Clinical and Biological Determinants of the Coronary Slow Flow Phenomenon

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THE UNIVERSITY
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Declaration

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* Kopetz V.A, Penno M.A.S, Hoffmann P, Wilson D.P, Beltrame J.F. Potential mechanisms of the Acute Coronary Syndrome Presentation – Insight from a plasma proteomic approach**.**

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Abbreviations

2D-DIGE = Two-dimensional Difference Gel Electrophoresis

AAT = Alpha-1 Anti-trypsin

ACE = Angiotensin Converting Enzyme

ACE-1 = Angiotensin Converting Enzyme Inhibitor

ACh = Acetylcholine

ACN = Acetonitrile

ACS = Acute Coronary Syndrome

ACT = Alpha-1 Anti-chymotrypsin

ADMA = Asymmetric Dimethylarginine

ADP = Adenosine Diphosphate

AF = Angina Frequency

AIx = Augmentation Index

ATP = Adenosine Triphosphate

BH_4 = Tetrahydrobiopterin

CAD = Coronary Artery Disease

CBG = Corticosteroid Binding Globulin

CCU = Coronary Care Unit

CFR = Coronary Flow Reserve

CHAPS = [3- (3 Cholamidopropyldimethylammonio) -1- propanesulfonate]

CHD = Coronary Heart Disease

Cl^- = Chloride ion

CK = Creatine Kinase

CMD = Coronary Microvascular Dysfunction

CRP = C-reactive protein

CSFP = Coronary Slow Flow Phenomenon

CSX = Coronary Syndrome X

CT = Computed Tomography

DDAH = Dimethylarginase

DNA = Deoxyribose Nucleic Acid

DTT = Dithiothreitol

E-selectin = Endothelial selectin

ECG = Electrocardiogram

EDHF = Endothelial Derived Hyperpolarising Factor

EDTA = Ethylenediaminetetraacetic Acid

ELISA = Enzyme-linked immunosorbent assay

ET-1 = Endothelin-1

eNOS = Endothelial NOS

FA = Formic Acid

FMD = Flow-mediated Dilatation

FN = Fibronectin

GTN = Glyceryl Trinitrate

H_2O_2 = Hydrogen Peroxide

HMG-CoA = 3-hydroxy-3-methyl-glutaryl-CoA

HOCl = Hypochlorous Acid

HPLC = High Performance Liquid Chromatography

hsCRP = High-sensitivity C-Reactive Protein

ICAM-1 = Intercellular Adhesion Molecule

IEF = Isoelectric Focusing

IFN-γ = Interferon Gamma

IL-1 = Interleukin 1

IL-6 = Interleukin 6

IL-8 = Interleukin 8

IPG = Immobilised pH gradient

iNOS = Inducible NOS

IP-10 = Inducible Protein 10

IVUS = Intravascular Ultrasound

LAD = Left Anterior Descending

LRα2GP = Leucine-rich alpha-2-glycoprotein

LV = Left Ventricular

MARS = Multiple Imunnoaffinity Removal System

MCP-1 = Monocyte Chemotactic Protein-1

MDA = Malondialdehyde

MMP = Matrix Metalloproteinases

MPO = Myeloperoxidase

MRI = Magnetic Resonance Imaging

MS = Mass Spectrometry

MVA = Microvascular Angina

mRNA = Messenger RNA

NADPH = Nicotinamide adenine dinucleotide phosphate

NO = Nitric Oxide

NO_2^- = Nitrite

NOS = Nitric Oxide Synthase

NMMA = N-monomethylarginine

NMR = Nuclear Magnetic Resonance

nNOS = Neuronal NOS

O_2^- = Superoxide

OD = Optical Density

$ONOO^-$ = Peroxynitrite

oxLDL = Oxidised Low-density Lipoprotein

PA = Persistant Angina

PAF = Platelet Activating Factor

PBS = Phosphate Buffered Solution

PBS-BSA = Phosphate Buffered Solution + 0.1% Bovine Serum Albumin

PBST + Phosphate Buffered Solution + 0.001% Tween

PCI = Percutaneous Coronary Intervention

PDGF = Platelet Derived Growth Factor

PET = Positron Emission Tomography

PGH_2 = Prostaglandin

PGI_2 = Prostacyclin

PON-1 = Paraoxonase -1

PTM = Post-translational Modification

PVD = Primary Microvascular Dysfunction

ROS = Reactive Oxygen Species

RCA = Right Coronary Artery

SAQ – Seattle Angina Questionnaire

SDMA = Symmetric Dimethylarginine

SDS- PAGE = Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SF-36 = Short-Form 36

SNC = Sublingual Nitrate Consumption

SOD = Superoxide Dismutase

SPECT = Single-photon Emission Computed Tomography

TBA = Thiobarbituric Acid

TBST = Tris-buffered saline + Tween

TIMI = Thrombolysis in Myocardial Infarction

TNF-α = Tumor Necrosis Factor Alpha

TXA_2 = Thromboxane A_2

TnT = Troponin T

U.S = United States

VSMC = Vascular Smooth Muscle Cell

VCAM-1 = Vascular Cell Adhesion Molecule

vWF = von Willebrand Factor

Abstract

Background

This thesis investigates the clinical and biological factors that contribute to the cardiovascular condition known as the Coronary Slow Flow Phenomenon (CSFP). From its initial description, little remains understood regarding the mechanisms contributing to this curious condition. The research efforts in this thesis have focused upon further characterising the CSFP and identifying an effective therapy, by investigating the mechanisms involved during different periods of presentation.

The specific objectives include:

1) Identifying the possible mechanisms of the acute coronary syndrome (ACS) presentation in CSFP patients by comparing plasma protein profiles from samples obtained from initial presentation and during a quiescent phase of the disorder;
2) Investigating the role of the endothelium during the chronic phase of the disorder. Specifically, this includes looking at mechanisms of endothelial dysfunction, inflammation and oxidative stress and comparisons with a healthy control group;
3) Evaluating the efficacy of a dual endothelin-1 (ET-1) receptor blocker (Bosentan) in ameliorating angina symptoms in CSFP patients. This project also involves monitoring improvements in health-related quality of life, clinical profiles, endothelial function, inflammation and oxidative stress following Bosentan treatment.

Methods

This thesis employed a number of methods to comprehensively assess the pathophysiological mechanisms contributing to CSFP aetiology. In order to identify possible protein biomarker candidates, a state-of-the-art proteomic approach was used to obtain plasma protein profiles. A paired-longitudinal study design was employed by which blood samples were obtained from CSFP patients during the ACS and compared to a quiescent phase. During the chronic phase of the condition, a cross-sectional study was conducted to assess endothelial function, inflammation and oxidative stress parameters compared with a healthy control group that had no history of chest pain or coronary disease. The clinical trial employed a randomised, double-blind, placebo-controlled, cross-over design that involved evaluating changes in chest pain, clinical characteristics, endothelial function, inflammation and oxidative stress parameters following treatment with bosentan therapy

Summary of major findings

The above studies yielded the following findings:

1) Proteomic investigations identified specific inflammatory and oxidative stress protein markers that were elevated during the ACS presentation compared to the chronic phase (Chapter 2).
2) There was no evidence of impairments in endothelial vasomotor function or increases in inflammatory and oxidative stress parameters during the chronic phase of the condition compared to control subjects (Chapter 3).
3) Bosentan therapy did not significantly improve angina symptoms, clinical profiles, endothelial function, inflammation and oxidative stress

parameters compared to placebo. Despite not reaching statistical significance, reductions in angina frequency and severity in addition to improvements in quality of life parameters were identified (Chapter 4).

Conclusion

This thesis provides a new platform for future investigations into the CSFP. Pathophysiological differences identified between the acute and chronic presentations have initiated the need to further conduct research on the specific mechanisms that contribute to both the ACS presentation and persistent symptoms. Additionally, investigating the role of ET-1 receptor blockade in CSFP patients has identified a number of inherent problems associated with clinical trial design in CSFP patients.

Statement of Authorship

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Published Abstracts and Presentations Arising from this Thesis

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Other Abstracts and Presentations

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Poster Presentations

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Plasma proteomic investigations into the mechanisms of ACS presentation in Coronary Slow Flow Patients.

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Kopetz V.A.

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Plasma Proteomic Investigations into the Human Coronary Vasculature: Exploring mechanisms of the acute coronary syndrome presentation. Presented at the 4th Asian-Oceanic Human Proteome Organisation Conference, June 22nd-26th, Cairns, Australia.

Preface

Coronary heart disease (CHD) is a disorder characterised by dysfunction in the large and/or small coronary vessels. Impairments in coronary vascular function result in reduced flow of oxygen and nutrient-rich blood to the myocardium thereby producing myocardial ischaemia. This in turn may manifest as chest pain (referred to as angina) or in severe cases may result in myocardial infarction or even death. Indeed, CHD is the leading cause of death globally, responsible for an estimated 17.3 million deaths worldwide in 2008 (1) and more than 2200 deaths every day in the United States of America (U.S.A) alone (2). Accordingly, it is imperative that we increase our knowledge and understanding of the condition that is responsible for considerable global morbidity and mortality.

This thesis will provide a comprehensive summary of cardiovascular pathology, with a particular focus on coronary microvascular disorders, namely the coronary slow flow phenomenon (CSFP). The introductory chapter will provide the necessary background relating to CHD and will begin by describing contemporary facets of CHD with a discussion on the contribution of both large (coronary artery disease) and small vessel dysfunction (coronary microvascular disease) in determining clinical outcomes. This will then be followed by an extended discussion of the clinical conditions associated with coronary microvascular dysfunction (CMD), with particular reference to coronary syndrome X (CSX) and the CSFP. Thereafter, the thesis objectives and proposed investigations will be outlined.

CHAPTER I

1.1. Basic Principles of the Coronary Circulation

1.1.1 Functional Anatomy

The coronary arterial system is divided into three main compartments comprising of large epicardial arteries, pre-arterioles and intramural arterioles (3). Each component plays an essential role in regulating adequate and efficient transport of nutrient-rich blood for maintenance of homeostasis (4). Abnormalities in these arterial systems have shown to contribute to the pathogenesis of many coronary vascular diseases.

1.1.1.1 Arteries

The proximal section of the coronary arterial supply comprises of large epicardial coronary arteries that are anatomically arranged to most effectively perfuse critical myocardial tissues (5). The left coronary artery originates at the aorta and divides into the left anterior descending (LAD) and circumflex arteries that extend to the apex of the heart. The right coronary artery (RCA) originates from the right coronary ostia and branches to perfuse the right atrium and ventricle. Structurally, these arteries have a diameter usually ranging from a few millimetres (mm) to approximately 500 micrometres (μm) and are clearly visible upon coronary angiography (6). These large arteries offer little resistance to coronary blood flow and thus have minimal contribution to adaptive circulatory responses during periods of increased myocardial oxygen demand, despite being regulated by endothelial vasomotor control mechanisms.

1.1.1.2 Pre-arterioles

Pre-arterioles are vessels directly preceding arterioles and are difficult to identify based on structural differences alone. Generally, pre-arterioles are characterised by their approximate anatomical sizes (ranging from 100 to 500μm in diameter), measurable pressure drops along their length and functional differences to arterioles. Similar to arteries, pre-arterioles are responsive to endothelial vasomotor changes in blood flow and thus are not regulated by local metabolites. However, unlike the large epicardial vessels, pre-arterioles to some extent contribute to vascular resistance and specifically function to regulate pressure at the arteriole origin when changes in coronary perfusion or flow occur (7). Pre-arterioles constitute the beginnings of the coronary microvasculature, as they are unidentifiable upon coronary angiography.

1.1.1.3 Arterioles

The most distal component of the coronary arterial tree is represented by arterioles. These vessels are characterised by a significant pressure drop, have a diameter <100μm and are responsive to local metabolic changes. The main role of the smaller arterioles is to match coronary perfusion with myocardial oxygen demands through regulation of coronary vascular resistance. As such, arterioles are the major resistance vessels in the vascular tree. Blood flow is regulated by local metabolites that change the calibre of the vessels, to generate the adequate pressure gradients required for necessary flow to tissues in instances of increased metabolic demand (8). Arterioles further branch out

into capillaries, the smallest of all vessels, which form the frontline for substrate exchange with surrounding cells.

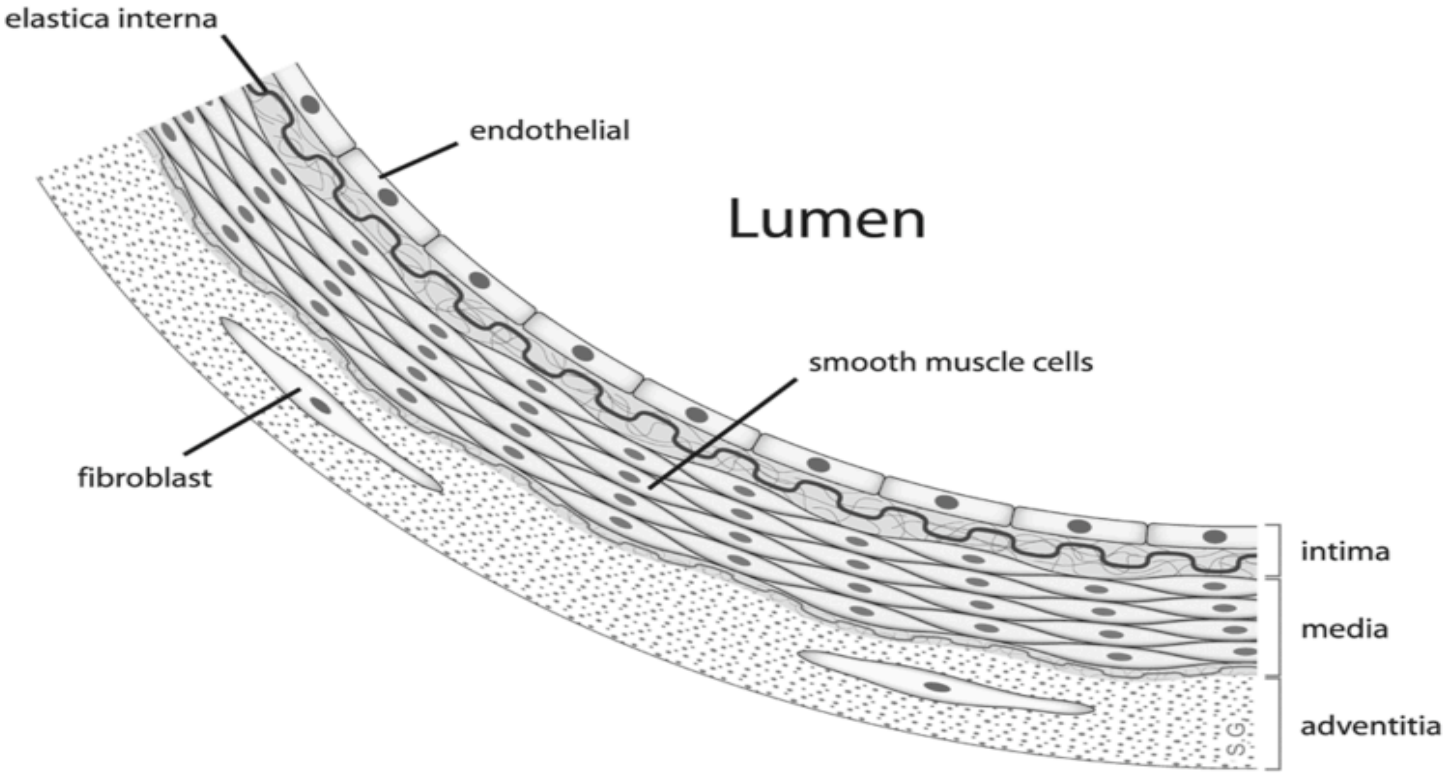
1.1.2 Functional Histology

Blood vessels consist of three layers of tissue known as the tunica intima, tunica media and tunica adventitia. Each tissue bed comprises of a specialised network of cells that work together to maintain the regulatory functions of the blood vessels. The thickness of each layer is dependent on the type and size of vessel (Figure 1.1, page 5).

1.1.2.1 Tunica intima

The tunica intima lines the lumen of blood vessels and consists of a layer of endothelial cells, a sub-endothelial layer comprising of connective tissue, and an elastic or fenestrated layer of longitudinal fibres. These cells form a slick surface that reduce friction, allow transfer of ions, solutes and fluids between the blood and interstitial compartments (9) and control vasomotor tone through endothelium-mediated release of vasoactive agents (10). Functional heterogeneity in endothelial cells is evident amongst different vascular beds, as specific vessel types both have basal or inducible permeability and undergo processes involving leukocyte trafficking, angiogenesis, haemostasis and vasomotor tone regulation (11, 12).

Figure 1.1. Graphical representation of coronary artery anatomy. Reproduced with permission from (13) under the Creative Commons Attribution-Share generic license.



1.1.2.2 Tunica media

The tunica media is the thick middle muscular layer of the blood vessel that primarily comprises of vascular smooth muscle cells (VSMC) and varying amounts of collagen and elastic fibres. It is clinically distinguishable from the tunica intima by its colour and its transverse arrangement of fibres. The primary function of VSMC is to alter the luminal diameter by contracting and dilating vessels to maintain adequate blood pressure. Additionally, VSMC also play a role in structural remodelling processes during periods of exercise or injury by changing cell number and connective tissue composition.

VSMC either have a contractile or synthetic phenotype, dependent upon differences in morphology, proliferation rates, migratory characteristics and expression of marker proteins (14). Contractile VSMC contain contractile filaments and are morphologically elongated, spindle-shaped cells in comparison to synthetic VSMC, which contain a high number of organelles involved in protein synthesis and have a rhomboid morphology (Figure 1.2, page 8). Furthermore, contractile VSMC generally exhibit lower growth and migration rates than synthetic VSMC and are histologically distinguishable by specific marker proteins such as smooth muscle-myosin heavy chain (15) and smoothelin (16). Numerous biochemical (platelet-derived growth factor [PDGF]) (17) and extracellular matrix factors (heparin) (18) have been shown to influence VSMC development, differentiation and phenotypic modulation.

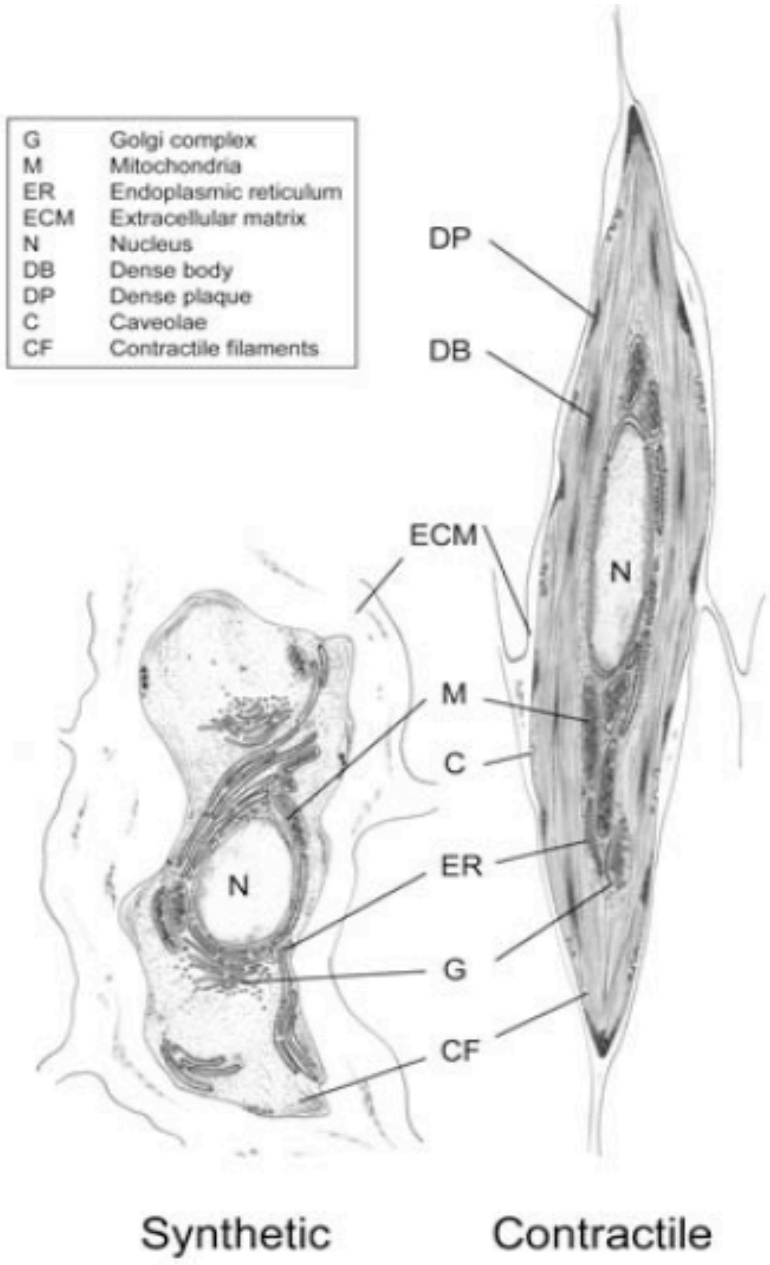
1.1.2.3 Tunica adventitia

The tunica adventitia is the outermost layer of a blood vessel surrounding the media that consists of a complex group of interacting cell types. This community of cells predominantly includes collagen-rich connective tissue, peri-vascular and autonomic nerves, fibroblasts, lymphatic vessels and inflammatory mediators. This important tissue layer has been shown to play numerous roles in regulation of blood vessel function by controlling cell trafficking through the artery wall (19, 20), participating in the growth and repair of the vessel wall (20, 21) and activation of fibroblasts for vascular remodelling processes (21). In addition, the adventitia has been identified as the vascular cell layer that controls the formation and regression of microvessels, which penetrate and nourish the media and intima cell layers (22).

1.1.3 Summary

The functional anatomy and histology of the coronary arterial system is structurally designed to maintain and regulate adequate blood flow. The individual components of the coronary vasculature contain a unique network of cells that specifically function to adapt to changes in the physiological environment. The next section will discuss the regulators of blood flow and how the different components of the vasculature work concurrently as a system to achieve homeostasis.

Figure 1.2. Structural characteristics of contractile and synthetic smooth muscle cells located in the tunica media. Reproduced with permission from (14).



1.2. Regulation of the Coronary Circulation

Myocardial blood flow rates are largely dependent upon the pressure gradients between the large and small vessels. Driving pressure is maintained along the large conduit vessels but decreases upon reaching the microvasculature. Physical factors that govern blood flow in the coronary circulation include intravascular pressure and resistance. The haemodynamic relationship between these parameters is summarised in the equation below. The flow of blood (F) in a vessel is determined by the pressure difference between arterial pressure (P_A) and venous pressure (P_V) divided by the vascular resistance (R).

$$F = \frac{\Delta P}{R} = \frac{(P_A - P_V)}{R}$$

The pressure gradient is the main driving force determining blood flow through a vessel. The large conduit vessels are subject to a high intravascular pressure generated from the cardiac contraction derived perfusion pressure. Changes in flow velocity contribute to greater shear stress on endothelial cells located in the arterioles, resulting in flow-mediated dilation of conduit vessels. Smaller arterioles are also regulated by changes in metabolic activity, resulting in vasodilatation and subsequent reduction in pressure upstream (23). Various interacting vasodilator mechanisms (neurohormonal, metabolic and endothelium-mediated) in addition to multiple vasoconstrictor mechanisms (α-adrenergic, humoral and myogenic) may influence the arterial tone in both the large and small coronary vessels.

1.2.1 Neural

Although local metabolites are the major players in coronary circulatory regulation, both large and small vessels exhibit adrenergic and cholinergic innervation that operate in conjunction with metabolic factors to adjust blood flow. Sympathetic neural stimulation of the heart results in tachycardia, increased cardiac contractility and subsequent peripheral vasoconstriction mediated through agonist binding upon adrenergic receptors (24). Parasympathetic innervation of the heart is controlled by the vagus nerve, which acts to indirectly cause vessel dilation through action via cholinergic receptors.

1.2.1.1 Sympathetic innervation

Sympathetic adrenergic receptors are a class of G-protein coupled receptors that are responsive to the actions of noradrenaline and adrenaline. These receptors are classified as either alpha or beta-adrenoreceptors which are further sub-divided into either (α_1/α_2) or ($\beta_1/\beta_2/\beta_3$) receptors, depending upon the heterotrimeric G protein to which it couples for activation of downstream signalling factors (25), and specific binding ligands (26, 27).

Alpha-adrenergic receptors tend to respond more to noradrenaline and are expressed at higher levels in vascular smooth muscle and at lower levels in cardiac myocytes. The small coronary resistance vessels (diameter <300 μm) are the main recipients of autonomic innervation and therefore exhibit changes in arterial diameter following neural stimulation. Early investigative studies demonstrated that α_1-adrenergic receptors stimulate vascular

constriction and therefore increase blood pressure due to increased intracellular calcium concentrations in VSMC (28, 29). During normal resting conditions, there is little α-adrenergic tone and an increase in blood flow from sympathetic innervation is blunted by local metabolites. However, if coronary circulation is impaired by atherosclerosis or impairments of auto-regulatory mechanisms, α-adrenergic influences may become sufficient to reduce coronary flow and potentially induce myocardial ischaemia. Both α_1- and α_2-adrenoreceptors are capable of initiating vasoconstriction, however, a location bias exists as α_1-adrenoreceptors are more commonly located in the larger conduit vessels in comparison to the α_2-adrenoreceptors, which are predominantly located in the microcirculation (30, 31).

β-adrenergic receptors also have a range of effects upon the heart and vasculature. β_1 receptors account for approximately 80% of all myocardial β-adrenergic receptors (32) and binding of these receptors with neurotransmitters causes an increase in heart rate and force of cardiac contraction. In contrast, β_2-adrenergic receptors have been shown to predominate in the vascular smooth muscle and play a role in mediating vasodilatation. As such, β_2-adrenergic receptor knockout mice have demonstrated abnormalities in vascular resistance following exercise-induced catecholamine release, despite the presence of normal basal cardiovascular function (33).

1.2.1.2 Parasympathetic innervation

Parasympathetic innervation of the heart is controlled by the actions of vagus nerve. Acetylcholine (ACh) released from parasympathetic nerve endings as a direct result of vagal nerve stimulation (24) elicits a nitric oxide (NO) mediated vessel dilation, following binding to M_3-muscarinic receptors located on endothelial and smooth muscle cells (34). Such effects are similar to those seen following intracoronary injections of ACh during angiography in patients with normal coronary arteries (35). Parasympathetic reflexes that influence coronary blood flow are mediated by carotid baroreceptors and chemoreceptor reflexes (36). Blood pressure increases result in reflex parasympathetic bradycardia, inhibition of sympathetic discharge to systemic vasculature and subsequent coronary vasodilation.

1.2.2 Humoral

In addition to neural factors, humoral factors such as hormones and autacoids also play important roles in modulating coronary vasomotor tone. Vasoconstrictor hormones such as angiotensin II, vasopressin, endothelin and neuropeptide Y, and vasodilator hormones including prostacyclin and neurotensin work in conjunction with metabolites released from the vascular endothelium layer to regulate vascular resistance and blood supply.

Specific peptides such as neurotensin (37) and neuropeptide Y (38) are present in high concentrations in the coronary vessels These humoral factors exert either a vasoconstrictor (neuropeptide Y) or vasodilator (neurotensin) effect that has been demonstrated in both in-vitro (39) and in-vivo (40)

investigations. Similarly, elevated plasma levels of angiotensin II have been shown to regulate coronary vasomotor tone by augmenting the sympathetic effects of α-adrenergic constriction and inactivating bradykinin (an agonist for NO release) (41). The vasodilator effects of atrial natriuretic peptide upon the coronary circulation have also been documented in canine studies as shown by increases in coronary artery diameter and blood flow (42, 43).

The autacoids histamine and bradykinin also exert a powerful effect upon vasomotor tone following ischaemic-related tissue damage. Histamine has been shown to cause direct vasoconstriction via H_1 receptors located in the vascular wall (44), vasodilation via H_1 receptors located in the endothelium (through NO release) (45) whilst H_2 receptor binding upon smooth muscle cells results in arterial vasodilation (46, 47). Similarly, bradykinin produced within the vascular wall has been shown to exert a potent vasodilator effect on both large and small vessels via stimulation of bradykinin receptors to produce the endothelium-derived relaxing factors - NO, prostacyclin and endothelium-derived hyperpolarising factor (EDHF) (48, 49).

Eicosanoids are signalling molecules formed in platelets and endothelial cells, derived from arachidonic acid metabolism and comprise of prostaglandins (PGH_2), prostacyclins (PGI_2), thromboxanes (TXA_2) and leukotrienes. These four metabolites produced by cyclooxygenase (prostaglandins, prostacyclins and thromboxanes) and lipoxygenase (leukotrienes) enzymes have a multitude of effects upon the coronary vasculature. PGH_2 and PGI_2 exert vasodilator effects that attenuate the vasoconstrictor effects of TXA_2 and leukotrienes as

demonstrated by studies showing enhanced vasoconstrictor responses in the presence of cyclooxygenase inhibitor indomethacin (50). Eicosanoid biosynthesis occurs on demand when the cells are activated either by mechanical stress, cytokines or a range of growth factors in the presence of myocardial injury (51).

1.2.3 Metabolic

Increases in myocardial metabolic activity are accompanied by increases in flow of nutrient and oxygen-rich blood to the myocardium. The mechanisms that trigger this cascade are multifactorial and involve adenosine, K^{+ATP} channel and NO pathways.

Adenosine is a key metabolite that has been implicated in coronary blood flow regulation (52). Human studies have shown that adenosine is released from the myocardium in the event of ischaemia (53) and can induce vasodilation of microvessels to an extent comparable to pacing studies (54). Several studies have suggested that the endothelial cell layer plays an important role in adenosine's vasodilator function, as removal of this layer has been shown to attenuate dilator activity (55). Accordingly, mechanistic studies have identified that adenosine induces vasodilation through binding to A_2 receptors located on endothelial cells and activating intermediate-conductance potassium channels via an adenylate-cyclase pathway (56).

Studies conducted on exercise-induced coronary blood flow responses in canines have demonstrated that K^+_{ATP} channels also act as critical regulators of

the vasodilator response, as individual blockade of NO and adenosine pathways alone does not abolish vasodilator responses (57). Similarly, further evidence supporting the notion that K^+_{ATP} is a critical regulator for dilation in resistance vessels has been shown following administration of K^+_{ATP} channel agonists (58) and antagonists (59, 60) in which potent vasodilator responses were observed upon K^+_{ATP} activation and diminished during blockade.

1.2.4 Endothelial

The critical obligatory role of the endothelium in maintaining cardiovascular homeostasis in health has been the research focus of many investigations. The vascular endothelium represents one of the largest organs in the body and the majority of cardiovascular diseases are associated with pathophysiological alterations in endothelial cell structure and function. This important cell layer modulates a number of physiological processes such as vasomotor tone, haemostasis and inflammation through release of a number of vasoactive [NO, endothelin-1 (ET-1)] (61) anti-aggregatory [NO, PGI_2] (62) and anti-inflammatory [NO] (63) mediators. These functions can be performed by sensing changes in haemodynamic environments and responding to these stimuli by producing a host of biologically active substances that modulate tone and structure of underlying vascular tissue.

Furchgott and Zawadzki, were the first to demonstrate the important role that endothelial cells play in arterial muscle tone through studies comparing denuded and intact ring preparations of rabbit thoracic aorta (64). In this investigation, aortic strips that had a denuded endothelium demonstrated an

impaired ACh mediated vasodilation compared to vessels with functional endothelial cells. This observation was subsequently confirmed in-vivo as ACh administration produced a paradoxical vasoconstrictor response in patients with advanced coronary stenoses (65). Vasodilators such as PGI_2, NO and EDHF are the three main metabolites known to exert vasodilator actions upon the vasculature and their associated vasodilator effects have been extensively studied. The endothelium also releases potent vasoconstrictors such as TXA_2, angiotensin II and ET-1 in response to endogenous substances (ACh, arachidonic acid, noradrenaline, PGH_2), pharmacological agents (calcium ionophores, nicotine, high K^+), physical forces (shear stress, pressure) and hypoxia that work in conjunction to regulate arterial tone and maintain a healthy endothelium (61).

1.2.4.1 Vasodilators

PGI_2 was the first endothelium-derived vasodilator discovered to bind to smooth muscle cells and cause relaxation (71). Synthesised in endothelial and smooth muscle cells from arachidonic acid by cyclooxygenase, prostacyclin has been shown to cause coronary vasodilation by inducing relaxation through the removal of intracellular calcium (66) as demonstrated in experiments using bovine coronary artery strips (67) and open chest anaesthetised dogs (68).

NO has received much attention since its discovery as being the main component of the endothelium-derived relaxing factor (69). In addition to its potent vasodilator effects, NO has been a small molecule of focus for many

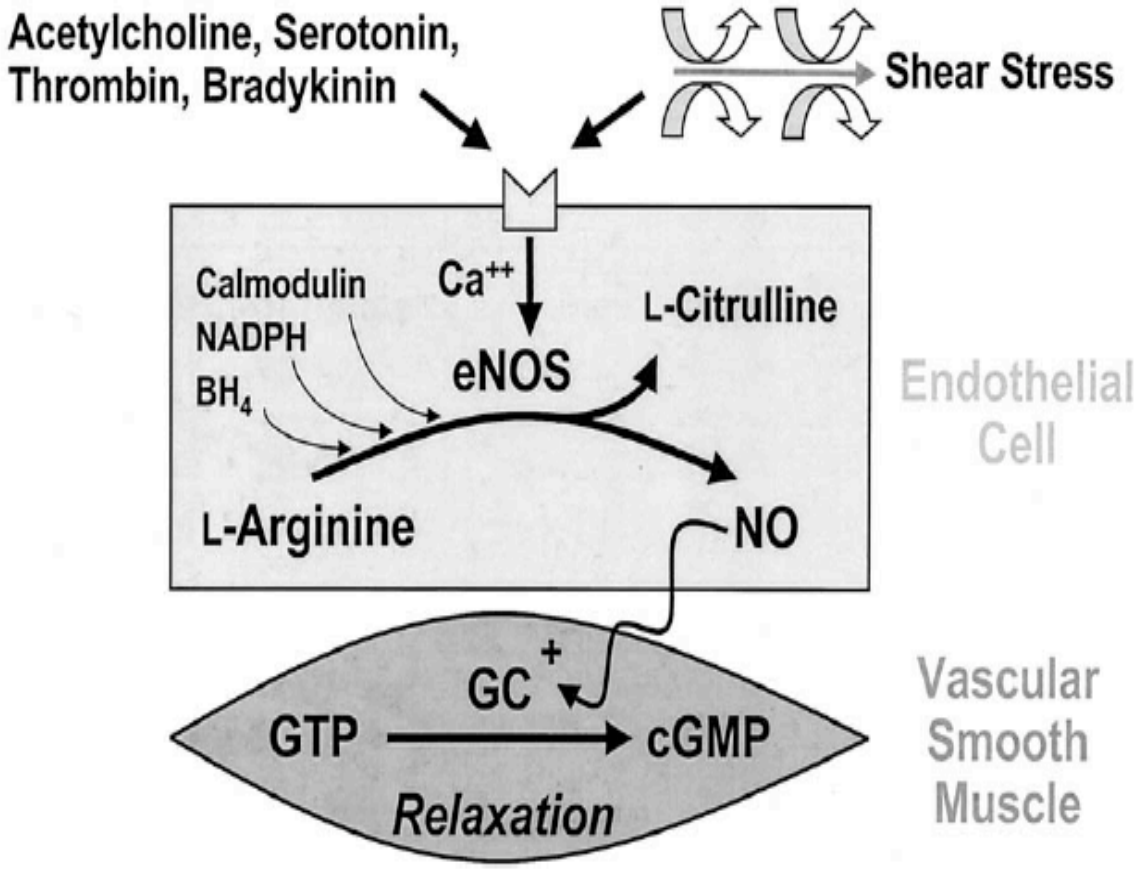
research studies due to its inhibitory effects upon platelet adhesion and aggregation (70), anti-inflammatory properties (71) and its ability to retard or prevent proliferation of vascular smooth muscle (72). As first discovered by Palmer, et al (69), NO is synthesised from L-arginine located in endothelial cells, through the actions of the calcium/calmodulin dependent enzyme nitric oxide synthase (NOS) and with the presence of co-factors such as tetrahydrobiopterin (BH_4) and nicotinamide adenine dinucleotide phosphate (NADPH) (73-75) (Figure 1.3, page 19).

Many cells are capable of producing NO and three isozymes of NOS have been identified as being responsible for its synthesis including NOS I, NOS II and NOS III (76). NOS I is found mainly in neuronal cells and is responsible for the production of nNOS. NOS II is found predominantly in macrophages and produces iNOS, whereas NOS III is mainly expressed in endothelial cells and produces eNOS. The continuous generation of NO occurs as a consequence of continuous blood flow or shear stress, which is detected by sensitive G-protein receptors located on endothelial cells that activate eNOS. Additionally, NO can also be released in response to humoral factors such as thrombin, serotonin, adenosine di-phosphate (ADP), histamine and bradykinin (77).

EDHF is the third major vasodilator that works in conjunction with prostacyclin and NO to achieve optimal vessel dilation. EDHF is synthesised by the endothelium in response to shear stress and acts to produce a vasodilator response by hyperpolarising VSMC. Early studies conducted on

vessels from heart transplant patients identified that bradykinin mediated release of EDHF in human coronary arteries causes an endothelium-dependent relaxation (78), an effect which is also evident in human microvessels (79, 80). Subsequent studies have identified that EDHF plays a more dominant vasodilator role in smaller vessels following inhibition of other endothelium-dependent vasodilators (NO and prostacyclin) in rat aorta, proximal and distal mesenteric arteries (81) as well as in human gastroepiploic and distal microvessels (82). Several mechanisms have been postulated for the vasodilator mechanism for EDHF and include involvement of calcium-activated K^+ channels (82) and endothelium derived hydrogen peroxide (H_2O_2) mediated vasodilation (83).

Figure 1.3. Mechanisms of nitric oxide (NO) production and its effects upon vascular smooth muscle. Reprinted from (84) with permission from Elsevier.



BH_4= tetrahydrobiopterin; Ca^{2+}= calcium ion; cGMP= cyclic guanosine monophosphate; eNOS= endothelial nitric oxide synthase; GC= guanylate cyclase; GTP= guanosine triphosphate; NADPH= nicotinamide adenine dinucleotide phosphate; NO= nitric oxide

1.2.4.2 Vasoconstrictors

ET-1 is a peptide derived upon endothelial activation from the metabolism of a large pre-cursor pro-endothelin peptide, which acts to stimulate vasoconstriction, leukocyte adhesion and recruitment, and smooth muscle cell migration (85). The product of endothelin gene transcription is prepro-ET-1, which is cleaved by an endopeptidase to form the active precursor pro-ET-1 and ET-1 end-product (Figure 1.4, page 22). Two ET receptors are present in the vasculature:

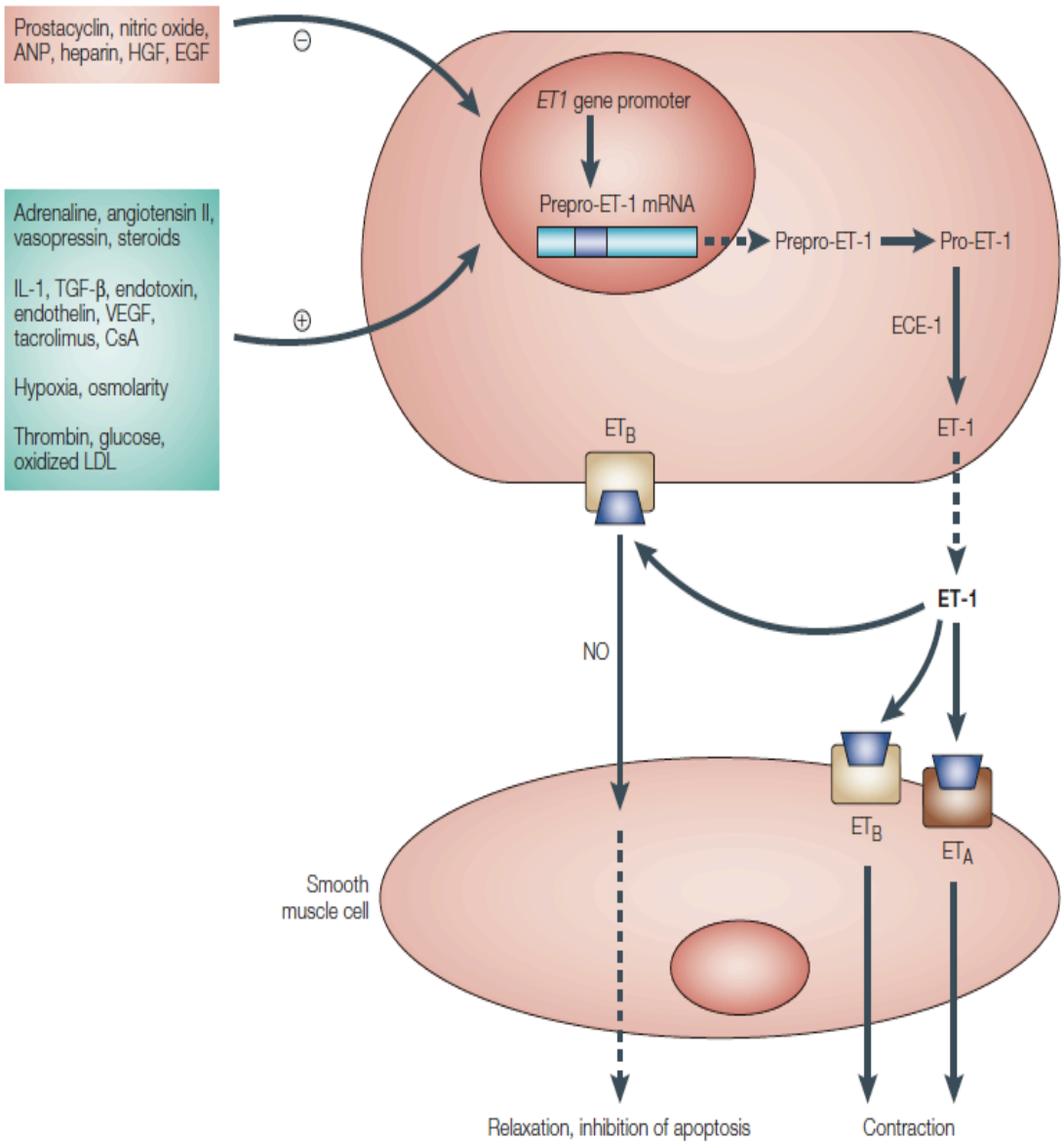
1) ET type-A receptors (ET_A) reside in vascular smooth muscle cells and mediate vasoconstriction and cell proliferation; and
2) ET_B receptors reside on both endothelial and smooth muscle cells.

Activation of ET_B receptors on endothelial cells by ET-1 mainly exerts vasodilator actions through the release of NO, whilst activation of ET_B receptors located on smooth muscle cells elicits vasoconstriction (86). In a healthy individual, the ET-1 gene is expressed constitutively and the ET-1 hormone primarily acts to intrinsically regulate homeostatic vasomotor tone (87, 88). In pathological conditions such as ischaemia or endothelial dysfunction, transcription of ET-1 messenger RNA (mRNA) is increased, which results in excessive vasoconstrictor responses.

TXA_2 is also a potent coronary vasoconstrictor and pro-thrombotic compound released by endothelial cells and platelets in response to endothelial injury. Since its initial discovery, TXA_2 has been shown to have a significant influence upon coronary vasomotor tone (89). TXA_2 has been identified as being a more potent vasoconstrictor than other prostaglandins in causing

contractions of swine coronary artery at small nanomolar concentrations (90). Furthermore, infusion of TXA_2 analogues have shown to significantly decrease cardiac output and reduce blood flow in coronary arteries (91).

Figure 1.4. Schematic representation of endothelin-1 synthesis and associated vascular effects. Taken from Remuzzi et al (86)



1.2.5 Mechanical

The endothelium also has the ability to sense changes in haemodynamic forces and regulate VSMC tone to adjust for increases in flow/shear stress and stretch/pressure. Shear stress upon the vascular wall results in a membrane hyperpolarisation of inward K^+ channels on endothelial cells to increase driving force of calcium entry, resulting in the release of PGI_2 and NO to cause vasodilatation (61). In contrast, elevated transmural pressure initiates a vasoconstrictor response via depolarisation of vascular smooth muscle cells and results in increased calcium influx into the cell and subsequent reduction of vessel diameter (92, 93). Thus, the endothelium modulates stretch- and pressure-induced vasoconstriction through reduced release of NO or EDHF or increased release of endothelium-dependent vasoconstrictors via mechanical receptors located on endothelial cells.

1.2.6 Summary

A large array of neural, humoral, metabolic, endothelial and mechanical factors act to regulate the coronary circulation through interactions with different anatomical components of the vasculature. The following section will focus on discussing the consequences of coronary circulatory dysfunction brought about by abnormalities in these mechanistic pathways. Specifically, it will focus on how endothelial dysfunction contributes to a multitude of cardiovascular disease states.

1.3. Coronary Circulatory Dysfunction

1.3.1 Endothelial Dysfunction

Maintenance of a healthy endothelium is essential for vascular homeostasis. If the endothelium becomes activated and/or damaged in response to numerous local or systemic neural, humoral or mechanical factors, abnormalities in endothelial cell function occur. This phenomenon is referred to as endothelial dysfunction, the pathological hallmark for initiation, progression and development of cardiovascular disease.

The endothelium serves as a protective barrier between tissues and the circulating blood. The main functions of the endothelial cell layer involve facilitating passage of macromolecules and blood gases in response to haemodynamic, blood-borne and tissue signals and regulation of these factors for maintenance of vascular homeostasis. In the presence of pathogens and internal physiological stimuli, the endothelial cell layer becomes activated and initiates a cascade of processes to remove pathogens and restore homeostasis. Endothelial cell activation is characterised by an increased expression of leukocyte adhesion molecules, change in phenotype from anti-thrombotic to pro-thrombotic states, cytokine and growth factor production and up-regulation of human leukocyte antigen molecules (94, 95). Excessive or chronic endothelial activation, however, may lead to physiological changes that cause damage to the underlying vasculature and result in endothelial injury and dysfunction.

Many factors have been shown to cause endothelial injury such as oxidised low-density lipoprotein (oxLDL) (96), coronary risk factors (hypercholesterolaemia, cigarette smoking) and haemodynamic stress (97). Prolonged exposure of these factors may result in endothelial dysfunction and compromise in maintenance of homeostatic mechanisms. In the pathology of many cardiovascular disease states, the severity of disease is determined by the extent of endothelial activation, the presence of dysfunction and associated imbalances in vasomotor, thrombotic or inflammatory processes. Such occurrences may result in an impaired blood supply to tissues and sequential formation and progression of atherosclerotic lesions (98) and ischaemic events. Accordingly, evidence of atherosclerotic lesions have been identified in patients with endothelial dysfunction (75) and conventional risk factors such as hypertension (99), hypercholesterolaemia (100) and smoking (101) .

The endothelium undergoes functional phenotypic changes in response to injury or activation that promote thrombosis, inflammation and vasoconstriction. Homeostatic thrombotic processes are disrupted following the release of cytokines (interleukin-1 [IL-1] and tumour necrosis factor [TNF]) that act upon the endothelial cell surface to activate platelet-activating factor (PAF) and platelet-derived growth factors (PDGF) (102). Similarly, endothelial injury stimulated release of TNF increases expression of inflammatory adhesion molecules such as ICAM-1 and HLA-A that promote mononuclear cell attachment and initiate the inflammatory cascade (103, 104). Endothelial injury has also been shown to impair endothelium-dependent vasomotor responses following administration of vasodilators ACh and

thrombin in both primates (105) and in humans (65, 106). Vasoactive impairments are generally thought to manifest from imbalances of potent vasodilators and vasoconstrictors.

The importance of a healthy vascular endothelium in the prevention of adverse cardiovascular events has been well defined in the literature and as such, current research objectives have focused on restoring endothelial cell function and preventing further damage. Current strategies applied to restore normal function include cholesterol lowering therapy, replenishing the supply of NO through administration of L-arginine and inactivation of superoxide free radicals with anti-oxidants. Initial studies conducted on hypercholesterolaemic rabbits and humans demonstrated that L-arginine administration improves endothelial function in hypercholesterolaemic patients and prevents development of atherosclerotic lesion formation in rabbits (107, 108). Additionally, a number of human clinical trials have shown improvements in endothelial function and reductions in the incidence of coronary events following administration of angiotensin-converting enzyme (ACE) inhibitors (109) and HMG-CoA (3-hydroxy-3-methylglutaryl coenzyme A) reductase inhibitors (110). Although these studies have demonstrated that certain drug therapies can have a beneficial effect on improving endothelial function, the precise mechanisms behind their actions are complex and are yet to be clearly defined.

1.3.1.1 Asymmetric Dimethylarginine (ADMA)

Increasing evidence in the literature has suggested that arginine residues such as ADMA, contribute to impaired endothelium-dependent vasodilator responses due to its inhibitory actions upon endogenous NOS. Formed from the proteolysis of post-translational methylated tissue proteins, ADMA has been shown to not only prevent NO production, through blockade of NOS, but also promote atherogenesis by enhancing endothelial dysfunction (111), increasing carotid intima-media thickening (112), endothelial oxidative stress and monocyte binding (113).

ADMA concentration is critically regulated by its rate of degradation by the enzyme dimethylarginine dimethylaminohydrolase (DDAH). A decrease in DDAH activity, as evident in patients with hypercholesteraemia (114), can result in increased plasma ADMA levels and subsequent vascular dysfunction. In situations where plasma ADMA concentrations are increased, treatment with L-arginine has been shown to reduce atherosclerosis and endothelial dysfunction in cholesterol fed rabbits (107). Based on these observational studies, it can be speculated that determining plasma ADMA levels may serve as a useful marker for the early detection of endothelial dysfunction and atherogenesis. Plasma ADMA measurements are currently used as research tools to assess endothelial dysfunction and its utility in routine clinical practice still needs to be defined.

1.3.2 Neural Mechanisms

The sympathetic and parasympathetic neural systems have complex interactions and effects upon the endothelium and coronary vascular smooth muscle in both epicardial and resistance vessels. During periods of shear stress, noradrenaline acts upon α_2-adrenoreceptors located on endothelial cells to release NO and attenuate α_1-adrenoreceptor mediated vasoconstriction upon smooth muscle cells (115). A healthy and intact endothelium therefore acts to reduce the prolonged vasoconstrictor effects of noradrenaline stimulation, as shown in studies examining the shear stress effect of adrenergic stimulation of epicardial coronary arteries in rabbits (116) and canines (117). However, in cases where endothelial function is compromised, vessels may become pre-disposed to enhanced α-adrenergic coronary vasoconstriction (118).

1.3.3 Inflammation

Inflammation is a complex, biological response involving intimate interactions between circulating and vascular cells, which are mediated by the presence of pathogens or damaged cells. The inflammatory system acts as the body's defence system by eliciting innate and adaptive protective responses to remove foreign entities and initiate body-healing processes. Prolonged periods of inflammation, however, may be detrimental to an individual's health and lead to the development of a number of pathologies. It is well established in the literature that associations between inflammation, the vascular endothelium and cholesterol exist and contribute to the initiation, progression and development of atherosclerotic plaques (119). Conventional

cardiovascular risk factors such as hypercholesterolaemia, diabetes mellitus and smoking have been also shown to initiate an inflammatory response in the vasculature by promoting leukocyte adhesion, endothelial cell activation and atherosclerosis (120).

1.3.3.1 C-reactive protein

Current interests in cardiovascular disease research have investigated the role of the inflammatory acute phase protein, C-reactive protein (CRP), in the vascular endothelium. Released in response to interleukin-6 (IL-6), CRP is a stable downstream marker of the inflammatory process that can increase >1000 fold in concentration upon detection of inflammatory stimuli (121). Initially believed to solely be a marker of inflammation, subsequent studies have identified that CRP plays a significant role in promoting endothelial dysfunction and atherogenesis as well as being a powerful predictor of future coronary events.

CRP promotes endothelial dysfunction by down-regulating and destabilising eNOS transcription (122), increasing IL-6 and monocyte chemotactic protein-1 (MCP-1) release and up-regulating adhesion molecule expression (123). Inside the vascular intima, CRP directly contributes to the atherogenic process by co-localising with monocytes (124), macrophages and lipoproteins (125), activating the classical pathway of the complement system (126), and inducing a pro-thrombotic effect through promotion of tissue factor expression (127). Evidence has also accumulated demonstrating that circulating CRP is one of the most important risk factors for development of

future cardiovascular events. Accordingly, in-vitro and in-vivo studies have confirmed that CRP is an independent risk factor for atherosclerosis (128), atherothrombosis (129), hypertension (130), myocardial infarction (131) and vascular death in women with no cardiovascular risk factors (131, 132). Hence, CRP plasma levels have become one of the most powerful inflammatory identifiers and predictors of atherosclerosis and future cardiac events (132, 133).

1.3.3.2 Myeloperoxidase

Myeloperoxidase (MPO) is a highly-abundant haem protein stored in azurophilic granules of neutrophils that is released in response to pathogen exposure and is frequently present in atherosclerotic lesions (134). Following activation, neutrophils release MPO from granulocytes into the phagasome and extracellular space to produce the microbicidal compound hypochlorous acid (HOCl) and chloride anion (Cl^-) from hydrogen peroxide (H_2O_2) (135). Additionally, MPO has been shown in-vivo to use H_2O_2 and nitrite (NO_2^-) to generate reactive nitrogen species such as 3-nitrotyrosine (136). In situations where an excess of oxidising species is present that overwhelms anti-oxidant defences, the vessel undergoes a state of oxidative stress that perpetuates atherosclerotic lesion formation.

MPO also modulates the inflammatory response by contributing to endothelial dysfunction and reducing NO bioavailability (137). Such an effect has been demonstrated in patients during ischaemic-reperfusion injury in which plasma levels of MPO were significantly elevated and addition of H_2O_2 accelerated

rates of NO consumption (138). As such, the prognostic and predictive value of MPO plasma levels has received considerable attention. The prognostic value of serum MPO has been shown to be predictive both of myocardial infarction and subsequent cardiac events in patients presenting with angina (139). Similarly, MPO serum levels have been shown to predict risk for subsequent cardiovascular events and extend upon the prognostic information provided by troponin T and other biochemical markers in patients presenting with an acute coronary syndrome (ACS) (140).

1.3.4 Atherosclerosis

The stages of atheroma formation are believed to involve a great array of different inflammatory cells, all interacting with one another in a complex system. A healthy endothelium under normal circumstances resists binding of circulating leukocytes, however, if the endothelium becomes activated, expression of vascular cell adhesion molecule (VCAM-1) (141), intercellular cell adhesion molecule (ICAM-1) as well as P- and E-selectins (142) are all increased. Adhesion molecule expression is initiated in the presence of chemo-attractant cytokines such as interleukin-8 (IL-8) (143), interferon-γ (IFN-γ), Il-1β and tumor necrosis factor-alpha (TNF-α) (144) as well as by oxLDL uptake via LOX-1 receptors (145) and CD40/CD40L interactions. Furthermore, imbalances in endothelial derived factors such as NO, ET-1 and angiotensin II contribute to the inflammatory response by increasing leukocyte adhesion, production of reactive oxygen species (ROS), expression of pro-inflammatory cytokines IL-6 and monocyte chemoattractant protein

(MCP-1) and up-regulate the expression of endothelial cell adhesion molecules (146).

Atherosclerotic lesions (atheroma) are focal thickenings arising from the intima that comprise of blood borne inflammatory, vascular and smooth muscle cells as well as connective-tissue elements, lipids and debris (147). The centre core of an atheroma comprises of foam cells and extracellular lipid droplets, surrounded by a cap of smooth muscle cells and a collagen-rich matrix. Atheroma formation is preceded by the presence of lipid-laden cells beneath the endothelium and progression of the lesion is further enhanced by the proliferation and migration of VSMC towards the intima.

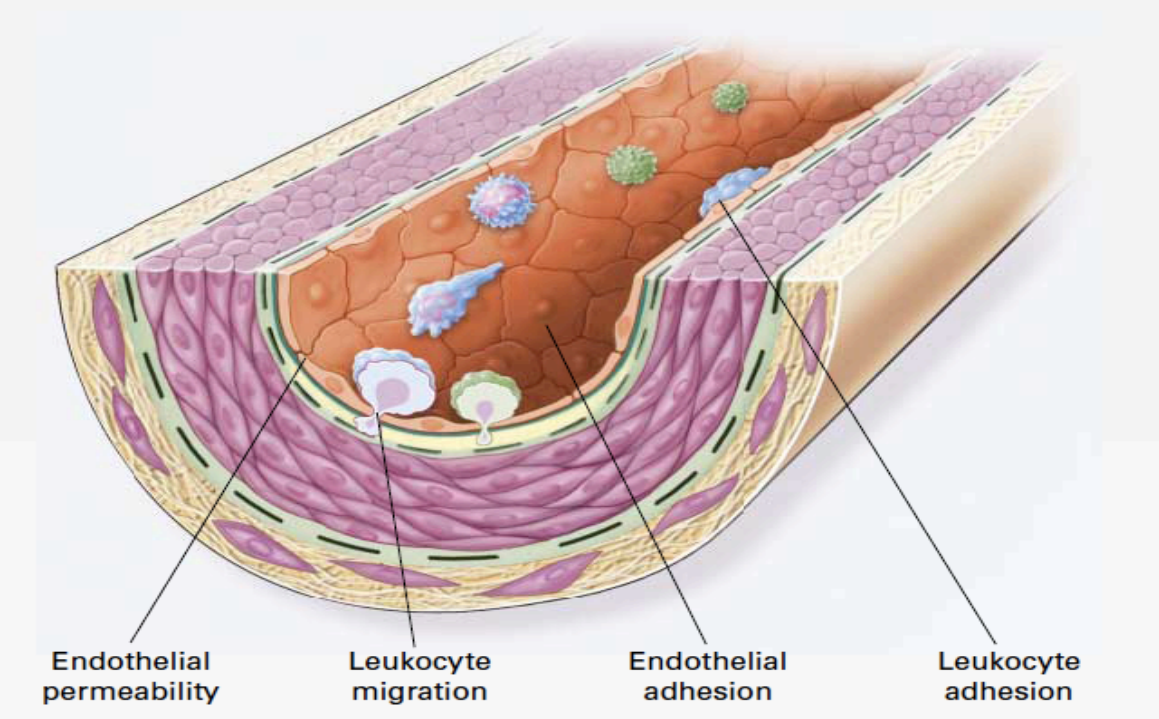
The initial steps of atherosclerotic plaque development begin from the presence of endothelial dysfunction. Upon adherence, leukocytes and monocytes migrate into the vascular intima via the monocyte receptor CCR2 (148) through a process known as diapedesis. Monocytes within the vascular intima develop into macrophages and begin to express scavenger receptors (SR-A, CD36) to internalise modified lipoproteins, which result in the formation of foam cells, the characteristic hallmark of atherosclerotic lesions (149). Foam cells act to augment the inflammatory response by secreting inflammatory cytokines for monocyte adhesion, enhancing expression of scavenger receptors and promoting macrophage replication (119). Other inflammatory mediators such as T-cells, dendritic cells and mast cells are also recruited into the atheroma in response to selective chemo-attractants such as inducible protein-10 (IP-10) and IFN-γ (144) and eotaxin (150). Cytokines

produced by activated endothelial cells, T cells and foam cells act to stimulate VSMC proliferation and promote collagen production (151). Such occurrences can lead to coronary vessel occlusion and subsequent reductions in blood flow (152). Collagen synthesis preserves the stability of the atherosclerotic plaque, however, release of pro-inflammatory cytokines such as IFN-γ (119) and MMP (153) can degrade and inhibit its formation, therefore making the plaque vulnerable to rupture by thinning the fibrous cap.

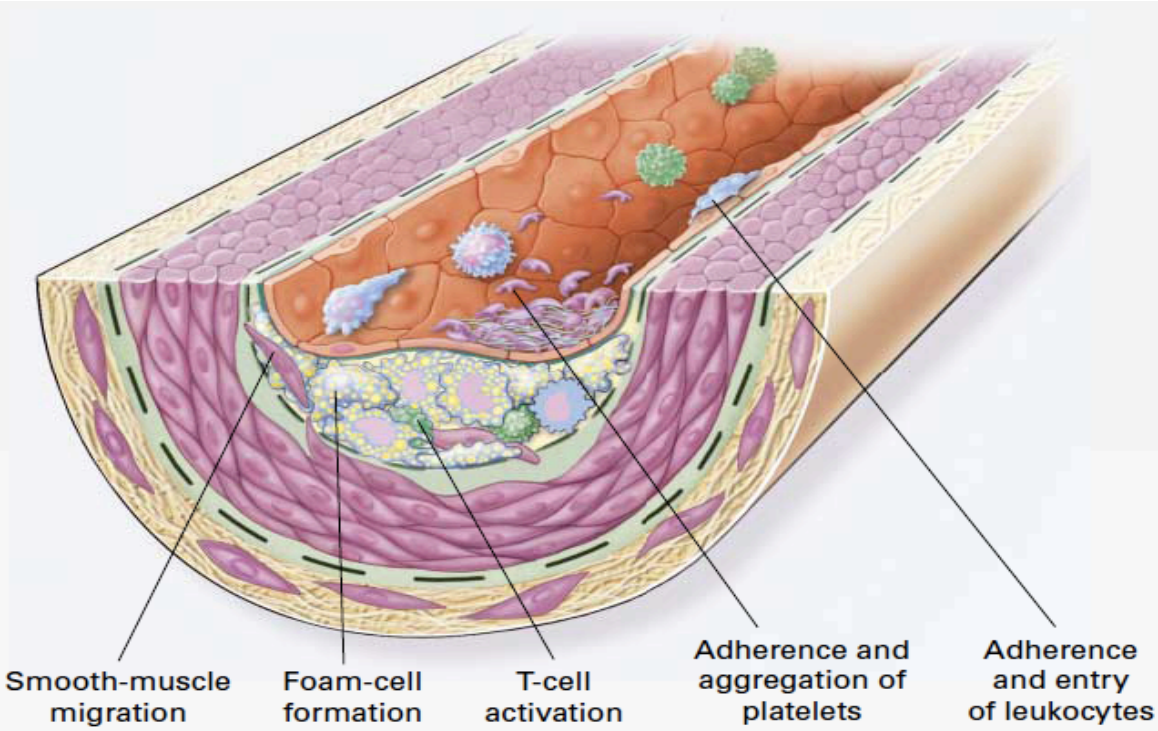
Plaque rupture is the precursor for thrombosis and subsequent acute myocardial infarction. Following disruption of the vulnerable plaque and spillage of the atheroma contents into the lumen, platelet activation and aggregation is triggered by interactions with thrombin, tissue factor and von Willebrand factor (vWF) (119). Furthermore, inflammatory mediators located within the plaque, such as IL-1, TNF-α, and CD40L enhance tissue factor over-expression and activate protease-dependent receptors on platelets, thereby promoting further thrombogenesis (154). If the thrombus is substantial in size to significantly compromise blood flow, myocardial infarction and subsequent death may occur. A representation of atheroma development and infarction resulting in myocardial cell death is depicted in four-stage process (Figure 1.5, pages 34-35). Due to the heavy involvement of inflammatory mediators in the atheroma process, numerous research studies have focused on elucidating these mechanisms in the development of coronary artery disease and also attempted to identify their individual clinical and prognostic significance.

Figure 1.5. Stages of atheroma development. Reproduced with permission from (155), Copyright Massachusetts Medical Society.

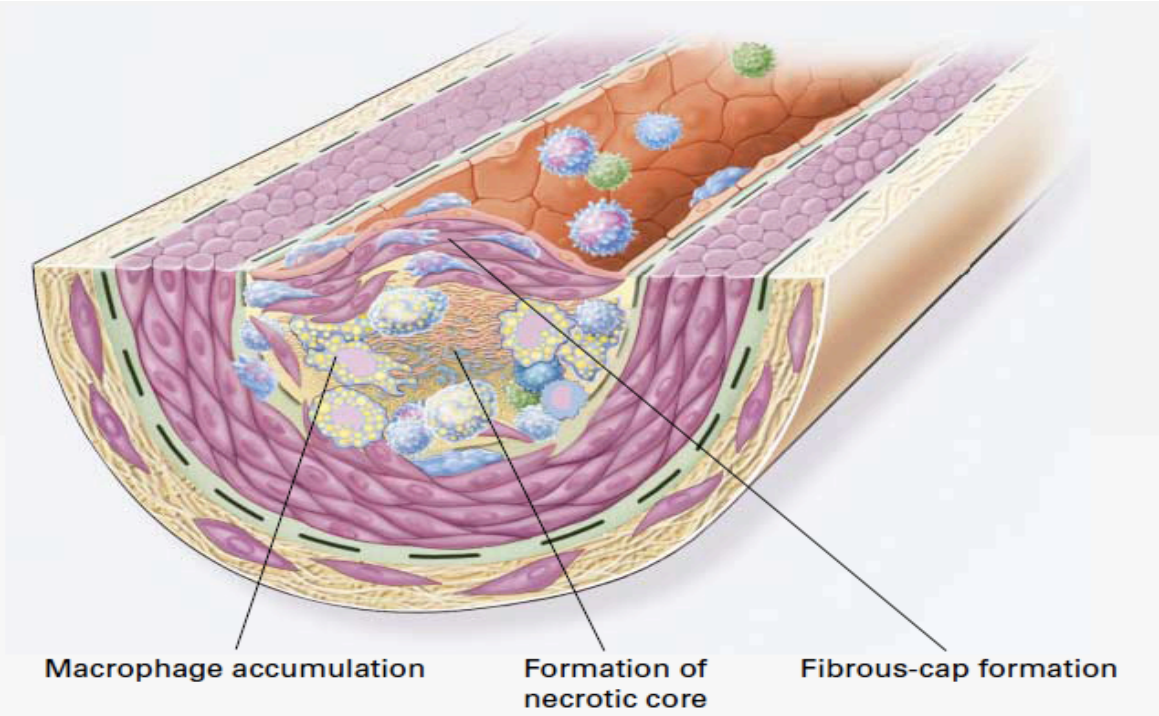
Stage 1. Endothelial Dysfunction



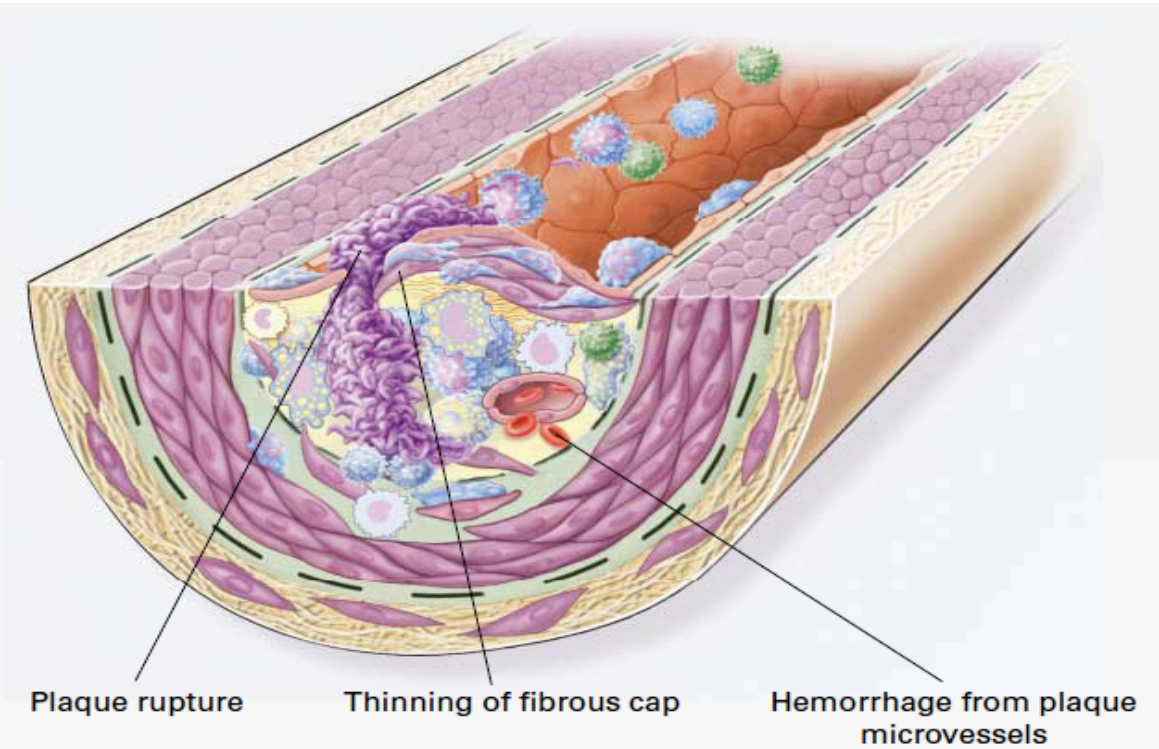
Stage 2. Fatty streak formation



Stage 3. Formation of advanced complicated atherosclerotic lesion



Stage 4. Rupture of fibrous plaque



1.3.5 Oxidative Stress

Normal cellular metabolism generates a great proportion of ROS, which have the potential to damage intracellular protein, and deoxyribose nucleic acid (DNA) structures. In a healthy individual, ROS that constitute various derivatives of oxygen such as superoxide (O_2^-), hydroxyl (OH^-), H_2O_2, and peroxynitrite ($ONOO^-$), are maintained at low concentrations due to the actions of innate anti-oxidant defences. In the event that the amount of circulating oxygen in tissues is reduced, such as in acute ischaemic or hypoxic events, the production of ROS greatly increases well above the anti-oxidant quenching capability, resulting in a state of oxidative stress. Reactive oxygen and nitrogen species can be sourced from numerous cellular mechanisms such as un-coupling of the mitochondrial electron transport chain, immune cell infiltration, and induction of oxidative enzymes.

Numerous studies have demonstrated that altered oxygen utilisation and/or increased formation of ROS contribute to atherosclerosis, by increasing monocyte adhesion, foam cell formation and increased vascular dysfunction. This has become evident in a number of investigations which have shown that superoxide anions (156), (157) and oxLDL (158) contribute to endothelial dysfunction and atherosclerotic lesion formation by inactivating NO. Such observations have been documented in a wide array of cardiovascular morbidities such as diabetes (159), hyperhomocysteinaemia (160) and hypertension (161).

Activation of cytosolic oxidases such as NADH/NADPH oxidase, xanthine oxidase and NOS isoforms have been shown to contribute to the oxidative stress environment by increasing production of O_2^- and $ONOO^-$ (162, 163). The enzymatic activity of these oxidases is increased in the presence of inflammatory cytokines, endothelial vasomotor autacoids and during hypoxic/ischaemic states. Specifically, NO avidly reacts with the superoxide anion to form the potent oxidant $ONOO^-$ and its clinical biomarker 3-nitrotyrosine, at diffusion-limited rates that exert a toxic effect upon endothelial cells (164). Scavenging enzymes such as superoxide dismutase (SOD) present in the mitochondria and cytoplasm function to prevent the production of $ONOO^-$ (165). Given the clinical importance of oxidative stress in cardiovascular conditions, identifying clinical biomarkers for detecting states of oxidative stress has been the subject of many research investigations.

1.3.5.1 Malondialdehyde

Lipid peroxidation is the process by which polyunsaturated fatty acids react with ROS to form reactive aldehydes and covalent adducts with various macromolecules. Aldehydes produced from lipid peroxidation reactions are usually end-products, however, some may behave as strong electrophiles that react with proteins to form adducts detectable in serum (166). Formation of aldehyde-protein adducts in vascular lesions is common and their contribution to atherosclerotic pathogenesis is denoted by the fact that:

i) Levels of reactive aldehydes increase in plasma in relation to the extent of atherosclerosis;

ii) Aldehyde concentrations are increased during oxidation of LDL phospholipids;

iii) Structural and functional changes of LDL oxidation can be reproduced by direct interaction of LDL and aldehydes; and

iv) Aldehyde reactions with lysine residues of LDL-apoB are detected within human monocyte-macrophages and lead to the accumulation of lipoprotein-derived cholesterol ester (167).

Malondialdehyde (MDA) is one of the main products formed from lipid peroxidation. It is a reactive aldehyde that primarily forms adducts with lysine protein residues or with amine head-groups of phospholipids and has shown clinical utility as a marker for oxidative stress (167, 168). Accordingly, MDA plasma levels have been used as clinical indicators of lipid peroxidation in a range of patients with cardiovascular disorders such as pre-eclampsia (169), non-insulin dependant diabetes mellitus (170), heart failure (171) and atherosclerosis (172). Thus, clinical investigations have utilised MDA as useful marker of oxidative stress status.

1.3.5.2 Homocysteine

Homocysteine is an intermediary product in the metabolism of dietary methionine to cysteine. Highly reactive, homocysteine is removed from the plasma by either conversion back to methionine or degradation to cysteine by a vitamin-B6-dependent enzyme. Individuals with genetic defects in homocysteine metabolism, nutritional vitamin deficits or renal impairments, show evidence of elevated plasma homocysteine levels. This condition,

known as hyperhomocysteinaemia, has been shown to be a cardiovascular risk factor (173), impair arterial endothelial function (174), a strong predictor of mortality in coronary artery disease patients (175) and an independent risk factor for vascular disease (176). Some of the postulated mechanisms of hyperhomocysteinaemia-induced endothelial dysfunction have included impaired release of NO (177), generation of superoxide anion radicals (178), increased leukocyte-endothelium interactions (179) and promotion of eNOS un-coupling due to reduced intracellular BH_4 availability (180). Since hyperhomocysteinaemia has been identified as an independent risk factor for cardiovascular disease development, current medical practices have focused on reducing plasma homocysteine levels, through appropriate vitamin supplementation.

1.3.6 Spasm

Coronary artery spasm is defined as a temporary, sudden, intense vasoconstriction of a coronary artery that results in vessel occlusion. It has been proposed by Lanza and colleagues that coronary artery spasm results from the interaction of two components, namely a localised abnormality of a coronary artery that makes it hyper-reactive to vasoconstrictor stimuli, and a vasoconstrictor stimulus that is able to induce spasm at the level of the hyper-reactive coronary segment (181). Specifically, this review article specifies that the mechanisms contributing to coronary artery spasm include endothelial dysfunction, hyper-reactivity of VSMCs, increased growth factor production, adventitial abnormalities and excessive intracellular calcium inflow. Other postulated mechanisms of spontaneous coronary artery spasm that have been

documented include enhanced phospholipase C enzyme activity causing smooth muscle cell hypersensitivity (182) and polymorphisms in endothelial NOS gene (183).

The diagnostic criteria of coronary artery spasm includes severe chest pain (usually without physical effort) and a concurrent electrocardiogram (ECG) showing transient ST-elevation (184). Several studies have demonstrated that the spasm can be induced by emotional stress, cold pressor tests, pharmacological agents (adrenaline and noradrenaline) (185) and most frequently occurs at rest in patients with normal or near-normal coronary arteries. Current treatments that have been shown to be effective in treating coronary spasm include nitrates and calcium-channel blockers (186).

1.3.7 Thrombosis

Thrombosis is the process of blood clot formation within the vasculature. Blood clots may form in the presence of certain pathologies that promote vessel occlusion and subsequent events such as myocardial infarction. Atherosclerosis and its accompanying pathology of lipid accumulation, macrophage infiltration and smooth muscle proliferation is generally a clinically silent process, manifesting only when plaque rupture with associated thrombus formation occurs. Platelets contribute to myocardial infarction development through multiple mechanisms (187), including:

i) Occlusion of an epicardial artery at a disrupted/eroded atherosclerotic plaque;

ii) Micro-embolisation of platelet-rich aggregates;

iii) Platelet-mediated vasoconstriction;

iv) Enhanced thrombus formation in the microcirculation; and

v) Platelet-mediated inflammatory reactions in the myocardium

The accumulation and prevalence of these events determines the extent of ischaemia, cardiac contractile dysfunction and associated clinical presentation of disease. Current therapeutic strategies for treating thrombotic vessel occlusion include either administration of fibrinolytic agents or through direct percutaneous coronary intervention (PCI).

Platelet-dependent formation of a thrombus at sites of atherosclerotic lesions involves a number of biological interactions with components of the sub-endothelial layer. Initial contact between circulating platelets and vessel wall lesion is established by the interactions of platelet receptors GPIb and GPVI with extracellular matrix proteins von Willebrand factor (vWF) and collagen (188). This adhesion activates and stabilises platelets by opening up platelet fibrinogen ($\alpha_{IIb}\beta_3$) and collagen ($\alpha_2\beta_1$) receptors and binding to extracellular matrix proteins (189). As a result, platelets spread, de-granulate and generate thrombin, adenosine di-phosphate (ADP) and TXA_2 to recruit additional platelets and form clots with the aid of fibrin and fibrinogen (188). Platelets also commonly form smaller emboli that lodge within the coronary microcirculation (190) to cause decreased regional coronary blood flow distal to a stenosis, vasoconstriction and transient ischaemia, as observed in patients with unstable angina and non-ST segment elevation myocardial infarction (191). In addition to microvascular obstruction, thrombotic emboli also induce temporary vasospasm through the release of serotonin, TXA_2 and free radicals

(187). Platelets also play a pivotal role in the inflammatory process by releasing chemotactic factors stored inside granules that act to recruit leukocytes at sites of endothelial injury (192).

1.3.8 Summary

Coronary circulatory dysfunction is attributed to a multitude of pathophysiological factors that work both independently and concurrently to contribute to impaired blood flow. Factors such as endothelial dysfunction, inflammation, atherosclerosis, oxidative stress, spasm and thrombosis all play a role in coronary dysfunction. These pathophysiological processes may arise from a range of mediating factors such as:

i) Presence of risk factors;

ii) Prolonged endothelial activation/injury;

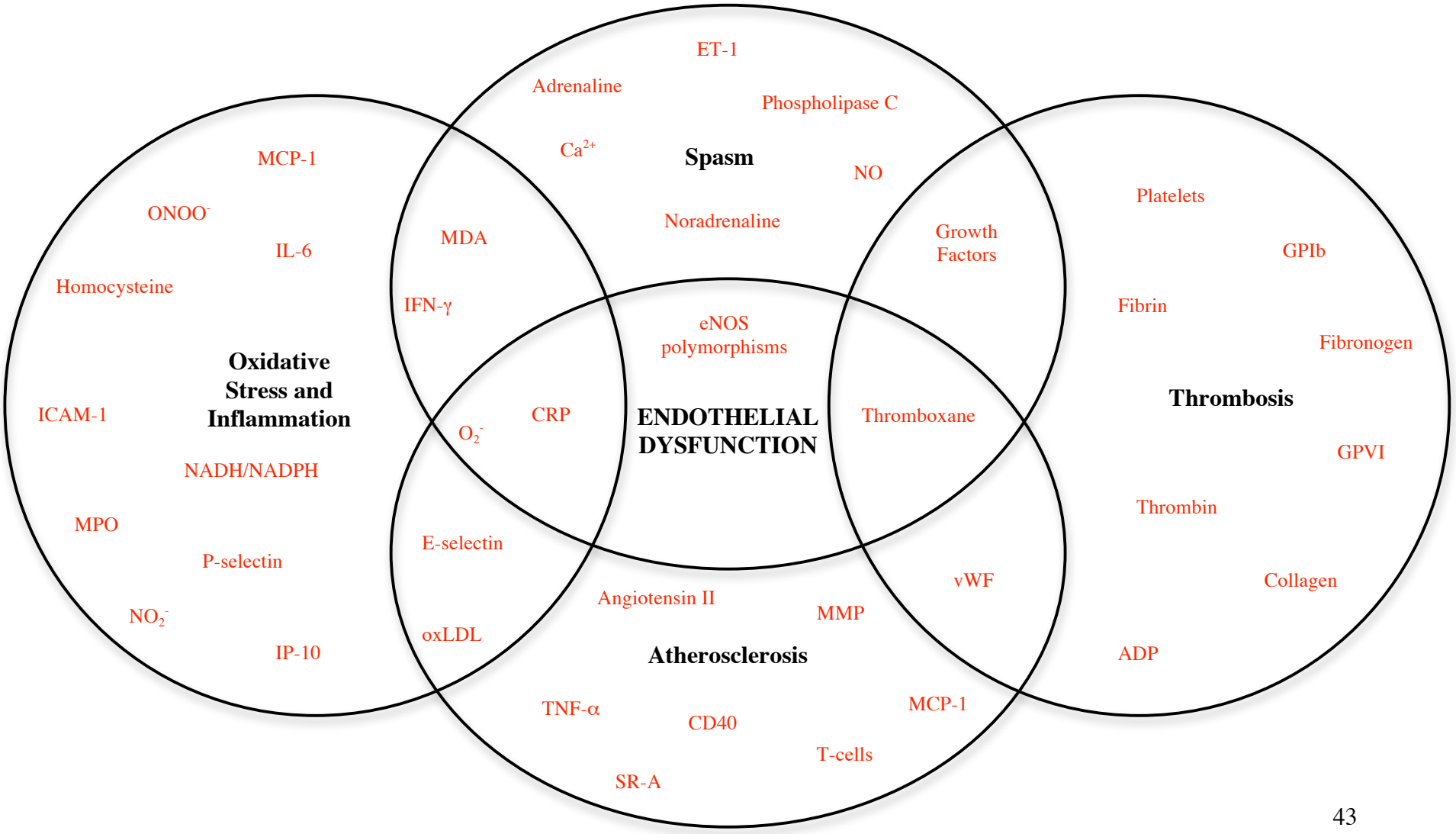
iii) Imbalances in autonomic nervous system innervation and vasomotor autacoid regulation;

iv) Haemodynamic stresses; and

v) Presence of pathogens

All of these factors work in an integrated network to initiate and sustain the mechanisms responsible for coronary circulatory dysfunction (Figure 1.6, page 43). The following sections will describe the consequences of coronary dysfunction from a clinical perspective and what quantitative methods are used to evaluate the abnormalities in the coronary circulation.

Figure 1.6. Venn diagram representing the biological mediators contributing to coronary circulatory dysfunction.



1.4. Consequences of Coronary Dysfunction

1.4.1 Myocardial Ischaemia

Myocardial ischaemia is the clinical endpoint arising from an insufficient blood supply to the heart tissues. The insufficient blood supply may be attributed to narrowing of the coronary arteries due to atherosclerosis, thrombotic occlusion, coronary spasm or microvascular dysfunction. Myocardial ischaemia clinically manifests as angina pectoris (majority of cases) but if prolonged, may give rise to myocardial infarction. Early detection of myocardial ischaemia is therefore critical to minimising tissue damage. However, as explained in the following sections, detecting the presence of ischaemia is not always an easy task given the limitations of current assessment methods and difficulties encountered in detecting the different stages of the ischaemic cascade.

1.4.2 Ischaemic Cascade

The ischaemic cascade is a sequence of events that describes the process of ischaemic development. Initiated by imbalances between myocardial oxygen supply and demand, the ischaemic cascade reflects different stages of cardiac abnormalities induced by ischaemia. The cascade begins with clinically unrecognisable physiological changes such as myocardial oxygen supply/demand imbalance, which lead to abnormalities in systolic and diastolic function (193, 194). Clinically identifiable indicators such as ECG abnormalities and angina do not occur until late in the ischaemic cascade (195). Angina is a late process in the ischaemic cascade and thus may not

occur in some patients despite evidence of impaired perfusion, myocardial dysfunction and ECG abnormalities (e.g. silent ischaemia). Thus, detection of abnormalities in perfusion and function, regardless of whether angina symptoms are present, is important for early detection of ischaemic events.

1.4.3 Angina Pectoris

Angina pectoris is a clinical manifestation arising from myocardial ischaemia characterised by severe pain radiating from the chest and surrounding areas. Typical clinical symptoms include chest, jaw, arm or back discomfort that is induced by either physical exertion, emotional stress or at rest. Angina may limit normal day-to-day activities and have a detrimental impact on quality of life, thereby posing a burden upon resources and resulting in substantial costs to the healthcare systems.

Several clinical angina syndromes have been described and include chronic stable angina, unstable angina, variant angina, decubitus angina and silent ischaemia. Chronic stable angina refers to a fixed arterial narrowing due to a stable plaque that manifests during times of increased myocardial oxygen demand and is relieved by rest or nitroglycerine. In contrast, unstable angina is typically characterised by crescendo or rest angina, and arises from partial or complete occlusion of an artery due to plaque rupture of an unstable atheroma and subsequent thrombus formation. Both stable and unstable angina symptoms may develop into myocardial infarction; however, the risk is significantly higher in the unstable angina group.

Prinzmetal and colleagues further described another form of angina (known as variant angina) that occurs as a result of coronary artery spasm and is characterised by recurrent episodes at rest associated with ST-elevation upon ECG (196). Decubitus angina refers to patients who experience chest pain and dyspnoea whilst lying down. Silent myocardial ischaemia is an unusual phenomenon characterised by ischaemia in the absence of angina symptoms (197). This condition is common in diabetic patients as it is postulated that the clinical manifestations from ischaemia are not experienced due to autonomic neuropathy (198). Significant research efforts are currently underway to further elucidate the mechanisms of this condition, as patients with silent ischaemia are more likely to experience adverse clinical events (199).

1.4.4 Summary

In order to accurately determine cardiovascular risk and apply appropriate clinical management strategies, quantitative assessment of the extent of myocardial ischaemia and underlying pathologies must be undertaken. Additionally, the impact of disease severity must be determined from a clinical perspective. The extent of physical limitation of the angina may be determined through reference to the Canadian Cardiovascular Society Angina Classification (CCSC) system (200) (Table 1.1, page 47). Current quantitative methods of assessment used in clinical practice include an array of imaging and electrophysiological modalities that can be used both independently and concurrently to ascertain the degree of myocardial ischaemia and vessel occlusion. These modalities will be further discussed in the following section.

Table 1.1 Canadian Cardiovascular Society Classification System for grading of angina pectoris. Adapted from (200).

Class I	Ordinary physical activity, such as walking or climbing stairs, does not cause angina. Angina occurs with strenuous, rapid or prolonged exertion at work or recreation.
Class II	Slight limitation of ordinary activity. Angina occurs when walking or climbing stairs rapidly, walking uphill, walking or stair climbing after meals, in cold weather, in the wind, or under emotional stress or only during the few hours after wakening. Angina occurs after walking more than 2 level blocks and climbing more than 1 flight of ordinary stairs at a normal pace and under normal conditions.
Class III	Marked limitations of ordinary physical activity. Angina occurs when walking 1-2 level blocks and climbing 1 flight of stairs at a normal pace under normal conditions.
Class IV	Angina occurs while walking less than 1 block or while walking in the house, or doing light chores or personal care.

1.5. Evaluation of the Coronary Circulation

Determining the presence and extent of disease in both the large and small coronary vessels is crucial for application of appropriate therapeutic management strategies. Accurate evaluation of the coronary vasculature requires both structural and functional assessments. Clinicians utilise a multitude of diagnostic techniques to identify the degree of large coronary artery occlusion and myocardial perfusion abnormalities. These techniques are designed to allow:

i) Structural imaging of the coronary arteries to identify obstructive atherosclerotic disease;

ii) Functional assessment of vascular reactivity; and

iii) Identification of ischaemia and myocardial perfusion dysfunction.

Although these techniques are clinically useful for identifying abnormalities in the large vessels, assessment of microvascular dysfunction is more reliant upon functional methods, as structural abnormalities in the microvasculature cannot be clinically imaged. These microvascular functional assessment methods can be invasive or non-invasive and include:

i) Detection of functional consequences in the ischaemic cascade (myocardial ischaemia, impaired perfusion and contraction); and/or

ii) Measurements of coronary blood flow.

1.5.1 Structural imaging of the coronary arteries

Accurate and quantitative assessments of large vessel diameter are required to determine the extent of atherosclerotic disease. Techniques such as coronary angiography, intravascular ultrasound (IVUS), computed tomography (CT) and magnetic resonance (MR) imaging are employed to provide detailed images of the large coronary arteries. Each quantitative method can be used both independently and in combination with others, to provide comprehensive information about the status of the vasculature for application of appropriate clinical therapeutic intervention.

1.5.1.1 Coronary angiography and intra-vascular ultrasound

Coronary angiography is an invasive diagnostic imaging technique used to identify obstructive stenoses and other pathological abnormalities in the coronary vasculature. Injection of radio-opaque contrast dye into the coronary arteries allows clinicians to quantify the degree of vessel blockage in the main coronary arteries and assess haemodynamic variables such as the blood flow rate. Additionally, IVUS techniques are also used in conjunction with angiography to visualise the intimal layer of epicardial blood vessels and assess the extension of atherosclerotic disease both cross-sectionally and longitudinally (201). Such imaging techniques allow clinicians to determine whether revascularisation procedures need to be performed.

1.5.1.2 CT and MR angiography

Coronary CT imaging is commonly used to quantify the degree of coronary artery calcification and detect the presence of atherosclerotic plaques (202).

Detailed images of coronary arteries are obtained using multi-slice CT scanners in conjunction with retrospective ECG gating techniques. Although this imaging modality is limited in its ability to quantitate the severity of coronary artery stenosis, its clinical utility lies in its non-invasive approach and ability to exclude the presence of coronary artery disease. MR angiography techniques use intravenously injected contrast dye to generate vessel images in order to visualise and quantify impairments in blood flow rates. Recent studies comparing MR angiography with conventional angiography have shown that non-invasive MR coronary angiography techniques have a sensitivity and specificity of >90% for identifying individual vessels with ≥50% stenosis (203).

1.5.2 Ischaemia detection techniques

Detecting the presence and extent of myocardial ischaemia is an important functional indicator of coronary heart disease. Techniques such as ECG, positron emission tomography (PET), single-photon emission tomography (SPECT), in addition to magnetic resonance imaging (MRI), are frequently used to: i) detect the presence of ischaemia; and ii) identify the extent and region of ischaemia so that clinical interventions can be appropriately targeted.

1.5.2.1 ECG and metabolic indicators

The twelve lead ECG test is the most common non-invasive technique used to assess the electrical activity of the heart and detect ischaemic tissue damage. Based on abnormalities seen in the ST-segment and/or T wave, the ECG can

be used to perform a cardiac stress test as a means to assess a patient's physical condition. In the presence of a stenosis, oxygen-rich blood flow may be reduced, thereby resulting in conversion to anaerobic metabolism and subsequent accumulation of waste products such as lactate. As such, metabolic indicators such as myocardial lactate concentrations can also be used in clinical practice to determine the presence of ischaemia (204, 205).

1.5.2.2 Myocardial Perfusion Imaging

Myocardial perfusion imaging techniques provide information regarding the presence of ischaemia by identifying perfusion defects through application of techniques such as (SPECT or PET) or MRI (206). SPECT nuclear imaging techniques utilise gamma-emitting radiopharmaceuticals to assess cardiac function and blood flow distribution following induction of myocardial stress. PET imaging involves positron emissions and detection of gamma photons that generate higher resolution images for assessment of regional metabolism and myocardial perfusion (207). Contrast MRI imaging techniques also allow perfusion imaging and the estimation of the myocardial perfusion reserve (208).

1.5.2.3 Echocardiography

Echocardiography is a commonly applied non-invasive technique used to assess myocardial contractility, through application of ultrasound techniques. Utilising the ischaemic cascade principle that systolic dysfunction is an early marker of myocardial ischaemia; echocardiography has evolved to include 'stress echocardiography' as an important diagnostic technique. In this

technique, left ventricular function is assessed at rest and following an ischaemic stress stimulus such as exercise, or pharmacological stimulation (dobutamine, adenosine or dipyridamole). Stress echocardiography has been shown to be comparable to myocardial perfusion scans in the detection of coronary artery disease (209). Contrast echocardiography is another technique where contrast agents that create microbubbles are used and allow echocardiographic imaging of myocardial perfusion (210). These agents are administered at rest and following stress stimuli (as above) thereby allowing stress contrast echocardiographic studies to be performed.

1.5.3 Coronary blood flow assessment in the microvasculature

The assessment of resting coronary blood flow is highly variable as it is dependent upon myocardial oxygen demand, heart rate, myocardial contractility and cardiac preload. Thus, the concept of coronary flow reserve (CFR) was developed and refers to the maximum ability of coronary arteries to increase blood flow above normal resting flow in response to vasoactive mechanisms. In a healthy individual, blood flow can be increased up to four-to-five times over baseline, in comparison to an individual with significant stenosis (>70% narrowing) by which resistance vessels become maximally dilated and thus are unable to further increase blood flow (211). An impaired CFR, as defined by the inability to increase blood flow to at least twice above the baseline flow, therefore indicates maximal dilation of resistance vessels and implies the presence of a significant coronary lesion or diseased microcirculation. Several non-invasive and invasive techniques have been used to measure coronary or myocardial blood flow and thus estimate

coronary/myocardial flow reserve. These include PET using blood flow tracers, quantitative perfusion MRI techniques, contrast echocardiography, Doppler flow-wire techniques (212), and pressure wire techniques.

1.5.4 Summary

Several established techniques are used in clinical practice to evaluate the extent of large and small coronary disease. However, the majority of techniques focus on evaluating the extent of stenosis in the large epicardial vessels. The following section will specifically focus on describing the role of microvascular dysfunction in disease and its importance in a range of cardiovascular pathologies.

1.6. Role of Microvascular Dysfunction in Disease

The majority of coronary disorders discussed thus far have focused on abnormalities in the large epicardial vessels. Given that the coronary circulation comprises of both large and small vessels, the importance of coronary microvascular dysfunction in disease pathology must also be considered. Generally, it is thought that myocardial ischaemia is caused by abnormalities in the epicardial coronary arteries. However, recent studies have demonstrated that abnormalities in the coronary microcirculation may also contribute to myocardial ischaemia in several conditions. Delineating the extent of large and small vessel dysfunction is problematic given the limits of current techniques. Microvascular dysfunction may play a larger role than the smaller vessels in certain clinical conditions. Thus, in order to ascertain the extent of microvascular dysfunction and understand the role it plays in disease pathology, a greater understanding of the different types of microvascular dysfunction must be established.

1.6.1 Coronary Microvascular Dysfunction

CMD frequently manifests as angina pectoris and is clinically defined based on abnormalities in coronary microvascular resistance, impaired myocardial perfusion and/or myocardial ischaemia not attributable to abnormalities in epicardial coronary arteries. Recently published landmark reviews (3, 213) propose that coronary microvascular dysfunction can be classified into four types as outlined in Table 1.2 (page 55).

Table 1.2. Clinical Classification of Coronary Microvascular Dysfunction.

Reproduced from (213) with permission from Elsevier.

CMD without associated myocardial/coronary artery disease

- Smoking
- Hyperlipidaemia
- Diabetes
- Hypertension
- Angina with normal coronary arteries

CMD associated myocardial disease

- Hypertensive heart disease
- Hypertrophic cardiomyopathy
- Tako-Tsubo cardiomyopathy
- Dilated cardiomyopathy
- Myocarditis
- Amyloid heart disease
- Anderson-Fabry disease

CMD associated epicardial coronary artery disease

- Obstructive atherosclerotic coronary artery disease
- Vasospastic angina
- Acute myocardial infarction

Iatrogenic CMD

- Post-angioplasty vasoconstriction
- No-reflow phenomenon

All types of microvascular dysfunction may be sustained by changes in structural, functional and extravascular pathogenetic mechanisms. The importance of each of these mechanisms varies amongst different clinical settings and in some instances, may co-exist in the same condition. Given the vast range of clinical conditions that coronary microvascular dysfunction is attributed to, the following sections will focus on reviewing the first type of microvascular dysfunction (coronary microvascular dysfunction in the absence of myocardial coronary artery disease), its associated clinical presentation, and impact upon patient health outcomes.

1.6.2 Defining angina and normal coronary arteries

Patients with chest pain suspicious of angina frequently undergo coronary angiography. In the majority of cases, obstructive lesions would be identified in at least one of the large epicardial vessels and revascularisation procedures would follow. However, it has been observed that approximately 10-20% of these patients do not have evidence of obstructive plaques, despite initially presenting with angina-like chest pain (214). Such an anomaly has become the subject of many research investigations and led to the discovery that coronary microvascular dysfunction is the key culprit behind this phenomenon.

Extensive investigations into patients with angina and normal coronary arteries have provided many important insights into disease pathology. Definitive conclusions regarding the extent of CMD and appropriate patient management strategies have been difficult to make given the heterogeneous nature of the condition. Several attempts have been made to further

characterise these patients based on differences in clinical and biological parameters. As a result of these investigations, the terms “cardiac syndrome X” (CSX), “microvascular angina” (MVA) and the “coronary slow flow phenomenon” (CSFP) have been coined in order to delineate between the clinical profiles of patients that present with angina and normal coronary angiograms.

1.6.2.1 Cardiac Syndrome X

In a pioneer study conducted in 1973, Arbogast and colleagues identified that similar to patients with angina and obstructive coronary artery disease (Group C), patients presenting with angina and normal coronary angiograms (Group X), also display evidence of myocardial ischaemia (215). Kemp and colleagues published an editorial further to this initial study defining this group of patients as having “syndrome X”. In its generic form, the term cardiac “syndrome X” encompasses patients that have angina-type pain that is cardiac in nature and associated with a normal angiogram. More specifically, CSX defines a clinically-distinct set of patients who demonstrate: i) effort induced angina; ii) ST-segment depression during spontaneous or provoked angina; and iii) angiographically normal arteries (216).

Both the generic and specific definitions have commonly been used interchangeably to characterise patients with angina and normal coronary arteries. However, not all patients that present with this clinical profile fall into the strict CSX definitions, such as those presenting with rest angina, hypertension or diabetes, or those with no ischaemic-suggestive ECG

changes. Additionally, several studies have demonstrated that only a minority of CSX patients have myocardial ischaemia upon exercise stress testing and that these symptoms may also be brought on by rest angina (217). Such observations generated controversy and ambiguity within the literature that made diagnosis and patient management difficult.

1.6.2.2 Microvascular Angina

Given the controversies regarding the differing clinical CSX profiles, further investigations were conducted to identify and characterise a clinically distinct group of patients. In the early 1980s, Cannon and Epstein described a group of patients who presented with angina-like symptoms and had abnormal vasomotor responses to metabolic and pharmacologic stimuli, despite a normal coronary angiogram (218). Only a minority of these patients had ischaemic ST-segment ECG changes, thereby suggesting that this group of patients were clinically distinct to those traditionally defined as having CSX. As such, the term "microvascular angina" was coined to describe this clinically distinct group of patients who had:

i) Angina-like pain;

ii) Angiographically determined normal coronary arteries; and

iii) Impaired myocardial blood flow responses to provocative stimuli (impaired coronary flow reserve).

1.6.2.3 Coronary Slow Flow Phenomenon

The CSFP is another disorder characterised by microvascular dysfunction. Tambe and colleagues were the first to report the condition, describing a

peculiar angiographic phenomenon characterised by slow flowing dye despite no significant coronary artery disease, in patients presenting with angina-like symptoms (219). Initial studies of patients with slow dye flow in normal epicardial coronary arteries speculated that functional obstruction of the small microvessels contributed to the pathology of this condition, as dipyridamole infusion tended to relieve this anomaly (220). In addition, histopathological myocardial biopsy studies have demonstrated the presence of structural microvascular disease in these patients (220, 221).

Beltrame and colleagues were the first to clinically and angiographically characterise the CSFP as a distinct coronary microvascular disorder. Beltrame et al, provided a quantitative definition of the CSFP and associated clinical features upon presentation (222). Clinically, these patients were distinct from CSX and MVA patients as they generally presented with:

i) An acute coronary syndrome;

ii) Rest-induced and prolonged angina;

iii) Recent onset angina that required urgent hospital re-admission;

iv) Normal exercise stress tests;

v) Occasional episodes of ST elevation; and

vi) Failure to induce coronary artery spasm following provocative testing.

Thus, although the CSFP resembles some similarities to CSX and MVA, it remains a separate clinical entity.

Two approaches have been used to quantitate coronary angiographic blood flow and clinically define the CSFP. These angiographic techniques were developed by the TIMI (Thrombolytics in Myocardial Infarction) group and comprise the TIMI flow grade and TIMI frame count (223). The TIMI flow grade comprises of:

i) TIMI-0 (no perfusion beyond a coronary occlusion);

ii) TIMI-1 (faint antegrade flow beyond an occlusion with incomplete filling of the distal coronary bed);

iii) TIMI-2 (delayed flow and usually requiring three of more cardiac cycles to opacify the distal vasculature); and

iv) TIMI-3 (normal flow).

TIMI-2 flow has commonly been used as a diagnostic parameter in the CSFP as it corresponds to delayed distal vessel opacification and usually requires three or more beats to opacify the distal vasculature (222). The TIMI frame count method consists of assessing the number of angiographic frames required to opacify a coronary vessel and has also been used to diagnose the CSFP during angiography (224). Although a more quantitative measure than the TIMI flow grade, the TIMI frame count requires dedicated analyses and does not adjust for heart rate.

1.6.2.4 Primary Coronary Microvascular Dysfunction

Lanza et al, recently published a review to describe an alternative means of classifying microvascular dysfunction in the absence of coronary artery disease, based on differences in clinical presentation (225). The term primary coronary microvascular dysfunction (PCVD) was coined to suggest a

classification of angina and normal coronary arteries in two main forms, as either stable (chronic) or unstable (acute). Stable PCVD refers to patients that present predominantly with exertion or effort angina, whereas unstable PCVD is defined on a de-novo angina and is associated with prolonged and worsening recurrent angina pain at rest.

1.6.3 Summary

Given the ambiguity in the literature, it is important to clinically distinguish between the different coronary microvascular disorders. Delineating between these microvascular disorders will be important in determining the presence/extent of ischaemia, identifying the exact pathophysiological mechanisms responsible for each condition and applying appropriate therapeutic strategies. The following section will focus on describing the differences predominantly between CSX and the CSFP, in regards to pathophysiological mechanisms, clinical features and current treatments.

1.7. Cardiac Syndrome X

1.7.1 Pathophysiological mechanisms

Following the initial description of CSX, controversy still exists regarding the responsible mechanisms behind this disorder. Whether myocardial ischaemia is the cause of chest pain in CSX patients remains debatable given the presence of unobstructed arteries, poor responses to nitrate administration and negative stress echocardiograms identified in the majority of patients. Evidence of transient ST-segment depression on ECG, abnormalities in myocardial perfusion (226), net lactate production (215, 227), and decreased coronary sinus oxygen saturation, (228) are examples supporting an ischaemic basis for the chest pain. Additionally, evidence of myocardial ischaemia has also been demonstrated in CSX patients using phosphorus-31 nuclear-magnetic-resonance (NMR) spectroscopy techniques (229) and by identifying increased levels of ischaemia-reperfusion oxidative stress markers following pacing-induced tachycardia (230).

CMD is implicated in the pathophysiology of CSX by the presence of ischaemia and/or impaired flow reserve. Several studies have suggested that the mechanism of CMD may be attributable to:

i) Structural abnormalities;

ii) Endothelial dysfunction;

iii) Inflammation; and/or

iv) Oxidative stress.

Evidence of structural abnormalities such as fibro-muscular vessel thickening (221), narrowing of capillary lumen (due to swollen endothelium) (231), increases in media thickness to lumen diameter artery ratios (232), and reductions in skin capillary density (233) have also been reported in CSFP patients.

Functional abnormalities in the coronary microvasculature have also been extensively documented. Initial studies identified that CSX patients had markedly impaired coronary vasodilator capacity in response to the dipyridamole administration (234). Intracoronary Doppler investigations have also confirmed this by demonstrating that both endothelium-dependent and endothelium-independent vasodilation is impaired in CSX patients compared to control subjects (235). Imbalances in regulatory endothelial vasomotor factors have been demonstrated by elevated ADMA levels (236) and increased circulatory levels of ET-1 (237). Additionally, ET-1 concentrations have been shown to be correlated to abnormal coronary microvascular responses in CSX patients (238). Other postulated mechanisms include enhanced sodium-hydrogen exchanger activity (239) in cell membranes and enhanced vasoconstriction due to the actions of intracellular rho-kinase (240).

Inflammation and oxidative stress mechanisms have also been implicated in CSX pathophysiology. Lanza and colleagues were the first to identify the presence of low-grade systemic inflammation in CSX patients attributable to increased plasma concentrations of CRP and IL-1 receptor antagonist (241). Furthermore, others have identified that elevated CRP levels in CSX patients

are associated with attenuated coronary blood flow responses to ACh (242) and increased carotid artery intima-media thickness (243). Increased concentrations of serum CRP have also been documented (243), and correlated with number of ischaemic episodes, ST-segment changes and vascular abnormalities (242, 244). Similarly, oxidative stress biomarkers have been identified in CSX patients as demonstrated by increased plasma homocysteine levels (245), reduced anti-oxidant activity of paraoxonase-1 (246) and increased production of oxidative hydroperoxides in the coronary sinus following atrial pacing (230).

A large body of evidence also suggests that abnormal pain perception is partly responsible for anginal pain in CSX patients. Clinical studies have demonstrated that CSX patients develop chest pain during intra-atrial injection of saline (247) and that abnormal cardiac adrenergic nerve function is evident post meta-iodobenzylguanidine scintigraphy studies (248). Furthermore, abnormal pain perception responses in women with CSX have been associated with inappropriate endogenous opioid responses (249). Crea and colleagues hypothesised that given most CSX patients exhibit pronounced functional abnormalities in cardiac adrenergic nerve fibres, repeat episodes of myocardial ischaemia might induce functional alterations in cardiac afferent nerve endings, resulting in increased activity to stimuli (216).

1.7.2 Clinical features

A clear clinical profile of CSX is difficult to obtain given the heterogeneous nature of the condition. Kaski and colleagues studied the clinical characteristics and evolution of symptoms in CSX patients using a strict homogenous definition (angina pectoris, positive exercise stress test and normal coronary angiograms) (250). In this study, it was identified that CSX was:

- More prevalent amongst post-menopausal females;
- Chest pain was mainly exertional, indistinguishable in character, location and radiation compared to patients with obstructive coronary artery disease;
- Had an average duration of >10 minutes;
- Sublingual nitrates were effective in only 42% of patients; and
- Transient ST-segment depression was observed in most patients during ambulatory ECG monitoring.

Furthermore, a sizeable proportion of these patients had a family history of coronary disease, elevated plasma cholesterol levels, presented with both effort-induced and rest angina and were associated with ST-segment depression suggestive of myocardial ischaemia.

The short and long-term clinical prognosis of CSX has been shown overall to be excellent. Long-term follow up studies demonstrate that CSX patients experience a low occurrence of major cardiovascular events (cardiac death, acute myocardial infarction)(251). Despite this good clinical prognosis, several studies have identified that approximately 20-30% of patients still

continue to experience progressive and recurrent angina symptoms following their initial diagnosis (250, 251). The worsening of symptoms is consistent with worsening of coronary microvascular dysfunction and/or enhanced pain perception (287, 288).

1.7.3 Treatments

Considering the difficulties in diagnosis, it is not surprising that multiple therapies have been described. Kaski et al, (252) proposed four treatment approaches, which include the use of:

i) Anti-ischaemic agents;

ii) Analgesic therapy;

iii) Hormone replacement therapy; and

iv) Psychological intervention.

These therapies should be tailored to the patient's symptoms, psychosocial state and co-existing cardiovascular risk factors.

Treatment with anti-ischaemic agents generally involves administration of conventional anti-anginals including nitrates, calcium-channel antagonists, and beta-blockers. Although nitrate therapy is extremely beneficial for the management of large vessel coronary disease, it is effective in only 40-50% in patients with typical chest pain and normal coronary angiograms (250). Calcium-channel antagonists seem to have mixed benefits, as some studies have shown that administration of these agents reduces nitroglycerine consumption, chest pain frequency and improves the exercise capacity of CSX patients (253), whereas some show no reduction in chest pain frequency

following treatment with verapamil (254). Beta-blockers such as atenolol and propanolol have also been shown in a number of randomised, placebo-controlled trials, to improve angina, ST-segment depression during continuous ECG monitoring, exercise-induced ST-segment changes and left ventricular diastolic function in CSX patients (254, 255). Furthermore, treatment with proponalol has been shown to significantly reduced ischaemic burden and improve ST-segment depression in comparison to verapamil treatment (254).

Unconventional anti-anginal agents such as ACE-inhibitors and alpha-blockers have also been assessed in this condition. In a randomised, placebo-controlled trial in patients with CSX and microvascular angina, the ACE inhibitor enalapril significantly improved total exercise duration and time to 1mm of ST-segment depression compared to placebo therapy (256). Similarly, cilazapril has also improved exercise-induced ST-changes and exercise capacity in CSX patients (257).

Given the abundance of evidence surrounding altered pain perception in CSX patients, it has been suggested that analgesic therapy may be useful in treating symptoms. The beneficial effect of imipramine, a drug with analgesic activity, was tested in large, randomised, non-crossover, placebo-controlled trials in patients with chest pain and normal coronary angiograms. Compared to control subjects, imipramine significantly reduced daily chest pain and pain provoked by manipulation of a stimulation catheter in the right ventricular cavity (258). Additionally, when co-prescribed with conventional anti-anginal

therapies, imipramine reduced episodes of chest pain in CSX compared to those on placebo (258).

Due to CSX commonly occurring in peri- and post-menopausal women, oestrogen deficiency has been suggested to play a role in the pathogenesis of this condition (259). As such, acute administration of transdermal oestrogen has shown to improve endothelium-dependent coronary vasomotion (260) and alleviate cardiac anginal symptoms (261). A randomised, double-blind, placebo-controlled trial performed on CSX patients using transdermal oestrogen demonstrated a significant improvement in angina symptoms and ECG parameters following exercise testing (262). A similar study also showed a significant reduction in daily chest pain although no changes in exercise test variables were evident (263). Given the varied results from these studies, further investigation is required to establish a clear beneficial therapeutic role of hormone replacement therapy in female CSX patients.

Psychological abnormalities also contribute to chronic chest pain and disability in patients with chest pain and normal coronary arteries. Appropriate intervention should thus be considered as studies have identified that chest pain episodes are reduced and improvements in autonomic symptoms are evident following structured cognitive behavioural therapy (264, 265). Others have similarly demonstrated significant reductions in frequency and severity of chest pain using a group cognitive behavioural approach (266), as well as reductions in psychological morbidity and hyperventilation in CSX patients (267).

1.7.4 Summary

The large body of evidence described above suggests that the pathophysiological mechanisms responsible for CSX are multifactorial. The heterogeneous nature of the CSX supports the notion that different pathophysiological factors may play a role in different variants of the condition. Appropriate therapeutic management of CSX patients is challenging given the heterogeneous nature and good prognostic outcomes. The clinical focus of CSX therapeutic management involves focusing on treating chest pain symptoms and improving quality of life. Such a task has its inherent problems as an array of pathogenic mechanisms may be responsible and can vary between individuals. As such, successful implementation of an effective therapy should therefore be tailored based on an individual basis and exclude extra-cardiac causes of chest pain.

1.8. Coronary Slow Flow Phenomenon

1.8.1 Pathophysiological Mechanisms

Identifying the pathophysiological mechanisms responsible for the CSFP has proven to be a difficult task. Several studies have attempted to characterise the pathogenic mechanisms contributing to microvascular dysfunction in the CSFP with conflicting results. Potential mechanisms that have been suggested include endothelial dysfunction, autonomic and platelet dysfunction, inflammation and oxidative stress.

Endothelial dysfunction studies conducted in CSFP patients have focused upon investigating impaired endothelial NO responses and the role of vasoconstrictors. Several studies have demonstrated impairments in NO-dependent flow-mediated vasodilatation and plasma NO bioavailability following angiography (268), reduced plasma NO concentration after exercise (269), increased ADMA levels (270, 271) and polymorphisms in eNOS (272) in CSFP patients. In contrast, microvascular vasodilator function studies have found that endothelium-dependent vasodilator and flow-mediated dilation responses are intact (273, 274) and that coronary sinus NO levels are normal (275).

The potential role of vasoconstrictors in the pathophysiology of the CSFP has also warranted investigation. In particular, ET-1 may play an important role in CSFP pathogenesis, as intracoronary infusion in both canines (276) and

rabbits (277) mimics the angiographic features of CSFP. In a human in-vivo model, intravenous ET-1 infusion in healthy individuals has been shown to decrease coronary oxygen sinus rates to similar levels observed in CSFP patients (278). As such, increased plasma ET-1 levels both at baseline (279) and following rapid right atrial pacing (275) have been demonstrated in this cohort. Additionally, studies have identified polymorphisms in the gene encoding ACE in CSFP patients thereby implicating its role in the condition (280).

Autonomic and platelet dysfunction has also been implicated in the CSFP. Heart-rate variability studies have suggested that CSFP patients have augmented sympathetic neural influences due to depressed heart-rate variability domains (279) and increased plasma levels of adrenaline and noradrenaline (281). Platelet investigations undertaken have identified increased platelet numbers (282), platelet volume (283) and aggregation potential (284) in this group of patients. The relevant importance of autonomic and platelet dysfunction in CSFP pathology is yet to be elucidated.

Inflammation and oxidative stress markers have also somewhat been shown to be associated with the CSFP pathogenesis. Increased plasma levels of soluble adhesion molecules ICAM-1, VCAM-1 and E-selectin (285), as well as high sensitivity CRP plasma levels have been documented (286, 287). In contrast, studies have also failed to demonstrate an inflammatory role in CSFP patients as demonstrated by unchanged hsCRP (271, 288) and MPO (289) levels compared to control subjects. Assessment of oxidants associated with the

CSFP have shown that this group of patients have increased serum MDA and superoxide dismutase (289) as well as elevated homocysteine (290-292) levels compared to those presenting with chest pain and normal coronary flow. Furthermore, the activities of the lipoprotein bound anti-oxidant enzyme, paraoxonase, have been found to be significantly lower in CSFP patients and be independently associated with mean TIMI frame counts (302).

1.8.2 Clinical Features

Few studies have attempted to create an accurate and distinct clinical profile for the condition. Initial investigations conducted by Beltrame and colleagues identified that although CSFP patients exhibit standard coronary risk factors such as hypertension, diabetes and hypercholesterolaemia, they tend to be younger, male and current smokers compared to patients with chest pain and normal angiograms (222). Another distinctive clinical feature was that these patients had recent onset angina and presented to the hospital emergency room mimicking an ACS that warranted urgent angiography. Furthermore, the anginal pain experienced by CSFP patients is of sufficient concern given that 33% of these patients re-present to the emergency department with prolonged pain and 20% are admitted to the coronary care unit for intravenous nitrates (222). The association between ACS and the CSFP has also been documented by others, which have shown the majority of patients present with unstable angina and/or myocardial infarction in the absence of coronary spasm (293, 294).

The clinical features of the CSFP differ to other coronary microvascular disorders such as CSX. Whereas CSX patients present with stable exertional angina, CSFP patients present with rest angina mimicking an ACS. Other clinical features of the CSFP have been characterised following studies attempting to identify the presence of coronary microvascular dysfunction. A range of ECG, scintigraphic and biochemical studies performed to identify the presence of myocardial ischaemia have produced conflicting results. Ischaemic ST changes during exercise stress testing have shown to occur in less than 50% of CSFP patients (222), despite some studies documenting 70% (224, 295). Only a minority of CSFP patients undergoing continuous ST-segment monitoring during an ACS demonstrate ischaemic ST-segment (24%) changes, whereas 86% display T-wave changes (296). Biochemical lactate production (gold standard marker of myocardial ischaemia) during atrial pacing has also failed to identify evidence of ischaemia in the majority of CSFP patients (297, 298).

Few epidemiological studies have been conducted on CSFP patients from its initial description (219, 222). Beltrame et al, identified that the incidence of the CSFP is approximately 1% of diagnostic angiograms (222), whereas others have reported a CSFP incidence varying from 3% (299) up to 7% of angiograms (220). Similar to CSX patients, the long-term prognosis of CSFP is good in terms of adverse cardiovascular events. Despite this good prognosis, follow-up studies in CSFP patients have shown that the angiographic phenomenon is persistent for up to 10 years following initial diagnosis, 84% of patients continue to experience recurrent chest pain, 33%

re-present to hospital emergency and 19% require re-admission to hospital (222). It has also been reported that CSFP patients are more likely to experience recurrent chest pain than those with chest pain and normal angiograms (300).

1.8.3 Treatments

Finding an effective treatment for CSFP symptoms has become an important area of research. Given that the majority of currently available conventional therapies are ineffective in treating symptoms, there is a need to explore alternate therapeutic approaches. Not only will a suitable therapy provide supporting evidence that the CSFP is a unique and distinct clinical condition, but it will also potentially provide insights into the mechanisms behind this disorder.

Few studies have been successful in identifying effective therapies for CSFP patients from conventional and novel anti-anginal agents. The effectiveness of the novel therapeutic agent, nebivolol (B_1 receptor blocker), has been shown in observational, open-label studies to improve flow-mediated dilation (301), mean exercise duration (302), oxidative stress markers (303) and left ventricular diastolic indices (304). Kurtoglu and colleagues demonstrated in CSFP patients that improvements in angiographic flow and decreases in chest pain frequency were evident following daily oral dipyridamole therapy (305). Other anti-anginal agents such as Nicorandil (K^+ channel opener) (306) and Trimetazidine (fatty-acid metabolism inhibitor) (307) have also shown to have some benefit in improving contrast flow, heart-rate variability, exercise

stress test performance and endothelin/nitric oxide levels in CSFP patients. The ACE inhibitor, perindopril, has been shown to improve coronary haemodynamic parameters by reducing heart rate and prolonged QT intervals on ECG (308). Similarly, statin therapy has been shown to improve myocardial perfusion abnormalities (309) and improve coronary flow reserve parameters in CSFP patients (310).

The therapeutic benefits of calcium-channel blockers in treating CSFP patients have also been well investigated. Beltrame and colleagues performed a randomised, placebo-controlled study investigating the angiographic and clinical benefits of dual calcium L- and T-channel blocker, mibefradil, in CSFP patients (311). This study demonstrated that mibefradil reduced total angina frequency by 56%, decreased prolonged angina frequency by 74% and sublingual nitrate consumption by 59% and improved quality of life in CSFP patients compared to treatment with placebo. The unique and potent effects of mibefradil have not been demonstrated with other conventional calcium-channel blockers such as verapamil and this is thought to be due to its effects upon both L- and T-type calcium channels in the microvasculature. As such, in-vitro studies have been conducted to compare the effectiveness of single L-channel blockers (verapamil and nifedipine) with mibefradil and another combined L- and T-type channel blocker, efondipine, in inhibiting ET-1 constrictor responses in both large and small vessels. In this study, it was demonstrated that despite all four calcium channel blockers effectively inhibiting ET-1 mediated constrictor responses in rat conduit arteries, only mibefradil and efondipine produced a greater inhibitory response in

mesenteric microvessels (312). The greater effectiveness of combined L- and T-type channel blockade was also demonstrated in human subcutaneous microvessels with an associated greater abundance of T-channels in the microvasculature identified.

1.8.4 Summary

Only a few studies have been successful in improving clinical outcomes in CSFP patients through use of a therapeutic agent. Two studies thus far have employed randomised, placebo-controlled studies (Mibefradil and Trimetazidine) and demonstrated promising results. Although Mibefradil was effective in ameliorating symptoms, it has been withdrawn from therapeutic availability due to extensive interactions with existing medications. Thus, the quest for an effective therapy for treating angina symptoms in CSFP remains incomplete. Therapeutic studies identifying effective treatments for the recurrent chest pain therefore warrant further investigation.

1.9. Thesis Objectives

The overall aim of this thesis is to investigate the clinical and biological factors contributing to the pathogenesis of the CSFP. Additionally, it will aim to identify an effective treatment for resolution of symptoms in this unique patient cohort. Specific parameters that have been focused upon include:

a) Identification of pathophysiological mechanisms contributing to the ACS presentation;

b) Examination of the role of endothelial dysfunction, inflammation and oxidative stress during the chronic phase of the condition; and

c) Determining the efficacy of a non-selective ET-1 blocker in ameliorating symptoms.

The specific hypotheses for the proposed projects include:

a) Circulating proteins mediate the ACS presentation in the CSFP;
b) Endothelial activation contributes to the persistence of the CSFP;
c) Anginal symptoms in CSFP patients are resolved following intervention with ET-1 receptor blockade.

Detailed synopses of all projects undertaken are outlined below.

Chapter 2

Potential mechanisms of the acute coronary syndrome presentation in patients with the coronary slow flow phenomenon – Insight from a plasma proteomic approach.

As discussed above, CSFP patients frequently present with an ACS although the pathophysiological mechanism/s responsible are unknown. Due to the

nature and frequency of CSFP patients presenting to the coronary care unit, it is logistically difficult to capture patients and perform analysis of suspect biomarkers. The aim of this study is to identify potential mechanisms for the ACS presentation associated with the CSFP using a plasma proteomic profiling approach. Utilising this technique allows the systematic screening of potential proteins that may be implicated in the ACS syndrome presentation.

Chapter 3

Endothelial function, oxidative stress and inflammatory studies in chronic Coronary Slow Flow Phenomenon patients.

The mechanisms responsible for the microvascular dysfunction in the CSFP are unknown. Endothelial dysfunction, inflammation and oxidative stress have been implicated in the pathology of many cardiovascular disorders. Although few studies have attempted to identify potential mechanisms, many of these investigations have been performed during the acute phase of CSFP presentation shortly following angiography. Only a small amount of studies have been performed on CSFP patients during a symptomatically quiescent state (chronic) and thus it is difficult to apply appropriate long-term treatment strategies when mechanisms of persistent pain are unknown. Thus, the objective of this study is to perform a comprehensive assessment of endothelial function in chronic CSFP patients through investigation of endothelial vasomotor responses and changes in plasma inflammatory and oxidative stress markers.

Chapter 4

The anti-anginal efficacy of Bosentan in the Coronary Slow Flow Phenomenon.

Currently, there is no available effective therapeutic intervention for amelioration of symptoms in CSFP patients. ET-1 has previously been implicated in the pathophysiology of the CSFP in both animal and human investigations. Bosentan is a clinically available therapeutic agent that acts as a potent, non-selective ET-1 receptor antagonist. Thus, the aim of this investigation was to determine whether orally-administered bosentan therapy (250mg/day) reduces angina frequency and severity, prolonged angina episodes (>20min) and sublingual nitrate consumption. Additionally, we aimed to investigate endothelial function and inflammatory/oxidative status changes in this group of patients following bosentan therapy. A randomised, double blind placebo-controlled, crossover study was performed to determine the clinical benefit of this therapeutic.

CHAPTER II

CHAPTER II

Potential mechanisms of the acute coronary syndrome presentation in patients with the Coronary Slow Flow Phenomenon - Insight from a plasma proteomic approach

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This results chapter is reproduced in the exact form as it appears in the manuscript, "Potential mechanisms of the acute coronary syndrome presentation in patients with the Coronary Slow Flow Phenomenon – Insight from a plasma proteomic approach" authored by Victoria A. Kopetz, Megan A.S. Penno, Peter Hoffmann, David P. Wilson and John F. Beltrame, and accepted for publication in *International Journal of Cardiology* 2012;156:84-91. (Appendix A)

In keeping with the style of this thesis, the abstract has been removed, the table and figures have been re-numbered, the references incorporated into the thesis' master reference list, the content formatted to English (AUS), the manuscript repaginated and abbreviations utilised following initial definition.

2.1. Introduction

The CSFP is a coronary microvascular disorder, angiographically defined by delayed distal vessel opacification in the absence of obstructive coronary artery disease (313). It is clinically distinct from other coronary microvascular disorders (314) and important to diagnose as effective therapies have been described (315). A distinguishing clinical feature of this disorder is its associated ACS presentation typically observed as the index manifestation (222). Furthermore, over 80% of affected patients experience recurrent rest angina with almost 20% requiring readmission to the coronary care unit for intravenous nitrate therapy. Despite the frequent episodes of rest pain and ACS readmissions, patients seldom experience acute myocardial infarction; however, their symptoms are disabling and the burden to community and health resources significant. The mechanisms responsible for the coronary microvascular dysfunction in patients with the CSFP require further clarification. Biopsy studies have demonstrated structural abnormalities of the microvasculature but clearly there is a dynamic component given the chronic episodes of rest angina and the frequent ACS presentations. Most mechanistic CSFP studies have focused on the chronic phase of the disorder considering the inherent difficulties with studying the acute presentation.

Although it is important to elucidate the underlying mechanisms responsible for the chronic angina, understanding the processes responsible for acute exacerbation are equally important. These acute processes may be

independent of the underlying chronic pathology and therapies directed towards these targets may prevent deterioration. Although theoretically attractive, studying ACS is logistically problematic as new CSFP cases account for 1-4% of angiographic studies and capturing affected patients requires considerable logistic clinical co-ordination. Also, the methodological approach to identifying the acute pathophysiological processes involved is difficult since multiple screening assays would need consideration.

An alternative strategy is to utilise a proteomic profiling approach. Proteomics is an emerging technology that permits investigation of protein profiles of a patient at a particular point in time. By employing a paired sample design, this technology can identify the change in the protein profile associated with a change in clinical status thereby providing insights into protein/enzymatic processes contributing to the clinical outcome. This technology has been further enhanced with the development of 2-D DIGE methods that reduce gel-to-gel variation by using a fluorescently labelled internal standard common to all samples on the gels. This internal standard provides a normalising variable constant that is comparable within and between gels thus reducing assay variability and sample size requirements (316). With this improvement, 2-D gel based proteomics is a sensitive approach to identify potential mechanisms for uncommon conditions. The objective of this study was to compare the plasma proteome of CSFP patients during an acute coronary syndrome presentation with a control sample during the chronic phase (symptomatically quiescent) of the disorder.

2.2. Methods

We used a prospective, controlled, paired-sample design with proteomic profiling by 2-D DIGE technology to compare the plasma proteome of six CSFP patients during an ACS admission with a sample taken one month later during the chronic phase of the disorder. Supporting clinical data, biochemical analyses and validating western blot assessments were also conducted on these 6 patients as well as an additional 3 patients.

2.2.1 Patient Recruitment and Blood Collection

CSFP patients admitted to the coronary care unit with an ACS were invited to participate in the study, which was approved by the Central Northern Adelaide Health Service and University of Adelaide Ethics Human Research Committees. The CSFP was angiographically defined as: [a] requiring ≥3 beats to opacify pre-specified branch points in the distal vasculature of any of the three major epicardial coronary arteries (TIMI-2 flow); and [b] the absence of obstructive CAD (i.e. <40% in any epicardial coronary artery). Both recently diagnosed and established CSFP patients were recruited, with diagnosis confirmed by two independent angiographic observers. A diagnosis of ACS was made on the basis of: (a) prolonged rest angina (> 20 minutes); associated with (b) ST/T wave changes; and (c) requiring admission to the coronary care unit (CCU) for intravenous nitrates. Exclusion criteria included: (a) coronary ectasia; (b) coronary intervention associated slow flow (no-reflow); (c) myocardial infarction, surgery or an infective illness within the

past six months.

Patients underwent venesection within three days of an ACS admission, following an overnight fast and cessation of intravenous nitrates and heparin infusions for at least 12 hours. Baseline clinical details including recent angina frequency and current medications were recorded. Plasma samples for hsCRP, CK and TnT levels were determined using validated immunoturbidmetric, colourmetric (Olympus Analyser, Japan) and immunoassays (Roche Diagnostics, Australia), respectively. Blood for proteomic analysis was collected in specialised tubes containing K_2 ethylenediaminetetraacetic acid (EDTA) and a propriety protein stabilizer (BD P100 Blood Collection System). These samples were then centrifuged at 2500g for 5 minutes at room temperature (23°C) and the plasma frozen at –80°C. One month following the ACS admission, patients returned to have clinical details recorded and a repeat venesection.

2.2.2 Proteomic Analysis

Identification of significant changes in specific proteins within the plasma proteome between the two samples involved: 1) depletion of the abundant plasma proteins; 2) separation of the sample proteins by 2D-DIGE; 3) identification of protein spots with >1.5 fold change between samples; 4) mass spectrometry based identification of proteins; and 5) western blotting validation.

2.2.2.1 Depletion of abundant plasma proteins

A Multiple Immunoaffinity Removal System (MARS 4.6 x 100mm, Agilent Technologies) column coupled with high performance liquid chromatograph [HPLC] (Hewlett Packard 1090) was used to deplete seven abundant plasma proteins (albumin, immunoglobulin G and A, transferrin, haptoglobin, alpha 1 anti-trypsin and fibrinogen). Depleted serum was de-lipidated and precipitated using ice cold acetone, washed twice with ice-cold 80% acetone and proteins re-solubilised with TUC4% buffer (2M thiourea, 7M urea, 30mM Tris and 4% CHAPS [3-[(3 Cholamidopropyldimethylammonio)-1-propanesulfonate]. Samples were desalted using continuous buffer exchange with TUC4% using Vivaspin 500 centrifugal devices (10kDa molecular weight cut-off, GE Healthcare) until conductivity was <300μS as measured by a Horiba Twin Cond conductivity meter (model B-173, Horiba). Protein concentrations were determined using the EZQ protein quantitation kit assay (Invitrogen).

2.2.2.2 Fluorescence labelling

Working solutions of DIGE Fluor minimal Cy2, Cy3 and Cy5 CyDyes (GE Healthcare) were prepared in anhydrous *N,N*-Dimethylformamide (Sigma) at 200 pmol/μL. Depleted plasma from each patient (acute & chronic) containing 100 μg of protein were labeled with 200 pmol of either Cy3 or Cy5 flurochromes. Labelling of samples with specific dyes was alternated between patients. An internal standard was prepared by pooling 50 μg of protein from each sample, which was then labelled with Cy2 using a 100 μg protein/200 pmol ratio. All labelling reactions were conducted on ice in darkness for 30 min and stopped with the addition of 1 mM lysine.

2.2.2.3 Two-dimensional electrophoresis

Patient samples labelled with Cy3 and Cy5 were combined with the Cy2 labelled internal standard and 65mM of the reductant dithiothreitol [DTT, Sigma], 0.5% of 3-11 pH non-linear carrier ampholytes (GE Healthcare) and iso-electric focusing (IEF) buffer (7M urea, 2M thiourea, 30mM Tris, 2% CHAPS, 0.5% ampholytes, 1.2% Destreak (GE Healthcare), trace bromophenol blue) to a final volume of 150µl. Prior to first dimensional isoelectric focusing, 6 x 24cm immobilised pH 3-11 gradient (IPG) strips (GE Healthcare) were re-hydrated overnight with 450µl of IEF buffer. Samples were introduced to the IPG strips via cup loading and IEF was continued for 70000 total volt hours. Focused IPG strips were equilibrated, placed into 12.5% 2D SDS-Gel DALT NF pre-cast gels (Gel Company), overlayed with 1% low melting agarose and subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using an Ettan DALT12 chamber (GE Healthcare). Gels were run at 25°C using a three-step protocol: 5 mA/gel for 1 hour, 10 mA/gel for 1 hour and 30 mA/gel for 14 hours.

2.2.2.4 Gel imaging

Gels were scanned using an Ettan DIGE Imager (GE Healthcare) at exposure times of 0.4 (Cy2), 0.07 (Cy3) and 0.1 (Cy5) seconds. Individual gel images were analysed using Differential In-gel Analysis and Biological Variation Analysis components of DeCyder V 6.5 software (GE Healthcare) for protein spot quantitation and paired comparison. Spot intensities were calculated, normalised and standardised to the Cy2 internal standard that was included on each gel. Paired t-tests were applied to standardised spot abundance values

and matched spots present on at least four out of the six gels demonstrating a ±1.5 fold change ($p<0.05$) in abundance were considered significant.

2.2.2.5 Power analysis

Based on previous two-dimensional gel electrophoresis studies, a power analysis was performed to determine the number of patients required to detect a significant difference in protein expression [6]. Assuming a biological variation of 20% between patients, then to detect a 1.5 fold change in protein expression, six patients would be required to achieve a power of 80% at the α=0.05 level.

2.2.2.6 Liquid chromatography mass spectrometry

Spots of interest were excised from the three analytical gels using an Ettan Spot Cutting Robot (GE Healthcare), digested overnight with trypsin (Promega, 100 mM in 5 mM NH_4HCO_3/10% acetonitrile [ACN]) and extracted in 50% ACN/1% formic acid [FA]. Solvent was removed from peptide extracts by vacuum centrifugation and peptides were re-constituted in 3%ACN/1%FA. Five microlitres of each sample was subjected to liquid chromatography using an 1100 HPLC system equipped with a 40 nL C-18 analytical column (Agilent Protein Chip Column) which directly interfaced a HCT Ultra 3D-Ion-Trap mass spectrometer (Bruker Daltonics) operating in positive ion mode. The column was equilibrated with 4% ACN/0.1%FA at a flow rate of 0.5μL/min and samples were eluted with an ACN gradient 4%-31% over 32 minutes. Ionisable peptides (with a mass-to-charge ratio between 300 and 1200) were trapped and the two most intense ions underwent

collision-induced association fragmentation. Mass spectra were subjected to peak detection and de-convolution using DataAnalysis (Version 3.4, Bruker Daltonics) and submitted to an in-house Mascot database-search engine (version 2.2 Matrix Science) for protein identification using the SwissProt 56.4 database. The specifications were: taxonomy = Mammalia; enzyme = trypsin; fixed modifications = carbamidomethylation of cysteine; variable modifications = oxidation of methionine; peptide mass tolerance = ±0.3 Da; fragment mass tolerance = ±0.4 Da, missed cleavages = 1; and peptide charge = 1+, 2+ and 3+. Protein identifications were made on the basis of having at least two matching peptides with ion scores above the specified threshold.

2.2.3 Western Blotting Validation

Following success of the 2D-DIGE screens for protein abundance changes, traditional western blot analysis was employed to compare the sensitivity of the two approaches. During this subsequent validation study we were able to recruit an additional 3 CSFP patients thereby establishing a total recruitment of 9 patients with an ACS presentation. Neat plasma samples were diluted 1/600 with sample buffer (2%SDS, 50mM Tris/HCl (pH 6.8) 30% Glycerol and 10mM DTT), subjected to SDS-PAGE using 10% polyacrylamide mini gels (BioRad) and subsequently electrophoretically transferred onto nitrocellulose membranes (0.22 μm). Following transfer, the nitrocellulose membrane was stained with Ponceau-S and scanned to enable normalisation of protein load to 67kDa serum albumin. The membrane was blocked using 5% non-fat dried milk powder in Tris-buffered saline (20mM Tris, 150mM NaCl pH 7.5) containing 0.05% Tween 20 (TBS-T). Antibody solutions were

made containing 1% non-fat dried milk powder in TBS-T. For detection of ∝-1-antitrypsin, a three-step antibody binding protocol was performed that involved sequential hour incubations with primary chicken anti-AAT antibody (1:1000 dilution, Chemicon, Millipore), secondary rabbit anti-chicken IgY (1:10000 dilution, Upstate) and mouse anti-rabbit IgY conjugated to horseradish-peroxidase (HRP) (1:10000 dilution, SantaCruz Biotechnology). For detection of paraoxonase-1, membranes were probed with primary antibody α-PON1 (1:1000 dilution) and secondary antibody bovine anti-goat HRP conjugate (1:10000 dilution, SantaCruz Biotechnology). Protein abundance was visualised using ECL western blotting detection reagent (Amersham, GE Healthcare) and autographic film. Membranes were scanned using an imaging densitometer (BioRad GS-710), normalised to serum albumin to account for any differences in protein load and quantified using QuantityOne software (BioRad).

2.3 Results

2.3.1 Patient Characteristics

Table 2.1 (page 94) summarises the clinical characteristics of the nine consecutive CSFP patients recruited. The patients were aged between 38 – 69 years [mean = 55 ± 10 years (SD)] with five being male. Risk factors were similar between patients with all having at least one conventional risk factor.

Patients were generally prescribed aspirin, calcium-channel blockers, nitrates, statins and angiotensin-converting-enzyme inhibitors (ACE-I) both in-hospital and as outpatients. Two patients had ceased taking calcium channel blockers and statins at the chronic phase. Nitrate therapy was stopped for another four patients with one initiating an ACE-I and another commencing a statin during the chronic phase. One patient ceased taking aspirin during the chronic phase and for two of the patients, medications did not change between the phases. Hence, therapies remained relatively similar between the acute and chronic phase samples. There was no evidence of myocardial injury in either the acute or chronic phase of the disorder as assessed by plasma cardiac markers. In contrast, hsCRP levels were significantly elevated in the ACS (14.9 ± 3.9mg/L) compared with the chronic phase (4.23 ± 1.37mg/L; $p = 0.05$).

2.3.2 Proteomic Analysis

To minimise technical variance when comparing the acute and chronic plasma proteomes from individual patients, both samples were evaluated on the same gel. A representative gel image (Figure 2.1, page 98) reveals that approximately 1240 protein spots were detected. Protein spots were matched

across the six gels that represented the plasma proteome of the six patients, however, only 17 protein spots showed a ≥1.5 fold change in abundance between the acute and chronic phase samples. Bio-informatic analysis of these spots successfully identified 16 peptides that corresponded with known proteins in the database. Proteins identified with ≥2 unique peptides were considered to have sufficient coverage of the amino acid sequence to provide definitive identification and thus were selected for further analysis (Table 2.2, page 96). The proteins showing increased expression in the acute relative to the chronic samples, included: (a) inflammatory proteins ∝-1-antitrypsin (AAT), ∝-1-antichymotrypsin (ACT), corticosteroid binding globulin (CBG) and leucine-rich alpha-2-glycoprotein (LRα2GP) and (b) the oxidative stress-related protein paraoxonase-1 (PON-1) (Figure 2.2, page 100). In contrast, there was a decrease of the plasma protein fibronectin (FN) evident during the acute phase. Four of the proteins were found to have multiple identifications on adjacent spots at the same molecular weight suggestive of a post-translational modification that may have included oxidation. These proteins included FN (5 spots), AAT (2 spots) and ACT (2 spots).

2.3.3 Western blot analysis

In order to account for differences in loading, a Ponceau-S stain of endogenous serum albumin in each sample was used as in internal loading control (Optical density [OD] of antibody detected protein/OD albumin). Quantitative western blot analysis demonstrated a significant increase in abundance of AAT during the acute phase (mean 1.33 ± 0.17 [SEM] Optical Density Ratio [Acute/Chronic], p=0.05) compared to the chronic phase

(Figure 2.3A & 2.3B, page 102). The mean change in optical density between phases for AAT was 0.61 ± 0.21 [SEM] acute-chronic OD. PON-1 abundance levels demonstrated a more heterogeneous effect, as differences in optical density ratios did not reach statistical significance (mean 1.47 ± 0.36 Optical Density Ratio [Acute/Chronic], $p = 0.19$)(Figure 2.4A & 2.4B, page 104). The mean change in optical density between phases for PON-1 was 0.1 ± 0.03 [SEM] (acute-chronic) OD.

Table 2.1. Clinical Characteristics of the Study Patients.

	1	2	3	4	5	6	7	8	9
Clinical Profile									
-Age (years)	38	53	49	69	61	66	55	45	60
-Gender	M	F	M	M	F	M	F	M	F
-BMI	42.3	36.3	37.2	25.5	26.4	30.7	28.6	27.3	26.4
-Risk Factors	HT, DM, HC, Cigs	DM, HC, Cigs	HC	HT	HT, HC	Ex-cigs	HC	Cigs	HT, HC
CSFP Features									
-Diagnosis	De-novo	De-novo	Established	Established	De-novo	De-novo	Established	Established	Established
-Vessels involved	2	3	3	3	1	2	3	1	1
-No. readmissions	0	0	3	1	0	0	0	0	0
Acute Presentation									
-Angina frequency	<1/week	10-20/week	10-20/week	3/week	<1/week	<1/week	10-20/week	2-3/day	<1/week
-Angina duration	≥24 hours	1-2 hrs	2-4 hrs	≥24 hours	≥24 hours	≥24 hours	≥24 hours	5-6 hrs	1-2 hrs
-Admission ECG	Inferior nsST	NS ST change	Inferior ↑ST	Lateral nsST	NAD	Anteroseptal ↓T waves	NAD	NAD	NAD
-Cardiac Meds	Stat, CCB ACE-I, ISDN	ASA, Stat ACE-I, ISDN	ASA, Stat CCB, ISDN	ASA, Stat, CCB, ISDN	ASA, Stat, CCB, ACE-I, ISDN	ASA, Stat, CCB, ISDN	ASA, CCB	ASA, CCB	ASA, Stat, CCB, ISDN
-Troponin T (μg/L)	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02
-CK (U/L)	124	46	56	152	97	87	48	99	76
--hsCRP (mg/L)	18	12	21	2	42	17	8	5	8
-WCC (x10*9/L)	5.1	9.1	5.3	6.7	8.3	9.3	6.3	7.2	5.9
1 month follow-up									
-Angina frequency*	0	1-2-/week	3/week	3/week	0	0	10/week	1-2/week	0
-Angina duration	0	2-3 min	10-15 min	2-3 min	0	0	2-30 min	< 1 min	0
-Cardiac Meds	ACE-I, ISDN	ASA, Stat, ACE-I	ASA, Stat, CCB, ACE-I,	ASA, Stat CCB, ISDN	Stat, CCB, ACE-I	ASA, ISDN,	Stat, CCB	ASA, CCB	ASA, Stat, CCB
-Trop T (μg/L)	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02
-CK (U/L)	118	55	109	407	177	87	72	107	88
-hsCRP (mg/L)	3.8	3.3	3.4	1.7	1.7	1.8	3.82	15^{Δ}	3.6

Table Legend 2.1

Clinical Characteristics of CSFP patients. *Angina Frequency assessed as episodes/week over the preceding 4 weeks. Δ Patient developed an acute infection in preceding week. nsST= non-significant ST elevation, NAD= No Abnormality Detected. Risk factors: ASA=Aspirin, HT=Hypertension, DM=Diabetes, HC=Hypercholesterolaemia [Total Cholesterol > 5.5mmol/L], Cigs=Current Smoker, Ex-Cigs=Ex-smoker. Medications are denoted as: ACE=Angiotensin Converting Enzyme Inhibitor, Stat=Statin, CCB=Calcium-Channel Blocker, ASA=Aspirin, ISDN=Isosorbide Dinitrate. Results are represented as mean ±

Table 2.2. Summary of proteins identified in the excised spots by mass spectrometry.

Protein	No. Of Unique Peptides	Combined Ion score [Threshold score/cut]	Fold Change ± SD [Acute vs Chronic]	% Sequence coverage MS/MS	Predicted pI	MW [kDa]
fibronectin*	6	224/34	-1.7 ± 0.54	4.1	5.38$^{\Delta}$	266
fibronectin*	13	575/35	-2.23 ± 0.72	10.2	5.38$^{\Delta}$	266
fibronectin*	18	862/35	-2.18 ± 0.79	11.1	5.38$^{\Delta}$	266
fibronectin*	12	541/35	-1.88 ± 0.63	7.6	5.38$^{\Delta}$	266
fibronectin*	23	1077/34	-2.28 ± 1	14.7	5.38$^{\Delta}$	266
∝-1-antichymotrypsin*	4	239/35	1.65 ± 0.13	9.2	5.32$^{\Delta}$	47.8
∝-1-antichymotrypsin*	5	321/34	1.57 ± 0.23	17	5.32$^{\Delta}$	47.8
∝-1-antitrypsin*	7	376/35	2.5 ± 0.34	24	5.37$^{\Delta}$	46.9
∝-1-antitrypsin*	2	153/35	2.26 ± 0.3	11	5.37$^{\Delta}$	46.9
leucine-rich alpha-2-glycoprotein $^{\#}$	5	247/34	1.6 ± 0.13	23	5.66	38.4
serum PON1/arylesterase 1$^{\#}$	3	144/34	1.6 ± 0.13	11	5.08	39.9
corticosteroid binding globulin	4	311/34	1.57 ± 0.23	17	5.64	45.3

Table Legend 2.2

Summary of proteins identified by mass spectrometry [n=6]. pI denotes isoelectric point. MW denotes molecular weight. "*"Signifies that peptides corresponding to protein were identified on adjacent gel spots. "#" Denotes ≥1 proteins were identified from the same gel spot. "Δ" indicates that pI is slightly varied between peptides likely due to post-translational modifications (PTM) or variation in isoforms.

Figure 2.1. A representative 2D-DIGE gel from a CSFP patient.

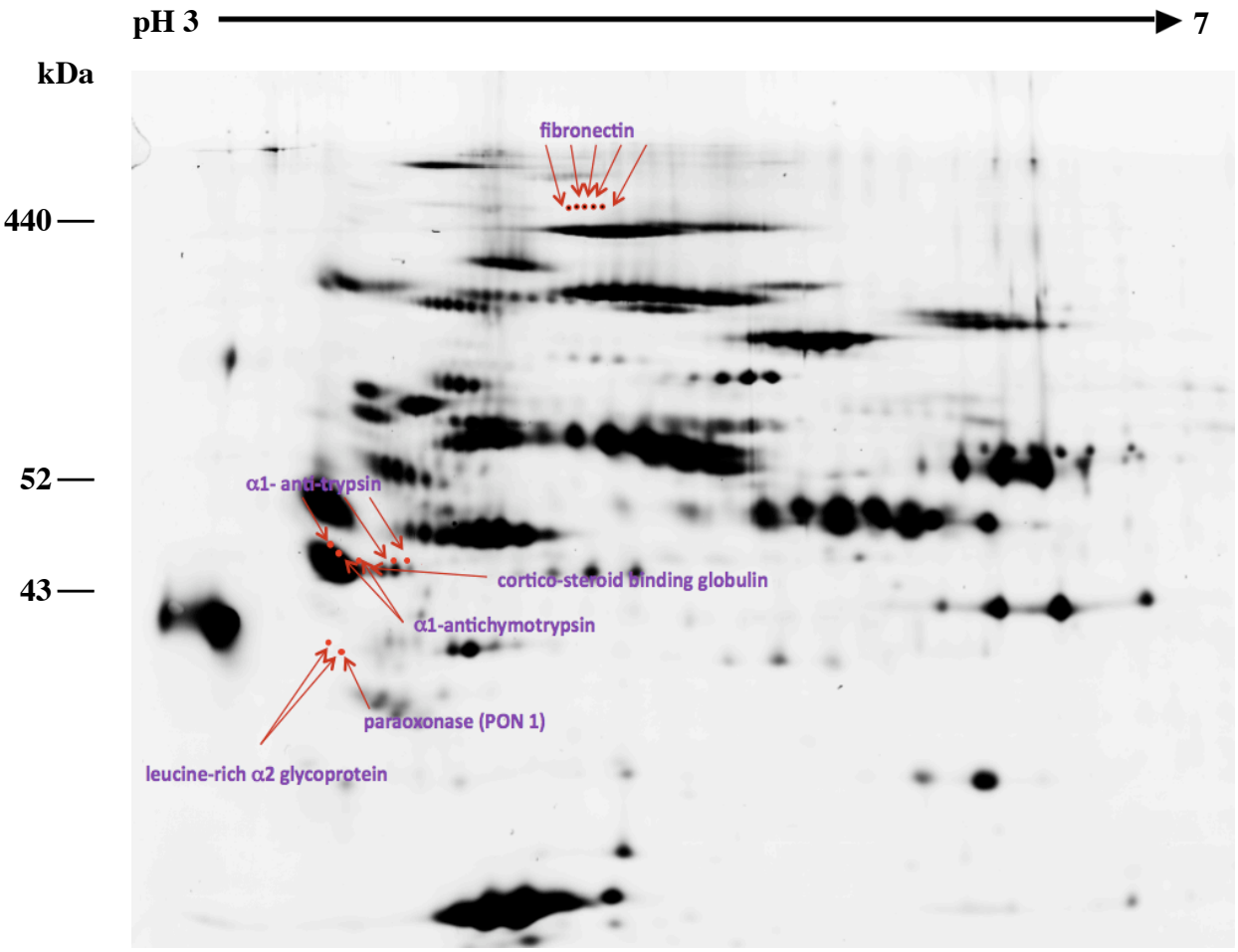


Figure Legend 2.1

Protein spots were separated across a pH range of 3-11 and a molecular weight range of 15-500 kDa. Spots highlighted represent the location of the differentially displayed spots identified between the two phases. Multiple protein identifications of specific proteins were evident on adjacent spots.

Figure 2.2. Standardised abundance values for proteins identified with ≥1.5 fold change between acute and chronic samples in CSFP patients.

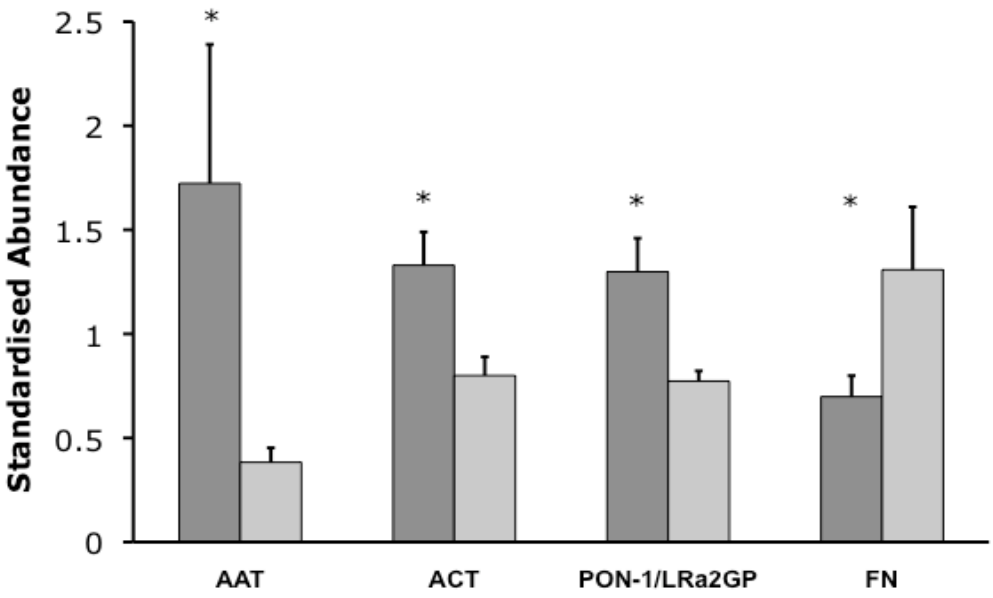
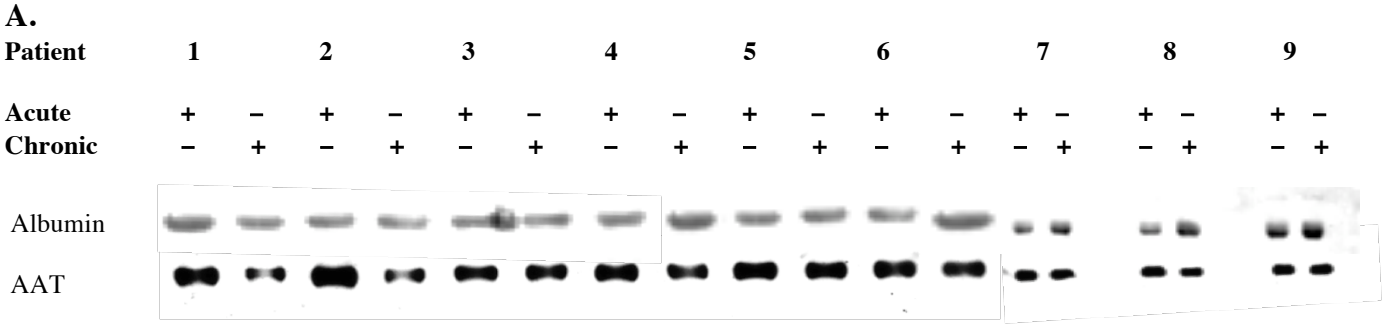


Figure Legend 2.2

2-D DIGE data represents average standardised abundance values obtained from a single protein spot for all patients [n=6] and are presented as mean ± SEM. Values with a "*" are indicative of significant ≥ 1.5 fold changes [p>0.05] in abundance between time points. AAT=∝-1-antitrypsin, ACT=∝-1-antichymotrypsin, PON-1=paraoxonase, LRα2GP=leucine-rich alpha-2-glycoprotein, FN=fibronectin. PON-1/LRα2GP are shown together as peptides were identified from one gel plug. Dark grey boxes represent protein abundance during acute phase and light grey boxes represent protein abundance during chronic phase.

Figure 2.3. AAT abundance as assessed by [A] Qualitative and [B] Quantitative Western Blot Analysis



B.

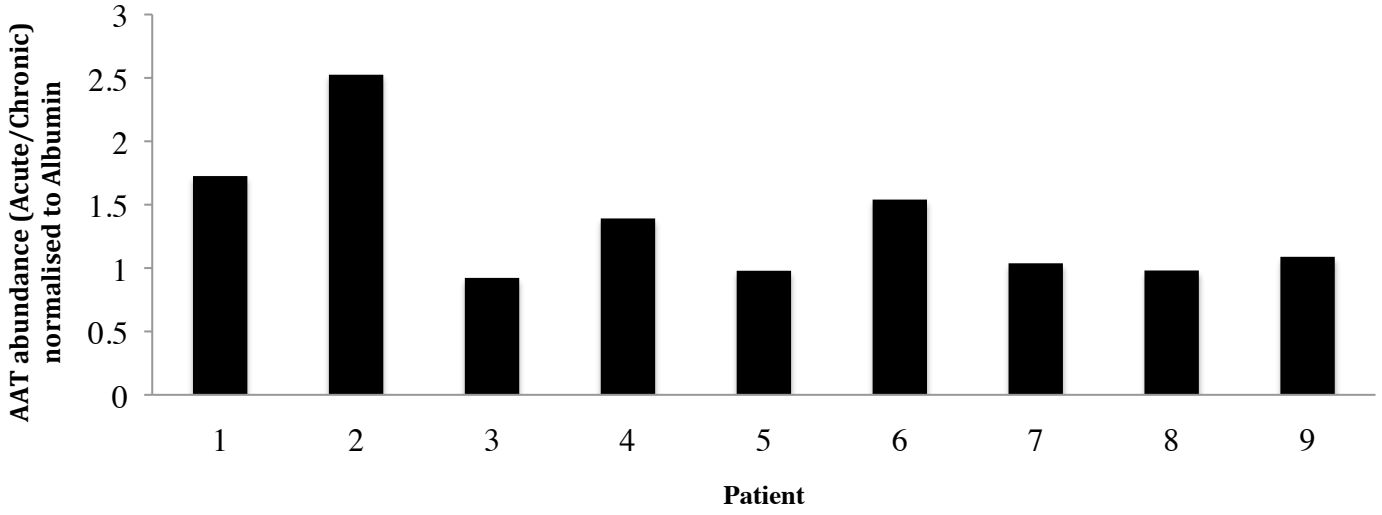
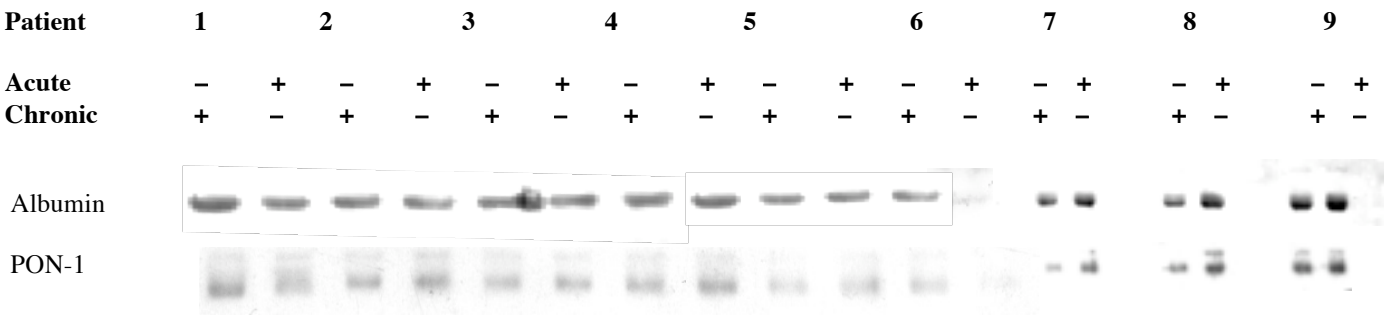


Figure Legend 2.3

Western blot analysis of AAT abundance in neat serum obtained from nine CSFP patients during acute and chronic phases. Optical density of AAT was normalised to albumin to account for slight differences in loading.

Figure 2.4. PON-1 abundance as assessed by [A] Qualitative and [B] Quantitative Western Blot Analysis

A.



B.

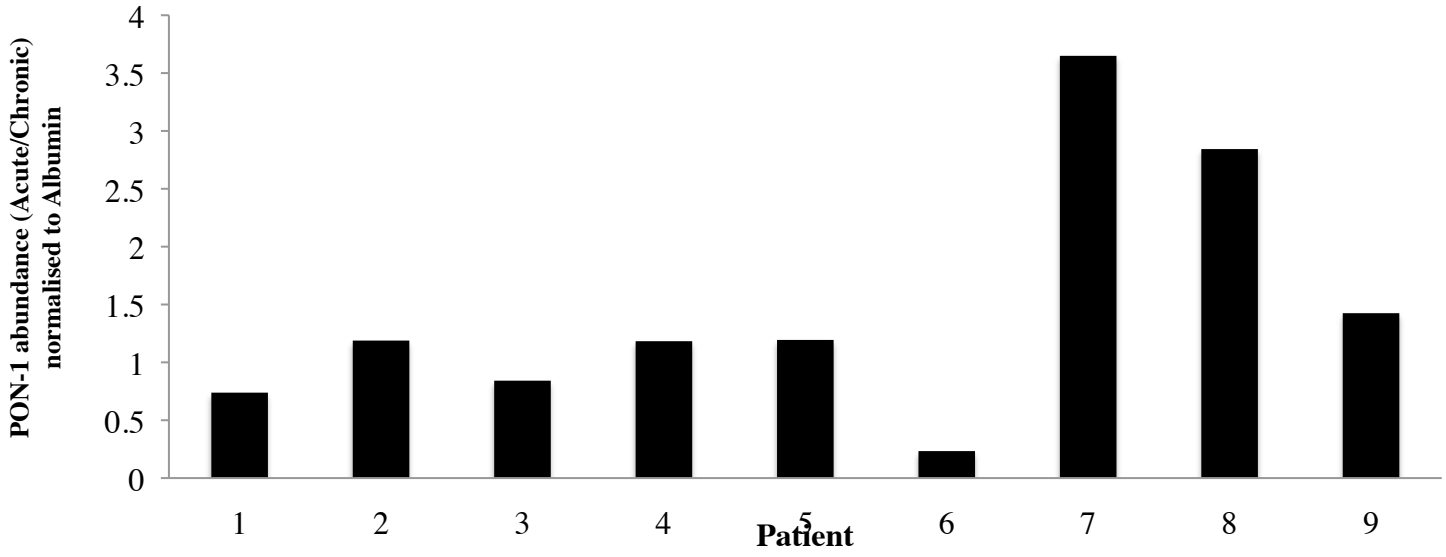


Figure Legend 2.4

Western blot analysis of PON-1 abundance in neat serum obtained from nine CSFP patients during acute and chronic phases.

Optical density of PON-1 was normalised to albumin to account for slight differences in loading.

Figure 2.5 Proposed roles of identified proteins in the acute coronary syndrome presentation of CSFP patients.

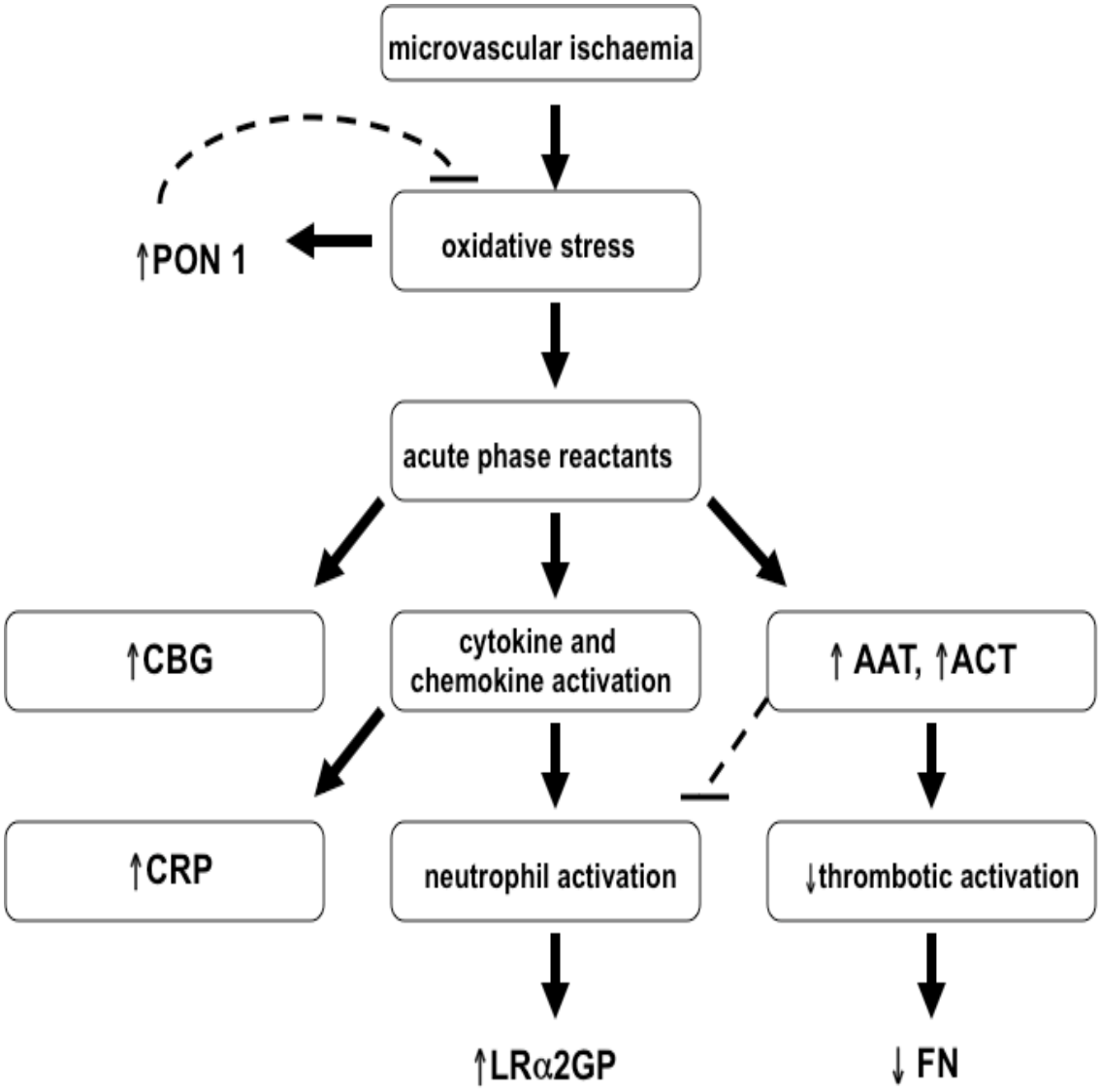


Figure Legend 2.5

Proposed role of identified proteins in the ACS presentation of CSFP patients. Following ischaemia, an oxidative stress environment populated with ROS is created that initiate innate inflammatory acute phase responses and result in the activation of key pro-inflammatory cytokines and chemokines, as well as neutrophil granulocyte differentiation. PON-1 acts as an anti-oxidant to prevent damage caused by ROS, whereas the proteins ACT, AAT, CBG released during the acute phase response act to prevent further cellular damage by inhibiting neutrophil activation. LRα2GP is a marker of neutrophil granulocyte differentiation and thus is higher in abundance. Serine protease inhibitors AAT and ACT also act to prevent activation of thrombotic pathways, which is evident by the decrease in FN level.

2.4. Discussion

Using a plasma profiling proteomic approach, this study has provided insights into the potential mechanisms involved in the ACS presentation of CSFP patients. In particular, the study demonstrates that inflammatory markers (ACT, AAT, CBG and LRα2GP) and oxidative stress-related proteins (PON-1) were up regulated during the ACS admission. Consistent with the proteomic results, there was a significant increase in hsCRP and AAT during the ACS admission. These findings direct future molecular mechanistic studies towards investigating inflammatory and oxidative stress pathways in the pathogenesis of the acute deterioration in these patients.

The quest to identify the patho-physiological mechanisms of the CSFP has thus far uncovered a range of risk factors and morbidities associated with the anomalies seen upon angiography. Clinical investigations have identified that these cohorts of patients have low resting coronary sinus oxygen saturation rates, (297) impaired coronary flow reserve, (317) and increased platelet activation (318) compared with control subjects. Basic molecular studies have also identified a range of elevated cardiovascular disease plasma markers such as homocysteine (319), endothelin-1 (269) and soluble adhesion molecules ICAM-1, VCAM-1 and E-selectin (285) in this group of patients. These findings suggest that CSFP patients show evidence of microvascular dysfunction and conventional cardiovascular risk factors and thus may be at risk for further disease progression.

2.4.1 Potential role of inflammation in the ACS associated with the CSFP

The serine protease inhibitors ACT, AAT and CBG regulate the inflammatory process by inhibiting circulating proteases such as neutrophil elastase. More specifically, ACT inhibits mast cell chymases and angiotensin converting enzyme proteases but stimulates production of interleukin-6, (320) which consequently increases CRP production. AAT inhibits key pro-inflammatory cytokines, namely tumor necrosis factor-α [TNF-α] and IL-1β, as well as stimulating the release of the anti-inflammatory cytokine IL-10 (321). LRα2GP is a neutrophil activation marker that is released following neutrophil granulocyte differentiation. Although the precise physiological function of LRα2GP is unclear, it is known to inhibit cytochrome C (322), a molecule often found to be elevated in plasma following myocardial necrosis. A proposed role of these identified proteins in the ACS presentation of CSFP patients is shown in Figure 2.5, page 106.

Multiple adjacent protein spots on the gel corresponded to AAT, ACT and FN. This observation is indicative of the presence of low molecular weight post-translational modifications (PTM) such as oxidation leading to shifts in isoelectric point. PTM play a pivotal role in governing and initiating numerous biological processes such as activation/inactivation of enzyme activity (oxidation/phosphorylation), regulation of gene expression (methylation) and cell-to-cell recognition (glycosylation) (323). Additionally, specific isoforms of AAT have been evident in both unstable angina and acute myocardial infarction patients relative to healthy controls (324). Thus, it may be inferred that multiple AAT isoforms show a greater specificity towards the acute clinical presentation.

It is important to note that the MARS-7 column was used to deplete the seven most abundant serum proteins, however, in the current study this approach removed ~90% of the most abundant proteins, leaving ~ 10% of albumin and AAT in the "depleted" serum. Despite the removal of as much as 90% of the AAT, we were still able to identify significant differences in AAT abundance between the acute/chronic phases of CSFP.

Inflammation has been implicated in the pathogenesis of the chronic phase of the CSFP with studies demonstrating increased inflammatory markers and adhesion molecules (285). Barutcu et al, (286) described elevated hsCRP in 32 CSFP patients compared with 30 control patients [60±0.58 vs 24±0.1 mg/L, p = 0.03]. In contrast, Yazici et al, were unable to detect a significant difference in CRP levels between 51 CSFP and 44 control patients [7.26+/-4.2 ng/dl vs. 6.43+/-2.8 ng/dl, p>0.05 (288). The variability between these studies may reflect the timing of sample collection in relation to a recent ACS presentation. Also, whether subclinical inflammation is present in the chronic phase and exacerbated in the ACS presentation is open to speculation and requires further investigation. Alternatively, acute inflammation during the ACS phase may be a process independent from the chronic pathophysiology of the CSFP.

Since almost half of the patients had the acute plasma samples taken within 24 hours of coronary angiography (de-novo patients), the possibility that this intervention caused an inflammatory response needs to be considered. However, several previous studies have consistently demonstrated that coronary

angiography per se does not usually alter CRP levels (325-327). Interestingly, Liuzzo et al, (325) demonstrated that coronary angiography did not alter CRP in stable angina patients but did precipitate a further rise in the unstable angina patients who already had an elevated CRP pre-angiography. These investigators speculated that the latter group was in a pro-inflammatory state and thus hypersensitive to an otherwise innocuous stimulus. Whether this has also occurred in the CSFP patients in this study is speculative.

2.4.2 Potential role of oxidative stress in the ACS associated with the CSFP

PON-1 is a potent antioxidant protein that plays an important role in preventing LDL oxidation (328) and thus is associated with the extent of CAD (329). Its other roles include modulating endothelial function (330, 331) and coronary vasomotor reactivity (331). Consistent with these roles, PON-1 activity is reduced among patients with coronary microvascular dysfunction including CSX (332) and the CSFP (333). Furthermore, a PON-1 polymorphism has been described in Japanese microvascular angina patients (334) that result in the synthesis of a defective PON-1.

The above-published findings relate to the chronic phase of these microvascular disorders. Interestingly, using proteomic methodology we have observed increased PON-1 levels during an ACS presentation in CSFP patients. Although this was not confirmed by western blotting, this anomaly may be explained by differences in patient variation and more likely sensitivity between techniques and

sample treatment (un-depleted plasma used for western blotting experiments). It is certainly conceivable that PON-1 is increased in response to an oxidative stress environment during the ACS. It is also noteworthy to mention that in this study we measured the abundance of PON-1 protein whereas previous studies assessed PON-1 activity (332, 333). Thus, there may be a compensatory increase in PON-1 protein to account for the reduced activity.

2.4.3 Fibronectin and the CSFP

During the ACS presentation, FN levels were reduced. FN functions as a pro-thrombotic agent in blood vessels, increasing the stability of adherent platelet aggregates and thus promoting haemostasis (335). Reduced plasma FN levels have been associated with delayed thrombus initiation (336). It is conceivable that the low FN levels contribute to the low incidence of myocardial infarction in the CSFP, despite the prolonged chest pain.

2.4.4 Study Limitations

The current study has identified proteins involved in the acute presentation of CSFP patients. Although it can be concluded that the differential proteins have an association with the acute presentation, it remains to be identified whether they play a causal role in this disorder. Also, whether the conventional, arbitrarily defined 1.5-fold change in abundance denotes a cut-off value for biologically relevant changes is still unclear. Another limitation of the current study is that small proteins and peptides less than 10kDa were not assessed. Accordingly, important peptides such as endothelin-1 were not measured, giving us an

incomplete plasma proteome profile. In addition, the relative contribution of other biological factors [e.g. catecholamines] in the pathophysiology of acute presentation remains unknown.

The day-to-day and month-to-month variability of proteins shown in the chronic phase could possibly reflect *n*-fold differences as part of spontaneous variability. However, previous biochemical investigations on AAT have demonstrated that although this acute phase protein increases concentration during an inflammatory state, it returns to a normal daily unchanged state four-to-five days following an acute event (337). Similarly, changes in plasma PON-1 protein concentrations over a six-week time period have been shown to be minimal following an acute presentation (338), thereby highlighting the specificity of the 2D-DIGE/MS protocols to detect large fold change differences in these proteins and allow for clear clinical distinctions between acute and chronic symptomatic status.

Western blotting analysis demonstrated a significant difference in AAT expression between acute and chronic phases, thereby confirming the results of the 2D-DIGE analysis. In contrast, western blot analysis of PON-1 did not reveal the *n*-fold changes observed in the 2D-DIGE analysis. These differences may be due to enhanced sensitivity of the 2D-DIGE/MS approach or differences in sample treatment (i.e. 2D-DIGE samples were depleted of abundant proteins whereas samples used for western blot experiments were not). Overall, these findings highlight the improved sensitivity of 2D-DIGE/MS techniques over

conventional western blotting in identifying changes in a small subset of biologically heterogeneous patients.

2.4.5 Clinical use of Proteomic Profiling Strategy

In this study we have used state-of-the-art proteomic methodologies in a clinical paired-sample design to identify potential mechanisms of an acute presentation. The use of appropriate sequential paired samples with 2-D DIGE technology provides a useful tool to assess potential mechanisms and permits the use of a small sample size. Consequently, the approach has been ideal for the study of the CSFP where samples of patients with ACS are difficult to obtain.

An important advantage of the proteomic profiling strategy is its non-selective approach thereby providing perspective on the mechanisms involved. An alternative strategy would be a more targeted approach as used in gene or protein array experiments, where suspect culprit proteins are specifically assayed. However, such a selective strategy may result in pathophysiologically important proteins being missed and/or possibly inappropriate confounding proteins being identified. The non-selective strategy utilised herein has resulted in an important discovery in understanding the CSFP, resulting in a change in our research direction with inflammation becoming a more central focus; this may not have arisen if inflammatory proteins were not assessed in a targeted approach. These same principles are equally applicable to other acute cardiovascular processes.

2.5 Conclusion

In conclusion, using contemporary 2D-DIGE proteomic analysis methodologies, this study has implicated an inflammatory/oxidative stress process in the pathogenesis of the ACS presentation associated with the CSFP. More specific studies are required to elucidate the precise mechanisms involved.

CHAPTER III

CHAPTER III

Endothelial function, oxidative stress and inflammatory studies in chronic coronary slow flow phenomenon patients.

This results chapter is reproduced in the exact form as it appears in the manuscript, "Endothelial function, oxidative stress and inflammatory studies in chronic Coronary Slow Flow Phenomenon patients." authored by Victoria Kopetz, Jennifer Kennedy, Tamila Heresztyn, Irene Stafford, Scott Willoughby and John F. Beltrame, and accepted for publication in *Cardiology* 2012;121:197-203

In keeping with the style of this thesis, the abstract has been removed, the table and figures have been re-numbered, the references incorporated into the thesis' master reference list, the content formatted to English (AUS), the manuscript repaginated and abbreviations utilised following initial definition.

3.1. Introduction

The CSFP is an angiographic finding characterised by delayed distal vessel opacification in the presence of near-normal or normal coronary arteries (222). Patients diagnosed with the CSFP frequently experience recurrent episodes of angina at rest that may limit their activities of daily living and thus impair their quality of life. The pathophysiology underlying the CSFP remains largely undefined. Previous myocardial biopsy studies of the CSFP patients have shown structural changes in the microvessels implicating microvascular dysfunction in the pathogenesis of this disorder (221). This has been further supported by coronary haemodynamic studies demonstrating an increased resting coronary microvascular resistance in these patients (297), although the mechanisms responsible for this microvascular dysfunction remain elusive.

Microvascular dysfunction may arise from endothelial and/or vascular smooth muscle cell dysfunction of the microvessels. No studies have focused on isolated vascular smooth muscle cell function but several have evaluated endothelial function in patients with the CSFP. Sezgin et al., demonstrated that flow-mediated vasodilation was impaired in patients with the CSFP and suggested that endothelial dysfunction played an important role in the pathogenesis of this disorder (339). Moreover, this group demonstrated that biomarkers associated with endothelial activation were also elevated including inflammatory proteins (285) and oxidative stress markers (292). In contrast, Shirani et al., (274) reported

that endothelial function was intact in patients with the CSFP and others have found more heterogeneous responses (297). In most of these small studies, comparisons were made with patients who underwent angiography for the evaluation of chest pain and who were subsequently shown to have no obstructive coronary artery disease or the CSFP; hence the absence of a microvascular disorder was not excluded in these 'control patients'. A more appropriate control group would be 'healthy controls' selected from patients who have never experienced chest pain or had a history of cardiac disease.

Considering the heterogeneity in reports assessing endothelial function in the CSFP, this study sought to comprehensively assess endothelial function and markers of endothelial activation in a large group of patients with the CSFP. Specifically, the objective of the study was to compare patients with the CSFP to healthy controls in relation to: (a) endothelial function assessed by applanation tonometry; (b) the endogenous inhibitor of nitric oxide – ADMA; and (c) biomarkers of endothelial activation including inflammatory proteins hsCRP and MPO and oxidative stress markers MDA and homocysteine.

3.2. Methods

This prospective, controlled trial assessed peripheral endothelial function and biomarkers of endothelial activation in patients with established CSFP as compared with age-matched healthy controls. The Human Research Committees of the Central Northern Adelaide Health Service and University of Adelaide approved the study.

3.2.1 Study Subjects

Patients with the CSFP were recruited from outpatient clinics whereas the healthy control subjects were recruited via public advertisements. All participants were above the age of 35 years, had no history of diabetes and all prescribed vasoactive medications (ACE inhibitors, angiotensin-receptor blockers, calcium-channel blockers, long-acting nitrates) were ceased 48 hours prior to the study interventions, following consent from their treating physician. The diagnosis of the CSFP for this study was made on the basis of angiographically documented TIMI-2 flow (i.e. requiring ≥3 beats to opacify an epicardial vessel) in the absence of obstructive coronary artery disease (<30% stenosis in all epicardial vessels), as defined by two blinded observers (222). The healthy control patients had no history of cardiac disease or chest pain. The exclusion criteria for the study included pregnancy, hypotension (systolic blood pressure < 100mmHg) or intolerance to sublingual nitrates.

3.2.2 Study Protocol

After informed consent was obtained, study participants underwent the following protocol. Clinical details concerning their cardiac history, current medications, and any cardiac investigations previously performed were obtained by interview or clinical record extraction. On the study day, patients underwent venesection following an overnight fast (12 hours) for analysis of plasma biomarkers. Patients also abstained from consuming caffeine one hour prior to pulse wave analysis studies.

3.2.3 Endothelial Function Assessment

The assessment of endothelial function was undertaken using applanation tonometry via the method described and validated by Wilkinson et al (340). This technique involved placing a pressure-transducer probe (Sphygmocor Software, Atcor Medical, Sydney, Australia) over the maximal arterial pulsation in the radial artery, so that adequate applanation of the vessel could be achieved to derive the radial pressure waveform. Aortic pressure waveforms were obtained from the peripheral pulse waveform utilising a validated generalised transfer function (341) in the integrated software. Systemic endothelial function was assessed through analysis of changes in aortic pressure waveforms and quantitative measurement of AIx following serial administration of endothelium-independent (glyceryl trinitrate, GTN) and endothelium-dependent (salbutamol) vasodilators (340). The AIx represented the difference in values between ventricular ejection (first systolic peak, P_1) and systolic peak pressure (second

systolic peak, P_2). The AIx is expressed as a percentage of the pulse pressure and corrected for heart rate.

The following procedure was adopted for the applanation tonometry assessment of endothelial function. On arrival, subjects rested for 30 minutes in a supine position during which serial baseline blood pressure and pulse rate were non-invasively measured from the left brachial artery. Pulse-wave analyses of the right radial artery waveforms were also measured during this rest period to ascertain the baseline AIx values. After establishing baseline parameters, 50μg GTN was administered sublingually and blood pressure, pulse and AIx values recorded at 3, 5, 10, 15 and 20-minute intervals. The protocol was repeated for 400μg salbutamol administration after AIx readings had returned to baseline values. For each time point, three AIx values were obtained and averaged. The arterial responses to GTN and salbutamol administration were defined as the maximum change in AIx over the total time interval compared to baseline as previously described (340).

3.2.4 Plasma Biochemical Assays

Fasting blood samples were placed into lithium heparin or EDTA pre-treated tubes and centrifuged for 10 minutes to obtain plasma aliquots. Homocysteine and hsCRP concentrations were assessed at the institutional diagnostic laboratory facility. The remaining plasma was stored at -80°C for subsequent batch analysis of MPO, MDA and ADMA. The techniques employed for each assay are described below.

3.2.4.1 Homocysteine and high-sensitivity CRP assays

Plasma homocysteine was assessed using a well-established and validated chemiluminescence immunoassay (Bayer ADVIA Centaur Assay, Australia) (342). Concentrations of plasma hsCRP were determined using a validated immuno-turbidimetric assay (Olympus OSR6199 Analyzer, Australia) (343).

3.2.4.2 Myeloperoxidase assay

MPO concentration was determined using the Zen™ Myeloperoxidase 96-well ELISA Kit (Invitrogen, Australia) as per the specified protocol. All antibody solutions, standards and patient samples were diluted with phosphate buffered saline/0.1%BSA. Briefly, individual ELISA wells: 1) were hydrated with PBS + 0.001% Tween (PBST) and incubated with 500ng/mL (100μL) solutions of mouse anti-MPO primary capture antibody; 2) underwent sequential washing steps with PBST; 3) incubated with standards, samples (100μL) and 1.0μg/mL of secondary rabbit anti-MPO capture antibody; 4) washed and incubated with 100μL goat anti-rabbit HRP conjugate; 5) and incubated with 100μL of Amplex UltraRed reagent mix (30 min) followed by addition of Amplex® stop solution. Fluorescence was measured using filters at $\lambda_{excitation}$ = 530nm and $\lambda_{emission}$ = 590nm.

3.2.4.3 Asymmetric dimethylarginine assay

Plasma ADMA, SDMA and arginine concentrations were determined using a modified version of a previously described protocol (344). Briefly, 150μL of plasma was combined with internal standard N-monomethylarginine [NMMA (5μg/mL)] and extracted on pre-conditioned Bond Elut Strong Cation Exchange

(Varian) cartridges loaded on an automated solid phase extraction system (Gilson Aspec GX-274). Arginine and the methylargines were eluted and prepared for HPLC as per published protocol. ADMA and SDMA were quantified by HPLC as described previously, from a 20μL injection volume. Arginine was quantified by injecting 2.5μL of mixture onto the HPLC.

3.2.4.4 Malondialdehyde assay

Plasma MDA levels were determined based upon a slightly modified protocol that involved fluorescent detection of an extracted MDA-thiobarbituric (TBA) acid adduct (345). Briefly, plasma samples were thawed and underwent protein precipitation (2.3M perchloric acid) and lipid removal (200μL chloroform) protocols, respectively. For the adduct reaction to occur, 200μL of supernatant was aliquoted into conical glass tubes containing 0.15M H_3PO_4 (750μL), 42mM TBA (250μL) and made up to 1.5mL with MilliQ water. Samples were boiled for 60 minutes at 100°C and cooled on ice thereafter. Extraction of the MDA adduct was performed by addition of 70:30 chloroform:methanol (vol/vol) and the top aqueous layer was collected for detection of MDA fluorescence at $\lambda_{excitation}$ = 530nm, $\lambda_{emission}$ = 547nm.

3.2.5 Data and Statistical Analysis.

The primary endpoint of the study was the change in AIx in response to salbutamol. Secondary endpoints included AIx response to GTN, and the biochemical markers. The study sample size was based upon the maximum changes in AIx following salbutamol administration as determined in previous

investigations (340). In order to detect a 30% difference between groups, it was calculated that a minimum of 22 patients would be required in each cohort for 80% power at an α=0.05. The study endpoints were normally distributed and thus the data have been expressed as mean ± SEM. Plasma biomarker comparisons between the CSFP patients and healthy subjects were conducted with unpaired Student t-tests. Comparison of categorical variables (risk factor and gender) between cohorts was conducted with Fisher's exact tests.

3.3. Results

Sixty-three subjects participated in the study with 40 fulfilling the angiographic definition for the CSFP and 23 healthy controls that had no history of chest pain or cardiac disease. The study groups were age and gender-matched but the CSFP patients had a higher body mass index as compared with the controls (Table 3.1, page 129). The CSFP patients were more likely to have been on maintenance cardiac medications (anti-platelet agents, statins, nitrates and calcium-channel blockers) considering their angina symptoms and non-significant tendency to have a history of hypertension or hypercholesterolaemia, compared with the healthy controls.

3.3.1 Endothelial Function Studies

There were no differences between groups in the baseline AIx measurements (CSFP = 22.4 ± 1.6% vs Controls = 22.9 ± 1.7%, p=0.81). In the assessment of endothelium-dependent responses, salbutamol administration reduced AIx values in both CSFP and control subjects over the total 20 minute time period (Figure 3.1.A, page 131). Maximal arterial responses to salbutamol were found not to be significantly different between cohorts ($\Delta_{CSFP:}$ -2.28 ± 0.88% vs $\Delta_{CONT:}$ -3.22 ± 0.70%, p=0.4).

Arterial responses to the endothelium-independent vasodilator, GTN, as assessed through changes in aortic AIx are depicted in Figure 3.1.B (page 131). During the twenty-minute period, maximal arterial responses to GTN were similar for both

cohorts, demonstrating equivalent endothelium-independent responses to an exogenous NO donor. Furthermore, the CSFP patients demonstrated no significant differences in maximal arterial responses to GTN over the total study time period compared to controls ($\Delta_{CSFP:}$ -11.3 ± 0.75% vs $\Delta_{CONT:}$ -13.3 ± 1%, p=0.12).

Considering the differences between the CSFP and control groups in cardiovascular risk factors that may influence endothelial function, a sensitivity analysis was conducted. All patients with a history of hypertension, hypercholesterolaemia or smoking were excluded thereby reducing the sample population to 11 CSFP patients and 14 healthy controls. Comparison of maximal arterial responses to salbutamol in these subgroups also demonstrated no significant differences ($\Delta_{CSFP:}$ -4.3 ± 0.9% vs $\Delta_{CONT:}$ -4.0 ± 0.8%, p=0.79). Similarly, there was no difference between these subgroups in maximal GTN responses ($\Delta_{CSFP:}$ -10.8 ± 1.2% vs $\Delta_{CONT:}$ -13.9 ± 1.4%, p=0.12).

3.3.2 Plasma Biochemical Markers

A summary of the plasma biochemical results is depicted in Table 3.2 (page 130). There was no significant difference between CSFP and control subjects in relation to ADMA or SDMA. Similarly, comparisons of arginine concentrations revealed that there were no significant differences in plasma levels between the two cohorts. Comparison of MPO plasma concentration between cohorts identified that CSFP patients had slightly lower levels of MPO, although statistical significance was not reached. Analysis of plasma hsCRP levels also did not yield

any statistical differences between cohorts. Investigation of MDA and homocysteine in CSFP patients also did not demonstrate any evidence of oxidative stress. No significant differences in plasma MDA or homocysteine were found between cohorts.

Table 3.1 Baseline characteristics of CSFP patients and control subjects.

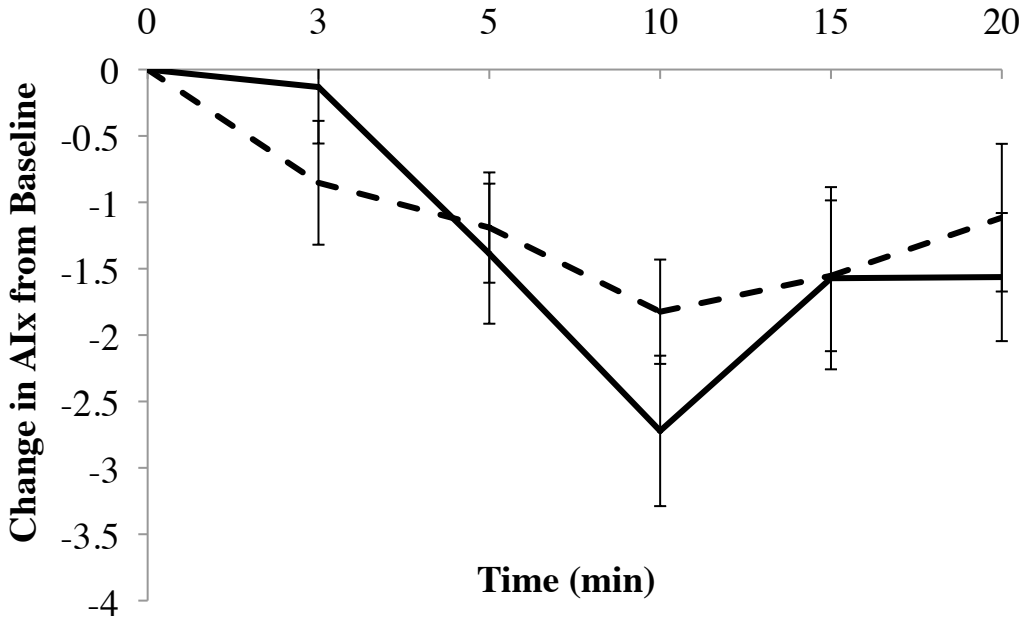
	Controls (n=23)	**CSFP (n=40)**	**p value**
Coronary risk factors			
Age, years	56 ± 7.4	55 ± 9.6	0.86
Male Gender	13 (56%)	25 (62%)	0.99
Hypertensive	4 (17%)	15 (38%)	0.15
Current smoker	4 (17%)	7 (18%)	1.0
Hypercholesterolemia	6 (22%)	21 (53%)	0.06
Body Mass Index (BMI)	27 ± 0.9	30 ± 1.0	0.03
Medications			
Long-acting Nitrates	0	16 (40%)	<0.05
Statins	3 (13%)	19 (48%)	<0.05
Anti-platelet agents	0	24 (60%)	<0.05
ACE inhibitors	3 (13%)	9 (23%)	0.5
Calcium channel blockers	0	24 (60%)	<0.05
Angina frequency			
>3 episodes/week over past month	0	26 (65%)	<0.05
TIMI frame counts			
Corrected left anterior descending (LAD)	N/A	55±5	
Circumflex	N/A	46±3	
Right coronary artery (RCA)	N/A	43±5	

Table 3.2. Plasma biomarker concentrations in CSFP patients and control subjects. Values expressed as mean ± SEM.

	Controls (n=23)	**CSFP (n=40)**	**p value**
Inflammatory markers			
MPO (ng/mL)	24.9 ± 4.8	16.9 ± 2.3	0.14
CRP (mg/L)	2.63 ± 0.77	3.23 ± 0.59	0.54
Oxidative stress markers			
MDA (ng/mL)	0.19 ± 0.05	0.19 ± 0.08	0.74
Homocysteine (μmol/L)	8.30 ± 0.34	9.20 ± 0.39	0.08
Endothelial markers			
ADMA (μM)	0.59 ± 0.01	0.57 ± 0.01	0.33
SDMA (μM)	0.53 ± 0.01	0.55 ± 0.01	0.38
Arginine (μM)	108.7 ± 5.5	99.8 ± 4.2	0.20

Figure 3.1. Change in AIx values in control subjects (n=23) and CSFP patients (n=40) from baseline following administration of Salbutamol (A) and GTN (B).

A.



B.

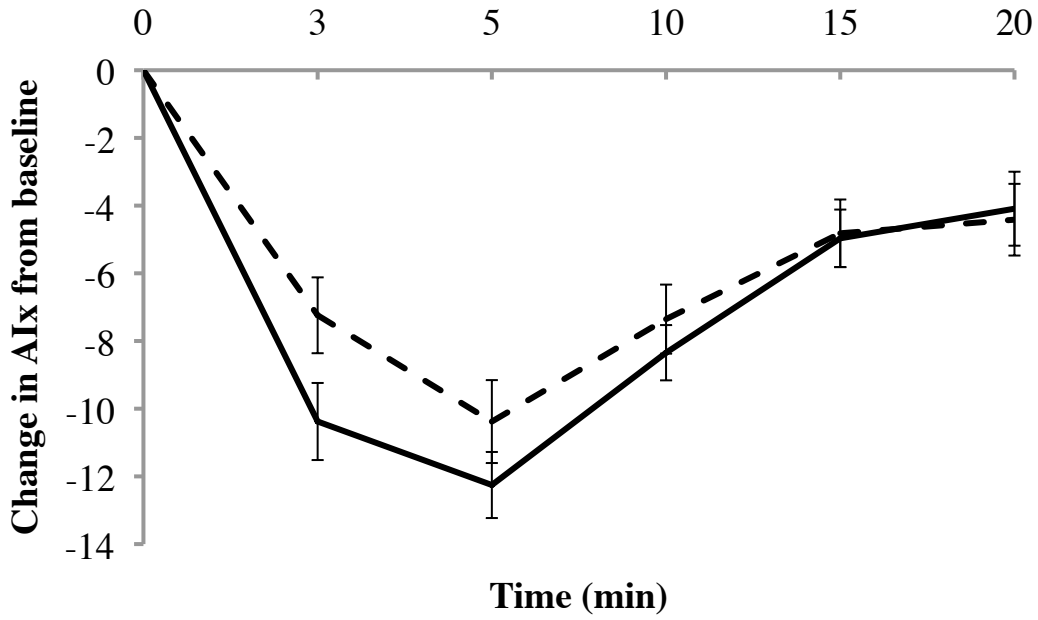


Figure Legend 3.1

Solid lines represent average AIx values obtained from baseline for control subjects. Broken lines represent average AIx values obtained from baseline for CSFP patients. Values expressed as mean ± SEM.

3.4. Discussion

This study is the first to comprehensively assess endothelial function and biomarkers of endothelial activation in a large group of CSFP patients as compared with a healthy control cohort. Its key finding is that endothelial function, as assessed by pulse wave analysis, is not impaired with similar responses in the CSFP patients and healthy controls. Consistent with this finding, endothelial activation was not detected as suggested by the measured inflammatory and oxidative stress biomarkers. Hence, this study would suggest that abnormalities in endothelial function do not play a major role in the pathogenesis of the CSFP in the cohort studied.

3.4.1 Previous Endothelial Studies of the CSFP

The previous studies investigating endothelial function in CSFP patients have produced divergent results due to differences in the study groups and endothelial function assessment method. In contrast to the present study and others (274), Sezgin et al., (339) reported impaired brachial artery flow mediated dilatation (FMD) in patients with the CSFP as compared with patients with chest pain, normal angiography and no slow flow. In the latter study, the presence of hypertension, dyslipidaemia or cigarette smoking may have influenced the findings as these were not reported for each study group, yet have been associated with impaired FMD. Moreover, cigarette smoking has been shown to be more prevalent amongst patients with the CSFP and hence the study may have been

confounded by unmeasured variables affecting endothelial function. Also of importance, FMD is a marker of large vessel endothelial function whereas AIx-assessed endothelial function has been validated against venous plethysmography, which is a measure of microvascular responses (340). Thus, even if FMD is impaired in patients with the CSFP, it is possible that microvascular endothelial function is intact.

Previous studies have reported that the CSFP is associated with elevated levels of homocysteine (290-292, 319), MDA (290), hsCRP(346), *Helicobacter pylori* infection (291) and plasma soluble adhesion molecules (285). In contrast, Yazici et al, (288) found no difference in C-reactive protein between 'active control patients' and those with CSFP. These differences may reflect heterogeneity in the recruited patients (i.e. differences in the inclusion criteria for both the CSFP and control cohorts) but may also be indicative of the natural history of the condition. Patients with the CSFP frequently exhibit episodes of rest pain mimicking an acute coronary syndrome. In a recent study, we have demonstrated increased inflammatory and oxidative stress markers in the CSFP patients during an acute coronary syndrome presentation, which subsequently resolved one month later (347). Thus, the observed differences between studies may reflect the current symptomatic/pathophysiologic status of the recruited patients.

Although no difference in ADMA or SDMA levels was observed in this study, Selcuk et al, (270) reported increased ADMA levels in their CSFP patients, which is consistent with the above endothelial dysfunction studies. However, similar to

the above biomarkers, ADMA and SDMA levels may fluctuate with disease activity and indeed have been shown to return to baseline levels following an acute coronary syndrome presentation (348), as well as not being elevated in chronic stable angina (349).

3.4.2 Alternative Potential Mechanisms for the CSFP

Since the above-mentioned factors do not appear to play a major role in the pathogenesis of the CSFP, other factors need to be considered. Future studies should focus on the role of other endothelial factors and platelet dysfunction as these have been implicated in the pathogenesis of coronary microvascular dysfunction. EDHF is a vasodilator substance whose exact chemical nature is controversial. Studies have suggested that EDHF is hydrogen peroxide and that it plays a more significant role than nitric oxide in vasodilatation of the microvasculature (83). Moreover, a recent study has confirmed its role in the human coronary microvasculature (350) although further studies are required to determine its contribution to coronary microvascular dysfunction.

Endothelin is an endothelium-derived vasoconstrictor that may play a role in the pathogenesis of the CSFP. When infused into the coronary arteries in animal studies, it mimics the angiographic appearance of the CSFP (276). Furthermore, in-vitro studies of human coronary artery segments have demonstrated that the distal coronary vasculature is more sensitive to endothelin than the proximal segments (351). Also, increased coronary sinus endothelin levels have been demonstrated in patients with the CSFP, both at rest and during rapid atrial pacing (275).

In addition to the above endothelium-derived vasoactive substances, platelet dysfunction may play an important role. Sen et al, demonstrated that mean platelet volume, a marker of platelet activation, is elevated in patients with the

CSFP (283). Thus, further investigations are required to elucidate the relative importance of these additional biological factors in the pathophysiology of the CSFP.

Currently, there are no clinically available therapies that have been shown to effectively reduce the angina frequency in patients with the CFSP. The T-type calcium-channel blocker, mibefradil, was shown to be effective but has been withdrawn by the manufacturer (30). Further studies are required to elucidate the molecular mechanisms responsible for this coronary microvascular disorder, as this will enable the development of targeted therapeutic strategies.

3.4.3 Study Limitations

In this study, healthy controls were recruited who had no history of chest pain or cardiac disease. This averted the problems with other previous study designs where patients with chest pain and normal angiography were recruited as controls. These 'active controls' do not have angiographic evidence of the CSFP but the presence of other coronary microvascular disorders (e.g. CSX) cannot be excluded. In the present study, patients without a history of chest pain or cardiac disease were recruited as controls, however, we cannot exclude that they may have angiographic evidence of coronary heart disease or the CSFP, since angiography was not undertaken. Despite this limitation, we can conclude that they did not have symptomatic coronary heart disease.

3.5. Conclusion

The present study has demonstrated similar endothelial function in stable CSFP patients and healthy controls. Furthermore, associated endothelial, inflammatory and oxidative stress biomarkers were not different between groups. This would suggest that the endothelial nitric oxide pathway is not dysfunctional in these patients and thus less likely to contribute to the pathogenesis of this intriguing disorder. Moreover, there may different pathophysiological mechanisms involved during the stable phase and acute exacerbations of this disorder. Thus, future studies should address these possible differences as well as examining other endothelial factors, such as EDHF and ET-1.

CHAPTER IV

CHAPTER IV

The anti-anginal efficacy of Bosentan in the Coronary Slow Flow Phenomenon

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This results chapter is written in publication format intended for submission for the *International Journal of Cardiology*.

In keeping with the style of this thesis, the abstract has been removed, the table and figures have been re-numbered, the references incorporated into the thesis' master reference list, the content formatted to English (AUS), the manuscript repaginated and abbreviations utilised following initial definition. The methodology for endothelial function assessment and plasma biochemical assays has been replicated as first described in Chapter III.

4.1. Introduction

The CSFP is a coronary microvascular disorder characterised by delayed distal vessel opacification in the absence of obstructive coronary artery vessel disease (222). Clinically distinct from other microvascular disorders such as CSX (297), the mechanisms responsible for the documented microvascular dysfunction in this disorder remain elusive. Although impaired endothelium-dependent vasodilator function has been implicated (339), others have refuted these findings (274).

An alternate potential mechanism for the increased microvascular resistance is an exaggerated response to vasoconstrictor stimuli. ET-1 is a potent endogenous vasoconstrictor peptide released from the endothelium that has been implicated in the pathogenesis of the CSFP. Observations supporting its role in the CSFP include: (a) intracoronary ET-1 administration mimics the angiographic appearance of the CSFP in canine (276) and rabbit (277) studies; (b) ET-1 levels have been shown to be elevated in patients with the CSFP (269); (c) intravenous ET-1 infusion into healthy individuals produces a fall in coronary sinus oxygen saturation (278) (a marker of coronary blood flow), similar to that observed in CSFP patients; and (d) recently we have observed a selective hyper-responsiveness to ET-1 in isolated subcutaneous microvessels of patients with the CSFP (312).

Considering the above evidence implicating ET-1 in the pathogenesis of the CSFP, this study sought to determine if ET-1 blockade with bosentan (a

combined ET-A and ET-B receptor antagonist) would improve the symptoms of patients with the CSFP. Thus, the primary objective of the study was to assess if bosentan administered orally at a dose of 125mg twice daily affected the angina frequency in patients with documented CSFP. Supporting secondary endpoints included the effect of bosentan therapy on: (i) other clinical endpoints (prolonged angina episodes & sublingual nitrate consumption); (ii) endothelial function assessment; (iii) endothelial vasoactive biomarkers (ADMA); (iv) inflammatory proteins (hsCRP & MPO); and (v) oxidative stress (MDA & homocysteine) biomarkers.

4.2. Methods

To achieve the above objectives, the study employed a randomised, double-blind, placebo-controlled, cross-over study design involving symptomatic CSFP patients. The Central Northern Adelaide Health Service and University of Adelaide Ethics Human Research Committees approved the study.

4.2.1 Study Patients

Both recently diagnosed and patients with established CSFP were recruited provided they fulfilled both of the following criteria: (1) stable recurrent angina episodes ≥ 3 times/week; and (2) angiographic evidence of the CSFP as defined by (a) requiring ≥3 beats to opacify pre-specified branch points in the distal vasculature of any of the three major epicardial coronary arteries (i.e. equivalent of TIMI-2 flow), and (b) the absence of obstructive CAD (i.e. <30% in any epicardial coronary artery). Exclusion criteria were based upon contra-indications to bosentan therapy and included: (a) elevated plasma hepatic transaminases twice the upper normal limit; (b) concurrent use of cyclosporine A or oral contraceptives; (c) anaemia (haemoglobin ≤ 100g/L); and (d) pregnancy.

4.2.2 Study Protocol

The study protocol is outlined in Figure 4.1 (Page 153). Following an initial visit when clinical history and informed consent were obtained, patients were randomised to either twice-daily bosentan (125mg) or matching placebo treatment using a computer-generated algorithm, with the sequence known only to the

hospital clinical study pharmacist who had no contact with the patients. After two weeks of bosentan/placebo therapy, patients were assessed for any adverse events and withdrawn from the study if transaminases demonstrated a ≥5-fold change from baseline. Patients continued with the assigned therapy for a further two weeks (four weeks total) and thereafter were clinically re-evaluated, venesection performed for biomarker determination and endothelial function assessed. Subsequently, patients commenced a one-week washout period and then crossed-over to the alternative bosentan or placebo therapy with the above study protocol repeated. The study medication utilised was bosentan monohydrate 125 mg tablet (Actelion Pharmaceuticals, Australia), administered orally twice daily. Actelion Pharmaceuticals prepared a corresponding placebo, which was indistinguishable in appearance to the active drug.

4.2.3 Study Endpoints

The study endpoints were all evaluated at the 4-week time point, while on study treatment. The primary endpoint was the frequency of total angina episodes recorded during the 4 weeks of treatment. Secondary endpoints included: (i) *clinical endpoints* – frequency of prolonged anginal episodes (>20 minutes) sublingual nitrate consumption, health status as assessed by the short form-36 (SF-36) and Seattle angina questionnaires (SAQ); (ii) *endothelial function endpoints* - as assessed by changes in AIx using pulse-wave analysis to endothelium-dependent vasodilators; (iii) *endothelial vasoactive biomarkers* – including plasma arginine, ADMA and SDMA levels; and (iv) inflammatory and oxidative stress biomarkers – including hsCRP, MPO, MDA and homocysteine.

4.2.4 Clinical Assessments

Throughout the study period, patients were asked to maintain an angina diary and record angina frequency, duration and sublingual nitrate consumption. During each visit, clinical observations were recorded, the angina diary reviewed and any changes in medication or occurrence of adverse events documented. In the last week of each phase, in addition to the clinical observations and review of the angina diary, an ECG was performed and patients completed the generic (SF-36) and disease-specific (SAQ) health-related quality of life questionnaires. These instruments have been previously validated for patients with angina and provide important insights into the impact on health status (352). Patient medication compliance was also assessed at the end of each phase by pill count.

4.2.5 Endothelial Function Assessment

The assessment of endothelial function was undertaken using applanation tonometry via the method described and validated by Wilkinson et al (340). This technique involved placing a pressure transducer probe (Sphygmocor Software, Atcor Medical, Sydney, Australia) over the maximal arterial pulsation in the radial artery, so that adequate applanation of the vessel could be achieved to derive the radial pressure waveform. Aortic pressure waveforms were obtained from the peripheral pulse waveform utilising a validated generalised transfer function (341) in the integrated software. Systemic endothelial function was assessed through analysis of changes in aortic pressure waveforms and quantitative measurement of AIx following serial administration of endothelium-independent (glyceryl trinitrate, GTN) and endothelium-dependent (salbutamol)

vasodilators (340). The AIx represented the difference in values between ventricular ejection (first systolic peak, P_1) and systolic peak pressure (second systolic peak, P_2), expressed as a percentage of pulse pressure and corrected for heart rate.

The following procedure was adopted for the applanation tonometry assessment of endothelial function. On arrival, subjects rested for 30 minutes in a supine position during which serial baseline blood pressure and pulse rate were non-invasively measured from the left brachial artery. Pulse wave analysis of the right radial artery waveforms was also measured during this resting period to ascertain the baseline AIx values. After establishing baseline parameters, 50μg GTN was administered sublingually and recordings of blood pressure, pulse and AIx values occurred at 3, 5, 10, 15 and 20-minute intervals. The protocol was repeated for 400μg salbutamol administration after AIx readings had returned to baseline values. For each time point, three AIx values were obtained and subsequently averaged. The arterial responses to GTN and salbutamol administration were defined as the maximum change in AIx over the total time interval compared to baseline as previously described (340).

4.2.6 Plasma Biochemical Assays

Fasting blood samples were placed into lithium heparin or EDTA pre-treated tubes and centrifuged for 10 minutes to obtain plasma aliquots. Homocysteine and hsCRP concentrations were assessed at the institutional diagnostic laboratory facility. The remaining plasma was stored at -80°C for subsequent batch analysis

of MPO, MDA and ADMA. The techniques employed for each assay are described below.

4.2.6.1 Homocysteine and high-sensitivity CRP assays

Plasma homocysteine was assessed using a well-established and validated chemiluminescence immunoassay (Bayer ADVIA Centaur Assay, Australia)(342). Concentrations of plasma hsCRP were determined using a validated immuno-turbidimetric assay (Olympus OSR6199 Analyzer, Australia) (343).

4.2.6.2 Myeloperoxidase assay

MPO concentration was determined using the Zen™ Myeloperoxidase 96-well ELISA Kit (Invitrogen, Australia) as per the specified protocol. All antibody solutions, standards and patient samples were diluted with phosphate buffered saline/0.1%BSA. Briefly, individual ELISA wells: 1) were hydrated with PBS + 0.001% Tween (PBST) and incubated with 500ng/mL (100μL) solutions of mouse anti-MPO primary capture antibody; 2) underwent sequential washing steps with PBST; 3) incubated with standards, samples (100μL) and 1.0μg/mL of secondary rabbit anti-MPO capture antibody; 4) washed and incubated with 100μL goat anti-rabbit HRP conjugate; 5) and incubated with 100μL of Amplex UltraRed reagent mix (30 min) followed by addition of Amplex® stop solution. Fluorescence was measured using filters at $\lambda_{excitation}$ = 530nm and $\lambda_{emission}$ = 590nm.

4.2.6.3 Asymmetric dimethylarginine assay

Plasma ADMA, SDMA and arginine concentrations were determined using a modified version of a previously described protocol (344). Briefly, 150μL of plasma was combined with internal standard N-monomethylarginine [NMMA (5μg/mL)] and extracted on pre-conditioned Bond Elut Strong Cation Exchange (Varian) cartridges loaded on an automated solid phase extraction system (Gilson Aspec GX-274). Arginine and the methylargines were eluted and prepared for HPLC as per the published protocol. ADMA and SDMA were quantified by HPLC as described previously, from a 20μL injection volume. Arginine was quantified by injecting 2.5μL of mixture onto the HPLC.

4.2.6.4 Malondialdehyde assay

Plasma MDA levels were determined based upon a slightly modified protocol that involved fluorescent detection of an extracted MDA-TBA acid adduct (345). Briefly, plasma samples were thawed and underwent protein precipitation (2.3M perchloric acid) and lipid removal (200μL chloroform) protocols, respectively. For the adduct reaction to occur, 200μL of supernatant was aliquoted into conical glass tubes containing 0.15M H_3PO_4 (750μL), 42mM TBA (250μL) and made up to 1.5mL with MilliQ water. Samples were boiled for 60 minutes at 100°C and cooled on ice thereafter. Extraction of the MDA adduct was performed by addition of 70:30 chloroform:methanol (vol/vol) and the top aqueous layer was collected for detection of MDA fluorescence at $\lambda_{excitation} = 530nm$, $\lambda_{emission} = 547nm$.

4.2.7 Data Analysis

The study was undertaken using double-blind methodology and all analyses were conducted blinded to the study treatment. The clinical angina parameters were analysed using cross-over trial methodology with comparison between patients and respect to treatment order (353). Large differences in angina frequency between phases for individual patients were observed and therefore the data was log transformed and non-parametric (Mann Whitney U) tests used for comparison. The continuous, normally distributed health status questionnaires, endothelial function and biomarker assay endpoints were expressed as mean ± SEM and analysed by unpaired Student t-tests. Statistical values with $\propto=0.05$ were considered as significant.

The study sample size was calculated based upon the primary endpoint of total angina frequency. From a previous study examining the anti-anginal efficacy of mibefradil in the CSFP (311), the total angina frequency during placebo therapy was 28 ± 31 episodes/month with 22 less episodes on active therapy. Thus, to detect a difference of 22 episodes or more with bosentan therapy, a minimum of 22 patients would be required for 90% power at $\propto=0.05$ level.

4.3 Results

Over a thirty-month period, 26 patients were recruited into the study with 3 patients subsequently withdrawing, before completion of the study protocol. Two of these patients were withdrawn because of concerns with the study drug (mild elevations in hepatic transaminases and potential drug interactions with existing medications). A further patient was withdrawn due to non-compliance with study visits. Amongst the patients completing the study protocol, there were no major adverse effects and medication compliance was over 95% on pill count.

4.3.1 Patient Characteristics

The recruited patients were more often male, with over half having at least 2 atherosclerotic risk factors (Table 4.1, page 154). All of the patients were on conventional anti-anginal therapy, including long-acting nitrates (68%), calcium-channel blockers (68%) and/or beta-blockers (14%); yet their mean angina frequency at baseline was 15±3 episodes/week.

4.3.2 Clinical Endpoints

The primary study endpoint of total angina frequency did not statistically differ between active and placebo therapy although 39% (median) less angina episodes occurred on bosentan therapy (Table 4.2, page 155). Similarly, there was no significant difference in prolonged angina episodes and sublingual nitrate consumption with bosentan therapy although these were reduced 67% and 56% (median), respectively. Assessment of health-related quality of life with the SF-36

showed no improvement in physical or mental summary scores with the bosentan therapy (Table 4.3, page 156). Furthermore, SAQ-assessed health status did not show significant improvements although there was a non-significant 7-point improvement in angina frequency with bosentan.

4.3.3 Endothelial Function Studies

Pulse-wave analysis data was successfully obtained from sixteen CSFP patients. Baseline AIx values were found to be similar for each phase (placebo: 19.9 ± 2.1% vs bosentan: 15.7 ± 2.3%, p=0.1). Maximal arterial responses to the endothelium-independent vasodilator, GTN and endothelium-dependent vasodilator, salbutamol, as assessed through changes in aortic AIx are depicted in Figure 4.4 (page 156). Comparison of maximum changes in AIx following GTN administration during placebo and bosentan treatments demonstrated no significant differences (Placebo: -10.3 ± 1.2% vs bosentan: -10.0 ± 1.1%, p=0.87). Maximal changes in AIx following salbutamol administration tended to be larger with bosentan therapy (Placebo: -1.4 ± 1.2% vs bosentan: -2.2 ± 1.3%, p=0. 61) despite not reaching statistical significance.

4.3.4 Endothelial, Inflammatory and Oxidative Stress Biomarkers

ADMA, SDMA and arginine plasma levels were assessed in eleven CSFP patients. As shown in Table 4.3 (page 156), bosentan therapy was associated with a significant increase in plasma ADMA levels but did not influence either SDMA or arginine concentrations. Plasma inflammatory markers for MPO and hsCRP were obtained from seventeen patients and demonstrated no significant changes

following bosentan therapy (Table 4.3, page 156). Similarly, there were no changes in oxidative stress markers including plasma MDA and homocysteine concentrations.

Figure 4.1. Study Protocol. Summary of the nine-week protocol including double-blind randomisation to bosentan (125mg)/placebo, twice-daily therapy for four weeks followed by cross-over to the alternate therapy after a one-week washout period.

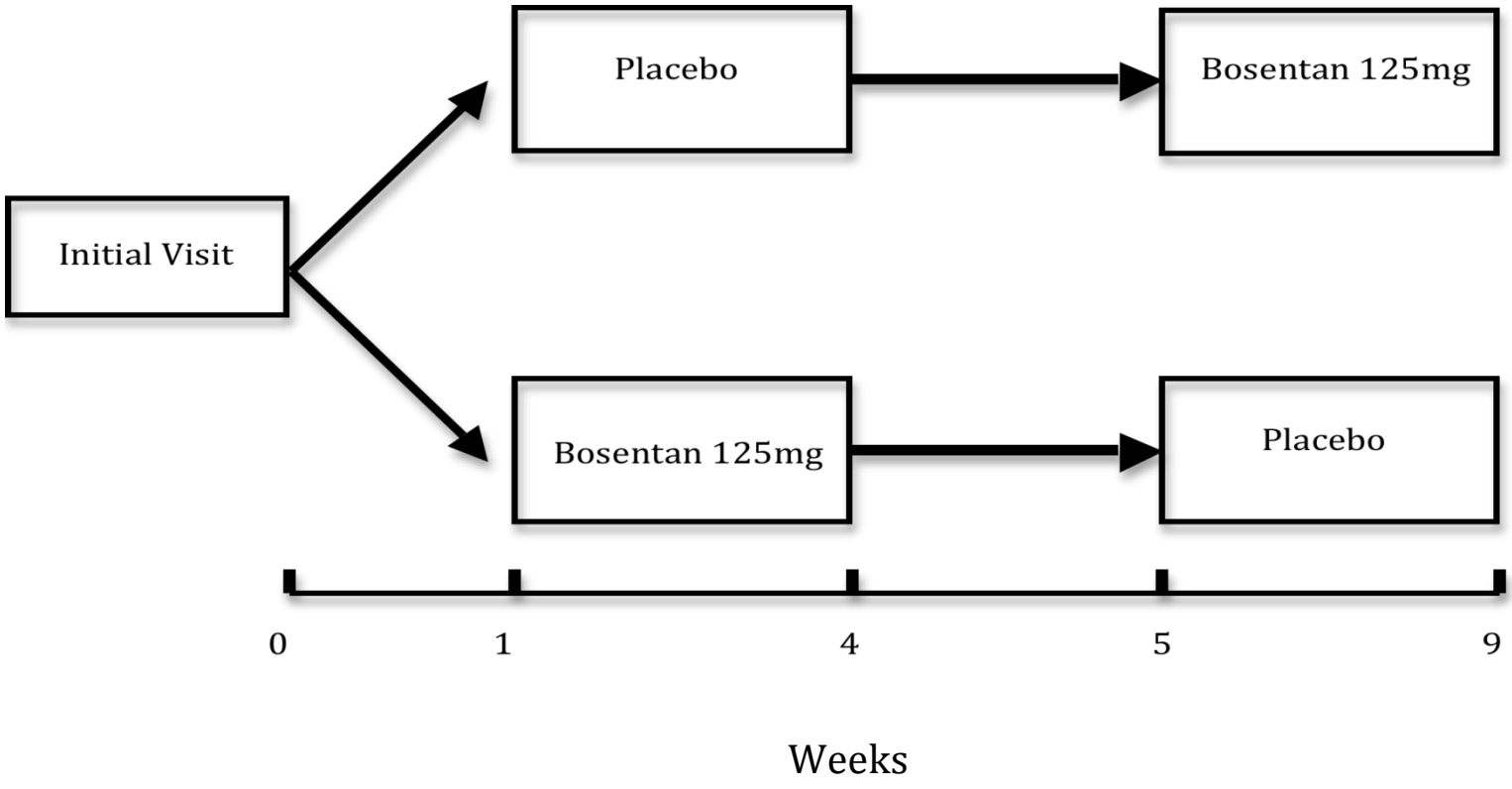


Table 4.1. Baseline clinical characteristics of CSFP study patients.

Coronary Risk Factors	**CSFP (n=23)**
Age (years ± SEM)	56 ± 2.2
Gender	13M: 9F
Current Smoker	2 (9%)
Hypertensive	10 (45%)
Diabetic	5 (23%)
Positive Family History	8 (35%)
Hypercholesterolaemia	14 (64%)
≥2 risk factors	13 (59%)
Current Medications	
Anti-platelet agents	19 (86%)
Statins	14 (64%)
Long-acting nitrates	15 (68%)
Calcium-channel blocker	15 (68%)
Beta-blocker	3 (14%)
ACE inhibitor	6 (27%)
Angiotensin II receptor blocker	4 (18%)
ECG findings	
ST/T wave changes at baseline	2 (9%)
CSFP Characteristics	
Recently diagnosed (angiogram < 1 month)	9 (39%)
Time since initial diagnosis (months ± SEM)	42 ± 12
Baseline angina frequency (mean weekly ± SEM)	15 ± 3

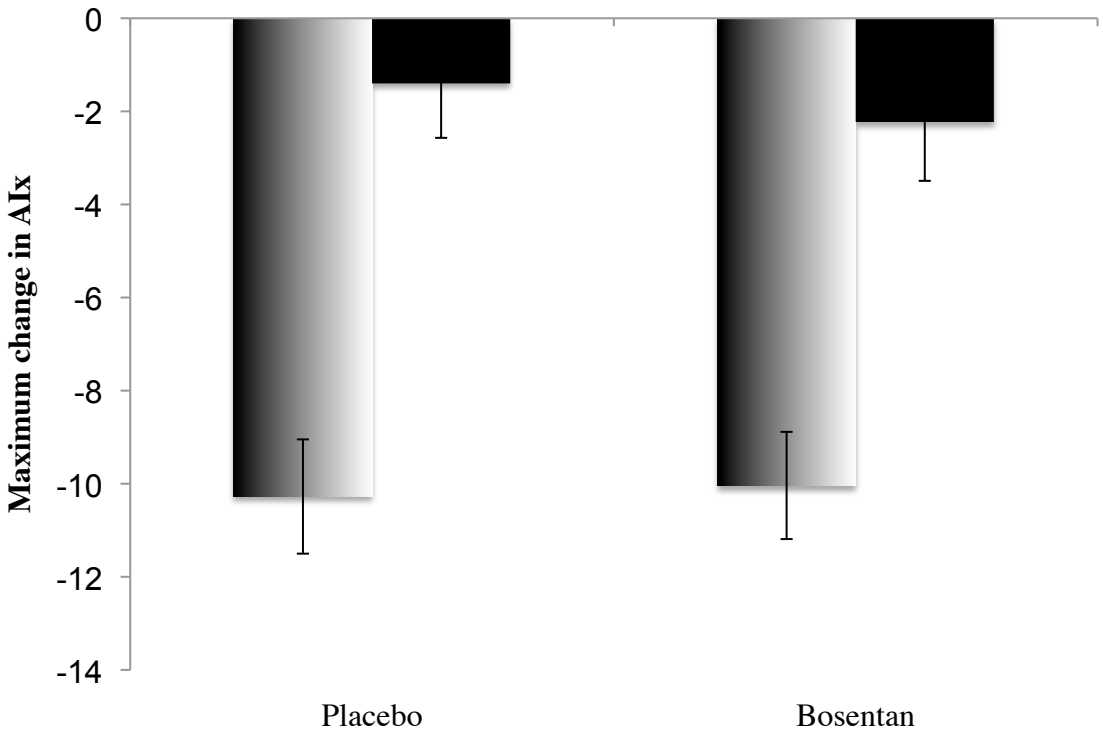
Table 4.2. Median values (25%, 75% interquartile ranges) of total angina episodes, prolonged angina episodes (>20 min) and sublingual nitrate use during four weeks of placebo and bosentan (125mg) twice daily therapy in 23 CSFP patients.

Parameter	**Placebo**	**Bosentan**	**% Change (Median)**	**p Value**
Total angina (episodes/month)	25 (9,55)	14 (5,36)	39	0.32
Prolonged angina (episodes/month)	5 (0,19)	1.5 (0,7)	67	0.31
Sublingual nitrate use (tablets or spray/month)	11 (0,35)	7.5 (0,19)	56	0.86

Table 4.3. Summary of secondary endpoints, including health status questionnaires, endothelial, inflammatory and oxidative stress biomarkers following four weeks of treatment with placebo or bosentan therapy.

SF-36 Health Survey Concept	**Placebo**	**Bosentan**	**p Value**
Physical Health Concept			
Physical Functioning	58 ± 5	61 ± 5	0.3
Bodily pain perception	47 ± 4	50 ± 5	0.5
General health perception	46 ± 5	46 ± 4	0.9
Role limitation due to physical health	39 ± 10	40 ±9	0.9
Physical health summary score	38 ± 2	38 ± 2	0.9
Mental Health Concept			
Vitality	41 ± 5	41 ± 6	0.9
Social functioning	66 ± 5	64 ± 4	0.7
General mental health	60 ± 4	67 ± 5	0.1
Role limitation due to emotional problems	56 ± 10	55 ± 10	0.9
Mental Health summary score	40 ± 2	41 ± 2	0.6
SAQ Questionnaire			
Physical limitation score	63 ± 5	64 ± 5	0.81
Angina severity	54 ± 7	58 ± 7	0.76
Angina frequency	42 ± 5	49 ± 7	0.19
Treatment satisfaction	89 ± 2	88 ± 3	0.57
Disease perception	51 ± 4	57 ± 6	0.37
Inflammatory markers			
MPO (ng/mL)	24.2 ± 7.1	17.9 ± 3.2	0.43
CRP (mg/L)	5.75 ± 1.78	4.28 ± 1.08	0.39
Oxidative stress parameters			
MPO (ng/mL)	0.17 ± 0.02	0.16 ± 0.02	0.79
Homocysteine (μmol/L)	10.13 ± 0.6	10.15 ± 0.6	0.96
Endothelial markers			
ADMA (μM)	0.58 ± 0.02	0.53 ± 0.01	0.03
SDMA (μM)	0.54 ± 0.01	0.53 ± 0.02	0.76
Arginine (μM)	104.3 ± 8	104.7 ± 9.1	0.93

Figure 4.2. Maximal change in AIx during the 20 minute time period of GTN and salbutamol administration, following placebo or bosentan therapy (n=16). Values expressed as mean ± SEM. Grey shaded boxes represent arterial responses to GTN. Solid black boxes represent arterial responses to salbutamol.



4.4 Discussion

This novel study was designed to assess the anti-anginal efficacy of bosentan in the CSFP based upon the accumulating evidence that ET-1 plays an important role in the pathogenesis of this disorder. Furthermore, the secondary endpoints were designed to explore potential mechanisms for any observed improvement as well as examining the impact of the therapy on health status. Unfortunately, the study demonstrated no benefit in the clinical endpoints of total angina frequency, prolonged angina episodes, nitrate consumption or health status with bosentan therapy. Moreover, there was no change in inflammatory/oxidative stress biomarkers, endothelial biomarkers (except for a small improvement in ADMA) or endothelial function measures, consistent with the clinical responses.

ET-1 has been implicated in the pathogenesis of the CSFP. Previous studies have shown increased plasma ET-1 and decreased concentrations of NO in CSFP patients following exercise testing and rapid atrial pacing activities (275). Pernow et al, intravenously infused ET-1 into healthy control subjects and observed a fall in coronary sinus oxygen saturations from 35±1% to 22±2%, thereby suggesting increases in coronary microvascular resistance (278). Moreover, the post-infusion coronary sinus saturations observed were similar to those measured in patients with the CSFP (23±4%) and significantly lower than controls (31±4%) (297). Unpublished studies by our group utilising isolated subcutaneous microvessels obtained from healthy controls and CSFP patients, have also demonstrated a

selective increase in vascular reactivity to ET-1 but not other vasoconstrictor agonists in the CSFP patients as compared with controls.

Despite this substantive evidence implicating ET-1 in the pathogenesis of the CSFP, the results of this study suggest that ET-1 receptor blockade with bosentan, does not modulate clinical or biological responses in patients with stable symptomatic angina associated with the CSFP. Hence, although ET-1 infusion appears to induce the CSFP, dual ET_A and ET_B receptor blockade does not alleviate angina symptoms, suggesting that alternative mechanisms may be responsible for these symptoms. In contrast to previous studies (268-271), we have recently reported that CSFP patients have normal endothelial function, inflammatory and oxidative stress markers (Kopetz et al, *in press*). In the current study, these biomarkers were not altered by bosentan, similar to the clinical symptoms. Thus, the findings would suggest that the ET-1 pathway has a limited role in the pathogenesis of symptoms in the CSFP.

An alternative interpretation is that ET-1 does play a role in the pathogenesis of the CSFP but ET-1 blockade with bosentan is ineffective. ET_A and ET_B receptors are found on vascular smooth muscle cells and produce a vasoconstrictor response when stimulated. Additionally, ET_B receptors are also found on endothelial cells and when activated, stimulate the release of nitric oxide as well as having direct constrictor effects. Whether ET_B receptor blockade limited the anti-anginal efficacy of bosentan is unknown and perhaps specific ET_A receptor

blockers should be considered in the future. Also, whether higher doses of bosentan may have been effective is unknown.

The sample size determined in this study was based upon our previous experience with this trial design in CSFP patients treated with mibefradil. In that study, mibefradil reduced angina frequency by 56% compared with placebo (median total angina frequency [25,75QI]: placebo = 34 [11,56]; mibefradil = 8 [3,25]). The negative findings in the present study would suggest that bosentan is not as effective as mibefradil in reducing angina frequency in patients with the CSFP. However, whether bosentan has some mild anti-anginal effects in this condition cannot be excluded, since there is a possibility that the study is limited by a Type-II error. Post-hoc power calculations would suggest that the study was only sufficiently powered to detect a 28% change in angina frequency. Thus, a larger study may show a mild anti-anginal effect but whether this would be clinically relevant is subject to speculation.

4.5. Conclusion

In conclusion, this study has not demonstrated a large anti-anginal benefit of administering bosentan 125mg twice/daily in CSFP patients. However, a small benefit cannot be excluded and requires a larger, more appropriately powered study. Thus continued investigations are required into this intriguing disorder, which still does not have an effective therapy available for its associated recurrent angina.

CHAPTER V

5. Thesis Conclusion

The overall aim of this thesis was to investigate the clinical and biological factors contributing to the pathogenesis of the CSFP, and identify a potential effective therapy for the treatment of its associated symptoms. The results obtained from these studies provide further directions to our understanding of this curious condition.

This thesis employed a variety of experimental designs and techniques to achieve the above aims including a novel plasma proteomic analysis, a controlled study examining potential biological factors responsible for the condition, and a randomised, placebo-controlled, double-blind, cross-over trial evaluating the anti-anginal efficacy of bosentan. The specific objectives and main findings for each investigation are summarised below.

Chapter 2 investigated the potential mechanisms responsible for the acute coronary syndrome presentation in patients with the CSFP by obtaining plasma samples from 6 affected patients during their coronary care unit admission, and repeating the analysis one month later when the acute symptoms had resolved. Plasma proteomic analyses identified up-regulation of inflammatory markers (ACT, AAT, CBG and LRα2GP) and the oxidative stress-related protein (PON-1) during the ACS admission. These findings were validated using clinical immunoturbidmetric and western blotting analysis techniques, for hsCRP and AAT respectively (n=9). This study therefore implicates an

inflammatory/oxidative stress process in the pathogenesis of the ACS presentation associated with the CSFP, and is consistent with previous acute studies.

In Chapter 3, attention was focused on the possible pathophysiological mechanisms involved in the chronic phase of patients with the CSFP. Specifically, the role of endothelial dysfunction, inflammation and oxidative stress were investigated using a validated pulse-wave analysis method to assess endothelial function and established assays for MPO, hsCRP, MDA, homocysteine, and ADMA. Compared with age-matched healthy controls, the CSFP patients had intact endothelial function, and no significant abnormalities in ADMA, inflammatory molecules (MPO, hsCRP), or oxidative stress markers (MDA, homocysteine). Thus, unlike the acute presentation, this study suggests that abnormalities in inflammation or oxidative stress do not play a major role in the pathogenesis of the chronic phase of the CSFP.

Chapter 4 was designed on the basis of previous studies that have implicated endothelin in the pathogenesis of the CSFP. This study investigated the biological and anti-anginal efficacy of a non-selective endothelin antagonist (bosentan) in patients with established CSFP. Using a randomised, double-blind, placebo-controlled, cross-over study design, the study demonstrated that bosentan therapy did not significantly reduce total angina frequency, prolong angina episodes or nitrate consumption, compared to placebo treatment. Furthermore, bosentan did not significantly change health-related quality of life parameters (SF-36, Seattle

angina questionnaire) endothelial function (endothelium-dependent vasodilation) or alter inflammatory/oxidative stress markers (MPO, MDA, hsCRP, homocysteine), despite a significant increase in ADMA plasma levels. Accordingly, based upon these findings, bosentan does not have a significant role in the treatment of the CSFP although a small effect cannot be excluded.

Based on the results of all three studies, we propose that: a) inflammation and oxidative stress mechanisms play a key role in the acute presentation in CSFP patients; b) the same inflammatory processes are not evident in the chronic phase (as hsCRP plasma levels were normal); c) the regulatory mechanisms explored during the chronic phase failed to identify abnormalities in endothelial function and oxidative stress; and d) bosentan therapy does not have a major clinical or biological impact on this condition although benefits in a small sub-group of CSFP patients cannot be excluded.

For the first time, this thesis has demonstrated that different pathophysiological mechanisms are involved in the acute and chronic phases of the CSFP. Whereas inflammatory and oxidative stress markers are elevated in the acute phase, these biomarkers are at similar levels to controls in the chronic phase. This pathophysiological observation has important clinical implications, as different therapeutic targets may need to be considered in both the acute and chronic phases.

As previously discussed, the endothelium is intimately involved in inflammatory and/or oxidative stress parameters. The question that remains to be answered, however, is whether endothelial dysfunction is responsible for the increased inflammatory/oxidative stress profile identified in the acute phase of the CSFP. The purpose of the study outlined in Chapter 3 was to identify whether specific biological markers of endothelial dysfunction were evident in a chronic sub-set of CSFP patients. Although it was discovered that there were no significant changes in the assessed endothelial dysfunction parameters, these patients did not present with an ACS. Thus, it remains unknown whether endothelial dysfunction is present specifically in the acute phase of the condition. Following confirmation of an increased inflammatory and oxidative stress status in the acute phase of the CSFP, future studies should now focus on elucidating the specific mechanisms responsible during the acute presentation.

Consideration should also be made as to whether high-throughput proteomic investigations should continue being used for future acute phase studies. Given the relative ease of obtaining blood samples for analysis, proteomic analysis provides a unique opportunity to investigate plasma protein profiles for a multitude of conditions. Although such an approach may provide valuable insights, careful consideration must be taken to the study design parameters and desired research objectives. Gel electrophoresis techniques have the technical limitations of only separating proteins within a certain molecular range and thus may not identify clinically significant differences in important peptide molecules (such as endothelin or neuropeptide Y). A combined approach of gel

electrophoresis, specialised mass spectrometry (to target small peptides) and targeted bioassay techniques could provide a more comprehensive overview of complete protein profiles during an acute phase study.

The quest to identify an effective treatment for anginal symptoms in the CSFP patients remains a challenging task. Given the substantial amount of evidence surrounding the role of ET-1 in CSFP pathology, a study investigating the efficacy of an ET-1 receptor blockade in improving symptomatic status was warranted. Bosentan was chosen as the therapeutic agent in the current clinical study due to the fact that it is a non-selective, dual ET-1 receptor blocker. Although a significant change in anginal symptoms was not evident, the benefit of bosentan therapy in selected groups of patients still needs further investigation. Decreases in angina frequency and severity when compared to placebo treatment (as identified by the median values) were evident despite not reaching statistical significance. This result suggests that the various mechanisms that cause anginal pains may not be solely attributable to ET-1 mediated vasoconstriction. Differences in receptor binding affinity, anatomical location of ET-1 receptors and endogenous ET-1 levels may have played a role in the varied responses observed. An array of other vasomotor mechanisms in addition to other neuronal, hormonal, metabolic and psychological factors that contribute to actual and perceived pain, also need to be taken into account both individually and together as a whole. Furthermore, the possibility that bosentan may exert a different effect in the acute phase compared to the chronic phase also needs consideration.

Designing future clinical trials that will adequately assess the efficacy of a therapeutic agent in CSFP patients will require forethought. Some important factors to consider include: identifying sub-populations of CSFP patients that will respond better to bosentan and other therapeutic agents; experimentation with different bosentan dosages; administering selective ET-1 receptor blockers; using a combination therapy approach with other therapeutic agents; and assessing changes in angina symptoms during either acute or chronic symptomatic status. More detailed investigations into the mechanisms and molecular pathways involved in the pathophysiology of the CSFP will enable a framework for future studies to be created.

The results of the studies in this thesis provide a new platform for future investigations. Differentiating between the acute and chronic presentations provide opportunities for subsequent studies to be conducted that focus on identifying the specific mechanisms behind the unique clinical profiles. During the acute phase, future studies should focus on determining the mechanisms behind the enhanced inflammatory and oxidative stress state by further elucidating the role of the specific markers identified. Future studies in the chronic phase should focus on the role of other non NO-mediated vasomotor pathways in the pathophysiology of the condition in addition to the role of neuronal, humoral and psychological influences. Given the heterogeneity of the CSFP patients, further work should also focus on identifying sub-populations within both the acute and chronic patient cohorts that could potentially make them more susceptible to developing severe and frequent chest pain symptoms, and

non-respondent to specific therapeutic interventions. Achieving such a task would involve undertaking a multitude of different studies (e.g. genetic polymorphism, electrophysiological, mechanistic, psychological) in order to obtain a comprehensive clinical and biological profile of CSFP aetiology.

Based upon the results of the current work, it is evident that further studies are required to fully elucidate the clinical and biological determinants of the CSFP. This thesis has set a new direction for future studies by clinically distinguishing between acute and chronic presentations, eliminating plausible mechanisms responsible for the pathophysiology during the chronic phase of this unique condition and identifying problems in clinical trial designs. Further re-defining the CSFP and attempting to understand the causes of heterogeneity involved in this disorder may greatly aid future research efforts in identifying an effective therapy.

APPENDICES

Appendix A – Publication 1

Potential Mechanisms of the acute coronary syndrome presentation in patients with the Coronary Slow Flow Phenomenon – Insight from a plasma proteomic approach.

International Journal of Cardiology 2012;156:84-91

Appendix B – Publication 2

Endothelial function, oxidative stress and inflammatory studies in chronic Coronary Slow Flow Phenomenon patients

Cardiology 2012;121:197-203

Appendix A

Kopetz, V.A., Penno, M.A.S., Hoffmann, P., Wilson, D.P. & Beltrame, J.F. (2012) Potential mechanisms of the acute coronary syndrome presentation in patients with the coronary slow flow phenomenon - insight from a plasma proteomic approach.
International Journal of Cardiology, v. 156(1), pp. 84-91

Appendix B

Kopetz, V., Kennedy, J., Heresztyn, T., Stafford, S.R. & Beltrame, J.F. (2012) Endothelial function, oxidative stress and inflammatory studies in chronic coronary slow flow phenomenon patients. *Cardiology, v. 121(3), pp. 197-203*

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