 5 SA	0	CD31+CD42-	EMP higher in patients with severe stenosis compared to milder stenosis (15212 vs. 5834 counts/µL) EMP 2.5-fold greater in patients with higher risk lesions compared to low risk lesions

Table 1.9 continued

Reference	Disease	Cases	Controls	EMP markers	Findings
(Simak *et al*., 2006)	Acute ischemic stroke	41	23	CD105+ EMP CD105+CD144+ EMP PS+ EMP CD105+CD54+ EMP	PS+ EMP higher in cases compared to controls, [59(28–86) vs. 28(14–36)] μL^{-1} EMP higher in moderate-severe stroke patients compared to controls: CD105+ [808(541-1122) vs. 415(201-625)], CD105+144+ [260(127-419 vs. 140(79-247)], PS [63(46-110) vs. 28(14-36)], CD105+CD54+ [97(76-166) vs. 56(41-85)] μL^{-1} EMP higher in moderate-severe stroke compared to mild stroke patients: CD105+CD144+ [260(127-449) vs. 154(99-182)], CD105+CD54+ [97(76-166) vs. 54(32-86)] μL^{-1}
(Jung *et al*., 2009)	Acute stroke	73	275	CD31+ CD42b- CD31+ annexin V+ CD62E+	EMP higher in cases compared to controls CD31+ CD42b+/µL 2.15±0.51 vs. 1.99±0.47 CD31+ annexin V+/µL 2.08±0.55 vs. 1.90±0.40 CD62E+/µL 2.01±0.44 vs. 1.75±0.40

EMP; endothelial microparticles, PS; phosphatidylserine, ACS; acute coronary syndrome, UA; unstable angina, MI; myocardial infarction

Platelet microparticles

Platelet MP are small vesicles that are released from the plasma membrane following platelet activation (Flaumenhaft *et al*., 2009, Cramer *et al*., 1997, Rozmyslowicz *et al*., 2003) or as a result of platelet apoptosis (Flaumenhaft, 2006). They are the most abundant in the circulation (Diamant *et al*., 2004). PMP express several receptors present on platelets themselves such as integrin glycoprotein IIb/IIIa, glycoprotein IX (CD42a), and glycoprotein Ib (CD42b), CD62P (P-selectin, a marker of activated platelets), CD40 and CD63. PMP also contain calcium-dependent phospholipid-binding protein annexin V (placental anticoagulant protein I) (Thiagarajan and Tait, 1991), TF (platelet procoagulant protein) (Panes *et al*., 2007), platelet-activating factor (Iwamoto *et al*., 1996), β-amyloid precursor protein (Nomura *et al*., 1994), Ca^{2+}-dependent protease calpain (Kelton *et al*., 1992, Pasquet *et al*., 1996), arachidonic acid (Barry *et al*., 1999), and phospholipids such as aminophospholipid translocase (Comfurius *et al*., 1990). These molecules are particularly important because they are involved in the function of PMP. PMP may reflect the functional state of platelets, as well as the nature of various stimuli for their release (Biro *et al*., 2005). The PMP characteristics give them the ability to facilitate atherogenesis by enhancing expression of cellular adhesion molecules, promoting proliferation of SMC, and stimulating inflammation.

Platelet microparticles in atherogenesis and atherothrombosis

Platelet MP are involved in all stages of atherosclerosis pathobiology. They have pro-inflammatory and prothrombotic effects and mediate many biological

processes due to the fact that they possess various platelet membrane proteins and bioactive lipids. PMP are highly procoagulant (Sinauridze et al., 2007). They express procoagulant molecules on their surface following activation or apoptosis of platelets. PMP express PS, TF and contain surface receptors for factor VIII (Gilbert et al., 1991) and factor Va (Comfurius et al., 1990) which can exert procoagulant effects from the site of platelet activation. These molecules can promote pathogenesis of arterial thrombotic disease via the assembly of coagulation complexes (Morel et al., 2008, Italiano et al., 2010, Wolberg et al., 1999).

PMP initiate atherogenesis by promoting monocyte-EC interaction. PMP are a cellular source of TXA_2. They participate in the transcellular metabolism of arachidonic acid to TXA_2 in the presence of phospholipase A_2 (Barry et al., 1997). This results in the transactivation of platelets and EC and promotion of monocyte-EC interactions (Barry et al., 1998). PMP also stimulate inflammation and atherosclerosis through release RANTES which promotes monocyte recruitment on endothelium (Mause et al., 2005).

Platelet microparticles as vascular biomarkers

Circulating PMP may provide a potential prognostic marker for atherosclerotic vascular disease. Elevated levels of circulating PMP reflecting apoptosis/activation of platelets have been reported in patients with atherosclerotic vascular disease. There is a significant independent association between large PMP and thrombin generation suggesting that formation of PMP are important for increased coagulation activation in myocardial infarction patients (Michelsen *et al*., 2008), in atherogenesis (Michelsen et al., 2009) and in promoting thrombosis (Tan et al., 2005a), PMPs may be a marker of

widespread inappropriate platelet activity (Tan et al., 2005b) and hence could be indicative of platelet hyperreactivity and activation, and may be predictive markers for platelet activation in peripheral arterial disease patients (Zeiger et al., 2000). In summary, plasma levels of PMP could be of prognostic value for the occurrence of atherosclerotic vascular diseases and may be viewed as markers of platelet activation.

Studies that have compared levels of platelet microparticles between atherosclerotic patients and healthy controls

Several studies have found increased levels of circulating PMP in patients with atherosclerotic vascular diseases compared to control subjects (table 1.10). Michelsen *et al., (2009)* found significantly higher levels of large PMPs in patients with carotid artery disease compared to subjects without carotid plaques. Circulating PMP levels remained significantly different between the two groups after adjustments for traditional cardiovascular risk factors and the use of cardiovascular drugs. This was the first study to report higher plasma concentrations of large PMP in plasma from individuals with carotid atherosclerosis (Michelsen *et al*., 2009).

Other studies found elevated CD61+ CD41+ PMP - coronary artery disease patients versus age- and sex-matched controls (Tan *et al*., 2005b), total and larger PMP - coronary artery disease patients versus age-matched and sex-matched healthy controls (Michelsen *et al*., 2008) and PMP - coronary artery disease patients versus healthy volunteers with no history of vascular occlusive disorders (Bernal-Mizrachi *et al*., 2003). Similar results have been reported in patients with peripheral arterial disease (Tan et al., 2005a), (Zeiger et al., 2000). Increased PMP formation in

atherosclerotic diseases may indicate platelet hyperreactivity and the presence of activated platelets in circulation during atherosclerosis. Thus, PMP may be good indicators of platelet activity *in vivo* and may be used to evaluate the state of platelets *in vivo.*

Only a few studies have found increased levels of circulating PMP in patients with unstable atherosclerotic vascular diseases. Increased PMPs are related to the severity of symptomatic peripheral arterial disease. PMP were raised in stable peripheral arterial disease (intermittent claudication) with an additional increase in severe peripheral arterial disease (critical limb ischemia) (Tan *et al*., 2005a). They were also higher in acute coronary syndrome patients than stable angina patients and in patients with myocardial infarction compared to those with unstable angina (Bernal-Mizrachi *et al*., 2003).

These data suggests that PMP are related to the severity of peripheral arterial disease and degree of coronary artery disease supporting the role of PMP in the progression of atherosclerosis from stable to unstable disease. In conclusion, circulating levels of PMP are related to the severity of atherosclerosis and may be used as a marker of inflammation.

Table 1.10: Summary of studies that compared cell-derived microparticles in atherosclerotic vascular disease patients and controls

Reference	Disease	Cases	controls	PMP markers	Findings
(Michelsen *et al*., 2009)	Carotid artery disease	40	20	Large CD61+CD41+	Large PMP higher in cases than controls (96.7±50.4 vs. 56.1±34.9 μg/L)
(Tan *et al*., 2005b)	Coronary artery disease	54	35	CD61+CD41+	PMP higher in cases than controls [38.8 (21-91.8) vs. 19.7 (11.3-40.8) ml/10^5]
(Zeiger *et al*., 2000)	PAD	50	50	CD61+CD41+	PMP higher in cases than in controls
(Tan *et al*., 2005a)	PAD	23 CLI 36 IC	30	-	PMP higher in cases than controls [CLI 7.13 (1.86-13.9), IC 2.82 (1.56-4.38) vs. 1.26 (0.67-2.95) mL/10^5]
(Michelsen *et al*., 2008)	Myocardial infarction	61	61	PMP	PMP higher in cases than controls Total PMP (314.3 , 273.1-361.4 vs. 225.8, 168.8–273.1) μg/L] Larger PMP (181.3, 160.7–204.3 vs. 134.3, 104.6–174.9 μg/L]
(Bernal-Mizrachi *et al*., 2003)	Coronary artery disease	38 MI 26 UA 20 SA	42	PMP	PMP higher in cases compared to controls PMP higher in ACS patients than SA patients and controls PMP higher in MI patients than UA patients

PMP; platelet microparticles, ACS; acute coronary syndrome, SA; stable angina, MI; myocardial infarction, PAD; peripheral artery disease, IC; intermittent claudicant, CLI; critical limb ischemia

1.4.5.2 Endothelial Progenitor Cells

Overview and history

Endothelial progenitor cells (EPC) are bone marrow derived cells that are recruited into the peripheral blood for endothelial regeneration (Real *et al*., 2008). EPC derive from one of the three primary germ cell layers in the very early embryo, the mesoderm. They originate from precursor cells that are able to differentiate into both hematopoietic and endothelial cell lineages (La Vignera *et al*., 2011).

The first molecular evidence for circulating EPC with angiogenic potential was provided by Asahara and colleagues in 1997. They were first described as putative EPC or angioblasts, and isolated from human peripheral blood on the basis of cell surface antigen expression of CD34 (a surface marker common to hematopoietic stem cells) and vascular endothelial growth factor receptor (VEGFR-2) or kinase domain receptor (KDR) (a surface marker specific to EC). These progenitor cells did not express other markers seen on fully differentiated EC and were able to differentiate into EC and incorporate into sites of active angiogenesis. The findings by Asahara *et al* (1997) suggest that these circulating cells may be useful in ischemic tissue regeneration (Asahara *et al*., 1997).

Although Asahara's work was touted as ground breaking, reports published as early as 1963 had demonstrated the existence of EPC (Stump *et al*., 1963). More recently, comparable results were obtained in 1998 by Shi *et al., (*1998*)* who characterized bone marrow derived EPC. They demonstrated a CD34+ subset of EPC

capable of differentiating to EC *in vitro* in the presence of bFGF, insulin-like growth factor-1 (IGF-1) and VEGF (Shi *et al*., 1998). Other studies have also provided proof of the existence of EPC capable of differentiating into mature vascular EC and form blood vessels (Ingram et al., 2004, Alessandri et al., 2001). The results by Asahara *et al*., (1997) confirmed that new blood vessels were formed differently in embryos and adults. In adults, formation of new blood vessels relied on the division of mature resident EC and EPC. This lead to creation of the term "postnatal vasculogenesis", which describes the generation of vascular EC from the precursors in addition to mature resident EC (Asahara et al., 1997).

Since the first evidence of the presence of EPC in adults, EPC have been extensively characterised based on their phenotype. Attempts to quantitate EPC began to appear in the literature within a few years after the initial description of EPC, centring on the use of CD34 and VEGFR-2 to describe EPC (Walter and Dimmeler, 2002). In addition, the role of EPC in vascular repair and neoangiogenesis have also been examined in detail (Kirton and Xu, 2010). However, despite this extensive research, questions still persist regarding their phenotypic and functional characteristics. Issues that have yet to be addressed include; the exact role of EPC in vascular repair, genuine sources of EPC and how to define EPC in research studies.

Characteristics of endothelial progenitor cells

Endothelial progenitor cells share various characteristics with stem cells and endothelial cells (table 1.11). However, there is no accepted standard method or criteria for defining EPC. The conventional definition of circulating EPC has used

surface markers including CD34, CD133 and VEGFR-2 (KDR) (Peichev *et al*., 2000). Other methods of characterization are based on *in vitro* behaviour (Prater *et al*., 2007)

Table 1.11: Characteristics of stem cells and endothelial cells shared by endothelial progenitor cells

Stem Cell Characteristic	**Endothelial Cell Characteristic**
Presence of the surface molecules CD34 and CD133	Presence of the surface molecules CD144, CD146, vWf, and VEGFR-2
Cluster Formation in culture	Response (proliferation) to endothelial growth factors in culture
Highly Proliferative	Adhesion to macromolecules
Resistance to Stress	Uptake of acetylated LDL and binding of UEA (*Ulex Europaeus* Lectin)
	Tube formation of and migration driven by VEGF

EC; endothelial cells, LDL; low density lipoprotein, VEGF; vascular endothelial growth factor, VEGF-R, vascular endothelial growth factor receptor, vWf; von Willebrand factor.
Urbich and Dimmeler, 2004

Measurement of endothelial progenitor cells

The two most widely used methods for EPC characterization and enumeration are flow cytometry and *in vitro* cell culture assays. Flow cytometry quantifies EPC obtained following cell culture or circulating EPC in peripheral blood. It is used for more quantitative analyses. *In vitro* cell culture assay examines EPC functional capacity and EPC quantities as colony forming units (CFU). It is mostly used to determine the proliferative potential (migratory and adhesive capacity) of EPC. Although extensively used in many EPC studies flow cytometry and cell culture assays often conflict and correlate poorly (Powell *et al*., 2005, Van Craenenbroeck *et al*., 2008). These two methods remain controversial because EPC correspond to a rather heterogeneous population of multiple origins and phenotype (progenitor, myeloid and endothelial

markers) (Hristov and Weber, 2008). None of these methods are standardized leading to different research groups employing significantly different methods.

The heterogeneity in methods and definition complicates cross-study comparisons with some studies suggesting increased EPC in coronary artery diseases (George *et al*., 2004, Guven *et al*., 2006), whereas others present decreased numbers in the presence of vascular risk factors and coronary artery diseases (Fadini *et al*., 2005, Hill *et al*., 2003, Vasa *et al*., 2001). Moreover, because circulating EPC represent 0.0001 % to 0.01 % of peripheral blood mononuclear cells (Werner *et al*., 2005, Shaffer *et al*., 2006) counting of such extremely rare events poses a technical challenge. Accurate detection and enumeration of events occurring at such low frequencies requires procedures not routinely used when analysing relatively common cells (Khan *et al*., 2005). Some of the challenges include background noise, non-specific binding and autoflourescence, which may lead to false positives.

***In vitro* cell culture assays**

Two major culture-based approaches have been described to identify EPC; i) early CFU assay that form the early EPC (table 1.12) and ii) late outgrowth endothelial cell (OEC) assay that form the late EPC (table 1.12) (Hill *et al*., 2003, Urbich and Dimmeler, 2004, Sieveking *et al*., 2008). In CFU assay mononuclear cells are plated on fibronectin coated plates. After a few days of culture non-adherent cells are removed and adherent cells remain in culture for the next 5-7 days (Hill *et al*., 2003). In late outgrowth endothelial cell (OEC) assay mononuclear cells are let to grow for 14-21 days forming characteristic cobblestone colonies (Yoon *et al*., 2005). The OEC assays,

most likely, identify vascular or, more specifically, EC progeny, whereas, ECFU assays identify the monocytic/macrophage cell lineage (Dotsenko, 2010).

Table 1.12: Summary of properties of early and late endothelial progenitor cells phenotypes

Property	**Early EPC**	**Late EPC**
Morphology	Form colonies with sprouting cells / spindle shaped cells (Cheng et al.)	Form flat cobblestone monolayer
Proliferative capacity	Low (lasts up to 4 weeks)	High
Marker expression	CD34+/-, VEGFR-2+, CD133+, CD31+, CD14+, CD45+, CD115+, uptake of Ac-LDL, vWF+, CD144-	CD34+, VEGFR-2+, CD31+, uptake of Ac-LDL, vWF+, CD144+, CD105+, CD146+, CD14-, CD45-, CD115-, CD133-
Phagocytosis ability	Can phagocytise bacteria	Cannot phagocytise bacteria
Vessel formation ability	Lack vessel formation ability *in vivo*	Can contribute to vessel formation *in vitro* and *in vivo*
Angiogenic factors secretion	Secrete angiogenic factors	-

EPC; endothelial progenitor cell, CAC; circulating angiogenic cells, VEGFR-2; vascular endothelial growth factor receptor 2, Ac-LD;L acetylated low density lipoprotein, vWf; von Willebrand factor.
Kirton and Xu, 2010

Early EPC augment angiogenesis in a paracrine fashion by releasing angiogenic factors whereas the late EPC provide new EC and vessels by forming tubules and incorporating them into developing blood vessels (Sieveking *et al*., 2008). Based on their functional characteristics, only the late EPC may be regarded as 'true EPC'. They are capable of generating EC and exhibit vessel forming ability (Hristov and Weber, 2004, Ingram et al., 2004, Lin et al., 2000, Yoon et al., 2005, Rehman et al., 2003, Yoder et al., 2007). However, these 'true EPC' make up <1% of circulati(Hristov and Weber, 2004)ng EPC, and an even smaller fraction of the CD34-positive bone marrow cells (Prater et al., 2007).

Flow cytometry

Flow cytometry works on the principle of detecting and quantifying surface antigens expressed on EPC. It can count EPC and quantify the expression of surface antigens (antigenic phenotype). Flow cytometry is a sensitive, specific and reproducible method for counting peripheral blood EPC (Khan *et al*., 2005). However, it is hampered by lack of a precise antigenic phenotype (Ingram et al., 2005).

In order to define the antigenic phenotype of EPC, EPC should express at least one marker of immaturity (such as CD34 and CD133), and an additional endothelial specific marker (such as KDR/VEGFR2, CD31 and vWf). Without a precise antigenic phenotype, EPC have been described differently; CD34+KDR+ (Werner *et al*., 2005, Schmidt-Lucke *et al*., 2005), $CD45^{low}$CD34+CD133+KDR+ (Kondo *et al*., 2004), $CD45^{-}$ CD34+ (Case et al., 2007) and CD45dimCD34+KDR+ (Schmidt-Lucke *et al*., 2010). Nonetheless, these EPC definitions are not definitively proven.

Roles of endothelial progenitor cells in vascular repair

Accumulating data over the past decade suggests that EPC from different origins are released into the circulation and contribute to vascular repair (Kirton and Xu, 2010). Studies have shown that EPC may regenerate injured endothelial cells by either differentiating into mature EC that integrate into damaged vessels or by locally activating mature EC by increasing supply of angiogenic growth factors to activate the resident mature EC (figure 1.20).

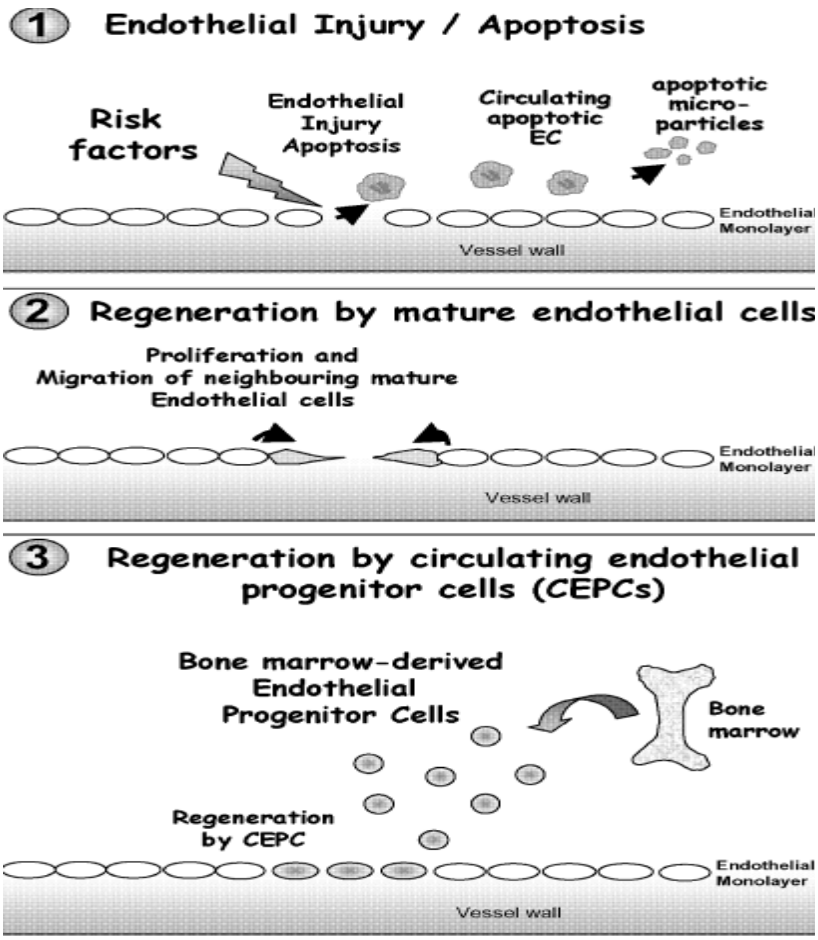


Figure 1.20: Regeneration of the endothelial monolayer after injury.
After induction of endothelial injury two possibilities exist to regenerate the injured endothelial monolayer (2, 3). (2) Regeneration by mature endothelial cells which migrate and proliferate to regenerate the endothelial layer. (3) Regeneration by bone marrow derived endothelial progenitor cells.
Dimmeler and Zeiher, 2004

To achieve either of these strategies, mobilized EPC must home to the "angiogenic active" sites, adhere to the activated/damaged EC or to the ECM and participate in the endothelial activation/repair process (Real *et al*., 2008). The efficiency of vascular repair may not solely involve the incorporation of EPC in newly formed vessels, but may also be influenced by the release of proangiogenic factors. However, it is not known whether the effects of EPC are a result of endothelial differentiation and subsequent proliferation of EPC or secondary to secretion of angiogenic growth factors.

Endothelial progenitor cells may act by releasing potent proangiogenic growth factors such as VEGF, hepatocytes growth factor, granulocyte colony stimulating factor (G-CSF) and granulocyte-macrophage colony stimulating factor (GM-CSF), that mediate their angiogenic effects (Rehman *et al*., 2003). EPC may increase angiogenesis by providing cytokines and growth factors which in turn may influence the classical process of angiogenesis, namely the proliferation and migration as well as survival of mature EC (Folkman, 1995). It has been speculated that early EPC have a more important role in vascular repair via paracrine mechanisms such as secretion of angiogenic cytokines than late EPC. Hur *et al.,(2004)* used *in vitro* cell culture, to demonstrate that early EPC secreted angiogenic cytokines (VEGF and interleukin 8) more so than late EPC (Hur *et al*., 2004).

In settings of tissue ischemia resulting from a damaged endothelium, EPC facilitate vascular repair by translocating from the bone marrow into circulation to where they proliferate and differentiate into EC. EPC have the capacity to physically contribute to vessel-like structures (Urbich *et al*., 2003). They have been shown to form three dimensional tubular structures that could rescue and preserve function of the ischemic tissue (Urayama *et al*., 2008). In addition, EPC can home to the site of endothelial damage, and incorporate into foci of endothelial regeneration thereby improving the flow of blood and recovery of the ischemic tissue (Rafii and Lyden, 2003, Werner *et al*., 2002).

Recruitment and incorporation of EPC into ischemic tissue is a coordinated multistep. However, the mechanisms and signals causing their migration and homing to sites of

injured endothelium or extravascular tissue remain largely unknown. It has been suggested that this process includes mobilization, chemoattraction, adhesion, transmigration, migration, tissue invasion, and in situ differentiation regulated by different factors (figure 1.21) (Urbich and Dimmeler, 2004).

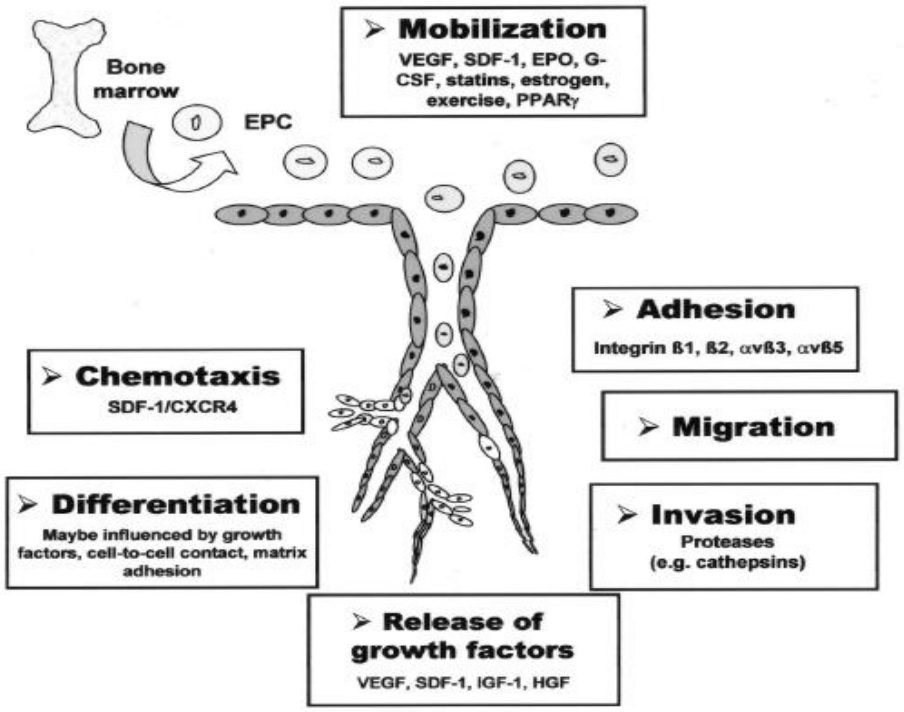


Figure 1.21: Mechanism of endothelial progenitor cells (EPC) homing and differentiation
Recruitment and incorporation of EPC into ischemic tissue requires a coordinated multistep process including mobilization, chemoattraction, adhesion, transmigration, migration, tissue invasion, and in situ differentiation. Factors that are proposed to regulate the distinct steps are indicated
(Urbich and Dimmeler, 2004)

Mobilization of endothelial progenitor cells from the bone marrow

The mobilization of EPC from the bone marrow into the blood circulation is a complex process, regulated by a variety of factors (figure 1.22).

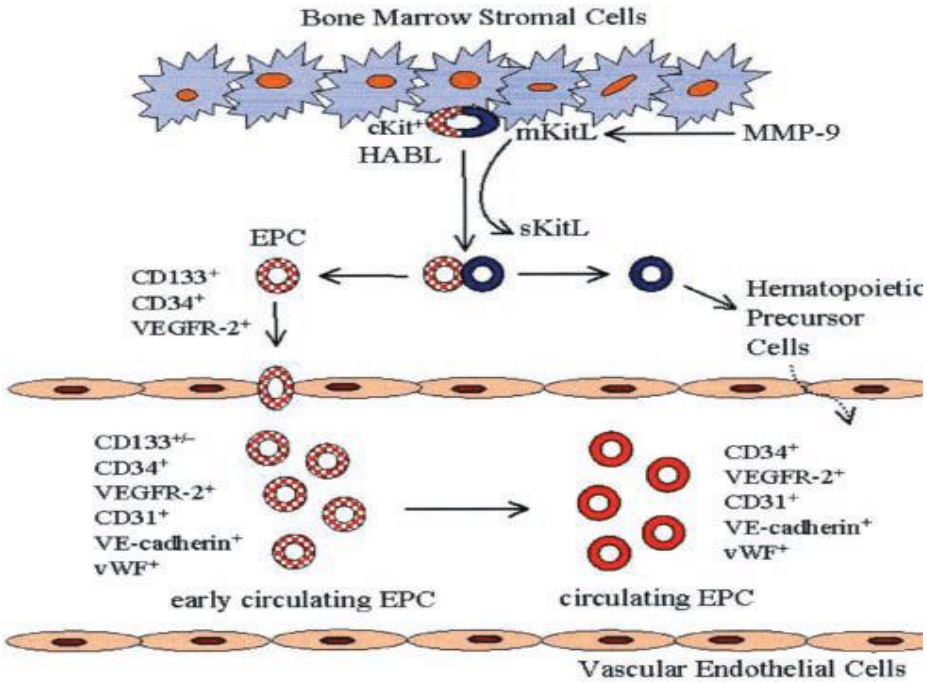


Figure 1.22: Mobilization of endothelial progenitor cells (EPC) from the bone marrow. The mobilization of EPC from the bone marrow into the blood circulation is a complex process, regulated by a variety of factors shown in the figure. Tyrosine-protein kinase Kit positive cell (cKit+), hematopoietic and angioblast precursor cell (hemangioblast, HABL), membrane-bound Kit ligand (mKitL), soluble Kit ligand (sKitL).
Hristov et al., 2003

Endothelial progenitor cells may increase with exogenous stimuli such as ischemia through activation of angiogenic factors and increase of MMP-9 activity (Heissig *et al*., 2002). The angiogenic factors include VEGF, angiopoietin-1, G-CSF, GM-CSF and stromal-derived factor-1 (SDF-1) (Asahara and Kawamoto, 2004, Aicher *et al*., 2005, Barber and Iruela-Arispe, 2006, Hattori *et al*., 2001, Takahashi *et al*., 1999, Lyden *et al*., 2001). In addition to their role in the mobilization of EPC, these factors may also play a role in homing and differentiation of EPC on endothelial sites where they are required (Hattori *et al*., 2002).

The first step in mobilization of EPC is activation of MMP-9. Once activated, MMP-9 transforms membrane-bound Kit ligand (mKitL) to a soluble Kit ligand (sKitL). mKitL is normally attached to the bone marrow stromal cells. After transformation,

sKitL acts on stem and progenitor cells which then translocate to a permissive vascular niche favouring differentiation. The cells then transmigrate through the endothelium into the blood stream where they mature to circulating EPC (Hristov *et al*., 2003).

Endothelial progenitor cells and atherosclerosis

There is intense discussion regarding the role of EPC in atherosclerosis. Two schools of thought have been proposed suggesting that EPC are involved in atherosclerosis progression as well as regression (figure 1.23).

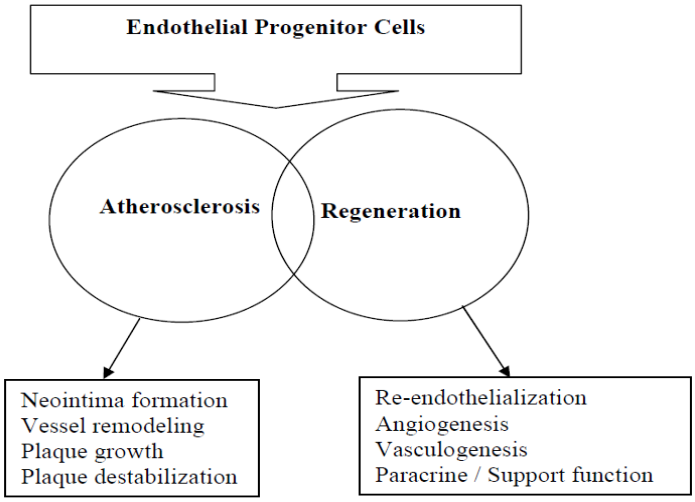


Figure 1.23: Schematic presentation of endothelial progenitor cells (EPC) involvement in vessels regeneration and atherosclerosis progression.
EPC maintain the integrity of the endothelial monolayer by re-endothelialisation, angiogenesis, vasculogenesis and paracrine support. EPC promote atherogenesis by mediating neointima formation, vessel remodelling, plaque growth and destabilization. Adapted from Dotsenko, 2010

Endothelial progenitor cells maintain the integrity of the endothelial monolayer, thereby preventing development of atherosclerotic lesions and subsequent thrombotic complications. They also promote vascular regeneration, inhibiting progression of atherosclerosis. By inhibiting atherosclerosis, EPC are able to prevent development of lesion (Ma et al., 2009, Werner et al., 2002, Tanaka et al.,

2003, Rauscher et al., 2003, Xu et al., 2003). Several studies have demonstrated the role of EPC in atherosclerosis development. EPC enhance endothelialisation and blood vessel formation within the atherosclerotic plaques. This in turn increases plaque formation (Bhattacharya et al., 2000, George et al., 2005, Langwieser et al., 2009).

Studies that have compared levels and function of endothelial progenitor cells between atherosclerotic patients and healthy controls

A number of studies have evaluated the relation between EPC concentrations or functional capacity in peripheral blood and ACVD such as coronary artery diseases and cerebrovascular disease (table 1.13). These flow cytometric or cell culture studies found a significant relation between the various atherosclerotic vascular diseases and the number, adhesion and migration capacities of EPC. The changes in EPC levels in the different clinical conditions display one major pattern, i.e. EPC depletion by means of consumption in patients with ACVD compared to controls. These studies found significantly lower levels of EPC in patients with cerebrovascular diseases (Ghani et al., 2005, Zhou et al., 2009, Yip et al., 2008) and coronary artery disease (Schmidt-Lucke et al., 2010, Vasa et al., 2001, Powell et al., 2005, Wang et al., 2007a) compared to controls. This may be as a result of continuous endothelial damage associated with atherosclerotic diseases and subsequent vascular repair initiated in attempting to resist the progression of atherosclerosis. Besides having lower EPC numbers, EPC isolated from ACVD patients had impaired migratory capacity (Vasa et al., 2001, Wang et al., 2007a).

Table 1.13: Summary of flow cytometry and cell culture studies that compared EPC in atherosclerotic vascular disease patients and healthy controls and found a reduction in EPC counts

References	Disease	Cases	Healthy controls	EPC markers	Effect on EPC numbers and function
(Zhou *et al*., 2009)	Cerebrovascular disease	136	67	CD133+KDR+	Lower EPC in cases compared with controls 0.037±0.001 vs. 0.06±0.002 %
(Ghani *et al*., 2005)	Cerebrovascular disease			EPC colonies	Lower EPC in cases compared with controls
(Lau *et al*., 2007)	Carotid atherosclerosis	30	30	CD34+/KDR+	Lower EPC in stroke patients compared with controls (235.7±45.5 vs. 400.4±56.8 cells/µL)
(Schmidt-Lucke *et al*., 2010)	Coronary artery disease	65	25	$CD45^{dim}CD34^{+}KDR^{+}$	Lower EPC in cases compared with controls
(Wang *et al*., 2007a)	Coronary artery disease	60	44	KDR+CD133+	Lower EPC in cases compared with controls EPC isolated from cases revealed impaired migratory capacity
(Powell *et al*., 2005)	Coronary artery disease	16	7	CD133+/VEGFR-2+ CFUs	Lower EPC in cases compared with controls 0.00033±0.00015 % vs. 0.0017±0.0006 % mononuclear cells Fewer CFU in cases compared with controls 2±1/well vs. 22±3/well
(Vasa *et al*., 2001)	Coronary artery disease	45	15	CD34+KDR+	Lower EPC in cases compared with controls EPC isolated from cases revealed an impaired migratory response.

ACS; acute coronary syndrome, SCAD; stable coronary artery disease, SA; stable angina, CSA; chronic stable angina, MI; myocardial infarction, IS; ischemic stroke, HS; hemorrhagic stroke, AIS; acute ischemic stroke, SIS; stable ischemic stroke, TIS; transient ischemic stroke, CFUs; colony forming units, MVD; multiple vessel disease, SVD; single vessel disease, NV: normal vessel.

1.4.5.3 Platelet leukocyte aggregates

Intracellular interactions between activated blood cells can lead to formation of cellular aggregates. An example of such formation involves activated platelets and leukocytes leading to formation of heterotypic aggregates, i.e. platelet leukocyte aggregates (PLA). PLA form when activated platelets degranulate and adhere to circulating leukocytes. The platelets interact predominantly with monocytes and polymorphonuclear leukocytes and only weakly with lymphocytes (Rinder *et al*., 1991b, Rinder *et al*., 1991a). Platelets bind via P-selectin (CD62p) expressed on the surface of activated platelets to the leukocyte receptor, P-selectin glycoprotein ligand-1 (PSGL-1) (Palabrica *et al*., 1992).

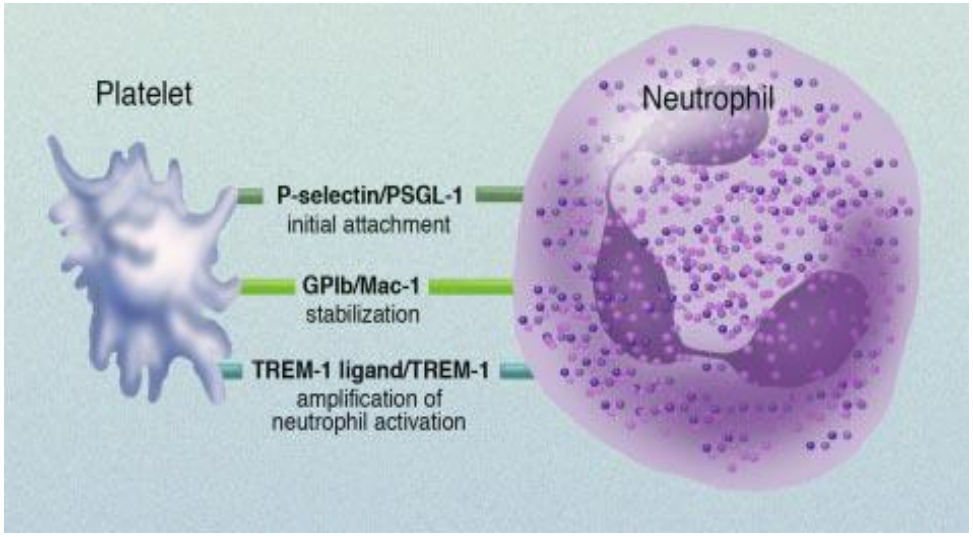


Figure 1.24: Receptor/counter receptor pairs involved in platelet-leukocyte interactions. Platelets interact with leukocytes through a number of factors as shown in the figure. (Haselmayer et al., 2007)

In addition to the interaction via P-selectin and PSGL-1, cation-independent interactions may play a role in platelet-leukocyte interactions (Sarma *et al*., 2002). Leukocyte Mac-1 (also known as integrin αMβ2 or CD11b/CD18) is responsible for

stabilizing the PLA. This integrin binds with platelet surface glycoprotein Ib (GPIb) (Lopez *et al*., 1999). There are additional receptors involved in platelet-leukocyte interactions. These are triggering receptor expressed on myeloid cells 1 ([TREM-1], a receptor expressed on neutrophils and TREM-1 ligand (TREM-1 counter receptor present on platelets) (Haselmayer *et al*., 2007). Monocytes and neutrophils may also bind non-activated platelets, although with lower affinity and independently of the expression of P-selectin (Rinder *et al*., 1991a).

Platelet leukocyte aggregates in atherosclerosis

Platelet leukocyte aggregates form in inflammatory states (Arber *et al*., 1991) representing amplification of inflammation (Michelson *et al*., 2001). When platelets interact with leukocytes, they release secretory granules that amplify leukocyte activation. The activated platelets and activated leukocytes produce various inflammatory substances and enzymes (cell-adhesion molecules, cytokines, chemokines and TF and MMP) (Vandendries *et al*., 2004). Adhesion and subsequent release of inflammatory substances by the activated cells up-regulates inflammatory responses in atherosclerosis (Neumann *et al*., 1997, Weyrich *et al*., 1996).

The inflammatory substances produced because of platelet-leukocyte interactions such as TF expressed on monocytes promotes procoagulation and fibrin deposition at sites of vascular injury (Palabrica *et al*., 1992, Lindmark *et al*., 2000). In addition, proteolytic enzymes such as MMP promote plaque rupture by degrading the basement membrane (Vandendries *et al*., 2004). PLA also plays a direct role in atherogenesis. Platelets supply cholesterol to monocytes which mature into lipid-

laden macrophages characteristic of coronary atherosclerosis (Munro and Cotran, 1988).

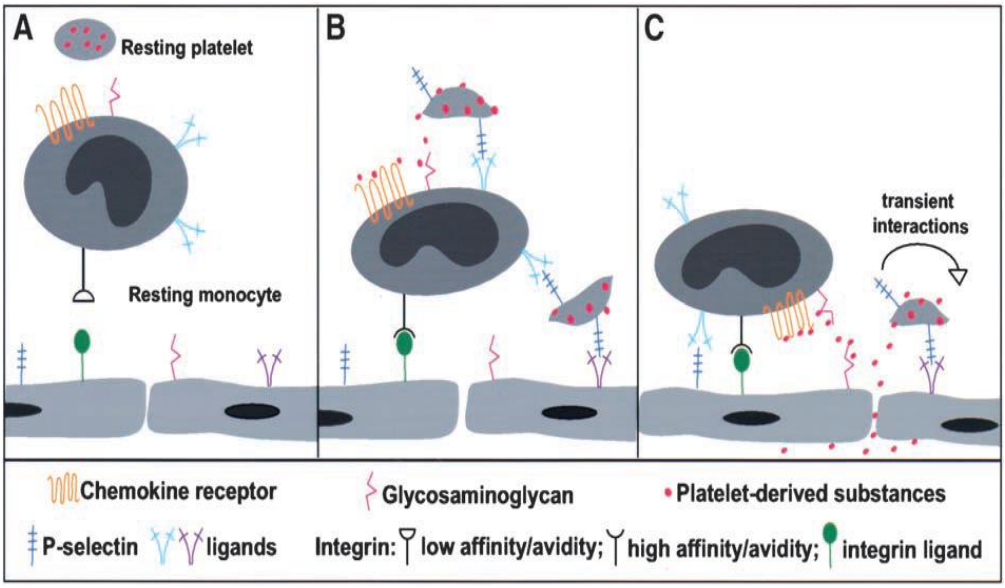


Figure 1.25: Mechanisms by which activated platelets and monocytes participate in the development of atherosclerosis
(A) No interactions occur between resting platelets and monocytes. (B) Activated platelets promote monocyte recruitment via platelet-monocyte interactions. Activated platelets interacting with monocytes deliver their proinflammatory factors to monocytes. Consequently, affinity and/or avidity of monocyte integrins are upregulated and monocytes arrest on endothelium. Additionally, monocyte-platelet aggregates may employ platelet P-selectin to mediate aggregates to interact with endothelium. (C) Activated platelets promote monocyte recruitment via platelet-endothelial interactions. Activated platelets transiently interacting with endothelium may deposit their proinflammatory factors on the surface of endothelium, causing subsequent rolling monocyte arrest. Also, platelet-derived proinflammatory factors may infiltrate into the vessel wall, triggering vascular cell proliferation, migration, and inflammation.
Huo and Ley, 2004

In summary, PLA formation may represent targeting of platelets and leukocytes to specific inflammatory or thrombotic sites (Palabrica et al., 1992, Kirchhofer et al., 1997). Activated platelets can support the arrest of monocytes onto inflamed endothelium thus driving in vivo progression of atherosclerosis (figure 1.25) (Freedman and Loscalzo, 2002). Platelet interaction with leukocytes has been shown

to be one of the strongest factors associated with carotid atherosclerosis (Shoji et al., 2006).

Analysis of platelet leukocyte aggregates

Analysis of PLA can be used to determine *in vivo* platelet and/or leukocyte activation, and the aggregation of blood cells (Catellier *et al*., 2008). Analysis of PLA as a vascular biomarker has several advantages over other known vascular biomarkers. PLA such as platelet-monocyte aggregates (PMA) enumerated by flow cytometry are more sensitive as markers of *in vivo* platelet activation than platelet surface P-selectin (Lindmark et al., 2001, Furman et al., 2001b, Furman et al., 1998, Michelson et al., 2001). PMA remain detectable in peripheral blood for a longer time period than P-selectin (Michelson *et al*., 2001). Activated platelets rapidly lose P-selectin whilst continuing to circulate and function (Michelson *et al*., 1996, Burger and Wagner, 2003). PMA have been shown to be an early marker of acute myocardial infarction compared with creatine kinase-MB (Furman et al., 2001b).

Studies that have compared levels of platelet monocyte aggregates between atherosclerotic patients and healthy controls

Several previous studies (table 1.14) have used flow cytomety to quantify and characterize circulating PLA and shown their association with ACVD in human. These studies have demonstrated increased PLA in patients with coronary artery disease (Wang et al., 2007b, Zhang et al., 2007, Michelson et al., 2001, Michelson and Furman, 1999, Furman et al., 1998, Furman et al., 2001a) and cerebrovascular diseases (Zeller et al., 2005, McCabe et al., 2004, Garlichs et al., 2003) compared to controls without

the disease (table 14). Further increases were observed in patients with advanced diseases such as acute coronary syndromes (Wang *et al*., 2007b, Zhang *et al*., 2007, Michelson *et al*., 2001) and acute cerebrovascular disease (McCabe *et al*., 2004).

Table 1.14: Summary of flow cytometric studies that have compared platelet-leukocyte aggregates in atherosclerotic vascular disease patients and healthy controls

Reference	Disease	Cases	Controls	Markers	Findings
(Zeller *et al*., 2005)	Cerebrovascular Disease	58	58	CD41 CD45	PMA higher in cases compared with controls 7.05±3.57 vs. 5.07±3.15 %
(McCabe *et al*., 2004)	Cerebrovascular Disease	149	27	CD42b CD45	PMA higher in cases compared with controls [acute group (5.9%), convalescent group (5.6%), and control group (4.9%)].
(Garlichs *et al*., 2003)	Cerebrovascular Disease	77	15	CD61 CD40	PMA higher in cases - stroke patients (55.6±18.7 MFI) and TIA patients (51.9±18.2 MFI) compared with controls,
(Wang *et al*., 2007b)	Coronary artery disease	132	46	CD42a CD14	PMA higher in ACS patients (36.5±12.2%) compared with SA patients (22.8±5.5%) and controls (12.3±3.3%)
(Zhang et al., 2007)	Coronary artery disease	127	46	CD42a CD14	PMA higher in NSTEMI (35.7±11.5%) compared with SA patients (22.8±5.5%) and controls (12.3±3.3%)
(Michelson *et al*., 2001)	Coronary artery disease	9	94	CD14 CD61	PMA higher in AMI patients (34.2±10.3%) compared with non-AMI patients (19.3±1.4%) and controls (11.5±0.8%)
(Furman et al., 2001b)	Coronary artery disease	61	150	CD61 CD14	PMA higher in cases (11.6±11.4%) compared with controls (6.4±3.6%).
(Furman *et al*., 1998)	Coronary artery disease	19	19	CD41/CD61 CD11b	PMA higher in cases (15.3±3.0%) compared with controls (6.3±0.9%).

PMA; platelet monocyte aggregates, ACS; acute coronary syndrome, SA; stable angina, MI; myocardial infarction, NSTEMI; non-ST elevation acute coronary syndromes, TIA; transient ischemia attack

1.5 Objectives

Protein and cellular biomarkers, quantified using flow cytometry, represent putative novel biomarkers with the potential to predict vascular disease and monitor changes in vascular health status. The key biomarkers of interest in the following series of studies are MP, shed from endothelial cells and platelets. There is considerable interest in the potential of cell-derived MP as vascular biomarkers. Microparticles have been isolated from atherosclerotic plaques and may also act as mediators of disease, harbouring surface antigens, cytoplasmic proteins and nucleic acids originating from their parent cells. Other cellular biomarkers of interest are endothelial progenitor cells and platelet-monocyte aggregates on which preliminary data has been collected.

Prospective biomarkers should have some of the following characteristics; they should be capable of predicting individuals with established disease, predicting disease stage or monitoring changes in cardiovascular risk and disease state in response to treatment. Novel biomarkers must add predictive and monitoring value beyond that of more established markers. The ability of biomarkers to predict vascular diseases should not be influenced by lipid lowering, anti-inflammatory and anti-platelet medications which are often prescribed only when a considerable disease burden is present. However, if biomarkers are to be used to monitor vascular health, this should not be the case. Such biomarkers should be sensitive to changes following interventions such as therapeutic or lifestyle. In addition, biomarkers should be

capable of being quantified reproducibly and objectively in simple, cost-effective assays. A series of studies is proposed that will examine the potential of cell-derived microparticles and other markers to act as biomarkers of vascular disease and vascular risk. The specific objectives of the study are to;

1. Quantify and determine the source of variability when enumerating MP
2. Determine the ability of MP to distinguish individuals with (cases) and without (controls) documented carotid artery disease
3. Determine the ability of MP to distinguish stable from unstable carotid artery disease.
4. Determine the additive value of MP over protein vascular biomarkers including soluble adhesion molecules, soluble selectins, proteolytic markers, angiogenic markers and inflammatory proteins.
5. Determine the influence of commonly prescribed medications on MP concentrations.
6. Determine the influence of a 24 week low carbohydrate diet on protein vascular biomarkers including soluble adhesion molecules, soluble selectins and inflammatory proteins.

Chapter 2

General Methods

2. General methods

2.1 Blood collection

After overnight fasting, blood samples were drawn from the participant's antecubital vein using a 23-gauge needle. In total 22 mL of blood was collected from each participant; 9 mL sodium citrate (9NC coagulation 3.2%, 3 mL), 5 mL EDTA (K3E K_3EDTA liquid, 2.5 mL) and 8 mL serum (Z serum Sep Clot activator, 4 mL) (Vacuette Greiner Bio-One tubes). Blood collection was carried out with minimal use of tourniquet to avoid frank endothelial damage and activation (Jy et al., 2004). The first 3.0 mL were discarded to minimise contamination by endothelial injury induced microvesiculation (Goon et al., 2006). The blood samples were processed within 2 h after collection. Unprocessed bloods were kept at room temperature.

2.2 Processing blood samples

2.2.1 Double centrifugation protocol - sodium citrate anti-coagulated bloods (Dignat-George et al., 2004)

The bloods samples were first centrifuged for 15 min at 1500 x g at 20^{o}C. The upper part of the plasma was collected by pasteur pipette which was carefully kept just below the surface, without disturbing the white buffy coat layer on top of the cell compartment and leaving exactly 0.5 cm of plasma above the buffy coat. This platelet rich plasma was centrifuged for 2 min at 13000xg at 20^{o}C to produce platelet poor plasma. Again the upper part of plasma was collected by pasteur pipette which was

carefully kept just above the 1 mL mark of the vessel. Aliquots of ~350 µL were stored at -80^{o}C for later analysis.

2.2.2 Single centrifugation protocol - EDTA anti-coagulated and serum clot activated bloods

The EDTA anti-coagulated and serum clot activated bloods were centrifuged for 15 min at 1500xg at 20^{o}C. The upper part of the plasma was collected, without disturbing the white buffy coat layer on top of the cell compartment and aliquots of ~350 µL stored at -80^{o}C until analysed at the end of the study.

2.3 Flow cytometric analysis of progenitor cells and endothelial progenitor cells

Whole blood samples (EDTA anti-coagulated blood) were used to quantify EPC. Each sample was analysed once. Monoclonal antibodies and their matching isotype controls directly labelled with fluorochromes were purchased from different companies. The following monoclonal antibodies were used (i) CD45-FITC (Beckman Coulter), (ii) KDR/VEGFR-2-PE (R&D Systems), CD34-PC7 (Beckman Coulter), (iv) Mouse IgG1k-PE (R&D Systems) and (v) MsIgG1-PC7 (Beckman Coulter). Red blood cells were lysed using a commercially available reagent IOTest®3 10x, (Beckman Coulter). Blood samples were analysed for EPC within 2 h of blood collection using a modified International Society of Hematotherapy and Graft Engineering (ISHAGE) protocol (Sutherland et al., 1996). VEGFR-2, CD34 and CD45 expression patterns as well as their morphologic qualities were used for detection. For this purpose 100 µL of peripheral blood collected in EDTA-containing tubes was stained with the following

fluorochrome-conjugated monoclonal antibodies: 20 µL CD45-FITC (a pan leukocytes marker), 12 µL KDR/VEGFR-2-PE (an endothelial specific marker) and 10 µL CD34-PC7 (a progenitor cell marker). An isotype sample was also prepared in order to set the appropriate regions. To 100 µL peripheral blood, 10 µL each of CD34-PC7, CD45-FITC and IgG1k-PE and was added. These were incubated in the dark for 30 min at room temperature. Following incubation, RBC were lysed with 2000 µL of lysing solution for 15 min and analysed immediately for 20 min at high speed on a flow cytometer (Beckman Coulter FC500, USA). Absolute cells counts (cells/µL) were calculated using the dual platform method in conjunction with the leukocyte count from the haematology analyser (coulter® A$^{C.}$T diff™ analyzer, Beckman Coulter).

For example:

$$\text{EPC (Counts/µL)} = \frac{\text{EPC count during 20 min collection}}{\text{CD45 count during 20 min collection}} \times \text{Leukocyte count from haematology analyser}$$

EPC were defined as describe in figure 2.1. EPC and progenitor cells counts are presented as cells/mL. In addition, EPC are expressed as a % of total progenitors and total CD45+ events. Progenitor cells are also presented as a % of total CD45+ events.

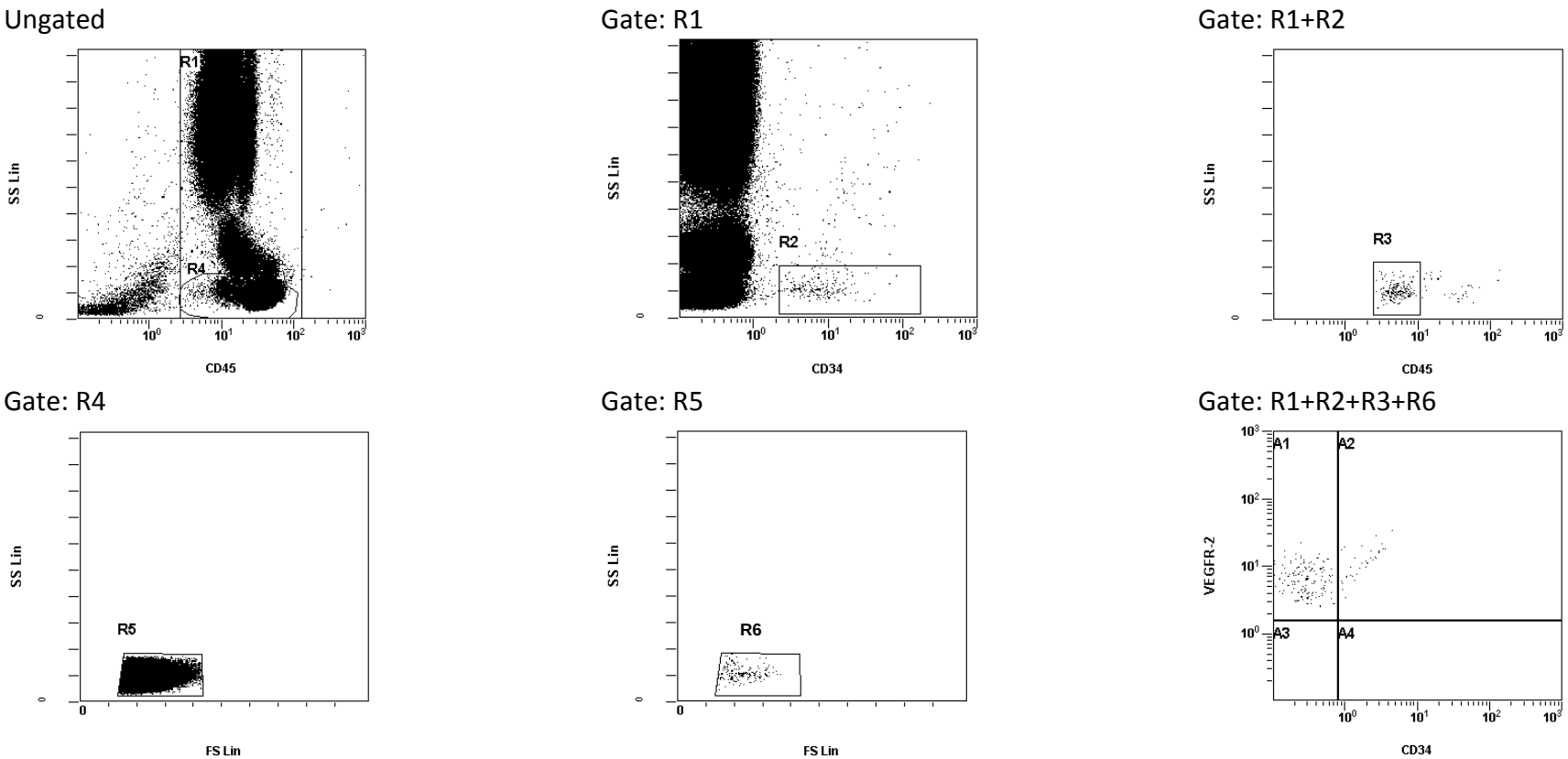


Figure 2.1: Scattergrams illustrating definition of endothelial progenitor cells

Endothelial progenitor cells (EPC) were identified using a modified version of the International Society of Hematotherapy and Graft Engineering (ISHAGE) protocol for progenitor cells (Sutherland et al., 1996) with the addition of the VEGFR-2 marker to identify progenitors of endothelial origin. Thus EPC were defined as $CD34^{+}VEGFR2^{+}CD45^{dim}$. Total EPC events were converted to cells·mL^{-1} using the dual platform method in conjunction with the haematology analyser ($A^{C}T$ Diff, Beckman Coulter, USA) determined leukocyte count.

2.4 Flow cytometric analysis of platelet monocyte aggregates

Whole blood samples (EDTA anti-coagulated blood) were used to quantify platelet monocyte aggregates. Each sample was analysed once. Monoclonal antibodies and their matching isotype controls directly labelled with fluorochromes were purchased from different companies, (i) CD41-PE (Biocytex), (ii) CD45-FITC (Beckman Coulter), (iii) CD14-PC5 (Beckman Coulter), and (iv) mouse IgG1k-PE (Beckman Coulter). Red blood cells (RBC) were lysed using a commercially available reagent IOTest®3 10x, (Beckman Coulter). Within 2 h of collection, 100 µL of sodium citrate anticoagulated whole blood was stained with the following fluorochrome-conjugated monoclonal antibodies 10 µL CD45-FITC (a pan leukocyte marker), 10 µL CD14-PC5 (a monocytes specific marker) and 10 µL CD41-PE (a platelet marker). In distinguishing CD41 positive and negative regions, one further sample was stained with 10 µL CD45-FITC, 10 µL CD14-PC5 and 10 µL IgG1k-PE isotype control. These were incubated in the dark for 30 min at room temperature. Following incubation, RBC were lysed with 2000 µL of lysing solution for 15 min and analysed immediately for 3 min at medium speed on a flow cytometer (Beckman Coulter FC500, USA). Monocytes were defined as $CD45^{+}CD14^{+}$ events with moderate side scatter. Platelet monocyte aggregates were determined as the percentage of CD14 events also expressing the platelet antigen CD41 (figure 2.2).

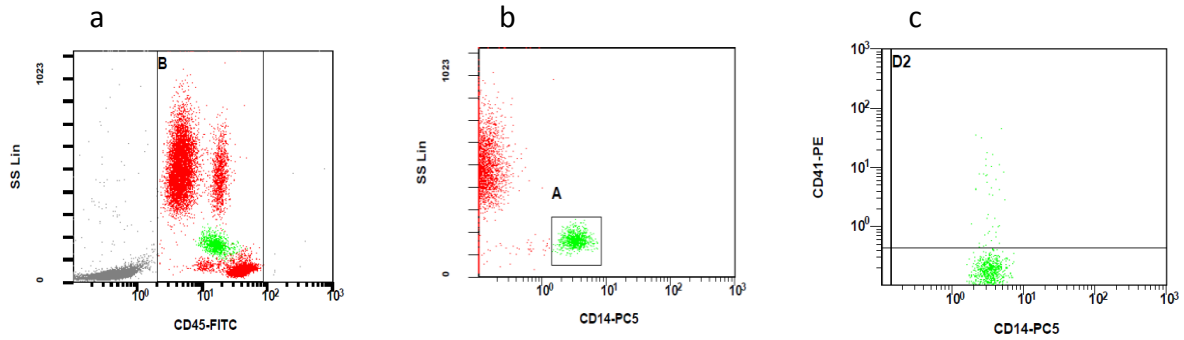


Figure 2.2: Scattergrams illustrating definition of platelet monocyte aggregates
$CD45^+$ events were gated according to their CD45 expression and light-scattering characteristics (figure 27a). Monocytes were identified by their characteristic binding of CD14 and light-scattering characteristics gated on all particles expressing CD45 (gate B) (figure 27b). Platelet monocyte aggregates were determined as the percentage of monocytes expressing platelet antigen CD41 (figure 27c).

2.5 Flow cytometric analysis of microparticles

2.5.1 Definitions of microparticles

Cell-derived microparticles were quantified based on size and fluorescence on a flow cytometer (Beckman Coulter FC500, USA). Each sample was analysed once. Three analyses were carried out (i) annexin V+CD41+ (PMP) which also used to determine annexin V+ MP (total apoptotic MP, mainly PMP) and CD41+ (PMP) counts separately, (ii) CD31+CD41+ which were used to determine CD31+ MP and CD31+CD41- EMP, and (iii) CD105+CD144+CD146+ monochrome multimarker which allowed us determine CD105/144/146+ EMP.

2.5.2 Reagents and reagent preparation

Monoclonal antibodies and their matching isotype controls directly labelled with fluorochromes were purchased from different companies, (i) annexin V-FITC

(Beckman Coulter), (ii) CD 41-PE-Cy™5 (BD Bioscience), (iii) PE-Cy™5 Mouse IgG1κ Isotype Control (BD Bioscience), (iv) CD 105-PE (BD Bioscience), (v) CD 144-PE (BD Bioscience), (vi) CD146-PE (BD Bioscience), (vii) CD 31-PE (BD Bioscience) and (viii) PE Mouse IgG1κ Isotype Control (BD Bioscience).

Antibodies were prepared by diluting in filtered phosphate buffered saline [PBS] (Beckman Coulter) as follows; (i) annexin V-FITC (1:10), (ii) CD41-PE CyTM5 (1:4 and 1:2 for washed samples and unwashed samples respectively), (iii) CD31-PE (1:3), (iv) CD105-PE (1:86), (v) CD144-PE (1:6), and (vi) CD146-PE (1:4). All isotype control samples had matched antibody concentrations. These dilutions were based on titrations. The antibodies were spun (for 5 min at 19000×g and 20^{o}C) to remove aggregates and supernatant used for incubations. Aggregates may result in considerable greater noise to signal ration.

Samples were stained with CD31-PE and CD41-PE in order to obtain CD31+CD41- EMP (MP expressing CD31 but negative for CD41). Samples were also stained with three endothelial specific monochrome antibodies (CD105-PE, CD144-PE and CD146-PE) in order to increase EMP counts. PBS-citrate buffer was prepared by first preparing 3.2% citrate solution by dissolving 1.6 g of sodium citrate in 50 mL of distilled water. PBS-citrate was then prepared in the ratio 1:9 i.e. 9 mL of PBS mixed with and 1 mL 3.2% citrate solution. The pH of the PBS-citrate was regulated to 7.4 using 0.1 M HCl and then filtered using a 0.2 μm filter.

2.5.3 Sample preparation

Platelet MP were analysed from thawed (at room temperature) plasma samples. The sample was vortexed thoroughly and 30 µL incubated for 30 min with, 5 µL of diluted annexin V-FITC and 10 µL of diluted CD41-PE. After 30 min in the dark, 500 µL of calcium containing binding buffer (Beckman Coulter) was added. A negative control was prepared by incubating 10 µL of diluted IgG1k-PE isotype and 5 µL of diluted annexin V-FITC and 500 µL of PBS (Beckman Coulter) [annexin V does not bind to MP membrane in the absence of Ca^{2+}] added after 30 min in the dark. These were analysed immediately for 2 min at low speed on a flow cytometer (Beckman Coulter FC500, USA).

Endothelial MP were analysed from thawed (at room temperature) and then washed plasma sample. The samples were washed twice with PBS-citrate. Exactly 310 µL of sample (2 vessels for each sample) was transferred into a new vessel and centrifuged for 30 min at 19000×g at 20^{o}C. After centrifugation 280 µL of supernatant was discarded appropriately and the remaining 30 µL diluted with 280 µL PBS-citrate and centrifuged for 30 min at 19000×g at 20^{o}C. After the second centrifugation 280 µL of supernatant was discarded and the remaining 30 µL pellet re-suspended in 25 µL PBS-citrate and vortexed thoroughly. The contents of the two vessels were then combined, vortexed thoroughly and used for analysis. Exactly 20 µL of washed sample was incubated with 10 µL of diluted CD105/CD144/CD146-PE cocktail or 10 µL of diluted CD31-PE and 10 µL of diluted CD41-PE-Cy™5. Two negative controls were prepared by incubating 20 µL of washed sample with 10 µL PE isotype or 10 µL PECy5

isotype. After 30 min in the dark 270 µL PBS-citrate was added and analysed immediately for 3 min at medium speed on a flow cytometer (Beckman Coulter FC500, USA).

2.5.4 Size calibration and gating strategy

Microparticles were defined as <1.0 µm and above the noise of instrument (approximately 0.5 µm). Size calibration was achieved using 0.5 µm and 0.9 µm sizing beads (Megamix, Biocytex, Marseille, France). A gate was established just outside the 0.9 µm bead population (figure 2.3) on forward scatter vs. side scatter and all events inside this gate were considered to be <1.0 µm in diameter. The 0.5 µm bead population represented the lower limit of detection of the instrument; the discriminator was set on each day so that that ratio of 0.5-0.9 µm beads was 1:1. Events excluded by the discriminator which was set to 0.5 µm were in the noise region of the instrument. We were able to standardise this lower limit of detection by ensuring the ratio of 0.5-0.9 µm beads was 1:1 on each analysis day.

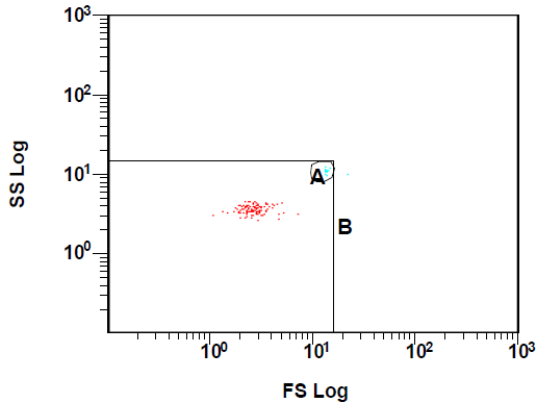


Figure 2.3: FS Log/SS Log plot used for size calibration and gating. A is the population of 0.9 µm beads and B is the gate that represents all events <1.0 µm. FS; forward scatter, SS; side scatter

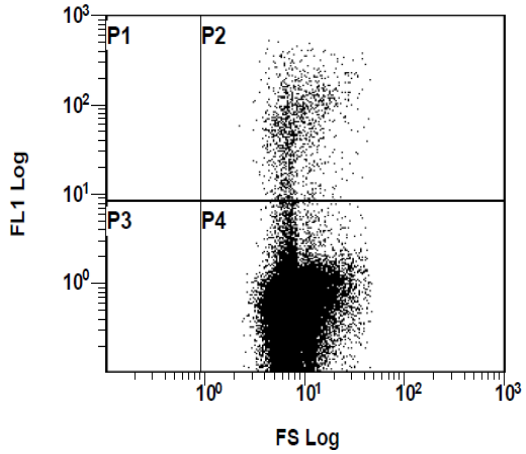
Annexin V+ MP were defined as events within this sizing gate that were positive on fluorescent channels for annexin V. Events defined as annexin V+ MP were selected for their annexin V binding determined by positivity for annexin V (y-axis). Total events in quadrant P2 were considered as the number of annexin V+ MP counted by flow cytometer on a low speed during sample acquisition for 2 min (figure 2.4a).

Platelet MP were defined as events within this sizing gate that were positive on fluorescent channels for CD41 or annexin V+CD41. Events defined as CD41+ MP were selected for their CD41 binding determined by positivity for CD41-PE-Cy™5 (y-axis). Total events in quadrant X1 and X2 were considered as the number of CD41+ MP counted by flow cytometer on a low speed during sample acquisition for 3 min (figure 2.4b). Events defined as annexin V+CD41+ PMP were selected for their annexin V and CD41 binding determined by positivity for annexin V-FITC (x-axis) and CD41-PE-Cy™5 (y-axis). A gate (AC) was drawn around the distinct cluster of events with high co-expression of annexin V and CD41. Total events in gate AC was considered as the total events counted by flow cytometer on a low speed during sample acquisition for 2 min (figure 2.4b).

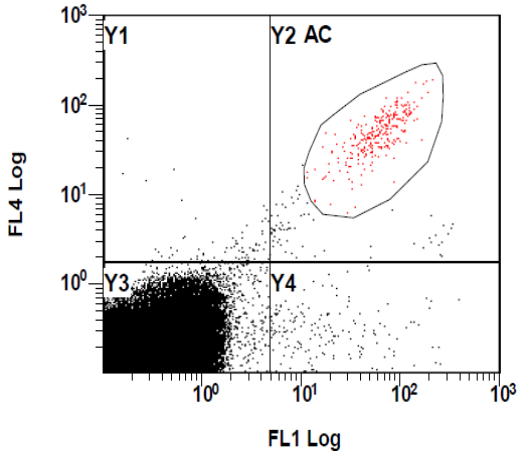
CD31+ MP were defined as events within this sizing gate that were positive on fluorescent channels for CD31. Events defined as CD31+ PMP were selected for their CD31 binding determined by positivity for CD31-PE (x-axis). Total events in quadrant X2 and X4 were considered as the number of CD31+ MP counted by flow cytometer on a low speed during sample acquisition for 3 min (figure 2.4c).

Endothelial MP were defined as events within the sizing gate that were positive on fluorescent channels for endothelial marker CD31 (but negative on fluorescent channels for CD41) or positive for either CD105, CD144 or CD146 (Samples were stained with these three endothelial specific monochrome antibodies in order to increase EMP counts). Events defined as CD31+CD41- EMP were selected for their CD31 binding and CD41 non-binding determined by positivity for CD31-PE (x-axis) and negativity for CD41-PE-Cy™5 (y-axis). Total events in quadrant X4 were considered as the number of CD31+CD41- EMP counted by flow cytometer on a low speed during sample acquisition for 3 min (figure 2.4c). Events defined as CD105/CD144/CD146+ EMP were selected for their CD105, CD144 and CD146 binding determined by positivity for either CD105-PE, CD144-PE and/or CD146-PE (y-axis). Total events in quadrant J2 were considered as the number of CD105/CD144/CD146+ EMP counted by flow cytometer on a low speed during sample acquisition for 3 min (figure 2.4d).

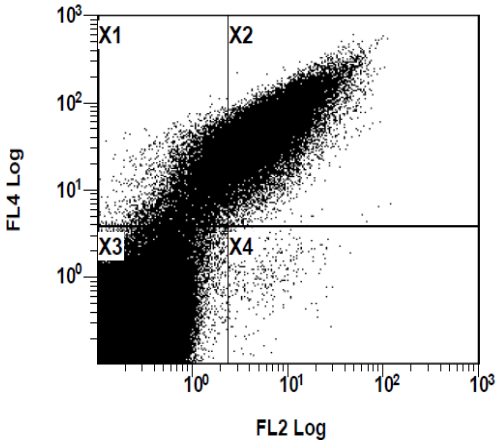
a. Annexin V-FITC/FS Log plot



b. Annexin V-FITC/CD41-PE-Cy™5 plot



c. CD31-PE/CD41-PE-Cy™5 plot



d. FS Log/CD105/144/146-PE plot

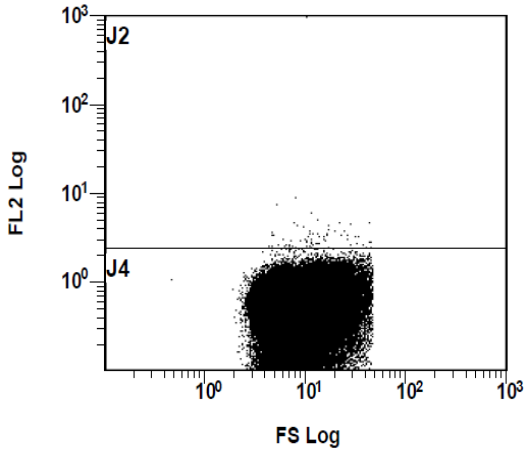


Figure 2.4: Figures illustrating definition of cell-derived microparticles
Total events in quadrant P2 were considered as the number of annexin V+ MP (a), total events in gate AC was considered as the number of annexin V CD41+ MP (b), total events in quadrant X1 and X2 were considered as the number of CD41+ MP (c), total events in quadrant X2 and X4 were considered as the number of CD31+ MP (c), Total events in quadrant X4 were considered as the number of CD31+CD41- EMP (c) and total events in quadrant J2 were considered as the number of CD105/CD144/CD146+ EMP (d).

2.5.5 Calculating events/µL

Events/µL were calculated as follows:

$$\text{Events}/\mu\text{L} = \frac{\text{Events/min}}{\text{Instrument flow rate } (\mu\text{L/min})} \times \text{dilution factor}$$

The flow rate was quantified using SPHERO^{TM} AccuCount particles (Spherotech) and calculated as follows;

$$\text{Instrument flow rate} = \frac{\text{Total events collected in 1 min in AccuCount particle gate}}{\text{Concentration of AccuCount particles in tube (particles/µL)}}$$

For microparticles quantified from unwashed plasma samples, the dilution factor was calculated as;

$$\text{Dilution factor} = \frac{\text{Total volume in tube; sample (µL)} + \text{antibodyvolume (µL)} + \text{PBS/BB (µL)}}{\text{Volume of sample (µL)}}$$

For microparticles quantified from washed plasma samples, a further adjustment was necessary to take account of the initial concentrating step when thawed plasma was centrifuged at high speed to produce a microparticle-rich pellet. Thus;

$$\text{Events/µL} = \frac{\text{Events/min}}{\text{Concentration factor}} \times \text{dilution factor}$$

$$\text{Concentration factor} = \frac{\text{Initial volume of plasma washed}}{\text{Volume of pellet produced after resuspension}}$$

Chapter 3

3. Analysing Variability in Flow Cytometry Assays Used to Enumerate Cell-Derived Microparticles

3.1 Introduction

The National Institute of Health, Working Group defines a biomarker as a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention (NIH, 2001). Biomarkers are generally considered to be systemic measurements of cells, molecules, genes or gene products, hormones, enzymes, or other proteins that provide independent predictive value by reflecting health or disease state or condition (Paramo et al., 2007). Biomarkers are measurable in tissues and body fluids and can be objectively evaluated to act as indicators of physiological or pathological processes therefore providing vital information in determining health status, disease risk and disease progression. For any biomarker to be utilised clinically, it should be accurate, reproducible and reliable. It should provide good sensitivity, specificity and predictive value. It has to add predictive value to traditional biomarkers (Paramo et al., 2007). Reproducibility reflects the degree of agreement between measurements or observations conducted on replicate specimens in different locations by different people. Good reproducibility is a key feature of any biomarker. It is typically determined from the co-efficient of variation statistic. In general, research studies will only be able to detect significant differences between populations or significant changes over time, that are greater than the co-efficient of variation (CoV) of the biomarker of interest.

There is increasing interest in cell-derived MP as vascular biomarkers. However, assay protocols used in enumeration and characterization of MP are not

standardized. Assay protocols vary significantly between laboratories making comparison of data between laboratories difficult (Jy et al., 2004). Standardization is essential for successful development of MP technologies. The development of standardized MP technologies would allow for direct comparison of results between studies and lead to a greater understanding of MP in health and disease. Although the standardization of pre-analytical and analytical variables for enumeration of MP remains a significant challenge newer approaches are being investigated. An international collaboration is working on standardization of flow cytometric detection and quantitation of MP. Issues under investigation are sizing, probing and counting (Lacroix *et al*., 2010).

Despite varied assay protocols, distinct common themes exist in MP analysis. The double centrifugation protocol eliminates large cells while minimising activation of MP. It depletes plasma of small platelets and cell debris. An example of a double centrifugation protocol include the 15 min medium speed spin at 1500×g followed by a 2 min high speed spin at 13000×g. Small platelets and large MP are of the same size order and therefore the protocol must eliminate platelets while conserving most MP. This is particularly important when MP are to be frozen as freezing and thawing will activate any remaining platelets increasing MP counts. This 1500×g/13000×g double centrifugation protocol is not the only protocol used in MP processing but is common and has been used in International Society on Thrombosis and Haemostasis working group studies which is attempting to standardize MP enumeration (Robert et al., 2009). Other common themes include minimal use of tourniquet during blood

collection to avoid frank endothelial damage and activation (Jy et al., 2004), discarding the first 3 mL to minimise contamination by endothelial injury induced microvesiculation (Goon et al., 2006), use of samples anticoagulated with sodium citrate as other anticoagulants increase MP counts (Shah et al., 2008, Kim et al., 2002), processing blood samples within 2-4 h after collection to minimise cellular activation and release of MP (Ayers et al., 2011).

It is very difficult to enumerate MP of endothelial origin due to their low frequency in the circulation (Horstman and Ahn, 1999). Several methods have been employed to improve EMP detection and enumeration using flow cytometry. One of the methods involves washing plasma samples which reduces noise and improves signal to noise ratio because it purifies sample by removing debris and other factors that circulate in the plasma. It also concentrates the sample and therefore increases counts (Simak et al., 2006). A second method involves simultaneous labelling of more than one antigen on EMP using cell-specific combinations of antibodies all labelled with the same fluorochrome (e.g. CD105-PE, CD144-PE and CD146-PE in same sample). The combination of monochrome composite markers improves sensitivity by increasing the signal to noise ratio on particles that have low surface expression of endothelial markers (Duval et al., 2010, Lacroix et al., 2010).

There are many threats to reproducibility when using flow cytometry to enumerate PMP and EMP. Sources of variability in MP analysis may include instrument, operator and biological variability. MP are 0.1-1 µm in diameter (Thery *et al*., 2009) but the lower cut-off remains to be established (Yuana *et al*., 2011). Small

size makes enumeration problematic. Conventional flow cytometers cannot reliably discriminate particles smaller than 0.3-0.5 µm from electronic noise (Perez-Pujol *et al*., 2007). In addition, because of their small size MP have a low degree of expression of specific markers. Current flow cytometers only enumerate a small portion of the total MP (Chandler et al., 2011). For MP to be considered as ideal vascular biomarkers, this small portion of MP that is detected by flow cytometry has to be enumerated reproducibly. One method of countering this problem is the use of size-calibrated fluorescent beads (Megamix, BioCytex) of diameters 0.5 µm and 0.9 µm and examining the ratio of 0.5 µm to 0.9 µm that the flow cytometer detects. Small changes in the small portion of MP that the flow cytometer actually sees could make a big difference to MP counts. This is standardised using the size-calibrated fluorescent beads (Robert et al., 2009).

Standardisation of sample processing during the double centrifugation and plasma sample washing protocols are also critical for reproducibility. There is no certainty that MP are distributed evenly in vessels after spinning. If the concentration of MP increases at increasing depths within the vessel in which plasma is processed, then standardising the volume of supernatant that is removed is absolutely critical. In addition to instrument limitations and processing, there is potential biological variability. As with any biomarker, day to day biological fluctuations are a source of variability that is not frequently quantified. Biological fluctuations are likely to increase variability above instrument limitations and sample processing. The co-efficient of variation (CoV) between samples taken on different days will not be less than that

from samples taken sequentially on the same day. Differences between samples taken from the same person on different days will reflect biological variation superimposed on sample processing variability and instrument limitations. Despite all the shortcomings that exist with MP analysis, it is possible to validate the efficacy of assays. One way is to undertake linearity checks by spiking samples with increasing volumes of MP to check if increasing counts are observed.

The purpose of these sub studies was to examine the variability involved in enumerating PMP and EMP using a protocol common in the literature and developed by expert body working groups. Four small sub studies will now be presented examining the distribution of PMP post-centrifugation within two different vessel vessels, (2) the variability in PMP and EMP counts associated with each link in the sample collection/processing/analysis chain, (3) biological variability in PMP and EMP counts over the course of one month and (4) the linearity of PMP and EMP counts when samples are spiked with increasing volumes of MP concentrate. These sub studies used blood samples from 14 apparently healthy adult volunteers (13 men and 1 woman). Blood samples were collected, processed and analysed as described in the general methodology sections 2.1, 2.2 and 2.5. Ethical approval for these sub studies was obtained from Waterford Institute *of* Technology Research Ethics Committee.

3.2 Sub study 1: Distribution of platelet microparticles post-centrifugation in two different vessels

3.2.1 Aim

To examine the distribution of PMP within the vessels in which the second centrifugation spin occurred.

3.2.2 Hypothesis

There is a layered distribution of PMP within the vessels in which the second centrifugation spin occurred.

3.2.3 Methods

In order to examine the possibility of a layered distribution of PMP within vessels following the second spin (2 min at 13,000×g) in the double centrifugation protocol, sufficient plasma was obtained from the first spin (15 min at 1,500×g) to completely fill one tapered 1.5 mL vessel and one cylindrical 1.5 mL vessel (figure 3.1). Following the second spin at 13,000×g, aliquots at increasing depths were carefully aspirated by keeping the pipette tip just below the surface of the plasma and carefully removing without disturbing layers below. Four different aliquots were aspirated from each vessel. PMP counts within each aliquot were then enumerated for 2 min at low speed on flow cytometer (Beckman Coulter FC500, USA).

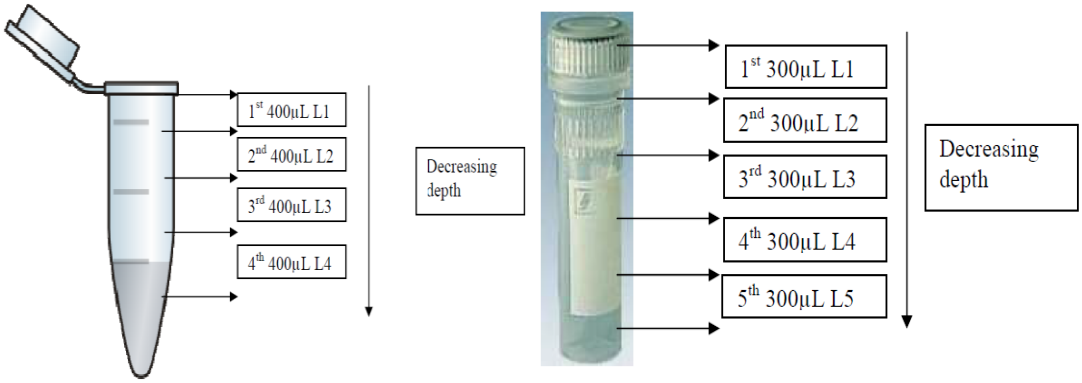


Figure 3.1: Diagram representing decreasing levels of platelet free plasma aliquoted from the tapered vessel (left) and diagram representing decreasing levels of platelet free plasma aliquoted from the cylindrical vessel (right)
Adapted from Google images
Accessed May 23rd 2010

3.2.2 Results and discussion

Table 3.1: Distribution of platelet microparticles (PMP) in a tapered vessel when platelet free plasma is spun at 1,500×g for 15 min followed by 13,000×g for 2 min (n = 1)

Descending levels	PMP (events/µL)
1 (top 300 µL)	372
2	381
3	1400
4 (bottom 300 µL)	4958

Table 3.2: Distribution of platelet microparticles (PMP) in a cylindrical vessel when platelet free plasma is spun at 1,500×g for 15 min followed by 13,000×g for 2 min (n = 1)

Descending levels	PMP (events/µL)
1 (top 300 µL)	243
2	208
3	725
4	2177
5 (bottom 300 µL)	6358

Both tapered and cylindrical vessels had different concentrations of PMP at the top and bottom of the vessel, with concentrations at the bottom being higher. For the

tapered vessel (table 3.1), levels 1 and 2 had the same PMP concentrations increasing thereafter in levels 3 to 4. Level 3 is the point at which tapering in the vessel begins suggesting that PMP tend to increase towards the tapered end of the vessel. For the cylindrical vessel (table 3.2), levels 1 and 2 had the same PMP concentrations increasing thereafter from level 3, and considerably from levels 4 to 5.

The reason for this distribution may be the short second spin (13000×g for 2 min) which does not give sufficient time for a proper pelleting of platelets and large PMP. This experiment reinforces the importance of standardisation. Two decisions were made in response to the findings. Firstly, it was decided to aspirate platelet poor plasma from the cylindrical vessel because it yielded more platelet poor plasma prior to the point where rapid increases in counts commenced. Secondly, the cylindrical vessel was filled completely and only platelet poor plasma above the 1 mL mark was aspirated to avoid going into the layer with progressively increasing counts.

A limitation of this small sub study was that it was undertaken in just one subject (n=1). We cannot be certain that a similar distribution would be found in the PMP counts of other individuals in these vessels following the 2 min high speed spin. However, even if the distribution differs from individual to individual, it is not possible or indeed valid to aspirate different volumes of plasma from vessels for different individuals. The key finding of this small sub study is that it highlights the potential for considerable variation in PMP counts using this double centrifugation protocol, even though the protocol has been adopted for multicentre standardisation exercises by the International Society for Thrombosis and Haemostasis (Lacroix *et al*., 2010). The key

implication of this sub study was the need for precise standardisation with all aspects of the double centrifugation protocol, given the potential for a layered distribution evident in this subject. In all subsequent analyses, vessels were filled to the brim and only platelet poor plasma above the 1 mL mark was aspirated. Although increasing counts were evident in both vessels at increasing depths, we decided to aspirate platelet poor plasma from the cylindrical vessels because it yielded more platelet poor plasma before the point where rapid increases in counts commenced.

3.3 Sub study 2: The variability in PMP and EMP counts associated with each link in the sample collection/processing/analysis chain

3.3.1 Aim

To determine the sources of variability in flow cytometric analysis of MP

3.3.2 Hypothesis

Microparticle sample collection, MP sample processing and the instrument used to quantify MP affect MP counts

3.3.3 Methods

3.3.1.1 Variability associated with the flow cytometer

When a single flow cytometer tube is ran twice (consecutively) through the flow cytometer, any differences in counts will be due to instrument error. A single frozen vessel containing ~350 µL plasma was removed from the freezer for each subject. Platelet MP and endothelial MP were analysed as described in the general methods (section 2.5). One flow cytometry tube was prepared for PMP analysis by removing 30 µL of sample from the vessel and incubating with annexin V-FITC and CD41-PE. After addition of binding buffer, the single tube for PMP analysis was then ran twice (consecutively) for 2 min at low speed through the flow cytometer (Beckman Coulter FC500, USA). One flow cytometry tube was prepared for EMP analysis by washing 310 µL of the remaining plasma twice and incubating 20 µL of the re-

suspended pellet with CD105/144/146-PE or 10 µL PE isotype (negative control). After addition of PBS, the single tube for EMP analysis was ran twice (consecutively) for 3 min at medium speed through the flow cytometer (Beckman Coulter FC500, USA). Reproducibility was determined by calculating CoV of the duplicate values for each individual.

3.3.1.2 Variability associated with duplicate samples

When duplicate flow cytometer tubes are prepared from the same frozen vessel, variability between tubes will be due to operator pipetting in conjunction with the sensitivity of the assay to small pipetting errors. In order to determine the variability between duplicate flow cytometer tubes prepared from the same frozen vessel, a single vessel containing ~350 µL plasma was removed from the freezer for each subject. Platelet MP and endothelial MP were analysed as described in the general methods (section 2.5). For PMP analysis, 30 µL was pipetted into two different flow cytometer tubes, which were then incubated with annexin V-FITC and CD41-PE. After addition of binding buffer, the 2 samples were then run for 2 min at low speed through the flow cytometer (Beckman Coulter FC500, USA).

Two flow cytometry tubes were prepared for EMP analysis after washing 310 µL of the remaining plasma twice in PBS-citrate. From the re-suspended pellet (90 µL), 20 µL aliquots were pipetted into separate flow cytometry tubes and incubated with CD105/144/146-PE or PE-labelled isotype control (negative control). After addition of PBS, the two tubes and the corresponding negative control tubes were ran for 3 min at

medium speed through the flow cytometer (Beckman Coulter FC500, USA). Reproducibility was determined by calculating CoV of the values for each individual.

3.2.1.3 Variability associated with blood collection and processing

When two blood tubes are taken consecutively from the same individual and processed independently using the double centrifugation protocol, some variability is likely to arise, due to small differences in sample processing. In order to determine the reproducibility associated with sample processing, two blood samples were taken consecutively from each volunteer and processed independently through the double centrifugation protocol, i.e. plasma from each blood tube was never mixed. A vessel containing ~350 µL plasma prepared from each original blood tube was subsequently removed from the freezer for MP analysis. PMP and EMP were determined from these vessels as previously described (section 2.5). Reproducibility was determined by calculating CoV of the values for each individual.

3.3.2 Results and discussion

3.3.2.1 Variability of platelet microparticle (PMP) and endothelial microparticle (EMP) within the same flow cytometry tube run consecutively through the flow cytometer

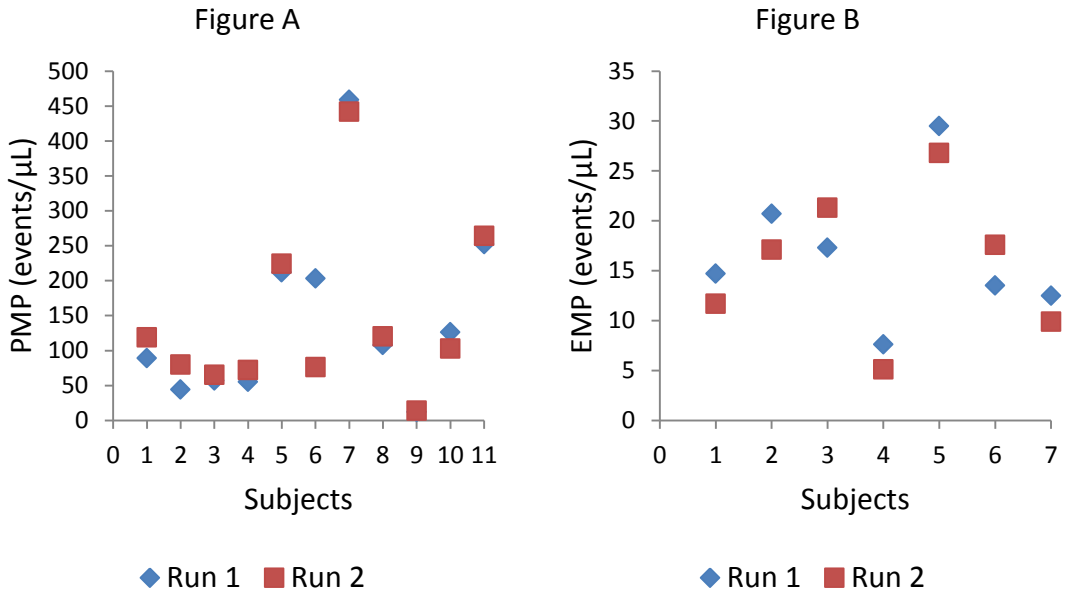


Figure 3.2: Variability of PMP (figure A) and EMP (figure B) within the same flow cytometry tube run consecutively through the flow cytometer

Figure 3.2 illustrates the PMP and EMP counts of each participant determined from the same tube ran consecutively through the flow cytometer. The median CoV between the first and second tube runs was 10.5% and 16.0% for PMP and EMP, respectively. Although there was a slight variation in both PMP and EMP counts between the first and second tube run the results suggest that the flow cytometer is capable of quantifying PMP and EMP with acceptable reproducibility. This acceptable reproducibility can be attributed to the use of MegaMix beads (Biocytex) which helps

the instrument “see” the same signal each time, even though the sample is close to the lower limit of detection of the instrument.

Previous studies found that using MegaMix beads on three different Beckman-Coulter Cytomics FC500 flow cytometers in different laboratories, gave CoV % values of PMP measurements as <12 % (Robert et al., 2009).

3.2.2.2 Variability of platelet microparticle (PMP) and endothelial microparticle (EMP) counts in two different flow cytometer tubes prepared independently from the same frozen plasma sample

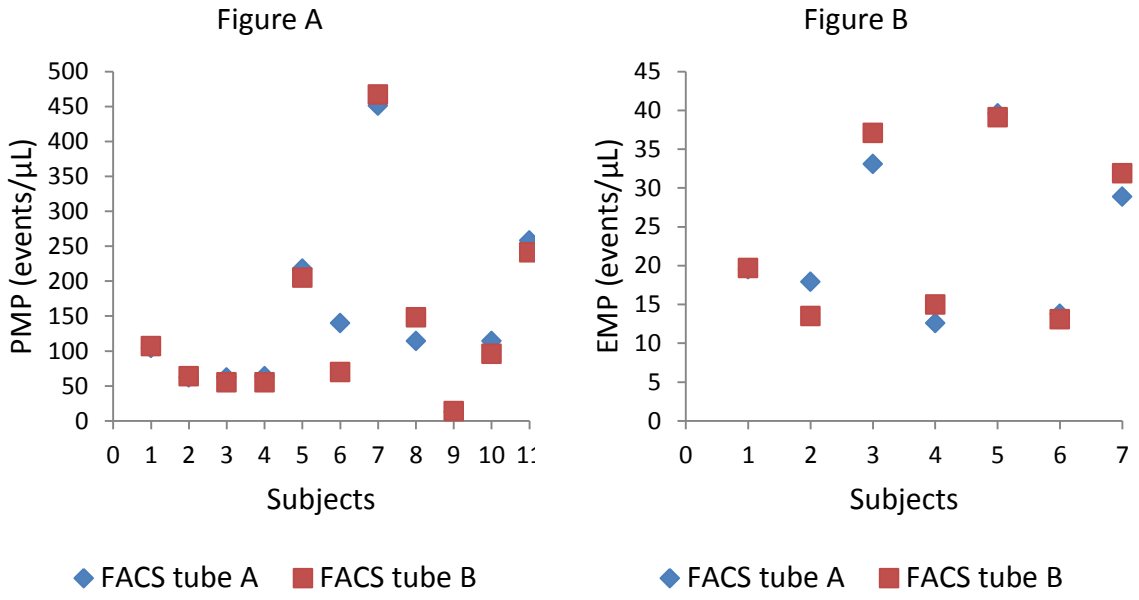


Figure 3.3: Variability of PMP (figure A) and EMP (figure B) counts in two different flow cytometer tubes prepared independently from the same frozen plasma sample

Figure 3.3 illustrates the PMP and EMP counts of each participant determined in two different flow cytometer tubes prepared independently from the same frozen plasma sample. The median CoV between the two separate flow cytometer tubes is 4.8 % and 6.9 % for PMP and EMP respectively. There was a slight variation in PMP and EMP counts in the two different flow cytometer tubes that likely reflects the additional error due to operator pipetting and the sensitivity of the assay to such minor variations. Since the CoV was very low it can be assumed that the pipetting procedure was good and that the assay was robust. The fact that the CoV was less

than that when same tube was ran twice (experiment one) is surprising considering that the pipetting error was superimposed on instrument error.

3.2.2.3 Variability of platelet microparticles (PMP) and endothelial microparticles (EMP) between two tubes of citrated blood collected consecutively from the same individual but processed independently

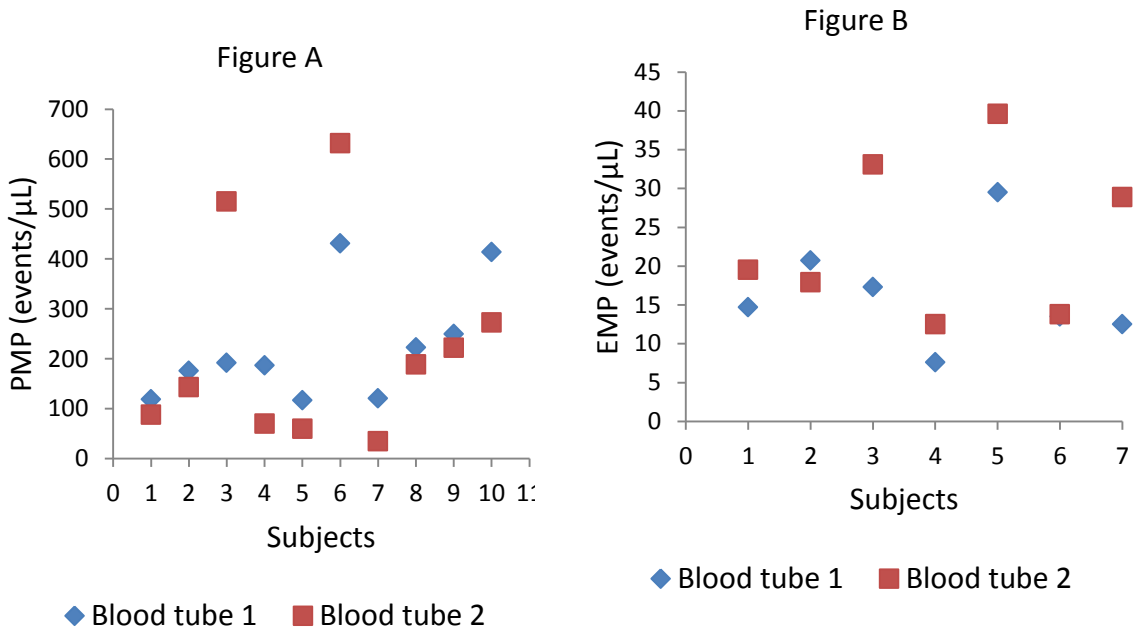


Figure 3.4: Variability of PMP (figure A) and EMP (figure B) between two tubes of citrated blood collected consecutively from the same individual but processed independently

Figure 3.4 summarised the PMP and EMP counts of each participant determined from two different blood tubes, collected consecutively from the same individual and processed independently. The median CoV was 29.0 % and 20.7 % for PMP and EMP respectively. These CoV are inclusive of outliers. The results indicate that sample processing has a considerable effect on MP counts. This has major implications for standardisation of blood collection and processing of the plasma

samples and is a major methodological issue that needs to be addressed if MP are going to be used as biomarkers to predict the risk of ACVD.

Based on the findings of this experiment the different platelet poor plasma aliquots from the same sample after the first (1500×g) and second (13000×g) spins were mixed in future experiments. This ensured that the aliquot from different vessels but from the same sample were representative of the sample.

3.4 Sub study 3: Biological variability

3.4.1 Aim

To examine the biological variability of MP counts in healthy individuals over the course of one month

3.4.2 Hypothesis

There is biological variation of MP counts in healthy individuals over the course of one month

3.4.3 Methods

In order to examine biological variability over the course of one month blood samples were obtained from participants at days 1, 3, 7, 14 and 28. These were processed as described in general methodology (section 2.2.1) and the plasma samples stored at -80 ^{o}C until analysis. One aliquot containing ~350 µL plasma was removed from the freezer for each participant for each of the five days of interest. Platelet MP and endothelial MP were analysed as described in the general methods (section 2.5). Each sample was analysed once. Reproducibility was determined by calculating CoV of the values for each individual.

3.4.2 Results and discussion

3.4.2.1 Variability in platelet microparticle (PMP) and endothelial microparticle (EMP) counts in subjects across a one month period

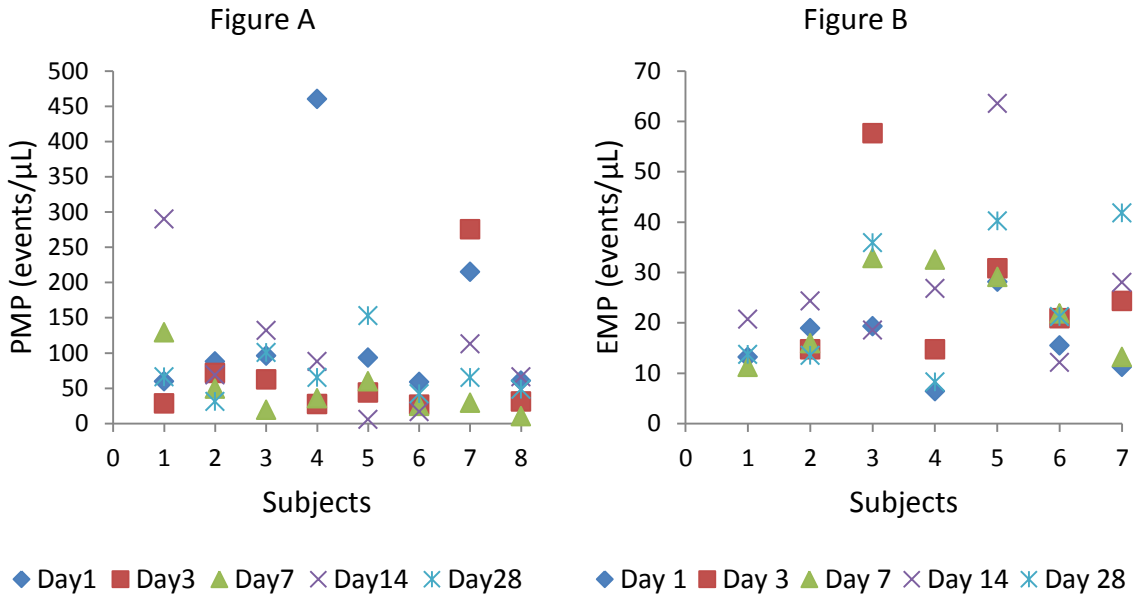


Figure 3.5: Variability in PMP (figure A) and (EMP (figure B) counts in subjects across a one month period

Figure 3.5 shows variation in PMP and EMP counts over the course of one month. The median co-efficient of variation over the course of one month was PMP 63.4 % and EMP 38.7 %. The CoV are inclusive of a number of outliers.

The biological variability of PMP and EMP counts was investigated over the course of one month and large variation in MP counts were observed over this period which likely represents biological variation superimposed on the instrument limitations, pipetting error, sample collection and processing error. The variation was minimized by ensuring that none of participants recruited in this sub study had

significant vascular risk factors, medical history, active disease or were on prescribed medication. Without controlling for these variables, the CoV may have been much higher. Although the CoV appears very high, this type of information is not readily available with other commonly used circulating biomarkers. To the best of our knowledge, this is the first study to investigate the biological variability of PMP and EMP counts in healthy individuals over the course of one month. Future work needs to investigate the cause of this variation further.

In general, research studies will only be able to detect significant differences between populations or significant changes over time that are greater than the CoV of the biomarker of interest. High variabilities have implications for the smallest difference that can be detected and may limit progress in the field. The results reported in MP studies must be interpreted in light of the large variability found in the present study. Inspection of individual participant data in the figures indicates large inter-individual variability suggesting that reproducibility was satisfactory for many of the participants but poor for others. Thus, standardisation is absolutely critical. Several steps were undertaken to standardize the assay protocols when measuring MP in the present study; (i) the first 3 mL of blood were discarded, (ii) vessels were completely filled following the first and second spins, (iii) the volume of supernatant that was removed from the vessel in which plasma was processed was standardised, and (iv) size-calibrated fluorescent beads (Megamix, BioCytex) of diameters 0.5 µm and 0.9 µm were used. It should be noted that much of the variability relates to the double centrifugation protocol used to process blood samples. Other technologies

besides centrifugation may need to be developed to isolate MP from whole blood. For example, the use of filtration of whole blood or plasma following a gentle spin of whole blood through 1 µm filters may greatly assist in reducing error.

3.5 Sub study 4: Linearity of platelet and endothelial microparticle counts in spiked assays

3.5.1 Introduction

A general principle when analysing blood samples is that the signal should increase in proportion to the amount of the analyte present in the sample. This can be checked with a linearity plot, when samples are spiked with increasing concentration of the analyte of interest.

3.5.2 Aim

To check if the flow cytometer was quantifying increasing MP counts with increasing amount of MP in the sample

3.5.3 Hypothesis

The quantity of MP detected by the flow cytometer should increase in proportion to the amount of the MP present in the sample

3.5.4 Methods

Platelet MP and endothelial MP were analysed as described in the general methods (section 2.5). Four different vessels were removed from the freezer and washed twice to produce four MP pellets. These four pellets were then combined into one vessel, vortexed thoroughly and mixed, resulting in a stock of MP concentrate. To four different flow cytometer tubes 5, 10, 15 and 20 µL of MP concentrate was added

respectively. To each tube annexin V-FITC was added. To another four flow cytometer tubes 5, 10, 15 and 20 µL of MP concentrate was added respectively. To each tube CD144-PE was added. These tubes were then analysed once for 3 min at medium speed through the flow cytometer (Beckman Coulter FC500, USA).

3.5.5 Results and discussion

3.5.2.1 Linearity of MP counts obtained by spiking samples with increasing volumes of MP concentrate

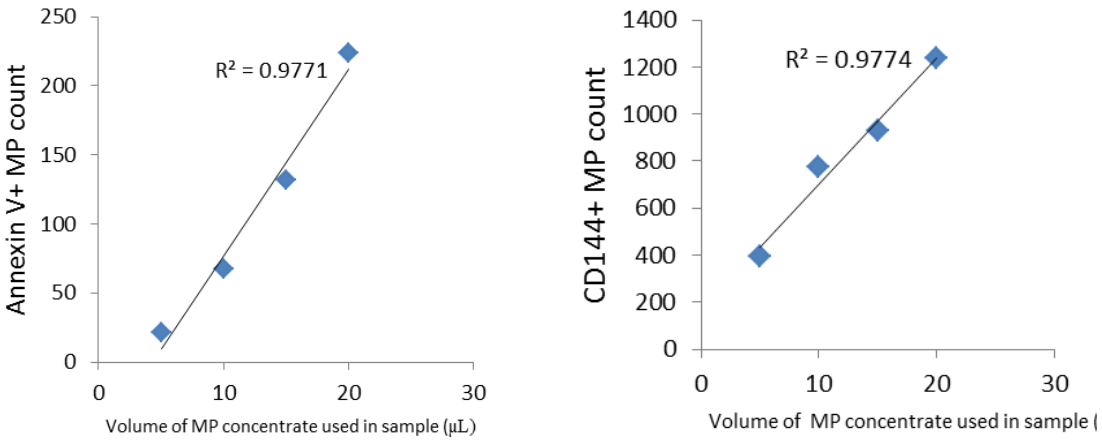


Figure 3.6: The linearity checks using annexin V and CD144.
Linearity of MP counts was obtained by spiking samples with increasing volumes of MP concentrate. The R^2 values were 0.9771 and 0.9774 for annexin V+ MP count and CD144+ MP count respectively.

Linearity checks were performed using annexin V and CD144 to determine if the increasing concentration of MP was being detected by the flow cytometer. There were increasing PMP and EMP counts with increasing volumes of MP concentration. The signal was proportional to the number of events both with PMP and EMP assays. Despite the fact that flow cytometry only detects a small proportion of total MP

events, increasing MP volumes results in an increasing signal. This provides validity to the assay, techniques and instrument and is the basis for proceeding with the experimental studies.

Chapter 4

4. The Predictive Potential of Cell-Derived Microparticles and Soluble Vascular Injury Biomarkers in Carotid Artery Disease

4.1 Introduction

Atherosclerosis is a complex multifactorial disease developing in the arterial wall in response to various forms of injurious stimuli. It occurs at the interface between blood and the inner arterial wall (endothelium) when physical or metabolic injury disrupts endothelial integrity (Hansson et al., 2006, Mallika et al., 2007, Libby et al., 2009). The triggers of endothelial cell injury and damage include bacterial or viral infection; oxidative stress through abnormal regulation of reactive oxygen species, hypoxia, turbulent blood flow and environmental irritants such as tobacco. These factors all lead to the generation of an inflammatory process and endothelial cell activation (Ross, 1999). However, lipids are regarded as the indispensable and essential trigger of atherosclerosis. Certain lipids such as low density lipoproteins (Calin et al.), very low density LDL and intermediate density lipoproteins can be retained in the intima artery wall. These lipids can then undergo oxidative modification (Berliner et al., 1997, Williams and Tabas, 1995) thereby inducing the expression of adhesion molecules, chemokines, proinflammatory cytokines, and other mediators of inflammation in macrophages and vascular wall cells. In addition, the modification may render the lipoproteins antigenic and capable of activating immune responses (Stemme et al., 1995) and inflammatory functions of vascular endothelial cells (Dichtl et al., 1999).

Following injury the endothelium transduces the perturbation into a biochemical signal altering expression of cellular adhesion molecules and other surface receptors. The adhesion molecules and surface receptors facilitate the transmigration of leukocytes, in particular monocytes and T-lymphocytes, through the endothelium

into the underlying tissue. Monocytes differentiate into macrophages that express a variety of scavenger receptors that recognize oxidised LDL-C. The macrophages take up the oxidised LDL through the scavenger receptors, accumulate the lipid, and are converted into the lipid-rich foam cells that are the hallmark of atherosclerosis. The foam cells that in turn aggregate to form fatty streaks that progress to an intermediate lesion and ultimately to a fibrous plaque (Hansson et al., 2006).

A common manifestation of generalized atherosclerosis is which involves the build-up of plaque in the carotid arteries. Carotid artery disease is closely associated with increased risk of stroke and myocardial infarction. The disease remains one of the leading causes of cerebral ischemic events (Kadoglou et al., 2008, O'Leary et al., 1999) and is responsible for 20-30% of ischemic strokes (Timsit et al., 1992). It tends to develop later in life remaining largely asymptomatic in most individuals. In advanced cases, some individual will experience warning symptoms of a transient ischemic attack.

Biomarkers are cells, molecules, genes or gene products, hormones, enzymes, or other proteins that can provide vital information in determining health status, disease risk and disease progression. A simplistic way to think of biomarkers is as indicators of disease trait (risk factor or risk marker), disease state (preclinical or clinical), or disease rate (progression) (Prentice, 1989). Accordingly, biomarkers can be classified as precursor biomarkers (identifying the risk of developing an illness), screening biomarkers (screening for subclinical disease), diagnostic biomarkers (recognizing overt disease), staging biomarkers (categorizing disease severity), or prognostic biomarkers (predicting future disease course and recurrence) (NIH, 2001).

Cells in the vasculature may release molecules into the bloodstream that can reflect the pathological processes within the vasculature (Martin-Ventura et al., 2009). Several biomarkers have been extensively studied in atherosclerosis. These biomarkers include i) protein biomarkers that are secreted or cleaved into the circulation from vascular cells following endothelial injury, ii) circulating cells involved in endothelial regression and repair, and more recently iii) plasma membrane derived vesicles that are shed into circulation from vascular cells following activation or injury.

Several molecules secreted or cleaved into the circulation from vascular cells have been shown to contribute to atherogenesis (Lind, 2003, Kampoli et al., 2009, Papazafiropoulou and Tentolouris, 2009) and may be evaluated as systemic biomarkers reflecting alterations of the main regulatory functions of the endothelium. These vascular biomarkers include soluble intercellular adhesion molecule 1 (sICAM-1), soluble vascular adhesion molecule 1 (sVCAM-1), P-selectin and E-selectin. Increases in the circulating levels of soluble molecules is accompanied by an increase in proinflammatory cytokines, including interleukins-1 (IL-1), -6 (IL-6), and tumour necrosis factor-α (TNF-α), originating at the site of pathology (Hirschfield and Pepys, 2003, Gabay and Kushner, 1999. The proinflammatory cytokines stimulate the release of inflammatory proteins such as C-reactive protein (CRP) and serum amyloid A (SAA) from the liver (Malle et al., 1993, Hirschfield and Pepys, 2003). Elevated levels of circulating inflammatory markers (Jousilahti et al., 2001, Liuzzo et al., 1994) and adhesion molecules (Guray et al., 2004, Lu et al., 2010) have been demonstrated in patients with atherosclerotic vascular disease.

There are a number of circulating vascular cells that may reflect the dynamics between injury and repair of the endothelium that may serve as vascular biomarkers. These include EPC and PMA. EPC are bone marrow derived cells that are mobilized as an endogenous repair mechanism to maintain the integrity of the endothelial monolayer (Dong and Goldschmidt-Clermont, 2007). PMA are formed when activated platelets degranulate and adhere to circulating monocytes (Palabrica *et al*., 1992). The platelet-monocyte complex is an *in vivo* functional measure of platelet and monocyte activation (Michelson et al., 2001). EPC circulate in lower numbers and have been found to have an impaired functional capacity (adhesive and migratory) in patients with atherosclerotic vascular diseases (Zhou et al., 2009, Ghani et al., 2005, Lau et al., 2007, Schmidt-Lucke et al., 2010, Wang et al., 2007a, Powell et al., 2005). Increased levels of PMA have been found in patients with atherosclerotic vascular disease (Zeller et al., 2005, McCabe et al., 2004, Garlichs et al., 2003, Wang et al., 2007b, Zhang et al., 2007, Michelson et al., 2001).

There is a growing interest in measuring the number of cell-derived MP that are shed into the circulation from vascular cells following activation or injury. In addition to acting as specific and non-invasive biomarkers, these vesicles may help to improve our understanding of the dynamics of endothelial injury (Leroyer et al., 2008). The vesicles are derived from the plasma membrane of a variety of cell and are shed into circulation (Burnier *et al*., 2009). Vascular cells increase release of MP during atherosclerosis (Tushuizen et al., 2011). Increased circulating levels of endothelial microparticles (EMP) (Bernal-Mizrachi et al., 2003, Mallat et al., 2000, Simak et al., 2006, Jung et al., 2009) and platelet microparticles (PMP) (Tan et al., 2005b, Zeiger et

al., 2000, Tan et al., 2005a, Michelsen et al., 2008) have been found in the plasma of patients with atherothrombotic diseases.

To date, only one study has compared MP concentrations between individuals with advanced carotid artery disease and age/sex-matched subjects without carotid plaques. Individuals with carotid atherosclerotic plaques had significantly higher levels of PMP than those without carotid plaques, independent of traditional cardiovascular risk factors and medical treatment (Michelsen et al., 2009).

The purpose of this study was to investigate the predictive potential of cellular and protein vascular biomarkers in identifying individuals with advanced carotid artery disease. In particular, we wanted to determine the additional predictive value of cellular biomarkers including MP, EPC and PMA over protein vascular biomarkers in a multivariable model. These biomarkers might complement each other and thus improve assessment of vascular diseases such as carotid artery disease (Koenig, 2010).

4.2 Aims

1. Determine the predictive potential of cell-derived vascular biomarkers, specifically PMP and EMP, and protein-derived vascular biomarkers in identifying individuals with advanced carotid artery disease in univariable and multivariable models.
2. Determine the additional predictive value of PMP, EMP, EPC and PMA over vascular injury biomarkers of protein origin

4.3 Hypotheses

1. Microparticles, EPC, PMA and soluble vascular injury biomarkers can predict individuals with carotid artery disease from controls without duplex ultrasound assessed carotid artery disease.
2. Microparticles, EPC and PMA add predictive value to biomarkers of protein origin in identifying advanced carotid artery disease.

4.4 Methods

4.4.1 Study overview

This was a case-control study that recruited men and women with (cases) and without (controls) documented carotid artery disease. The cases (n=42) were all patients undergoing carotid endarterectomy at Waterford Regional Hospital. The controls (n=73) were matched for age and sex and recruited from a local general practice and the locality. The absence of carotid artery disease was verified by ultrasound. A single fasting blood sample was collected from cases and controls from which the concentrations of cellular and protein biomarkers were determined (figure 4.1).

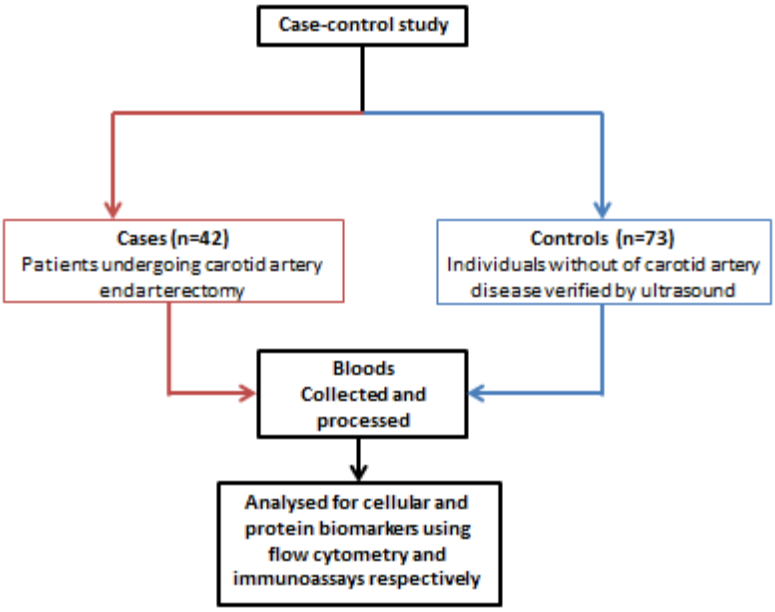


Figure 4.1: Case-Control Study Design
Forty two patients undergoing carotid artery endarterectomy and 73 individuals without carotid artery disease were recruited into this study from which bloods were collected, processed and analysed for various protein and cellular biomarkers.

4.4.2 Study population

The descriptive characteristics of the participants are summarized in table 17. Clinical and paraclinical data for the cases was obtained from their medical chart. The only exclusion criterion for this group was an inability to consent because of greatly diminished cognitive function. The majority of cases were men, smokers (current or former), with hypertension and/or diabetes and on anti-hypertensive, anticoagulant, lipid lowering and/or glucose control medications. The exclusion criteria for controls included a history of coronary artery disease, cerebrovascular disease, peripheral arterial disease, aneurysmal disease or cancer. In both groups, a sample was not taken if the participant was suffering from acute infection or inflammatory disease flare-up at the time of recruitment. Written informed consent was obtained from the

participants, and the study was approved by the Waterford Institute of Technology and Waterford Regional Hospital Research Ethics Committees.

Table 4.1: Descriptive characteristics of controls (n = 73) and cases (n = 42)

	Controls	**Cases**
Gender (%)		
Male	55	69
Female	45	31
Age (years) mean±SD	71.1 ± 0.9	69.3 ± 1.2
Smoking status (%)		
Former	33	54
Current	13	27
Conditions associated with atherosclerosis (%)		
Hypertension	22	90
High cholesterol		81
Diabetes	6	24
Medications (%)		
Hypertension	33	88
Anticoagulant	20	100
Lipid lowering	46	93
Glucose control	4	12

4.4.2 Vascular imaging of arteries

Vascular imaging was undertaken at the Waterford Regional Hospital Vascular Laboratory by a clinical ultrasound technologist using LOGIQ9 ultrasound (General Electric Healthcare, USA). Cases were imaged prior to surgery whereas controls we imaged on after recruitment. All were scanned with the carotid preset values in colour and B- modes and measurements were taken at the standard 60-degree angle of interrogation. The carotid system was imaged in cross-sectional view in colour and B-mode to check for obvious plaque especially at bifurcation level. The vertebral artery was scanned in longitudinal view - waveform was compared to common carotid artery waveform to determine whether disease was present. Common, internal and external carotid arteries were scanned in longitudinal view for plaque and any stenosis found

was graded using the Strandness criteria. All vessels were interrogated at standard 60-degree angle of interrogation.

One participant with >70 % stenosis was excluded from the study.

4.4.3 Blood sampling, processing and analysis

Sodium citrate anti-coagulated, EDTA anti-coagulated and serum clot activated bloods samples were taken from a prominent forearm vein by venepuncture with the first 3.0 mL discarded. Further details of blood sampling, processing and analyses are contained in the general methodology (section 2.1). Briefly, a complete blood cell count (appendix J) including white cells and sub fractions, red cells and platelets was undertaken in duplicates on a haematology analyser (Coulter® A$^{C\cdot}$T diff™ analyzer, Beckman Coulter, USA).

Endothelial progenitor cells and PMA were analysed as described in the general methodology (section 2.3 and 2.4 respectively). EPC were analysed using a modified ISHAGE protocol. Briefly, 100 µL of EDTA anticoagulated whole blood was stained with 20 µL CD45-FITC, 12 µL KDR/VEGFR-2-PE and 10 µL CD34-PC7 for 30 min at room temperature in the dark, then RBC lysed with 2000 µL of lysing solution for 15 min and analysed immediately for 20 min at high speed on a flow cytometer (Beckman Coulter FC500, USA). Each sample was analysed once. For PMA analysis, 100 µL of sodium citrate anticoagulated whole blood was stained with 10 µL CD45-FITC, 10 µL CD14-PC5 and 10 µL CD41-PE, incubated for 30 min in the dark at room temperature then RBC lysed with 2000 µL of lysing solution for 15 min and analysed immediately for 3 min at

medium speed on a flow cytometer (Beckman Coulter FC500, USA). Each sample was analysed once.

Cell-derived MP were analysed as described in the general methodology (section 2.5). Each sample was analysed once. For EMP analysis, plasma samples were washed twice with PBS-citrate. Two different EMP subsets were enumerated, EMP expressing CD31 but not expressing the platelet marker CD41 (CD31+CD41- EMP) and also EMP expressing CD105, CD144 or CD146 using a monochrome multimarker approach (CD105-PE/CD144-PE/CD146-PE). For determination of CD31+CD41- EMP, 20 µL of washed sample was incubated in the dark for 30 min with 10 µL of diluted CD31-PE and 10 µL of diluted CD41-PE-Cy™5. Negative controls were prepared by incubating 10 µL of matched concentrations of PE isotype to 20 µL of washed plasma sample. For determination of CD105/144/146 EMP, 20 µL of washed sample was incubated in the dark for 30 min with 10 µL of diluted CD105-PE/CD144-PE/CD146-PE cocktail. Negative controls were prepared by incubating 10 µL of matched concentrations of PECy5 isotype to 20 µL of washed plasma sample. This was followed by addition of 270 µL PBS-citrate and immediate analysis for 3 min at medium speed on a flow cytometer (Beckman Coulter FC500, USA).

For determination of PMP, frozen plasma samples were thawed at room temperature and 30 µL incubated with 10 µL of diluted CD41-PE-Cy™5 antibody and 5 µL of diluted annexin V-FITC. Negative controls were prepared by incubating 5 µL of diluted annexin V-FITC and 10 µL of matched concentrations of PE-Cy5 isotype to 30 µL of plasma sample. These were incubated in the dark for 30 min. To the positive samples, 500 µL binding buffer was added, and to the negative control samples 500 µL

of phosphate buffered saline was added. Samples were then analysed immediately for 2 min at low speed on a flow cytometer (Beckman Coulter FC500, USA).

The protocols for analysis of soluble vascular injury biomarkers, apoliproteins and lipoproteins are available in appendices K, L, M, N, O and P. Soluble vascular injury biomarkers were analysed in duplicates using commercially available immunoassays (Meso Scale Discovery, Rockville, MD, USA) which employ multiplexing technology and electrochemiluminescence detection. The COV were; C-reactive protein (4 %), sVCAM-1 (4 %), sICAM-1 (4 %), serum amyloid A (6 %), soluble E-selectin (1 %), soluble P-selectin (3 %), soluble intercellular adhesion molecule 3 (5 %) and soluble thrombomodulin (2 %). Serum cholesterol, direct HDL cholesterol, direct LDL cholesterol, triglycerides, apolipoproteins A, apolipoproteins B and glucose were determined via spectrophotometric assays performed on an automated clinical chemistry system (ACE® Wassermann B.V., Netherlands) using appropriate reagents, calibrators and controls (Randox Laboratories, UK). Each sample was analysed once.

4.4.4 Statistics

All data was checked for normality using the Kolmogorov-Smirnov test. Data that was not normally distributed was natural log transformed. One way analysis of covariance was used to compare cell-derived microparticle counts and concentrations of other biomarkers between cases and controls. The influence of lipid lowering and antiplatelet/anticoagulant medication on the biomarkers was determined in the control group. Biomarkers that were influenced by medication in the control group were then adjusted for medication usage (medication usage entered as a covariate) in

the comparison of cases and controls to determine if they were influenced by these medications. The covariates were lipid lowering and antiplatelet/anticoagulation medications. The ability of promising biomarkers to predict the presence of carotid artery disease was determined using binary logistic regression. Carotid artery disease state (case/control) was the dependent variable. The various cell-derived biomarkers and vascular injury biomarkers of protein origin constituted the independent variables. Single binary logistic regression analysis was used to determine predictive ability of individual biomarkers. Multiple binary logistic regression analysis with forward stepwise entry of variables (likelihood ratio) was used for combinations of biomarkers. Only biomarkers with $p<0.1$ in single binary logistic regression analysis were entered in the model. Four different models were used; (i) all eligible biomarkers, (ii) all biomarkers except soluble P-selectin and soluble thrombomodulin which were excluded because of biological implausibility, and (iii) all protein vascular biomarkers excluding MP. The use of different models was used to compare the predictive value of MP and protein vascular biomarkers, and to determine the predictive value of biologically plausible biomarkers only. The sensitivity and specificity of various multivariable models was also determined using receiver operator characteristic curves. The C-statistic of each receiver operator curve (ROC) represents the area under the curve (AUC). Significance was set at $p<0.05$.

4.2 Results

The influence of lipid lowering and antiplatelet/anticoagulant medication on biomarkers of interest was determined in the control group. This analysis was necessary so that the comparison of cases and controls could be adjusted for

medication usage, should it be shown to influence biomarker concentrations. Participants currently taking lipid lowering medication had lower ($p<0.05$) levels of soluble P-selectin (sP-selectin) and CRP compared to those not on lipid lowering medications (table 4.2). Participants on antiplatelet/anticoagulant medication had lower ($p<0.05$) CD31+ MP and annexin V+ CD41+ MP levels than those not on antiplatelet/anticoagulant medications (table 4.3).

When mean values are compared between carotid artery disease cases and controls, a number of biomarkers were significantly different ($p<0.05$). Annexin V+ MP, CD41+ PMP, CD31+ PMP and annexin V+CD41+ PMP were higher in cases than controls (figure 4.2), and remained significantly higher ($p<0.05$) after adjustment for antiplatelet/anticoagulant medications. Other MP analysed; CD31+CD41- EMP and CD105/CD144/CD146+ EMP (figure 4.2), circulating total PC and EPC counts and percentages (table 4.4), and PMA (figure 4.3), were not different between cases and controls.

C-reactive protein, SAA, and sVCAM-1 were higher ($p<0.05$) in cases than controls (table 4.6). C-reactive protein remained higher ($p<0.05$) after adjustment for lipid lowering medications. sP-selectin and sTM were lower ($p<0.05$) in cases compared to controls. sP-selectin was no longer significant after adjustment for lipid lowering medication. Other soluble vascular injury biomarkers analysed; soluble E-selectin (sE-selectin), sICAM-1 and sICAM-3 (table 4.6) were not different between cases and controls.

Serum APOA1, APOB, cholesterol, HDL-cholesterol and LDL-cholesterol were lower ($p<0.05$) in cases compared to controls (table 4.5). Non esterified fatty acid was higher ($p<0.05$) in cases compared to controls (table 4.5).

In single analysis using binary logistic regression sTM, sP-selectin, SAA, soluble vascular adhesion molecule 1 (sVCAM-1), CRP, annexin V+ MP, CD31+ PMP, CD41+ PMP and annexin V+CD41+ PMP had predictive value in identifying cases (table 4.7). Multiple binary logistic regression analysis with forward stepwise entry of variables (likelihood ratio) was used for combinations of biomarkers. The biomarkers with $p<0.1$ in single binary logistic regression analysis that were entered in the model were sTM, sP-selectin, SAA, sVCAM-1, CRP, annexin V+ MP, CD31+ PMP, CD41+ PMP, annexin V+CD41+ PMP, CD31+ CD41 EMP and CD105/CD144/CD146+ EMP).

In multivariable model 1 annexin V+ MP, CD105/CD144/CD146+ EMP and sP-selectin were significant in the model to predict cases (table 4.8). The C-statistic for the ROC (figure 4.4) was 0.739; 95% confidence interval: 0.638-0.841. Using the ROC in figure 4.4, when the cut-off was low enough to give 80% sensitivity (80 % of true positives correctly identified), the false positive rate (1-specificity) was 56%. Alternatively, when the cut-off was high enough to reduce the false positive rate to 20%, the sensitivity was 57%. In multivariable model 2 annexin V+ MP and CD105/CD144/CD146+ EMP were significant in the model to predict cases (table 4.9). The C-statistic for the ROC (figure 4.5) was 0.694; 95% confidence interval: 0.585-0.803. Using the ROC in figure 4.5, when the cut-off was low enough to give 80% sensitivity (80% of true positives correctly identified), the false positive rate (1-specificity) was 72%. Alternatively when the cut-off was high enough to reduce the

false positive rate to 20%, the sensitivity was 42%. In multivariable model 3 (table 4.10) sVCAM-1, sTM and SAA were present in the final model. The C-statistic (figure 4.6) for the ROC was 0.629; 95% confidence interval: 0.514-0.743. Using the ROC in figure 4.6, when the cut-off was low enough to give 80% sensitivity (80% of true positives correctly identified), the false positive rate (1-specificity) was 69%. Alternatively when the cut-off was high enough to reduce the false positive rate to 20%, the sensitivity was 19%.

4.2.1 Comparison of various biomarkers between controls and cases

Table 4.2: Influence of lipid lowering medication on cell-derived microparticles and soluble vascular injury markers in controls

	Medication		p-value
	No (n=40)	Yes (n=33)	
Soluble thrombomodulin (ng/mL)	2.9±0.1	2.7±0.1	0.46
sICAM-3 (ng/mL)	1.1±0.1	0.9±0.1	0.06
Soluble E-selectin (ng/mL)	14.1±0.7	15.2±1.1	0.41
Soluble P-selectin (ng/mL)	83.3±3.7	71.4±3.1	0.02
Soluble serum amyloid A (ng/mL)	3748.0±1264.0	3042.0±834.0	0.65
sVCAM-1 (ng/mL)	401.0±22.0	356.0±22.0	0.17
C-reactive protein (mg/L)	8.9±3.0	2.0±0.4	0.04
sICAM-1 (ng/mL)	131.0±10.2	111.0±9.0	0.16
Annexin V+ MP (events/µL)	688.0±51.0	579.0±49.0	0.13
CD31+ PMP (events/µL)	116.0±10.0	99.0±10.0	0.26
CD41+ PMP (events/µL)	91.8±8.0	80.7±8.0	0.34
Annexin V+ CD41+ PMP (events/µL)	348.0±39.0	388.0±103.0	0.69
CD31+CD41- EMP (events/µL)	0.9±0.1	2.0±1.1	0.29
CD105/CD144/CD146+ EMP (events/µL)	1.4±0.1	1.7±0.2	0.32

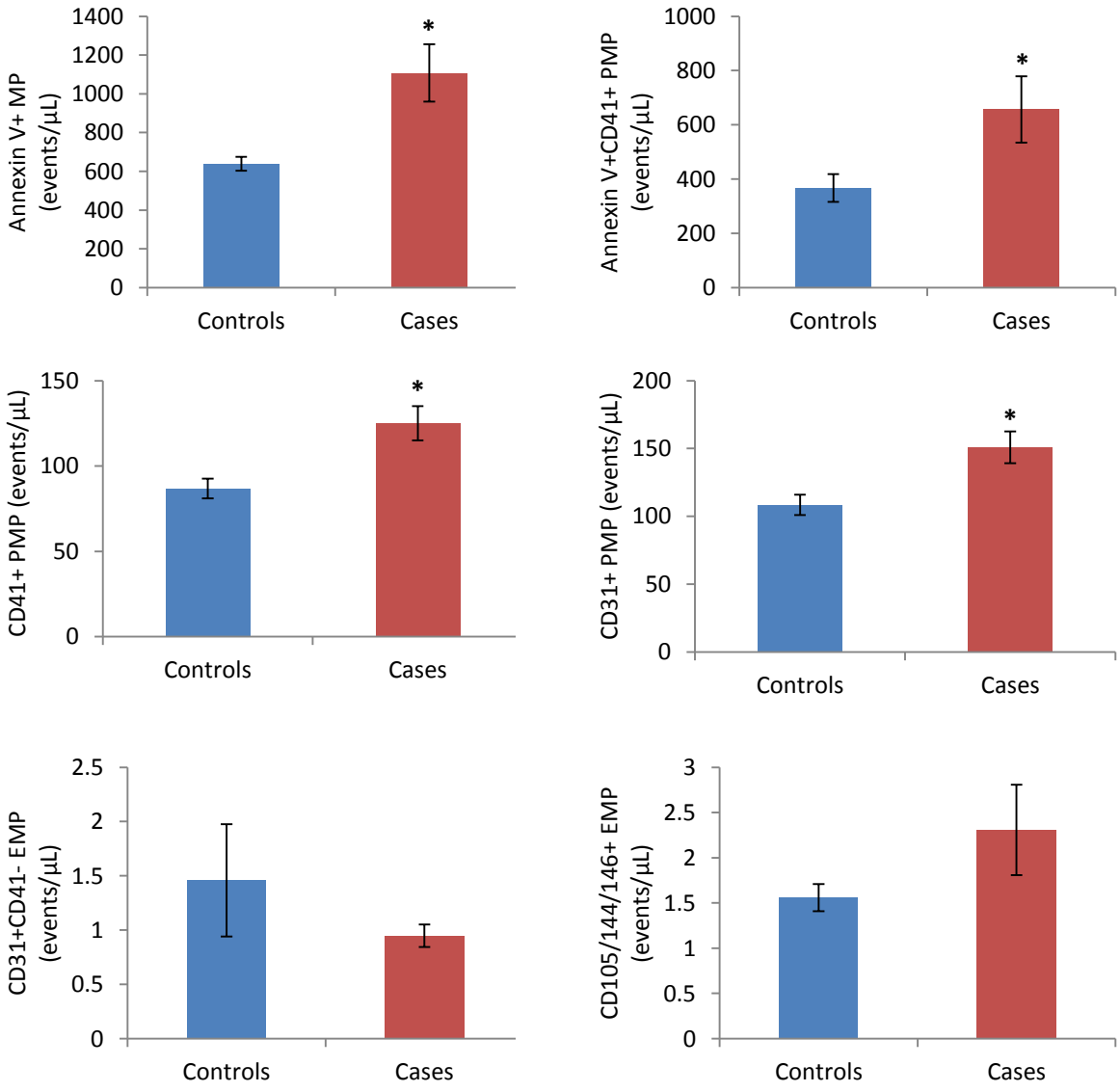
Values are mean±SEM. EMP = endothelial microparticles, MP = microparticles, PMP = platelet microparticles, SEM = standard error mean, sICAM = soluble intercellular adhesion molecule, sVCAM = soluble vascular adhesion molecule.

Table 4.3: Influence of antiplatelet/anticoagulation medication on cell-derived microparticles and soluble vascular injury markers in controls

	Medication		p-value
	No (n=59)	**Yes (n=14)**	
Soluble thrombomodulin (ng/mL)	2.80±0.10	2.90±0.20	0.86
sICAM-3 (ng/mL)	1.10±0.04	0.90±0.09	0.05
Soluble E-selectin (ng/mL)	14.10±0.60	16.30±2.00	0.21
Soluble P-selectin (ng/mL)	79.10±2.90	72.90±5.50	0.35
Soluble serum amyloid A (ng/mL)	3640.0±961.0	2541.0±567.0	0.58
sVCAM-1 (ng/mL)	369.00±16.00	427.00±45.00	0.15
C-reactive protein (mg/L)	5.50±1.90	7.10±3.80	0.71
sICAM-1 (ng/mL)	117.00±6.00	140.00±26.00	0.20
Annexin V+ MP (events/µL)	669.00±41.00	511.00±66.00	0.08
CD31+ PMP (events/µL)	114.00±8.00	82.00±13.00	0.04
CD41+ PMP (events/µL)	91.10±6.40	68.50±11.50	0.12
Annexin V+ CD41+ PMP (events/µL)	401.00±61.00	220.00±39.00	0.01
CD31+CD41- EMP (events/µL)	1.50±0.60	1.00±0.10	0.71
CD105/CD144/CD146+ EMP (events/µL)	1.40±0.10	0.10±0.30	0.29

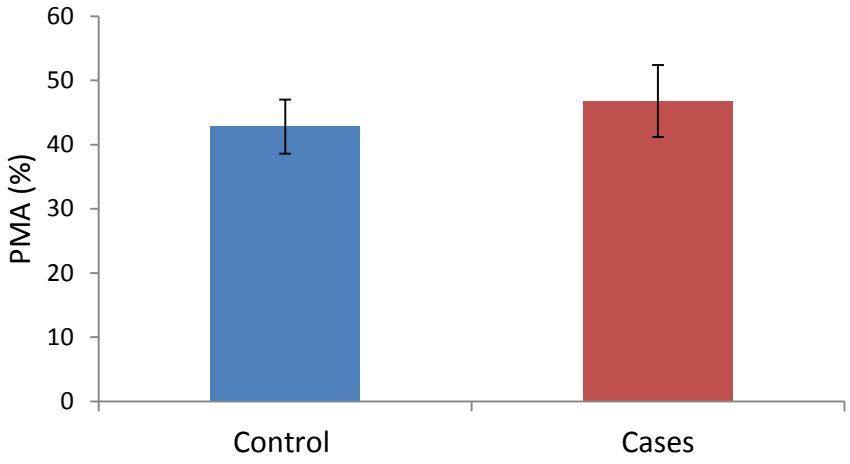
Values are mean±SEM. EMP = endothelial microparticles, MP = microparticles, PMP = platelet microparticles, SEM = standard error mean, sICAM = soluble intercellular adhesion molecule, sVCAM = soluble vascular adhesion molecule.

Figure 4.2: Annexin V+ MP, annexin V+CD41+ PMP, CD31+ PMP, CD41+ PMP, CD31+CD41- EMP and CD105/CD144/CD146+ EMP in controls (n = 69) and cases (n = 42)



**p<0.05 compared to controls. Values are mean±SEM. EMP = endothelial microparticles, MP = microparticles, PMP = platelet microparticles, SEM = standard error mean. CD105/144/146 EMP represents microparticles positive for either CD105, CD144 or CD146. Samples were stained with these three endothelial specific monochrome antibodies in order to increase EMP counts*

Figure 4.3: Platelet monocyte aggregates (PMA) in controls (n=28) and cases (n=20)



Values are mean±SEM. PMA = platelet monocyte aggregates, SEM = standard error mean

Table 4.4: Circulating progenitor cells in controls (n=28) and cases (n=20)

	Control (N=28)	**Cases (n=20)**
Total PC (events/µL)	256.0±30.0	210.0±23.0
EPC (events/µL)	21.5±3.9	24.1±5.0
Total PC (as % of total $CD45^+$ events)	0.030±0.003	0.030±0.003
EPC (as % of total $CD45^+$ events)	0.0030±0.0005	0.0030±0.0007

Values are mean ± SEM. EPC = endothelial progenitor cells ($CD34^+VEGFR\text{-}2^+CD45^{dim}$), zPC = progenitor cells ($CD34^+CD45^{dim}$), SEM = standard error mean.

Table 4.5: Serum lipids, apolipoproteins and glucose in controls and cases

	Control (N=73)	**Cases (n=42)**
Apolipoprotein A1 (mg/dL)	126.0±2.6	100.0±3.0 *
Apolipoprotein B (mg/dL)	107.0±3.0	81.1±4.4 *
Cholesterol (mmol/L)	5.0±0.1	3.8±0.1 *
HDL-cholesterol (mmol/L)	1.6±0.1	1.2±0.1 *
LDL-cholesterol (mmol/L)	3.0±0.1	2.2±0.2 *
Triglycerides mmol/L	1.2±0.1	1.4±0.1
NEFA (mmol/L)	0.60±0.03	0.80 ±0.10 *
Glucose (mmol/L)	5.6±0.1	5.2±0.2

*Values are mean±SEM. *p<0.05 compared to controls. Values are mean±SEM. HDL = high density lipoprotein, LDL = low density lipoprotein, NEFA = non-esterified fatty acid, SEM = standard error mean*

Table 4.6: Protein vascular biomarkers in controls and cases

	Control (N=73)	**Cases (n=42)**
CRP (mg/L)	5.8±1.6	14.7±4.1 *
Soluble E-selectin (ng/mL)	14.6±0.6	13.6±0.9
Soluble P-selectin (ng/mL)	77.9±2.5	68.6±3.8 *¥
SAA (ng/mL)	3429.0±784.0	11591.0±4112.0 *
sICAM-1 (ng/mL)	122.0±7.0	140±12
sICAM-3 (ng/mL)	1.07 ±0.04	1.04 ± 0.04
sVCAM-1 (ng/mL)	381.0±16.0	445.0±28.0 *
sTM (ng/mL)	2.8±0.1	2.4±0.1 *

*Values are mean±SEM *p<0.05 compared to controls. ¥Significance no longer present after adjustment for lipid lowering medication. Values are mean±SEM. CRP = C-reactive protein, sICAM = soluble intercellular adhesion molecule, SAA = serum amyloid A, SEM = standard error mean, sVCAM-1 = soluble vascular cell adhesion molecule 1, sTM = soluble thrombomodulin*

4.2.2 Predictive value of selected biomarkers

Table 4.7: Single logistic regression analysis to predict patients with carotid artery disease

Biomarker	Wald[a]	p-value[b]	Odds Ratio[c]
Soluble thrombomodulin	5.902	0.002	0.560
Soluble P-selectin	4.081	0.043	0.982
Serum amyloid A	4.122	0.042	1.000
sVCAM-1	4.035	0.045	1.003
C-reactive protein	4.381	0.036	1.024
Annexin V+ MP	12.327	0.001	1.056
CD31+ PMP	8.619	0.003	1.009
CD41+ PMP	10.005	0.002	1.012
Annexin V+ CD41+ PMP	4.547	0.033	1.032

Values are mean±SEM. MP = microparticles; PMP = platelet microparticles; sVCAM-1 = soluble vascular cell adhesion molecule 1. [a]Wald = Logistic regression statistic calculated for the variables in the model to determine whether a variable should be removed. Higher values denote greater predictive value in univariable model. [b]p-value = Significance of Wald statistic. [c]Odds ratio = increase in likelihood that an individual is a case for every one unit increase in the biomarker

Table 4.8: Multiple logistic regression model containing all eligible biomarkers from single logistic regression analysis to predict patients with carotid artery disease

	B	SEM	Wald	p-value	Odds Ratio
Annexin V+ MP	0.002	0.001	15.654	<0.001	1.002
CD105/CD144/CD146+ EMP	0.293	0.124	5.577	0.018	1.34
Soluble P-selectin	-0.027	0.012	5.712	0.017	0.973
Constant	-1.106	0.904	1.496	0.221	0.331

EMP = endothelial microparticles; MP = microparticles; SEM = standard error mean. p<0.05 indicates that a biomarker significantly adds predictive value to the multivariable model

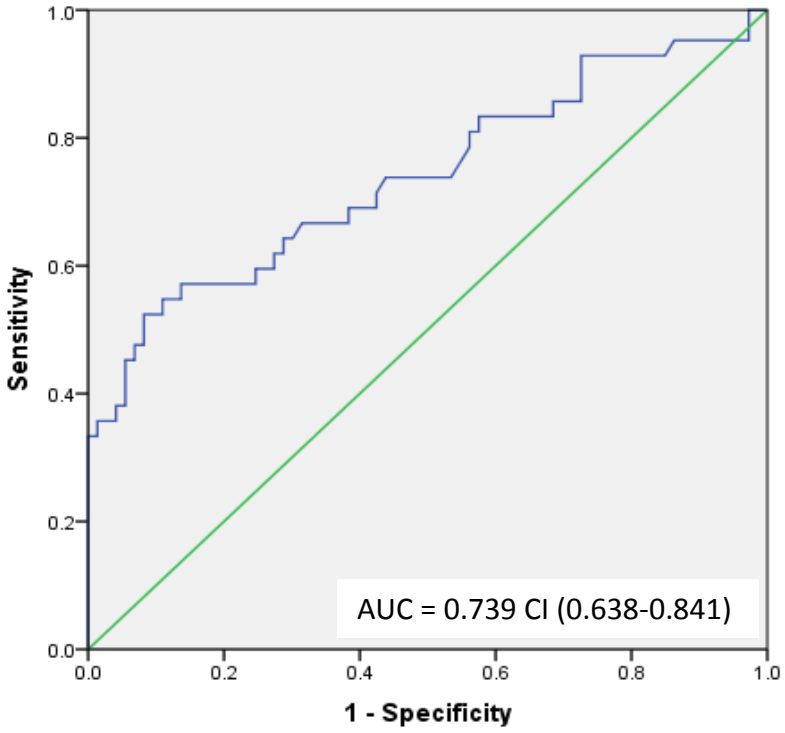


Figure 4.4: Receiver Operator Curve to predict patients with carotid artery disease based on all eligible biomarkers from single logistic regression analysis to predict patients with carotid artery disease.

AUC = area under curve; CI = confidence interval

Table 4.9: Multiple logistic regression model containing all biomarkers except soluble P-selectin and soluble thrombomodulin to predict patients with carotid artery disease

	B	SEM	Wald	p-value	Odds Ratio
Annexin V+ MP	0.002	0.001	13.082	<0.001	1.002
CD105/CD144/CD146+ EMP	0.213	0.102	4.337	0.037	1.237
Constant	-2.466	0.533	21.416	<0.001	0.085

EMP = endothelial microparticles; MP = microparticles; SEM = standard error mean. p<0.05 indicates that a biomarker significantly adds predictive value to the multivariable model

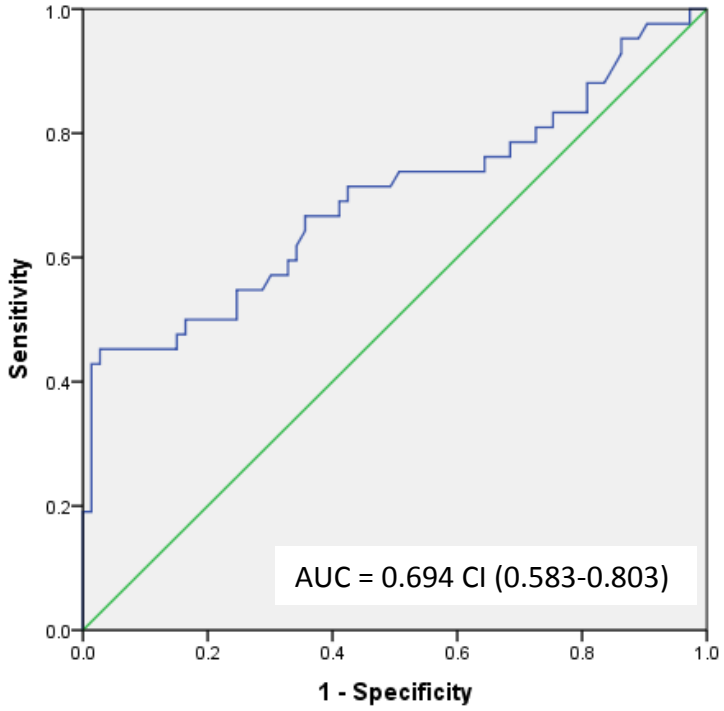


Figure 4.5: Receiver Operator Curve to predict patients with carotid artery disease based on all biomarkers except soluble P-selectin and soluble thrombomodulin

AUC = area under curve; CI = confidence interval

Table 4.10: Multiple logistic regression model containing all protein vascular biomarkers excluding cell-derived microparticles to predict patients with carotid artery disease

	B	SEM	Wald	p-value	Odds Ratio
Serum amyloid A	0.000	0.000	2.558	0.11	1
sVCAM-1	0.004	0.002	4.943	0.026	1.004
Soluble thrombomodulin	-0.873	0.299	8.54	0.003	0.418
Constant	-0.183	0.761	0.058	0.81	0.832

SEM = standard error mean; sVCAM-1 = soluble vascular adhesion molecule 1. p<0.05 indicates that a biomarker significantly adds predictive value to the multivariable model

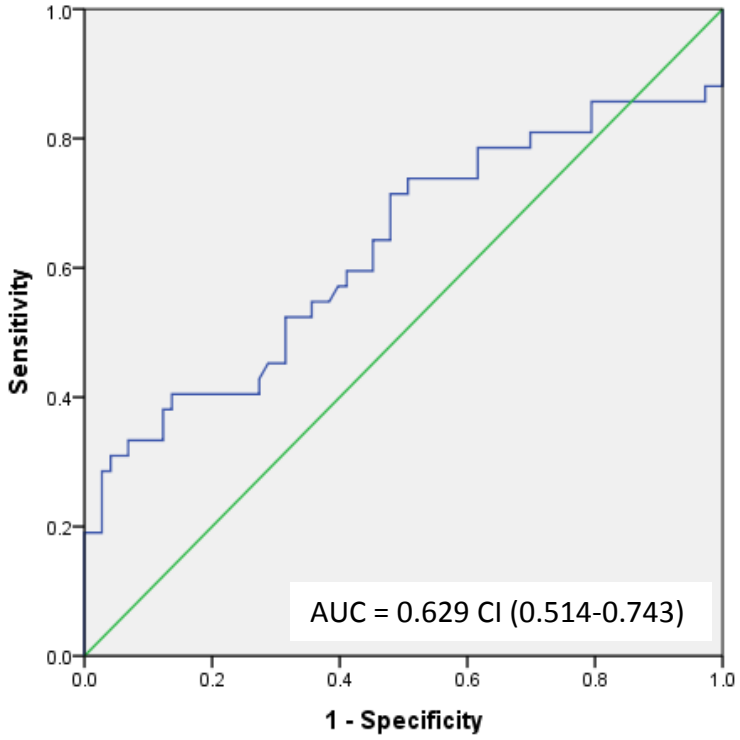


Figure 4.6: Receiver Operator Curve to predict patients with carotid artery disease based on all protein vascular biomarkers excluding cell-derived microparticles

AUC = area under curve; CI = confidence interval

4.3 Discussion

4.3.1 Introduction

This study investigated the predictive potential of MP as biomarkers of carotid artery disease. MP had the highest predictive ability compared to soluble vascular injury biomarkers, and added predictive value to soluble vascular injury biomarkers. Whether sP-selectin and sTM are included or excluded from models, annexin V+ MP and CD105/144/146 EMP were significant in multivariable models and these multivariable models had higher predictive ability compared to models that relied solely on soluble vascular injury biomarkers.

4.3.2 The discriminative and predictive value of cell-derived microparticles

Annexin V + MP and PMP were higher in cases compared to controls. Annexin V+ MP were 2-fold higher in cases that controls whereas CD31+ MP and CD41+ MP were 1.5-fold higher in cases compared to controls. Annexin V + MP, annexin V CD41+ MP, CD31+ MP and CD41+ MP had predictive value in identifying men and women with carotid artery disease. However, annexin V+ MP had the highest Wald values of any predictor in univariate analysis. Although other platelet derived MP were analysed and added in the multivariate logistic regression, only annexin V+ MP remained positive in multivariate logistic regression analysis. This is not surprising as nearly all annexin V+ MP are derived from platelets. Moreover, annexin V+ MPs were higher in the cases despite the fact that nearly all participants in this group were on antiplatelet/anticoagulant medication. The elevated levels of annexin V+ MP and PMP

in patients with advanced carotid artery disease may be indicative of pronounced platelet activation.

The multivariable models containing annexin V+ MP and CD105/CD144/CD146+ EMP, had a higher C-statistic if MP were omitted from the model and only soluble vascular injury biomarkers were. This holds whether or not sTM and sP-selectin were included in the multivariable model. To our knowledge this has not been reported previously.

This is the first study to evaluate the ability of annexin V+ MP and PMP to discriminate and predict carotid artery disease, in addition to adding predictive value to the multivariable models. The findings are consistent with Nozaki *et al., (2009)* who reported that a combination of Framingham risk, hsCRP and EMP predicted future cardiovascular events in patients at high risk for coronary heart disease. The inclusion of EMP in the model gave the highest C-statistic value (0.763). This is the only study that has used a prospective follow-up to examine the prognostic potential of MP in (Nozaki et al., 2009).

Combining vesicles shed by vascular cells with proteins secreted or cleaved into the circulation by similar cells as biomarkers may be a novel approach of predicting cardiovascular diseases and a reflection of the pathological process within the vasculature. Taken together, these findings support the view that MP as a single biomarker or in a multiple biomarker strategy may identify individuals with advanced carotid artery disease. However, multiple biomarker approaches containing MP having the highest predictive ability. These novel biomarkers might complement traditional

biomarkers by adding to their predictive value thus improving the assessment of vascular diseases such as carotid artery disease.

This study did not find a significant difference in CD105/CD144/CD146+ EMP levels between cases and controls. One possible reason may be due to the fact that EMPs are very low in the circulation. However, washing and triple-labelling plasma samples with endothelial specific monochrome protein markers increased the number of EMP counts due to a reduction in instrument electronic noise thus. Although CD105/CD144/CD146+ EMP levels were not significantly different in simple comparisons and were just short of statistical significance using simple logistic regression, they do add predictive value in multivariable models. CD105, CD144 and CD146 are constitutive markers expressed following endothelial cell apoptosis (Jimenez et al., 2003). The increase in EMP bearing these constitutive markers normally represents an increase in EC apoptosis. I t is therefore tempting to speculate that increased atherosclerotic processes lead to apoptosis of EC and release of apoptotic EMP.

4.3.3 The predictive value of soluble vascular injury biomarkers

Cases had lower levels of soluble P-selectin and soluble TM compared to controls. These findings are distinct from previous studies that have reported increased levels of soluble P-selectin in patients with carotid artery disease (Blann et al., 1997b, Frijns et al., 1997). However, there are contradictory reports in relation to soluble TM. Studies have reported elevated levels (Blann et al., 1997a, Blann et al., 1996, Seigneur et al., 1993, Blann et al., 1997b) and reduced levels (Laszik et al., 2001)

of soluble TM in patients with atherosclerosis. The lower levels of P-selectin and soluble TM in the carotid artery disease cases may be due to their medication regimen. The influence of both lipid lowering and antiplatelet/anticoagulant medications on the full range of biomarkers was examined by comparing biomarker concentrations to healthy age-matched controls. Participants in the control group on lipid lowering medications had significantly lower levels of soluble P-selectin compared to those not on the medication. The significant difference between cases and controls was no longer present after adjusting lipid lowering medications. It is likely therefore; that the lower levels of soluble P-selectin found in the present study may be due to the medications. It is questionable therefore whether or not P-selectin should be included into the multivariable models. Moreover, it is unclear whether sTM levels are increased or decreased in the presence of endothelial damage. Until the issue as to why it is lower in disease states in some studies and higher in others is clarified, it may be unwise to include it in predictive models.

This study also quantified levels of inflammatory biomarkers CRP and SAA, and soluble molecules secreted or cleaved into circulation from vascular cells, sVCAM-1, sICAM-1 and sICAM-3. Inflammatory markers CRP and SAA were elevated in the cases compared to the controls. Previous studies have reported elevated CRP in carotid artery disease (Rerkasem et al., 2002, Alvarez Garcia et al., 2003) and SAA in coronary disease (Liuzzo et al., 1994, Johnson et al., 2004, Jousilahti et al., 2001, Fyfe et al., 1997). As CRP and SAA are acute phase proteins that may increase 10000- and 1000-fold respectively in various inflammatory diseases, increased concentration in the cases may be indicative of the inflammatory nature of carotid artery disease (Pepys et

al., 1985). Soluble VCAM-1 is up-regulated on the vascular endothelium in the injured or inflamed state and soluble concentrations reflect endothelial surface expression (Blankenberg et al., 2003, Olivot et al., 2004). In addition to being significantly different; CRP was significant in single logistical regression analysis but was eliminated by other factors in the multivariable logistic regression models. Soluble ICAM-1 and sICAM-3 were not different between the two groups.

4.3.4 The predictive value of circulating vascular cells

There was no difference in the number of progenitor cells, circulating endothelial progenitor cells and platelet monocyte aggregates between cases and controls. These biomarkers were however only quantified in a subset of cases and controls. Additional numbers of cases and controls are therefore highly unlikely to yield different results. Previous studies have reported conflicting findings in patients with atherosclerosis. EPC were found to be lower in patients with cerebrovascular disease compared to age- and gender-matched volunteers (Zhou et al., 2009), carotid atherosclerosis compared to age- and sex-matched subjects who had no history of cardiovascular or cerebrovascular diseases (Lau et al., 2007), coronary artery disease compared to healthy subjects without any evidence of coronary artery disease by history and physical examination (Schmidt-Lucke et al., 2010) and coronary artery disease compared to subjects with normal coronary arteries (Wang et al., 2007a). In contrast, EPC were higher in patients with cerebrovascular disease (Yip et al., 2008). Findings from this study confirm that EPC are extremely rare and representing only a minute cell population in peripheral blood.

This study also measured levels of circulating platelet monocyte aggregates. There was no difference in percentage of monocytes expressing platelet antigen CD41 between cases and controls. Previous studies have found increased levels of platelet monocyte aggregates in cerebrovascular disease (Zeller et al., 2005, McCabe et al., 2004) and coronary artery disease (Wang et al., 2007b, Zhang et al., 2007). However, it is difficult to explain the difference between the present study and results from previous studies.

4.3.5 Lipids, apolipoproteins and glucose as biomarkers of carotid artery disease

Apolipoprotein A1, apolipoprotein B, cholesterol, HDL-cholesterol, LDL-cholesterol and glucose were lower in cases compared to controls. NEFA was higher in cases compared to controls. These findings are consistent with a study by Zenker and colleagues who found that patients with cerebrovascular disease had lower total cholesterol, HDL-cholesterol, LDL-cholesterol than controls without coronary artery disease (Zenker et al., 1986). However, other studies have found higher levels of lipids, and apoliproteins in patients with carotid artery and cerebrovascular diseases compared to age-matched and sex-matched controls (Debing et al., 2008, Zhou et al., 2009). In the present study 93% of patients were on lipid lowering medication compared to 46% of controls. In addition, 12% of patients were on glucose control medication compared to 4% of controls. This difference in medication use might to explain why cases had lower levels of lipids, apolipoproteins and glucose. In any case, lipids and apolipoproteins have effectively little role as biomarkers or predictors of carotid artery disease in populations in which the use of lipid lowering medications are very prevalent.

4.3.6 Study limitations

The majority of cases recruited in this study were symptomatic. It would have been more advantageous assessing the potential of these biomarkers using asymptomatic patients rather than symptomatic patients. Of greater value would be an examination of the potential of these biomarkers to predict carotid artery disease before patients exhibit TIAs and other neurological symptoms.

The majority of the cases were also heavily medicated on lipid lowering and antiplatelet/anticoagulant medications. It is well established that these medications influence the levels of circulating biomarkers. Although we added medication usage as a covariate in the analysis, this may not have completely accounted for the medication effect as we were unable to capture the specific drug type and dosage for the majority of the study participants. It should also be emphasised that flow cytometry can only measure a small portion of total MP. For MP to be considered as ideal cardiovascular biomarkers, this 'tip of iceberg' that is detected by flow cytometry has to be enumerated reproducibly. Nonetheless, MP assays are among the most promising cardiovascular biomarkers because MP can be measured in platelet-poor plasma and appear stable even if frozen for months provided that platelets are removed prior to freezing.

4.3.7 Conclusion

Taken together, the study findings suggest that annexin V+ MP, PMP, CD105/CD144/CD146+EMP, sP-selectin, sTM, CRP, SAA and sVCAM-1 may act as mediators and biomarkers of carotid artery disease. They may be involved in carotid

artery disease from initiation to onset of complications, and may be evaluated as biomarkers to depict adverse changes accompanying advanced carotid artery disease. In conclusion, this study has demonstrated the predictive potential of MP and soluble vascular injury biomarkers in single or multiple biomarker strategies. MP had the strongest predictive ability, and added predictive value in multivariable models. Despite the limitations, MP are worth pursuing as biomarkers of carotid artery disease and further studies are therefore warranted.

Chapter 5

5. The Predictive Potential of Cell-Derived Microparticles in Assessing the Stability of Carotid Artery Plaques

5.1 Introduction

Atherosclerotic plaque stability is determined by the level of SMC proliferation, rate of collagen synthesis relative to degradation by proteolytic enzymes such as MMP (Welgus et al., 1990, Galis et al., 1994). Reduced collagen synthesis and increased degradation results in the development of unstable or vulnerable plaques that are more likely to weaken and rupture (Halvorsen et al., 2008, Arroyo and Lee, 1999) leading to thromboembolic events and cerebrovascular ischemia (Naghavi et al., 2003). Stable plaques although unlikely to rupture may impair cerebral blood flow.

Atherosclerosis is a multifocal systemic disease that is associated with diffuse lesions in the arterial tree. Consequently it is likely that the quality and content of carotid artery plaque is reflective of plaque throughout the vascular tree (Chen et al., 1995, Kaski et al., 1995, Goldstein et al., 2000, Rioufol et al., 2002, Kerensky et al., 2002).

Although carotid artery disease can be easily evaluated using ultrasound imaging and treated surgically, it is currently difficult to determine whether the disease will become symptomatic or to predict when symptoms will occur. Simple quantification of carotid artery disease based on the degree of stenosis has limitations (Sharma et al., 2009) and does not always provide information on the degree of plaque vulnerability. There are also risks associated with carotid endarterectomy or carotid artery stenting (Hermus et al., 2010). The ability to identify carotid plaques which confer excess risk of neurologic events in symptomatic or asymptomatic patients would greatly assist in the selection of patients for vascular intervention.

Circulating biomarkers are molecules secreted from vascular cells or cleaved from vascular cell membranes. These include a number of atherogenic mediators involved in plaque stability. To date only a few atherogenic mediators have been investigated as risk markers for unstable carotid plaque. These include CRP (Alvarez Garcia et al., 2003), MMP-9 (Alvarez Garcia et al., 2004), MMP- 1,2,3,7 and 9 (Pelisek et al., 2009), (Sapienza et al., 2005). There are other atherogenic mediators that participate in lesion formation, propagation, and rupture, that are worthy of investigation as risk markers of unstable carotid disease. These include SAA (Liao et al., 1994), adhesion molecules (Brevetti et al., 2006), proteolytic enzymes (Ardans et al., 2001) and angiogenic factors (Inoue et al., 1998, Hauer et al., 2009, Arici and Walls, 2001).

Circulating biomarkers investigated to date are unable to identify carotid plaques which confer excess risk of neurologic events in symptomatic patients. There is therefore increasing interest in novel protein and cellular biomarkers that may identify symptomatic patients with unstable plaques that would benefit most from treatment. One cellular protein that has generated a lot of interest in the recent past is MP. Cell-derived microparticles are <1.0 μm diameter plasma-derived vesicles that are shed into circulation by cells such as platelets, leukocytes and EC following activation or injury. These have been identified as a putative novel cellular biomarker to assist with identifying symptomatic patients with unstable plaques that would benefit most from treatment (Burnier *et al*., 2009).

Higher levels of thrombogenic MP of different cellular origin have been found in human atherosclerotic plaques than in plasma (Leroyer et al., 2007). MP may

contribute to plaque destabilisation as they are proinflammatory (Barry *et al*., 1997, Mause *et al*., 2005, Jy *et al*., 1995, MacKenzie *et al*., 2001, Mesri and Altieri, 1999), procoagulant (Forlow et al., 2000, Simoncini et al., 2009, Gilbert et al., 1991, Spek, 2004) and proangiogenic (Barry *et al*., 1997, Barry *et al*., 1999).

There is some evidence that MP can predict the more advanced and acute stages of cardiovascular disease. Higher circulating levels of MP have been found in patients with (i) acute coronary syndrome than patients with stable angina (Bernal-Mizrachi et al., 2003, Mallat et al., 2000), (ii) severe stenosis and high risk lesions compared to those with milder stenosis and low risk lesions (Bernal-Mizrachi et al., 2004), (iii) moderate-severe stroke patients compared to mild stroke patients (Simak *et al*., 2006) and (iv) critical limb ischemia compared to those with intermittent claudication (Tan et al., 2005a). To date no study has examined the ability of MP to distinguish stable from unstable carotid artery disease.

This study therefore investigated the predictive potential of MP (platelet and endothelial MP), and protein vascular biomarkers (vascular injury, angiogenic and proteolytic markers), in identifying individuals with unstable carotid artery disease. In particular, we wanted to determine the additional predictive value of MP over protein vascular biomarkers in a multivariable model. These biomarkers might complement each other and thus aid in evaluating risk of unstable plaques, particularly in asymptomatic patients.

5.2 Aims

1. To investigate the predictive potential of Cell-derived microparticles and soluble cell adhesion molecules, angiogenic and proteolytic factors in identifying individuals with unstable carotid artery disease in univariable and multivariable models.
2. To determine the additional predictive value of PMP and EMP over protein based biomarkers, specifically soluble cell adhesion molecules, angiogenic and proteolytic factors in identifying unstable carotid plaques.

5.3 Hypotheses

1. Microparticles and protein vascular biomarkers can predict unstable carotid artery disease
2. Microparticles add predictive value to protein vascular biomarkers in identifying unstable plaques

5.4 Methods

5.4.1 Study overview

This was a comparative study that involved 41 men and women undergoing endarterectomy for occlusive disease of the carotid artery. Plaques were analysed post-surgery using immunohistochemistry and classified as stable or unstable using an established technique. A single fasting blood sample was taken on the morning of surgery from which the concentration of cell-derived microparticles and protein biomarkers were determined.

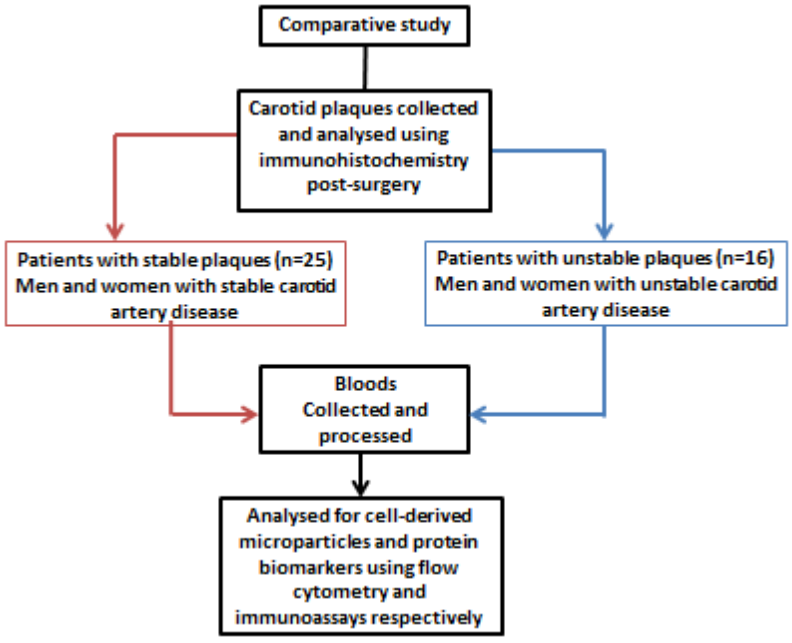


Figure 5.1: Stable-unstable plaque study design
Forty one patients were classified into stable or unstable plaques based on immunohistochemical analysis of their carotid plaques. 25 patients had stable plaques whereas 16 had unstable plaques.

5.4.2 Subject recruitment

A total of 42 patients were recruited, however only 41 patients were included in the statistical analysis because plaque from one patient was not collected. The

descriptive characteristics of the participants are summarized in table 5.1. The only exclusion criterion was an inability to consent because of greatly diminished cognitive function. Only the first surgery in those cases that had both left and right carotid endarterectomy was included in the analysis. Informed written consent was obtained from the participants, and the study was approved by the Waterford Institute *of* Technology and Waterford Regional Hospital Research Ethics Committees.

Table 5.1: Descriptive characteristics in cases with stable (n = 25) and unstable (n = 16) plaques

	Stable	**Unstable**
Gender (%)		
Male	72	69
Female	28	31
Age (years) mean±SD	70.3 ± 1.6	68.1 ± 2.0
Surgical site (%)		
Right carotid artery	52	44
Left carotid artery	48	56
Main diagnosis (%)		
Asymptomatic	16	0
Amaurosis fugax	20	6
Transient Ischaemic attack	64	94
Smoking (%)		
Former	57	53
Current	19	40
Conditions associated with carotid artery disease (%)		
Hypertension	92	87
High cholesterol	80	87
Diabetes	28	19
Medications (%)		
Antihypertensive	88	87
Antiplatelet/anticoagulant	100	100
Lipid lowering	92	94

5.4.2 Blood sampling, processing and analysis

Sodium citrate anti-coagulated, EDTA anti-coagulated and serum clot activated bloods samples were taken from a prominent forearm vein by venepuncture with the first 3.0 mL discarded. Further details of blood sampling, processing and analyses are

contained in the general methodology (section 2.1). Briefly, a complete blood cell count (appendix J) including white cells and sub fractions, red cells and platelets was undertaken on a haematology analyser (Coulter® AC.T diffTM analyzer, Beckman Coulter, USA). Each sample was analysed once.

Cell-derived MP were analysed as described in the general methodology (section 2.5). Each sample was analysed once. For EMP analysis, plasma samples were washed twice with PBS-citrate. Two different EMP subsets were enumerated, EMP expressing CD31 but not expressing the platelet marker CD41 (CD31+CD41- EMP) and also EMP expressing CD105, CD144 or CD146 using a monochrome multimarker approach (CD105-PE/CD144-PE/CD146-PE). For determination of CD31+CD41- EMP, 20 µL of washed sample was incubated in the dark for 30 min with 10 µL of diluted CD31-PE and 10 µL of diluted CD41-PE-Cy™5. Negative controls were prepared by incubating 10 µL of matched concentrations of PE isotype to 20 µL of washed plasma sample. For determination of CD105/144/146 EMP, 20 µL of washed sample was incubated in the dark for 30 min with 10 µL of diluted CD105-PE/CD144-PE/CD146-PE cocktail. Negative controls were prepared by incubating 10 µL of matched concentrations of PECy5 isotype to 20 µL of washed plasma sample. This was followed by addition of 270 µL PBS-citrate and immediate analysis for 3 min at medium speed on a flow cytometer (Beckman Coulter FC500, USA).

For determination of PMP, frozen plasma samples were thawed at room temperature and 30 µL incubated with 10 µL of diluted CD41-PE-Cy™5 antibody and 5 µL of diluted annexin V-FITC. Negative controls were prepared by incubating 5 µL of diluted annexin V-FITC and 10 µL of matched concentrations of PE-Cy5 isotype to 30 µL

of plasma sample. These were incubated in the dark for 30 min. To the positive samples, 500 µL binding buffer was added, and to the negative control samples 500 µL of phosphate buffered saline was added. Samples were then analysed immediately for 2 min at low speed on a flow cytometer (Beckman Coulter FC500, USA).

The protocols for analysis of soluble vascular injury biomarkers and lipids are available in the appendices K, L, M, N, O and P. Soluble vascular injury, angiogenic and proteolytic biomarkers were analysed in duplicates using commercially available immunoassays (Meso Scale Discovery, MD, USA) which employ multiplexing technology and electrochemiluminescence detection. The COV were; CRP (4%), sVCAM-1 (3%), sICAM-1 (4%), serum amyloid A (6%), sE-selectin (1%), sP-selectin (3%), sICAM-3 (5%), sTM (2%), bFGF (4%), PLGF (3 %), soluble fms-like tyrosine kinase-1 (sFlt-1) (3 %), Tie-2 (7%), VEGF (3%), VEGF-C (3%), VEGF-D (3%), MMP-2 (5%), MMP-10 (7%), MMP-1 (5%), MMP-3 (5%) and MMP-9 (4%). Serum cholesterol, direct HDL-cholesterol, direct LDL-cholesterol, triglycerides, APOA1, APOB and glucose were determined via spectrophotometric assays performed on an automated clinical chemistry system (ACE® Wassermann B.V., Netherlands) using appropriate reagents, calibrators and controls (Randox Laboratories, UK). Each sample was analysed once.

5.4.3 Immunohistochemical analysis of plaques

Plaques were collected immediately after excision, and preserved in 10% formalin until processed. Processing of the plaques was undertaken not more than 24 h after collection. The plaque was fixed in 10 % formalin for 24 h and decalcified using formic acid for a further 24 h. Decalcification was only undertaken if it was deemed

necessary following macroscopic examination by a histopathologist. The plaque was then embedded in paraffin wax in an automated embedder (Tissue-Tek® AutoTEC® Sakura). The blocks were sectioned at ~3µm and immunostained on the Leica BOND-MAX™ automated immunostainer using the Bond Polymer Refine Detection kit (appendix Q). The sections were analysed for the presence of macrophage and T-cells using the marker CD68 (Dako Denmark, clone PG-M1, dilution 1:50) and CD3 (Leica Clone LN-10, Prediluted) respectively.

5.4.4 Grading of the plaques

Plaques were graded using a modified version of the semi-quantitative scale previously used by Redgrave *et al., (2006)* (Redgrave et al., 2006). For this study, four immunohistological features were associated with stable and unstable plaques. These were the presence of inflammatory cells (macrophages or lymphocytes stained respectively with CD68 or CD3 antibody), cap rupture, lipid core and surface thrombus (figure 5.2). The inflammatory cell proliferation in the plaque core and in the cap was graded separately on a scale of 1-3 for both CD68 and CD3 content. Grade 1, 2 and 3 represented occasional cells, 1 group of >50 cells and 2-5 groups of >50 cells respectively, in both the plaque core and the cap. This gave a total inflammatory cell score of between 4 and 12, with 12 representing high CD68 and CD3 content in both the plaque core and the cap area. Plaques were regarded as unstable if they had any of the following: inflammatory cell score of between 9 and 12, evidence of cap rupture or evidence of surface thrombus.

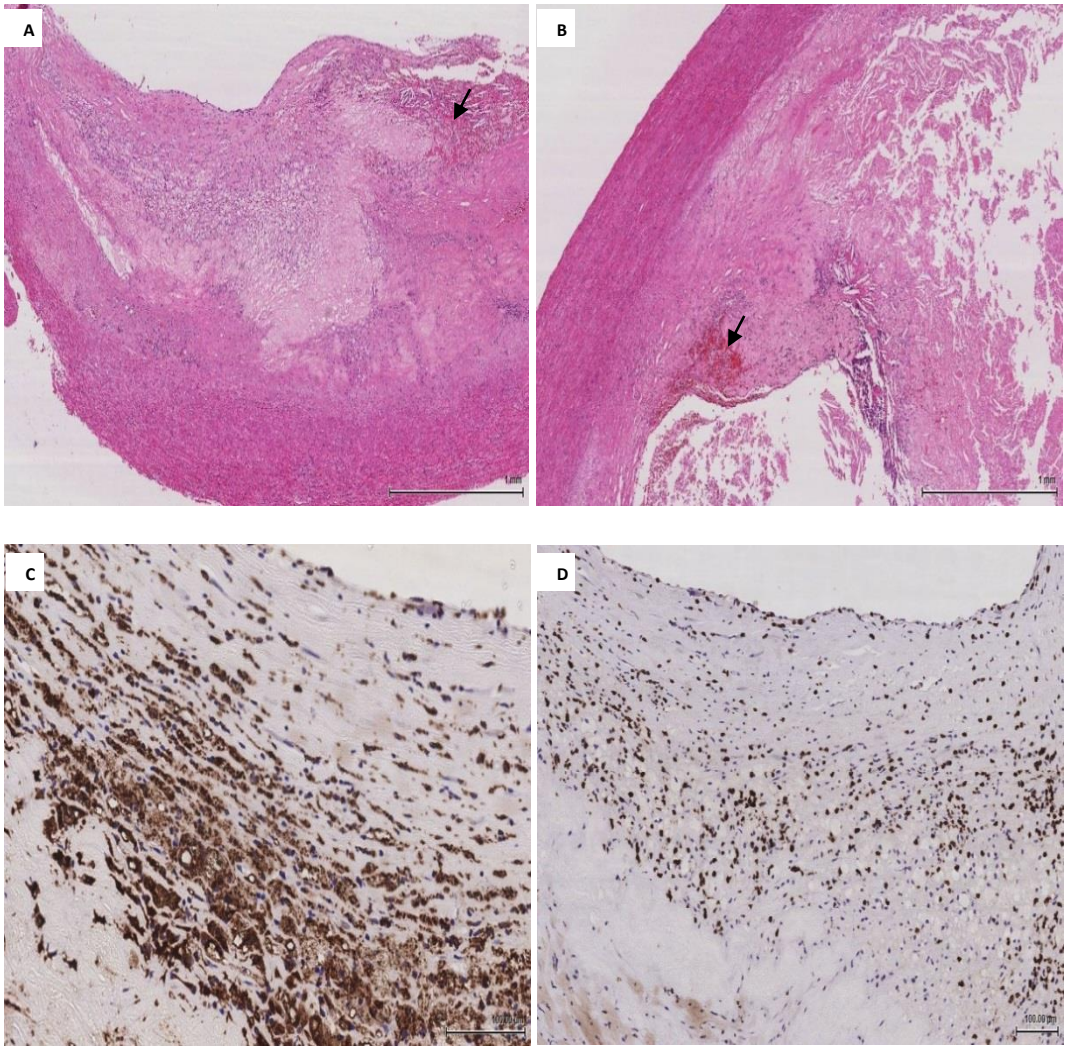


Figure 5.2: Examples of plaque immunohistological features in carotid endarterectomy specimens
A: Unstable plaque with rupture (arrow). B: Unstable plaque with haemorrhage (arrow). C: Macrophages staining brown with CD68 antibody. D: Lymphocyte nuclei staining brown with CD3 antibody.

5.4.4 Statistics

All data was checked for normality using the Kolmogorov–Smirnov test. Data that was not normally distributed was natural log transformed. The independent-samples t-test was used to compare cell-derived MP counts and concentrations of other biomarkers between stable and unstable plaques. The ability of promising biomarkers to predict the presence of unstable carotid artery disease was determined using binary logistic regression. Carotid artery disease state (stable/unstable) was the

dependent variable. The various cell-derived biomarkers and protein vascular biomarkers constituted the independent variables. Single binary logistic regression analysis was used to determine predictive ability of individual biomarkers. Multiple binary logistic regression analysis with forward stepwise entry of variables (likelihood ratio) was used for combinations of biomarkers. Only biomarkers with $p<0.1$ in single binary logistic regression analysis were entered in the multivariable model. Three different models were used; (i) all eligible biomarkers from single logistic regression analysis (ii) all eligible biomarkers excluding MP (iii) all eligible biomarkers excluding those that are not biologically plausible because of the direction of the difference. Different models were used to compare the predictive value of MP and protein vascular biomarkers, and to determine the predictive value of biologically plausible biomarkers only. The sensitivity and specificity of various multivariable models was also determined using receiver operator characteristic curves. The C-statistic of each ROC represents the AUC. The significance value was set at $p<0.05$.

5.2 Results

Out of the 41 plaques classified, 25 were stable plaques and 16 were unstable plaques. Six patients had both left and right plaques analysed. However, only the first surgery was included in the analysis as these were treated as duplicates. In 5 of the 6 cases both the left and right plaques from the same patient were of the same grade.

CD31+CD41- EMP and CD105/CD144/CD146+ EMP, were higher ($p<0.05$) in the participants with unstable compared with stable plaques (figure 5.3). sP-selectin and sTM were lower ($p<0.05$) in those with unstable compared with stable plaques (table 5.3). There was no significant difference in Annexin V+ MP, CD31+ PMP, CD41+ PMP, annexin V+CD41+ PMP (figure 5.3), lipids, apolipoproteins and glucose between the groups (table 5.2). The remaining angiogenic, vascular and proteolytic markers were not significantly different in patients with stable plaques compared to those with unstable plaques (table 5.3).

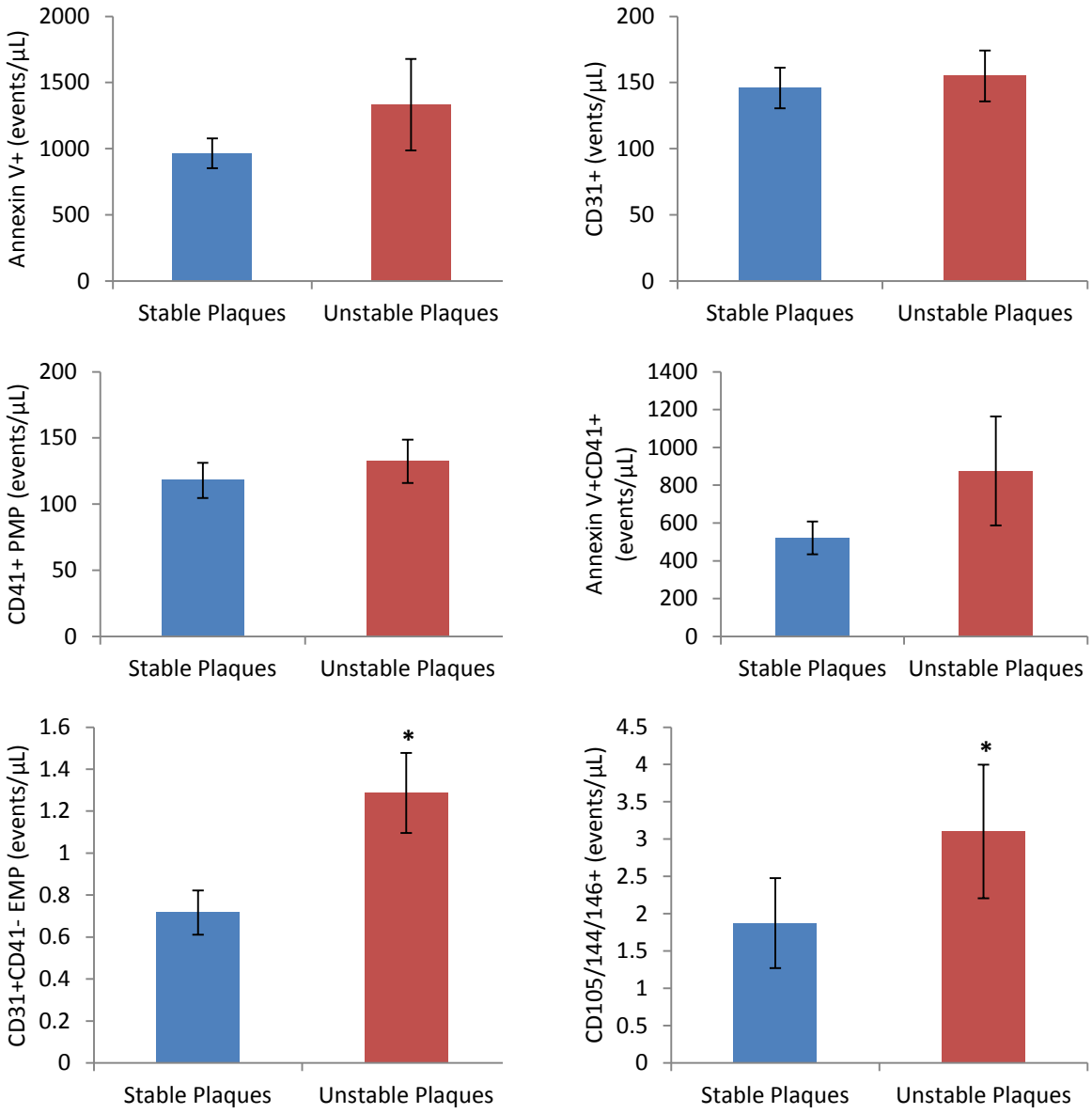
In single analysis using binary logistic regression sTM, sP-selectin, CD31+CD41+ EMP and CD105/CD144/CD146+ EMP had predictive value in identifying patients with unstable plaques (table 5.4). Multiple binary logistic regression analysis with forward stepwise entry of variables (likelihood ratio) was used for combinations of biomarkers. The following biomarkers with $p<0.1$ in single binary logistic regression analysis were entered in the model; CD31+CD41- EMP, CD105/144/146+ EMP, sP-selectin and sTM.

In multivariable model 1 CD31+CD41- EMP, sP-selectin and sTM were significant in the model to predict patients with unstable plaques (table 5.5). The C-statistic for the ROC (figure 5.4) was 0.856; (95% confidence interval = 0.722-0.990).

Using the ROC figure 5.4, the false positive rate (1-specificity) was 16% when the cut-off was low enough to give 80% sensitivity (80% of true positives correctly identified). In multivariable model 2 only sP-selectin remained significant in the model to predict patients with unstable plaques (table 5.6). The C-statistic for the ROC (figure 5.5) was 0.735 (95% confidence interval = 0.567-0.903). Using the ROC in figure 5.5, the false positive rate (1-specificity) was 48 %when the cut-off was low enough to give 80% sensitivity (80 % of true positives correctly identified). Alternatively when the cut-off was high enough to reduce the false positive rate to 20 %, the sensitivity was 68%. In multivariable model 3 only CD31+CD41- EMP was significant in the model to predict patients with unstable plaques (table 5.7). The C-statistic for the ROC (figure 5.6) was 0.729 (95% confidence interval = 0.562-0.895). Using the ROC in (figure 5.6), the false positive rate (1-specificity) was 52 % when the cut-off was low enough to give 80% sensitivity (80% of true positives correctly identified). Alternatively when the cut-off was high enough to reduce the false positive rate to 20 %, the sensitivity was 56 %.

5.2.1 Comparison of various biomarkers in patients with stable and unstable plaques

Figure 5.3: Annexin V+ , annexin V+CD41+ PMP, CD31+ PMP, CD41+ PMP, CD31+CD41- EMP and CD105/CD144/CD146+ EMP in patients with stable (n = 25) and unstable plaques (n = 16).



**p<0.05 compared to patients with stable plaques (natural log transformed data). Values are mean±SEM. EMP = endothelial microparticles, MP = microparticles, PMP = platelet microparticles. CD105/144/146 EMP represents MP positive for either CD105, CD144 or CD146. Samples were stained with these three endothelial specific monochrome antibodies in order to increase EMP counts*

Table 5.2: Serum lipids, apolipoproteins and glucose in patients with stable and unstable plaques

	Stable plaques (n-25)	**Unstable plaques (n=16)**
Apolipoprotein A1 (mg/dL)	98 ± 4	101±4
Apolipoprotein B (mg/dL)	80.2±5.1	83.3±8.6
Cholesterol (mmol/L)	3.7±0.2	4.0±0.3
HDL-cholesterol (mmol/L)	1.2±0.1	1.2±0.1
LDL-cholesterol (mmol/L)	2.2±0.2	2.2±0.3
Triglycerides mmol/L	1.4±0.1	1.3±0.1
Non-esterified fatty acid (mmol/L)	0.7±0.1	0.8±0.1
Glucose (mmol/L)	5.2±0.4	5.3±0.1

Values are mean±SEM. HDL = high density lipoprotein; LDL = low density lipoprotein

Table 5.3: Protein vascular biomarkers in patients with stable and unstable plaques

	Stable plaques (n-25)	**Unstable plaques (n=16)**
VEGF (pg/mL)	634.0±101.0	556.0±110.0
VEGF-C (pg/mL)	458.0±20.0	468.0±54.0
VEGF-D (pg/mL)	886.0±45.0	949.0±80.0
Tie-2 (pg/mL)	4851.0±293.0	5089.0±382.0
sFlt-1 (pg/mL)	107.0±5.0	121.0±15.0
bFGF (pg/mL)	7.6±1.0	6.5±2.7
PlGF (pg/mL)	24.4±1.0	23.5±2.3
MMP 2 (pg/mL)	84792.0±2549.0	77411.0±5669.0
MMP 10 (pg/mL)	1532.0±175.0	1847.0±172.0
MMP 1 (pg/mL)	23129.0±4596.0	23386.0±5778.0
MMP 3 (pg/mL)	16236.0±3527.0	19249.0±5823.0
MMP 9 (pg/mL)	296116.0±38407.0	249243.0±38055.0
sTM (ng/mL)	2.6 ± 0.1	2.0±0.1 *
sICAM-3 (ng/mL)	1.1 ± 0.1	1.01±0.09
Soluble E-selectin (ng/mL)	13.5±1.0	13.4±2.0
Soluble P-selectin (ng/mL)	77.3 ± 3.9	55.6±6.7 *
Serum amyloid A (ng/mL)	11453.0±6340.0	12319.0±5325.0
sVCAM-1 (ng/mL)	470.0±44.0	414.0±33.0
C-reactive protein (mg/L)	15.2±5.9	14.9±6.1
sICAM-1 (ng/mL)	139.0±13.0	144.0±24.0

*Values are mean±SEM. ICAM = intercellular adhesion molecule; MMP = matrix metalloproteinase; sFlt-1 = Soluble fms-like tyrosine kinase-1; VEGF = vascular endothelial growth factor; VCAM-1 = vascular cell adhesion molecule-1, bFGF = basic fibroblast growth factor, PlGF=p*lacental growth factor, sTM = soluble thrombomodulin

5.2.2 Predictive value of selected vascular biomarkers

Table 5.4: Single logistic regression analysis to predict unstable plaques

Biomarker	Wald[a]	p-value[b]	Odds Ratio[c]
sTM	4.037	0.045	0.371
sP-selectin	6.222	0.013	0.959
CD31+CD41+ EMP	5.860	0.015	3.982
[§]CD105/CD144/CD146+ EMP	4.665	0.031	2.078

[§]Natural log transformed data. EMP = endothelial microparticles, sP-selectin = soluble P-selectin, sTM = thrombomodulin. [a]Wald = Logistic regression statistic calculated for each variable in the model. Higher values denote greater predictive value in univariable model. [b]p-value = Significance of Wald statistic. [c]Odds ratio = increase in likelihood that an individual has unstable carotid plaque.

Table 5.5: Multiple logistic regression analysis containing all eligible biomarkers from single logistic regression analysis to predict patients with unstable plaques

	B	SEM	Wald	p-value	Odds Ratio[c]
CD31+CD41- EMP	2.211	0.917	5.816	0.016	9.128
Soluble P-selectin	-0.048	0.026	3.454	0.063	0.953
Soluble thrombomodulin	-2.014	0.861	5.468	0.019	0.133
Constant	4.854	2.654	3.345	0.067	128.271

EMP = endothelial microparticles, SEM = standard error mean. p<0.05 indicates that a biomarker significantly adds predictive value to the multivariable model

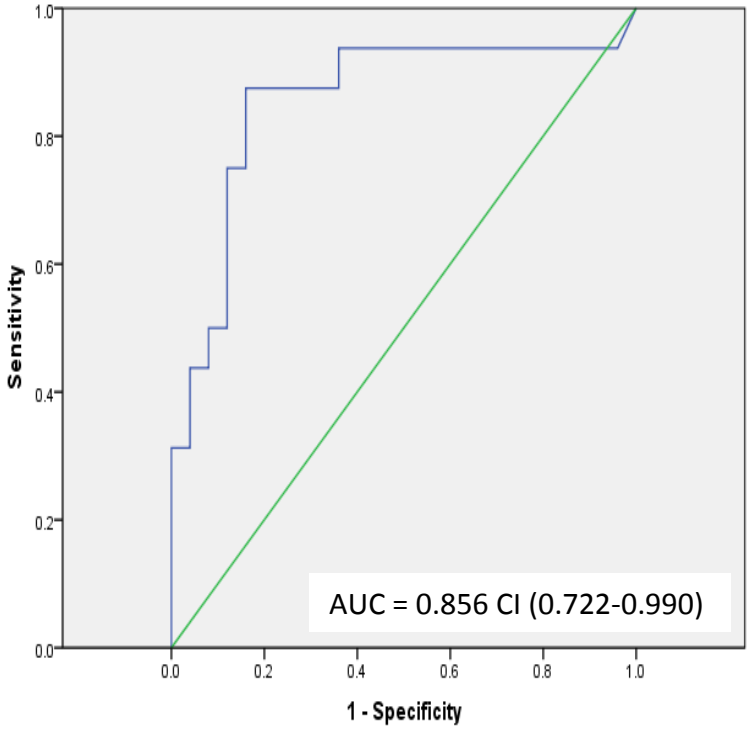


Figure 5.4: Receiver Operator Curve based on all eligible biomarkers from single logistic regression analysis to predict patients with unstable plaques

AUC; area under curve, CI; confidence interval

Table 5.6: Multiple logistic regression analysis containing all eligible soluble vascular injury biomarkers from single logistic regression analysis but excluding MP to predict patients with unstable plaques

	B	SEM	Wald	p-value	Exp(B)
Soluble P-selectin	-0.056	0.02	7.661	0.006	0.946
Constant	3.117	1.337	5.439	0.02	22.578

SEM = standard error mean. p<0.05 indicates that a biomarker significantly adds predictive value to the multivariable model

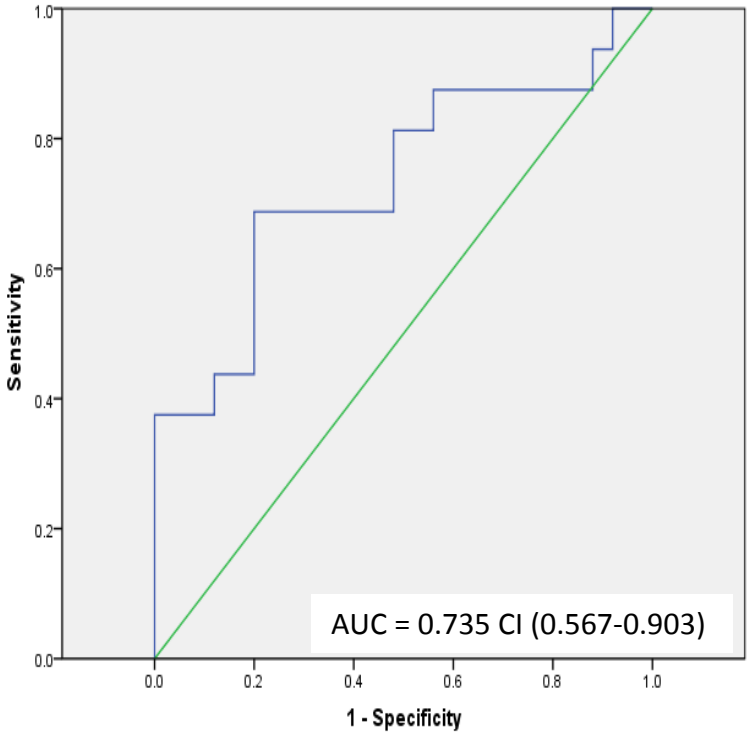


Figure 5.5: Receiver Operator Curve based all eligible soluble vascular injury biomarkers from single logistic regression analysis but excluding microparticles to predict patients with unstable plaques

AUC; area under curve, CI; confidence interval

Table 5.7: Multiple logistic regression analysis containing all eligible biomarkers from single logistic regression analysis that are biologically plausible to predict patients with unstable plaques

	B	SEM	Wald	p-value	Exp(B)
CD31+CD41- EMP	1.382	0.571	5.86	0.015	3.982
Constant	-1.783	0.653	7.466	0.006	0.168

EMP = endothelial microparticles, SEM = standard error mean. p<0.05 indicates that a biomarker significantly adds predictive value to the multivariable model

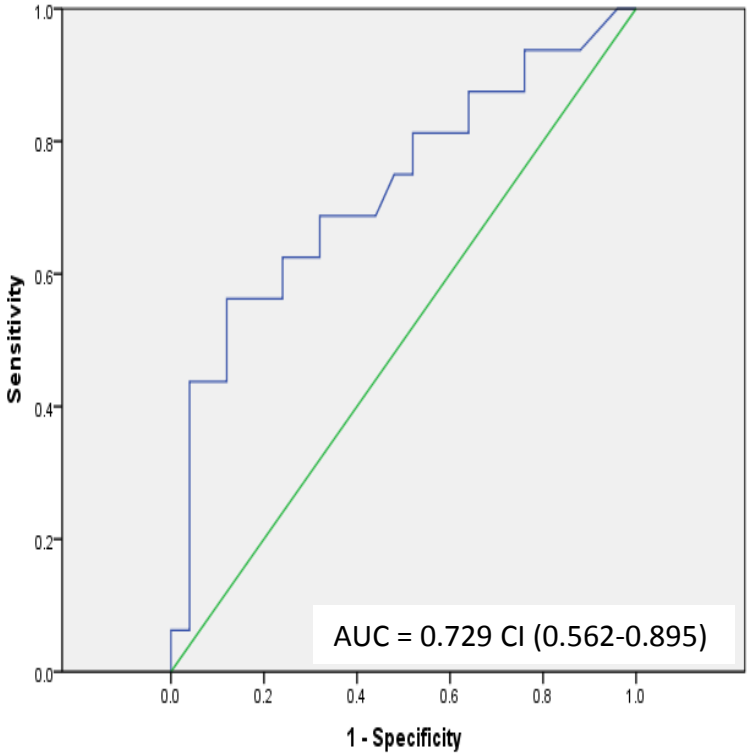


Figure 5.6: Receiver Operator Curve based on all eligible biomarkers from single logistic regression analysis that are biologically plausible to predict patients with unstable plaques

AUC; area under curve, CI; confidence interval

5.3 Discussion

5.3.1 Introduction

This study investigated the predictive potential of MP as biomarkers for predicting carotid plaque stability. Plaque stability was determined post-endarterectomy using immunohistochemistry. EMPs were significant in univariable model, and added to predictive ability in a multivariable model that included circulating vascular injury biomarkers. Indeed EMP was the only biomarker significant in univariable or multivariable models that was biologically plausible

5.3.2 Atherosclerosis is a multifocal systemic disease

Out of a total of 41 plaques collected and analysed, 25 patients had stable plaques whereas 16 had unstable plaques. Six patients had both left and right plaques analysed. In 5 of the 6 patients both the left and right plaques from the same patient were of the same grade which confirms evidence that although only one of the plaques may be identified as the main culprit, others plaques may be distant from this culprit plaque. It is therefore likely that the quality and content of carotid artery plaque is reflective of plaque throughout the vascular tree.

5.3.3 The predictive value of cell-derived microparticles

CD31+CD41- EMP and CD105/CD144/CD146+ EMP were higher in patients with unstable plaques compared to those with stable plaques. Both had predictive value in identifying patients with unstable carotid artery disease. However,

CD105/CD144/CD146+ EMP were eliminated by CD31+CD41- EMP in the multivariable model. CD31+CD41- Previous studies have found a close link between EMP and disease stage in patients with acute coronary syndrome. Bernal-Mizrachi *et al., (2003)* reported a 2.5-fold increase in CD31+ EMP in myocardial infarction and unstable angina patients compared to stable angina patients (Bernal-Mizrachi et al., 2003) and higher CD31+ CD42- EMP in patients with high risk angiographic lesions compared to those with low risk angiographic lesions (Bernal-Mizrachi et al., 2004). Simak *et al., (2006)* found higher CD105+ CD144+ EMP and CD105+ CD54+ CD45- EMP in patients with moderate-severe stroke compared to patients with mild stroke (Simak et al., 2006). This is the first study to demonstrate higher levels of EMP in patients with unstable carotid artery disease.

There appears to be a link between EMP and disease stage in patients with atherosclerosis. By definition in this study, patients with unstable plaques had higher inflammatory cell count (T-lymphocytes and activated macrophages), cap rupture and surface thrombus which are an indication of a more active atherosclerotic process. These plaque features produce molecules that continuously drive the progression and remodelling of the atherosclerotic plaque. Underlying the plaque are endothelial cells that when exposed to these perturbations may be activated and become apoptotic Consequently, EMP may be released in response to the hypercoagulability and proinflammatory state taking place within the plaque. The pattern of the EMP released may reflect the functional state of the parental endotheliocytes (Shantsila et al., 2010). EMP expressing the constitutive antigens CD31+, CD105, CD144 and CD146

markers are released upon apoptosis (Chironi *et al*., 2009) suggesting that elevated levels of EMP in patients with unstable plaques reflects pronounced endothelial cell death. Thus, assessment of the EMP might serve as valuable biomarkers of the nature and extent of endothelial injury in carotid artery disease.

This is the first study to evaluate the ability of CD31+CD41- EMP to predict unstable carotid artery disease both as a univariable marker and in multivariable models. In a multivariable model with CD31+CD41- EMP the C-statistic was higher than if EMP were omitted from the model and soluble vascular injury biomarkers were used independently. This holds whether or not sTM and sP-selectin were included in the multivariable model. This finding has not been reported previously.

There was no difference in the levels of annexin V+, CD31+, CD41+ and annexin V+ CD41+ MP (essentially PMP) between patients with stable plaques compared to those with unstable plaques. These results differ from those by Michelsen *et al., (2009)* that reported higher PMP levels in individuals with echogenic (surrogate for stable) compared with echolucent (surrogate for unstable) plaques (Michelsen et al., 2009). At the present time it is difficult to explain the difference in the levels of annexin V+, CD31+, CD41+ and annexin V+ CD41+ MP in the present study and the study by Michelsen*s* group. However, the present results indicate that EMP are a more sensitive marker of unstable plaques than PMP.

5.3.4 The predictive value of soluble angiogenic, proteolytic and vascular injury biomarkers

Contrary to biological plausibility sP-selectin and sTM were lower in patients with unstable plaques than those with stable plaques. They had predictive value in identifying those with unstable carotid artery disease. Soluble P-selectin had the highest Wald values of any predictor in univariable analysis. Distinct from the present study, previous studies have found higher levels of soluble P-selectin in patients with advanced atherosclerosis. Studies have found higher levels of soluble P-selectin in patients with acute myocardial infarction and unstable angina pectoris compared to those with stable angina pectoris (Guray et al., 2004, Lu et al., 2010). The results from chapter 2 indicate that medications influence these markers. It is acknowledged that not all the patients were on lipid lowering or antiplatelet/anticoagulant medications. However, it is possible that patients on medications may have been on different doses and it was not possible to control for this variation. In agreement with the present findings, lower levels of soluble P-selectin have been found in coronary artery disease (Danzig et al., 2010).

Studies have also reported contradictory findings in relation to soluble TM with some reporting elevated levels (Blann et al., 1997a, Blann et al., 1996, Seigneur et al., 1993, Blann et al., 1997b) and others reduced levels (Laszik et al., 2001) in patients with atherosclerosis. It is therefore questionable as to whether P-selectin and TM should be included in multivariable models. Moreover, it is unclear whether soluble TM levels increase or decrease in atherosclerosis. Until the issue as to why it is lower

in disease states in some studies and higher in others is clarified, it may be unwise to include it in predictive models. However, research efforts are justified to clarify the reasons for the contradictory results with respect to both biomarkers given that the Wald value for each was high in the univariable models.

The inflammatory biomarkers C-reactive protein (CRP) and serum amyloid A (SAA) were not different between patients with stable plaques and those with unstable plaques. The angiogenic biomarkers basic fibroblast growth factor, placental growth factor, soluble fms-like tyrosine kinase-1, tyrosine-protein kinase receptor, vascular endothelial growth factor, vascular endothelial growth factor-C and vascular endothelial growth factor-D were not different between the two groups. Similarly, the proteolytic biomarkers; MMP- 1, 2, 3 and 9 were not different between the two groups of patients. These findings are contrary to those reported in previous studies that have shown higher levels of CRP (Alvarez Garcia et al., 2003) and MMP (Pelisek et al., 2009, Sapienza et al., 2005, Alvarez Garcia et al., 2004) in patients with unstable carotid artery disease. The patients in this study were heavily medicated with lipid lowering and antiplatelet/anticoagulation medications. It is possible that these medications may reduce levels of circulating inflammatory molecules such as CRP and MMP.

5.3.5 Study limitations

This study had several limitations. Firstly, the C-statistic of CD31+CD41- EMP without sTM and sP-selectin was 0.729, which is an acceptable predictive value.

However, a biomarker combination with a C-statistic of 0.729, only has a specificity of 48% when the sensitivity is 80% and is not ready to be used as a diagnostic test for predicting unstable carotid artery disease. The second limitation is based on the classification of plaques as stable and unstable. Although an objective method was employed to categorise plaques based on the work of Redgrave *et al., (2006)* (Redgrave et al., 2006), it cannot be assumed with certainty that plaques with an inflammatory content just above our cut-off were definitely unstable and likely to rupture. There is no single definition of an unstable plaque based on immunohistochemistry. However, plaques that were classified as unstable in the present study had the highest inflammatory burden, including the area of the fibrous cap. The results indicate that MP are not yet ready to be used as a diagnostic test for unstable plaques. The small sample size is a further limitation. Significant differences may have been observed in the many soluble cell adhesion molecules, angiogenic and proteolytic factors with a greater sample size.

5.3.6 Conclusion

In conclusion, the study findings indicate that CD31-CD41+ EMP, CD105/CD144/CD146+ EMP, sP-selectin and sTM are suitable biomarkers in single or multiple biomarker strategies to identify unstable carotid artery disease. EMP discriminated patients with unstable plaques from those with stable plaques and added predictive value when included in multivariable models. The use of these emerging biomarkers in a multimarker strategy to indicate plaque activity may allow far more individualized risk assessment and provide a powerful opportunity to

adequately initiate targeted prevention strategies. While several of the investigated novel markers are of limited, value, particularly in asymptomatic individuals, the study findings support the value of multimarker strategies with EMP in diagnosis and prognosis of individuals with unstable carotid artery disease. Despite occurring in very low levels in the circulation, EMP are worth pursuing as biomarkers of carotid artery disease and further studies are warranted.

Chapter 6

6. Influence of a low carbohydrate diet on cellular and protein vascular health biomarkers

6.1 Introduction

Biomarkers are systemic measurements of cells, molecules, genes or gene products, hormones, enzymes, or other proteins that provide independent diagnostic or predictive value by reflecting health or disease states or conditions (Paramo et al., 2007). Some biomarkers are more efficacious at identifying early stages of disease, others at identifying later stages of disease and others in monitoring the change in risk status. An important feature of any biomarker is its ability to monitor changes in disease or risk status following a medical or health-enhancing intervention.

Changes to dietary macronutrient and micronutrient content of the diet can have both favourable and adverse effects on vascular health. Low carbohydrate (CHO) diets are an increasing in popularity, but their effect on vascular health has been questioned (Frigolet et al., 2011). The Atkins diet and the carbohydrate addicts' diet limit the carbohydrate intake to <20 g/day (Atkins, 1992) and only 1 meal/day (Heller, 1991), respectively. In contrast, the protein power diet limits CHO intake to <30 $g \cdot day^{-1}$ during the induction phase and 55 $g \cdot day^{-1}$ thereafter (Eades, 1996). The sugar busters' diet involves avoidance of sucrose and high-glycaemic index food (Steward et al., 1995). Although various theories exist to explain the effects of low CHO diets on body weight, it is generally accepted that they reduce in total energy intake and ultimately weight loss (Bilsborough and Crowe, 2003).

A reduction in body weight of 5-10 % following dietary restriction has the potential to improve vascular risk profile (Institute, 1998). However, individuals on

these diets obtain a greater proportion of energy from fat and protein which may adversely atherogenic blood lipids and cardiovascular risk profile (Freedman et al., 2001a, Lichtenstein et al., 2006, Parks and Hellerstein, 2000). Overweight populations typically have elevated levels of cardiovascular and inflammatory risk markers (Poirier et al., 2006). More biomarkers are needed that are sufficiently sensitive to identify changes in cardiovascular risk following modest weight loss in order to establish the efficacy and safety of such dietary strategies.

Endothelial MP are plasma membrane derived vesicles shed into the circulating blood by endothelial cells following activation or injury (Burnier et al., 2009) reflecting endothelial damage and impaired endothelial function. These vesicles may aid in understanding the dynamics of endothelial injury in addition to acting as specific and non-invasive biomarkers (Leroyer et al., 2008). EPC are bone marrow derived cells that are recruited into the peripheral blood as an endogenous repair mechanism to maintain the integrity of the endothelial monolayer (Dong and Goldschmidt-Clermont, 2007). They may aid in understanding the dynamics between injury and repair of the endothelium and thus may serve as vascular biomarkers. To date, only one study, involving animals has specifically examined the influence of a low CHO diet on circulating levels on EPC and found a significant decrease in cell count. (Foo et al., 2009). No study has examined the influence of a low carbohydrate diet or weight loss on MP.

The purpose of this study was to examine changes in MP and EPC resulting from a 24-week low carbohydrate diet. MP and EPC were measured in conjunction

with protein vascular biomarkers that are more routinely employed in research studies, in order to determine the direction and magnitude of the change in MP and EPC relative to the change in protein vascular biomarkers. Serum lipids and apolipoproteins were also measured as potential mediators of positive or negative changes. Overweight women represent an at-risk population who are likely to have elevated risk factors with potential for improvement.

6.2 Aims

1. To determine the influence of a 24 week low CHO diet on cellular and protein vascular health biomarkers
2. To determine the direction and magnitude of the change in PMP, EMP and EPC relative to the change in protein vascular biomarkers in response to a 24 week low CHO diet
3. To determine role of serum lipids and apolipoproteins in mediating change vascular biomarkers following a 24 week low CHO diet

6.3 Hypotheses

1. Cellular and protein vascular health biomarkers; PMP, EMP, EPC and protein vascular biomarkers change following a low CHO diet in a manner consistent with improved vascular health
2. The change in EMP and EPC is at least as great as that in protein vascular biomarkers following a 24 week low CHO diet

3. The influence of the low CHO diet on these cellular and protein biomarkers is mediated by changes in lipids and apolipoproteins.

6.3 Methods

6.3.1 Study overview

This was a randomised cross over study examining the influence of a 24 week low CHO diet on cellular and protein vascular biomarkers. Twenty eight overweight women were recruited and randomised to either 24 weeks on their normal diet or a low CHO diet after which they crossed over to the alternative diet (figure 46). A fasting blood sample was obtained at the start of the study, at the cross-over point and at the end of the study from which cellular and protein biomarkers were determined (figure 46). Body composition was also evaluated at these time points.

6.4.2 Study population

The women in late reproductive and post-menopausal stages, between the age of 39-65 years who were overweight with body mass index (BMI) between 25 and 30 kg/m^2 and had not engaged in dieting practices in the previous 6 months. Exclusion criteria included women that were engaging in intense physical activity (> 3 times per week of vigorous physical activity) or excessive alcohol drinking (> 14 units weekly). In addition, women that were pregnant or lactating, taking hormone replacement therapy, had a history of chronic menstrual irregularities, diabetes mellitus, kidney disease, chronic illness, inflammatory conditions, renal, gastrointestinal or hormonal disorders or had a hysterectomy were also excluded from the study. Each participant completed a health screening form (appendix G) to determine eligibility for the study. Ethical approval was obtained from Waterford Institute *of* Technology Research Ethics

Committee. Each participant signed a written consent form (appendix F) which outlined the procedures involved and any potential health risks.

Ninety women were screened for eligibility. Only 40 were eligible and 28 of these agreed to participate in the study. The 28 women were randomized to their normal diet or a low CHO diet during the initial 24 week period and then completed the other diet during the next 24 weeks. However, 4 dropped out at various stages so the final analysis was based on 24 participants who completed both phases of the study (figure 6.1).

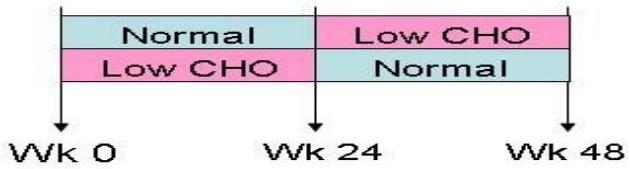


Figure 6.1: Low Carbohydrate Diet Study Design
Although 28 women were randomized to their normal diet or a low CHO diet during the initial 24 week period, only 24 completed both phases of the study. CHO; carbohydrate

6.3.2 Diet

A 3 day food diary (appendix H) was completed at baseline, week 12 and week 24 during each experimental condition. Participants were instructed to maintain normal dietary habits and to estimate the food quantities. During the low CHO period participants were instructed to reduce their carbohydrate intake considerably but not below 40 g/day. The range of intakes during the low CHO phase was 39-144 g/day. Four individuals exited the study because of an inability to adhere to a low CHO diet. In contrast, the normal diet group were instructed to keep their dietary habits as close to

the baseline estimates as possible. Participants crossed over at 24 weeks. Those who had been consuming their normal diet changed to the low CHO diet and vice-versa.

Participants completed weekly food frequency questionnaires (appendix I) for self-monitoring purposes and to assist with compliance. These were discussed with participants at the mid-point of each phase but were not analysed for research purposes. Participants were also provided with information on what constituted CHO foods, a list of the macronutrient content in the most commonly consumed foods and a booklet with low CHO recipes, meal plans and advice. In addition, they attended cooking classes that demonstrated how to make low CHO meals that were not readily available in stores.

6.3.3 Dietary assessment

The food diaries were analysed using CompEat^{TM} analysis software. Intake of selected macro-nutrients (energy, protein, fat and carbohydrate), micro-nutrients (calcium, magnesium, potassium, phosphate, vitamin C, vitamin D, sodium) and essential and non-essential amino acids during the normal diet and low CHO phases were calculated by averaging daily values at the mid-point and end of each phase from the 3 day food diary analyses.

6.3.4 Physiological Measurements

Height, weight, waist circumference and blood pressure were measured at baseline, 12 weeks and 24 weeks in each experimental condition. The height was

measured in metres with a SECA Leicester Portable Height Measure (Seca Ltd. Birmingham). The weight was measured in kilograms using a SECA 700 Mechanical Column Scale (Seca Ltd. Birmingham). Both measurements were taken with the individual standing in an upright position with bare feet. The height and weight measurements were used to calculate the BMI (kg/m^2), [weight (kg)/height m^2]. Waist circumference was measured by placing a measuring tape around the abdomen, above the hip bone. The tape measure was placed horizontally and snug but not compressing the skin and the measurements recorded in centimetres. The blood pressure was measured using a manual digital blood pressure sphygmomanometer with appropriate cuff size (Omron Healthcare, USA). The individual was rested in a quiet room for 5 min before taking measurements. They removed tight-fitting clothing from the left arm, sat in a chair with feet flat on the floor. The cuff was wrapped firmly around the left arm. The arm was rested on the table so that the cuff was at the same level as the heart and the measurements taken.

6.3.5 Blood sampling, processing and analysis

Sodium citrate anti-coagulated, EDTA anti-coagulated and serum clot activated bloods samples were taken from a prominent forearm vein by venepuncture with the first 3.0 mL discarded. Further details of blood sampling, processing and analyses are contained in the general methodology (section 2.1). Briefly, a complete blood cell count (appendix J) including white cells and sub fractions, red cells and platelets was undertaken on a haematology analyser (Coulter® $A^{C.}$T diff™ analyzer, Beckman Coulter, USA).

EPC were analysed using a modified ISHAGE protocol described in the general methodology (section 2.3). Each sample was analysed once. Briefly 100 µL of EDTA anticoagulated whole blood was stained with 20 µL CD45-FITC, 12 µL KDR/VEGFR-2-PE and 10 µL CD34-PC7 for 30 min at room temperature in the dark. RBC were lysed with 2000 µL of lysing solution for 15 min and immediately analysed for 20 min at high speed on a flow cytometer (Beckman Coulter FC500, USA).

MP were analysed as described in the general methodology (section 2.5). Samples were analysed once. For EMP analysis, plasma samples were washed twice with PBS-citrate. Two different EMP subsets were enumerated, EMP expressing CD31 but not expressing the platelet marker CD41 (CD31+CD41- EMP) and also EMP expressing CD105, CD144 or CD146 using a monochrome multimarker approach (CD105-PE/CD144-PE/CD146-PE). For determination of CD31+CD41- EMP, 20 µL of washed sample was incubated in the dark for 30 min with 10 µL of diluted CD31-PE and 10 µL of diluted CD41-PE-Cy™5. Negative controls were prepared by incubating 10 µL of matched concentrations of PE isotype to 20 µL of washed plasma sample. For determination of CD105/144/146 EMP, 20 µL of washed sample was incubated in the dark for 30 min with 10 µL of diluted CD105-PE/CD144-PE/CD146-PE cocktail. Negative controls were prepared by incubating 10 µL of matched concentrations of PECy5 isotype to 20 µL of washed plasma sample. This was followed by addition of 270 µL PBS-citrate and immediate analysis for 3 min at medium speed on a flow cytometer (Beckman Coulter FC500, USA).

For determination of PMP, frozen plasma samples were thawed at room temperature and 30 µL incubated with 10 µL of diluted CD41-PE-Cy™5 antibody and 5 µL of diluted annexin V-FITC. Negative controls were prepared by incubating 5 µL of diluted annexin V-FITC and 10 µL of matched concentrations of PE-Cy5 isotype to 30 µL of plasma sample. These were incubated in the dark for 30 min. To the positive samples, 500 µL binding buffer was added, and to the negative control samples 500 µL of phosphate buffered saline was added. Samples were then analysed immediately for 2 min at low speed on a flow cytometer (Beckman Coulter FC500, USA).

The protocols for analysis of soluble vascular injury biomarkers and lipids are available in appendices K, L, M, N, O and P. Briefly, soluble vascular injury biomarkers were analysed in duplicates using commercially available immunoassays (Meso Scale Discovery) which employs multiplexing technology and electrochemiluminescence detection. The mean COV were; CRP (7%), sVCAM-1 (9%), sICAM-1 (10%), SAA (11%), sE-selectin (4%), sP-selectin (3%), sICAM-3 (7%) and sTM (3%). Serum cholesterol, HDL-cholesterol, LDL cholesterol, triglycerides, APOA1, APOB and glucose were determined via spectrophotometric assays performed on an automated clinical chemistry system (ACE® Wassermann B.V., Netherlands) using appropriate reagents, calibrators and controls (Randox Laboratories, UK). Samples were analysed once.

6.3.6 Statistics

All data was checked for normality using the Kolmogorov–Smirnov test. Data that was not normally distributed was natural log transformed. The principal technique

used to determine the influence of low CHO diet was to compare values at the end of the normal diet and low CHO diet phases using one way repeated measures analysis of covariance. The low CHO was the dependent variable. The various cell-derived biomarkers and protein vascular biomarkers constituted the independent variables. When differences between the end of the normal and low carbohydrate phases were significant, they were also adjusted for differences in body weight (the covariate) between these time points in order to determine if the effect of the low carbohydrate diet was dependent on, or independent of, body weight changes. This was preferable to comparing delta values during the normal and low CHO phases as there was no wash out between phases. The magnitude of the low carbohydrate diet effect on protein and cellular vascular biomarkers was assessed using Cohen's effect size statistic. Significance was set at $p<0.05$.

6.2 Results

The mean daily intake of energy and macronutrients on the normal and low CHO diets are shown in figure 6.2. Energy and carbohydrate intake were lower ($p<0.05$) on the low CHO diet. Carbohydrate intake was 92±60 g/day lower (mean±SD) on the low CHO compared to the normal diet. Intake of fat, saturated fat and protein was not different on normal and low CHO diets. However the percentage of energy obtained from fat and protein was higher ($p<0.05$) on the low CHO diet compared to the normal diet (figure 6.3).

Body weight, body mass index and waist circumference were 3.6 kg, 2.5 kg/m^2 and 3.5 cm lower respectively ($p<0.05$) at the end of the low CHO diet compared to the normal diet phases (table 6.1). Systolic blood pressure was not significantly different at the end of the low CHO diet compared to the normal diet phases. Diastolic blood pressure was lower at the end of the low CHO diet compared to the normal diet phases (table 6.1).

The levels of MP following 24 weeks on the normal diet and 24 weeks on the low carbohydrate diet are shown in figure 6.4. CD31+CD41- EMP were lower at the end of the low CHO diet compared to the end of the normal diet and remained significantly different after adjustment for the difference in body weight between each diet phase. Annexin V+CD41+ PMP, annexin V+ MP and CD105/CD144/CD146+ EMP were not significantly different at the end of the low CHO diet and normal diet phases. Total progenitor cell and EPC whether expressed in counts/mL or as a percentage of

total leukocytes (figure 6.5), were not significantly different at the end of the low CHO diet and normal diet phases.

Concentrations of protein vascular biomarkers following 24 weeks on normal diet and 24 weeks on low CHO diet are shown in figure 6.6. Concentrations of sE-selectin, sP-selectin, sTM, CRP and SAA were significantly lower ($p<0.05$) following 24 weeks on low CHO diet compared to 24 weeks on normal diet. sP-selectin and SAA remained significantly lower after adjustment for the difference in body weight between each diet phase. E-selectin, sTM and CRP were not different after adjustment for difference in body weight between each diet phase. The magnitude of the low CHO diet novel and established biomarkers was compared using Cohen's effect size statistic (table 6.2). The effect size was greatest for CD31+CD41- EMP (0.47) and SAA (0.42), with smaller effect sizes for the remaining soluble vascular biomarkers. sICAM-3, sICAM-1 and sVCAM-1 were not different following 24 weeks on normal diet and 24 weeks on low CHO diet. Serum lipids, apolipoproteins and glucose (table 6.3) were not significantly different at the end of the low CHO diet compared to the normal diet phases.

6.2.1 Mean daily intake of various nutrients

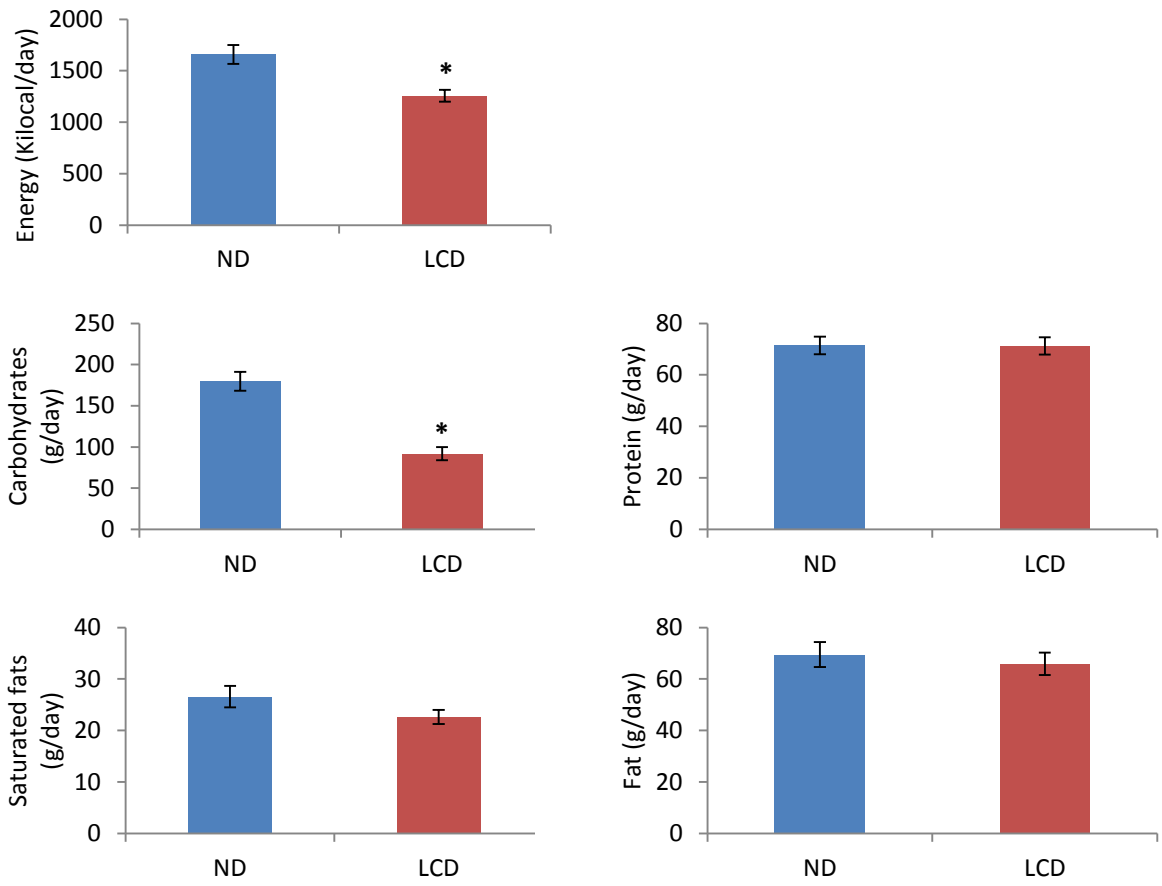


Figure 6.2: Mean daily intake of energy, carbohydrate, protein, saturated fat and fat when following a normal diet (ND) and a low carbohydrate diet (LCD)
** p < 0.05 compared to normal diet. Values are mean ± SEM.*

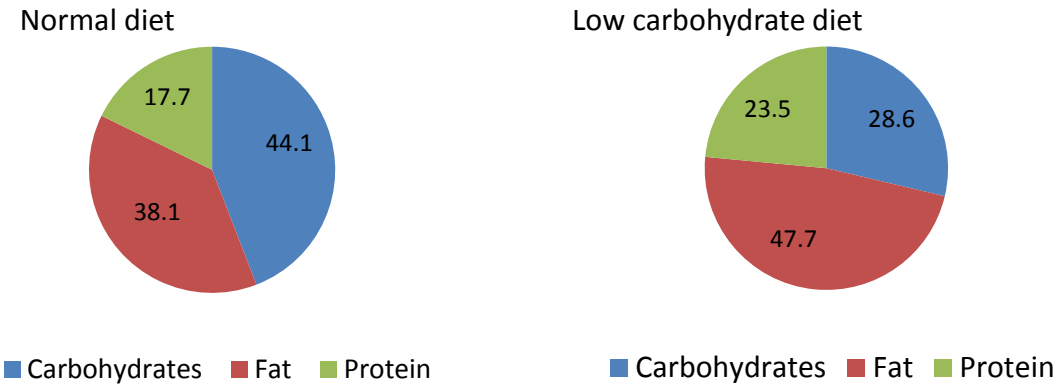


Figure 6.3: Percentage of total calories from carbohydrates, fats and proteins when following a normal diet (ND) and a low carbohydrate diet (LCD)

6.2.2 Body composition and blood pressure

Table 6.1: Body composition and blood pressure following 24 weeks on normal diet and 24 weeks on low carbohydrate diet

	Normal diet	Low carbohydrate diet
Weight (kg)	73.8 ± 1.8	70.1 ± 1.8*
Body Mass index (kg/m^2)	27.1 ± 0.6	25.7 ± 0.6 *
Waist circumference (cm)	85.8 ± 0.5	82.3 ± 0.6 *
Systolic blood pressure (mmHg)	130.2 ± 2.0	128.3 ± 3.1
Diastolic blood pressure (mmHg)	84.7 ± 1.7	80.0 ± 2.2*

*Values are mean±SEM; *p < 0.05 compared to normal diet.*

6.2.3 Cell-derived microparticles

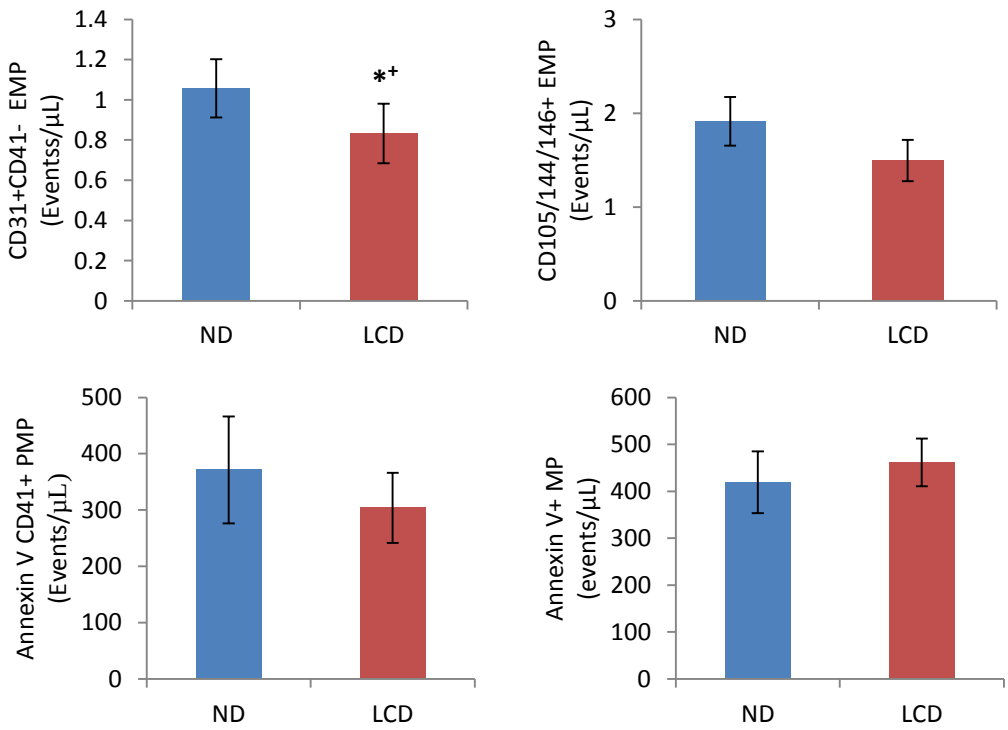


Figure 6.4: Cell-derived microparticles following 24 weeks on normal diet (ND) and 24 weeks on low carbohydrate (LCD) diet
**p<0.05 compared to normal diet. $^+$p<0.05 after adjustment for difference in body weight between each diet phase. Values are mean±SEM. EMP = endothelial microparticles, PMP = platelet microparticles. CD105/144/146 EMP represents microparticles positive for either CD105, CD144 or CD146. Samples were stained with these three endothelial specific monochrome antibodies in order to increase EMP counts*

6.2.4 Circulating cells

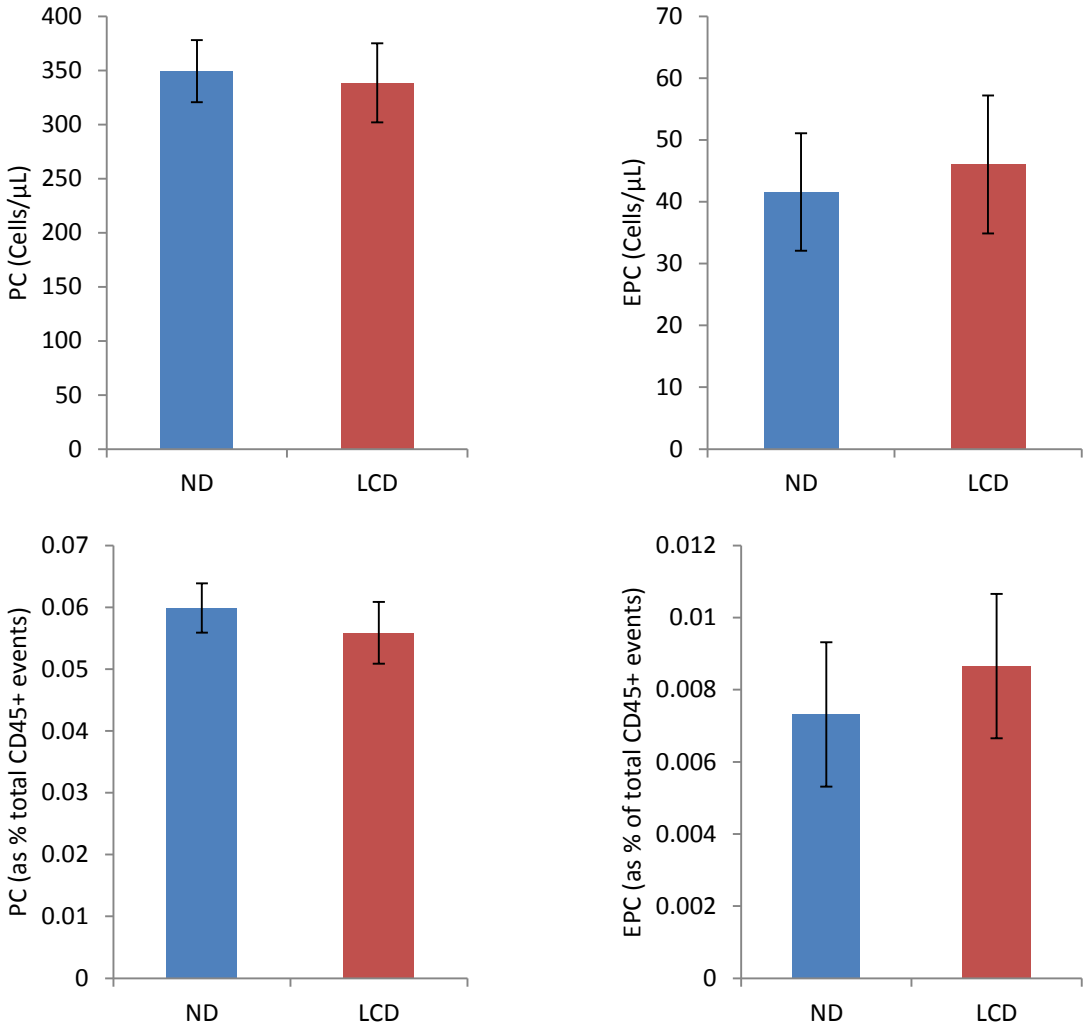


Figure 6.5: Total progenitor cell counts and percentages following 24 weeks on normal diet (ND) and 24 weeks on low carbohydrate diet (LCD)
Values are mean ± SEM; PC = progenitor cells ($CD34^+$ $CD45^{dim}$)*, EPC = endothelial progenitor cells* ($CD34^+$ $VEGFR\text{-}2^+$ $CD45^{dim}$)

6.2.5 Soluble vascular biomarkers

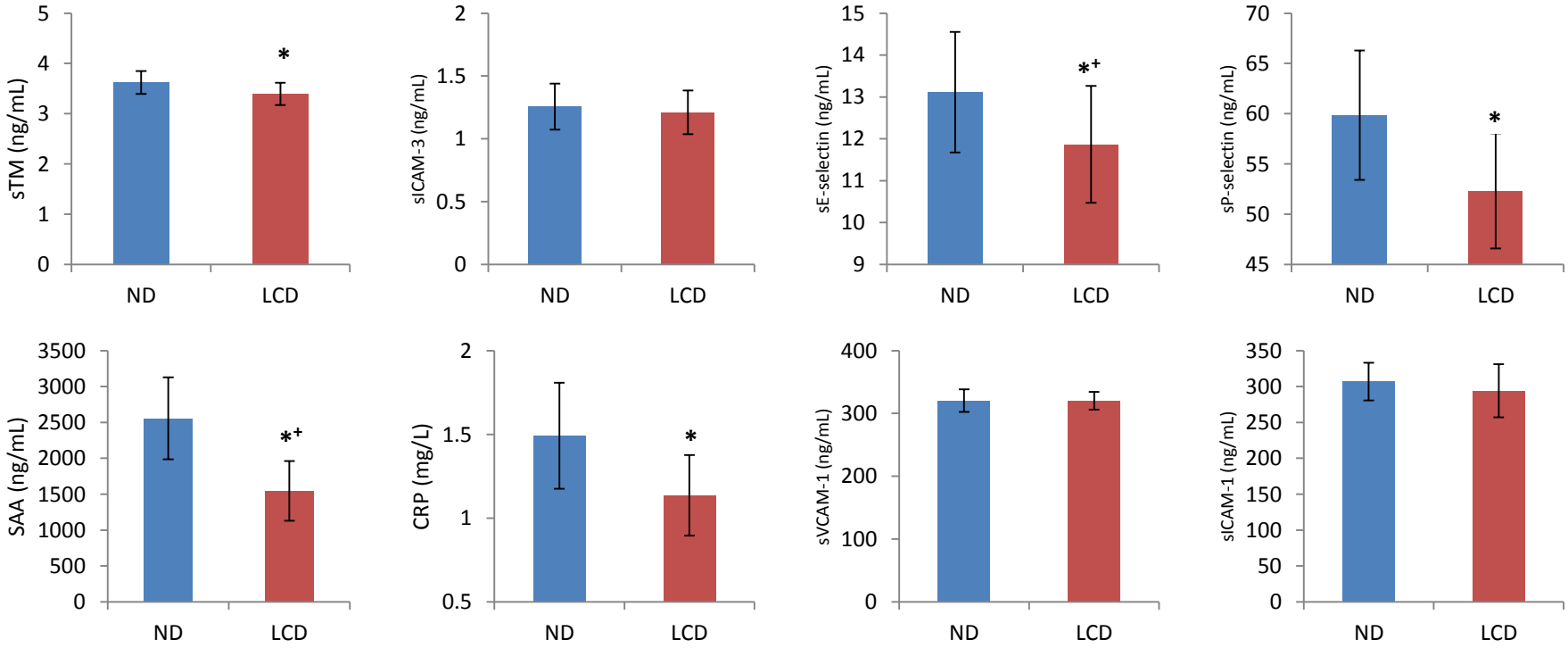


Figure 6.6: Protein vascular biomarkers following 24 weeks on normal diet (ND) and 24 weeks on low carbohydrate (LCD) diet
**p<0.05 compared to end of control phase. $^{+}$p<0.05 after adjustment for difference in body weight between each diet phase. Values are mean±SEM. CRP = C-reactive protein, SAA = serum amyloid A, sE-selectin = soluble E-selectin, sICAM-3 = soluble intercellular adhesion molecule 3, sICAM-1 = soluble intercellular adhesion molecule 1, sP-selectin = soluble P-selectin, sTM= soluble thrombomodulin and sVCAM-1 = soluble vascular cell adhesion molecule*

6.2.6 Effect sizes of significant biomarkers

Table 6.2: Magnitude of the low carbohydrate diet effect on vascular biomarkers

	Effect size (Cohen's d)*
Soluble E-selectin	0.18
Soluble P-selectin	0.24
Soluble thrombomodulin	0.20
C-reactive protein	0.26
Serum amyloid A	0.42
CD31+CD41- EMP	0.47

**Calculated from the difference in values between the end of the normal diet and low carbohydrate diet phases divided by the baseline standard deviation.*

6.2.7 Serum lipids, apolipoproteins and metabolic markers

Table 6.3: Serum lipids, apolipoproteins and metabolic markers following 24 weeks on a normal diet and 24 weeks on low carbohydrate diet

	Normal diet	**Low carbohydrate diet**
Apolipoprotein A1 (mg/dL)	138 ± 3	141 ± 4
Apolipoprotein B (mg/dL)	112 ± 3	110 ± 4
Cholesterol (mmol/L)	5.5 ± 0.1	5.4 ± 0.1
HDL-cholesterol (mmol/L)	1.67 ± 0.07	1.66 ± 0.07
LDL-cholesterol (mmol/L)	3.3 ± 0.1	3.2 ± 0.2
NEFA (mmol/L)	0.69 ± 0.06	0.61 ± 0.03
Triglycerides (mmol/L)	0.92 ± 0.06	0.9 ± 0.1
Glucose (mmol/L)	5.2 ± 0.1	5.2 ± 0.1

Values are mean±SEM. HDL = high density lipoprotein; LDL = low density lipoprotein; NEFA = non-esterified fatty acid

6.3 Discussion

The purpose of this study was to determine the influence of a 24 week low CHO diet that reduced body weight on cellular and protein vascular health biomarkers; PMP, EMP, EPC and protein vascular injury biomarkers in overweight women. Body weight decreased by 3.6 kg and waist circumference decreased by ~4 cm in response to the low CHO diet. EMP were reduced by 21% but EPC were unchanged. The low CHO diet resulted in a reduction in the circulating levels of the protein vascular biomarkers including sTM, sE-selectin, sP-selectin, SAA and CRP. However, serum lipids, apolipoproteins, glucose and insulin were unchanged.

This dietary intervention targeted a reduction in CHO intake. Participants were not permitted to reduce CHO intake below 40 g/day in order to avoid ketogenesis. Otherwise the participants were free to devise their own strategies to help them achieve the reduction in CHO intake. CHO intake was ~90 g/day lower on the low CHO diet compared to the normal diet. The fact that there was no meaningful increase in fat or protein intake when consuming the low CHO diet resulted in a daily energy deficit of ~400 Kcal. However, the percentage of energy obtained from CHO decreased from 44 % to 28 % with the percentage of energy from fat increasing from 38 % to 47 % and protein from 17 % to 23 %. There was no difference in saturated fat intake between the low CHO and normal diet phases, whether expressed in g/day or as a % of total fat intake. The energy deficit over the course of the 24 weeks resulted in a moderate reduction in body weight and waist circumference, respectively. The percentage reduction in body weight was ~5 %, which according to some sources (Bilsborough and Crowe, 2003) is the threshold at which health benefits are observed.

Microparticles and EPC were measured along with vascular protein biomarkers, namely CRP and SAA, soluble cell adhesion molecules and soluble selectins. CD31+CD41- EMP, sTM, sE-selectin, sP-selectin, SAA and CRP protein were lower at the end of the low CHO phase than the normal diet phase. However, CD105/144/146+ EMP, PMP and EPC were not significantly between the normal diet and the low CHO diet.

Findings from previous studies examining the effects of low CHO dietary interventions in individuals at high risk for developing CVD have been equivocal. Low CHO diets have resulted in both favourable and unfavourable changes to endothelial function and vascular risk factors. Golan *et al*., (2011) found a decrease in CRP levels in healthy obese participants but not in obese individuals with type 2 diabetes in response to a 2 year low CHO diet observed (Golan et al., 2011). In another study sICAM and sP-selectin were decreased but CRP and IL-6 remained unchanged in diabetic individuals on a 2-week low CHO diet of ~25 g/day (Davis et al., 2011). Keogh *et al., (2008)* found reduced circulating levels of E-selectin, P-selectin and sICAM-1 and increased sVCAM-1 in overweight and obese participants following an 8-week low CHO diet ((Keogh et al., 2008). Similarly, Brinkworth *et al., (*2004*)* reported decreased levels of sICAM-1 and CRP in obese hyperinsulinemic individuals consuming a low carbohydrate for more than one year (Brinkworth et al., 2004). In a study by Wycherley *et al., (*2009*)* involving overweight and obese subjects, sICAM-1 decreased but VCAM-1 and E-selectin were unchanged following 52 weeks low CHO diet (4 %) (Wycherley et al., 2009).

It appears that CRP, sICAM-1, sP-selectin, sE-selectin are reduced following a low CHO diet, at least in some studies, with sVCAM-1 unchanged. These results are not dissimilar to the current study with respect to protein vascular biomarkers. However, none of the previous studies measured EMP. CD31+41- EMP are also sensitive to low CHO diets that reduce body weight. Indeed, CD31+CD41- EMP had the greatest change in terms of effect size and it is recommended that should be measured in future dietary interventional studies.

Obesity is associated with dyslipidemia, which includes elevated total cholesterol, low density lipoprotein cholesterol, triglycerides, ratios of LDL-cholesterol /HDL-cholesterol and total cholesterol/HDL-cholesterol in addition to decreased HDL-cholesterol (Institute, 1998). However, the modest 3.6 kg loss of body mass and ~4 cm reduction in waist circumference were not accompanied by a change in blood lipids. Although other lipid sub-fractions such as small dense LDL and oxidised LDL were not measured, it does not appear that the changes in endothelial and inflammatory markers were mediated by blood lipids.

Several randomised interventional studies have shown improvement in lipid-related factors, fasting glucose levels, fasting insulin levels and insulin resistance following a restricted or reduced CHO intake in diabetic, overweight and obese individuals (Sacks et al., 2009, Keogh et al., 2008, Brinkworth et al., 2004, Brinkworth et al., 2009, Foster et al., 2003, Samaha et al., 2003, Golan et al., 2011, Stern et al., 2004, Dansinger et al., 2005, Westman et al., 2006). The absence of a change in the present study may be due to the modest change in body composition. However, it is also possible that changes in endothelial and inflammatory markers may occur

independent of changes in blood lipids. Adipose tissue is known to release pro-inflammatory cytokines including IL-6 and TNF-α that stimulate increases in the acute phase proteins, CRP and SAA (Hirschfield and Pepys, 2003, Gabay and Kushner, 1999). On the other hand, CRP can induce endothelial activation directly (Wang et al., 2011). It is possible that the reductions in EMP and other vascular biomarkers are attributable to a reduction in these inflammatory mediators following weight loss. Future studies examining the effect of weight loss on vascular health such measure endothelial and inflammatory biomarkers such as CD31+CD41- EMP in addition to blood lipids.

The influence of the low CHO diet on endothelial and inflammatory biomarkers was determined using analysis of covariance with and without the addition of body weight difference between the two phases as a covariate. CD31+CD41- EMP, sTM, sE-selectin, sP-selectin, SAA and CRP were different but only CD31+CD41- EMP, sP-selectin and SAA were significant after adjustment for the difference in body weight. This suggests that the effect of a low CHO diet on vascular risk in mediated in part by a reduction in body weight, though there may be some independent effect on selected biomarkers including EMP.

Unlike EMP, annexin V+ MP (mainly PMP) were unchanged at the end of the low CHO and normal diet phases. PMP have shown promise as biomarkers in being able to distinguish disease status. In other studies in this dissertation, PMP were able to predict the presence of carotid artery disease. PMP are released from platelets following cell activation. However, platelets are sometimes viewed as a biosensor of endothelial health, due to their frequent interaction with the endothelium, resulting in considerable cross talk (Warkentin et al., 2003). PMP can increase parallel to a

reduction in endothelial dependent dilation after a high fat meal (Tushuizen et al., 2006). It was not unreasonable that changes in endothelial health status might have been reflected in the levels of circulating PMP. Compared to EMP, circulating levels of PMP were more numerous and their enumeration is not beset with the difficulties of rare event analysis. However, PMP do not appear to change in response to a low CHO diet that result in moderate weight loss. It may be important to measure a number of microparticle subpopulations in research of this nature to get an overall estimate of intervention efficacy rather than relying on one isolated biomarker.

EPC which are involved in endothelial regeneration were not different at the end of either dietary phase. Other dietary interventional studies have demonstrated elevated EPC levels. Marin *et al., (*2011*)* randomised participants to a 2 week dietary intervention of Okinawan vegetables, and a control group and found that EPC numbers had increased significantly in the interventional group. No previous studies involving human volunteers had examined the influence of a low CHO diet on EPC. Foo et al., (2009) found a significant decrease in both circulating and bone marrow resident EPC in mice in response to a low-CHO (12%) high-protein (43%) diet (Foo et al., 2009). It is possible that magnitude of the weight loss was not sufficient to increase EPC and the extent of the changes in macronutrient intake were not sufficient to decrease EPC in the present study. EPC, whether expressed as cells/mL or as a % of total progenitors and total CD45+ events, are clearly not a sensitive biomarker of endothelial health following moderate weight loss but may be more useful as a candidate in mechanistic research studies where intervention effect is greater.

There were no adverse effects of the low CHO diet on any of the novel or established vascular biomarker or on any blood lipid markers. This study does not however, demonstrate the safety of all low CHO diets in all populations. The participants targeted a reduction in CHO intake but not to reduce intake below 40 g/day. The diet was not as extreme as other low CHO diets (Freedman et al., 2001b). In reality, CHO intake was considerably higher than the threshold of 40 g/day, though participants still reduced intake by ~90 g/day so that the CHO provided <30% of the total daily energy intake, sometimes regarded as the definition of a low CHO diet (Bilsborough and Crowe, 2003). More extreme ketogenic diets may have had adverse effects on endothelial health, regardless of the magnitude of weight loss. Although the magnitude of weight loss may have been greater, we cannot be sure that we would repeat these findings in more obese cohorts or those with impaired glucose tolerance.

This study had a number of limitations. The study design did not involve a washout between trial phases with the mid-trial blood sample representing the end of the initial phase (normal or low CHO phase) and the start of the next phase. This was in part due to the absence of data indicating the duration of a wash out necessary following the low CHO phase to return biomarkers to pre-study concentrations but also a desire not to prolong the study beyond 48 weeks. It was therefore not possible to compare delta values, i.e. the changes in biomarker concentrations that occurred during the normal diet phase to the changes during the low CHO phase. Although all women reduced CHO intake, the variance in this reduction was large. The women were set a lower limit for CHO intake and not a specific target. More targeted statements for the effect of specific CHO intakes on vascular health may be possible with greater

control of dietary intake. This may necessitate a trial in which all meals are provided given the difficulties in controlling dietary intake under free living conditions over a 48 week period. Weight loss was not targeted in this study though it is frequently observed despite instructions to increase fat and protein intake to prevent weight loss. Although we statistically adjusted for differences in body weight between trials, it would be intriguing to examine the influence of low CHO diets in the absence of weight loss. Finally, it was not possible to employ physiological measures of vascular function such as endothelial dependent dilation. Future studies should examine the effect of specific low CHO diets on vascular function and vascular health biomarkers.

The low CHO model was a useful model with which to examine biomarker responses as these diets could in theory have positive consequences for vascular health, related to weight loss or negative consequences related to increased fat intake. The study has demonstrated a decrease in specific EMP subpopulations in response to this lifestyle intervention. The reductions in CD31+CD41- EMP were observed in along with a reduction in a range of protein vascular biomarkers though the greatest effect of the diet was in CD31+CD41- EMP. Circulating levels of EPC were unchanged and may not be as sensitive a vascular health biomarker. The study also emphasises the value of a moderate weight loss, achieved via reduced carbohydrate intake on vascular health. These changes were evident in the absence of changes in serum lipids and lipoproteins indicating other mechanisms must mediate the effect.

Chapter 7

General Discussion and Recommendations

7. General Discussion and Recommendations

A series of studies was undertaken to (i) quantify and determine the source of variability when enumerating MP, (ii) determine the ability of MP to distinguish individuals with (cases) and without (controls) documented carotid artery disease, (iii) determine the ability of MP to distinguish stable from unstable carotid artery disease, (iv) determine the additive value of MP over protein vascular biomarkers including soluble adhesion molecules, soluble selectins, proteolytic markers, angiogenic markers and inflammatory proteins, (v) determine the influence of commonly prescribed medications on MP concentrations and (vi) determine the influence of a 24 week low CHO diet on protein vascular biomarkers including soluble adhesion molecules, soluble selectins and inflammatory proteins.

In each of the three major studies undertaken, a MP subset, though not always the same subset, was found to have the greatest predictive value or risk monitoring potential. Annexin V+MP distinguished cases of carotid artery disease from controls and had the highest predictive of any biomarker in univariable analysis. Although not the primary focus of these studies, some interesting data was found in relation to the effect of commonly prescribed medications on MP. When controls were analysed separately, those on antiplatelet/anticoagulation medications had significantly lower CD31+ and annexin V+CD41+ MP. In the study comparing stable and unstable carotid artery disease from post-surgical immunohistochemistry CD31+CD41- EMP distinguished patients with unstable plaques from those with stable plaques, and gave additional predictive value over soluble vascular biomarkers in identifying unstable carotid artery disease. In the low CHO diet study CD31+CD41- EMP were significantly

lower following a low CHO diet with the magnitude of the reduction, in terms of the effect size, greater than for any other biomarker. However, there was considerable sample processing and biological variability in the procedures used to enumerate MP.

Results from our studies, indicate that MP have potential as biomarkers of vascular disease and vascular risk. The studies undertaken in the present study show that MP can be used to predict advanced carotid artery disease, predict disease stage and decrease in response to a lifestyle intervention. It is not clear why CD31+CD41- EMP were not different in the carotid artery disease case-control study. This EMP subset was found to have the greatest value in predicting the most serious form of disease (unstable) and was significantly decreased in response to a lifestyle intervention in those who were apparently healthy. However, EMP were not useful in distinguishing carotid artery disease cases from controls.

These studies did not find a role for CPC or PMA as potential biomarkers. There were no difference in CPC or PMA between carotid artery disease cases and controls. In addition, CPC did not change following the low CHO diet. These results suggest that progenitor cells, EPC and PMA may have no value either in predicting vascular disease or in determining the influence of lifestyle intervention.

Protein vascular biomarkers were also assessed. Biomarkers that had potential in predicting disease and disease stage, and determining influence of lifestyle intervention included CRP, SAA, sVCAM-1. SAA was higher in carotid artery disease cases compared to controls and was considerably reduced following a low CHO diet.

The effect size for the reduction was second only to EMP. This acute phase protein is measured less frequently compared to its counterpart CRP.

The findings in relation to sTM and sP-selectin were contradictory. Both of these protein biomarkers were higher in controls compared to carotid patients, and higher in patients with stable compared to unstable plaques. Both decreased significantly along with all other cellular and protein vascular biomarkers following the weight reducing low CHO diet. It is difficult to explain the inconsistencies in data from the current series of studies and other studies. The majority of previous studies have reported elevated levels of sTM and sP-selectin in patients with atherosclerosis. We can only speculate that the lower levels of sP-selectin in the carotid artery disease cases-controls study was due in part to differences in medication regimens as majority patients in the present study were on both lipid lowering and antiplatelet/anticoagulant medications. These observations need further attention. It is questionable as to whether biomarkers that may be influenced by medications have clinical utility as predictors in heavily medicated populations. Such biomarkers may be considered in interventional studies such as those that intend to monitor change following a therapeutic intervention. On the other hand, it would be unwise to regard biomarkers that lack clarity as to whether they are significantly lower or higher in disease states as valuable vascular biomarkers. Until such issues are clarified, these findings should be interpreted cautious.

Studies are needed to evaluate the ability of biomarkers to identify asymptomatic carotid artery disease patients. Although the present series of studies have identified valuable vascular biomarkers, nearly all of the carotid artery disease

cases were symptomatic with transient ischemic attacks. Ideally, MP measured in the present series of study should have utility as predictors of disease and disease stage, and change in response to lifestyle intervention. Future studies should examine the ability of different MP subsets to identify individuals with asymptomatic carotid artery disease and particularly those with unstable plaques who would benefit most from treatment. These studies should also investigate the effects of different medication regimens on MP. In addition, prospective studies are needed to investigate the efficacy of MP (this work has already taken place for other biomarkers) to predict future cardiovascular outcomes including mortality and cerebrovascular events including strokes.

Considerable work is also required in characterizing MP, particularly their detection and accurate measurement. Methodological issues that require considerable attention include centrifugation protocols that ensure reproducibility, techniques that enrich endothelial MP samples and factors affecting biological variability. Previous attempts have focused on removing as many platelets as possible while conserving platelet MP. Other technologies worth investigating include filtration to eliminate large cells such as platelets while conserving MP. There is also need to investigate different techniques to enrich samples with EMP, which are present in considerably lower numbers, than PMP. The current technique of double washes at 19000×g for 30 min is very time consuming and a threat to objectivity. In addition, future studies need to investigate factors affecting day to day variability of MP.

In summary, MP have the potential to act as biomarkers of vascular disease. Despite technical limitations, the series of studies undertaken as part of this PhD thesis

demonstrates the superiority of MP over other vascular biomarkers in identifying individuals with disease, distinguishing disease stage and monitoring changes in vascular health with treatment.

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Appendices

Appendix A: Written informed consent form for carotid artery disease cases

WATERFORD REGIONAL HOSPITAL/WATERFORD INSTITUTE OF TECHNOLOGY
DEPARTMENTS OF VASCULAR SURGERY (WRH)/HEALTH EXERCISE SPORTS SCIENCE (WIT)
RESEARCH - INFORMED CONSENT FORM

I. Project Title:

Circulating microparticles: A potential predictive marker for unstable atherosclerotic plaques

II. Introduction to this study:

Atherosclerosis is a disease affecting the arterial blood vessels, leading to heart attack and stroke. However, advanced plaques are often relatively stable and unlikely to rupture due to the thickening of the overlying fibrous cap. There is increasing interest in the use of biomarkers (measurable from a blood sample) to identify at-risk individuals with unstable vulnerable plaques. Microparticles are one such biomarker with predictive potential. These vesicles are shed into the circulation from a range of vascular cells, with potential to act as sensitive and discriminatory biomarkers of disease activity.

III. I am being asked to participate in this research study. The study has the following purposes:

1. To determine if the presence of atherosclerosis in the carotid artery and the aorta be predicted, from circulating microparticles measured in a simple blood sample
2. To determine if unstable atherosclerotic plaques be distinguished from stable plaques from circulating microparticles and/or other plasma biomarkers measured in a simple blood sample

IV. This research study will take place at Waterford Regional Hospital and Waterford Institute of Technology

V. This is what will happen during the research study:

1. Your artery will be imaged using vascular ultrasound to determine the extent to which the artery has been narrowed or weakened by atherosclerosis.
2. The atherosclerotic plaque (area of hardened artery) removed during surgery will be graded in the pathology laboratory to determine plaque stability (likelihood of rupture).
3. A blood sample will be taken prior to surgery to quantify microparticles and other blood biomarkers.

VI. My confidentiality will be guarded:

Waterford Regional Hospital / Waterford Institute of Technology will protect all the information about me and my part in this study. My identity or personal information will not be revealed, published or used in future studies. The study findings will form the basis for preparation of a postgraduate thesis, academic publications, conference papers and other scientific publications.

VII. <u>If I have questions about the research project, I am free to call a member of the vascular surgery team at telephone no. 051-842308 or Dr. Michael Harrison at telephone no. 051-302161</u>

VIII. <u>Taking part in this study is my decision</u>.

If I do agree to take part in the study, I may withdraw at any point. There will be no penalty if I withdraw before I have completed all stages of the study. My medical treatment will not be affected in any way. However, once I have completed the study I will not be allowed to have my personal information and results removed from the database.

IX. <u>Signature:</u>

I have read and understood the information in this form. My questions and concerns have been answered by the researchers, and I have a copy of this consent form. Therefore, I consent to take part in this research project entitled: *"Circulating microparticles: A potential predictive marker for unstable atherosclerotic plaques"*

Signed: _____

Date: _____

Witness: _____

Appendix B: Written informed consent form for controls

WATERFORD REGIONAL HOSPITAL/WATERFORD INSTITUTE OF TECHNOLOGY
DEPARTMENTS OF VASCULAR SURGERY (WRH)/HEALTH EXERCISE SPORTS SCIENCE (WIT)
RESEARCH - INFORMED CONSENT FORM

I. Project Title:

Circulating microparticles: A potential predictive marker for unstable atherosclerotic plaques

II. Introduction to this study:

Atherosclerosis is a disease affecting the arterial blood vessels, leading to heart attack and stroke. However, advanced plaques are often relatively stable and unlikely to rupture due to the thickening of the overlying fibrous cap. There is increasing interest in the use of biomarkers (measurable from a blood sample) to identify at-risk individuals with unstable vulnerable plaques. Microparticles are one such biomarker with predictive potential. These vesicles are shed into the circulation from a range of vascular cells, with potential to act as sensitive and discriminatory biomarkers of disease activity.

III. I am being asked to participate in this research study. The study has the following purposes:

3. To determine if the presence of atherosclerosis in the carotid artery and the aorta can be predicted, from circulating microparticles measured in a simple blood sample

IV. This research study will take place at Waterford Regional Hospital and Waterford Institute of Technology

V. This is what will happen during the research study:

4. Your artery will be imaged using vascular ultrasound equipment to determine the extent to which your artery is narrowed by atherosclerosis.
5. A blood sample will be taken to quantify microparticles and other blood biomarkers.

VI. There are no envisaged risks or side effects associated with participation in this study:

VII. There may be benefits from my participation in this study. These are:

The vascular imaging (ultrasound) may reveal the presence of advanced atherosclerosis in your artery. Analysis of your blood sample may show elevated levels of various risk factors (e.g. cholesterol). If this occurs, you will be informed and advised of the appropriate course of action.

VI. My confidentiality will be guarded:

Waterford Institute of Technology will protect all the information about me and my part in this study. My identity or personal information, will not be revealed, published or used in future studies. The study findings will form the basis for preparation of a postgraduate thesis, academic publications, conference papers and other scientific publications.

VII. <u>If I have questions about the research project, I am free to call Dr. Michael Harrison at telephone no. 051-302161:</u>

VIII. <u>Taking part in this study is my decision</u>.

If I do agree to take part in the study, I may withdraw at any point. There will be no penalty if I withdraw before I have completed all stages of the study. My medical treatment will not be affected in any way. However, once I have completed the study I will not be allowed to have my personal information and results removed from the database.

IX. <u>Signature:</u>

I have read and understood the information in this form. My questions and concerns have been answered by the researchers, and I have a copy of this consent form. Therefore, I consent to take part in this research project entitled: "*Circulating microparticles: A potential predictive marker for unstable atherosclerotic plaques"*

Signed: _____

Date: _____

Witness: _____

Appendix C: Plain language statement for carotid artery disease cases

Plain Language Statement

Doctors at Waterford Regional Hospital and researchers at Waterford Institute of Technology have teamed up on a medical research project. We hope to develop a test that might indicate the presence of cardiovascular disease in the arteries, from a simple blood sample. We are particularly interested in the concentration of small particles in the bloodstream known as microparticles.

Shortly, you will be undergoing surgery at Waterford Regional Hospital in order to remove a fatty deposit from the artery wall in your neck area. We are seeking your permission to analyse this fatty deposit in the lab. We also wish to take a blood sample in advance of surgery in order to measure microparticles and other substances that may be related to cardiovascular disease. We hope to be able to predict the severity of cardiovascular disease from the concentration of these microparticles in the bloodstream.

We hope to be able to predict the presence of cardiovascular disease from the concentration of these microparticles in the bloodstream.

If you have any further questions, please call

Michael Harrison (WIT), 051-302161

Appendix D: Plain language statement for controls

Plain Language Statement

Doctors at Waterford Regional Hospital and researchers at Waterford Institute of Technology have teamed up on a medical research project. We hope to develop a test that might indicate the presence of cardiovascular disease in the arteries, from a simple blood sample. We are particularly interested in the concentration of small particles in the bloodstream known as microparticles. A number of patients undergoing surgery at Waterford Regional Hospital will have the severity of their cardiovascular disease determined prior to surgery using ultrasound. They will also have a blood sample taken in order to measure microparticles and other substances that may be related to cardiovascular disease.

We are seeking individuals of similar age who have no history of cardiovascular disease to act as comparisons. Your participation in the study will involve a visit to your GP to have a blood sample taken in the fasted state. You will also be called at a later date to have the arteries in your neck scanned using ultrasound. If disease is detected in your arteries, you and your GP will be informed.

In order to make contact with you for the ultrasound, it will be necessary to release your name and contact details to the research team at WRH / WIT. They also need to know if you suffer from blood pressure or diabetes or if you are taking medication to treat high blood pressure, diabetes, high cholesterol or to thin the blood.

We hope to be able to predict the presence of cardiovascular disease from the concentration of these microparticles in the bloodstream.

If you have any further questions, please call

Michael Harrison (WIT), 051-302161

Appendix E: Questionnaire for controls

The Carotid Artery Biomarker (CAB) Study

(To be filled out for every participant)

Patients are not eligible for study if there is a history of coronary artery disease, cerebrovascular disease, peripheral arterial disease, aneurysmal disease or cancer.

Sample should not be taken if patient is suffering from acute infection or inflammatory disease flare-up

Date of blood sample:

Patient Name:

Contact details (please include contact telephone number)

Year of birth:

Smoker Yes □ No □ Former □

Diagnosis of

Diabetes (type I or II)	Yes □	No □
Hypertension	Yes □	No □

Medications

Anti-hypertensive	Yes □	No □
Lipid-lowering	Yes □	No □
Glucose control or insulin	Yes □	No □
Anti-platelet or anti-coagulant	Yes □	No □

Appendix F: Written consent form for low carbohydrate diet participants

Waterford Institute *of* Technology
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Consent by Subject for Participation in Research Protocol

Subject Number: _____ Name of Volunteer: _____

Title of Protocol: The effect of a low-carbohydrate diet on the biomarkers of bone health in pre and postmenopausal women.

Researcher: Doreen Fitzmaurice Supervisor: Dr. Lorna Doyle

You are being asked to participate in a research study. The researchers at Waterford Institute of Technology study the impact of dietary practices on possible disease development in an attempt to reduce further disease incidence. In order to decide whether or not you want to be part of this research study, you should understand enough about its risks and benefits to make an informed judgment. This process is known as informed consent. This consent form gives detailed information about the research study which will be discussed with you. Once you understand the study, you will be asked to sign this form if you wish to participate.

Osteoporosis and bone fragility affects one in three women and one in five men in Ireland. The incidence of osteoporosis is increasing among females. Low-carbohydrate diets continue to be a popular choice in weight loss; these diets involve increasing protein and fat intakes to maintain adequate energy levels. Increased protein intake increases urine acidity, calcium excretion, bone resorption, and ultimately may reduce bone health. Studies have demonstrated the effectiveness of low-carbohydrate diets in loosing weight, but few have examined low-carbohydrate diets and their potential effect on bone health. Due to increasing osteoporosis incidence in women, this study investigates the influence of low-carbohydrate consumption, on urine acidity, calcium excretion and bone health.

What does it involve?

Each subject will be screened to ensure they have no factors which could affect bone health and are well enough to take part in the study. Each subject will have a DEXA scan to ensure normal bone health and have FSH and oestrogen levels measured to establish hormonal status. If any risk factors are identified during the screening process a follow up letter will be sent by the researcher to the subject's G.P. informing them of the findings.

After screening each subject will complete a physical activity questionnaire (osteogenic index measurement) to assess their physical activity level, since this could have a beneficial effect on bone health. Each subject will then complete a food diary for 3 days (2 weekdays and 1 weekend day) and a Food Frequency Questionnaire (FFQ)

which will be analyzed on the dietary analysis programme CompEat. While the 3 day diary is being completed each subject will collect 3 first morning urine samples for measurement of urine pH. A blood sample of 10ml (one tube) will be taken from each subject by a trained phlebotomist (doctor or nurse). This sample will be used to analyze serum creatinine (indicator of kidney function) bone formation and resorption indicators.
Subjects will be advised to continue with their normal diet for 6 months, and return each month to complete a FFQ and monitor bone health and kidney function at 8 week intervals.
or
Subjects will be advised on the low-carbohydrate diet to follow. Support for adherence to the diet will include quantities of carbohydrates in various foods, recipes, meal plans, and cooking classes to produce low carbohydrate foods that are not readily available. They will also return each month to complete a FFQ and monitor bone health and kidney function at 8 week intervals.
At the end of 6 months the subjects will give a blood sample for measurement of biomarkers of bone turnover and kidney function, complete a 3 day food diary, physical activity questionnaire, and 3 day urine sample for measurement of urinary pH. They will then be crossed over.
Subjects who have been following their normal diet will then follow the low-carbohydrate diet, and subjects who have been on the low-carbohydrate diet will revert to their normal diet for 6 months with the same reporting procedures as in the previous 6 months, in place. At the end of 12 months the subjects will give a blood sample for measurement of biomarkers of bone turnover, complete a 3 day food diary, physical activity questionnaire, and 3 day urine sample for measurement of urinary pH.

How inconvenient will this study be to you?
Taking blood sometimes may cause bruising. Very rarely it may cause inflammation of the vein and possible infection. The doctor makes every effort to avoid these situations. You will be asked to fast overnight on occasions that you give blood samples, this entails not eating from approximately 9.00 pm the night before and delaying breakfast until after the blood sample (between ~8.00 - 9.00 a.m.) which will be taken here in Waterford Institute of Technology.
We will be glad to provide you with the results of this study including your dietary intakes. The information that we collect is only for our research and will be confidential. This information will be stored in a secure place and in any publications that arise from this research; volunteers will be identified by number codes only.
The DEXA scanner used to measure bone density emits a very small dose of radiation, about 0.01 mSv, which is about the same as the average person receives from background radiation in one day, so the potential carcinogenic effect of exposure to radiation is minimal.
A low-carbohydrate diet will initially induce rapid weight loss, this is mainly due to water loss. If carbohydrate levels go very low the body will use protein and fat for energy and a condition known as ketosis may occur. The diet in this study is designed to prevent this. The symptoms of ketosis may include tiredness or fatigue, headache, bad breath, metallic taste in the mouth, weakness, dizziness, nausea or stomach ache, sleep problems. Drinking plenty of water can prevent or ease ketosis. If any symptom

occurs eat an appropriate mod/high carbohydrate food, such as a carrot or some red pepper to alleviate the symptoms.
We consider this study to involve only "minimal risk", that is we think the worst thing to happen would be minor bruising after the taking of blood.

Benefits to the volunteer
As a result of taking part in this research volunteers can get some benefits in terms of weight loss. Feedback will also be available on:

- Dietary intake and advice
- Bone health status
- Body fat %
- Hormonal status
- Kidney function
- Blood pressure

Your decision to take part in this study is entirely voluntary. You may leave the study at any time. If you have any questions concerning the study, you may contact Ms. Doreen Fitzmaurice at 086 3028743 who will deal with any queries you have.

Agreement to Consent
The research project and the treatment procedures associated with it have been fully explained to me. All experimental procedures have been identified and no guarantee has been given about the possible results. I have had the opportunity to ask questions concerning any and all aspects of the project and any procedures involved. I am aware that participation is voluntary and I may withdraw my consent at any time. Agreement to consent to take part in this study adheres to the regulations of the Data Protection Act. Confidentiality of records concerning my involvement in this project will be maintained in an appropriate manner. No subject in this research will be referred to and will be assigned a code (subject number) when dealing with result presentation, in order to ensure confidentiality. When required by law, the records of this research may be reviewed by government agencies and sponsors of the research.
I, the undersigned, hereby consent to participate as a subject in the above-described project conducted at the Department of Sport and Exercise Science, Waterford Institute of Technology. I have received a copy of this consent form for my records. I understand that if I have any questions concerning this research, I can contact the researchers listed above.
After reading the entire consent form, if you have no further questions about given consent, please sign where indicated.

Researcher:_____ Signature of Subject:_____

Witness:_____ Date:_____ Time:_____

am/pm (circle)

Appendix G: Health screening questionnaire

Waterford Institute *of* Technology
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Health Screening Questionnaire

All information provided will remain confidential

Personal details

Name: _____

Address: _____

Phone: _____ Email: _____

Height: _____ Weight:_____

Age: _____

GP's name: _____

GP's address: _____

GP's number: _____

Medical History

1. Have you ever broken any bones or experienced stress fractures?

Yes **No**

If yes please give details.....
.....
.....

2. Have you ever been immobilized for more than two weeks?

Yes **No**

If yes please give details.....
.....
.....

3. Has anyone in your family suffered from osteoporosis?

Yes **No**

If yes please give details.....
.....
.....

4. Do you or have you ever suffered from any problems concerning your bones or joints (ie osteoarthritis, rheumatism, lower back pain, metabolic bone diseases)?

Yes **No**

If yes please give details.....
.....

.....

5. Have you undergone a hysterectomy? **Yes** **No**

If yes please give details: .....

.....

6. Are you suffering from any of the following conditions?

Thyroid or parathyroid disorder **Yes** **No**

Kidney disease **Yes** **No**

Digestive/hormonal disorder **Yes** **No**

Diabetes **Yes** **No**

Drug History

7. Are you now, or have you ever taken or used any of the following and if so for how long and at what age?

Mirena coil **Yes** **No**.....

H.R.T **Yes** **No**.....

Oral contraceptives **Yes** **No**.....

General Nutrient supplements **Yes** **No**.....

Soya products **Yes** **No**.....

Calcium supplements **Yes** **No**.....

8. Are you currently taking any other medication?

Yes **No**

If yes please give details

.....

.....

.....

9. Do you smoke?

Yes **No**

If YES;

How old were you when you started? .....

How many do you smoke per day on average over the last year?.....

If NO;

Have you ever smoked? **Yes** **No**

How old were you when you started? .....

How old were you when you stopped?.....

How many did you smoke/day on average?.....

10. Do you drink? Coffee **Yes** **No**

If YES; How many per day? .....

Tea **Yes** **No**

If YES; How many per day? .....

Coke/cola **Yes** **No**

If YES; How many per day? .....

11. Do you drink alcohol?

Yes **No**

If YES; How many days per week?.....

What do you drink and how much on an average day/night?

During week: .....

.....

At weekend: .....

.....

Menstruation history

12. Is there a possibility that you may be pregnant? **Yes No**

If no: please give details.....

13. Approx what was the length of your menstrual cycle between the ages of 25 and 35 years?.....

.....

14. Did you have chronic irregularities at any stage during menstruation between the ages of 25 and 35 years? **Yes No**

If yes please give details.....

.....

.....

15. Has your menstrual cycle ever become 7 days longer or shorter than normal? **Yes No**

If yes: please state how long ago this started to occur .....

: If it still occurs .....

16. Has the length of time between your menstrual cycles ever changed by seven days?

Yes No

If yes: please state how long ago this started to occur .....

: If it still occurs .....

17. Have you ever had an interval of 60 days or more without having a period (not including pregnancy)? **Yes No**

If yes: please state how long ago this started to occur .....

: If it is still occurring .....

18. Have you been 12 months or more without having a period? **Yes No** (not including pregnancy)

If yes how long is it since your final menstrual period?.....

Activity and Dieting history

19. Do you or have you ever engaged in intense physical training? **Yes No**

If yes please give details .....

.....

20. How would you describe your level of activity between the age of 25 and 35 years?

Leisure: **very active** **moderate** **non active**

Household: **very active** **moderate** **non active**

Occupational: **very active** **moderate** **non active**

21. How would you describe your level of activity now?

Leisure: **very active** **moderate** **non active**

Household: **very active** **moderate** **non active**

Occupational: **very active** **moderate** **non active**

22. Do you use self-administered and self-monitored diets?

Yes No

If YES;
How old were you when you started? .....
How many times were you on a diet on average?.....
How many times have you been on such a diet over the last year?.....
If NO;
Have you ever? **Yes No**
How old were you when you started? .....
How old were you when you stopped?.....
How many times were you on a diet on average?.....
23. Have you been on a diet that was monitored by a health professional? **Yes No**
If yes please give details.....
.....
.....
24. Have you ever been on a Low-Carbohydrate diet? **Yes No**
If yes : approx how many times?.....
: How long ago?.....
: For how long?.....
: What were it's effects?.....
.....

In accordance with the data protection act the subjects' name will appear on the screening form and on the consent form. In all other incidences the subject is allocated a code. The screening and consent forms will exit only as a single hard copy which will be confidential. The data will be destroyed seven years after the publication date of the study.

Appendix H: Food diary

Food Diary

How to fill out the diary

Fill in everything you eat on three different days, they do not have to be consecutive but must include one weekend day

-Include everything you eat and drink throughout the day, both at home and outside the house

-**Be specific**. E.g:

i. If you had toast – white/brown, a thick/medium/thin slice? Butter/spread, thickly/thinly spread? Jam/marmalade, was it spread thickly/thinly?
ii. If you had tea/coffee – with milk? Skimmed/low-fat/whole? Sugar/artificial sweetener? How many?
iii. If you had fruit was it small/medium/large?

-**Include extras** – gravy, butter/cheese on vegetables etc.

-**Write down as you go** through the day, it is difficult to remember at the end of the day

-**Tell the truth** as this is aimed to be a positive guide to understand what you eat.

Portion sizes

Portion sizes can be determined in a number of different ways

-**By weight**. At first you will have to weight out the food, but very quickly you will be able to judge the amount by eye.

A *digital* scale makes this easier. Recipes can use grams (g) or ounces (oz). To help convert 1oz = 25g

-**By tea/tablespoons**

A teaspoon (tsp) = 5ml/5g. A tablespoon (tbls) = 25mls/25g

-By cup measures, teacup, mug. Cup measures are available in most shops. But you can use a teacup or a mug from your kitchen.

Liquids -A teacup = approx 100mls

-A mug = approx 300mls

1 cup = 3oz/75g celery

1 cup = 4oz/100g carrot

1 cup = 3oz/75g broccoli

 It can be more difficult to gauge portions sizes when you eat out so here are some guidelines that might help.

 To estimate the amount of a serving of vegetables, cooked cereals or even fruit that would be considered 1 cup, make a fist with your hand. Now visualize the portion size of a vegetable on your plate. It should be placed within the area of your fist.

 Your proteins are usually measured in ounces. With your palm facing you visually draw a circle starting where your fingers join your hand. This area is an example of how much room on your plate 3 oz/75g. should cover.

 Place your hand in front of you with palm up. Now cup your hand. The amount of nuts/cereals to equal 1 oz/25g should fit in the cup of your hand and not roll out.

Dietary Analysis

Record all foods eaten yesterday and the portion size of each.

	Food (Include all foods, drinks and supplements)	Amount/Portion Size (for example ½ cup, 3oz)	**Symptoms** (for example gas, bloating, diarrhoea, none)	**Mood** (for example anxious, calm, angry, sad, happy, excited, worried, bored)
Breakfast or 1st meal Time:				
Snack Time:				
Lunch or 2nd meal Time:				
Snack Time:				
Dinner or 3rd meal Time:				
Snack Time:				

Appendix I: Food frequency questionnaire

Food Frequency Questionnaire

Nutrition Systems COMP-EAT

Question 1

Did you eat any breakfast cereals last week	Yes	NO

Tick or highlight selection

Question 2 (ignore if you answered NO to question 1)

Indicate which cereals most closely represent the type you ate last week and how often.

All-bran		Bran flakes		cornflakes	
Coco pops / honey smacks / crunchy nut c'flakes		Fruit'n fibre		Muesli no added sugar	
porridge		Puffed wheat / shredded wheat		Rice krispies	
Special K		Sugar puffs / frosties		weetabix	

Question 3

If you eat bread, indicate the type of bread, rolls, pitta etc. you ate last week and how much.

Slices – brown / wholemeal		brown / wholemeal large roll		brown / wholemeal small roll	
brown / wholemeal pitta		croissant		Slices white	
White large roll		White small roll		White pitta	
crumpet		Plain muffin / scone		Chapattis – without fat	
naan		Fried bread		Crisp bread / rice cakes	
oatcakes					

Question 4

How many times last week did you use butter, margarine or a low fat spread? Count each slice of bread, roll, biscuit and what you put on potatoes and other vegetables.

0	1	2	3	4	5
6	7	8	9	10	11
12	13	14	15	16	17
18	19	20	25	30	35
40	45	50	55	60	65+

Tick or highlight selection

Question 5

Which of the following spreads do you usually eat?

Butter	**Hard Margarine**
Soft Margarine	**Monounsaturated Marg.**
Low Fat Spread	**Very Low Fat Spread**
A Mixture Of Spreads	**Polyunsaturated Marg.**
Soya Margarine	

Tick or highlight selection

Question 6

Do you spread:

Thickly	**Medium**
A Thin Scrape	

Tick or highlight selection

Food Frequency Questionnaire

Nutrition Systems COMP•EAT

Question 7

How many teaspoonfuls of marmalade, jam or honey did you eat last week?

0	1	2	3	4	5
6	7	8	9	10	11
12	13	14	15	16	17
18	19	20	25	30	35
40	45	50	55	60	65+

Tick or highlight selection

Question 8

If you eat pasta (noodles / spaghetti) or rice, indicate the type eaten and how often last week

White rice		Brown rice		White / orange / green pasta	
Wholewheat pasta					

Question 9

If you eat potatoes / cassava / yams / plantain (include potatoes in soups, shepherd's pie etc), indicate the type and how often you ate them last week. (For chips see next question)

Boiled / mashed		Jacket / boiled in skins		Mashed with spread	
roast		Croquettes / waffles			

Question 10

How often last week did you eat any of the following?

Oven chips		Retail / home-made chips		Shallow fried potatoes	
Fried plantain					

Question 11

Indicate the vegetables (fresh, frozen, tinned) which you ate last week and the number of servings. Count a portion of salad or home-made vegetable soup as a serving. Vegetables in cooked dishes e.g. stews and curries should be included as a serving of vegetable mixture.

Vegetable mixture		A portion of salad		Vegetable stir-fry mix	
Aubergine / artichoke		Beans: green, broad, runner		Broccoli / peppers	
Cabbage / cauliflower		carrots		Courgette / leeks	
Peas, fresh / frozen		mushrooms		onions	
Sprouts / parsnips / okra		Spring greens / spinach / kale		Swede / turnip / pumpkin	
Sweetcorn / sweet potato		Tomato, other than salad			

Question 12

If you ate any vegetables last week, were they fried?	Yes	NO

Tick or highlight selection

Question 13

How often do you eat Quorn, Tofu or TVP?

Daily	**3 – 5 Times a week**
1 – 3 Times a week	**1 – 3 Times a fortnight**
Fortnightly	**Occasionally / Never**

Tick or highlight selection

Question 14

How often did you eat beans (including baked beans), split peas, dahl or lentils last week?

Canned in water only		Canned with added salt only		Canned with added salt and	
Canned in sauce e.g. tomato		Canned in sauce – reduced sugar		Canned in sauce – reduced sugar / reduced salt	
dried					

Question 15

If you eat any of the following vegetarian dishes, how often did you eat them last week?

beanburgers		falafel		Vegetable pie – pastry top	
Lentil rissoles		Nut cutlets / roast		Vegebanger / vegeburger	
Vegetable samosa					

Question 16

Indicate the fruits (fresh, frozen, tinned) and dried fruit, e.g. raisins, you ate last week and how many portions.

Fruit salad, a bowl		Dried fruit, a small handful		Apples / Apricots	
avocado		Bananas		Small bunch grapes / plums	
Kiwi / nectarines		Melons, Mangoes		Oranges / Grapefruit	
Peaches / Pears		Pineapple / Rhubarb		Satsumas / Tangerines	
Soft fruits e.g. strawberries					

Question 17

How much milk, including soya milk and made-up powdered milk, do you have in a day? Include what is used in tea, coffee and sauces. You will be asked about milk drinks and milk puddings in another question.

1 pint or more	**¾ Pint**
½ Pint	**¼ Pint**
I seldom use milk	

Tick or highlight selection

Question 18

How much milk, including soya milk and made-up powdered milk, do you have in a day? Include what is used in tea, coffee and sauces. You will be asked about milk drinks and milk puddings in another question.

Gold Top (breakfast milk)	**Whole Milk (silver; red top)**
Semi-Skimmed (red and silver striped)	**Skimmed (blue and silver checked)**
A mixture of different cows milk	**Goats Milk**
Soya Milk	**Don't Know**

Tick or highlight selection

Food Frequency Questionnaire

Nutrition Systems COMP-EAT

Question 19

If you use cream, canned milks or coffee whitener, what kind did you use and how often last week? (Include what is used in cooking.)

Double cream (1 tbsp)		Single cream (1 tbsp)		Whipping cream (1 tbsp)	
Sour cream (1 tbsp)		Imitation creams (1 tbsp)		Condensed Milk, whole (1 tbsp)	
Condensed Milk, skim (1 tbsp)		Evaporated Milk (I small tin)		Coffee whitener (per tea / coffee)	

Question 20

If you eat cheese, what kind did you eat last week and how often? A portion of hard cheese is equivalent to the size of a small match box. Include the cheese in sauces, etc.

Blue cheese (stilton)		Hard cheese (cheddar type)		Hard cheese, reduced fat	
Vegetarian cheddar		Cheshire / Caerphilly		Gouda / emmental / edam	
Brie / camembert		Fetta / mozzarella / ricotta		cream cheese (1 tbsp)	
Half fat cream cheese (1 tbsp)		cottage cheese (3 tbsp)		Plain fromage frais / quark (3 tbsp)	
Processed cheese		Soya cheese		Don't know the type	

Question 21

If you eat eggs, how are they cooked and how many did you eat last week?

Boiled / Poached		Fried		scrambled	
2 egg omelette / soufflé		Egg mayonnaise filling		Scotch eggs	
Cheese and egg quiche					

Question 22

If you eat meat, indicate the types which you ate last week and the number of times you ate them. Remember to include what you ate in sandwiches.

2 bacon rashers or 1 sausage		Low fat sausage		Sausage rolls	
Beef / lamb / pork – lean + fat		Beef / lamb / pork – no fat		Chicken / turkey – with skin	
Chicken / turkey – no skin		Breaded veal / chicken		ham	
Liver / kidney etc		Luncheon meat		Meat pies / pastie	
Mince / stews		Beefburgers / corned beef		Low fat beefburgers	
pâté / liver sausage		Blackpudding			

Question 23

If you ate any meat last week, was any of it fried?	Yes	NO

Tick or highlight selection

Question 24

If you eat fish (fresh, frozen or tinned), indicate what you ate last week and how often.

Fried fish		Fish steamed / grilled		Fish fingers / coated fish	
Kipper/herring/mackerel/salmon		Pilchards / sardines		Shellfish (prawns / crab)	
trout		Tuna in water		Tuna in oil	
Fish pâté / fish paste					

Food Frequency Questionnaire

Question 25

Did you eat any of the following RESTAURANT or TAKE-AWAY meals last week and if so how often?

Chinese meal / prawn meal		Chinese vegetable meal		Meat / prawn curry meal	
Tandoori chicken meal		Chicken tikka meal		Vegetable curry meal	
Kebab, shish		McDonalds / Burger King		Home made / shop pizza	

Question 26

If you eat nuts and seeds, how many times last week did you eat the equivalent of one handful?

plain		salted		Dry roasted	
A mixture					

Question 27

How many times last week did you eat a level tablespoonful of nut or seed butter (e.g. peanut)?

0	1	2	3	4	5
6	7	8	9	10	11
12	13	14	15	16	17
18	19	20	25	30	35
40	45	50	55	60	65+

Tick or highlight selection

Question 28

How many times last week did you eat a packet of potato crisps or other savoury nibbles (e.g. Asian snacks)?

0	1	2	3	4	5
6	7	8	9	10	11
12	13	14	15	16	17
18	19	20	25	30	35
40	45	50	55	60	65+

Tick or highlight selection

Question 29

How many times last week did you eat tinned or packet soups and sauces?

0	1	2	3	4	5
6	7	8	9	10	11
12	13	14	15	16	17
18	19	20	25	30	35
40	45	50	55	60	65+

Tick or highlight selection

Question 30

If you eat any of the following, how many times last week did you eat the equivalent to 1 tablespoonful? Don't forget what you added to sandwiches.

Hummus / taramasalata		Pickle / brown sauce / ketchup		French dressing / mayonnaise	
Low calorie dressing		Salad cream		coleslaw	
Chocolate spread		Scrape of marmite			

Food Frequency Questionnaire

Nutrition Systems COMP•EAT

Question 31

If you eat biscuits and crackers, how many did you eat last week?

Cereal bars		Chocolate coated biscuits		Digestive biscuit, plain	
Digestive biscuit, chocolate		Semi-sweet / rich tea		shortbread	
Custard creams / bourbons		Jaffa cakes		Garibaldi / fig rolls	
Wholemeal crackers		Cream crackers		Bread sticks / water biscuits	
flapjack					

Question 32

If you eat cakes and puddings, how many did you eat last week?

Doughnut / Danish pastry		gateau		Fruit cake / mince pie	
Fruit pie or crumble		Cheese cake		trifle	
Steamed / baked puddings		Sponge cake		Asian pastries	
Cream cakes		Fancy iced cakes		Currant buns / teacakes	

Question 33

If you eat milk-based desserts, how many servings did you eat last week?

Custard on pudding		Ice cream		mousse	
Milk puddings		Plain low fat / diet yoghurt		Fruit low fat yoghurt	
Plain whole milk yoghurt		Fruit whole milk yoghurt		Greek yoghurt	
Soya yoghurt		Fromage frais, fruit			

Question 34

Indicate which, if any, of the following confectionery you ate last week, and how many times.

Milky way / fudge / kitkat (2 bar)		Flake / maltesers / crème egg		Aero / wisper / crunchie	
Twirl / spira		Double decker / kitkat (4 bar)		Caramel / toffee crisp / lion bar	
Bounty / drifter		Mars / snickers / twix		yorkie	
Smarties / m & m's / minstrels		Plain or milk chocolate (50g bar)		toffees	

Question 35

How often did you eat a packet of sweets last week? Include mints, pastilles, boiled sweets, liquorice, fruit chews, fruit gums, etc.

One packet a day	**More than two packets a week**
Two packets a week	**One packet a week**
Occasionally / never	

Tick or highlight selection

Food Frequency Questionnaire

Nutrition Systems COMP-EAT

Question 36

How many teaspoonfuls of sugar do you eat a day on cereals and in hot drinks? Do not include artificial sweeteners.

0	1	2	3	4	5
6	7	8	9	10	11
12	13	14	15	16	17
18	19	20	25	30	35
40	45	50	55	60	65+

Tick or highlight selection

Question 37

How many drinks of the following did you have last week?

Milky drinks e.g. cocoa		Fizzy drinks (not low cal.)		Fruit juice, unsweetened	
Glass of milk		Squash / cordial		Fruit drinks	
Water / low calorie drinks		Tea / coffee		Low cal. Hot instant drinks	

Question 38

If you drink alcoholic drinks, how many of the following did you drink last week?

Half pint of beer / larger / cider		Glass of wine		Single measure of spirits	
sherry		Martini / port		liqueurs	
Low alcohol beer		Low alcohol wine			

Personal Details

Email address		*Optional*
Full Name		*Optional*
Height		
Weight		
Age		
Sex		
Occupation		*Light, moderate or heavy*
Lifestyle		*Non-Active, Moderate or Very Active*
country		*Optional*
Nearest city		*Optional*

Office Use

Appendix J: Protocol for haematology analysis

Protocol for haematology analysis (COULTER® A^{C}●T diff™ haematology analyser [Beckman Coulter])

Running controls (COULTER® 4C PLUS/4C-ES® cell controls [Beckman Coulter])

i. Enter in the instrument the lot number, expiration date, assigned values and expected ranges from assay sheet supplied with controls (refer to QUALITY CONTROL SET UP instructions in the User's Guide)
ii. Remove control vials from refrigerator. Confirm that lot numbers and expiration dates on vial match information on the table of expected results.
iii. Warm at room temperature 10-15 minutes.
iv. At instrument main screen, touch QA icon then 4C Run icon.
v. Select correct control level: L-low, N-normal or H-high. Make sure that level of control you are testing matches the one selected (L, N, or H)
vi. Verify control cap is secure. Mix each control vial 8 x 8 x 8 times according to package insert instructions. Inspect vial contents to ensure uniform cell distribution. If contents are not well distributed, repeat this mixing procedure.
vii. Cover top of control vial with lint-free tissue and remove cap. Place well-mixed vial under probe. Press the aspirate switch. When you hear the beep remove the vial and recap it.
viii. Results appear on screen. Review control results. Rerun any controls not within expected ranges. If rerun control is still out of range, follow your laboratory's protocol for troubleshooting out-of-range controls. Reject the control, if necessary.
ix. Repeat steps ii through vii for each required control level.

Running patient samples / whole blood mode

i. At main screen select whole blood mode. Touch sample results screen icon. Touch patient range icon until the desired range (1, 2, or 3) appears. Enter sample ID
ii. Mix sample thoroughly according to your laboratory's protocol. Be sure you are in whole blood mode.
iii. Place a lint-free tissue over the top and remove cap. Place well-mixed sample at probe and press aspirate switch. Remove tube when you hear the beep.
iv. Instrument automatically saves results.
v. Results appear on screen. Print results.

Calibration (COULTER® A^{C}●T diff™ haematology analyser [Beckman Coulter])

Preparation for calibration

Refer to A^{C}●T diff 2 Operator's Guide for information about frequency of calibration and other reasons to calibrate the instrument.

To prepare the instrument, before calibrating, perform

i. Pre-calibration Checks
ii. Reproducibility
iii. Carryover

Pre-calibration Checks

i. Required maintenance has been performed on the instrument.
ii. Perform Cleaning (Bleaching) the Baths procedure.
iii. Average room temperature is within the system's operating temperature range.
iv. Sufficient reagent supply to complete these procedures
v. Perform Startup.

Reproducibility

NOTE: Refer to the Operator's Guide for patient sample criteria for performing reproducibility.

i. At Main screen select whole blood mode
ii. Touch QA icon the reproducibility icon
iii. Thoroughly mix sample. Remember to mix sample gently between each cycle.
iv. Analyse sample in whole blood mode for your instrument.
v. When Reproducibility sample result displays, touch Trash icon to delete the first (prime) sample manually
vi. Repeat step iv until an N of 11 is reached. (Look at upper left corner of screen for N#)
vii. Verify PASSED for all parameters.
viii. Touch the Print Summary icon to print a reproducibility summary report for your records

Carryover

NOTE: Refer to the Operator's Guide for patient sample criteria for performing Carryover. You may use 4C cell control as an alternative to normal whole-blood sample

i. At Main screen select whole blood mode
ii. Touch QA icon the carryover icon
iii. Thoroughly mix sample and cycle in whole blood mode two times.
iv. Repeat steps 3 for the second sample.
v. Run a blank sample by pressing aspirate switch.
vi. Repeat step v twice for a total of three blank samples.
vii. Touch Summary icon to view carryover summary screen.
viii. High-to-Low Carryover on the A^{C}●T diff System should meet these limits: ALL parameters should show that Carryover is <= 2.0%
ix. Touch Print Summary icon to print a carryover summary report for your records.

Auto calibration with COULTER S-CAL® calibrator

i. Make sure that you have performed all pre-calibration checks. Beckman Coulter recommends using S-CAL calibrator.
ii. Prepare S-CAL calibrator according to instructions in S-CAL package insert
iii. Confirm that lot number and expiration date on vial match information in the table of assigned values.
iv. Do use Calibrator if it has expired.
v. Print calibration setup report.
vi. At main screen, touch setup icon touch setup report icon.
vii. After calibration setup report prints, touch Exit icon.
viii. (These are the "current" calibration factors in the instrument.)
ix. At Main screen, select whole blood mode
x. At Main screen, touch QA icon. Calibration icon
xi. Calibration assay screen appears.
xii. Refer to Table of Assigned Values supplied with your calibration material.

xiii. On screen, use the keypad to enter values from table of assigned values.
xiv. When you have entered all values, touch continue icon.
xv. The run screen appears.
xvi. Mix the S-CAL calibrator according to the package insert instructions.
xvii. Run S-CAL 11 times in whole blood mode.
xviii. Mix S-CAL vial gently between each cycle.
xix. The instrument does not use the result from the first run. It performs statistics on runs 2 through 11 for a total of 10 runs. The instrument automatically saves the results.
xx. The instrument displays but automatically rejects non-numeric result.
xxi. If you choose to reject a result, you can only reject the last sample analysed.
xxii. After 11 acceptable results, the summary icon will appear.
xxiii. Touch the to view the calibration summary screen and print for records
xxiv. Review results status on the Report Summary.
a. PASSED for all parameters means calibration adjustment is not required. Touch Return icon.
b. NEEDED for any of the parameters means calibration adjustment is required.
c. If calibration is NEEDED, be sure to perform the next four steps!
- Touch Save and Exit icon to automatically replace NEEDED (current) calibration factor with new calibration factor.
- Print new calibration factor for your records (log book).
- At Main screen, touch Setup Print Setup Report icon.
- Verify calibration by analyzing three levels of 4C-ES cell control.

d. If FAILED appears, the % diff value and/or %CV exceeds high acceptable limit. DO NOT calibrate.
e. You will not be able to save the changes for the parameters that show NEEDED
f. Call your Beckman Coulter Representative for assistance.
xxv. IMPORTANT! After you have finished Calibration, be sure that you have the following printouts for your records:
- Reproducibility Summary results
- Carryover Summary results
- Current CAL factors (prior to Calibration)
- Calibration Summary results
- New CAL factors (after Calibration)

Appendix K: Protocol for lipids, apolipoproteins, glucose and high sensitivity C-reactive protein analysis

Protocol for lipids, apolipoproteins, glucose and high sensitivity CRP (hsCRP)

Consumables

i. Cuvettes
ii. Sample cups
iii. Seglets

Reagents, calibrators and controls

i. Triglyceride reagent
ii. Direct HDL-cholesterol (HDL-C) reagent
iii. Direct LDL-cholesterol (LDL-C) reagent
iv. Apolipoprotein A1 reagent
v. Apolipoprotein B reagent
vi. Glucose HK reagent
vii. NEFA reagent
viii. hsCRP reagent
ix. Lipid control level 2
x. hsCRP control level 2
xi. Gemcal serum calibrator
xii. Direct HDL/LDL calibrators
xiii. Apolipoprotein A1 and B calibrator
xiv. NEFA standard
xv. CRP calibrators

Preparation and running of reagents, calibrators and controls

i. Prepare reagents, calibrators and controls by following manufacturer's instructions.
ii. Load and run calibrators and verify the results
iii. Load and run controls and verify the results
iv. Run samples

Running samples

Samples (≥200 µL of serum for each sample) can be loaded and run individually or as a batch. Samples must be run within two hours after they have been loaded. A requisition must be created before samples can be loaded. Test results are printed for record keeping. Instructions can be found in ACE Clinical Chemistry System Operator's Manual.

For our analysis calibration was carried out before any tests and whenever; (i) a new lot of reagent was used; (ii) at intervals, after running 10 patient samples and (iii) control values were out of range. At least two levels of controls were tested each day the instrument was run to verify acceptable performance of the system. Samples were only run after verifying results of calibration and control runs. Reagents were used for quantitative determination of lipids, apolipoproteins, glucose and hsCRP.

Control requisition report

Test	Results	Acceptable Range	Control
Apolipoprotein A1 (mg/dL)	128.38	116-168	Lipid control level 2
Apolipoprotein B (mg/dL)	104.90	93.3-136	Lipid control level 2
Cholesterol (mmol/L)	5.43	4.75-6.17	Lipid control level 2
Direct HDL-cholesterol (mmol/L)	1.30	0.95-1.29	Lipid control level 2
Direct LDL-cholesterol (mmol/L)	3.32	2.96-4.00	Lipid control level 2
Triglycerides (mmol/L)	2.14	1.81-2.49	Lipid control level 2

Appendix L: Protocol for thrombomodulin, intercellular adhesion molecule-3, E-selectin and P-selectin analysis

Protocol for thrombomodulin, intercellular adhesion molecule-3 (ICAM-3), E-selectin and P-selectin analysis

MSD Materials

i. Read Buffer T (4X), with surfactant
ii. Blocker A Kit
iii. MULTI-SPOT 96-well 4 Spot Vascular I Plate(s)
iv. SULFO-TAGTM Anti-Human Vascular Injury I Detection Antibody Blend (50X)
v. Diluent 10
vi. Human Vascular Injury I Calibrator Blend

Other Materials & Equipment (not supplied)

i. Deionized water for diluting Wash Buffer and Read Buffer
ii. Phosphate buffered saline + 0.05% Tween-20 (PBS-T) for plate washing.
iii. Adhesive plate seals
iv. Microtiter plate shaker
v. Plate washer or other efficient multi-channel pipetting equipment for washing 96-well plates
vi. Liquid handling equipment for desired throughput that must accurately dispense 10, 25, 40, and 150 µL into a 96-well micro plate

Protocol

i. Add 150 µL/well of Blocker A Solution and incubate on a plate shaker at room temperature for 1 hour or without shaking, overnight at 4 oC.
ii. Wash plates 3 times with 200 µL per well phosphate buffered saline with 0.05% Tween-20 (PBS-T).
iii. Add 40 µL Diluent 10.
iv. Add 10 µL/well calibrator or sample and incubate at room temperature with shaking for 2 hours.
v. Wash plates 3 times with 200 µL per well PBS-T.
vi. Add 25 µL/well of 1X Detection Antibody Solution and incubate at room temperature with shaking for 1 hour.
vii. Wash plates 3 times with 200 µL per well PBS-T.
viii. Prepare SECTOR Imager so that plate can be read immediately after Read Buffer addition.
ix. Add 150 µL/well 1X Read Buffer T. Avoid bubbles.
x. Read plate immediately following Read Buffer T dispense on the SECTOR Imager.

Appendix M: Protocol for serum amyloid A, C-reactive protein, vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 analysis

Protocol for serum amyloid A (SAA), C-reactive protein (CRP), vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) analysis

MSD Materials

i. Read Buffer T (4X), with surfactant
ii. Blocker A Kit
iii. MULTI-SPOT 96-well 4 Spot Vascular II Plate(s)
iv. SULFO-TAGTM Anti-Human Vascular Injury II Detection Antibody Blend (50X)
v. Diluent 15
vi. Human Vascular Injury II Calibrator Blend

MSD Materials

i. Read Buffer T (4X), with surfactant RT
ii. Blocker A Kit RT
iii. MULTI-SPOT 96-well 4 Spot Vascular II Plate(s) 2-8 ºC
iv. SULFO-TAGTM Anti-Human Vascular Injury II Detection Antibody Blend (50X)1 2-8 ºC
v. Diluent 15 ≤-10 ^{o}C
vi. Human Vascular Injury II Calibrator Blend

Assay Protocol

i. Add 150 µL/well of 5% Blocker A Solution and incubate at room temperature for 1 hour with shaking (or overnight at 4 oC).
ii. Wash plates 3 times with 200 µL per well phosphate buffered saline with 0.05% Tween-20 (PBS-T).
iii. Add 40 µL/well of Diluent 15.
iv. Add 10 µL/well Calibrator or diluted sample and incubate at room temperature with shaking for 2 hours.
v. Wash plates 3 times with 200 µL per well PBS-T.
vi. Add 25 µL/well of 1X Detection Antibody Solution and incubate at room temperature with shaking for 1 hour.
vii. Wash plates 3 times with 200 µL per well PBS-T.
viii. Prepare SECTOR Imager so that plate can be read immediately after Read Buffer addition.
ix. Add 150 µL/well 1X Read Buffer T. Avoid bubbles. The use of an electronic multi-pipettor at moderate speed setting is recommended.
x. Analyze immediately with SECTOR Imager. This is important because the VCAM-1 assay signal decreases about 20% within the first 5 minutes of Read Buffer T incubation.

Appendix N: Protocol for vascular endothelial growth factor (VEGF), VEGF-C, VEGF-D, tyrosine kinase-2 (Tie-2), soluble fms-like tyrosine kinase-1 (sFlt-1), placental growth factor (PlGF), basic fibroblast growth factor (bFGF) analysis

Protocol for VEGF, VEGF-C, VEGF-D, Tie-2, sFlt-1, PlGF, bFGF analysis

MSD materials

i. MULTI-SPOT 96-Well 7-Spot Angiogenesis Panel 1 (human) Plate
ii. SULFO-TAG Anti-hu VEGF Antibody1 (50X)
iii. SULFO-TAG Anti-hu VEGF-C Antibody1 (50X)
iv. SULFO-TAG Anti-hu VEGF-D Antibody1 (50X)
v. SULFO-TAG Anti-hu Tie-2 Antibody1 (50X)
vi. SULFO-TAG Anti-hu sFlt-1 Antibody1 (50X)
vii. SULFO-TAG Anti-hu PlGF Antibody1 (50X)
viii. SULFO-TAG Anti-hu bFGF Antibody1 (50X)
ix. Angiogenesis Panel 1(human) Calibrator Blend (20X)
x. Diluent 7
xi. Diluent 11
xii. Blocker A Kit (Blocker A [dry] in 250 mL bottle and 50 mL bottle of 5X Phosphate Buffer)

Required Material and Equipment (not supplied)

i. Appropriately sized tubes for reagent preparation
ii. Microcentrifuge tubes for preparing serial dilutions
iii. Phosphate buffered saline plus 0.05% Tween-20 (PBS-T) for plate washing
iv. Liquid handling equipment for desired throughput, capable of dispensing 10 to 150 µL/well into a 96-well microtiter plate
v. Plate washing equipment: automated plate washer or multichannel pipette
vi. Adhesive plate seals
vii. Microtiter plate shaker
viii. Deionized water

Protocol

i. Add Blocker A Solution: Add 150 µL of Blocker A solution to each well. Seal the plate with an adhesive plate seal and incubate for 1 hour with vigorous shaking (300–1000 rpm) at room temperature.
ii. Wash and Add Sample: Wash the plate 3 times with 300 µL/well of PBS-T. Add 50 µL of sample (standards, controls, or unknowns) per well. Seal the plate with an adhesive plate seal and incubate for 2 hours with vigorous shaking (300–1000 rpm) at room temperature.
iii. Wash and Add Detection Antibody Solution: Wash the plate 3 times with 300 µL/well of PBS-T. Add 25 µL of 1X detection antibody solution to each well. Seal the plate with an adhesive plate seal and incubate for 2 hours with vigorous shaking (300–1000 rpm) at room temperature.
iv. Wash and Read: Wash the plate 3 times with 300 µL/well of PBS-T. Add 150 µL of 2X Read Buffer T to each well. Analyze the plate on the SECTOR Imager. No incubation in read buffer is required before reading the plate.

Appendix O: Protocol for matrix metalloproteinase (MMP) -2 and MMP-10 analysis

Protocol for MMP-2 and MMP-10 analysis

MSD Materials

i. MULTI-SPOT 96-well 4 Spot
ii. Human MMP 2-Plex Plate
iii. SULFO-TAG™ Detection Antibody Blend1 (50X)
iv. Human MMP 2-Plex Calibrator Blend (5 µg/mL of MMP-2 and 1 µg/mL of MMP-10)
v. Diluent 2
vi. Diluent 3
vii. Read Buffer T (4X)

Required Materials and Equipment - not supplied

i. Deionized water for diluting concentrated buffers
ii. 50 mL tubes for reagent preparation
iii. 15 mL tubes for reagent preparation
iv. Microcentrifuge tubes for preparing serial dilutions
v. Phosphate buffered saline plus 0.05% Tween-20 (PBS-T) for plate washing
vi. Appropriate liquid handling equipment for desired throughput, capable of dispensing 10 to 150 µL into a 96-well microtiter plate
vii. Plate washing equipment: automated plate washer or multichannel pipette
viii. Adhesive plate seals
ix. Microtiter plate shaker

Assay Protocol

i. Dispense 25 µL of Diluent 2 into each well. Seal the plate with an adhesive plate seal and incubate for 30 min with vigorous shaking (300–1000 rpm) at room temperature.
ii. Dispense 25 µL of sample or Calibrator into separate wells of the MSD plate. Seal the plate with an adhesive plate seal and incubate for 2 hours with vigorous shaking (300–1000 rpm) at room temperature.
iii. Wash the plate 3 times with PBS-T. Dispense 25 µL of the 1X Detection Antibody Solution into each well of the MSD plate. Seal the plate and incubate for 2 hours with vigorous shaking (300–1000 rpm) at room temperature.
iv. Wash the plate 3 times with PBS-T. Add 150 µL of 2X Read Buffer T to each well of the MSD plate. Analyze the plate on the SECTOR Imager. Plates may be read immediately after the addition of Read Buffer.

Appendix P: Protocol for matrix metalloproteinase (MMP) -1, MMP-3 and MMP-9 analysis

Protocol for MMP-1, MMP-3 and MMP-9 analysis

MSD Materials

i. MULTI-SPOT 96-well 4 Spot Human MMP 3-Plex Plate
ii. SULFO-TAG™ Detection Antibody Blend1 (50X)
iii. Human MMP 3-Plex Calibrator Blend (1 µg/mL of MMP-1 and MMP-3, 5 µg/mL of MMP-9)
iv. Diluent 2
v. Diluent 3
vi. Read Buffer T (4X)

Required Materials and Equipment - not supplied

i. Deionized water for diluting concentrated buffers
ii. 50 mL tubes for reagent preparation
iii. 15 mL tubes for reagent preparation
iv. Microcentrifuge tubes for preparing serial dilutions
v. Phosphate buffered saline plus 0.05% Tween-20 (PBS-T) for plate washing
vi. Appropriate liquid handling equipment for desired throughput, capable of dispensing 10 to 150 µL into a 96-well microtiter plate
vii. Plate washing equipment: automated plate washer or multichannel pipette
viii. Adhesive plate seals
ix. Microtiter plate shaker

Assay Protocol

i. Addition of Diluent 2: Dispense 25 µL of Diluent 2 into each well. Seal the plate with an adhesive plate seal and incubate for 30 min with vigorous shaking (300–1000 rpm) at room temperature.
ii. Addition of the Sample or Calibrator: Dispense 25 µL of sample or Calibrator into separate wells of the MSD plate. Seal the plate with an adhesive plate seal and incubate for 2 hours with vigorous shaking (300–1000 rpm) at room temperature.
iii. Wash and Addition of the Detection Antibody Solution: Wash the plate 3 times with PBS-T. Dispense 25 µL of the 1X Detection Antibody Solution into each well of the MSD plate. Seal the plate and incubate for 2 hours with vigorous shaking (300–1000 rpm) at room temperature.
iv. Wash and Read: Wash the plate 3 times with PBS-T. Add 150 µL of 2X Read Buffer T to each well of the MSD plate. Analyze the plate on the SECTOR Imager. Plates may be read immediately after the addition of Read Buffer.

Appendix Q: Immunohistochemical staining on the BOND-MAX^{TM} automated immunostainer

Immunohistochemical staining on the BOND-MAX^{TM} automated immunostainer

Reagents and equipment

i. CD68 (Dako Denmark, clone PG-M1, dilution 1:50)
ii. CD3 (Leica Clone LN-10, Prediluted)
iii. Microtome
iv. Slide dryer set at 60ºC
v. Leica Microsystems Plus Slides
vi. Bond universal slide labels
vii. Bond universal covertiles
viii. Bond polymer refine detection kit
ix. Bond Dab Enhancer
x. Bond epitope retrieval solution 1
xi. Bond epitope retrieval solution 2
xii. Bond wash solution 10X concentrate
xiii. Deionised water
xiv. Xylene
xv. Ethanol

Detailed steps in the staining process are as follows:

i. Sections were cut from paraffin blocks and baked onto glass slides for 60 minutes at 60ºC.
ii. A slide label was printed from the bond max and applied to the slide. The slide label contains an alpha numerical code that tells the Bond what protocol has been assigned to it.
iii. Slides were placed on the immunostainer for staining.
iv. Slides were dewaxed using the Bond dewax solution.
v. An epitope retrieval solution was applied to the slides and heated to 97ºC.
vi. After epitope retrieval the section was incubated for 15 minutes at room temperature with the antibodies CD68 and CD3.
vii. A dextran polymer molecule labelled with secondary antibody and an enzyme was applied.
viii. A DAB chromogen was applied producing a stable, coloured end product.
ix. Cover slips were applied and slides assessed for staining under a light microscope.

Interpretation of results

A qualified histopathologist evaluated controls and qualified the stained product before interpreting results. The specificity and sensitivity of antigen detection are dependent on the specific primary antibody utilised. To ensure desired staining, each specific antibody was optimised on the Bond system, varying the time of incubation and/or the specific antibody concentration. Failure to optimise the specific antibody may result in sub-optimal antigen detection.

i. Positive control tissue

The positive control was examined first to ascertain that all reagents were functioning properly. When using DAB-based systems, the presence of a brown (3, 3' diaminobezidine tetrachloride, DAB) reaction product with the target cells indicated positive reactivity. If the positive tissue control failed to demonstrate positive staining, results with the test specimens were considered invalid.

ii. Negative control tissue

The negative tissue control was examined after the positive tissue control to verify the specificity of the labelling of the target antigen/nucleic acid by the primary antibody/probe. The absence of specific staining in the negative control tissue confirmed the lack of antibody/probe cross-reactivity to cells/cellular components.

If specific staining (false positive staining) occurred in the negative tissue control, results were considered invalid. Non-specific staining, if present usually has a diffuse appearance. Sporadic staining of connective tissue was observed in sections from excessively formalin-fixed tissues. Necrotic or degenerated cells often stained non-specifically.

iii. Patient tissue

The patient specimens stained with the primary antibody/probe were examined last. Positive staining intensity was assessed within the context of any non-specific background staining of the negative reagent control.