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Assessing the Immune Phenotype of Cardiac Syndrome X: A Prospective Study of Biomarkers

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Thesis submitted for the degree of Doctor of Philosophy

Under the supervision of Prof. Timothy G. Dinan

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March, 2016





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Declaration

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism.

Author Contributions

All of the work within this thesis was conducted independently by the author with the following exceptions:

Chapter 5: Dr. Gerard Clarke assisted the author to perform the high performance liquid chromatography (HPLC) analysis of the plasma samples to produce the tryptophan and kynurenine data.

Chapter 6: Dr. Karen Scott assisted the author to perform the extraction of RNA from plasma samples to allow for miRNA analysis. Exiqon A/S performed the Next Generation Sequencing (NGS). Dr. Gerard Moloney assisted the author to perform the quantitative PCR used to validate the NGS results.

Chapter 7: Ms. Elaine O'Brien performed the gas chromatography used to analyse the fatty acid profiles of the plasma samples.

Signed

_____

James Dollard

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"Those masterful images because complete
Grew in pure mind, but out of what began?
A mound of refuse or the sweepings of a street,
Old kettles, old bottles, and a broken can,
Old iron, old bones, old rags, that raving slut
Who keeps the till. Now that my ladder's gone,
I must lie down where all the ladders start
In the foul rag and bone shop of the heart."

The Circus Animals' Desertion, W.B. Yeats

Publications and Presentations

Published Papers

1. Dollard J, Kearney P, Clarke G, Moloney G, Cryan JF & Dinan TG. A prospective study of C-reactive protein as a state marker in Cardiac Syndrome X. Brain, Behavior, and Immunity 2014.
2. Dollard J, Kearney P & Dinan TG. Cardiac syndrome X in Ireland: incidence and phenotype. Irish Journal of Medical Science 2015.

Manuscripts Submitted/In Preparation

1. Dollard J, Clarke G, Moloney G, Kearney P, Cryan JF & Dinan TG. Inflammation in Cardiac Syndrome X: Implications for tryptophan metabolism and relevance to pathogenesis. *To be submitted to Heart.*
2. Dollard J, Moloney G, Scott K & Dinan TG. The microRNA signature of Cardiac Syndrome X: a signal of microvascular remodelling. *To be submitted to Circulation Research.*
3. Dollard J, O'Brien E, Stanton C & Dinan TG. Fatty acids in Cardiac Syndrome X: a role for diet and omega-3 supplementation? *In preparation.*

Conference Posters

1. Dollard J, Kearney P and Dinan TG. Cardiac Syndrome X in Ireland: Incidence and Phenotype. *65th AGM of the Irish Cardiac Society. Athlone, Ireland. October 2014.*

Oral Presentations

1. Dollard, J. (2016) Elevator Pitch. Inflammatory Biomarkers in Cardiac Syndrome X. *APC Microbiome Institute Symposium (Invited talk).*
2. Dollard J. (2013) Diagnosing and Managing Cardiac Syndrome X in Ireland. *Cardiology Departmental Meeting, CUH, Cork.*
3. Dollard J. (2013) Incidence and phenotype of Cardiac Syndrome X in Ireland. *Cardiology Study Day. CUH, Cork. (invited talk).*

Abbreviations

AA	Arachidonic Acid
ACE	Angiotensin Converting Enzyme
AFS	Angina Frequency Scale
AT	Angiotensin
ATP	Adenosine Triphosphate
AUC	Area Under the Curve
BSH	Bon Secours Hospital
CAD	Coronary Artery Disease
CCS	Canadian Cardiac Society
CFR	Coronary Flow Reserve
CM	Chylomicron
CMR	Cardiac MRI
CNS	Central Nervous System
COX	Cyclooxygenase
CPNCA	Chest Pain Normal Coronary Arteries
CREC	Clinical Research Ethics Committee of the Cork Teaching Hospitals
CRP	C-Reactive Protein
CRT	Coronary Reactivity Testing
CSFP	Coronary Slow Flow Phenomenon
CSX	Cardiac Syndrome X
CUH	Cork University Hospital
CV	Co-efficient of Variation
CVA	Cerebrovascular Accident
DHA	Docosahexaenoic Acid
DTS	Duke Treadmill Score
EC	Endothelial Cell
ECG	Electrocardiogram
EET	Epoxyeicosatrienoic Acid
EMT	Epithelial-Mesenchyme Transition
EndMT	Endothelial-Mesenchyme Transition
eNOS	Endothelial Nitric Oxide Synthase
EPA	Eicosapentaenoic Acid
EST	Exercise Stress Test
ET	Endothelin
ETS	E26 Transformation Specific
FMD	Flow-Mediated Vasodilation
GC	Gas Chromatography
GTN	Glyceryl Trinitrate
HDL	High Density Lipoprotein
HPLC	High Performance Liquid Chromatography
IBS	Irritable Bowel Syndrome
ICAM	Intercellular Adhesion Molecule
IDO	Indoleamine-2,3-Dioxygenase
IFN	Interferon
IHD	Ischaemic Heart Disease
KLF	Krüppel-Like Factor
KTR	Kynurenine:Tryptophan Ratio
KYNA	Kynurenic Acid
LCFA	Long Chain Fatty Acids
LCSX	Loose Cardiac Syndrome X

LDL	Low Density Lipoprotein
LGI	Low Grade Inflammation
LHC	Left Heart Catheterisation
LLOD	Lower Limit of Detection
LLOQ	Lower Limit of Quantification
LOX	Lectin-like Oxidized LDL Receptor
LPS	Lipopolysaccharide
LTE-Q	List of Threatening Experience
MCFA	Medium Chain Fatty Acid
MCP	Monocyte Chemoattractant Protein
MI	Myocardial Infarction
MPI	Myocardial Perfusion Imaging
MRI	Magnetic Resonance Imaging
MSD	Mesoscale Discovery
MUFA	Monounsaturated Fatty Acid
MUH	Mercy University Hospital
MVA	Microvascular Angina
NFκB	Nuclear Factor kappa-light-chain enhancer of activated B cells
NGS	Next Generation Sequencing
NK	Natural Killer
NO	Nitric Oxide
NSAID	Non-Steroidal Anti-Inflammatory Drugs
PAH	Pulmonary Arterial Hypertension
PCR	Polymerase Chain Reaction
PET	Positron Emission Tomography
PGI_2	Prostacyclin
PLS	Physical Limitation Score
PSS	Perceived Stress Scale
PUFA	Polyunsaturated Fatty Acid
QOL	Quality of Life
RISC	RNA Induced Silencing Complex
ROC	Receiver-Operating Characteristic
RPP	Rate-Pressure Product
SAA	Serum Amyloid A
SAQ	Seattle Angina Questionnaire
SCFA	Short Chain Fatty Acid
SFA	Saturated Fatty Acid
SIRT1	Silent Mating Type Information Regulation 2 homolog 1
TDO	Tryptophan -2,3-Dioxygenase
TFA	Trans-fatty acid
TNF	Tumour Necrosis Factor
TRIG	Triglyceride
TRL	Triglyceride Rich Lipoprotein
TRP	Tryptophan
TRPV1	Transient Receptor Potential Cation Channel Subfamily V Member 1
TSS	Treatment Satisfaction Score
TXA_2	Thromboxane
VCAM	Vascular Cell Adhesion Molecule
VSMC	Vascular Smooth Muscle Cell

Abstract

Cardiac Syndrome X (CSX), the presence of angina pectoris with objective signs of myocardial ischaemia despite angiographically normal epicardial coronary arteries, appears to be due to coronary microvascular dysfunction and is known to be associated with an elevation of several inflammatory biomarkers, suggesting a possible role for inflammation in its pathogenesis. We aimed to further characterise this relationship by prospectively analysing a wide variety of molecular biomarkers in a cohort of CSX patients, thereby charting the changes in biomarkers throughout the natural history of CSX from its initial diagnosis to eventual disease quiescence. We followed a cohort of CSX patients from the time of their diagnosis through two further follow-up visits and compared their biomarkers to those of healthy age- and sex-matched controls.

We found that CSX patients have a persistent low-grade systemic inflammatory response characterised at all time points by an elevation of the cytokines Tumour Necrosis Factor (TNFα) and Interferon gamma (IFNγ), regardless of the presence or absence of contemporaneous signs or symptoms of disease activity. Interleukin-6 (IL-6) and C-reactive Protein (CRP), on the other hand, are only elevated when patients have clinical evidence of disease activity and may be state markers in CSX. Moreover, CRP levels appear to correlate with signals of disease severity such as the time taken to develop symptoms during exercise stress testing. Both IL-6 and CRP are capable of directly mediating endothelial dysfunction and we contend that they may be responsible for a temporary worsening of microvascular function during symptomatic periods. We have also demonstrated that the enzyme Indoleamine-2,3-dioxygenase is upregulated in active disease, with plasma tryptophan levels being reduced and the Kynurenine:tryptophan ratio being elevated in symptomatic CSX patients. This may provide an explanation for the increased burden of psychological disease encountered

in CSX patients in general and for the increased perceived stress and disproportionately reduced disease-related quality of life seen in our own CSX cohort.

In our analysis of the microRNA transcriptome we noted that miR-143 is significantly under-expressed in CSX patients. This might allow phenotype switching in vascular smooth muscle cells and lead to vascular remodelling. These cells may enter a proliferative and secretory phase responsible for increased extracellular matrix production and local medial hypertrophy. This would result in reduced vessel responsiveness to local rheological stimuli and reduced luminal diameter, resulting ultimately in relatively increased microvascular resistance during times of increased myocardial oxygen demand, thereby limiting maximal hyperaemia during exercise.

Taken together, these findings corroborate many previous hypotheses regarding the role of inflammation in CSX, generate new insights into possible pathogenic mechanisms and offer new therapeutic targets for the future management of this important cardiological condition.

Chapter 1: General Introduction



Cork University Hospital



Bon Secours Hospital, Cork.



Main Quadrangle, University College Cork.



Brookfield Health Sciences Complex, University College Cork.



Biosciences Institute, University College Cork.

1.1 Introduction

Cardiac Syndrome X (CSX) is the presence of typical anginal chest pain, with objective evidence of ischaemia, no evidence of epicardial coronary artery stenosis on coronary angiography and in the absence of structural heart disease [1]. CSX is the poor relation of epicardial Coronary Artery Disease (CAD). The latter has garnered an abundance of attention and well-funded research while CSX has received considerably less. This is partly due to the fact that, while CAD is one of the most potent killers worldwide, CSX has been shown to have a very favourable prognosis in terms of mortality. Otherwise, CSX does its best to imitate its kin. The pain it causes is indistinguishable from the angina from atherosclerotic CAD, its symptoms can also persist for years after diagnosis and its impact on the quality of life of its sufferers is equivalent to that of patients with CAD [2,3]. Additionally, all but the best invasive medical tests are unable to distinguish between CSX and CAD.

CSX imposes a large burden on the healthcare system by causing frequent hospitalisations and necessitating costly investigations, often repeatedly in the same patient. It also usually results in on-going medication requirements as well as necessitating long-term follow-up with primary care physicians while also causing patients to re-present to cardiology services. Given that it imposes such a burden on both patient and healthcare-system it is frustrating that after 46 years its aetiology and effective treatment strategies remain largely undetermined due in no small part to the vastly contradictory results of research in this area. The prevailing consensus now is that most of the patients with CSX likely suffer from microvascular angina, relative ischaemia of the myocardium during exercise due to dysfunctional coronary microvessels such as resistance arterioles, which may be affected by chronic low-grade inflammation. Another purported mechanism is the “Sensitive Heart” theory, which holds that CSX patients are especially sensitive to cardiac stimuli and can suffer from angina even in the absence of substantial ischaemia [4].

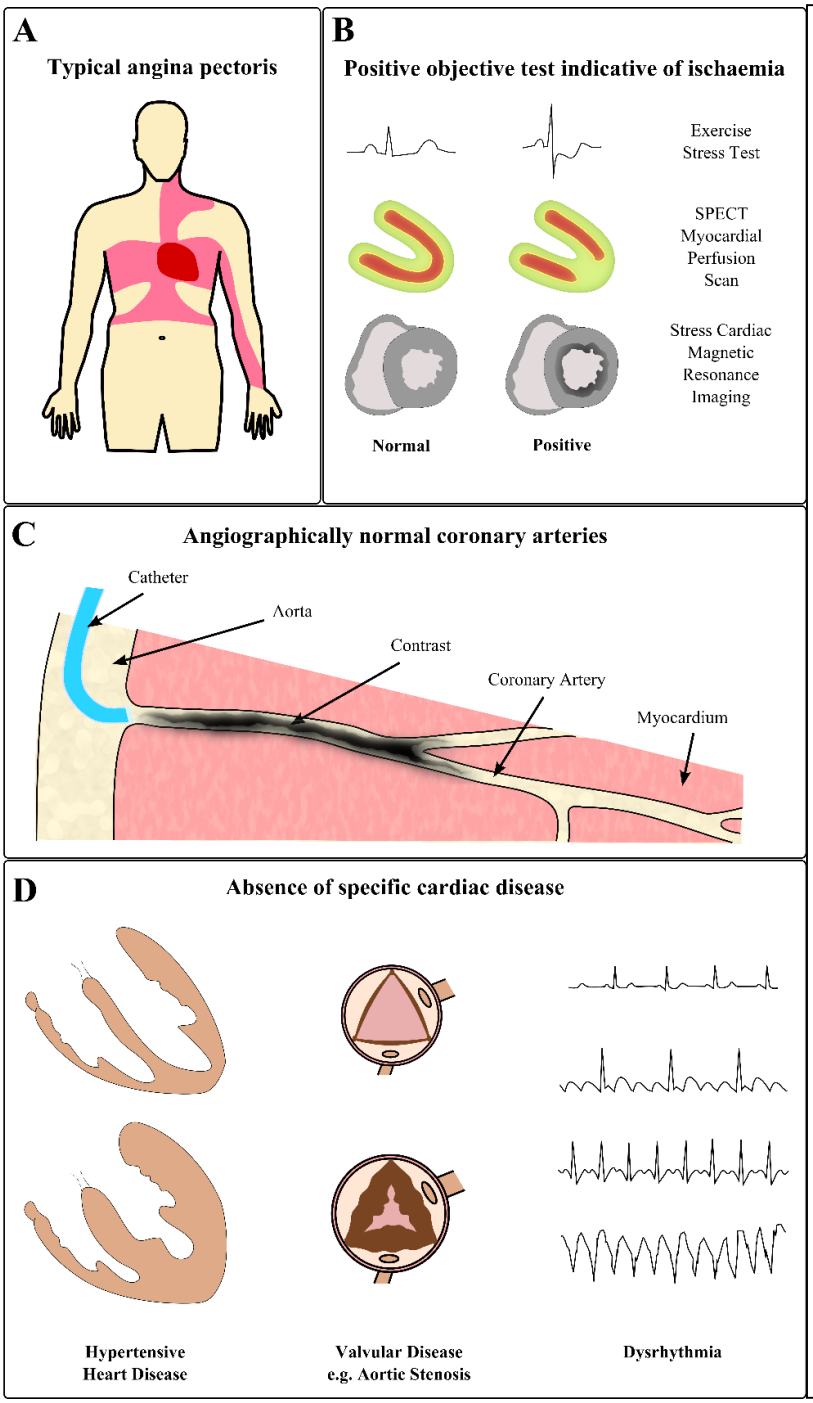
The ischaemic aetiology is questioned, however, due to the lack of irrefutable evidence of true myocardial ischaemia. Exercise Stress Testing (EST) and Myocardial Perfusion Imaging (MPI) using isotope scans are useful investigations and are sufficient to facilitate the diagnosis of CSX but both are susceptible to providing false-positive results. Furthermore, even positive results in these tests are only suggestive of ischaemia and not diagnostic of such. Studies looking at other indicators of potential ischaemia such as stress-induced wall-motion abnormalities during echocardiography and metabolic studies examining myocardial ischaemic metabolite production CSX have had contradictory results [5-9]. More modern imaging techniques such as Cardiac MRI (CMR) and Positron Emission Tomography (PET) studies have both demonstrated and failed to demonstrate myocardial hypoperfusion and reduced coronary flow reserve in patients with angina but normal coronary arteries [10-12]. The widely disparate findings in all of these studies is partly due to the varying inclusion and exclusion criteria used in each. All facets of the diagnostic criteria of CSX are susceptible to subjective biases and subtle changes in criteria may produce hugely different cohorts of patients. This will be discussed further in section 1.2.1, the diagnostic criteria.

Importantly, CSX is not in the everyday vocabulary of most practicing cardiologists and its true incidence in Ireland is undocumented. That notwithstanding, a typical cardiology department will encounter at least 30 of these patients every year but these will likely go undiagnosed due to the low profile of CSX.

1.2 Diagnosis

Unfortunately, research into CSX has been hampered by the inconsistency in its definition. There is no universal definition of CSX nor has it been codified by the two large cardiology associations, the American Heart Association (AHA) and the European Society of Cardiology (ESC). Despite this, the most commonly used contemporary diagnostic criteria were propounded by Lanza in 2007 and are described in section

1.2.1 below [1]. These criteria were utilised as the inclusion criteria for the patients described in this thesis. The most recent ESC guidelines on the management of stable coronary artery disease, however, describe the evolution of the diagnosis of CSX into 'microvascular angina,' (MVA) a term chosen to reflect the most likely pathogenic



A. Diagram showing the typical distribution of referred cardiac pain (in pink) with the surface overlying the heart being shown in red.

B. Diagram showing three commonly used diagnostic tests demonstrating ischaemia; the exercise stress test (with ST-depression), single-photon emission computed tomography (SPECT, with an apical defect) and cardiac MRI (with sub-endocardial hypo-enhancement).

C. Diagram showing invasive coronary angiography. The catheter is introduced in a retrograde fashion into the coronary arteries. Contrast is then injected and a fluoroscopic image is obtained to illustrate an outline of the vessel lumen.

D. Other Cardiac conditions that may lead to angina pectoris and a positive EST preclude a diagnosis of CSX.

Figure 1.1: Diagnostic Criteria for Cardiac Syndrome X

mechanism. While tests of microvascular function can be performed to further strengthen the diagnosis of CSX or MVA, at present they are not necessary to achieve the diagnosis, which is usually made by exclusion, as these tests carry not-inconsiderable procedural risks[13,14]. Thus, CSX and MVA have generally been used as synonyms, although not all CSX patients have demonstrable microvascular dysfunction.

1.2.1 Diagnostic Criteria

In order to make the diagnosis of CSX/MVA several criteria must be met (See *Fig. 1.1*). As alluded to in section 1.1, most of these criteria are open to interpretation and consequently many studies have had subtly but significantly different entry criteria. For this thesis it was decided to be quite strict in the adherence to stringent criteria so as to maximise the likelihood that all patients diagnosed with CSX truly had a cardiac cause for their pain. The most cogent criteria were codified by Lanza et al and are adhered to in this thesis[1]. Thus, to have a diagnosis of CSX the patients must have satisfied all of the following conditions:

a) Angina Pectoris

The cardinal symptom of CSX is angina pectoris. Angina in CSX is clinically indistinguishable from that seen in CAD. It was first described by Heberdon in 1772:

> *"They who are afflicted with it, are seized while they are walking, (more especially if it be up hill, and soon after eating) with a painful and most disagreeable sensation in the breast, which seems as if it would extinguish life, if it were to increase or continue; but the moment they stand still, all this uneasiness vanishes….. The pain is sometimes situated in the upper part, sometimes in the middle, sometimes at the bottom of the os sterni, and often more inclined to the left than to the right side. It likewise very frequently extends from the breast to the middle of the left arm.."*[15]

Chest pain is categorised by the AHA and ESC into typical angina, atypical angina and non-cardiac chest pain based on the presence or absence of the criteria shown in table 1.1 below. Patients with ≤ 1 of these criteria have non-cardiac chest pain and are generally at much lower risk of obstructive coronary artery disease, patients with 2 have atypical (but probable) angina and patients with all three have typical (or definite) angina pectoris, with increasing likelihood of significant CAD as more criteria are met (prevalence of >50% stenosis of 0.47, 0.75 and 0.92 for non-cardiac, atypical and typical angina respectively based on Diamond's data) [16]. Research by Kaski shows that CSX patients tend to have retrosternal chest pain of gradual onset, which may persist for >15 minutes in a third of cases and which is related in all cases to exertion but may also occur at rest in 41% [17].

Table 1.1 ESC Clinical Classification of Chest Pain, 2006, based on work by GA Diamond.

Typical Angina (Definite)	All 3 of the following characteristics: ▪ Substernal chest discomfort of characteristic quality and duration ▪ Provoked by exertion or emotional stress ▪ Relieved by rest and/or GTN
Atypical Angina (Probable)	Meets 2 of the above characteristics
Non-cardiac Chest Pain	Meets ≤1 of the above characteristics

Only patients with typical/definite angina were eligible for inclusion in the CSX group used in this thesis so as to homogenise the group and to minimise the potential of including patients with non-cardiac causes of chest pain. Even this was open to interpretation, however, as the "characteristic quality" is not specified. For this study, pain was noted to be uncharacteristic if it was of atypical character (e.g. pleuritic,

radicular), of excessive duration (e.g. lasting several hours without relief) or being atypical in location (e.g. cervical spine, sub-scapular).

b) Positive objective test suggestive of ischaemia

The vast majority of studies into CSX have included exercise stress testing (EST) as the non-invasive investigation of choice to illustrate possible ischaemia. A few studies, however, use radionucleotide myocardial perfusion imaging for enrolment while coronary reactivity testing and coronary sinus metabolite analysis have also been used. It should also be mentioned that in the past not all studies required positive ischaemic testing to diagnose CSX, with some requiring merely angina and a normal angiogram to make the diagnosis, further highlighting the inconsistency amongst research protocols in this area. An EST involves monitoring the surface 12-lead electrocardiogram (ECG) while a patient walks on a treadmill following a predetermined protocol. The most commonly used protocol is the Bruce protocol where the pace and incline of the treadmill increases at 3 minute intervals. ECG changes suggestive of ischaemia include ST-depression, ST-elevation and ventricular dysrhythmia.

For this thesis, an electrically positive treadmill-based EST was used as an inclusion criterion for CSX. An EST was considered electrically positive if there was ≥1mm of horizontal or down-sloping ST-depression 80ms after the j-point on the stress electrocardiogram. An EST was considered symptomatically positive if it elicited typical angina chest pain in the patient. ESTs have a sensitivity of 75-90% and a specificity of 70% for CAD and it is important to note that CSX would constitute a “false-positive” EST for CAD. The specificity of EST for ischaemia in CSX is unknown but indeed a “false positive” EST can be associated with microvascular dysfunction[18].

c) Angiographically normal coronary arteries

Coronary angiography or left heart catheterisation (LHC) is the gold standard investigation to investigate suspected coronary artery disease. It involves cannulating a major artery (e.g. femoral or radial) and passing catheters retrogradely until they reach the coronary arteries, where contrast is used under fluoroscopic guidance to outline the lumina of the arteries. Again the literature is somewhat hazy on what constitutes a normal coronary angiogram. Some studies have used <50% decrease in luminal diameter, others have used <20% while still others have insisted on perfectly smooth coronary arteries. For this thesis, an angiogram was considered normal if the coronary arteries were smooth or had minimal luminal irregularity (<10%) as determined by two independent reviewers. It should also be noted that a normal coronary angiogram only ensures the absence of macroscopic obstructive lesions in the epicardial coronary arteries. It does not imply complete normality of the arteries (as CSX patients do not have normal arterial vasomotor function, as will be discussed later). It should also be pointed out that fluoroscopic coronary angiography lacks the resolution to evaluate the coronary microvasculature, the proposed site of dysfunction in CSX.

d) The absence of other causative cardiac conditions

CSX can be a diagnosis of exclusion. Once a patient has appropriate symptoms, objective evidence of ischaemia and a normal angiogram they become a possible case of CSX/MVA. It is only if they do not have any other disease capable of causing angina and a positive EST that they are labelled CSX/MVA. Some of these conditions are described in table 1.2 below. In addition, one should always entertain the possibility that a patient has a non-cardiac cause of chest pain and a real false-positive EST. Some gastrointestinal conditions (such as oesophageal spasm) present with a retrosternal squeezing sensation, which can be relieved by nitrates. See section 1.2.2 for further discussion on this subject.

Table 1.2: Other potential causes of angina, a positive EST and a normal LHC.

Condition	Effect
Valvular Heart Disease	Stenotic lesions may result in myocardial hypertrophy, diastolic dysfunction and angina pectoris. Myocardial hypertrophy increases the chance of EST positivity.
Hypertensive Heart Disease	Increases ventricular afterload with consequent hypertrophy and diastolic dysfunction.
Dysrhythmia	Tachyarrhythmias may produce rate-related ischaemia during EST as well as leading to symptoms.
Diabetes Mellitus	Can lead to secondary microvascular dysfunction, which is considered a separate diagnosis to primary MVA/CSX.
Cardiomyopathy	Dilated, hypertrophic and restrictive cardiomyopathy can all lead to ventricular dysfunction, increased wall tension and symptoms of angina with positive ischaemic testing.

Thus, on the surface the diagnosis of CSX appears to be relatively straight forward. There are two chief problems relevant to this thesis, however. First, even patients with atypical angina can have obstructive CAD, implying that atypical angina of microvascular aetiology similarly exists. The exclusion of all patients with atypical angina from this study will almost certainly have excluded some patients with microvascular angina/CSX but with the trade-off that all patients in this study had typical symptoms of CSX, increasing the reliability of the diagnosis but perhaps limiting the generalisability of the results. Second, the degree to which exercise stress testing for CAD is actually falsely positive rather than diagnostic of microvascular angina is unknown. For example, the specificity of an EST for obstructive CAD is 75%. Of the 25% with a false-positive EST, some will have true CSX in which case the test wasn't actually falsely positive for ischaemia. Some authors have simply taken patients with typical

angina and a normal LHC, forgoing the EST. Some of these patients were also recruited for comparison in the present study and will hereafter be termed 'loosely' diagnosed CSX, or LCSX. Both CSX and LCSX are subsets of patients with chest pain and normal coronary arteries (CPNCA).

1.2.2 Clinical Features

The clinical characteristics of patients with CSX were well elucidated in a study by JC Kaski et al in 1995 [17]. In this study of 99 patients with CSX they noted that the chest pain was predominantly gradual in onset, lasted anywhere from <5 minutes (in 21%) to over a quarter of an hour (in 33%) and was related to exertion in 99% of cases and also occurred at rest in 42%. The chest pain was predominantly retrosternal (84%) with radiation occurring to the neck in 7%, left arm in 35% and arm and neck in 19%. Patients on average had 3-7 episodes of angina per week. Physical examination in CSX is usually normal. In Kaski's cohort 81% had normal baseline ECGs with 11% having flattened T-waves and a further 7% having ST-depression. Interestingly, only 24% actually had reversible perfusion abnormalities on myocardial perfusion scintigraphy. Non-invasive testing showed that the EST became positive at an average of 324 ± 246s (modified BRUCE protocol) while Holter monitoring revealed ambulatory ST-depression in 64% of cases, which were associated with pain in only 49% of cases. Similarly, many episodes of chest pain occurred in the absence of documented ST-depression on Holter monitoring. Echocardiography is also normal by case definition.

1.2.3 Differential Diagnoses

Chest pain with normal coronary arteries (CPNCA) encompasses a heterogeneous population with a myriad of causes of chest pain, both cardiac and non-cardiac. In fact, only 20% of patients with CPNCA will have typical angina pectoris, strongly suggesting a cardiac cause in only these cases. The remainder will have either a non-cardiac source

for their chest pain or cardiac chest pain with atypical features. Determining the cause of chest pain in these patients is oftentimes difficult and requires a systematic approach.

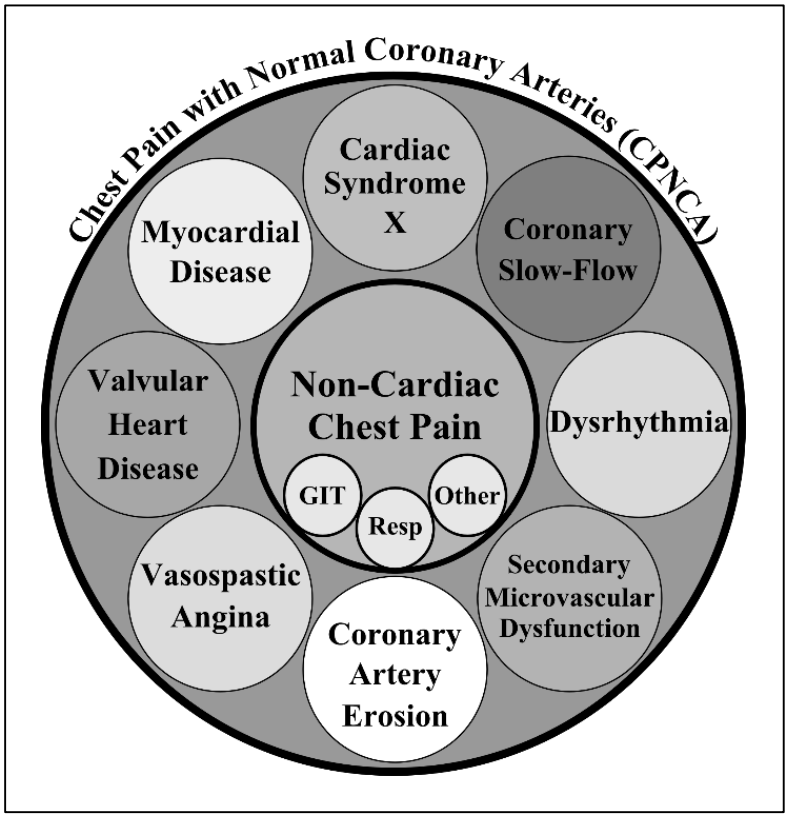


Figure 1.2: The Differential Diagnosis of Chest Pain with Normal Coronary Arteries. The majority will be non-cardiac (inner circle) in nature with normal stress testing and may originate from gastrointestinal (GIT e.g. GORD, cholelithiasis), respiratory (Resp. e.g. LRTI, PE) or psychological sources. Cardiac causes (outer perimeter) are legion and many of these will induce a positive EST.

The first step is to undertake a thorough history and examination looking for features of atypical chest pain or symptoms and physical signs to indicate an alternative diagnosis. Examples would include chest wall tenderness in musculoskeletal chest pain and pain brought on by ingestion of alcohol or fatty food in GI disturbances. The EST will further exclude many patients with truly non-cardiac sources of chest pain such as lower respiratory tract infection (LRTI). Unfortunately, the EST may be falsely-positive (mimicking the diagnosis of CSX) or negative (potentially missing patients with microvascular angina) and so cannot be relied on fully. Other tests such as holter

monitoring, echocardiography, provocation tests on coronary angiography, coronary reactivity testing (CRT) or oesophago-gastroduodenoscopy (OGD) may be indicated to exclude other causes of chest pain as directed by the history, examination, stress test or angiographic findings. Figure 1.2 shows the main differential diagnoses to be excluded.

1.2.4 Related Microvascular Conditions

CSX, Coronary Slow Flow and Microvascular Angina

The diagnostic criteria for CSX are designed to exclude patients with non-cardiac chest pain (by only selecting patients with typical symptoms) and to select patients with chest pain due to myocardial ischaemia that is not due to epicardial coronary artery disease or structural heart disease. It is believed that microvascular dysfunction is the main cause of symptoms in this group. Even this highly selected group is believed to be heterogeneous and there are several related conditions that are believed to be caused by microvascular dysfunction, namely Cardiac Syndrome X (CSX), Microvascular Angina (MVA) and the coronary slow flow phenomenon (CSFP), which has also been referred to as Cardiac Syndrome Y.

As mentioned above, CSX and MVA have been used synonymously, perhaps unwisely, since the inception of CSX as a condition. In recent times CSX has been equated to stable primary microvascular angina and may be renamed as such [14]. Most recently, however, the term microvascular angina has been reserved, at least in a research setting, for patients with demonstrable coronary microvascular dysfunction on invasive coronary reactivity testing (CRT). The only real difference between CSX and MVA is that all MVA patients have undergone confirmatory CRT to diagnose coronary microvascular dysfunction while CSX patients are only presumed to have it. This may be a reasonable approach, however, as reactivity testing is a potentially hazardous procedure and its

sensitivity for ischaemia-inducing microvascular dysfunction is not known. It currently has a Class IIb level C recommendation in the 2013 ESC Stable Coronary Artery Disease guidelines, meaning that, while it may be considered in special cases, its value is not well established. Furthermore, over 75% of patients with CSX will have reduced coronary flow reserve and an even higher proportion will have impaired peripheral flow-mediated dilatation, a marker of peripheral endothelial function[19-22]. Additionally, MVA is having the same identity crisis in terms of diagnostic criteria that CSX has endured and the necessity for objective evidence of ischaemia in the diagnosis is still under debate. If methods can be established to diagnose microvascular dysfunction more routinely and safely then it is likely that Cardiac Syndrome X will no longer exist as a diagnosis and the emphasis will be put on patients with proven microvascular angina[23]. Cardiac MRI and PET scanning are the most promising modalities that may fulfil this role in the future but at present are costly and time-consuming[24,25].

The coronary slow flow phenomenon (CSFP) is an angiographic diagnosis of presumed microvascular dysfunction. In this condition there is a slowing of the passage of contrast down the coronary arteries, taking 3 or more beats to fill the artery (so-called TIMI 2 flow) in the absence of obstructive coronary artery disease. Like the two other conditions above, there is disagreement on the diagnostic criteria for CSFP, including how many vessels need to exhibit slow flow to make the diagnosis and how stenosis-free the arteries must be. CSFP is believed to occur due to increased coronary microvascular resistance inhibiting the flow of contrast down the arteries. Like CSX, patients with CSFP have a high prevalence of recurrent chest pain (84% at 21 months) and high anxiety scores. A comparison between the 3 microvascular diagnoses is shown in the table 1.3 below.

Table 1.3: Conditions involving coronary microvascular dysfunction

Clinical Syndrome	Cardiac Syndrome X	Microvascular Angina	Coronary Slow Flow Phenomenon
Diagnostic Criteria	Typical Angina	Angina*	Angina
	Normal Coronary Arteries (<10% stenosis)	Non-obstructive coronary artery disease (<50% stenosis)*	Non-obstructive coronary artery disease (<50% stenosis)*
	Objective Evidence of ischaemia (EST, MRI, PET, MPI or metabolite studies) Absence of other cardiac disease	Evidence of impaired coronary microvascular function	TIMI 2 flow or corrected TFC>21±3 frames in the LAD, >22±4 in the LCx or >20±3 in the RCA
Nature of Angina	Typical exertional angina	Typical or atypical	Usually at rest
Stress Test	Positive by definition	Less commonly positive	Infrequently positive
Gender predominance	Female	Female	Male
Pathophysiology	Myocardial Ischaemia	Myocardial Ischaemia	Microvascular spasm
	Microvascular Dysfunction	Reduced microvascular vasodilatory responses	
	Endothelial Dysfunction	Abnormal cardiac pain perception	
	Abnormal cardiac autonomic regulation		
	Abnormal pain perception		
	Abnormal platelet function		

EST-Exercise Stress Test; **LAD**- Left Anterior Descending Artery; **LCx**. – Left Circumflex Artery; **MPI**-Myocardial Perfusion Imaging; **RCA**- Right Coronary Artery; **TFC**- TIMI frame count; **TIMI**- Thrombolysis in Myocardial Infarction. * Uncertain criteria at present.

1.2.5 Grading of Disease Severity

Once a diagnosis of CSX has been made, attempts should be made to quantify the burden of symptoms experienced by the patient. Symptoms may be graded using the simplest and most widely used classification of angina symptoms, the Canadian Cardiovascular Society (CCS) classification. This grades angina based on the minimum exertion required to elicit the chest pain and is numbered from I-IV. Table 1.4 illustrates this scale with an estimate of the metabolic equivalents (METS) of the inducing activity.

Table 1.4: Canadian Cardiovascular Classification of Angina

CCS Class	Physical Limitation	METS
CCS I	No limitation in everyday activities. Angina only brought on by strenuous or prolonged exertion	7-8
CCS II	Mild limitation of ordinary activity such as walking uphill, rapidly climbing stairs etc.	5-6
CCS III	Marked limitation of ordinary activity such as angina when walking on the flat or walking at a normal pace up one flight of stairs	3-4
CCS IV	Unable to carry out any physical activity without angina. Angina may occur at rest	1-2

METS-Metabolic Equivalents

Clinicians usually grade symptoms based on the history reported by the patient although the EST offers a more objective assessment by allowing the determination of several numerical estimates of physical limitation. These include total exercise duration, time to angina and ST-depression, Rate-Pressure Product (RPP) at first symptoms and at peak exercise and total METS achieved. Furthermore, several

questionnaires allow for self-assessment of disease burden. The Seattle Angina Questionnaire is a well-established questionnaire that provides the clinician with a validated estimate of disease burden. It generates 5 scores in different response categories (see table 1.5) with each scale ranging from 0 to 100, with higher scores being associated with better health outcomes.

Table 1.5: Seattle Angina Questionnaire (SAQ) Summary Scores and sample scores from the validation cohort of patients with coronary artery disease (CAD).

SAQ Summary Score	Sample Questions	CAD Score[26]
Physical Limitation (PL)	▪ Over the past four weeks, how much limitation have you had due to chest pain during the following activities? Climbing a hill, walking, cleaning etc.	50.2
Angina Stability (AS)	▪ Compared with 4 weeks ago how often do you have chest pain when doing your most strenuous activities?	52.0
Angina Frequency (AF)	▪ Over the past 4 weeks, on average, how many times have you had chest pain?	67.5
Treatment Satisfaction (TS)	▪ How satisfied are you that everything is being done to treat your pain? ▪ How bothersome is it for you to take your pills for your chest pain?	78.1
Quality of Life (QOL)	▪ Over the past 4 weeks, how much has your chest pain limited your enjoyment of life? ▪ How often do you think or worry that you may have a heart attack or die suddenly?	56.7

1.3 Epidemiology

Given the lack of consistency in the diagnostic criteria used in CSX it may come as no surprise that the incidence and prevalence of CSX worldwide is not known. The introductions to most studies in CSX contain the general statistic that up to 30% of LHCs performed to investigate angina pectoris are normal (CPNCA) but almost no study reports the number of patients screened in order to obtain the patient cohort. Any study that has recruited a large number of CSX patients has done so over several years (e.g. 164 patients in 6 years and 108 in 5 years [27,28]) In general, CSX is seen to be a condition that occurs in post-menopausal women.

1.3.1 Demographics

An analysis of the pooled results of studies into CSX found that 56% of patients with CSX were female and that the mean age was 53.8 ± 5.8 years [29]. The 57 studies included in this analysis, however, varied widely in their entry criteria. Table 1.6 below shows the demographics for CSX patients from studies in several different regions in the world all of which include the strictest entry criteria of typical chest pain, positive exercise stress testing and normal coronary arteries. These studies are in broad agreement that CSX predominantly occurs in middle-aged women who suffer from a variable number of cardiac risk factors.

Table 1.6: Selected studies from different global locations demonstrating the demographics of CSX patients

Study	Year	Country	n	Cardiac Risk Factor Prevalence					
				Gender	Age	HTN	Chol	Smoking	F.Hx.
Tritto	2009	Italy	350	72% F	61±10	65%	61%	22%	24%
Qing	2013	China	120	81% F	48±8	47%	36%	12%	9%
Kaski	1995	England	99	79% F	48.5±8	*	29%	29%	46%
Radice	1995	USA	30	73% F	61±6	-	17%	20%	-
Dollard	2014	Ireland	17	88% F	59±7	35%	82%	0%	65%
Ezhumalai	2015	India	35	50% M	53±9	53%	31%	18%	-

*Chol=Cholesterol, F.Hx.= Family History of ischaemia heart disease, HTN= Hypertension. * Patients with hypertension were specifically excluded from this study*

1.3.2 Incidence and Phenotype in Ireland

Only one paper specifically examining the incidence of CSX in a European setting has been published. This was performed in a Dutch hospital in 2003 and the investigators noted that 10% of patients attending for coronary angiography to investigate chest pain had normal coronary arteries and that only 3% had normal arteries and a positive exercise stress test thus achieving the diagnosis of CSX[29]. An Asian study showed that 3.5% of their coronary angiography patients had CSX by strict definitions[30]. In an effort to describe the incidence and phenotype in Ireland, we performed a prospective study of all patients attending the catheterisation laboratory in Cork University Hospital over a three-month period. This was published as a paper entitled, "Cardiac Syndrome X in Ireland: Incidence and Phenotype," and the results of this are discussed in chapter 2.

1.4 Pathophysiology

1.4.1 Mechanism of Angina

The pathways involved in the genesis and perception of angina pectoris deserve a brief description as an understanding of these allows one to speculate as to the possible pathogenesis of CSX. Angina is the pain caused by the visceral sensation of myocardial ischaemia. It is not proportional to the degree or distribution of ischaemia and so relatively mild ischaemia can lead to severe symptoms. An overview of the mechanism of angina is shown below in Figure 1.3.

a) Local Stimulus

The stimulus for angina begins at the myocardial cellular level. The accepted theory was propounded by Lewis in 1932 [31]. Known as the chemical theory, this states that local chemicals produced in ischaemic tissue trigger local nociceptive neurons resulting in perceived pain. Myocardial cells are highly metabolically active and require constant turnover of their high-energy phosphate stores. This demands a constant supply of oxygen and fuel (predominantly in the form of free fatty acids but also ketone bodies and carbohydrates). Indeed, even a brief interruption of this supply line for even 15 seconds results in the depletion of all ATP stores within the cardiac myocytes. The most usual cause of an interrupted supply of metabolites is by a reduced blood supply to the heart (ischaemia). This typically is the result of a stenosed epicardial coronary artery, which limits maximal hyperaemia during exercise, or a complete blockage as occurs in an acute myocardial infarction.

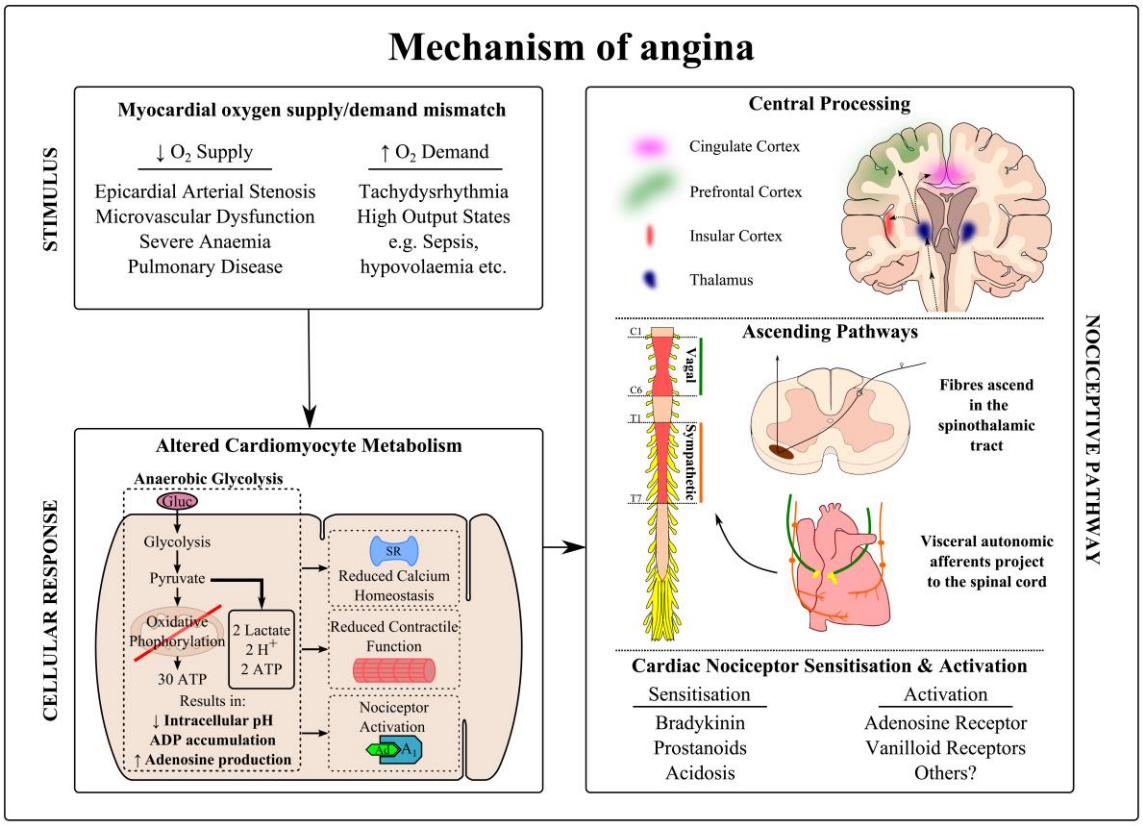


Figure 1.3: The mechanism of angina.

During exercise the metabolic rate of the myocytes increases and necessitates an increased blood supply. Control of regional myocardial perfusion predominantly rests with the microcirculation (vessels <500µm in diameter) which can dilate or constrict rapidly in response to the metabolic needs of the subtended territories. Under normal circumstances, the larger conduit arteries do not contribute to resistance to blood flow but with a functionally significant epicardial arterial stenosis they become the limiting factor to increased blood flow. The normal response to exercise is widespread vasodilation of the microvasculature as well as of the supplying arteries. When the response is normal, this results in up to an 8-fold increase over resting flow rates. If this is not normal, however, the end result is a mismatch between the supply and demand of oxygen. This results in a switch to anaerobic metabolism within the myocytes. Glycolysis increases but is not followed by oxidative phosphorylation and hence the pyruvate is metabolised in the cytosol into lactate and free hydrogen ions.

This reduces intracellular pH, which further worsens myocyte function by diverting energy use towards calcium homeostasis as well as impairing contractile function. Additionally, ATP production is reduced and there is a build-up of ADP, inorganic phosphate, and adenosine. This change in myocyte biochemistry triggers intracardiac sensory nerve endings, beginning the process of pain perception.

b) *Neural Pathways*

Both myelinated Aδ fibres and unmyelinated C fibres are present in the epicardial interstitium and contain chemo-, mechano- and thermo-sensitive channels (the TRPV1, transient receptor potential vanilloid 1, channel is believed to be an important player). These fibres are also sensitised by reduced pH and some ischemia-induced substances such as bradykinin and prostanoids. Furthermore, selective adenosine receptors (A_1 and A_2) are present in the perivascular sympathetic nerves and the activation of A_1 receptors by adenosine has been shown to stimulate angina. These fibres aggregate into bundles in the septa of the muscle and run alongside the coronary arteries.

These sensory afferents are believed to mainly run in the autonomic fibres (both sympathetic and vagal) coming from the heart, as surgical interventions on local sympathetic ganglia reduced anginal symptoms in >80% of patients[32]. These fibres project upwards towards the thalamus primarily in the spinothalamic tracts (although some fibres project in the dorsal columns), where viscerosomatic convergence occurs over a long segment of spinal cord. This results in the referral of visceral cardiac pain over a diffuse cutaneous area, usually involving the C2-C6 and T1-T5 dermatomes, but rarely involving C7 or C8. Thus, cardiac pain is felt in the throat, chest and inside of the left arm but rarely in the hand and fingertips. It has been noted that angina results in a bilateral increase in blood flow in the thalamus and that this occurs even in “silent” ischaemia. From here, the signals project cortically and are processed in the prefrontal

and cingulate cortices[33]. Interestingly, significantly greater activation of the right anterior insula is seen in CSX patients with angina pectoris than in patients with obstructive coronary artery disease or healthy controls, which might play a role in their apparent increased perception of cardiac stimuli[34].

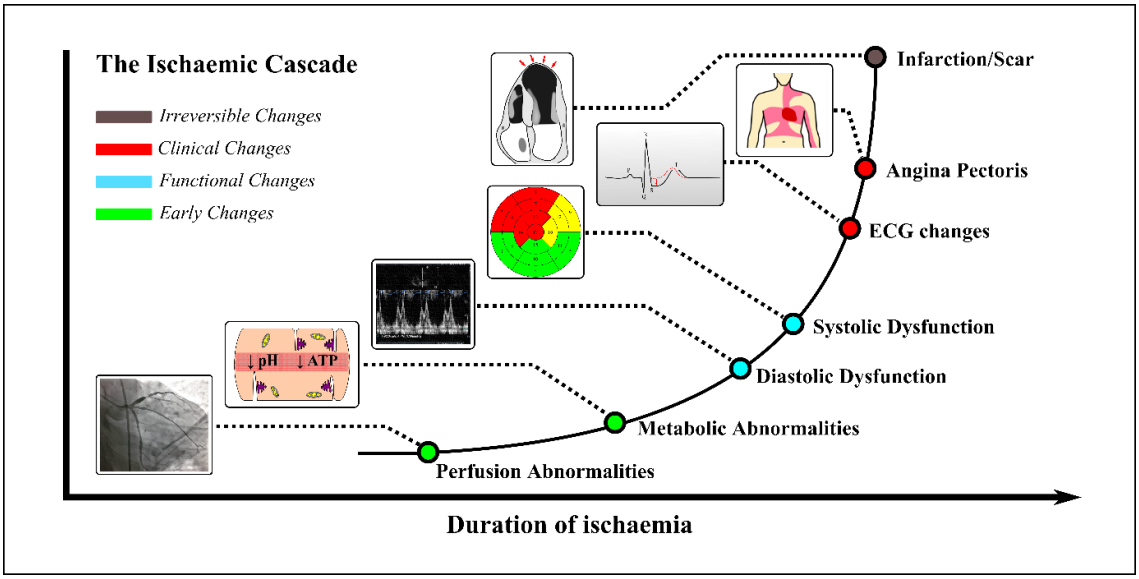


Figure 1.4: The Ischaemic Cascade

1.4.2 Myocardial Ischaemia in CSX

As angina is initiated by myocardial ischaemia it is reasonable to expect that ischaemia is present in CSX, a condition typified by angina. There is again, however, considerable debate and conflicting data regarding this. The majority of the evidence suggests, however, that myocardial ischaemia is indeed present in CSX. The ischaemic cascade is depicted in figure 1.4 above. The classical pathway indicates that the onset of ischaemia is followed first by metabolic evidence of ischaemia, then by evidence of impairment of myocardial mechanical function (in the form of reduced muscle relaxation and eventually contraction), then altered myocardial electrical activity (in terms of ST-segment depression or elevation on an ECG) and finally angina pectoris before irreversible myocardial necrosis occurs. It has been suggested that this model may not apply to ischaemia in CSX and that the pathway may even be mostly inverted

(i.e. that angina precedes ECG changes and mechanical evidence of ischaemia[35]) in CSX patients.

Evidence for myocardial ischaemia

Metabolic evidence of ischaemia is considered the gold standard for detection of true ischaemia. However, altered myocardial mechanical function and ECG changes also occur during the ischaemic cascade and are also considered as markers for myocardial ischaemia.

a) Evidence of Perfusion Abnormalities

CMR: Initial cardiac magnetic resonance (CMR) studies utilising myocardial perfusion techniques demonstrated possible subendocardial hypoperfusion in CSX patients. A small study by Panting et al (n=20) used gradient-echo, gadolinium-enhanced sequences in a 1.5T scanner, both at rest and after 6 minutes of adenosine infusion, to calculate a myocardial perfusion reserve index[10]. They demonstrated that CSX patients failed to substantially improve their endocardial perfusion in response to adenosine but did have a normal response in their epicardium. Additionally, they found that 95% of CSX patients developed chest pain on adenosine infusion compared with 40% of healthy controls (χ^2=26.1, p<0.001). These findings were contradicted by a larger, well-executed study 5 years later[11]. A further 20 CSX patients underwent spoiled echo gradient, gadolinium enhanced sequences in a 1.5T scanner both at rest and after 3 minutes of adenosine stress. This study failed to observe any impaired improvement of subendocardial perfusion during stress and pointed out that the selection criteria for patients in the two studies differed, with Panting relying on EST and Vermeltfoort on MPI. A subsequent CMR study involving 42 patients, however, also demonstrated reversible stress-induced subendocardial perfusion defects[36]. A CMR study in patients with proven microvascular dysfunction (by coronary flow reactivity testing) showed

very similar results and all of these patients also fulfilled the criteria for diagnosis with CSX[22].

PET/CT*:* Cardiac Positron Emission Tomography detects injected radionuclide tracer concentrations in the heart and is useful in accurately demonstrating myocardial perfusion. Studies involving PET have consistently demonstrated that CSX patients have reduced coronary flow reserve i.e. a diminished capacity to increase blood flow in response to an increase in demand[12,37].

Myocardial Perfusion Scintigraphy: Fragasso showed hypoperfusion in 77% of CSX patients during stress in his small cohort using thallium. Furthermore, 97% of the patients had defects at rest or stress. This was also seen in an older study of patients with CPNCA and CSX, where 98% had abnormal thallium scans[38]. These were more pronounced than the mere 40% of patients who had perfusion defects in a technetium labelled study[39].

Echocardiography: Non-invasive assessment of coronary blood flow and muscle perfusion may be achievable by transthoracic Doppler interrogation of the left anterior descending coronary artery coupled with myocardial contrast echocardiography. This was achieved in a small study and demonstrated reduced coronary flow reserve in CSX patients[40]. A more recent study also showed impaired coronary auto-regulation with increased baseline blood flow in addition to reduced coronary reserve in CSX patients[41].

Invasive Angiography: By definition, the coronary arteries of CSX patients are angiographically normal. Invasive assessment of coronary flow reserve using doppler wires and coronary reactivity testing, however, has demonstrated that reduced coronary flow reserve is common in CSX patients[19,22]. Furthermore, simple blush scoring of the myocardium after contrast injection during coronary angiography has been used to attempt to quantify the microvascular perfusion in CSX patients and has been shown to be reduced in this cohort[42].

In summary there is compelling evidence from multiple modalities that confirms that a stress-induced perfusion deficit does exist in patients with CSX.

b) Metabolic Evidence of ischaemia

The original paper in which the first CSX patients were described (10 patients with normal arteries, angina and ST-depression induced by atrial pacing; the so-called group X) demonstrated that CSX patients had similar myocardial lactate production to patients with obstructive CAD, with extraction rates of <10% (indicative of ischaemia) seen in roughly half of each group[43]. Lactate metabolism has also been examined in several other studies but these have widely had disparate results, with some studies showing no evidence of lactate production and other showing lactate production in only 30% of cases[8]. Similarly, coronary sinus pH is reduced in ischaemia and was shown to be reduced in 30% of patients with CSX. Oxygen extraction from the coronary blood increases during increased metabolic demand and a reduction in SpO_2 in the coronary sinus may also indicate possible myocardial ischaemia. One study showed that coronary sinus SpO_2 decreased in 50% of patients with CSX, but that this was only transient in a half of these cases where it returned to normal within 20s[44]. A study of 35 women with angina pectoris and normal coronary arteries using myocardial phosphorus-31 Nuclear Magnetic Resonance Spectroscopy during isometric handgrip exercises demonstrated a large decrease in phosphocreatine:ATP ratios in 20% of subjects indicating an abnormal metabolic response in these women. While not examining a CSX cohort specifically, the authors believed that the women with evidence of ischaemia constituted a likely cohort of patients with microvascular angina[45].

c) Evidence of mechanical dysfunction secondary to ischaemia

This area has cast the greatest doubt on the ischaemic aetiology of CSX when looked at through the paradigm of the ischaemic cascade. Several studies have examined the evidence for systolic mechanical dysfunction, a classical hallmark of ischaemia, and had consistently found it to be lacking in CSX until a very recent study using the modern technique of speckle tracking showed reduced longitudinal strain of the left ventricle during systole, indicating impaired systolic function[46]. A small, unblinded study showed no difference in left ventricular volumes or function during stress or rest between healthy controls and CSX patients while other studies showed no regional wall motion abnormality during stress (in the form of dobutamine or atrial pacing) echocardiography[5,6,47,48]. Another small study using adenosine stress echocardiography in patients with CSX demonstrated inducible diastolic dysfunction during stress indicating global dysfunction with reversal of the e' to a' ratio on tissue Doppler imaging (see *Fig. 1.5* below). Researchers have hypothesised that the hypoperfusion in CSX is likely to be patchy and diffuse rather than confluent and focal with the result that no regional wall motion abnormalities are demonstrable, unlike the highly regional area of hypoperfusion and impaired function seen in classical ischaemia seen in coronary artery disease[49].

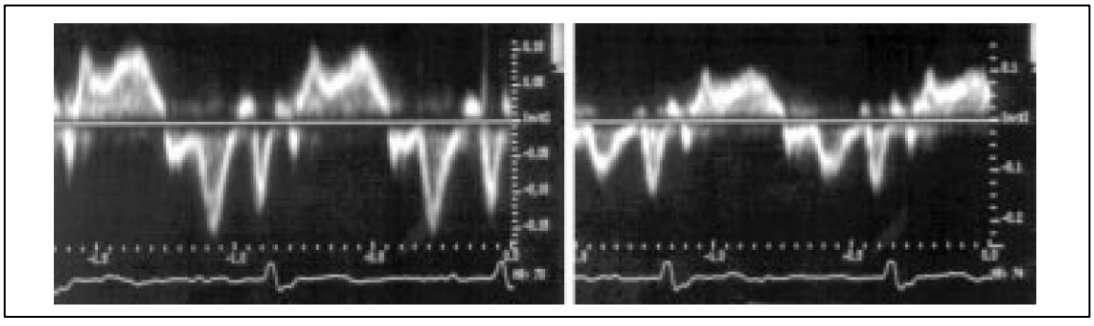


Figure 1.5: Pulse wave Doppler over the lateral mitral annulus during rest (left) and during adenosine stress (right) in a CSX patient. Note the reversal of the spectral profile indicating induced diastolic dysfunction.

d) Evidence of electrical dysfunction due to ischaemia

This is one of the hallmarks of CSX. In this thesis the presence of ECG evidence of ischaemia during EST is one of the diagnostic criteria for CSX. Electrocardiographic evidence of ischaemia is seen almost ubiquitously in exercise stress testing as well as during Holter monitoring of these patients[27,50-52]. The diagnostic value of ST-depression in EST is controversial, however, especially in the female population. The sensitivity and specificity of ST-depression in EST for obstructive coronary artery disease is about 60-70% and 70-80% respectively. Its sensitivity and specificity for myocardial ischaemia (including macrovascular and microvascular ischaemia) is unknown.

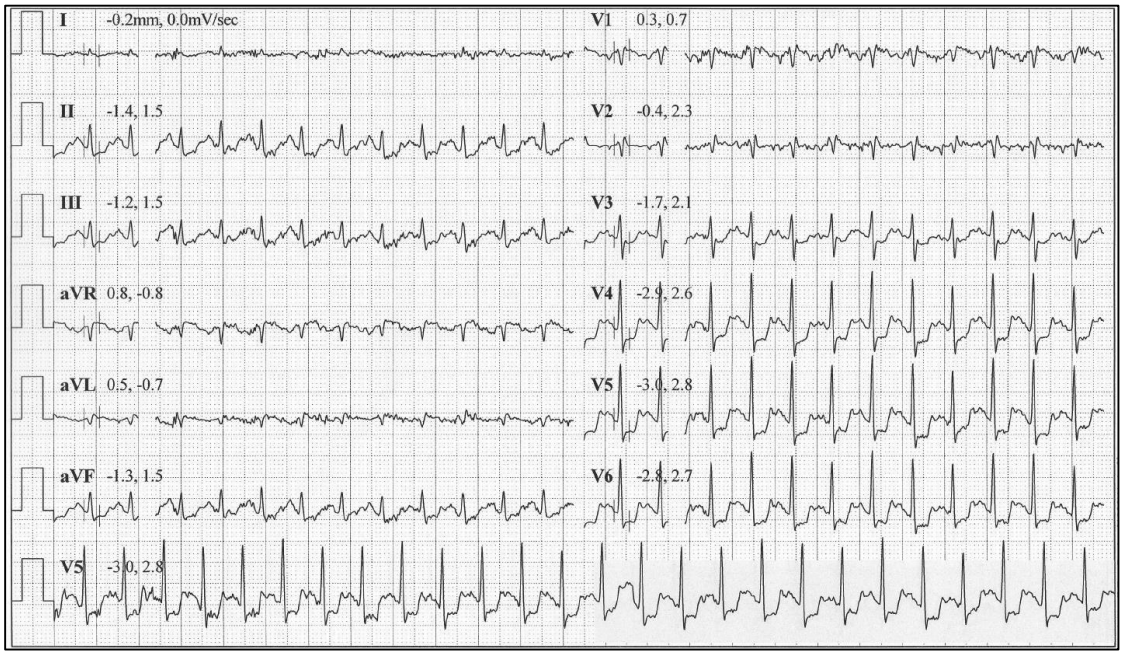


Figure 1.6: An ECG from an EST performed on one of the CSX patients in this thesis. Note the 2mm of horizontal ST-depression seen in leads V4-V6.

1.4.3 The Role of the Endothelium & Microvasculature in CSX

The endothelium is a cellular monolayer that forms the primary interface between the circulating blood and the various tissues around the body. There is a surprising degree of heterogeneity in endothelial phenotypes, with different vascular beds having different endothelial linings: the sinusoids in the liver and the glomerulus in the

nephron, for example. The endothelium in the coronary arteries is of particular clinical importance as dysfunction of this tissue is critical in the development of conditions such as atherosclerosis, myocardial infarction and CSX. Healthy endothelium is typified by several general features: being anti-thrombotic, anti-inflammatory, selectively permeable and capable of mediating the phenotype and activity of the underlying muscle. Endothelial dysfunction or activation may be the primary abnormality in CSX and thus the function of normal endothelium warrants detailed discussion.

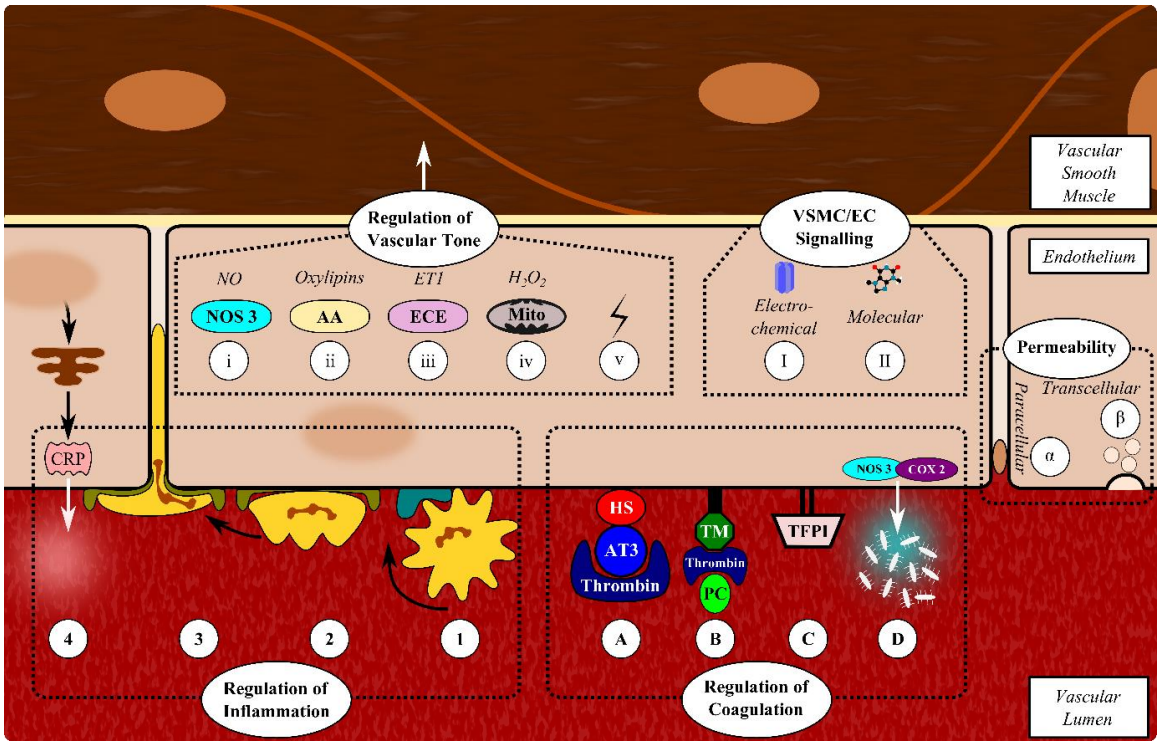


Figure 1.7: Functions of Normal Endothelium

Normal Endothelial Function

a) Immune modulation

Endothelium is responsible for the recruitment of inflammatory cells to the site of tissue damage. In health, the endothelium does not interact with the circulating immune cells. If a local pro-inflammatory stimulus (such as necrosis or oxidative

damage) is present, the endothelial cells are stimulated to express several molecules that recruit immune cells, such as macrophages and leucocytes, to the local tissue. Adhesion molecules play a crucial role here.

- *Figure 1.7 (1).* The initial interaction between endothelium and leucocyte is mediated by selectins and induces 'rolling' of the leucocytes. P-selectin, stored in the Weibel-Palade bodies (WPB), is released in response to cytokine stimulation of the EC (particularly histamine, IL-1 and TNFα) and binds with PSGL-1 (p-selectin glycoprotein ligand) on neutrophils and T-cells. E-selectin is produced via delayed transcription of the *SELE* gene in response to the cytokines and is then expressed on the cell surface. It further arrests the movement of the leucocytes, sticking them to the endothelial lining.
- *Figure 1.7 (2).* Once the leucocytes have come to a halt due to the interactions with the selectins, locally produced chemokines activate the leucocytes, which then display integrins. These integrins then proceed to anchor the leucocyte firmly to the endothelial cell through their interactions with Intercellular Adhesion Molecules (ICAMs) and Vascular Cell Adhesion Molecules (VCAMs).
- *Figure 1.7 (3).* Finally, the cells transmigrate through the endothelial cell layer to get to the inflamed tissue in a process termed diapedesis. This is believed to again be mediated by ICAM-integrin interactions and primarily takes place through the paracellular route, when local intercellular junctions become disrupted by inflammatory signalling.
- *Figure 1.7 (4).* C-reactive protein, an acute phase protein, has recently been shown to be produced by activated coronary artery endothelium in response to inflammatory cytokines and leptin, an adipokine [53,54]. This has been shown to increase platelet adhesion and CRP is known to strongly induce endothelial dysfunction through multiple pathways.
- In addition, local vasodilation and increased permeability occur in response to a pro-inflammatory stimulus.

b) Regulation of coagulation

A healthy endothelium is necessary to maintain the fluidity of blood by providing a constant anti-thrombotic microenvironment. It inhibits coagulation in several important ways.

- *Figure 1.7 (A).* Healthy ECs express heparan sulphate proteoglycans (HS), as part of the glycocalyx, which bind to and augment circulating anti-thrombin-III (AT3) activity. This protein inhibits the formation on thrombin, thereby preventing the activation and crosslinking of fibrin and thus clot formation.
- *Figure 1.7 (B).* Surface expression of thrombomodulin (TM) is also potently anti-thromobotic. Thrombomodulin binds thrombin and changes its target from fibrin to Protein C (PC), which it activates and which in turn inhibits factor VIII (intrinsic pathway) and factor V (common pathway) activation thereby preventing coagulation.
- *Figure 1.7 (C).* Healthy endothelium also expresses Tissue Factor Pathway Inhibitor (TFPI), which prevents the activation of the extrinsic coagulation cascade by inhibiting factor VII activation by local tissue factor.
- *Figure 1.7 (D).* Additionally, endothelium produces many factors that govern the activation of platelets. ECs can produce prostacyclin and nitric oxide, which can inhibit platelet aggregation, or von Willebrand Factor, CRP and Thromboxane A_2, which increase platelet activation.
- Finally, ECs can cause local vasoconstriction, which can improve haemostasis immediately.

c) Regulation of Permeability

As mentioned before, the ultrastructure of endothelial linings varies from tissue to tissue. In general, the coronary arterial endothelium is impermeable to plasma proteins due to the presence of intercellular tight and adherent junctions, which preclude the

movement of substances greater than 4Å through. The endothelial layer is capable of regulating its permeability, however.

- *Figure 1.7 (α).* The cells may increase paracellular transport of proteins (and indeed cells during diapedesis of leucocytes as mentioned above) by regulating the integrity of the intercellular junctions. The loosening of adherent junction binding allows the formation of intercellular gaps, through which substances may pass. The adherent junctions are enzymatically altered (usually by phosphorylation), which leads to their internalisation and indeed may also cause cytoskeletal contraction, further separating the cells. Inflammatory molecules (such as bradykinin and histamine) lead to such upregulation of paracellular transport.
- *Figure 1.7 (β).* The luminal surface of endothelial cells is pock-marked by multiple invaginations termed caveolae. These pits contain clusters of proteins aggregated in lipid rafts that may bind to and internalise extracellular substances and allow for vesicles-mediated transport from the luminal surface to the abluminal membrane in a process termed transcytosis. The caveolae also play a critical role in the activation of eNOS.

d) Paracrine Signalling

Endothelial cells interact closely with the other cells in the vessel wall. They are in intimate contact with pericytes (Rouget cells) and can modulate the activity of the underlying vascular smooth muscle cells (VSMC)[55]. In health, the VSMCs maintain a contractile phenotype but this can be dramatically altered into a non-contractile, secretory phenotype that can propagate the pro-inflammatory cascade.

- *Figure 1.7 (I).* ECs have direct connections to the underlying pericytes (through peg and socket contacts) involving gap junctions, thereby allowing any electrochemical signal to be propagated directly from cell to cell. Similar myoendothelial gap junctions may allow for electrical continuity between the EC and VSMC

populations. This may allow for immediate hyperpolarisation of VSMCs in response to laminar shear stress.

- *Figure 1.7 (II).* In addition, the endothelium produces many compounds that may influence the phenotype of underlying smooth muscle cells. Substances such as growth factors (e.g. Platelet-derived growth factors, insulin-like growth factor and basic fibroblast growth factor) and prostanoids such as prostacyclin are produced in the ECs and may modulate VSMC differentiation. Even microRNAs released from ECs in response to shear stress may also affect VSMC phenotype (e.g. miR-200b and miR-143). Furthermore, ECs may produce matrix metalloproteinases and pro-angiogenic substances, which may alter the local extracellular environment and contribute to vessel remodelling.

e) Alteration of Vascular Tone

Perhaps the function of endothelium that is most relevant to CSX is its ability to affect vascular tone, leading to vasodilatation or vasoconstriction. It is capable of producing many substances that lead to these vascular changes. Experimental evidence suggests that abnormal vasomotor responses are key to the pathogenesis of CSX. Given its importance, endothelial regulation of vascular tone will be discussed in more detail in the next section.

Vascular Tone Homeostasis

The regulation of the diameter of blood vessels is a dynamic and local process, which is influenced primarily by the characteristics of the blood flow through the lumen of the vessel as well as through neural inputs. The ultra-structure of the blood vessel wall is adapted to allow for rapid and sustained changes in vascular tone in response to altered haemodynamics and local stresses. The components of the tunica intima, primarily the endothelium, respond to changes in local rheology and signal the vascular

smooth muscle (VSMC) in the tunica media to relax. Autonomic inputs also modify these responses. Altered regulation of vascular tone is of great importance in CSX.

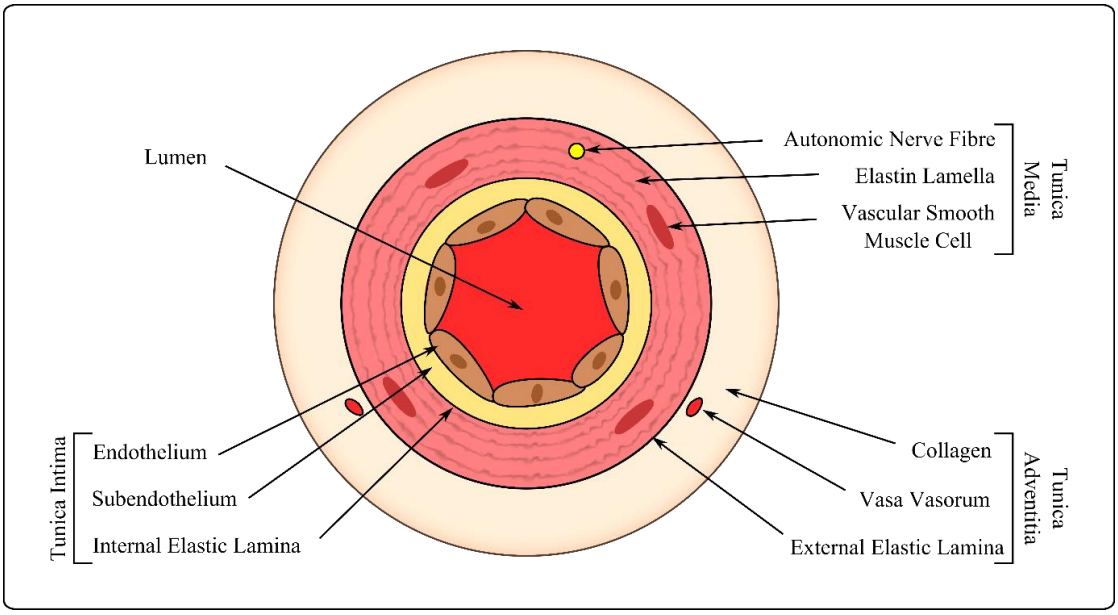


Figure 1.8: Cross-section of an artery

Endothelial Stresses

As blood flows through a blood vessel it exerts several forces on the endothelial lining. The hydrostatic blood pressure generated by the pumping action of the heart works normally (i.e. perpendicularly) to the endothelium, essentially compressing it, as well as creating a circumferential tension due to altering pulse pressures. The adventitia is also stretched outwards, creating a tensile stress. Taken together these create a wall stress that is distributed across the entire thickness of the vessel wall (i.e. intima, media and adventitia). At the same time shear stress is exerted on the endothelium by the blood as it flows past. It is a tangential stress that is exerted parallel to the flow of the fluid. In general, studies have found that shear stress is an important modifier of endothelial cell function although some suggest that the normal forces could be equally important[56].

Shear Stress

If one considers a blood vessel in cross-section, the region of maximal blood flow velocity is at the centre of the blood vessel. The blood layer nearest the endothelium is flowing at a much lower velocity due to friction between it and the endothelium. In between these two extremes the blood flow velocity changes gradually with each outer layer moving slightly more slowly than the layer immediately inside it. This tends to try to drag the endothelium along in the direction of blood flow and this creates the shear stress. The shear rate is a measure of how quickly the velocity changes from each layer of blood to the next and has a direct bearing on the magnitude of shear stress generated. The characteristics of the blood also directly affect the shear stress. Unlike the normal hydrostatic pressure, the effects of shear stress are mostly limited to the endothelial layer of the vessel wall.

Work by Lipowsky et al shows that the microcirculation experiences the greatest shear stress in the vascular tree (by a factor of 6 when compared with arteries and veins) implying that shear stress is critical in the regulation of microvascular tone and indicating that the increase in velocity and decrease in diameter outweigh the reduced viscosity of blood in these vessels[57]. Vessels work to autoregulate the shear stress they experience, endeavouring to keep it at 15 dynes/cm^2. At high levels of shear stress, the vessels dilate to reduce this stress while at low levels you can get some neointimal hyperplasia to reduce the vessel lumen. When the shear stress is at the desired level endothelial and smooth muscle proliferation is minimal. This does not tell the complete story, however, as not all shear stress is equal.

Laminar versus Oscillatory Shear Stress

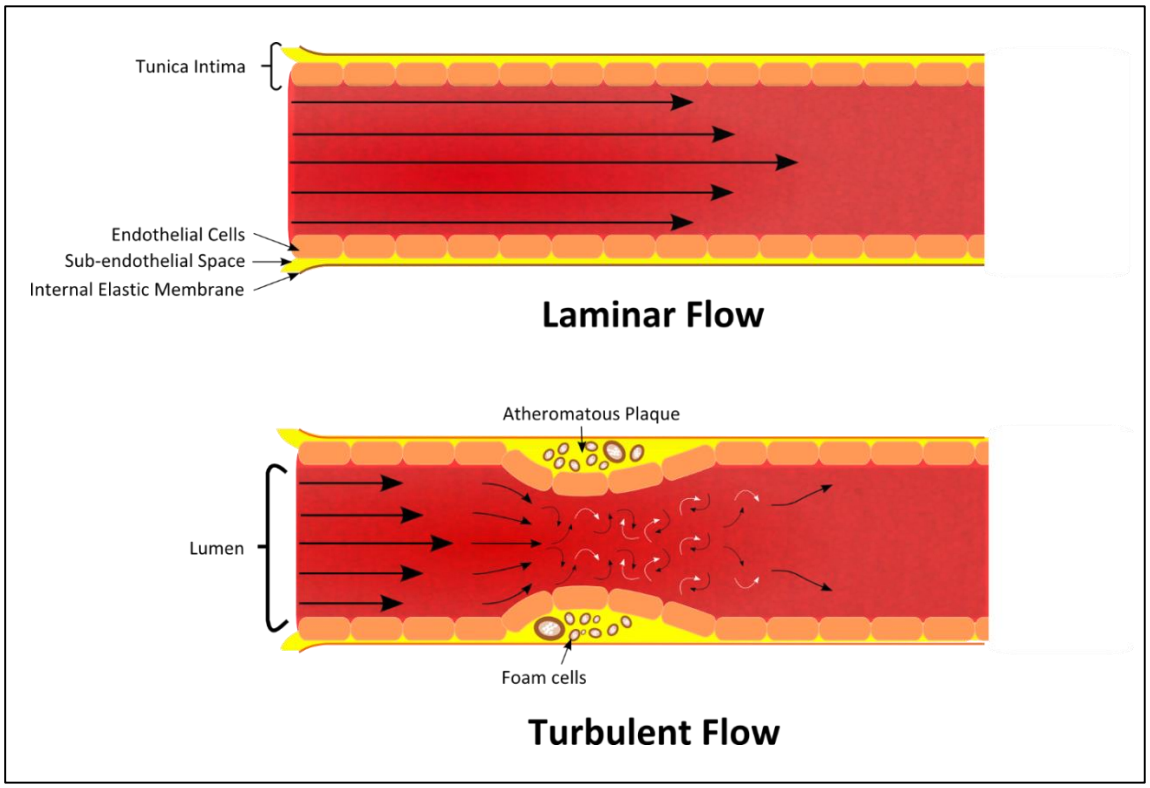


Figure 1.9: Comparison of Laminar and Turbulent blood flow in a blood vessel

Normal blood flow in undiseased, straight blood vessels is laminar and rhythmical and creates pulsatile laminar shear stress. Endothelial cells respond to this by a process called mechanotransduction (see below) and this activates transcription of many genes that protect the endothelium from damage, inducing vasodilatation, anti-inflammatory and anti-oxidant processes. Atherosclerosis starts at predictable points in an artery; where there is a bend, a bifurcation or an anatomical abnormality. All of these result in turbulent local blood flow, which disrupts the laminar shear stress and causes oscillatory shear stress. As described below, this changes the activity of many regulatory pathways in the endothelial cell, predisposing the local intima to atheroma formation.

Mechanotransduction

Endothelial cells respond rapidly to changes in local blood flow. Signals can be relayed almost instantly to affect vasomotor tone and gene transcription expression can be altered as quickly as within one hour. The mechanisms involved in the cellular response to this mechanical stimulus are interesting but remain to be fully determined.

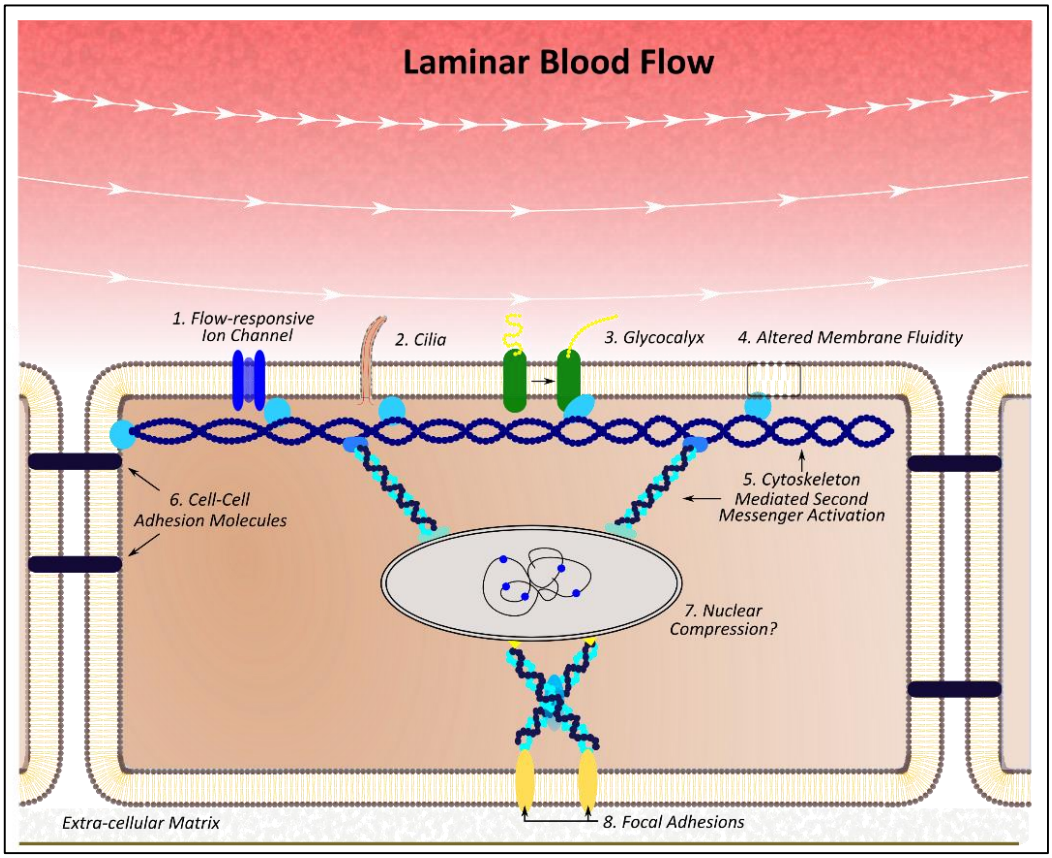


Figure 1.10: Methods of Mechanotransduction

The main theory is that ion channels respond to local shear thereby altering transcellular gradients and activating second messenger systems. The chief channel involved appears to be an inward rectifying K^+ channel, which ultimately leads to an increase in intracellular calcium which can activate signalling pathways including the cytosolic release of eNOS from caveolae. Several theories exist as to how these channels respond to shear stress.

a. *Direct deformation*: opening of the channel might occur as a direct result of the drag of blood across its extracellular surface. In silico models have suggested, however, that the physical drag of blood along the endothelium is unlikely to be of sufficient magnitude to cause this.
b. *Cytoskeletal- dependant activation*: endothelial cells are exposed to shear stress and circumferential stretch and this can cause activation of sensory proteins attached to the cytoskeleton. These may activate the ion channels in the cell membrane as well as G-protein coupled receptors even in the absence of the necessary ligand.
c. *Membrane Fluidity:* Blood shear can alter the local viscosity of the plasma membrane and this has been shown to activate ERK as well as potentially activating many second-messenger systems directly.

In addition to ion channels, ECs may monitor local stresses by other means.

a. *Cilia:* ECs can develop cilia, which may directly detect local blood flow characteristics. Indeed, laminar flow has been shown to reduce cilia density while cilia are upregulated in areas of turbulent flow
b. *Glycocalyx* consists of glycosylated transmembrane proteins that extend out beyond the cell membrane plane into the blood. These negatively-charged proteins are coiled when shear stress is low but unfurl when blood flow is fast, allowing Na^+ to bind and activating a second messenger system.
c. *Via attachments to the ECM and other cells:* The ECs are anchored to the ECM and basement membrane as well as to each other. Shear stress will be transmitted via the cytoskeleton to these anchors and may allow signal transduction to nearby structures.
d. *Nuclear Compression:* One interesting theory, without evidence to date, is that cellular compression can also result in nuclear compression with deformation of the chromatin, altering the access of transcription factors to genes.

Once the signal has been received by the cell, either by ion channel activation with resultant secondary messenger activation or by direct cytoskeletal-mediated non-ligand dependant G-protein activation, various cell-messenger systems become activated, altering cellular function and EC gene expression.

Mediators of Vasodilation

One of the primary effects of increased laminar shear stress is to cause vasodilation of the blood vessel in a process termed flow-mediated vasodilatation (FMD), which is an endothelial-dependent process (which is to say that FMD fails to occur in the absence of a healthy endothelium). This increase in vessel diameter reduces the shear stress back towards normal, thereby providing a negative feedback. In a typical scenario, increased cardiac workload results in the release of local metabolites (e.g. bradykinin) around the cardiomyocytes, which causes local capillary vasodilation. This in turn increases the pressure gradient across the resistance arterioles, which then increases the blood flow velocity through these vessels and consequently the shear stress experienced by the arteriolar endothelium. This is detected through the various means of mechanotransduction and then provokes resistance vessel vasodilation, increasing the blood supply to the cardiomyocytes and reducing shear stress to normal levels. When the metabolic demand of the tissue diminishes, the microvasculature reverts back to its basal state.

Table 1.7: Endothelium-Derived Mediators of Vasodilation

Mediator	Source	Mechanism of action
Nitric Oxide	eNOS	Activates GC and MLCP and opens potassium channels
Carbon Monoxide	Haem Oxygenase	Activates GC and opens potassium channels
EET	CYP	Opens potassium channels causing hyperpolarisation
Prostacyclin (PGI_2)	COX2	Activates PKA and inhibits MLCK
PGE_2	COX2	↑cAMP production
H_2O_2	Metabolism	Opens potassium channels causing hyperpolarisation
Electrochemical	Mechanotransduction	Hyperpolarisation via myoendothelial gap junctions

COX- Cyclo-oxygenase**; CYP-** Cytochrome P**; EET-** Epoxyeicosatrienoic Acid**; GC-** Guanylyl Cyclase**; MLCP-** Myosin Light Chain Phosphatase**; MLCK-** Myosin Light Chain Kinase**; PKA-** Protein Kinase A**.**

The endothelium mediates vasodilatation in a surprising variety of ways but in the main it either causes VSMC hyperpolarisation through the opening of potassium channels or affects the contractile apparatus in the VSMC through GPCR pathways. The chief mediators of the endothelium-dependent vasodilation are shown in the table 1.7 above. As previously mentioned, the VSMC and EC are in direct electrical continuity via gap junctions and indirectly via pericytes. Increased shear stress causes hyperpolarisation of the ECs and this also affects the membrane potential of the VSMCs, thereby preventing contraction. Laminar shear stress also promotes the production of many substances which are released by the endothelium and diffuse locally to promote local smooth muscle relaxation. Many of these substances are oxylipins and are discussed in more detail in chapter 7. The most important mediator of endothelium-dependent vasodilation is nitric oxide (NO). It seems that NO is important in macrovascular dilation while the other endothelial derived mediators have more of a role in the microvasculature.

Nitric Oxide

Nitric Oxide (NO) is synthesised through the action of Nitric Oxide Synthase (NOS) on the substrate L-arginine in the presence of several co-factors to produce NO and citrulline. The NO gas diffuses rapidly towards the VSMC, where it activates second messenger systems before being rapidly destroyed through interactions with local reactive oxygen species.

Synthesis

The endothelium harbours large quantities of NOS (termed endothelial NOS, eNOS or NOS3). eNOS is localised to membrane invagination called caveolae, where it is contained through myristoyl and palmitoyl lipid anchors. It is also intimately associated with a membrane protein, caveolin, which renders it inactive. Various stimuli lead to dissociation of the eNOS from the caveolin, allowing eNOS to be activated through phosphorylation. Activated eNOS has both reductase and oxygenase domains as well as a calmodulin-binding domain. The release of intracellular endothelial calcium allows further activation of eNOS through this calmodulin domain. eNOS requires the presence of several co-factors for its activity, including tetrahydrobiopterin (BH_4), NADPH, FAD and FMN. These co-factors allow the transfer of electrons along the enzyme. NOS mediates the oxygenation of arginine using electron donors. This reaction produces the NO radical and citrulline, an amino acid. The absence of any of the co-factors can lead to uncoupling of this reaction and the production of reactive oxygen species.

Regulation of activity

eNOS activity is altered by its interaction with caveolin, other proteins such as NSIP and NOSTRIN and through its phosphorylation by various kinases (e.g. Akt, AMPK, SIRT1).

Increased endothelial calcium concentrations in response to shear stress lead to calmodulin binding, which leads to conformational changes in eNOS, increasing its efficiency. Laminar shear stress, acetylcholine, bradykinin and oestrogen can lead to upregulation of NO production through phosphorylation pathways as well as releasing it from caveolin. Oxidative stress, endothelial dysfunction, ageing and increased competition from arginase lead to reduced NO production.

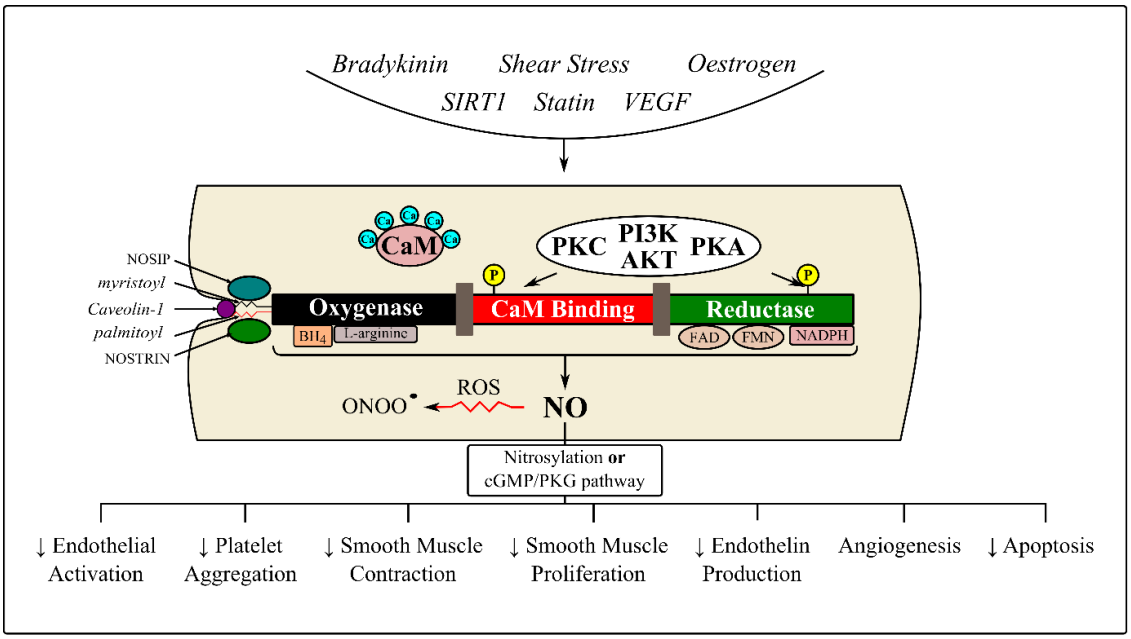
Effects

NO has many potent protective effects. It reduces formation of oxLDL, inhibits platelet aggregation, reduces endothelial activation and reduces Endothelin production. In addition, it diffuses into nearby VSMCs where it activates guanylyl cyclase. This increases cGMP formation that in turn leads to opening of potassium channels with resultant VSMC membrane hyperpolarisation and smooth muscle relaxation. cGMP also causes calcium sequestration in the sarcolemmas of the VSMCs, depriving the contractile elements of this crucial element and allowing for relaxation. It also seems to activate Myosin light chain phosphatase, which allows relaxation of smooth muscle. Furthermore, NO reduces VSMC proliferation and phenotype switching and through nitrosation reactions may inhibit apoptosis.

Termination of Signalling

NO has an extremely short half-life as it binds to haemoglobin in blood and is eradicated by reactive oxygen species in the ECF or cells, being converted to peroxynitrite. Oxidative stress and reactive oxygen species therefore increase the degradation of NO and reduce its bioavailability. Also, as will be discussed further in the next section, dysfunctional endothelium has a reduced capacity to produce nitric oxide and this has many deleterious effects on endothelial biology. Other extrinsic mediators of endothelial vasodilation include adenosine, histamine, bradykinin and

various neurohumoral inputs (e.g. circulating adrenaline effects via α and β adrenoceptors.



AKT- Protein Kinase B; **BH4-** Tetrahydrobiopterin; **CaM-** Calmodulin; **FAD-** Flavin Adenine Dinucleotide ; **FMN-** Flavin Mononucleotide; **NADPH-** Nicotinamide Adenine Dinucleotide Phosphate; **NOSIP-** Nitric Oxide Synthase Interacting Protein ; **NOSTRIN-** Nitric Oxide Synthase Trafficking; **PI3K-** Phosphoinositide 3 Kinase; **PKA-** Protein Kinase A; **PKC**- Protein Kinase C; PKG- Protein Kinase G **SIRT1-** Sirtuin; **VEGF-** Vascular Endothelial Growth Factor;

Figure 1.11: Regulation of endothelial Nitric Oxide Synthase and biological effects of Nitric Oxide.

Mediators of Vasoconstriction

The ability of the endothelium to mediate vasodilation is counterbalanced by its capacity to induce strong vasoconstriction. The endothelial derived factors that mediate vasoconstriction are listed in the table below. The most potent of these is Endothelin-1, a peptide that is locally produced through the activity of Endothelin converting enzyme (ECE) in the endothelium. Endothelin stimulates the release of sarcolemmal calcium into the cytoplasm of vascular smooth muscle cells, causing potent vasoconstriction. Furthermore, Endothelin has the capacity to increase platelet and leucocyte activation, having a pro-inflammatory effect. Stimuli for the formation of Endothelin include angiotensin II, cytokines and oscillatory shear stress while its release is impaired by prostacyclins and nitric oxide.

Mediator	Source	Mechanism of action
Thromboxane A_2	COX2	Activates MLCK and Protein Kinase C (PKC).
Endothelin -1	ECE	Calcium release from sarcoplasmic reticulum
H_2O_2	Respiration	Calcium influx and PKC activation
PGH_2	COX2	↑cAMP production
Angiotensin II	ACE	Activates PKC, inhibits AC, ↑ myosin phosphorylation
Electrochemical	Mechanotransduction	Depolarisation via myoendothelial gap junctions

Table 1.8: Endothelial Mediators of Vasoconstriction

The overall vessel tone is a summation of the counterbalancing effects of the vasodilators and vasoconstrictors on the vascular smooth muscle. It has been demonstrated that a dysfunctional endothelium is tipped towards a net vasoconstriction, due to an imbalance in the release of many of the aforementioned substances.

Endothelial Activation and Dysfunction

Healthy endothelium has adapted to respond to laminar shear stress by having anti-inflammatory, anti-apoptotic, anti-thrombotic and vasodilatory paracrine effects. Early stages of atherosclerotic vascular disease, however, are typified by a loss of this protective phenotype and the endothelium is referred to as being activated and dysfunctional. Many of the conventional risk factors for atherosclerosis may mediate their deleterious effects by inducing this endothelial dysfunction, which then alters local processes and promotes atherosclerotic vascular disease.

One hallmark of endothelial dysfunction appears to be the reduced bioavailability of nitric oxide, either by a reduction in its production or by increased degradation through interactions with reactive oxygen species. In addition to nitric oxide, many of the endothelial derived vasodilating compounds are downregulated in dysfunctional endothelium. This leads to a reduced capacity for endothelial-dependent vasodilatation, i.e. vasodilatation that is brought about through the secretion of factors from intact endothelium. In vivo studies have shown that diseased coronary arteries lose the normal vasodilatory response to intracoronary acetylcholine, a substance that is normally a potent stimulator of nitric oxide release and endothelium-dependent vasodilation. Indeed, these dysfunctional arteries undergo paradoxical vasoconstriction in response to acetylcholine. This effect is also caused by an increased secretion of vasoconstrictor compounds such as endothelin at the same time as the reduction in vasodilators.

Table 1.9: Features of Endothelial Dysfunction

Causes of Endothelial Dysfunction	Markers of Endothelial Dysfunction
Aging (Senescence)	Abnormal Coronary Reactivity Testing (CRT)
Diabetes	Reduced Flow Mediated Dilatation (FMD)
Dyslipidaemia	Increased Markers of Oxidative stress (ADMA)
Hyperhomocysteinaemia	Increased Pro-Coagulant Factors (vWF)
Hypertension	Increased Markers of Vascular Inflammation (e.g. CRP, ICAM, VCAM, Selectins)
Inflammation	
Oestrogen deficiency	
Oxidative Stress	
Smoking	

Another feature of endothelial dysfunction is the increased expression of surface markers such as chemokines, cytokines, adhesion molecules and platelet activators, in a state termed endothelial activation. Furthermore, the endothelium may trigger the release of many cytokines and inflammatory markers. In part this is due to activation of endothelial NFκB. The result of this change in surface expression is the recruitment and activation of many effector cells of the immune and haemostasis systems. Local immune cell infiltration is a feature of plaque formation. Thus, the induction of an activated and dysfunctional state in endothelial cells by diverse risk factors leads to diminution of endothelial-dependent vasodilation in the vasculature and the fomenting of a local pro-atherosclerotic milieu. This is believed to be the earliest step in the atherosclerotic process.

Measuring endothelial and microvascular function

There are several methods used to determine endothelial and microvascular function. The most direct of these is through coronary reactivity testing. This invasive procedure is performed during cardiac catheterisation and involves the intracoronary injection of substances designed to trigger endothelial-dependent vasodilation. The most commonly used substance is acetylcholine. The degree of vasodilation is determined by comparing the peak blood flow velocity using a Doppler wire before and after infusate delivery. These results are then compared with vasodilation induced by substances that do not require functioning endothelium, usually adenosine or GTN. The response to adenosine typically demonstrates the microvascular function while the response to GTN elucidates the macrovascular response. An indirect measure of endothelial and microvascular function is the degree of vasodilation in response to increased blood flow (and hence shear stress) in an artery. This is termed flow-mediated vasodilation and is an endothelium-dependent process. The most common FMD protocol involves measuring the change in the radial or brachial arterial diameter in response to reactive hyperaemia following a short period of ischaemia induced by the inflation of a

sphygmomanometer cuff to supra-systolic levels. It has been shown that peripheral endothelial function assessed in this way correlates with coronary artery endothelial function in population studies[58].

Aside from measuring the impact of endothelial dysfunction on vasomotor function, it is possible to gauge the state of the endothelium through the measurement of circulating biomarkers. Activated endothelium expresses many surface markers and secretes substances that may be measured in circulating blood.

- Vascular inflammation markers with the highest endothelial specificity include E-selectin and VCAM. Other suitable biomarkers include ICAM-1, CRP and SAA (although these latter two are not specific to the endothelium).
- Increased oxidative stress (a cause of endothelial dysfunction) may be indirectly measured by assessing the concentration of serum asymmetric dimethylarginine (ADMA), a potent inhibitor of nitric oxide synthesis, which is induced by oxidative stress.
- Pro-coagulant factors: von Willebrand Factor (vWF) is released from activated endothelium via the release of Weibel-Palade bodies. This substance promotes platelet adhesion and blood coagulation.
- General Inflammatory Markers: In the absence of other causes of active inflammation, endothelial dysfunction is associated with elevated levels of pro-inflammatory cytokines such as IL-6, TNFα and IL-1.

Endothelial Dysfunction in Cardiac Syndrome X

There is ample evidence that the endothelium in patients with CSX is dysfunctional and activated. Many markers of endothelial dysfunction have been assessed and the results of selected studies are shown in the table below. Serum levels of soluble markers of

activation, such as the selectins and ICAM-1, have been shown to be significantly elevated in CSX, while VCAM-1 is also high, albeit not significantly so [59,60]. This implies that the endothelium expresses higher levels of these substances and as such may be triggering cellular immune responses. It has furthermore been shown that markers of inflammation are elevated in CSX. Moreover, the symptom burden in CSX appears to directly correlate with the degree of elevation of inflammatory markers [2,27]. Other biomarkers hint that the endothelium in CSX patients is exposed to excessive oxidative stress and this might in part be responsible for the endothelial dysfunction seen in this condition[61]. The trigger for these changes may merely be the exposure to many traditional cardiovascular risk factors such as hypertension and dyslipidaemia, although some research has focussed on the possibility of an infectious cause of low-grade vascular inflammation, with chlamydia and helicobacter pylori as two potential causes [62,63].

Both invasive and non-invasive measures of peripheral and coronary vasomotion consistently show that endothelial-dependent vasodilation is impaired in CSX patients and many have inferred from this observation that inadequate coronary vasodilation in times of increased cardiac demands may be the aetiopathogenesis of the cardiac syndrome X phenotype[64]. These microvascular abnormalities have been confirmed through many different modalities including invasive coronary reactivity testing with acetylcholine, PET scanning and Doppler interrogation of flow velocities. Therefore, the prevailing theory for CSX, which may be more appropriately termed microvascular angina, is that endothelial dysfunction is induced in coronary vasculature in response to traditional cardiovascular risk factors and that this leads to inadequate vasodilation and hence inadequate coronary flow reserve to meet the metabolic needs of the myocardium during exercise, which in turn leads to angina.

Table 1.10: Summary of studies of endothelial dysfunction in CSX

Study Author	Year	Population	Measures	Results
Vascular Activation				
Desideri et al	2000	24 CSX 14 Controls	VCAM-1 Endothelin-1	VCAM-1 and baseline endothelin-1 levels did not significantly differ between the two groups. After a glucose tolerance test, ET-1 levels were significantly higher in the CSX group (p=0.03).
Tousoulis et al	2001	36 LCSX 11 Controls	ICAM-1 VCAM-1	ICAM-1 was significantly higher in CSX patients compared with healthy controls (362 ± 22 v 225 ± 29 ng/ml , p<0.01)while the difference in VCAM-1 did not reach statistical significance (656 ± 42 v 551 ± 60 ng/ml, p=0.09)
Lin et al	2002	32 CSX 17 Control	ICAM-1 VCAM-1 vWF	CSX patients had significantly increased serum levels of ICAM-1 compared to healthy controls (225 ± 61 v 180 ± 23 ng/ml, p<0.01) while there was no significant differences in terms of VCAM-1 and vWF.
Senen et al	2005	21 CSX 22 Controls	P-selectin E-selectin	P-selecin was elevated in CSX patients compared to healthy controls (43 ± 9 v 23 ± 6 ng/ml, P<0.001) while E-selectin was similarly elevated (68 ±15 v 36 ± 5, ng/ml, p<0.001). Levels were similar to those seen in CAD.
Inflammation				
Cosin-Sales et al	2003	137 CPNCA	CRP	CRP was higher in patients with more frequent and more severe chest pain episodes. It also correlated with the number of episodes of ST-depression on holter and with the magnitude of ST depression on stress testing.
Lanza et al	2004	55 CSX 60 Controls	CRP IL-1RA	CRP was significantly higher in the CSX group than in the control group (1.4 [0.9-4.5] v 1.2 [0.7 - 2.3] mg/L, p=0.008) while IL-1RA was also higher in the CSX group (261 [178-492] v 216 [111- 374] pg/ml, p= 0.013)
Li et al	2007	36 CSX 30 Control	IL-6	IL-6 was significantly elevated in CSX patients compared with healthy controls (13.4 ± 1.2 v 6.2 ± 0.6 pg/dl, p<0.01) IL-6 stimulates CRP release.
Dollard et al	2014	16 CSX 13 Controls	CRP	CRP was higher in CSX patients, correlated with time to symptoms on stress testing (-0.69, p=0.013) and was lower in the CSX patients whose symptoms resolved over a 1 year follow-up (1.2 ± 0.2 v 2.8 ± 0.6 mg/L, p=0,018)
Oxidative Stress				
Piatti et al	2003	9 CSX 14 Control	ADMA	Serum asymmetric dimethylarginine (ADMA) levels were higher in CSX patients. ADMA is an endogenous inhibitor of Nitric Oxide Synthase.
Gur et al	2007	33 CSX 20 Control	PON-1 Antioxidant	Basal paraoxonase (PON-1) levels were lower in the CSX group (p<0.001) while total antioxidant status was found to be low (p<0.001). These markers hint that there is excess oxidative stress in CSX.
Erdamar et al	2009	92 CSX 16 Control	MDA MPO SOD	CSX patients had increased myeloperoxidase activity with increased serum malondialdehyde, indicating inceased oxidative stress. Their superoxide dismutase activity was lower than that of healthy controls.
Flow-Mediated Dilatation				
Tondi et al	2011	42 CSX 20 Control	Brachial FMD CRP	Brachial arterial FMD was reduced in CSX patients versus controls (4.8 ± 4.4 % v 13.7 ± 4.0 %, p<0.001). The CRP levels showed a significant inverse correlation with FMD (r=-0.34, p=0.006).
Liu et al	2008	21 CSX 24 Control	Brachial FMD LDL	Brachial FMD was reduced in CSX patients versus controls (4.7 ± 1.9% v 12.8 ±3.7%. p<0.001) FMD correlated with serum LDL levels (-0.513, p<0.001).
Coronary Reactivity Testing				
de Vries et al	2006	24 CSX	CFR by PET	Ammonia positron emission tomography was used to evaluate coronary flow reserve in CSX at rest and during dipyridamole infusion. Basal flow and CFR were reduced in CSX compared with controls.
Galiuto et al	2007	17 CSX 17 Control	CFR by TTE	Transthoracic echocardiographic (TTE) assessment of CFR demonstrated a significant reduction in CSX patients (2.0 ± 0.6 v 2.9 ± 1.5, p<0.01)
Luo et al	2014	18 CSX 18 Control	CFR by CRT	Invasive coronary reactivity testing using adenosine showed a reduced coronary flow reserve (2.8 ± 0.8 v 3.7 ±0.7, p<0.001) and an increased coronary microvascular resistance (33 ± 8 v 19 ± 6 U, p<0.001) in CSX.

Structural Abnormalities of the Vessel in CSX

Quite apart from the activation and dysfunction of the microvascular endothelium in CSX, there is some evidence of overall structural and morphological abnormalities of microvessels in CSX. Studies examining the ultrastructure of peripheral capillaries in CSX patients show an overall decreased capillaries density and an increase in the thickness of the vessel media (due to increased VSMC mass) and a relative decrease in lumen size in the microvessels of CSX patients compared with those of healthy controls[65]. PET scanning shows that similar morphological changes are present in the coronary microvessels of CSX patients[66]. Biopsy studies of cardiac tissue in CSX patients also demonstrates perivascular monocytic and polymorphonuclear cell infiltration of the microvascular wall with endothelial oedema and ultimately perivascular fibrosis[67]. These findings suggest chronic vessel wall inflammation. The same study also demonstrated the abnormal presence of apoptotic endothelial cells in the CSX patients. This may demonstrate premature cell senescence or else severe cellular damage.

Calcium scoring on CT scanning has been used as a marker of the burden of coronary atherosclerosis with an Agatston score of zero being associated with low burden and with increasing scores correlating with larger plaque burden. 64-slice CT scanning of CSX patients has demonstrated that these patients have higher calcium scores than age and sex matched healthy controls despite the absence of coronary artery disease on invasive angiography[68]. This highlights the relative insensitivity of coronary angiography for early atherosclerosis and demonstrates that CSX patients do have cardiac pathology that may explain some of their symptoms, viz. early atherosclerosis with endothelial dysfunction. The same study also demonstrated an increase in the carotid intima-medial thickness in CSX patients compared to controls.

In short, the macrovasculature and microvasculature in both the peripheral and coronary circulations are structurally abnormal in CSX patients. This is in addition to the obvious functional abnormalities that have already been discussed.

Endothelial Senescence

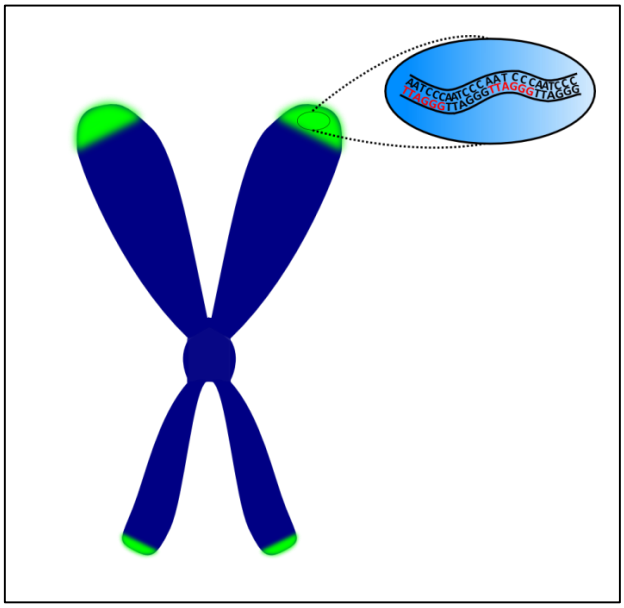


Figure 1.12: Telomeres

All cells in the body have a finite capacity to replicate. This is known as the Hayflick phenomenon and is believed to be due to telomere shortening. All chromatids have telomeres of varying lengths at each end. Telomeres are regions of repetitive nucleotide sequences that are many kilobases long. They contain many guanine-rich 6-8bp sequences (typically TTAGGG) that repeat. Due to the physical mechanics of DNA replication, the information at the ends of chromatids cannot be replicated and is lost. With each division, therefore, the chromatin strand shortens. Telomeres provide a reservoir of "junk" DNA that can be lost with each replicative cycle without loss of DNA that encodes genes. Typically, each cycle results in the loss of between 25 and 100bp. Once the telomeres run out, the cell is incapable of replicating without loss of critical

genetic material and successful division ceases. Studies by Hayflick on foetal cells showed that they could divide between 40-60 times on average. Some malignant cells can lengthen telomeres through the action of an enzyme called telomerase and this can result in immortalisation of the cells. Endothelial cells, like most somatic cells, have very low levels of telomerase.

When a cell can no longer replicate it enters a phase known as senescence with these cells irreversibly entering permanent cell-cycle arrest, with DNA content that is typical of the G1 phase. As well as undergoing telomeric senescence as described above, a cell can enter senescence prematurely in response to stresses such as oxidative damage or a continuous mitogenic stimulus. This appears to have evolved as a protective mechanism, possibly to minimise the risk of cancer. Regardless of the cause, senescence appears to involve activation of a p53/p21 or p16/retinoblastoma tumour suppressor pathways that then results in alteration of cellular structure and function. Senescent cells are usually enlarged and flattened and contain a high concentration of β-galactosidase, signifying an increase in cellular liposomal population. The cells are also functionally altered and, as in the case of endothelium, are no longer capable of carrying out some necessary functions. Some studies have shown that the endothelium overlying atherosclerotic plaque exhibits high levels of senescence-associated β-galactosidase (SA-β-gal) and further studies have also shown that telomere lengths in endothelial cells are shorter in atherosclerosis-rich regions than in healthy endothelium[69,70]. Despite the fact that the immune system appears to be able to remove senescent cells, ageing is associated with an increase in the population of senescent cells in various tissues and it is believed that these cells are responsible for the deleterious age-related changes seen in vivo.

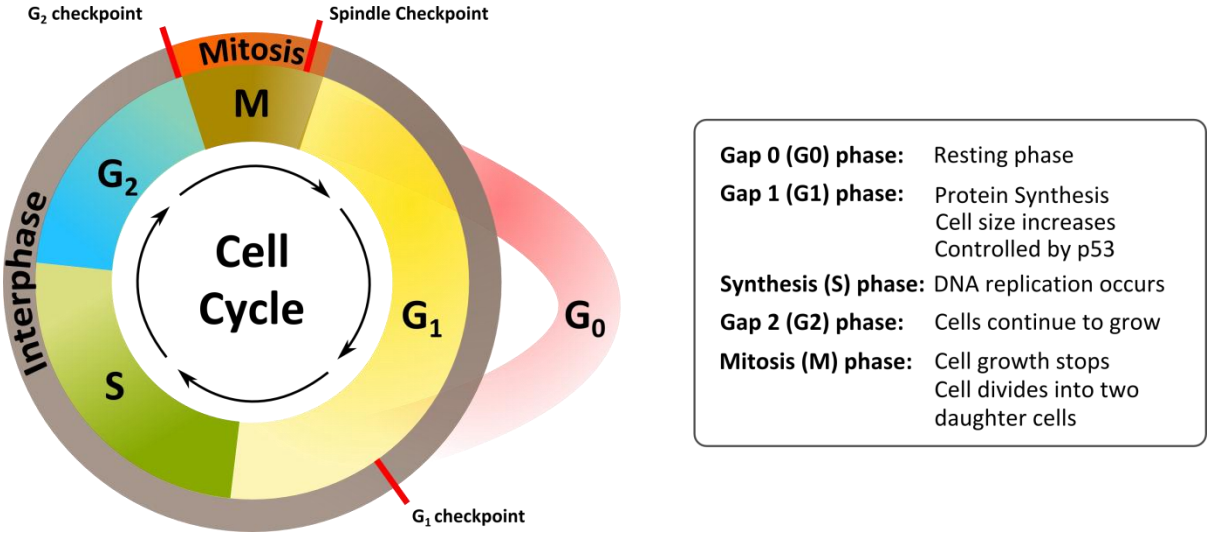


Figure 1.13: The Cell Cycle

Factors regulating endothelial senescence

Apart from simple replicative (or telomeric) senescence, endothelial cells are subject to multiple stimuli that can cause stress-induced (or premature) senescence.

a. **Oxidative Stress:** Normal mitochondrial metabolism produces many reactive oxygen species (ROS), which are normally neutralised by endogenous reducing agents such as glutathione. Endothelial cells can produce many ROS, including superoxide and hydrogen peroxide, with the main source being NADPH oxidase (NOX). ROS are capable of oxidising many substances in vivo, including proteins and nucleic acids. Telomeres, and the GGG repeats in particular, are particularly susceptible to oxidative damage and this may induce senescent similar to traditional telomeric senescence. In addition, oxidative stress can activate stress-response kinases that may induce p53 activity and senescence. Homocysteine also shortens telomeres while oxidised LDL has been shown to inhibit telomerase in endothelial cells.[71]

b. **Sustained mitogenic stimulus:** Oncogene overexpression can induce senescence through dysregulation of mitochondrial activity with an overflow of ROS into the cells, Miyauchi et al showed that this was the case with Akt-induced senescence in endothelial cells with Akt inhibiting FOXO3a's ability to regulate cellular levels of ROS with resultant p53 activation[72].
c. **SIRT1 activity:** Sirtuins are known to prolong cellular life. It seems as if they are capable of deacetylating and inactivating p53, thereby switching off the senescence apparatus. Sirtuin activity in the endothelium is mediated by nitric oxide and shear stress, with laminar flow upregulating its expression. Oscillatory shear, seen in atherosclerosis prone areas, downregulates SIRT1, which may result in endothelial senescence. Indeed, endothelium overlying atherosclerotic plaques has been shown to be populated by a high proportion of senescent cells[73].
d. **Nitric Oxide:** Quite apart from its ability to upregulate sirtuins, NO has also been shown to directly upregulate telomerase activity and delayed EC senescence [74].

Impact of endothelial senescence

Senescent endothelium demonstrates a pro-inflammatory phenotype and is dysfunctional in several ways. The most important of these features are tabulated below. It is essential to observe that many of the features of senescent endothelial cells are generally prominent in the endothelium of CSX patients, the implication being that EC senescence may be a pathological feature of CSX. It is possible that oxidative stress and other causes of endothelium dysfunction also cause premature endothelial senescence in CSX patients. This would explain the typical functional and structural abnormalities seen in the microvasculature in this condition. The specific presence of senescent endothelium has not been evaluated in CSX but histological studies have shown that a proportion of EC in CSX patients are apoptotic[67]. Senescent endothelial cells may ultimately undergo apoptosis in an effort to reduce the negative impacts of the senescent phenotype[75].

Consequences of Endothelial Senescence	
↑ **Vascular Inflammation**	Increased expression of adhesion molecules, chemokines and cytokines.
↑ **Atherogenesis**	Reduced lipoprotein degradation increased inflammation and increased ROS lead to increased plaque formation.
↑ **Thrombosis**	Increased PAI leads to a pro-thrombogenic state.
↓ **Endothelial Dependant Vasodilatation**	Limited NO synthesis and reduced prostacyclin production.
↓ **Angiogenesis**	Permanent cell cycle arrest prevents proliferation despite adequate stimulus and nutrients.

NO-Nitric Oxide; **PAI**-Plasminogen Activator Inhibitor; **ROS**-Reactive Oxygen Species

Table 1.11: Endothelial Senescence

1.4.4 Abnormal Autonomic Function

Another theory for the apparent microvascular dysfunction in CSX is the possibility of sympathetically mediated vasoconstriction. The autonomic innervation of the coronary microvascular is quite potent and is mediated mostly through adrenoceptors. α-adrenoceptors mediate vasoconstriction, with α_1 receptors predominating in the epicardial coronary arteries and α_2 found in the microvasculature. These are opposed by the activity of β-adrenoceptors. These receptors are found in highest concentrations in the arterioles and are mostly the β_2 subtype[76]. Under normal circumstances in healthy coronary arteries there is almost no activity through the α-adrenoceptors. It is believed that as atherosclerosis progresses, the relative contribution to vascular tone shifts from predominantly β-receptor driven to α-receptor driven with resultant vasoconstriction.

There is some evidence that α-adrenergic tone is indeed elevated in CSX. Indirect evidence of sympathetic overdrive, such as observations of QT dispersion, heart rate variability and resting heart rates, has been seen in CSX [77-80]. Imaging techniques using MIBG (an analogue of noradrenaline) have also shown frequent abnormalities involving cardiac MIBG uptake, indicating that there may be excessive competition with endogenous noradrenaline in the heart in CSX patients, hinting again at sympathetic overdrive[81]. One interventional trial showed that the use of doxazosin, an alpha-blocker, increased coronary vasodilator reserve in CSX patients[82].

There is, of course, some contradictory evidence regarding adrenergic tone in CSX. Use of doxazosin as a therapy for CSX has been unsuccessful, refuting the adrenergic theory somewhat [83]. In addition, PET scanning of CSX patients following pre-therapy with doxazosin failed to show any improvement in coronary blood flow or flow reserve. There is a caveat, however, that doxazosin is a selective α_1-blocker and it is the α_2-receptors that may be relevant to the microvascular dysfunction seen in CSX. Interestingly, non-selective inhibitors of alpha blockers (e.g. imipramine) have been shown to be effective in treating CSX[84].

1.4.5 Abnormal Nociception

As has been described in section 1.4.1, the neural sensation of angina is brought to the somatosensory cortex via autonomic afferent fibres. These fibres project cortically via the thalami bilaterally. An early theory into the aetiology of CSX suggested that the symptoms of the condition might be due to abnormal perception of cardiac stimuli, either at a peripheral nerve level or indeed centrally. One of the early investigators in CSX coined the phrase “sensitive heart syndrome” when a patient who presented with chest pain, a positive EST and normal angiogram had their pain reproduced by pacing of the right ventricle with a pacing lead. Interestingly, imipramine was used to successfully treat her symptoms[4].

There is some evidence that CSX patients may be more sensitive to cardiac stimuli. Normally people demonstrate differing levels of awareness of their own heart. Even an individual's ability to perceive their own heartbeat (ventricular ectopics for example) varies tremendously depending on their mental state, state of arousal etc. Studies in CSX patients have shown that these patients demonstrate a heightened awareness to intracardiac stimuli. For example, two studies showed that over 80% of the CSX patients developed chest pain upon mere manipulation of an angiography catheter in their right atrium and over 90% in response to manipulation of the catheter in their right ventricle [85-87]. Similarly, injection of contrast into the coronary arteries evoked pain in the majority of CSX patients studied.

Similarly, it has been shown that CSX patients have altered responses to peripheral painful stimuli. An early study showed that CSX patients had significantly lower pain thresholds compared to healthy controls when confronted with noxious stimuli such as forearm ischaemia and electrical stimuli. On average their pain threshold was 35-40% lower than that seen in healthy patients[88]. Furthermore, other studies using laser evoked potentials (a technique whereby a painful heat stimulus is delivered to the skin using a focused laser and the cortical response is recorded via electroencephalograph leads) have demonstrated a reduced habituation to repeated painful stimuli. The normal response to repeated noxious stimuli is a gradual reduction in amplitude of the evoked cortical potentials. This normal habituation is absent in CSX patients indicating abnormal cortical handling of painful stimuli [18,89]. This phenomenon is also observed in migraine and in fibromyalgia. Moreover, treatment of CSX with implantable spinal cord stimulators has been shown to restore normal habituation and to alleviate anginal pain in CSX patients[90].

Further evidence of abnormal pain processing is to be found in Positron Emission Tomography (PET) studies in CSX patients. These studies evaluated the increases in cerebral blood flow to (and hence activation of) various cortical regions in response to

dobutamine infusion. The general response to cardiac stimulation by dobutamine was activation of the hypothalamus, thalami, right frontal cortex and temporal poles, essentially delineating the normal sensory pathway. Some differences did exist between healthy controls and CSX patients, however. There was a relative increase in blood flow to the right insular cortex in CSX patients compared to controls, while the control patients had higher activation of the left insula and right anterior cingulate cortex[34]. These changes were not related simply to the presence of chest pain in the CSX patients during the study as further comparison with a coronary artery disease group also showed higher right insular activation in the CSX group despite similar chest pain. The insular cortex is believed to be responsible for interoception and has been implicated in irritable bowel syndrome[91]. The possibility of CSX being a central pain processing problem is intriguing.

A final consideration when evaluating the perception of pain by CSX patients is the frequent presence of psychosocial co-morbidity in these patients. CSX patients tend to have higher incidences of anxiety and depression compared to healthy controls and even patients with coronary artery disease[92-94]. Anxiety states have been shown to augment pain perception[95].

1.4.6 Summary

Although debate still exists as to the exact definition of Cardiac Syndrome X, let alone its pathogenesis, there is a growing realisation among cardiologists that it is a real pathological condition with demonstrable abnormalities on many investigations. Its aetiology likely involves the effects of many conventional cardiovascular risk factors on the endothelium, with endothelial and resultant microvascular dysfunction and perhaps an abnormal nociceptive processing overlay. A summary is presented below in figure 1.14.

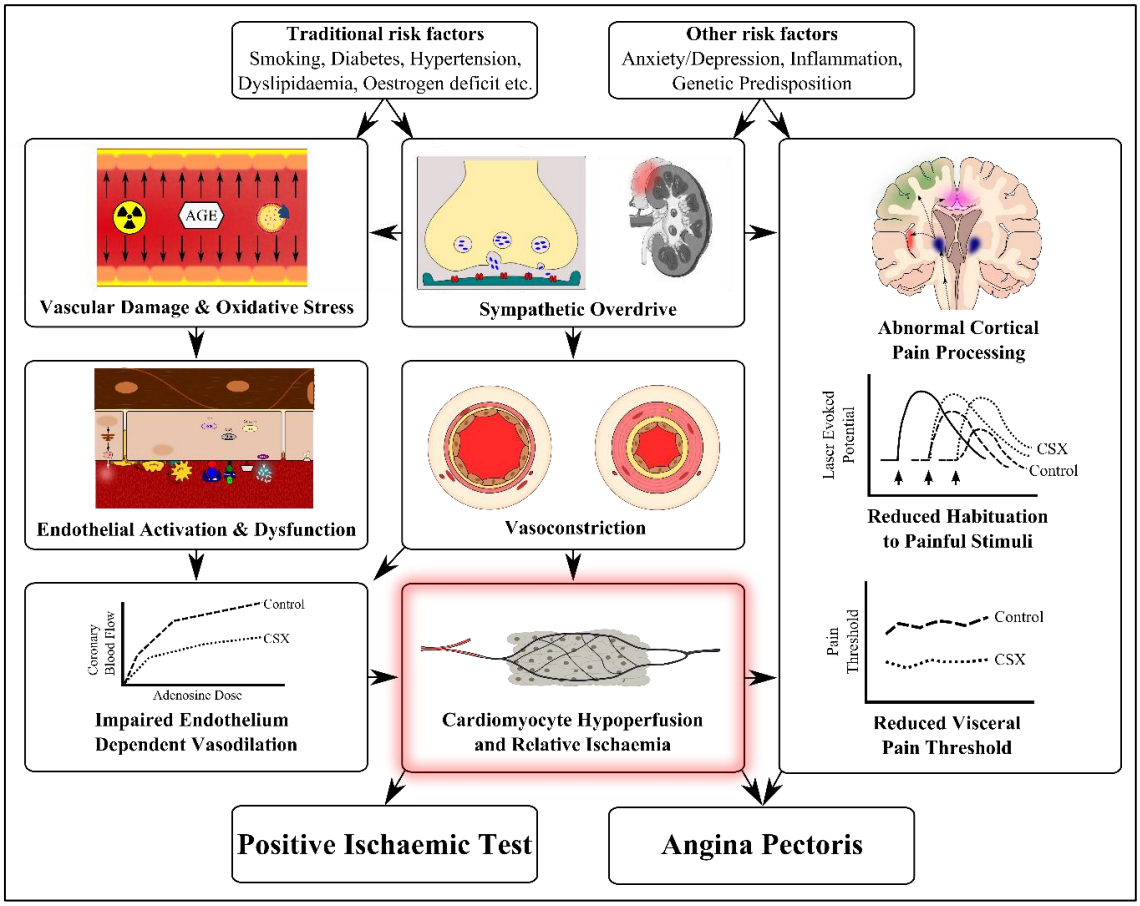


Figure 1.14: The possible pathogenesis of CSX

1.5 Prognosis

The overall prognosis in CSX has been perceived as being good and it may be because of this that research into the condition has been relatively sparse. The lack of consistency in case definition has also limited the generalizability of data from different studies. Recent studies have shown, however, that the prognosis for microvascular angina is worse than that of the general population with an increased risk of future stroke and vascular disease. Despite the generally favourable outcome, however, the burden of disease on the patients with CSX is similar to that of patients with coronary artery disease. A systematic review of the prognosis in CSX was undertaken for this thesis and follows here.

1.5.1 Systematic Review of Prognosis in Cardiac Syndrome X

1.5.1.1 Abstract

Objective

Cardiac Syndrome X (CSX) has been a challenging diagnosis since it was first described over 40 years ago, when the presence of angina with a normal angiogram was considered diagnostic. This heterogeneous group was shown to have an excellent prognosis. The modern diagnosis of CSX includes objective evidence of ischemia, defining a subset of the previously described group. This systematic review attempted to determine if prognosis in CSX with objective ischaemia remained benign.

Design and Outcomes Measured

A review of all English language literature in PubMed identified relevant studies of CSX patients with objective ischaemia. Data regarding outcomes, including death, myocardial infarction (MI), hospitalization and repeat angiography, were extracted and synthesised.

Results

Sixteen papers were included with a total of 1177 patients with a mean (SD) 6.17(3.06) years follow-up. The overall Level of Evidence was level 4. Thirteen deaths occurred in 6576 patient-years [median (IQR) 0.00(0.00-0.26) deaths/100 patient-years.] Few MIs occurred [0.00(0.00-0.09) MI/100 patient-years] but recurrent hospitalizations occurred frequently [Mean (SD) 4.8(5.0) hospitalizations/100 patient-years] with a median (IQR) 2.70 (1.93-7.91) angiograms/100 patient-years. Of these angiograms only 2.7% required revascularization. 70.1(25.7) % of patients at 6.1(3.4) years follow-up

had on-going pain with 77.0(18.5) % of patients on anti-anginal medications at 8.2(3.8) years.

Conclusions

This review confirms the benign prognosis of CSX with an exceptionally low mortality. Recurrent hospitalization, repeat angiography and on-going symptoms requiring medication are the main problems confronting the CSX population. The low incidence of revascularization in these patients might deter the desire for repeat angiography.

1.5.1.2 Introduction

Cardiac Syndrome X (CSX) is a clinical problem that has been confounding cardiologists since the early days of diagnostic coronary angiography. Initially described in 1973, CSX is the presence of typical anginal chest pain (burning, retrosternal discomfort which is worsened by exertion and relieved by rest or nitrates) despite the absence of demonstrable epicardial coronary stenosis on angiography [96]. Between 15-30% of all angiograms performed to investigate angina are normal[97,98]. Not all of these patients have CSX, as in the majority of these cases the chest pain is non-cardiac. The diagnostic criteria for CSX have evolved over the past 20 years and the general consensus now is that a diagnosis of CSX requires objective evidence of myocardial ischaemia in addition to the typical chest pain and angiographically normal coronary arteries. Ischemia may be implied by several means but most commonly by positive exercise stress testing (EST), myocardial perfusion imaging (MPI) or dobutamine stress echocardiography (DSE)[3]. Although the exact pathophysiology remains undetermined it is believed that the aetiology of CSX is microvascular dysfunction[37,99,100].

CSX is regarded generally as having a benign prognosis based on early case series[97,101]. These heterogeneous series did not include the strict criterion of demonstrable

ischemia. They indicated that these patients generally suffered no excess cardiac mortality but did continue to suffer from chest pain for many years after diagnosis. Also, traditional therapies for angina were poorly effective in the CSX populations. Importantly the optimal medical treatment of coronary artery disease has substantially evolved in recent years. However, the issue of benign prognosis in CSX has recently been called into question[102].

The objective of this review is to identify all studies that include prognostic data for patients with the modern definition of CSX (viz. typical angina pectoris, normal coronary angiograms and objective evidence of ischaemia) and to establish if these patients suffer from excess morbidity and mortality.

1.5.1.3 Methods

Criteria for Study Selection

All studies published in the English language up to January 2013 relevant to the review topic were eligible for consideration. Study populations met the strict diagnosis of CSX. All suitable participants were included regardless of age, gender or ethnicity. Anginal symptoms were required to be typical in nature in order to exclude patients with likely non-cardiac chest pain and to make the study population under review more homogeneous. “Normal” angiograms were those without any stenosis >20% severity, in line with most studies’ inclusion criteria. Objective evidence of ischemia included electrically-positive EST, positive DSE or MPI evidence of hypo-perfusion. Duration of follow-up of at least 2 years was chosen as necessary to allow time for the development of outcomes such as hospitalisations and revascularisations.

Outcomes Measured

The main outcomes of interest included the overall mortality (including deaths not attributable to cardiovascular disease) and the incidence of myocardial infarction and other vascular complications. Rates of re-hospitalisation during the period of follow-up as well as the need for repeat angiography and/or re-vascularisation were also recorded. Finally, the use of anti-anginal medication and the presence of on-going angina at the end of follow-up were noted. Not all studies included all of these outcomes but all data were extracted where available

Search Strategy

A review of EMBASE, PubMed, Web of Knowledge and the Cochrane database was undertaken to identify all relevant papers up to June 2013. MeSH terms used in Pubmed included "microvascular angina" and "/prognosis". Other keywords used with Pubmed and EMBASE were "microvascular", "long-term", "outcome", "Cardiac Syndrome X" and "complications". A search of the grey literature found one additional abstract, an Italian registry of CSX patients [Tritto et al, ESC Congress, Barcelona 2009].[103] Finally, all articles' references were evaluated and any other potentially relevant articles were reviewed.

Each study included was examined in detail by the authors of this review. The patient selection process was examined closely. All studies included stated that patients had typical chest pain. Some studies were excluded as they included patients with atypical chest pain (i.e. at rest, pleuritic etc.) These patients may not be diagnosed with CSX but possibly with non-cardiac chest pain. All included studies also specified that subjects had undergone testing, which had revealed objective evidence of ischaemia. EST was used fifteen studies and MPI was used in one.[104] The studies also needed to attempt to

account for all patients lost to follow-up and provide evidence that efforts were made to enrol all suitable patients.

Assessment of Study Quality

No. of centres	Was the case series collected in more than one center?
Hypothesis	Was the hypothesis/aim/objective clearly described?
Clear Criteria	Were the inclusion and exclusion criteria (case definition) clearly reported?
Clear Outcomes	Was there a clear definition of the outcomes reported?
Prospective	Were data collected prospectively?
Recruitment	Was there an explicit statement that patients were recruited consecutively?
Findings	Were the main findings of the study clearly described?
Stratification	Were outcomes stratified by test results, patient characteristics etc.

Table 1.11: NICE Quality Assessment Tool for Case Series: 0-3 = poor; 4-6 = fair; 7-8 = good

Studies were graded as being good, fair or poor based on the assessment of the NICE (National Institute for Health and Clinical Excellence) criteria for the assessment of case series (*Table 1.11*). Each criterion was worth 1 point in the assessment. A score of 0-3 would be poor, 4-6 fair and 7-8 good. Follow-up of at least 2 years' duration was required. At least 80% follow-up was expected from studies to be included.

Data extraction

Data was extracted independently by the authors. The mean, SD and range of follow-up in years was recorded. The number of patients and gender distribution of patients were also noted. The number of patient-years was determined by multiplying the number of patients by the mean duration of follow-up. Where specified, the numbers for death, myocardial infarction (MI), revascularization and repeat angiography were noted. Data on the proportion of patients with on-going symptoms was also noted if available.

Data analysis

All data from the studies was input into a database on SPSS (v20). The average point estimate per 1000 patient-years was calculated for each parameter in each study. The mean of these estimates from all studies was calculated as well as the standard deviation. If the data was not normally distributed, its central tendency and dispersion were described in terms of its median and interquartile range**.** The data itself is reported as mean (SD) in the form x (y) if normally distributed or as median (IQR) in the form x (y-z) if not normally distributed. It was intended that gender-specific outcome data would be calculated but only 13 of 16 studies gave population breakdown by gender and no study divided outcomes into subgroups by gender, thereby precluding synthesis of this data.

1.5.1.4 Results

Description of the Studies

The search strategy identified 318 total citations (see figure 1.15 below). Abstract and title review excluded 284 of these. Thirty-four full-text articles were examined and 18

of these were found to be unsuitable for inclusion. Six of the excluded articles included patients with atypical angina, 1 had >20% loss to follow-up, 8 had no objective evidence of ischaemia, 2 had no clear inclusion criteria, 1 had data that was unsuitable for extraction and 4 had two or more of the above issues. This left 16 studies for analysis with a total 1177 patients giving a median (IQR) of 43(26-97) patients per study with a mean (SD) 6.2 (3.0) years follow-up (*Table 1.12*). All of the included studies used the modern criteria for CSX diagnosis. Most included studies are of level of evidence 4 based on the Agency for Healthcare Research and Quality scale. No randomized controlled trials were found and 14 of the 16 studies were non-comparison observational studies (i.e. case series).[3,17,28,101,104-114] One of the remaining papers had a comparison group defined by the presence or absence of a second positive ischemic test (i.e. a positive MPI in addition to a positive EST).[108] The other paper was a case-control study assessing the efficacy of spinal cord stimulation.[111] The control group in this latter study had CSX and was included in this review. No paper had a healthy control group.

The median (IQR) quality score was 6 (5.25-7.00) out of a possible 8. Fifteen of the studies were prospective. In all cases, efforts were made to ensure 100% follow-up of patients. For the subsidiary end-points such as the degree of improvement in angina etc. appropriate questionnaires were used. Enrolment was complete in all studies although only 5 specifically stated that consecutive patients were enrolled. Each study had at least 2.5 years of active follow-up and >90% follow-up was maintained in all cases. The lack of healthy control groups impacts on the strength of data derived from these studies.

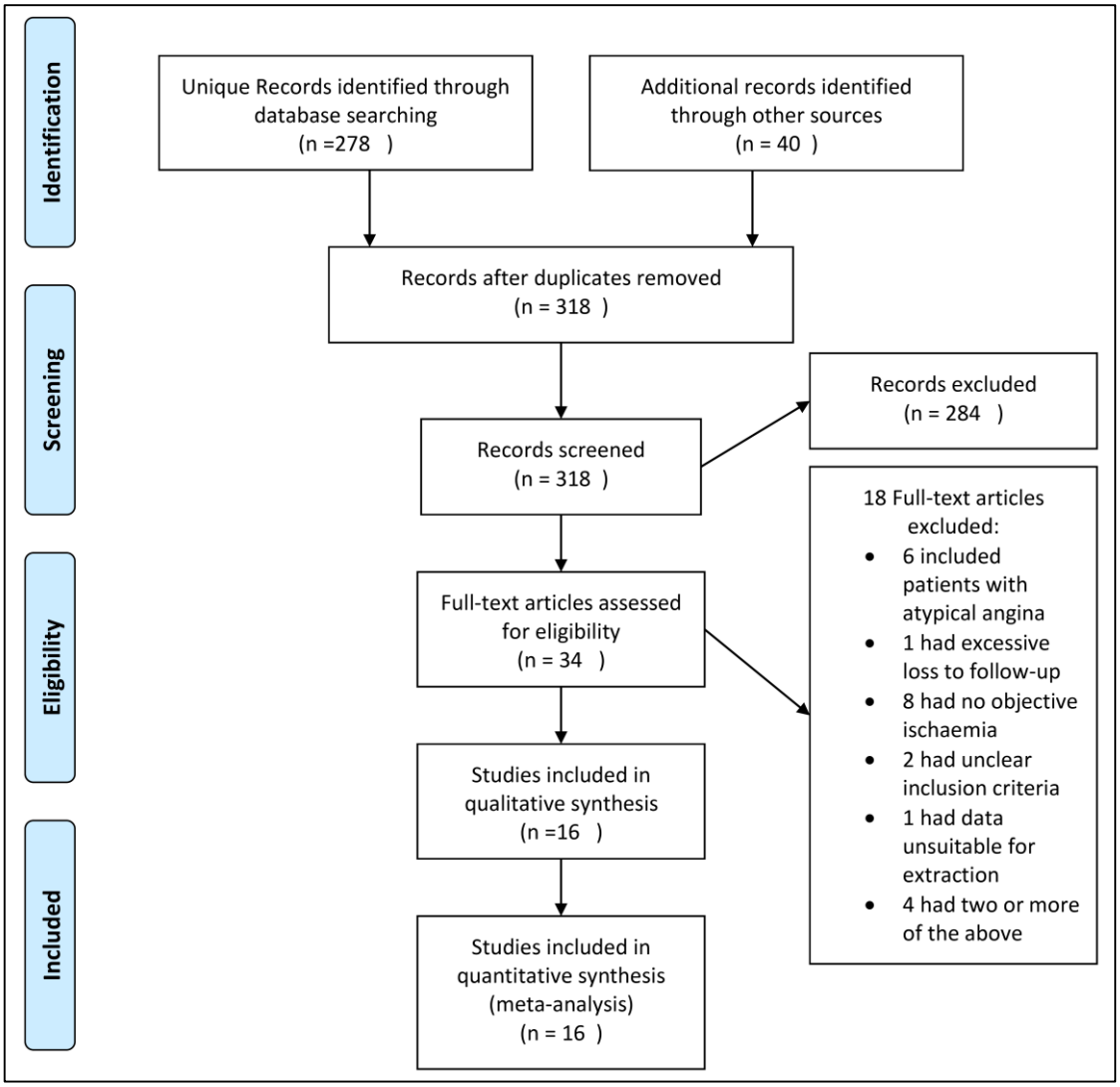


Figure 1.15: PRISMA diagram

Outcomes

Outcomes are summarized in *Table 1.13,* which highlights the percentage of the study populations that registered each individual end-point, and in *Table 1.14,* which shows summary descriptive data of the major outcomes noted by the majority of the trials.

Study	Year	Country	Quality score	Sample Size	Follow-up (years)	Outcomes Measured
Bemiller et al	1973	USA	4/8	12	4.1	MI
Bugiardini et al	2004	Italy	6/8	42	10.3	Death, MI, Pain, Hosp, Meds
Chauhan et al	1993	UK	7/8	82	3.0	Death, MI, Pain, Hosp, Meds
Delcour et al	2009	USA	5/8	6	7.4	MI, CVA
Di Monaco et al	2010	Italy	6/8	40	6.6	Death, MI, Pain, Hosp, Angio.
Huang et al	2010	Taiwan	7/8	108	5.0	Death, MI, Hosp, Revasc, CVA
Kaski et al	1995	UK	8/8	99	7.0	Death, MI, Pain, Hosp
Lamendola et al	2010	Italy	5/8	155	11.4	Death, MI, Pain, Hosp, Angio Revasc, Meds.
Leu et al	2006	Taiwan	7/8	92	2.7	Death, MI, Hosp, Angio, Revasc, CVA
Opherk et al	1989	Germany	6/8	25	4.0	Death, MI, Pain
Radice et al	1995	Italy	5/8	30	12.5	Death, MI, Pain, Hosp, Angio, Revasc, Meds, CVA
Romeo et al	1993	Italy	6/8	30	5.0	Death, MI, Pain, Meds.
Sgueglia et al	2007	Italy	6/8	9	3.0	Death, Pain
Shintani et al	2003	Japan	6/8	43	6.4	Death, MI, Pain
Sun et al	2001	Taiwan	7/8	54	7.1	Death, MI, Pain, Meds
Tritto et al	2009	Italy	5/8	350	3.3	Death

Table 1.12: Characteristics of Included Studies. CVA- Cerebrovascular Accidents, **Hosp**- Hospitalisations, **Meds**- Medications, **MI**- Myocardial Infarction, **Revasc**- Revascularisations.

Study	n	Death	MI	Hosp	Angio	Revasc	Pain	Meds	CVA
Bemiller et al	12	-	0	-	-	-	-	-	-
Bugiardini et al	42	0.02	0.02	0	-	-	31.0	42.0	-
Chauhan et al	82	0	0	23.2	-	-	47.6	45.0	-
Delcour et al	6	0	16.7	-	-	-	-	-	16.7
Di Monaco et al	40	0	0	67.5	62.5	-	-	-	-
Huang et al	108	4.62	0	9.25	-	0	-	-	4.62
Kaski et al	99	0	0	29.3	-	-	-	-	-
Lamendola et al	155	2.59	0	57.4	21.3	1.3	-	-	-
Leu et al	92	0	0	8.70	8.70	0	-	-	3.26
Opherk et al	25	0	0	-	-	-	100	-	-
Radice et al	30	3.33	0	13.3	26.6	0	67.7	21	10.0
Romeo et al	30	0	0	10.0	-	-	97	29	-
Sgueglia et al	9	0	-	-	-	-	100	-	-
Shintani et al	43	2.33	0	-	-	-	44.2	-	-
Sun et al	54	1.85	1.85	-	-	-	77.8	33	-
Tritto et al	350	0	0.29	-	-	-	71.2	-	-

Table 1.13: Outcomes at End of Follow-up as Percentage of Study Population. CVA- Cerebrovascular Accidents, **Hosp**- Hospitalisations, **Meds**- Medications, **MI**- Myocardial Infarction, **Revasc**- Revascularisations

Outcome	No. of studies	Patient Years	Absolute Count	Average Measure
Death	15	6577	13	0.0 (0.0-2.6)/1000pt-yrs
Myocardial Infarction	15	6599	4	0.0 (0.0-0.9)/1000pt-yrs
Hospitalisation	9	4592	189	47.8 (50.0)/1000pt-yrs
Repeat Angiography	4	2654	74	41.7 (35.8)/1000pt-yrs
Revascularisation	4	2930	2	0.0(0.0-0.8)/1000pt-yrs
On-going Pain	9	3021	450/665	70.6± 25.7% of patients at 6.1± 3.4 years' follow-up
Anti-Anginal Use	6	3231	293/393	77.0± 18.5% of patients at 8.2± 3.8 years
Cerebrovascular complications	4	1207	12	12.9± 0.7/1000pt-years

Table 1.14: Summary of Major Outcomes

1.5.1.5 Discussion

This review appears to confirm the benign nature of CSX in terms of mortality, even when using the widely accepted contemporary diagnostic criteria. The calculated mortality rate of 2.6 per 1000 patient-years compares favourably with even 2012 mortality data for the general unselected adult populations from the included study locations (range: 6.4-11.0 deaths/1000 pa). Some of this discrepancy will be explained by the fact that cardiovascular disease accounts for 30% of all deaths and CSX patients necessarily have normal epicardial coronary arteries and structurally normal hearts.

Additionally, patients in these series have also been thoroughly medically assessed, benefitted from regular specialist follow-up and have been selected as "pure" CSX patients. Many studies excluded diabetic patients and those with hypertensive heart disease, further narrowing the case definition and selecting out healthier patients. Finally, the use of cardio-protective medications, such as statins and beta-blockers, in these patient groups would further reduce cardiovascular risk. Clearly, however, a diagnosis of CSX does not seem to confer increased mortality.

The increased rate of cerebrovascular events in this population does warrant further scrutiny. A typical Western country would expect to have 1-3 strokes per 1000 patient-years but the incidence in the CSX population noted here is more than triple that. It must be noted that only 4 studies (18% of the 6626 patient-years) reported on the presence or absence of cerebrovascular events and in these only 12 events occurred. All of these studies specified that the events were not "procedure related" but did not elaborate as to how this was determined. CVAs are known to complicate 0.05-0.1% of all coronary angiograms. [115,116] It is possible that conventional risk factors that may result in endothelial dysfunction and CSX may also potentially increase the risk of concurrent cerebrovascular disease.

The true burden of CSX seems to come from the recalcitrant symptoms. Over two-thirds of patients suffer with on-going typical angina pectoris and this in turn leads to consumption of anti-anginal medications in a similar proportion of patients. It is somewhat reassuring then that only 4.8 per 100 patients per annum require re-hospitalization due to this persistent angina. The incidence of repeat angiography is, however, relatively high with 4.2 occurring per 100 patients per annum indicating that a large proportion (>85% of those studied) of CSX patients who are hospitalized during follow-up will undergo a further angiogram. The most relevant statistic is that <0.08 revascularizations occurred per 100 patients per annum implying that >97% of

angiograms performed did not indicate a need for intervention. This data is based on only 4 studies reporting on revascularizations.

1.5.1.6 Limitations

Whilst undertaking this review it became clear that there is a paucity of studies with robust design quality looking at prognosis in CSX. This is demonstrated by the absence of healthy comparison groups, the use of small sample sizes and the intermediate quality scores of the available studies and is borne out by the heterogeneity of the outcome data across all of the studies. This review attempted to include studies with similar patient cohorts in terms of case definition with particular emphasis on studies looking at typical angina pectoris to avoid encompassing subjects with probable non-cardiac chest pain and incidental false-positive ischemic testing. This means that the results of this review are applicable mostly to patients with typical chest pain CSX and not necessarily all patients with chest pain and CSX. A comprehensive review published last year included studies with a more heterogeneous population as well as patients without objective of ischaemia and reported favourable outcome data in terms of mortality and myocardial infarction in this group again showing the prognostic value of a normal coronary angiogram.[117]

1.5.1.7 Implications for Practice

Once the diagnosis of CSX has been made the patient should be reassured regarding prognosis. The vanishingly low rate of MI, revascularizations and death are welcome. The cardiology community does need to be aware of the fact that the majority of these patients will continue to suffer from angina even after 5 years and that over three quarters will require long-term treatment. Repeat angiograms should be avoided in these patients unless there is a strong clinical suspicion that something has changed.

1.5.1.8 Implications for Research

There is a lack of high-quality studies in this area. Inadequate case definition has led to heterogeneous study population and consequently heterogeneous results. The data generated by studies to date are quite convincing but the studies are undoubtedly subject to substantial bias. Higher quality research with consistent case definitions in terms of symptoms as well as more reliable measures of true ischemia (e.g. PET or coronary sinus lactate sampling) would allow more homogeneity across studies.

1.6 Treatment

Treatment of CSX is dependent on the ability to diagnose it. As the condition presents with symptoms identical to that seen in obstructive coronary artery disease many traditional anti-anginal therapies have been employed to affect symptom control in CSX patients. Results have been inconsistent, however, and the majority of patients are left with inadequate symptom control. This section will examine the evidence of the effectiveness of many treatment options and will attempt to provide a framework for controlling symptoms in these patients.

1.6.1 Conventional Anti-Anginal Pharmacotherapy

Anti-anginal therapy aims to reduce myocardial metabolic demand by reducing cardiac work as well as maximizing oxygen delivery to the myocardium. Several therapies that are useful in treating angina in obstructive coronary artery disease have also been shown to be of use in CSX.

***Beta-blockers**:* The sympathetic nervous system up-regulates several cardiac functions resulting in an increased metabolic demand. Normal myocardium contains an abundance of β_1- and β_2-adrenoceptors, which are involved in the activation of the

myocardium by the sympathetic nervous system. This results in increased chronotropic, inotropic, dromotropic and lusitropic effects. All of these increase myocardial oxygen demand, which can lead to ischaemia. Studies have shown that CSX patients have increased sympathetic drive as evidenced by a reduced heart rate variability [80], higher average heart rates [79] and prolonged QTc [77] compared to healthy controls. Abating this increased sympathetic tone could remedy some of the symptoms of CSX.

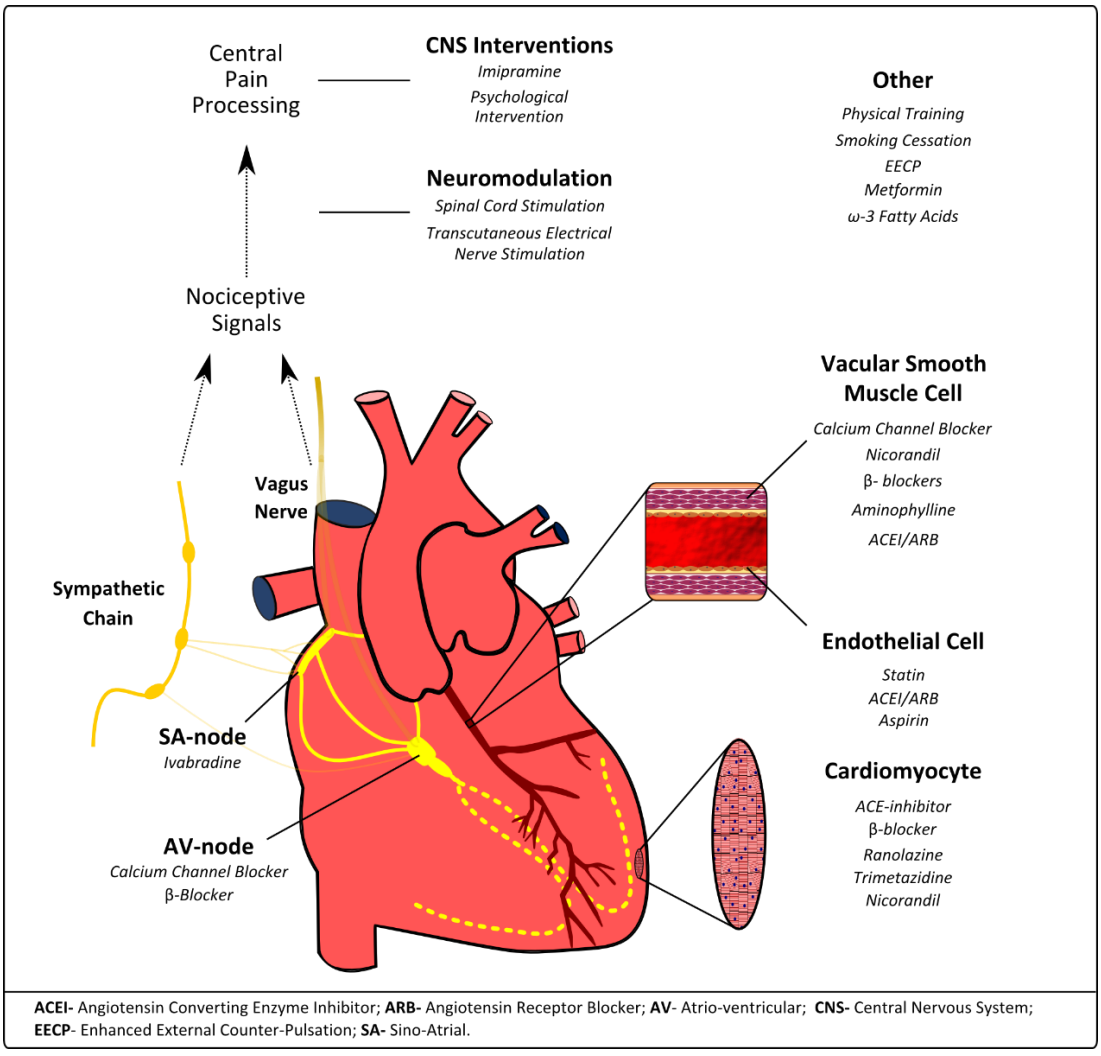


Figure 1.16: Treatment Options in CSX

Third-generation beta-blockers have been shown to increase baseline coronary blood flow as well as improving coronary flow reserve (CFR). These features may be of particular benefit in CSX where an impaired CFR is often seen. Nebivolol, a commonly prescribed 3rd generation beta-blocker, has a good dataset supporting its use in CSX. It has been shown to improve CCS anginal class in 70% of patients and significantly prolongs exercise duration as assessed on exercise stress testing (EST). In addition, it improves endothelial function with a 200% increase in NO release and also reduces the concentrations of inflammatory molecules such as vWF, hsCRP and ADMA (aspartate dimethylarginine, a potent inhibitor of NO activity.)

Despite their tendency to cause macrovascular vasoconstriction, 2nd generation beta-blockers have also been shown to be clinically effective in CSX. Two studies showed significant reductions in the frequency of angina attacks while on atenolol [118,119], while the latter study also noted that 18/22 (82%) of CSX patients developed electrically negative ESTs while taking atenolol. In summary, Beta-blockers appear to be effective in controlling symptoms and improving exercise capacity in CSX and Nebivolol may even modulate pathways allowing recovery of endothelial function.

Calcium Channel Blockers (CCBs): CCBs block slow voltage-gated calcium channels in the smooth muscle cells of vascular walls, preventing calcium influx and allowing vasodilatation. This reduces peripheral resistance and afterload, which accounts for much of their anti-anginal effects. Non-dihydropyridine CCBs also affect the myocardium and exert negative inotropic and chronotropic effects, thereby reducing metabolic demands. In aggregate, CCB use in CSX can be supported either after a trial of beta-blockade or perhaps in tandem with it. Studies have shown improvements in exercise duration on exercise stress test (along with a delay in time to ischaemia of 80%.) CCBs also reduce angina frequency and GTN use. [120,121]

Ranolazine: Ranolazine reduces the late transcellular sodium current thereby reducing calcium influx via sodium-dependant Calcium channels in cardiomyocytes. This improves lusitropic function and may improve diastolic filling parameters. This also reduces wall stress and end-diastolic pressure with a concomitant reduction in microvascular compression during diastole. Ranolazine appears to have a small significant benefit on coronary and systemic microvascular function (as measured by CFR in response to adenosine and cold-pressor test, and brachial artery FMD and nitoprusside-mediated dilation[122].) A pilot study in 20 women with CSX showed that 4 weeks of treatment with ranolazine improved several subjective parameters as measured by the Seattle Angina Questionnaire (SAQ). Quality of life (p=0.021), angina stability (p=0.008) and physical functioning scores (p=0.046) were all improved[123]. A similar study expanded on this with 30 CSX patients and found that ranolazine significantly improved all aspects of the SAQ and also improved time to 1mm ST-depression and total exercise time during EST. A study run in patients with a diagnosis of microvascular angina determined by invasive coronary reactivity testing failed to replicate these findings[124].

Nicorandil: Nicorandil is a novel anti-anginal agent with two distinct modalities of effect. It can activate the cGMP second messenger pathway, mimicking nitric oxide activity, in vascular smooth muscle cells. It also activates an ATP-sensitive K^+ channel, which causes hyperpolarization of the myocytes while also reducing intracellular calcium. At lower doses the nitrate-like activity allows epicardial vasodilatation but higher doses are needed to allow resistance vessel dilatation, resulting in reduced coronary arterial resistance. One study showed that oral administration of 5mg three times daily of nicorandil significantly prolonged total exercise time and time to 1mm ST-depression on EST in 13 patients with CSX[*125*]. The frequency of angina episodes, use of GTN and subjective feeling of improvement were also significantly improved.

Ivabradine: Ivabradine induces bradycardia by directly affecting the I_f channels of the sino-atrial node. Its effect is one of pure heart rate reduction. It is indicated for use in chronic stable angina and in heart failure. The only study into its effectiveness in CSX showed that ivabradine significantly improved all aspects of the SAQ. It did not, however, show any clinical improvement in terms of EST-induced ST-depression or exercise capacity. Also it showed no significant improvement in systemic or coronary microvascular function.[126] When taken with the fact that beta-blockers are almost universally useful in CSX, ivabradine's effectiveness tells us that heart rate control is a very important goal in the treatment of CSX.

Trimetazidine: The data regarding the usefulness of trimetazidine in CSX is underwhelming. The results have been mixed although two small studies have shown a marginal improvement in EST parameters while on trimetazidine.

1.6.2 Adjunctive Medical Therapy

The main aim in the management of CSX is to control the patient's symptoms but an important secondary goal should be to modify their vascular phenotype (which is typified by reduced NO availability and endothelium-dependant vasodilatation.) The main agents, beyond those mentioned above, that would be of useful in this regard are statins and ACE-inhibitors.

Renin-Angiotensin Aldosterone System antagonism: Angiotensin II is a potent vasoconstrictor and leads to increased myocardial contractility and heart rate due to AT_1 receptor activation. ACE inhibitors, as well as preventing the formation of Angiotensin II, prevent the degradation of bradykinin, itself a potent vasodilator. Therefore, ACEI may be of benefit in CSX by preventing both vasoconstriction and increased cardiac workload but also by facilitating bradykinin-mediated vasodilatation,

which may be central to their effectiveness. In addition to having a beneficial effect on markers of endothelial function, studies using exercise stress test outcomes suggest that ACE-inhibitors consistently improve exercise duration. Ramipril and Enalapril have both been shown to be particularly effective in this regard and ramipril also reduce the weekly angina burden by 66% with a tandem drop in GTN use in studies.[121,127,128] Both the Kaski and Pizzi studies also showed significant reductions in the magnitude of ST-depression at peak exercise whilst on ACEI therapy.

ARBs on the other hand have not been shown to be effective in CSX. One study showed that irbesartan failed to improve exercise duration in 24 CSX patients[129]. This lack of efficacy may highlight that the bradykinin-mediated vasodilation pathway may be responsible for the benefits of ACE inhibitors in CSX patients, as ARBs do not increase bradykinin levels in vivo.

Statins: HMG-CoA reductase inhibitors reduce mortality in many vascular conditions through direct reduction in LDL levels as well as through pleiotropic effects. The utility of statins in CSX has been studied in several small RCTs. Simvastatin use in CSX patients with pre-existing dyslipidaemia improved brachial artery FMD (indicative of improved endothelial-dependant vasodilatory function) as well as improving the time to 1mm ST-depression on exercise testing[130]. Statins can even be effective in CSX patients with normal plasma lipids[131]. This last study also noted clinical benefit in the form of increased Exercise duration on EST and time to 1mm ST-depression with significant improvements in FMD on pravastatin, changes which were absent in the placebo group. Additionally, patients reported feeling improved more when on pravastatin than those in the placebo group (p=0.014).

Aspirin: The current ESC guidelines advocate the use of aspirin in microvascular angina (CSX) alongside statin therapy in an effort to modulate endothelial dysfunction, although there is no evidence specifically supporting its use on a secondary prevention basis in CSX.

Aminophylline: Aminophylline is a non-selective adenosine receptor antagonist as well as being a phosphodiesterase inhibitor. Adenosine plays an important role in regulation of coronary blood flow, chiefly by causing local vasodilatation at the site of its release. Antagonism of adenosine has been shown to improve exercise capacity in patients with CAD but its use in this manner has never been widespread. Studies in CSX patients have shown that IV aminophylline infusion at the time of EST increases exercise capacity by 40% as well as rendering the test electrically negative in the majority of cases[132,133]. The effectiveness of oral aminophylline was also investigated and shown to significantly improve exercise time, time to ST-depression, magnitude of ST-depression and time to angina on EST [134].

1.6.3 Non-pharmacological Therapies

Some patients may be resistant to the use of medications, especially if they are prone to side-effects. Non-pharmacological therapies may be of benefit used alone or in conjunction with medications.

Exercise Training: The benefits of aerobic exercise and cardiac rehabilitation in Ischaemic Heart Disease and heart failure have been well demonstrated and there may be a role for it in CSX. Several small studies have been performed but are limited to female populations with CSX. Studies consistently show that women with CSX have a reduced functional capacity and that there is significant benefit to following a cardiac

rehabilitation program. Both exercise capacity itself and measures of psychological well-being (such as the HADS and HAQ scores) improve following these programs.[135-137] Exercise is safe, cheap and effective in women in CSX and as such should be recommended for all suitable female CSX patients. No studies have examined its effectiveness in a male population to date but it is likely to be similarly beneficial in that population.

Neuromodulation therapy: Many CSX patients have been noted to have increased sensitivity to a variety of cardiac stimuli (e.g. injection of contrast, intracoronary adenosine infusion or ventricular pacing) and it has been suggested that they have a dysregulation of cardiac nociceptive pathways. Modulation of these pathways may be achieved using TENS or, more invasively, Spinal Cord Stimulation (SCS). In aggregate, studies of neuromodulation therapy demonstrate some efficacy in CSX.

Psychological Intervention: CSX patients suffer from more anxiety and depression than healthy control and even people with CAD [92,138].Given the potential for psychological stress to cause ischaemia and even infarction, treatments aimed at controlling psychological factors might have a role in symptom management in CSX. A Cochrane Review into psychological interventions (including Cognitive Behavioural Therapy) in general non-cardiac chest pain (which included some CSX patients) identified 16 RCTs (n=803) and showed a significant reduction in reported chest pain in the first 3 months, Relative Risk of 0.68 (95% CI 0.57 to 0.81), which was sustained to 9 months[139]. In a small pilot study, *Cunningham et al*[140] showed that transcendental meditation reduced angina frequency in women with CSX while improving QOL scores. Therefore, psychological interventions can be of benefit in CSX, especially when psychological comorbidities have been identified.

1.6.4 Other Potential Therapies

Treatment may be unsuccessful despite trying the whole gamut of previously mentioned interventions. Further less usual treatments may be of benefit in selected patients.

Enhanced External Counterpulsation: EECP has been shown to increase shear stress in blood vessels and consequently improves endothelial function. Kronhaus and Lawson showed that 35 hours of EECP improved CCS class (from 3.57±0.4 to 1.42) and normalised perfusion defects in 28 of 30 patients with CSX, although many of their patients had comorbidities such as diabetes, CKD and CCF. Effects persisted at 1 year[141].

Metformin: One study showed that metformin improved peripheral microvascular function as well improving Duke Treadmill Score by 6.1 Units in 33 CSX patients without diabetes. Chest pain incidence tended to be less when on metformin (-30%, p=0.054)[142].

Proton-Pump Inhibitors: The PITFALL trial showed that proton-pump inhibitors significantly improved chest pain in a highly selected cohort of patients who had cardiac syndrome X. Only 34% of eligible patients were enrolled and 97% of these had gastritis/GORD on OGD. Unsurprisingly, PPIs were effective in this cohort[143].

Imipramine: *Cox et al* showed that imipramine significantly reduced the total number of chest pain episodes experienced by 47% but at the expense of a high incidence of side-effects (83% suffered from anticholinergic effects) in a group of 18 women with chest pain and normal coronary arteries (14 also met the criteria for CSX.) Quality of life was not improved by therapy[84].

ω-3 Fatty acids: One recent trial showed that dietary supplementation with omega-3 significantly reduced symptoms in a CSX cohort. It would be of great interest if dietary factors could influence CSX symptoms[144].

Vitamin D: One recent small study showed a significant benefit in terms of symptoms and EST parameters in CSX patients treated with vitamin D supplements[145].

1.6.5 Ineffective Therapies

Some drugs commonly used in cardiovascular disease have not been shown to be of benefit in CSX.

Alpha-blockers: Alpha-adrenoceptor blockade leads to arteriolar vasodilatation and as such may be useful in CSX. The only therapeutic trial looking at clinical effectiveness of doxazosin in CSX has shown no benefit[83]. Similarly, Prazosin and clonidine have been shown to be ineffective in CSX.

Nitrates: Findings across many studies consistently show that nitrates are not of substantial benefit in the CSX population. In fact, most of the studies show a disimprovement in exercise capacity as measured by EST, with a reduced time to ischaemic threshold and a prolonged recovery time. *[134,146-148]* Similarly studies into L-arginine (an amino-acid precursor of NO) supplementation also show no improvement in exercise duration on EST despite beneficial effects on CFR and FMD. The reason for this lack of benefit is likely to be a rebound increase in sympathetic tone with resultant vasoconstriction. Additionally, nitrates affect larger arteries with minimal effects on the microvasculature. Finally, they may precipitate a coronary steal phenomenon by preferentially promoting sub-epicardial vasodilatation at the expense of the endocardium.

Table 1.15: Evidence Base for Therapeutic Options

Intervention	Study	Year	Details	n	Results
α-blocker	Botker et al	1998	Doxazosin 1-4mg, 10/52, x-2B	16	No effect on EST parameters.
ACE Inhibitors	Chen et al	2002	Enalapril 5mg, 8/52. 2B	20	↑ exercise duration on EST by 16.8% and coronary flow reserve by 23%. ↓ vWF and ADMA. ↑ NOxlevels.
	Kaski et al	1994	Enalapril 10mg, 2/52, x-1B	10	↑ exercise duration by 13%.↑time to ST-depression by 42%.
	Pizzi et al	2004	Ramipril 10mg*, 6/12,	45	↑ exercise duration by 23%. ↑FMD by 91%. ↓ SOD. ↑ SAQ results by 64%.(* in combination with atorvastatin 40mg)
	Ozcelik et al	1999	Ramipril 2.5mg, 4/52,x	18	↓ angina frequency by 66% and GTN use by 71%. Modest 4.7% ↑ in execise duration.
Aminophylline	Radice et al	1996	400mg STAT. x	20	50% of ESTs became electrically and symptomatically negative. ↑ time to angina by 58% on the remaining positive ESTs.
	Elliott et al	1997	225 or 350mg BD, 3/52, x-2B	13	↑ time to angina by 21%. 15% withdrew because of side-effects. NS change in angina frequency.
β-Blockade	Kayaalti et al	2010	Nebivolol 5mg, 4/52	38 (20 T, 18C)	↑ brachial artery baseline and maximal diameter. No change in FMD. ↓ hsCRP, vWF, fibrinogen.
	Sen et al	2009	Nebivolol 5mg, 12/52, 1B	54 ($19T_1$, $19T_2$, 16C)	↑ NO bioavailability, ↑ L-arginine, ↓ ADMA. ↑ Exercise duration on EST by 10%. CCS class improved in 70%. (T_2=metoprolol 50mg).
	Leonardo et al	2000	Atenolol 100mg, 2/52, x-2B	16	↓ Angina frequency by 90%. ↑ time to ST-depression on EST by 25%. ↓E/A ratio on ECHO indicating improved diastolic function.
	Lanza et al	1999	Atenolol 100mg, 4/52, x-2B	10	↓ Angina frequency by 38%. improved QOL. (Versus wash out)
	Fragasso et al	1997	Atenolol 100mg, 10/7, x-1B	35 (22 CSX, 13HC)	↓ Angina frequency and all EST became -ve in 80% of CSX patients. Improved E/A ratio..
Calcium Channel Blockers	Cannon et al	1985	Verapamil/Nifedipine, 1/12, 2B	26	↓ Angina frequency by 40%, ↓ Nitrate consumption by 44%. ↑ EST duration by 20%
	Ozcelik et al	1999	Nisoldipine 5mg BD, 4/52	18	↓ Angina Frequency and GTN use by 90% (low baseline incidence in this cohort), ↑ QOL.. ↑t ime to ST-depression on EST by 80%
	Lanza et al	1999	Amlodipine 10mg, 4/52, x-2B	10	No significant effect. Compared to Atenolol and ISMN
Exercise Training	Asbury et al	2008	Phase III CR exercise program	64	↑ shuttle walk distance by 30%. Improved HADS and HAQ and physical functioning scores. Female only study population.
	Eriksson et al	2000	Bicycle ergometer* for 8/52	26	(* for 30 minutes, 3 days a week) ↑ time to angina on EST by 100%. ↑ Exercise capacity by 34%.Female only study population.
	Tyni-Lynne et al	2002	Physical training for 8/52	24	↑ 6-minute walk test distance by 6%. Improved Health-related QOL.
Ivabradine	Villano et al	2013	5mg BD, 4/52	46	↑ SAQ scores. No effect on FMD. Compared with Ivabradine and placebo.
Neuromodulation	Sgeuglia et al	2007	Spinal Cord Stimulation 36/12	28 (19T, 9C)	↓ in angina in 62% and GTN use in 52%. ↑ exercise duration by 18%, time to ST-depression by 33% and time to angina by 48%.
	Jessurun et al	2003	TENS, 4/52	8	↓ in angina frequency by 85% and GTN use by 80%
Nicorandil	Chen et al	1997	Nicorandil 5mg TDS, 2/52, x-2B	13	↑ Exercise time by 10% and time to ST-depression by 25%. ↓ Angina frequency and GTN use. No effect on heart rate variability.
Nitrate	Lanza et al	1994	ISDN 5m SL, STAT	18	↓ exercise time by 21% and time to ST-depression by 38%.
	Lanza et al	1999	ISMN, 4.52, x-2B	10	No significant effect on symptoms or QOL scores.
Psychological Intervention	Cunningham et al	2000	Meditation*, 12/52	9	(* 20 minutes, BD) ↓ angina frequency by 54%. No change in exercise duration but ↑ time to ST-depression by 24%. All female.
	Asbury et al	2011	Group Support, 12/12	49	No significant effects.
Ranolazine	Mehta et al	2011	Ranolazine, 500-1000mg, x-2B	20	↑ SAQ scores
	Villano et al	2013	Ranolazine 375mg BD, 4/52.	46	↑ SAQ scores. ↑ time to ST depression and overall EST duration. No effect on FMD. Compared with Ivabradine and placebo.
Statin	Fabian et al	2004	Simvastatin 20mg	40	Patients all had dyslipidaemia. ↑time to ST-depression. ↑ in FMD by 52%.
	Kayikcioglu et al	2003	Pravastatin 20mg, 3/12, 1B	40	Patients were all normolipidaemic. ↑ exercise time and time to ST-depression by 10% and 57% respectively. ↑ FMD by 68%
	Pizzi et al	2004	Atorvastatin 40mg*, 6/12.	45	↑ exercise duration by 23%. ↑FMD by 91%. ↓ SOD. ↑ SAQ results by 64%.(* in combination with ramipril 10mg)
Trimetazidine	Leonardo et al	1999	Trimetazidine 20mg TDS, 2/52, x-2B	16	No significant effect.
	Nalbantgil	1999	Trimetazidine 60mg, 4/52, 2B	35	↑ total exercise time and time to ST depression on EST.
	Rogacka	2000	Trimetazidine 20mg TDS 6/12	34	↑total exercise time on EST by 15%. Reduction in symptoms.

2B- Double Blind; **ADMA-** Asymmetric Dimethylarginine; **CCS-** Canadian Cardiovascular Society; **EST-** Exercise Stress Test; **FMD-** Flow Mediated Dilatation; **HC-** Healthy Control; **HADS-** Hospital Anxiety and Depression Score; **HAQ-** Health Assessment Questionnaire; **ISMN-** Isosorbide Mononitrate; **NOx**- Plasma Nitrite/Nitrate; **QOL-** Quality of Life; **SAQ-** Seattle Angina Questionnaire; **SOD**-Superoxide Dismutase; **vWF**- von Willebrand factor; **x-** Crossover study design.

1.6.6 Recommendations

- All patients with CSX should be commenced on an exercise programme with the aim of improving their physical conditioning as well as building confidence in their ability to exercise. This is cheap, safe and effective. Other lifestyle factors such as smoking and diet should also be modified.
- Patients with concomitant dyslipidaemia should be started on a statin if suitable.
- Treatment with ACEI if hypertension develops or renal dysfunction exists would be advisable.
- Aspirin should be commenced if no contra-indications exist.
- A reasonable first line therapy in CSX is a beta-blocker, particularly nebivolol, as these give good symptomatic benefit.
- A trial of ranolazine would be an acceptable alternative as the results of most trials to date show improved SAQ scores as well as improved EST parameters.
- Nicorandil, ivabradine and calcium channel blockers could be added if symptomatic control remains poor.
- Nitrates should be avoided in CSX.
- Other centres' experiences with SCS are needed before it can be recommended as a treatment.

A suggested treatment pathway is shown in the figure 1.17 below.

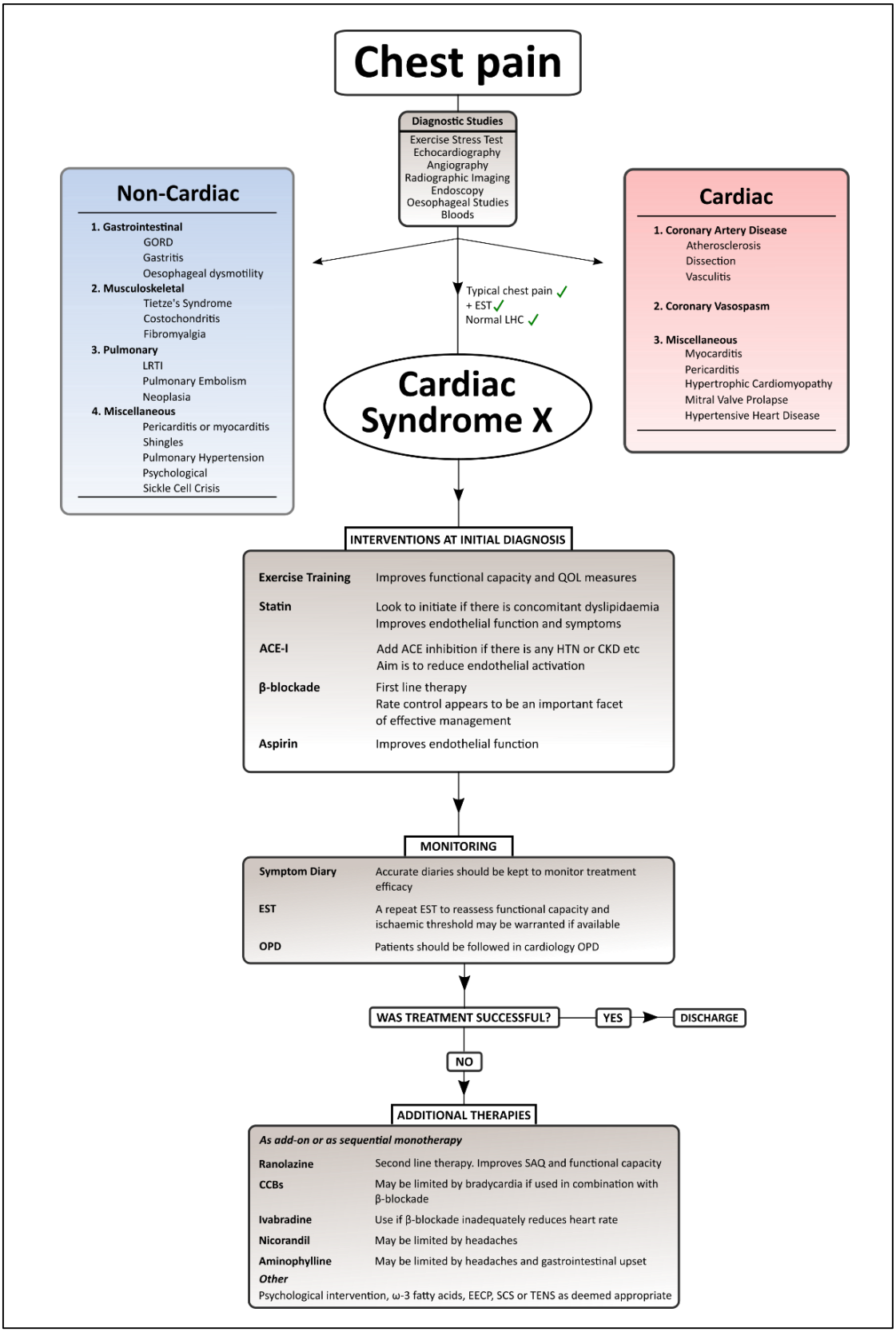


Figure 1.17: Suggested Management Flowchart

1.6.7 Discussion

The treatment and diagnosis of CSX has only recently been codified by cardiology organisations. It is clear that the guidelines regarding its management are based on the evidence detailed in table 1.15 and indeed some of the recommendations are based on trials with small numbers of patients and variable results. Management of CSX continues to require a trial and error approach on a patient-by-patient basis. Inconsistent results of trials into treatment options are likely due to contamination of the sample population by patients with non-cardiac chest pain. It is also possible that we still have not identified the true cause of microvascular angina, making the rational choice of best effective therapy difficult. Regardless, the reality is that approximately 70% of CSX patients will remain symptomatic despite therapy at an average of 7 years' follow-up.

All efforts must be made at the time of initial diagnosis to exclude other common mimickers of cardiac pain. Particular care must be taken to out rule GORD and other GIT disorders as these tend to be present long-term and do not respond to anti-anginal therapy. A further obstacle to effective treatment is that patients with normal coronary arteries and unexplained chest pain tend to be discharged from cardiology services with the result that their general practitioner undertakes their further management under the assumption that their pain is non-cardiac.

To enable progress in this field it must become easier to reliably diagnose CSX. At present the diagnosis mostly hinges on the result of an EST, an unreliable test with a high false-positive rate, which likely means that some patients with non-cardiac chest pain will erroneously be labelled as having CSX. Once the population becomes homogenised, meaningful and more consistent data regarding therapies may hopefully be obtained.

1.7 Biomarkers in CSX

The term “Biomarker” was originally entered as a MeSH (Medical Subject Heading) term in 1989 and is defined on Pubmed as a “measureable and quantifiable biological parameter which serves as an index for health- and physiology-related assessments such as disease risk, psychiatric disorders, environmental exposure and its effects, disease diagnosis, metabolic processes, substance abuse…. etc.” The National Institute of Health further clarified this by defining a biomarker as “A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic intervention”. These biomarkers can be anything from a physical sign, such as heart rate or temperature, to biological molecular biomarkers such as microRNAs or proteins, or to imaging findings such as coronary arterial calcium scores or wall motion abnormalities on echocardiography.

Biomarkers are most practically utilised to identify the presence or severity of a disease state and as such may be used as screening tools to rule in a disease (such as genotyping for long QT syndromes), as point of care tests to rule out a disease (such as an acute myocardial infarction in the case of high sensitivity troponins) or to monitor disease activity or response to therapy (such as tumour markers for various cancers). Good screening biomarkers require a high degree of specificity to rule a disease in (the spIN rule) and usually have a very high likelihood ratio (usually >12) for disease when positive. The specificity can be increased by requiring sequential positive tests (the AND rule). Diagnostic biomarkers used to rule diseases out should have a very high sensitivity (the snOUT rule) with a low LR (<0.10)[149]. The utility of a diagnostic biomarker may be evaluated using the c-statistic (analogous to the area under the curve of a receiver operating characteristic curve). The c-statistic is the likelihood that a particular test can predict the identity of a patient with the disease when it is used on a pair of patients where one has the disease and one does not. Finally, biomarkers may

also be used to help elucidate pathogenic mechanisms in diseases by implicating certain biological processes in disease activity and so may be useful to further characterise diseases.

Examples of biomarkers currently in use in cardiovascular medicine include high sensitivity troponin, blood pressure, urine albumin excretion, ankle-brachial index, carotid intimal-medial thickness, ADMA, hsCRP and lipoprotein (a). Many parameters have been assayed in CSX including markers of vascular inflammation, cardiac risk, vascular function and functional capacity. Some of these are summarised in table 1.16 below. Critically, these biomarkers have only been assessed at a single time point and as such can be poorly used to assess the natural history of the disease. The aim of this thesis was to evaluate biomarkers in Irish participants with CSX and in those without (i.e. to potentially discover diagnostic biomarkers) but also to see if the trajectory of certain biomarkers correlated with known clinical indices of disease activity (i.e. to determine type 0 or prognostic biomarkers) or if baseline biomarkers could predict future outcomes.

One of the most consistent findings in patients with CSX is the presence of low-grade inflammation. Whether this is cause or effect is unknown. Studies have regularly demonstrated elevated levels of high-sensitivity CRP while the data is inconsistent with respect to other markers such as intercellular adhesion molecules and tumour necrosis factor. Another relative constant in CSX is the demonstration of deranged markers of vascular function such as flow-mediated vasodilation (FMD) in the forearm or coronary flow reserve (CFR) determined by invasive or non-invasive methods. Several molecular biomarkers have been shown to correlate with clinical markers of disease burden such as evidence of subclinical atherosclerosis in the form of increased carotid intima-media thickness, duke treadmill score on EST or impairment of FMD[150-152]. Only two biomarkers, baseline bilirubin and basal superoxide production, have been shown to

predict long-term prognosis in CSX patients while it is known in general that CRP predicts outcome in patients with atherosclerosis [28,110,153].

Table 1.16: Selected studies into biomarkers in CSX

Biomarker	Study	Finding
hsCRP	Lanza et al[62]	Elevated with no evidence of increased pathogenic burden
	Atmaca et al[150]	Elevated and correlated with FMD measurements (r=-0.44, p<0.001)
	Tenekecioglu et al[154]	Elevated and associated with reduced HDL-C levels
ICAM-1	Tousoulis et al[59]	Elevated with non-significant elevation in VCAM-1
Selectins	Senen et al[60]	P-selectin and E-selectin were elevated in CSX.
Interleukin-6	Li et al[155]	Elevated and associated with an elevated white cell count
	Rasmi et al[63]	Elevated and associated with CagA+ H. Pylori infection
TNFα	Lin et al[156]	Elevated and associated with increased superoxide free radicals
Homocysteine	Timurkaynak et al[151]	Elevated and inversely correlated with Duke Treadmill Score (r=-0.506, p<0.001)
MCP-1	On et al[157]	Elevated and associated with decreased serum anti-oxidant levels
Bilirubin	Huang et al[28]	Patients with adverse events at follow-up had lower baseline bilirubin
Uric Acid	Acikgoz et al[158]	Elevated and associated with increased carotid intima-media thickness
	Elbasan et al[159]	Increased Uric Acid in CSX predicted coronary slow flow
ADMA	Sen et al[152]	Increased and correlated with increased carotid intima-media thickness
	Okyay et al[160]	Increased and associated with abnormal myocardial tissue perfusion
Superoxide	Leu et al[110]	Basal superoxide generation predicted future clinical events (OR 3.87, p<0.001)
Leptin	Jadhav et al[161]	Elevated with increased insulin indicating possible metabolic syndrome
Endothelin	Hoffman et al[162]	Elevated in patients with CSX
Calcium Score	Mizia-Stec et al[68]	Elevated in CSX and are age-related and independent of vascular function
LV function	Yagmur et al[46]	Reduced LV longitudinal strain on speckle tracing is seen in CSX
FMD	Huang et al[163]	Reduced in CSX and correlated with levels of EPCs (r=0.557, p=0.001)

ADMA- Asymmetric dimethylarginine, **EPC**- Endothelial Progenitor Cells, **FMD**-Flow-mediated Vasodilation, **HDL**-High Density Lipoprotein, **ICAM**- Intercellular Adhesion Molecule, **LV**- left ventricle, **MCP**- Monocyte Chemoattractant Protein, **TNF**- Tumour Necrosis Factor.

1.8 Primary hypothesis and aims of the thesis

This thesis aims to interrogate the hypothesis that immune activation and inflammation play a central role in the pathogenesis of Cardiac Syndrome X and that biomarkers indicative of the activation of these systems can be used to track disease activity, to predict prognosis and to implicate pathways relevant to this syndrome's causation. While biomarkers have already been evaluated in CSX, we aim to broaden the scope of this assessment to the longitudinal study of biomarkers in these patients, paying particular interest to changes in these biomarkers as disease activity waxes or wanes. We aim to further investigate the possibility of novel molecular biomarkers by investigating the transcriptome and lipidome in CSX patient. We also wished to see if inflammation was necessary or sufficient to cause disease in our cohort.

Aim 1: To determine if Cardiac Syndrome X is present in an Irish population and then to determine if these Irish CSX patients are broadly similar to previously described populations from other regions. CSX has not been investigated in an Irish setting. To establish its presence, we interviewed all-comers to the cardiac catheterisation laboratory in a tertiary cardiac centre during the study period. We adhered to a particularly strict definition of CSX to ensure reliable diagnosis. The demographics, laboratory data and cardiac investigation results were assessed in patients diagnosed with CSX and the phenotype of a typical Irish CSX patient was determined. Moreover, an additional group of patients with chest pain, angiographically normal epicardial coronary arteries and a normal EST (the so-called LCSX population) was recruited to determine if they were an immunologically and clinically distinct population and to therefore assess the importance of a positive EST in the diagnosis of CSX.

Aim 2: To investigate the role of life stress and the psychological impact of disease in CSX patients. CSX patients have a high burden of psychological comorbidity such as

anxiety and depression. We attempted to evaluate this psychological aspect of CSX by studying life stresses (both actual and perceived) and markers of disease-mediated psychological effects such as disease related quality of life and overall satisfaction with disease management using validated questionnaires. We wished to examine the impact of disease activity on life stress to see if there was any relationship between external factors, disease perception and molecular biomarkers of disease activity.

Aim 3: To establish the baseline immune phenotype of Irish CSX patients in terms of cell counts, acute phase reactants, endothelial adhesion molecules, cytokines and associated inflammatory processes. Having identified a population of Irish CSX patients using the rigorous application of the most stringent diagnostic criteria we attempted to define the nature and extent of the inflammation in our cohort by comparing the concentrations of various molecular biomarkers in their plasma to levels in age- and sex-matched healthy controls. We also intended to assess any relationship between the degree of immune activation and the severity of symptoms, thus determining if a biological gradient or dose-response relationship existed between inflammation and symptoms.

Aim 4: To explore the microRNA transcriptome in our CSX cohort to determine if our patients had a particular miRNA signature. As microRNAs provide a relatively specific insight into the possible mechanisms at play in many disease conditions we wished to use Next-generation sequencing to evaluate the differential expression of miRNAs in CSX patients. We hoped that this might shed some light on the unclear pathophysiology in play in CSX as well as perhaps providing a valid plasma biomarker for disease activity.

Aim 5: To prospectively follow our patients over the course of 2 further visits, re-evaluating symptoms, objective signs of disease activity and biomarker values at each visit, to allow the natural history of the disease to play out. Our expectation would be that as a patient's clinical status improves their inflammation would similarly abate. Additionally, repeated measures would allow us to assess the consistency of immune activation in CSX patients over time. We reasoned that the clinical status of some patients would change over time. If we could identify these patients and examine which biomarkers (if any) changed in parallel with their clinical changes it would give the opportunity to identify appropriate type 0 biomarkers in this population. It might also allow for further hypothesis generation with respect to the potential roles the various biological processes associated with the implicated biomarkers might have in the pathogenesis of CSX. Evidence of a resolution of immune activation in concert with symptom resolution would provide further evidence of possible causation of inflammation in CSX by demonstrating reversibility.

Aim 6: To hypothesise as to the cause of inflammation in CSX. By analysing the pattern of immune activation as well as the miRNA profile in CSX we hoped to generate a hypothesis as to the underlying cause of inflammation in the CSX population as this remains unknown despite decades of research. Additionally, we attempted to analyse diet as a potential trigger of CSX by obtaining information regarding patients' dietary habits using validated questionnaires and also by analysing the plasma fatty acid profile of our patients. If we could determine a cause for the inflammation, we could make recommendations as to potential future therapies in CSX and suggest further avenues of research to pursue.

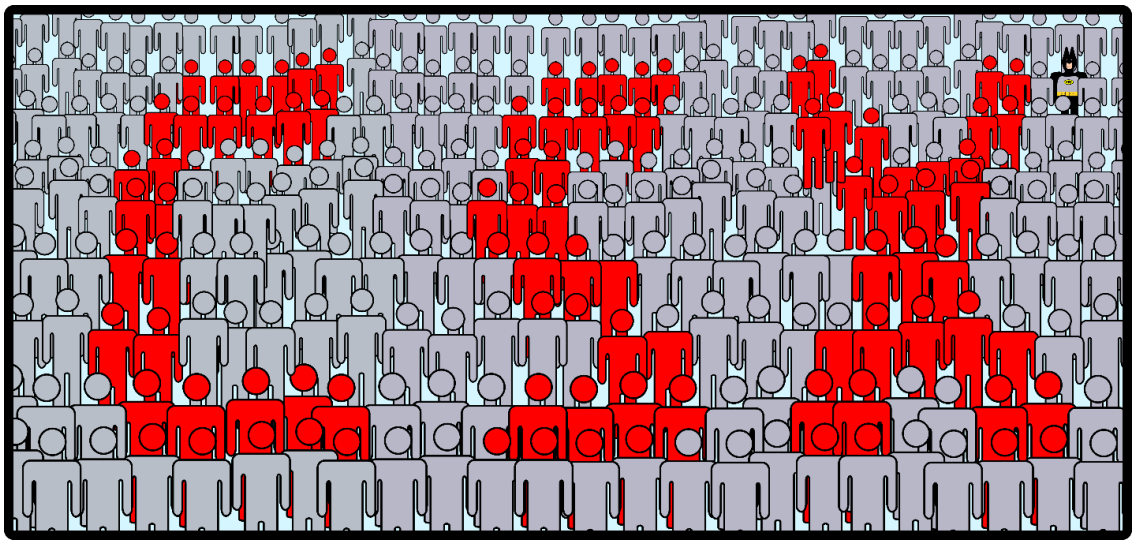
1.9 Summary

Cardiac Syndrome X is a clinical condition that is seen globally but which is often underdiagnosed. It is defined as the presence of angina pectoris, a positive non-invasive test suggestive of ischaemia and normal epicardial coronary arteries on angiography. In essence this means that it is true cardiac ischaemic pain but where the ischaemia is not attributable to disease of the larger distributing coronary arteries. The term Syndrome X is unfortunate as it implies a sense of mystery surrounding the condition. In truth much is now known about the condition and it should now be termed microvascular angina. The terms CSX and microvascular angina are used interchangeably in this thesis.

CSX is mostly seen in middle-aged women with a past history of dyslipidaemia as well as many other traditional cardiovascular risk factors. It may be diagnosed in approximately 1% of all coronary angiograms undertaken in Ireland and in 3% of those performed to investigate chest pain. It is likely due to microvascular dysfunction, specifically failed endothelium-dependent vasodilation of the resistance arterioles in the coronary vascular beds in response to exercise. An abnormality in pain processing and co-morbid psychological abnormalities may also contribute to the phenotype. The microvascular dysfunction is most likely secondary to endothelial dysfunction, which may in turn be as a result of the early impact of traditional cardiac risk factors (hypertension, dyslipidaemia, diabetes etc.) on the endothelium of the microvasculature. Many markers of endothelial activation and vascular inflammation are elevated in CSX, hinting at an inflammatory aetiology for this condition.

Overall the prognosis in CSX is good but patients are plagued by refractory symptoms and impaired quality of life despite proven benefit for some traditional anti-anginal therapies.

The purpose of this thesis is to longitudinally and prospectively study changes in various plasma molecular biomarkers in CSX patients. We hope to identify markers of disease activity that vary in response to changes in disease state. This may allow the development of potential diagnostic biomarker panels as well as providing further insight into the exact pathogenesis of CSX. Markers of vascular inflammation, cell-mediated immunity and general inflammation will be studied. Furthermore, the genetic pathway will be indirectly studied through the analysis of the presence of plasma microRNAs.



Chapter 2: Study Design and Participant Characteristics

Introduction

2.1 Chapter Overview

2.1.1 Chapter Objectives

1. The nature of the Irish CSX population is currently unknown. The main objective of this chapter was to **determine the phenotype of the typical Irish CSX patient** by reliably identifying them using strict diagnostic criteria and then analysing a variety of parameters such as demographics, traditional cardiac risk factor profiles, symptom burden and life stresses. In addition, routine laboratory urine and blood test results would be investigated to establish the usual values of these investigations in CSX patients and the results of their cardiac evaluations would similarly be collated to establish the normal results for this population. An age and sex-matched healthy control group would allow for the identification of significant deviations of CSX patients' baseline characteristics from "normal".
2. These CSX patients would be followed longitudinally to **assess changes in their clinical status** in terms of symptoms, EST parameters and markers of disease activity. This will be more relevant in other chapters concerned with variations in biomarkers with time.
3. As well as identifying and phenotyping this population, we also aimed to **establish the incidence of CSX** in patients undergoing coronary angiography in an Irish tertiary cardiac referral centre as this has not been previously explored. We wished to estimate the overall burden of CSX in Ireland as this may help to direct future diagnostic/treatment pathways.

2.1.2 Phenotype Study

A case-control study design was adopted to assess the baseline phenotype of the CSX population and to compare various risk factors to those of age and sex-matched healthy controls. It also allowed the assessment of potential biomarkers of disease activity. The study aimed to go beyond this, however, by following-up this initial study

with a longitudinal prospective observational study of the CSX patients to assess changes in their clinical status and biomarkers over time. Suitable patients were recruited from 3 participating hospitals; Cork University Hospital (CUH), a tertiary cardiology referral centre; the Mercy University Hospital (MUH), a regional hospital; and the Bon Secours Hospital (BSH) in Cork, a private hospital with a large volume throughput, with a combined overall catchment area of 660,000 people. A simple flow-chart illustrating the design is shown below in Fig. 2.1 overleaf. The study design was approved by the Clinical Research and Ethics Committee of the Cork Teaching Hospitals (CREC).

2.1.3 Incidence study

It is estimated that approximately 20-30% of coronary angiograms are normal but the majority of these patients will not be diagnosed with CSX as they may have atypical symptoms, equivocal stress testing, other conditions leading to angina or have a different indication for LHC (such as pre-transplant evaluation etc.). The only study specifically examining the incidence of CSX in a European hospital setting was a single-centre study performed in Holland where they noted an incidence of 3.2% in their 2003 study cohort [29]. It is not known if this is generalizable to a broader European population. During the design of this study it was estimated that over 40 patients with CSX could be recruited by screening patients three days per week in the designated centres for 1 year. This figure was based on an estimated 2500 angiograms occurring annually during regular working hours with an expected incidence of 3% in a European population. Unfortunately, it became clear quite early on that recruitment would be much slower than anticipated with ultimately only 17 patients being recruited over a 15-month period (a longer recruitment period was impossible due to study time constraints imposed by the necessary period of follow-up) as shown more clearly in figure 2.2 below. To investigate the apparently low incidence of CSX in Ireland we also performed a prospective incidence study over a three-month period.

Figure 2.1: Longitudinal study design showing the parameters that would be obtained at each time point. * and ** were not included in the study design ab initio but were added later.

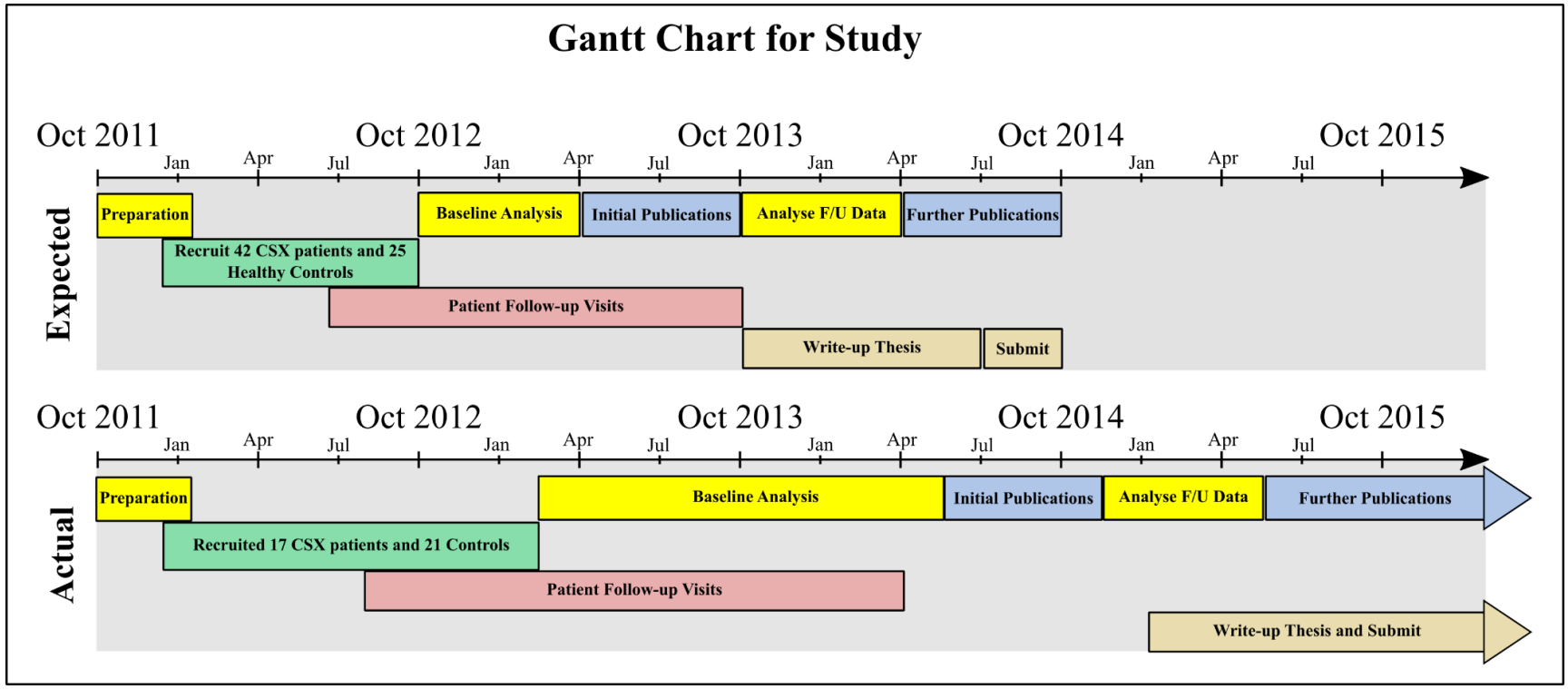


Figure 2.2 Gantt chart showing predicted timeline of study compared to the timeline that actually occurred.

Methods

2.2 Subject Recruitment

2.2.1 Cardiac Syndrome X patients for phenotyping

The principal investigator attended CUH 3 days a week from 0900 until 1600 and the BSH one day a week from 0900-1200 (MUH does not have a catheterisation laboratory). All consultant cardiologists in CUH, MUH and BSH were aware of the recruitment effort. Separate information sheets for patients and doctors were displayed in the catheterisation laboratory control room, waiting bay and outpatient clinics in CUH, MUH and BSH (see Appendix I). Patient recruitment ran from December 2011 until March 2013. Cardiac Syndrome X was diagnosed if the patient had (a) typical stable angina pectoris (i.e. sub-sternal discomfort of typical character and duration, which was exacerbated by exercise and relieved by rest and/or nitrate); (b) an electrically positive exercise stress test (defined as ≥1mm of horizontal or downsloping ST depression 80ms after the J-point); (c) no lesion >10% on coronary angiogram; and (d) no other explanation for the chest pain (e.g. valvular, hypertensive or myocardial heart disease or an obvious non-cardiac source for the chest pain). These criteria are the most commonly used contemporaneous criteria[1]. Exclusion criteria included other cardiac conditions, systemic inflammatory conditions (including infection, chronic kidney disease, connective tissue disease and depression) and the regular use of NSAIDS, corticosteroids or immunosuppressants. Due to requirements from the ethics committee, we limited the study to people under the age of 70 and excluded pregnant patients. The presumed mechanism for the CSX patients' pain was, as previously mentioned, microvascular angina (see Fig. 2.3).

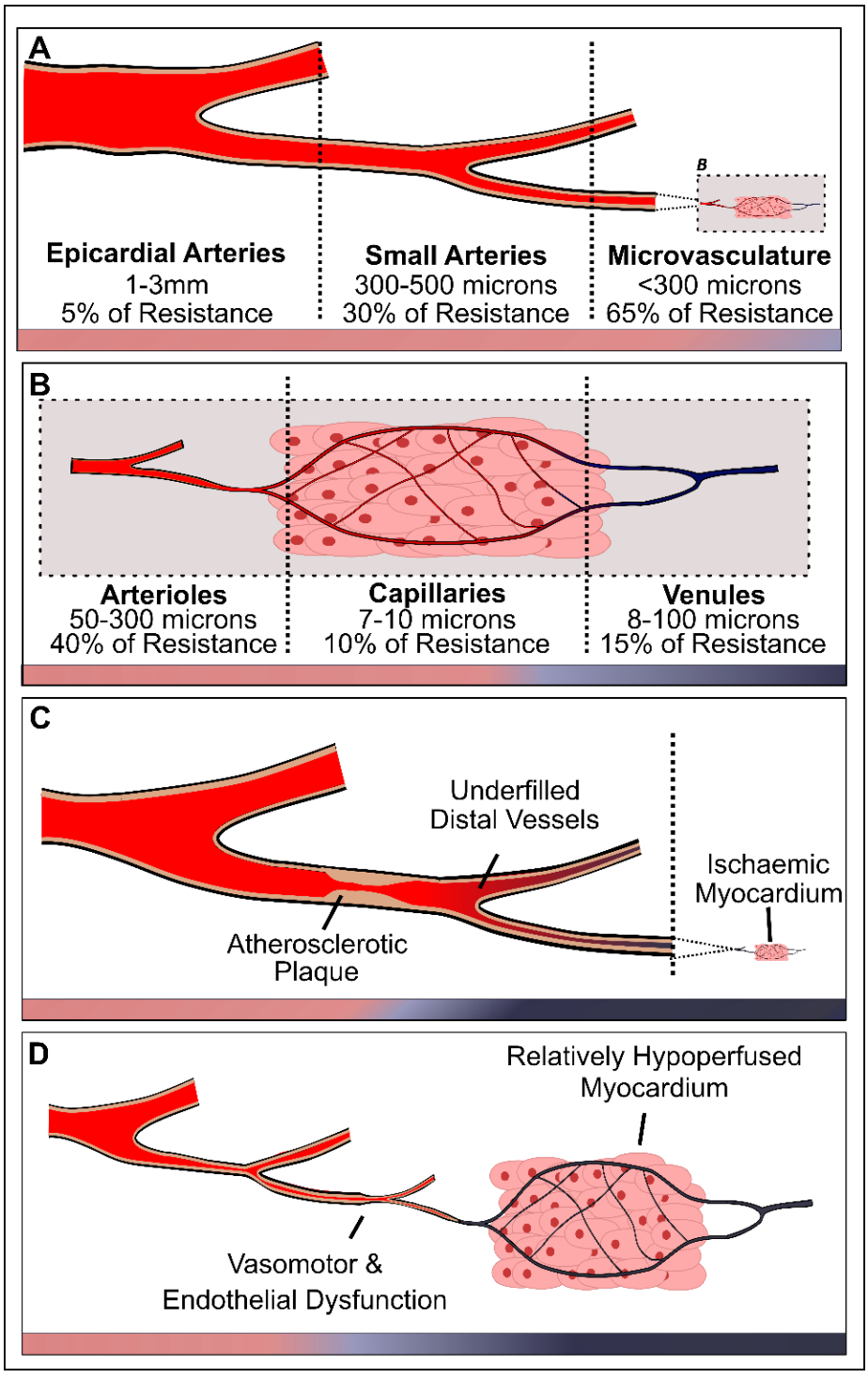


Figure 2.3 Possible pathophysiology of Cardiac Syndrome X. **a**. Longitudinal cross-section of the coronary circulation. Note that the microvasculature accounts for the majority of the resistance. **b.** Cross-section of the coronary microvasculature. **c.** Classical myocardial ischaemia resulting from the atherosclerotic stenosis. The slider shows the degree of myocardial perfusion longitudinally along the tissue. Pink= well perfused; blue=poorly perfused. d. Possible mechanism of ischaemia in Cardiac Syndrome X. The resistance arterioles fail to adequately dilate in response to increased cardiac workload with consequent relative hypo-perfusion of the distal myocardium.

The nQuery Advisor (Release 4.0) by Statistical Solutions Ltd, Cork, Ireland was used to power the study. Initial sample size calculations were based on previous studies into hsCRP in CSX populations, which showed a mean change in CRP of 2.3±3.8. A power of 80% and a two-tailed significance of 0.05 were sought and the projected sample size for each group was 44.

To identify suitable patients, all patients who attended for coronary angiography were interviewed individually by the principal investigator and completed the Rose Angina questionnaire, a WHO validated angina questionnaire (Appendix I). Their symptoms were categorised as being typical of angina, atypical or non-cardiac in nature as described in section 1.2.1. Only those patients with typical angina pectoris were assessed further. The results of ESTs or other non-invasive tests for ischaemia were then noted. Only those patients with an electrically positive EST or equivalent continued to be considered for inclusion. The ESTs were reviewed by the study author and the consultant responsible for the patient. Once the coronary angiogram was performed and found to be normal, the patients were again approached and full informed consent was obtained for inclusion in the study if no exclusion criteria were met (Appendix I). It should be noted that all patients who met the inclusion and exclusion criteria were approached and all of these patients consented to study enrolment.

2.2.2 Healthy Controls

Control patients were recruited from 2 local primary care centres by the principal investigator. All patients were in good health, had no previous cardiac history and conformed to the same exclusion criteria as CSX patients. Efforts were made to age and sex-match them for the expected study cohort, mainly by attempting to enrol middle-aged women with a history of hyperlipidaemia. Consecutive control patients adhered

to the same exclusion criteria as CSX patients and additionally had no history of cardiac disease or symptoms.

2.2.3 "Loose" CSX patients (LCSX)

Several patients were enrolled without review of the EST printouts as these tests had been performed at an outlying hospital before referral to CUH. As such on the day only the written report from the EST was available stating that it was positive. These patients were enrolled as possible CSX patients as they had typical angina pectoris and normal coronary arteries. The hardcopies of the EST were later obtained and in 7 cases were found to be electrically normal but had been deemed abnormal by inexperienced non-consultant hospital doctors who in most cases had misinterpreted normal up-sloping ST-depression as pathological, a common error. As blood samples had already been obtained and questionnaires administered to these patients we included them in a small cohort termed "loose CSX" (LCSX), where a patient has angina with normal coronary arteries but no objective evidence of ischaemia. It should be pointed out that this definition of CSX has also been used in many publications in the past but is now deemed insufficient for a true diagnosis of CSX (where a positive objective test for ischaemia is now required) as it led to a markedly heterogeneous population. It would be of interest to see if a positive EST defines a different population in terms of phenotype, clinical outcome or biomarkers compared with patients who may have microvascular angina.

2.2.4 Incidence Study subset

To determine the specific incidence of CSX in Ireland we examined all patients presenting for coronary angiography over a defined three-month period from December 2012-February 2013. This comprised a total of 55 weekdays from 0800-1800. Out-of-hours cases were not included as the study investigators were not present to interview patients at these times. CSX patients adhered to all of the usual

inclusion and exclusion criteria (apart from the upper age limit of 70 years). During this study period anonymised demographic data, cardiac risk factor profiles, EST results and angiography results were recorded for all comers, as opposed to only CSX patients. All patients signed full informed consent.

2.3 Initial Investigations

2.3.1 Baseline information

Once enrolled in the study, CSX patients were interviewed further and data was collected from the hospital notes and databases regarding traditional cardiovascular risk factors, such as blood pressure, cholesterol levels and smoking history while routine blood test results and results from previous cardiology investigations (such as electrocardiogram, echocardiography, cardiac MRI etc.) were also documented. EST parameters including the overall test duration, time to ECG changes, maximum rate-pressure product and time to symptoms were also noted.

2.3.2 Questionnaires

Several questionnaires were administered including the 10- point Cohen Perceived Stress Scale (PSS-10) and the Brugha List of Threatening Experiences (LTE-Q) questionnaire to examine the role of perceived and actual life stresses as a measure of stress and coping in CSX patients (see appendix I). Higher scores on the PSS-10 indicate increased perceived stress and indicate greater vulnerability to stressful life-event triggered depressive symptoms. It also correlates with depressive and physical symptomatology, social anxiety and the degree of utilisation of health services[164]. Higher scores on the LTE-Q indicate greater life stress while also being associated with an increased risk of depression (OR 1.64-2.57), anxiety (OR 1.35-1.97) and alcohol dependence (OR 2.86-4.80)[165].

The Seattle Angina Questionnaire was licensed from Cardiovascular Outcomes Inc. (Missouri, USA) and was administered to the CSX cohort. This well-validated questionnaire assesses the health status of the patient with regard to their angina[26]. It examines the severity of symptoms by providing 5 summary scores in several areas; a physical limitation score, stability and frequency of angina scores, treatment satisfaction score and a disease related quality of life score. The domains are marked from 0-100 with higher scores implying less disease impact (e.g. a physical limitation score of 100 would mean that the patient was asymptomatic). It has been used extensively in cardiovascular research. The scores from the various domains have been significantly linked with mortality rates at 1 year[166].

2.3.3 Biological samples management

Venous blood samples were taken from an ante-cubital fossa vein and drawn into a 10ml dipotassium EDTA tube. No patient had been fasting for more than 3 hours. The samples were immediately centrifuged at 112 RCF for 15 minutes at 4°C. The plasma was then aliquoted into 2ml microtubes, which were then transferred in an ice box to the Biosciences Institute in University College Cork where they were stored in a -80°C freezer until they were subsequently needed for analysis. Mid-stream urine samples were collected from patients in standard universal containers and were sent directly to the hospital laboratory for standard analysis.

2.3.4 Exercise Stress Testing

All CSX patients underwent a baseline treadmill EST following the BRUCE protocol. This standard protocol involves a staged increase in treadmill speed and incline until limited by patient factors (such as fatigue or limiting angina pectoris) or until at least 85% of an age-related target heart rate ([220- age in years] beats per minute) is achieved. The overall exercise duration, METS achieved, time to 1mm of ST-depression on ECG, time to angina, peak rate pressure product and the Duke Treadmill Score (DTS) were all

calculated. The Duke Treadmill score is a useful summary statistic as it uses EST-defined parameters such as exercise duration, electrocardiographic evidence of ischaemia and degree of induced angina to give a weighted score that predicts cardiovascular outcomes such as mortality and degree of coronary atheroma. DTS scores usually range from -25 (highest risk) to +15 (lowest risk) and is defined in the following equation:

$$DTS = Exercise\ time\ in\ minutes - 5(STdepression\ in\ mm) - 4(angina\ index)$$

The angina index is 0 when no angina is experienced, 1 when non-limiting angina occurs and 2 when the angina is limiting, i.e. the EST must be stopped due to the angina[167].

2.3.5 Data management

Each patient was given an anonymised identification code at enrolment (e.g. CSX13 or HC21, indicating CSX patient number 13 or healthy control number 21) and all data was coded and input into a database using SPSS for Windows v20 (Armonk, NY: IBM Corp.) and then encrypted. Hardcopies of data were kept in a locked filing cabinet in a locked office adjacent to the catheterisation laboratory in CUH. Continuous variables are expressed as mean ± SEM if normally distributed and median (IQR) if not normally distributed. Standard statistical tests were used. Categorical variables were compared using Fisher's exact test while the normality of the distribution of continuous variables was assessed using the Kolmogorov-Smirnov test (KS test) and Shapiro Wilk test. Summary statistics of normally distributed variables were compared using the student t-test or one-way ANOVA with Bonferroni post-hoc testing where appropriate. Transformations were attempted on non-normally distributed variables to enable parametric testing but the Mann-Whitney U test or Kruskal-Wallis tests were used if simple transformation did not render the distribution of the variables normal. Differences between repeated measures were assessed using the paired student t-test or Related Samples Wilcoxon Signed Rank test as appropriate to data distribution. Correlations were sought using Spearman's rank correlation co-efficient. Standard

Linear Regression and logistic regression analyses were performed with a limit of one predictor variable per 10 subjects. All reported p-values are two-tailed and reported confidence intervals are calculated to the 95% confidence level.

2.3.6 Follow-up visits

CSX patients were invited to return for two further reviews to chart the course of their self-reported symptoms, questionnaire results, EST parameters and blood tests. All follow-up visits were performed in the CUH outpatient department between 0900 and 1300. There was 100% follow-up at visit 2 and 94% at visit 3 for CSX patients. There was 100% follow-up of LCSX patients at visit 2 and they were not brought back for a third visit. Healthy controls were only seen at a single time-point.

Results

2.4 Incidence

2.4.1 Incidence Results

The published results of this study are in appendix II. During the 3-month incidence study period 485 patients underwent coronary angiography but only 372 of these actually presented with chest pain. Of those with chest pain, 258 (69%) had typical angina pectoris. Figure 2.4 shows the breakdown of patient numbers at each step of the diagnostic process. Angiograms investigating chest pain were normal in 77/372 (21%) of cases and of these 38 (49%) had undergone an EST, which was electrically positive in a mere 8/38 patients. Two of these patients had atypical chest pain while 6 complained of typical angina. One of the latter had severe hypertensive heart disease and thus was excluded from the diagnosis of CSX. Thus only 1.3% of patients with chest pain undergoing coronary angiography achieved a diagnosis of CSX. CSX patients comprised 1.0% of all angiograms (including those for non-cardiac indications), 4.1% of

normal coronary angiograms and 1.9% of angiograms performed to investigate typical angina pectoris (see table 2.1)

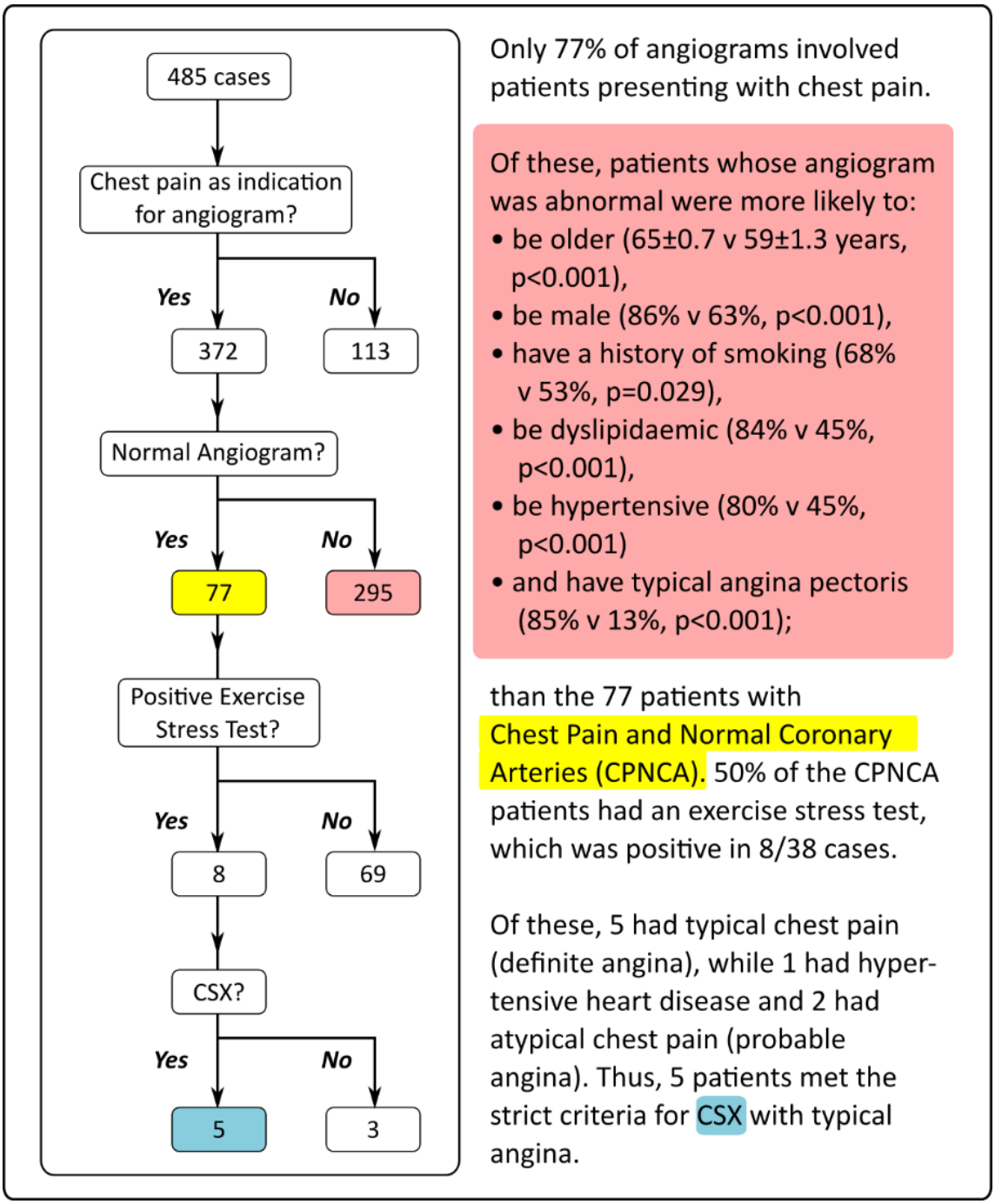


Figure 2.4: Diagnosing CSX in an Irish cohort of patients undergoing coronary angiography.

Diagnosis	Patient Features	Cohort or subset	Observed Incidence
CSX	1. Typical Angina	All-comers (n=485)	1.0%
n=5	2. Positive EST	Patients with chest pain (n=372)	1.3%
	3. Normal LHC	Patients with Typical Angina (n=258)	1.9%
		Normal Coronary Angiograms (n=123)	4.1%
Atypical	1. *Any chest pain*	All-comers	1.4%
CSX	2. Positive EST	Patients with chest pain	1.9%
n=7	3. Normal LHC	Patients with Typical Angina	2.7%
		Normal Coronary Angiograms	5.7%
LCSX	1. Typical Angina	All-comers	1.9%
n=9	2. *No EST*	Patients with chest pain	2.4%
	3. Normal LHC	Patients with Typical Angina	3.5%
		Normal Coronary Angiograms	7.3%
CPNCA	1. Any chest pain	All-comers	15.9%
n=77	2. Normal LHC	Patients with chest pain	20.7%
		Normal Coronary Angiograms	62.6%

Table 2.1: Observed incidence of CSX, Atypical CSX, LCSX and CPNCA in CUH.

2.4.2 Comparing patient groups by angiography results

Patients were categorised into 3 groups based on their angiogram result and overall diagnosis to allow for comparison between groups. Patients with ischaemic heart disease were placed in Group 1 if they had macroscopic coronary artery disease (stenosis >10% severity) and consisted of 331/485 patients. Patients with a normal angiogram but without a diagnosis of CSX were placed in Group 2 118/485. It should be noted that 31 patients had no epicardial coronary artery disease but had other abnormalities on angiography (such as dilated cardiomyopathy or a severely regurgitant valve) and were excluded from this analysis. The 5 CSX patients identified in the incidence study were added to the 12 already recruited for this thesis and together comprised Group 3. The main characteristics of these groups are shown below in table 2.2.

Parameter	Group 1 IHD (n=331)	Group 2 Normal LHC ex. CSX (n=118)	Group 3 CSX cases* (n=17)	$P^{1/3}$ CSX v IHD	$P^{2/3}$ CSX v Normal
Gender (M:F)	76%:24%	53%:47%	12%:88%	0.001	0.001
Age (years)	65.1 ± 0.6	59.6 ± 1.0	59.2 ± 1.6	0.003	0.870
BMI (kg/m^2)	28.2 ± 0.3	28.1 ± 0.5	27.7 ± 1.0	0.699	0.769
Hyperlipidaemia	277 (83.7%)	49 (41.5%)	14 (82.4%)	0.500	0.009
Hypertension	267 (80.7%)	56 (47.5%)	6 (35.3%)	0.003	1.000
Symptoms					
Chest pain	285 (86.1%)	72 (61.0%)	17 (100%)		
Typical	246 (74.3%)	5 (4.2%)	17 (100%)	0.143	0.001
Atypical	39 (11.8)	67 (56.7%)	0 (0.0%)	0.235	0.000
SOB	98 (29.6%)	40 (33.9%)	4 (23.5%)	0.786	0.581
Smoking Status				0.796	0.601
Current	72 (21.8%)	17 (14.4%)	0 (0%)		
Ex	139 (42.3%)	44 (37.3%)	9 (52.9%)		
Non	119 (35.9%)	57 (48.3%)	8 (47.1%)		

Table 2.2: Comparison between groups. Patients with IHD (Group 1); normal angiogram excepting CSX (Group 2); CSX patients (Group 3). $P^{1/3}$ is the p-value for differences between group 1 and group 3 using fisher's exact or student t-test test while $p^{2/3}$ refers to differences between groups 2 and 3. * CSX group includes 5 patients from incidence study period and 12 from extended recruitment.

All of the CSX patients were Irish and 88% were female, with the majority (87%) of these being post-menopausal. This significantly differed from the marked male majority seen in the IHD group ($p<0.001$) and the neutral gender split in the normal angiogram category ($p=0.001$). The mean age for CSX patients was 59.2 ± 1.6 years and was significantly lower than the mean age of patients with IHD ($p=0.003$) and no different to other patients with normal coronary angiograms ($p=0.817$). CSX patients were more likely to have hyperlipidaemia compared to other patients with normal coronary angiograms. They were also less likely to have hypertension than patients with IHD. Of note, no patient was discharged with a diagnosis of CSX or MVA, highlighting a lack of acceptance or awareness of the diagnosis.

2.5 Phenotype at Baseline

2.5.1 Basic Parameters

Over the prolonged 18-month period 17 CSX patients were identified and recruited with a further 7 LCSX patients included. Twenty-one age and sex-matched healthy controls were also recruited. The baseline characteristics between these groups are shown in table 2.3. These three groups are very similar in terms of most major criteria. The control group and CSX group in particular are very well matched with the only significant difference being the use of aspirin in the CSX group. It should be noted that these patients were recruited directly from the catheterisation laboratory where premedication with aspirin is routine. These patients would only have ingested 75mg daily over the week prior to enrolment.

As mentioned above, the CSX patients had an average age of 59 years and were predominantly female (88%). This is in keeping with the literature, which has observed that CSX is most commonly seen in post-menopausal women. The patients were, on average, mildly overweight with a BMI of 27.6 and there was also a high prevalence of dyslipidaemia in all groups. Curiously, the prevalence of treated hypothyroidism in this group was 24%, far higher than the usual prevalence of 1.0-1.4% in the general Irish population. This is likely attributable to the age and gender of the CSX patients although triiodothyronine (T_3) is known to have vasoactive properties including coronary arterial vasodilation and its role as a possible factor in the pathogenesis of CSX is an intriguing possibility.

Parameter	Healthy Control (n=21)	Cardiac Syndrome X (n=17)	Loose Cardiac Syndrome X (n=7)	p-value
Gender (M:F)	19%:81%	12%:88%	57%:43%	0.06[FE]
Age (years)	60.1 ± 1.4	59.2 ± 1.6	57.4 ± 4.1	0.70[A]
BMI (kg/m^2)	27.9 ± 0.8	27.6 ± 0.9	26.4 ± 1.1	0.63 [A]
Hypertension	9 (42.9%)	6 (35.3%)	3 (42.9%)	0.92[FE]
Hyperlipidaemia	18 (85.7%)	14 (82.4%)	6 (85.7%)	1.00 [FE]
Statin	8 (38.1%)	8 (47.1%)	5 (71.4%)	0.31 [FE]
ACEI	5 (23.8%)	2 (11.8%)	1 (14.3%)	0.77 [FE]
Aspirin	5 (23.8%)	11 (64.7%)	3 (42.9%)	0.05 [FE]
Smoker	11 (53.4%)	9(52.9%)	2 (28.6%)	0.57 [FE]

Table 2.3 Baseline characteristics of CSX, LCSX and healthy control groups. [A]=ANOVA, [FE]=Fishers Exact.

2.5.2 Traditional Cardiovascular Risk Factors

a) **Hyperlipidaemia**: Over 80% (14/17) of the CSX patients had a diagnosis of hyperlipidaemia (defined as the use of a statin and/or a total cholesterol >5.0mmol/L), which was matched in the control group. The mean cholesterol for the CSX group was 5.37 ± 0.3mmol/L, being 4.8 ± 0.6mmol/L for those on statin therapy and 6.2 ± 0.8mmol/L in the 9 patients who were not on a statin. LDL was modestly elevated at 3.6 ± 0.7 mmol/L while HDL levels were normal (1.49 ± 0.2 mmol/L).

b) **Hypertension**: It should be noted that patients with demonstrable hypertensive heart disease (electrocardiographic evidence of left ventricular hypertrophy using the Cornell criteria or echocardiographic LVH) or poorly controlled hypertension (systolic blood pressure of >180mmHg at the start of the EST) were excluded from a CSX diagnosis. With this caveat, 6/17 CSX patients were diagnosed with mild systemic hypertension.

c) **Smoking:** Remarkably none of the CSX patients were active smokers while 9 (52.9%) admitted to having smoked in the past with a mean 16.5 years since they had quit and a median pack year history of 15.0 (IQR 12.5 – 26.3). Three of the healthy controls were current smokers while 47% had never smoked. Only 2 of the LCSX group had ever smoked.

d) **Family History:** The majority of CSX patients had a significant family history of ischaemic heart disease (defined as diagnosed ischaemic heart disease before the age of 55 in male or 65 in a female first or second degree relative).

2.5.3 Co-morbidities

Apart from hypertension and hyperlipidaemia mentioned above, the CSX patients had relatively little comorbidity. A minority, 3/17 (18%), had some mild osteoarthritis. The prevalence of hypothyroidism in this group was 23.5%, far higher than the national average of 1.0-1.4%. Two of the CSX patients had IBS and one had coeliac disease. One LCSX patient went on to be diagnosed with Rheumatoid Arthritis over one year later

2.5.4 Reported Symptoms

CSX patients had developed symptoms 3.4 ± 0.8 years prior to angiography and most had symptoms 1-2 times per week. All of the CSX patients had central chest discomfort, which radiated to the throat in 29% of cases and to the left side of the chest and left arm in a third. The severity of angina was a median Canadian Cardiovascular Society (CCS) Class of 2 (IQR 1 to 2). Only 11.8% got angina on walking at their normal pace with the remainder getting symptoms on quickening their pace or walking up an incline. The pain eased on slowing down in 35.3% of cases but 52.9% of patients needed to stop entirely before getting relief while 11.8% experienced the “walk-through” phenomenon. Only 1 patient reported getting rest pain with that occurring only once during a time of emotional stress. Each bout of angina lasted for an average

of 5.0 ± 1.1 minutes. Almost a quarter of patients, 4/17 (23.5%), also complained of dyspnoea on exertion in tandem with their angina.

The LCSX group's pain had been present on average only 1.4 ± 0.3 years and lasted an average of 5.7 ± 1.9 minutes. The pain was predominantly retrosternal in all patients and radiated to the arm in over a half of cases and to the neck in 29%. Only 1 LCSX patient got pain while walking at their usual pace while the remainder required more strenuous exertion to induce the pain. The chest pain resolved on slowing down in 5/7 (71%) with only 1 having to stop to get relief and one walking through. The average severity of angina was CCS class 1, i.e. only on extreme exertion.

2.5.5 SAQ results

SAQ domain	Score (CSX[a])	Score (LCSX[b])	P[ab]	Score (IHD[∫])	p[a∫]
Physical Limitation	81.8 ± 2.1	83.3 ± 6.1	p=0.80	50.2	p<0.001*
Angina Stability	52.9 ± 4.2	46.4 ± 3.6	p=0.42	52.0	p=0.43
Angina Frequency	76.5 ± 1.9	65.7 ± 4.8	p=0.04*	67.5	p<0.001*
Treatment Satisfaction	86.0 ± 3.0	85.7 ± 4.4	p=0.95	78.1	p=0.01*
Quality of Life Score	53.4 ± 4.9	60.7 ± 7.2	p=0.42	56.7	P=0.52

Table 2.4: SAQ scores: Scores range from 0-100, with a higher score indicating improved health function. Values reported as mean ± SEM.

p[ab] Significance between CSX and LCSX groups by Mann-Whitney U

p[a∫] Reported significance between CSX and Spertus' IHD group using Wilcoxon Signed Rank test

The SAQ questionnaire was originally validated in an ischaemic heart disease cohort and there are reported values in this cohort. These values are shown above in the fifth column of table 2.4, labelled "score (IHD[∫])," and are used as a baseline comparison group for our CSX patients[26]. As can be observed from the table, the CSX patients have

significantly less physical limitation than patients with conventional stable angina ($p<0.001$) and also experienced less frequent angina ($p<0.001$) and greater treatment satisfaction ($p=0.01$). Most significantly, however, the CSX patients had similar disease impact on their quality of life as patients with obstructive coronary artery disease and angina ($p=0.52$). The QOL score is determined by the patient's responses to questions regarding their estimates of disease impacts on enjoyment of life as well as an assessment of their response to the prospect of life-long symptoms and their worries regarding the possibility of a heart attack or sudden cardiac death. This highlights the impact that CSX has on the psychological well-being of patients.

LCSX patients did not differ from CSX patients in terms of physical limitation or quality of life but did have more frequent angina pectoris.

2.5.6 PSS and LTE-Q

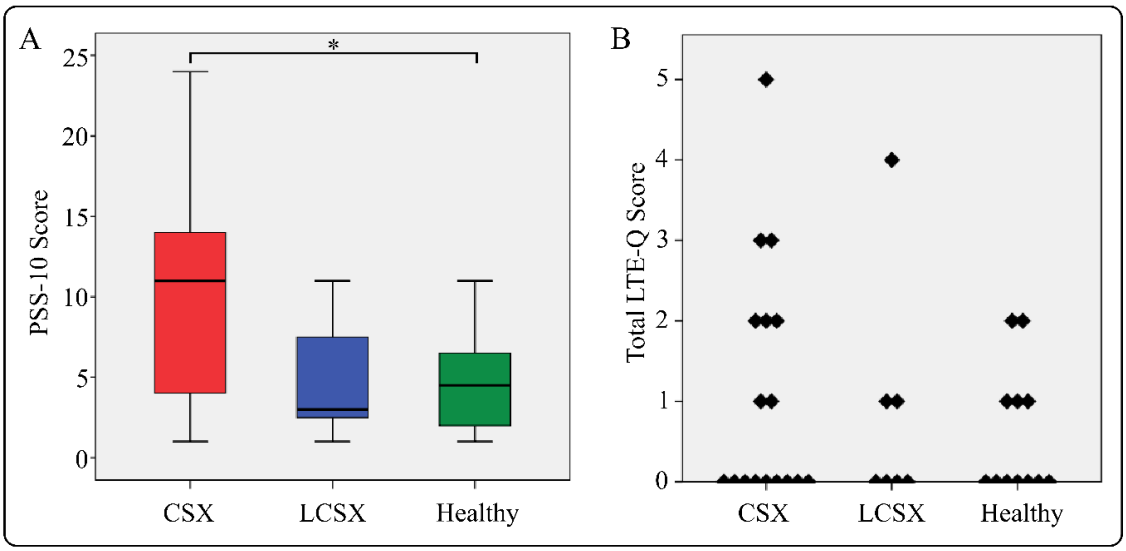


Figure 2.5 A. Perceived Stress Scale-10 item questionnaire scores. **B.** Brugha List of Threatening Experiences scores.

In general, recruited patients had relatively low 10-part Cohen questionnaire (PSS-10) scores with an average score of 9.8 ± 1.7 in the CSX group, a lower score of 5.0 ± 1.4 in

the LCSX group with the healthy controls having a mean score of 4.6 ± 0.8 (see Fig 2.5 Panel A above). It should be noted that only 12 healthy control patients completed the PSS-10 score and LTE-Q, perhaps introducing some bias. One-way ANOVA confirms a significant difference between CSX and Healthy Controls (mean difference of 5.2 ± 2.1, p=0.05) while the LCSX patients only trended lower (p=0.19) than CSX, although the t-test reached significance (t_{22}=-2.117, p=0.047). Thus, CSX patients do appear to have greater perceived life stress. This may indicate the impact of CSX on the psychological health of patients.

"Normal" scores for each demographic cohort in US populations are published, although the validity of extrapolating these normal scores to an Irish population is questionable[164]. For the given cohort (mean age of 59), the US normal score for PSS-10 is 11.9 ± 6.9. This is not significantly different from our CSX cohort, t_{16}=-1.172, p=0.259 but is higher than both our LCSX cohort t_6=4.82, p=0.003 and healthy controls, t_{11}=8.63, p=0.001. It would seem reasonable to conclude that, although they have greater perceived stress than their Irish controls, Irish CSX patients do not suffer from markedly high levels of stress by international standards.

Interestingly in the CSX group the PSS-10 score did negatively correlate with the SAQ domain scores for both treatment satisfaction (r_s=-0.517, df=16, p=0.040) and quality of life (r_s=-0.553, df=16, p=0.026) but not in the other domains, indicating that increased perceived life stress was associated with worsening markers of psychological disease impact rather than physical symptoms in CSX (see figure 2.6 below). Additionally, a multiple linear regression analysis was performed to investigate if these two domain scores could predict a participant's perceived stress. Test assumptions of normality, linearity and multicollinearity were confirmed. A significant regression equation was found (F (2,21)=9.0, p=0.001 with an adjusted R-squared of 0.411).

Participants PSS-10 score equalled 42.7 - 0.185 (QOL) - 0.278 (TSS). Both QOL and TSS were significant predictors of PSS-10 score (p=0.007 and 0.003 respectively).

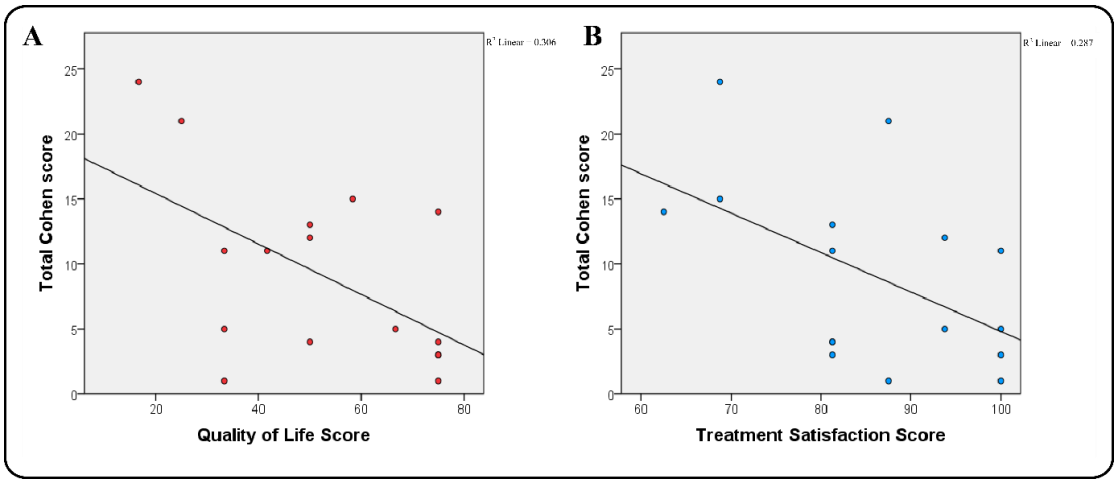


Figure 2.6: A. Correlation between the PSS-10 (Cohen) score and the SAQ quality of life score for CSX patients**. B.** PSS-10 and SAQ treatment satisfaction scores. Multiple regression analysis confirms significant interactions.

The LTE-Q scores were low in all groups with only 8 (47%) of the CSX, 5(42%) of healthy controls and 3 (43%) of the LCSX group listing any significant experiences in the preceding 6 months. This did not significantly differ (p=0.753, see Fig 2.5 Panel B). Predictably there was a moderate correlation between PSS-10 scores and scores from the LTE-Q, (r_s=0.537, df=36, p=0.001) reflecting the fact that stressful life experiences increase perceived stress. In those who had experienced at least one threatening experience, there was an even stronger correlation (r_s=0.769, df=16, p=0.001) as shown in the scatter plots in Fig 2.7 below.

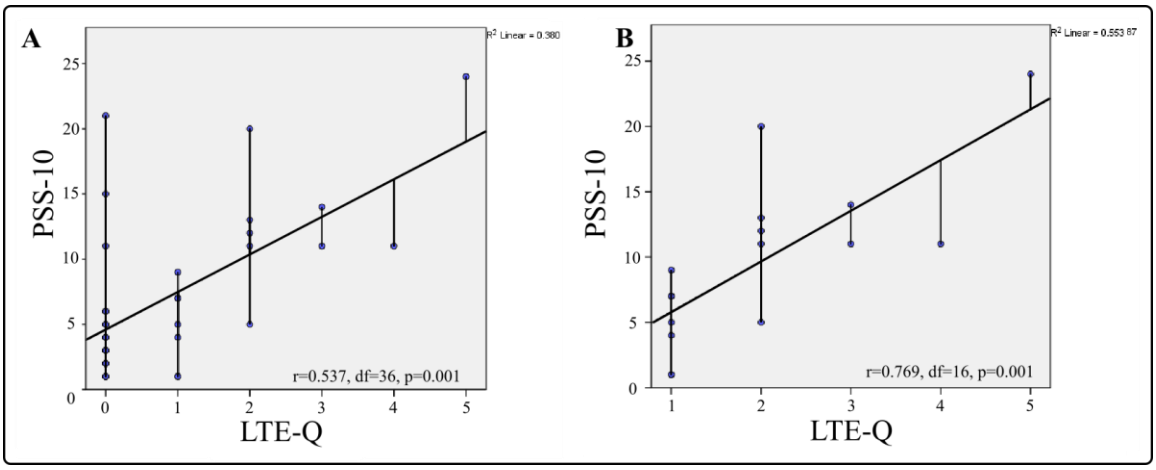


Figure 2.7 Scatter plots showing the correlation between PSS-10 scores and the LTE-Q scores at baseline for all subjects. Panel B shows this correlation in people who reported any threatening experience.

2.5.7 Results of Cardiac Investigations

Parameter	CSX (n=17)	LCSX (n=7)	p-value
EST duration (mins)	8.7 ± 0.5	8.3 ± 2.0	0.77
METS	9.4 ± 0.5	8.9 ± 2.5	0.75
RPP	28985 ± 977	27040 ± 2910	0.53
TTS	6.0 ± 0.7	5.6 ± 1.5	0.81
TTECG	5.7 ± 1.0	-	-
DTS	-2.2 ± 1.0	4.0 ± 1.6	0.006*

Table 2.5: EST parameters. RPP- Rate Pressure product. TTS- time to symptoms. TTECG- time to ECG changes. DTS- Duke Treadmill score.

CSX patients' pre-test 10-year cardiovascular risk score (chance of having a major cardiovascular event) as assessed by the SCORE calculator was low at 2% (IQR 1-2). Before stress testing the average pre-test probability for obstructive coronary artery disease was 54 ± 3.0% for CSX and higher at 63.7 ± 7.7% for LCSX patients (p=0.287), mostly due to the greater number of males in the latter group. The CSX group had a mean EST duration of 8.7 ± 0.5 minutes while the mean rate-pressure product was 28985 ± 997, signifying a high-intermediate haemodynamic response. Their average Duke Treadmill Score was -2.2 ± 1.0, indicating a post-test moderate risk of IHD (≈43%

chance of significant CAD and 33% chance of 3 vessel disease). The time to angina on the EST was 6.0 ± 0.7 minutes while the time to diagnostic ECG changes was slightly less at 5.7 ± 1.0 minutes, perhaps hinting that the CSX patients do follow the traditional ischaemic cascade (see section 1.4.2). There were no significant differences between the CSX and LCSX groups in terms of exercise time, METS achieved, rate-pressure product or time to symptoms. The Duke Treadmill score was significantly lower in the CSX group but this is to be expected given the absence of ECG changes by definition in the LCSX group.

During coronary angiography only 2 of the CSX patients reported chest pain on the injection of contrast while only 1 of the LCSX patients complained of such. Also, the CSX group mean Left Ventricular End-diastolic Pressure (LVEDP) was measured and found to be elevated at 18.1 ± 1.2mmHg (normal range 8-12mmHg) although this was not different to that seen in LCSX (19.7 ± 2.4, p=0.59) This might imply, however, that CSX patients have increased ventricular wall tension due to diastolic dysfunction, which would have ramifications for microvascular function. Other studies have shown evidence of diastolic dysfunction in CSX[168].

2.5.8 Blood results

The results of routine haematological and biochemical tests were normal in both CSX and LCSX and did not differ significantly. The CSX average haemoglobin was 13.6 ± 0.27g/dl, the WCC was 6.2 ±0.3 x 10^6 cells/l with normal platelets of 238 ±13.4 x 10^6 cells/ml. Interestingly, CSX patients had an elevated neutrophil to lymphocytes ratio (NLR) when compared with healthy controls at 2.22 ± 0.09 vs 1.47 ± 0.08 x 10^6 cells/l (t_{25}=3.5, p=0.002), although this had returned somewhat to normal at follow-up (1.89 ± 0.20, adj. p=0.07). The renal function was normal with an average creatinine of 70 ± 3.3 µmol/L as was hepatic function with an ALT of 29.2 ± 4.6 units/L and an INR of 0.99 ± 0.1. As part of their work-up, 9/17 CSX patients had an autoimmune panel sent. This

standard assay, performed by the hospital laboratory includes Anti-nuclear antibody, Rheumatoid factor and Extractable nuclear antibodies. Eight of the patients had negative rheumatoid factor and negative anti-nuclear antibodies (ANA) while one patient had a weakly positive ANA.

2.5.9 Urinalysis

Structural and functional changes have been seen in microvessels of CSX patients including rarefaction, medial hypertrophy etc [169]. Hypertensive and diabetic microvascular disease has been shown to lead to proteinuria. We wished to see if the microvessels in CSX lead to clinically detectable levels of proteinuria. Thus we assessed the urinary albumin:creatinine ratio in our CSX patients. The CSX population had normal albumin: creatinine ratios (ACR) with and average ACR of 1.04 +/- 0.6 (normal range 0-3.0). No patient had an elevated ACR and there was no significant difference in ACR between symptomatic and asymptomatic patients.

2.6 Second Visit

Each CSX patient was scheduled to be reviewed at two further review visits during which questionnaires, blood tests and exercise stress tests were repeated. The follow-up in the CSX patient group was excellent with all patients returning for at least one review (visit 2) at 9.7 ± 0.5 months. It is important to note that one patient had injured themselves before visit 2 and as such was unable to perform an EST while another patient was only 2 months after an operation and had not been mobilising well and as such was unable to give an accurate assessment of symptoms. All LCSX patients were reviewed at 8.2 ± 0.7 months. Control patients were not followed up.

2.6.1 Visit 2 SAQ

SAQ domain (CSX)	Visit 1	Visit 2	p
Physical Limitation	81.8 ± 2.1	86.3 ± 3.2	p=0.07
Angina Stability	52.9 ± 4.2	56.3 ± 4.8	p=0.42
Angina Frequency	76.5 ± 1.9	90.6 ± 3.0	p=0.001
Treatment Satisfaction	86.0 ± 3.0	91.8 ± 2.7	p=0.017
Quality of Life Score	53.4 ± 4.9	74.5 ± 3.4	p=0.003

Table 2.6: **CSX SAQ parameters at visit 2 compared with baseline at visit 1**. P-values reported using the paired t-test or related-samples Wilcoxon Signed-Rank test as appropriate

At follow-up, the SAQ domain scores had generally improved in the CSX cohort (see table 2.6). The average physical limitation score (PLS) trended towards improvement with a mean difference of 5.0 ± 2.7; t_{16}=1.89, p=0.07. In tandem with this improvement in PLS, the angina frequency score significantly improved over the follow-up period with the mean score increasing by 14.1 ± 2.7 (95%CI: 8.9 to 20.5). There was also a significant improvement in treatment satisfaction score of 5.8 ± 2.3 (95%CI: 1.3 to 11.2) while there was a marked improvement in disease related quality of life by 21.1 (95%CI: 9.6 to 33.5). A further point of interest is that the burden of physical symptoms, as measured either by SAQ (PLS) or EST (time-to-symptoms or DTS) did not appear to correlate with perceived quality of life indicating that the psychological burden of CSX may not be wholly related to physical symptom severity.

At visit 2, nine CSX patients claimed to have had no angina in the month prior to review but only four (24%) claimed to be completely asymptomatic with a PLS of 100. Ten patients, however, had improved at least marginally, with 7 showing worsening of symptoms from baseline. In terms of magnitude of change, 3 patients had significantly worsening symptoms (defined as a change in PLS score of -5 or greater), 6 had minimal change and 8 (47%) had significantly improved symptoms (PLS change of +5 or greater).

LCSX patients had generally improved dramatically at follow-up. They were more likely (p=0.06) to have complete resolution of their symptoms than CSX patients with the majority 5/7 (71%) being symptom free by visit 2. One other LCSX patient had significantly improved while the remaining one had significantly worsened.

2.6.2 Visit 2 Life Stress

CSX PSS-10 scores at follow-up were a mean 6.8 ± 1.4. Scores correlated strongly with the patients' visit 1 perceived stress scores (r_s=0.683, df=15, p=0.002) but as a group had significantly decreased by 3.1 (95%CI -5.8 to -0.3, p=0.03). PSS-10 scores were significantly lower in the patients whose SAQ physical limitation score had significantly improved (Δ of >5) compared to those whose symptoms had significantly worsened (p=0.012) but the difference between completely asymptomatic patients and symptomatic patients failed to reach significance (p=0.10), see Figure. 2.9 below. There was a moderate negative correlation between PSS-10 scores and Treatment Satisfaction Scores (r_s=-0.599, df=17, p=0.011). There remained very few threatening events noted in the CSX group, with only 5 patients having any significant event in the previous 6 months. The LTE-Q did not have any ability to discern patients with regards to SAQ or EST parameters.

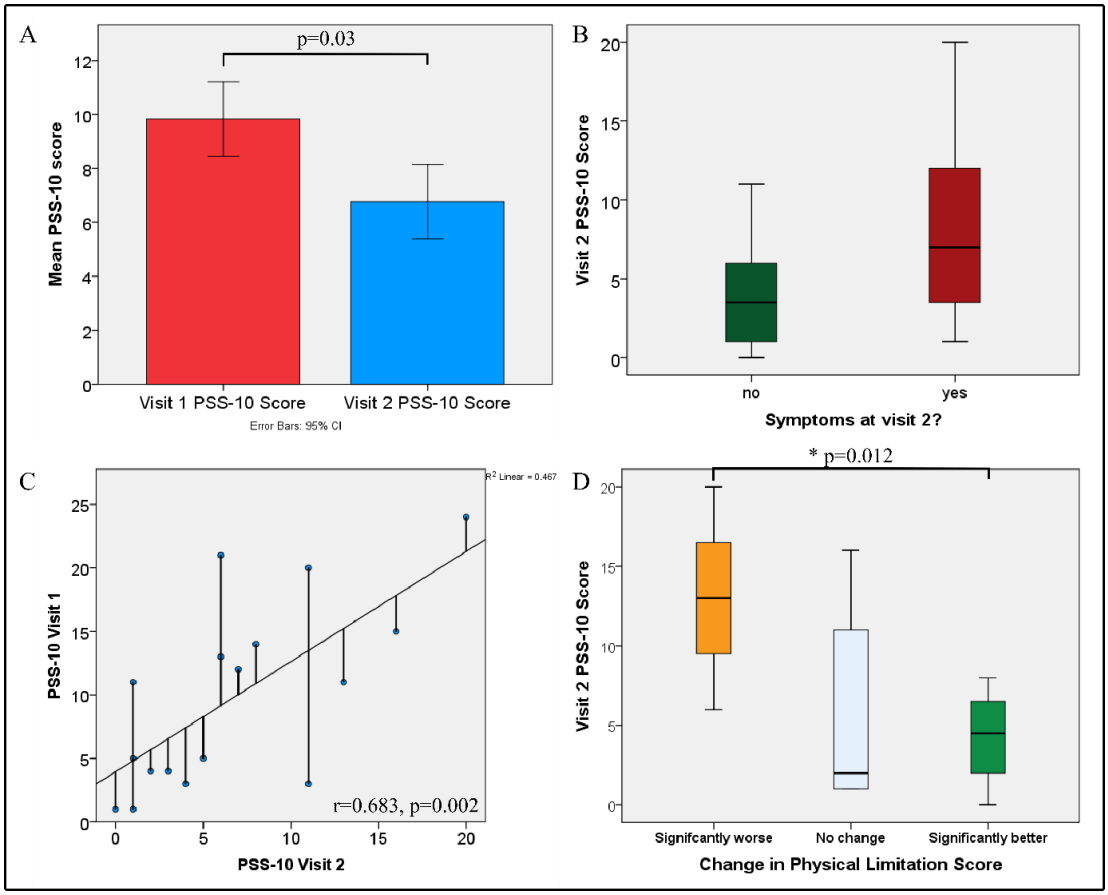


Figure 2.9: A. Comparison between Cohen Perceived Stress Scale scores (PSS-10) observed at visit 1 and at visit 2 in CSX patients. **B**. PSS-10 scores in symptomatic v asymptomatic patients at follow-up (p=0.10) **C.** Scatter plot illustrating the strong correlation between visit 1 and visit 2 PSS-10 scores indicating the consistency in patients' perceived stress. **D.** PSS-10 scores in patients whose Physical limitation scores had worsened >5, remained the same within ±5 or improved >5.

2.6.3 Visit 2 EST

As noted above in 2.6, only 15 CSX patients had a follow-up EST. Nine patients now had electrically negative ESTs while 6 had ongoing ischaemic changes and chest pain. Two patients could not get a follow-up EST, one because of a recent surgery and one because of an injury. One of these patients claimed no ongoing physical limitation from angina while the other continued to get symptoms. The average EST duration, rate-pressure product, time to ECG changes/symptoms (if present) were no different at visit 2 than they were at baseline. Predictably, the Duke Treadmill score was significantly better at visit 2 than at baseline as patients no longer had significant ST-changes (mean

difference 5.8 ± 1.7, p=0.005). CSX patients with a positive stress test at visit 2 had significantly worse physical limitation scores (t_{13}=2.93, p=0.04), angina frequency scores and treatment satisfaction scores at follow-up but did not have worse quality of life scores than those with normal ESTs (see table 2.7). The summary EST score (the Duke Treadmill score) did correlate with the PLS score at visit 2 (r_s=0.548, df=15, p=0.035) while the time to symptoms on EST also strongly negatively correlated with PLS (r_s=-0.873, df=7, p=0.01).

Visit 2 SAQ domain	Normal EST n=9	Positive EST n=6	p
Physical Limitation	92.7±2.2	76.4±6.0	0.04
Angina Stability	61.1±7.3	50.0±6.5	0.53
Angina Frequency	98.9±1.1	78.3±4.0	0.001
Treatment satisfaction	95.8±2.8	85.4±5.0	0.05
Quality of life	75.0±3.7	72.2±7.3	0.75

Table 2.7: CSX SAQ parameters at visit 2. Scores are compared between improved and worsened patients as determined subjectively (by SAQ PLS score) or objectively (by EST). EST- Exercise Stress Test. PLS- Physical Limitation Score.

Those patients whose EST became normal appeared to be less symptomatic even at enrolment (see Fig 2.10). It appears logical that the less severe your symptoms are (measured both objectively by EST or subjectively by SAQ) the more likely it is that you will improve at follow-up. Logistic regression analysis was performed to investigate the effect of baseline PLS on EST positivity at visit 2. The logistic regression model was significant, $\chi^2(1) = 10.625$, p=0.001. The model explained 71% (Nagelkerke R^2) of the variance in EST positivity at follow-up and correctly classified 92.9% of cases. Increasing baseline PLS was associated therefore with a decreased likelihood of ongoing EST positivity (OR 0.72 (95%CI 0.53-0.99).

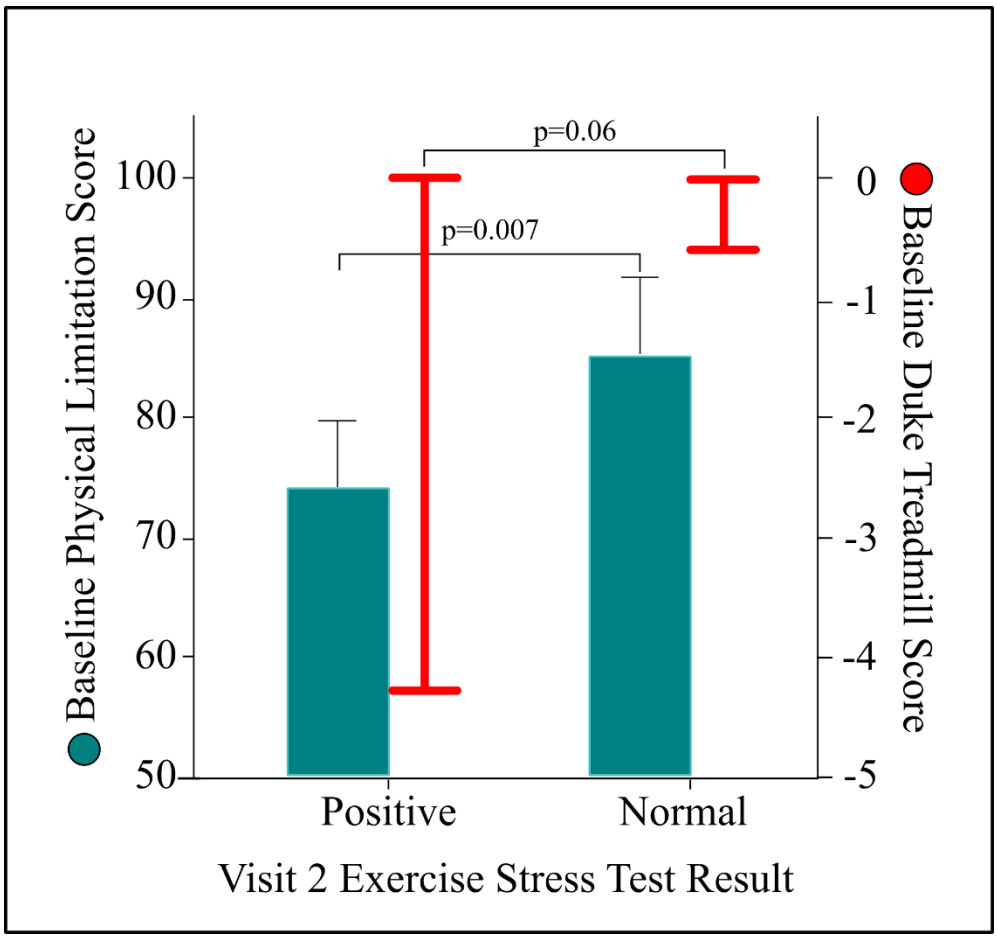


Figure 2.10: Bar chart comparing baseline PLS scores and DTS scores between CSX patients who would complete a normal EST at follow-up and those who would continue to have an electrically abnormal EST.

Explicitly, patients with normal follow-up EST had significantly higher baseline PLS (86.2±2.6 v 74.7±1.8, t_{13}=3.20, p=0.007) and they had less severe EST abnormalities at enrolment with borderline better Duke treadmill scores (U=5, p=0.06), longer time-to-symptoms (t_{12}=2.75, p=0.025) and a trend towards longer time to ECG changes (p=0.15). In essence, these patients had milder disease and it appeared to improve spontaneously. These normal patients did not differ from those with ongoing EST changes in terms of statin use (p=0.608), aspirin use (p=0.379) or ACE-inhibitor use (p=1.00) at follow-up.

2.7 Third Visit

Follow-up of CSX patients was 94% complete at visit 3 at an average 16.8 ± 0.7 months after initial enrolment with 16/17 patients returning for review with the remaining patients being uncontactable at last follow-up. No patient had died or suffered a major cardiovascular event (such as MI or cerebrovascular accident). Only 11/16, however, consented to performing a third EST.

2.7.1 Visit 3 SAQ scores

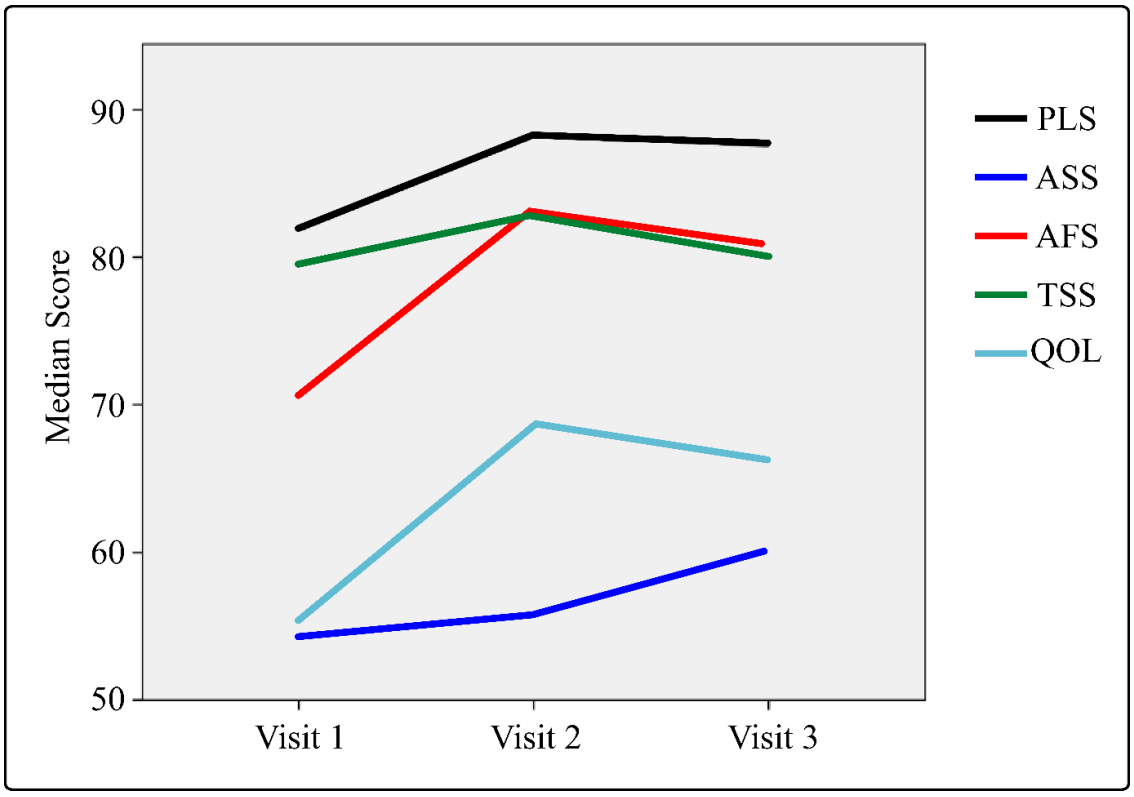


Figure 2.11: Line chart showing the changes in SAQ scores from visit 1 through to visit 3

The SAQ domain scores were not significantly different from visit 2 to visit 3 and in most cases had fallen back to an intermediate value between visit 1 and visit 2, perhaps signifying a fluctuating clinical course in CSX (see Fig. 2.11). The Angina frequency (p=0.01), Physical limitation (p=0.06) and Quality of life scores (p=0.07)

remained better than at baseline, however. By visit 3, only 4/15 patients claimed to be asymptomatic with a PLS of 100. Four patients had significantly disimproved since visit 2 in terms of symptoms (ΔPLS >-5) and 5 had significantly improved (ΔPLS >+5) while the remaining 6 had no significant change. The QOL score at visit 3 correlated strongly with markers of disability such as the EST Duke Treadmill Score (r_s=0.712, df=10, p=0.02), PLS (r_s =0.584, df=14, p=0.028), ASS (r_s =0.621, df=14, p=0.018) and TSS (r_s =0.638, df=14, p=0.014) as shown in figure 2.10. Simple linear regression analysis of the Visit 3 SAQ parameters reveals a significant interaction between Visit 3 PLS and TSS with the observed QOL scores (F(2,11)=12.47, p=0.001; adjusted R^2 of 0.638). Both TSS and PLS were significant predictors of QOL and all pre-test assumptions were met.

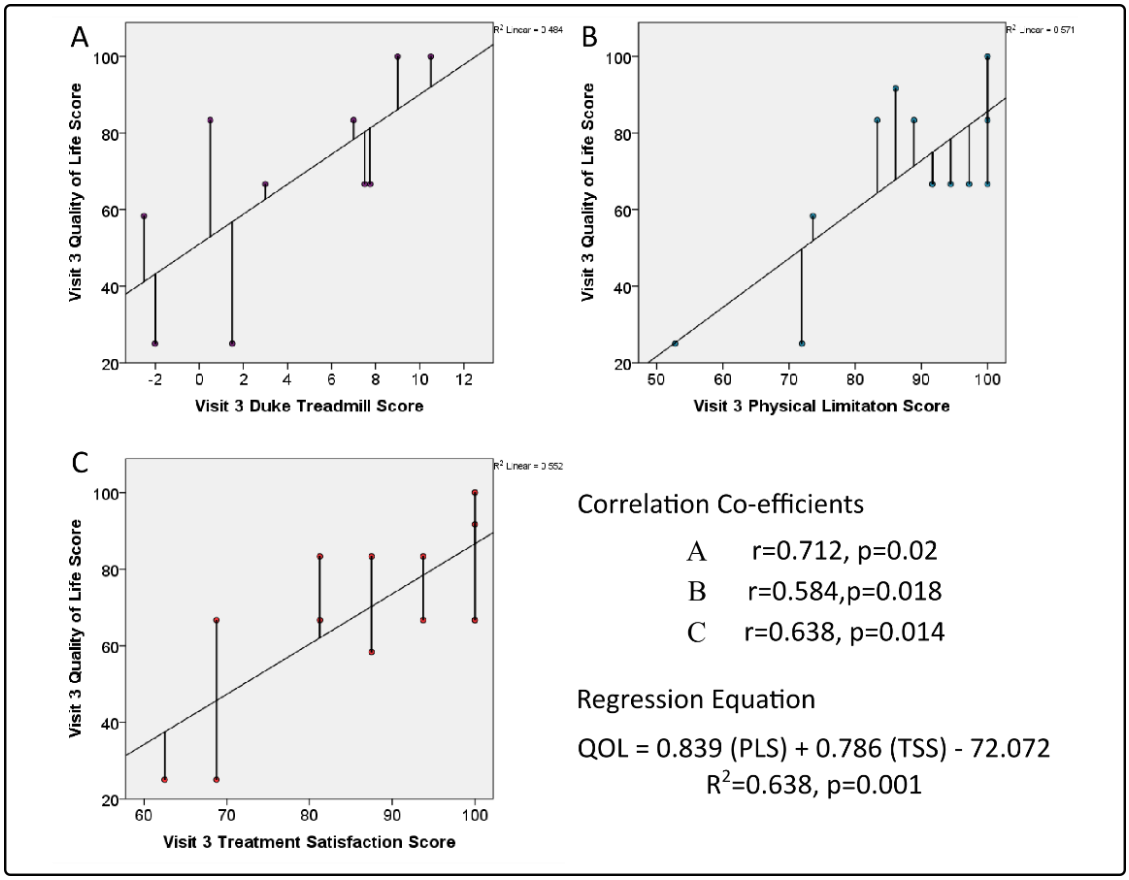


Figure 2.12: Scatter plots showing the correlations between quality of life and A. Duke Treadmill Score, B. Physical Limitation Score and C. Treatment Satisfaction score. D. Spearman rank correlation co-efficients and regression equation with adjusted R-square of the model.

By the end of the study only 4/16 (25%) of CSX patients followed to the end were asymptomatic while 5/7 (72%) of the LCSX patients were asymptomatic (p=0.06 for difference).

2.7.2 Visit 3 Life Stress

Despite a further diminution of LTE-Q scores, the PSS-10 score of CSX patients increased slightly from visit 2 to visit 3 (see Fig. 2.13). Despite this divergence, overall the PSS-10 scores and LTE-Q scores again correlated (r_s=0.594, df=15, p=0.02). The PSS-10 scores correlated closely across all 3 time points.

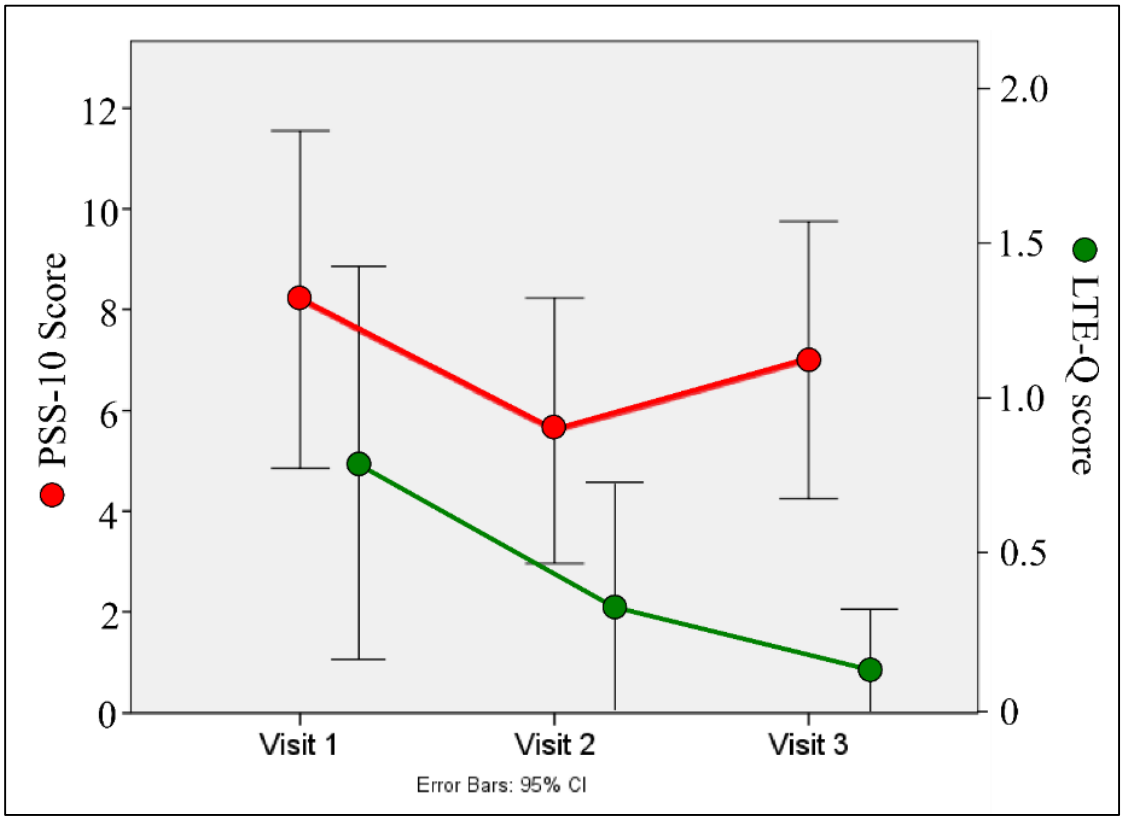


Figure 2.13 Mean PSS-10 and LTE-Q scores over the three visits.

2.7.3 Visit 3 EST

Only two of the 11 CSX patients performing a stress test now had an unequivocally electrically positive EST with a further 4 patients getting mild chest pain on the treadmill. Similarly, nine patients were now minimally symptomatic by PLS. Due to the reduced compliance, especially given the small starting population, the study began to lose statistical power. The average duration was 8.2 ± 0.4 minutes with an average time to ECG change of 6.5 ± 0.5 minutes (n=2) and time to symptoms of 5.5 ± 0.6 minutes (n=6). The average Duke Treadmill score remained higher than at baseline (average difference of 8.1 ± 1.4, p=0.001) and borderline higher than the visit 2 values (average difference 1.5 ± 0.8, p=0.077)

Discussion

2.8 Incidence Discussion

We are one of the first groups to attempt to prospectively investigate the incidence of CSX in a European population and we show that, although CSX exists in Ireland in a definable population, it is relatively uncommon. Approximately 1.3% of patients undergoing coronary angiogram during the incidence study period at an Irish tertiary referral centre met the diagnosis of Cardiac Syndrome X. This is substantially lower than the 3% observed by Vermeltfoort et al in their retrospectively identified 2003 cohort of Dutch patients with CSX. There are two main possible explanations for this difference, either a difference in the true incidence exists or else there is a difference in the diagnostic process.

Given that the exact pathophysiology of CSX remains the subject of debate it is difficult to be authoritative about the possible relevant contributory differences between the two populations in these studies, however several potentially relevant dissimilarities exist. Firstly, the Dutch cohort was taken from angiograms performed back in 2003

compared with our group, which are mainly from 2012. It is possible that the incidence of CSX is diminishing with time or that improved primary prevention interventions for vascular disease are having a beneficial impact on the potential patient population. Secondly, the Irish and Dutch populations differ somewhat in terms of diet, ethnicity, cardiovascular risk and population density.

The other main possibility, that we underdiagnosed CSX relative to the Dutch paper, is likely. In this study we used a strict definition of CSX, particularly with respect to the nature of the chest pain. Typical angina pectoris was a prerequisite for diagnosis, which excluded patients complaining of atypical pain (e.g. pain that was not exertional/stress related, was pleuritic in nature, was not characteristically substernal or that persisted for an excessively long duration despite rest.) The Dutch paper does not specify their definition of angina pectoris and the study was performed retrospectively, which limits the ability to identify atypical features of pain and may have led to the inclusion of patients with non-cardiac chest pain. In our study, the typical angina criterion excluded 2 patients with atypical chest pain, normal arteries and positive EST. Had they been included an overall incidence of 1.9% would have been seen, which is closer to that seen in the Netherlands.

Although our design limited our potential population, we believed it to be important to ensure that we define a homogeneous and reproducible cohort so as to optimise our ability to identify characteristics of that group and to allow a clear signal to be seen by cutting out noise from borderline or erroneous cases, as these patients may bear no resemblance to patients with true CSX. The result of this policy was that we had a smaller than anticipated number of patients and so did not meet our target sample size as estimated by our pre-study power calculations. Longer recruitment was precluded due to time constraints within the study design.

Another factor that limited our ability to diagnose CSX patients was that during the incidence study only 49% of patients with normal coronary arteries had undergone an EST. This was beyond our control. Only 10/77 patients with CPNCA had typical angina pectoris, however, and 9 of these had undergone stress testing (with the last being unable to due to physical limitations) indicating that the majority of appropriate patients were stress tested. The importance of the EST in diagnosing CSX will also be investigated further in this thesis. Had we ignored the requirement of a positive EST and simply included patients with typical angina and normal coronary arteries (the older classification of CSX, termed LCSX in this thesis) we would have doubled our intake. A positive EST, however, identified a distinct set of patients who were more likely to have ongoing symptoms at follow-up despite similar initial symptoms and angiography results (CSX v LCSX, p=0.06).

A final explanation for our relatively low incidence is that it may be possible that some CSX patients were missed in out-of-hours periods as acute cases such as acute ST-elevation myocardial infarctions would be prioritised during daytime hours, deferring CSX angiograms until a later time, and thus potentially masking its true incidence.

2.9 Phenotype discussion

The phenotype of the Irish CSX patient seemed to mirror that of previously reported patients in other CSX studies, whereby the significant majority of subjects were female with an average age of about 60. Irish CSX patient differed from IHD patients in that they tended to be younger by about 5 years (t_{346}=-3.34, p=0.003; 95%CI -2.3 to -9.5 years) and were less likely to have hypertension, although they had a similar prevalence of hyperlipidaemia. The increased prevalence of hypothyroidism in our CSX cohort may represent a red herring or may hint at a possible contribution to the pathogenesis of CSX. Triiodothyronine (T_3) is known to affect the microvasculature and

perhaps its lack (or the use of the synthetic L-thyroxine) may have an aetiological role. This deserves further study.

Irish CSX patients are only mild-moderately symptomatic physically but appear to suffer significant non-physical effects from their condition. Their SAQ-defined quality of life scores are disproportionately low when compared to their physical symptom scores and are equivalent to those of patients with obstructive ischaemic heart disease. The specific SAQ questions for this summary score include: “How much has your chest pain limited your enjoyment of life?”, “how would you feel if you had these symptoms for the rest of your life?” and “how often do you think or worry that you may have a heart attack or die suddenly?” indicating that these patients dwell on their physical symptoms even though they are less physically limited. Their QOL scores improved over time but, despite the reassurance of a normal angiogram, these patients remained concerned at follow-up, with low QOL scores. The follow-up QOL scores appeared to depend on the severity of ongoing symptoms, indicating the importance of making the diagnosis and instigating treatment in these patients to allow for the amelioration of disease burden.

As well as having a reduced disease-related quality of life, Irish CSX patients also had greater perceived life stress than controls despite no relative excess of notable life stressors. This perhaps may reflect the psychological toll of angina. Indeed, the PSS-10 score correlated with both QOL and treatment satisfaction scores, showing that CSX patients have a generally less favourable perception of life, stress and disease impacts. Stress didn’t seem to reliably predict symptoms but there was a trend towards greater stress in CSX patients with ongoing symptoms at follow-up compared with patients who had improved. It is known that stress can reduce pain thresholds and worsen patients’ coping skills so there may be a bidirectional interaction between stress and symptoms in CSX[170].

CSX patients in our cohort tended to show ECG changes on the treadmill before they developed chest pain, suggesting that the traditional model of the ischaemic cascade may be valid in CSX too. Another consideration regarding possible pathophysiology is that we found CSX patients to have elevated Left Ventricular end-diastolic pressures (LVEDP) during angiography. This implies increased wall tension during diastole (the time of maximal myocardial blood flow) and hence may lead to the compression of micro-vessels within the myocardium, which may contribute to the disease process. This has not been reported before.

Irish CSX patients generally improved during the course of an 18-month follow-up. Most parameters of physical symptoms such as SAQ scores and EST measures improved at follow-up. This was matched by an improvement in perceived stress scores. At the second visit 10/16 (63%) patients had symptomatically significantly improved while by the final visit this number reached 11/15 (73%). It should be noted that only 27% of patients claimed to be completely asymptomatic at study end highlighting the prolonged nature of the disease. It is also important to point out that EST electrical positivity dwindled as the study went on. Technically these patients could be considered to no longer have CSX and merely just chest pain with normal coronary arteries. Only 6 and 2 patients had ongoing chest pain and an electrically positive EST at visit 2 and 3 respectively.

The lack of reproducibility of EST results in this cohort may just follow the overall improvement in patients' clinical condition with time. The patients whose ESTs remained positive over time did have lower PLS at baseline (t_{13}=-3.20, p=0.007), at visit 2 (t_{13}=-2.54, p=0.04) and at visit 3 (t_9=-3.63, p=0.006) than those whose EST normalised, indicating that the patients with the most severe disease had persistent dynamic ECG changes. This might demonstrate the natural history of CSX; patients with

a positive EST being worst off and those with milder disease eventually developing normal ESTs and disease resolution.

As a by-product of the recruitment process we also identified a cohort of patients with typical angina a normal angiogram and a normal EST, which we labelled loose CSX (LCSX), as this has historically been used as an alternative definition of CSX. We will use this to examine the clinical utility of the EST in the initial diagnosis CSX. The effectiveness of the EST for clinically defining a distinct cohort of patients is uncertain in patients with normal coronary arteries as the EST is generally useful in predicting the presence of obstructive CAD, having a modest sensitivity and specificity of 68% and 77% therein. The values for these parameters in microvascular angina are unknown. A false positive EST may just demonstrate myocardial ischaemia in a CSX patient as opposed to being truly "falsely" positive. The distinction therefore between LCSX and CSX patients in terms of baseline biomarkers will be interesting going forward. We found that these patients resembled the CSX patients in most respects (such as their demographics, symptoms and cardiac risk factors) and differed only in their PSS-10 scores and the fact that they were more likely to be symptom free at follow-up ($p=0.06$).

Finally, it is important to note that we have recruited controls who are well matched for age and sex. They also ended up being well matched in terms of statin use, which may be important as we will be investigating sensitive inflammatory markers and the pleiotropic effects of statins include an anti-inflammatory effect.

2.10 Limitations

Our observational study prospectively acquired data from a cohort of patients undergoing coronary angiography in two centres. The prospective nature allowed for the pre-diagnosis assessment of symptoms and risk factors, allowing for unbiased assessment of symptom character. There are several limitations in the execution of this study, however:

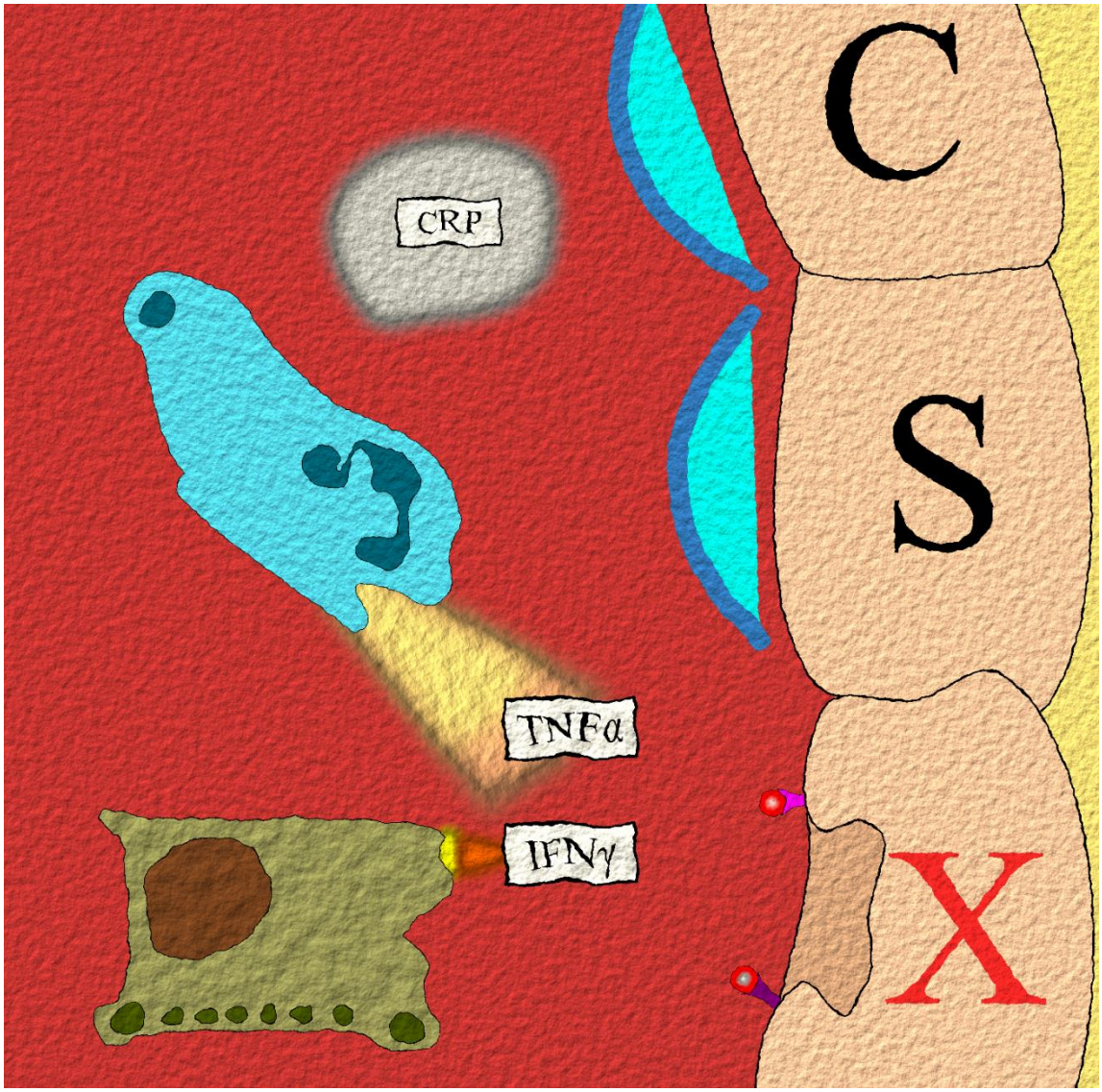
- The patients are non-consecutive. This was unavoidable as resources were not available to allow for 24-hour coverage of the catheterisation laboratory by study investigators. Patient interview was a necessary part of the study to allow accurate documentation of symptoms. Therefore, patients with angiograms performed after hours could not be enrolled in the study as the method of data acquisition for these patients would not be consistent with other patients.
- The determination of the 'typicalness' of the angina is partly subjective, despite the checklist provided for its characterisation by the ESC, due to the ambiguity of the phrase "typical character and duration." Great efforts were made to be consistent in the characterisation of chest pain. Another facet of this is that significant coronary stenosis can present with atypical angina, thus the exclusion of patients with these symptoms may exclude some CSX patients with ischaemic pain. This was necessary, however, to ensure the exclusion of patients with a correct diagnosis of non-cardiac chest pain.
- Finally, exclusion criteria in the CSX patients may have affected some of the measured characteristics of the group. For example, the upper age limit of 70 years was imposed by the local ethics committee, however only 1 patient during the incidence study period was over 70 and would have qualified for a diagnosis of CSX. Most studies into CSX exclude patients with diabetes mellitus or systemic hypertension. We excluded patients with DM or evidence of hypertensive heart disease (LVH by ECG or ECHO) but included patients with mild or controlled hypertension. As hypertension also leads to endothelial dysfunction, the hallmark

of CSX, the exclusion of hypertensive patients would probably exclude people with true microvascular angina. This may account for some of the reduced prevalence of hypertension noted in the CSX group. Conversely, diabetic microvascular disease probably represents a separate cohort of patients phenotypically and warrants exclusion.

Summary

In summary, we identified 17 Irish CSX patients with moderate symptoms and followed them for approximately 17 months with repeated EST, questionnaires and blood tests. We also enrolled 21 healthy controls from 2 primary care practices. A small 7 patient cohort of patients with chest pain, normal coronary arteries and normal stress tests was also recruited for comparison. CSX patients had a reasonable degree of psychological disease impact and almost three-quarters of CSX patients remained symptomatic until the end of follow-up, although most patients improved from baseline.

We also found that in Ireland CSX is a relatively uncommon diagnosis made after only about 1.3% of all angiograms investigating chest pain and is most likely to occur in middle-aged women with dyslipidaemia and a moderate overall risk categorisation based on pre-angiography non-invasive testing. Despite the low incidence, it should be stressed that CSX is no less common than many other notable cardiac conditions such as anomalous coronary arteries (seen in 1-2% of angiograms) or coronary arterial dissection (in 0.1%). It deserves greater exposure and cardiologists should be aware of it. The application of the diagnosis to appropriate patients was low in CUH and thus CSX patients in Ireland are underdiagnosed and therefore lacking sufficient treatment. They warrant specialist follow-up in order to appropriately manage their condition.



Chapter 3: Markers of General and Vascular Inflammation in Cardiac Syndrome X

Introduction

3.1 Chapter Overview

In this chapter we will examine the status of general inflammation in the Irish CSX patient and establish its relationship with endothelial activation and clinical features. Many studies have confirmed the presence of a low-grade systemic inflammatory response in CSX[59,60,171]. Our first step is to confirm that our patients conform to this body of evidence. We then wish to examine biomarker evidence of endothelial dysfunction in the form of endothelial adhesion molecules. Having done this, we aim to investigate the temporal relationship between inflammation, vascular and clinical features, including symptoms and EST parameters.

3.1.1 Acute Phase Reactants

APRs are generally plasma proteins that are primarily but not exclusively synthesised by the liver as part of the innate immune response. They are typified by an acute and sustained rise in plasma concentrations mere hours after a suitable stimulus. They remain elevated for the duration of this stimulus, which is usually either infective or inflammatory, and as such can be used as a non-specific barometer of inflammatory activity. We have decided to examine the role in CSX of two of the most studied APRs, C-reactive Protein (CRP) and Serum Amyloid A (SAA), to establish overall inflammatory activity.

C-Reactive Protein

CRP has been extensively investigated in the field of cardiovascular medicine. It is a 23kDa pentameric protein that is released from the liver in response to IL-1, IL-6 and TNFα amongst other signals. Even within its normal range of 1-10mg/L, CRP has been shown to have strong predictive prognostic power in patients with acute coronary syndromes and is a powerful non-traditional cardiovascular risk factor in otherwise

"healthy" populations. It is known to be modestly elevated in CSX and has been shown to correlate with the degree of endothelial dysfunction (as assessed by flow-mediated dilation of the brachial artery and coronary flow reserve on invasive angiography) and somewhat with disease severity in terms of Duke Treadmill Score (see chapter 2)[172,173]. Its role in CSX is interesting because it may be a direct mediator of disease as well as being an indicator of disease severity.

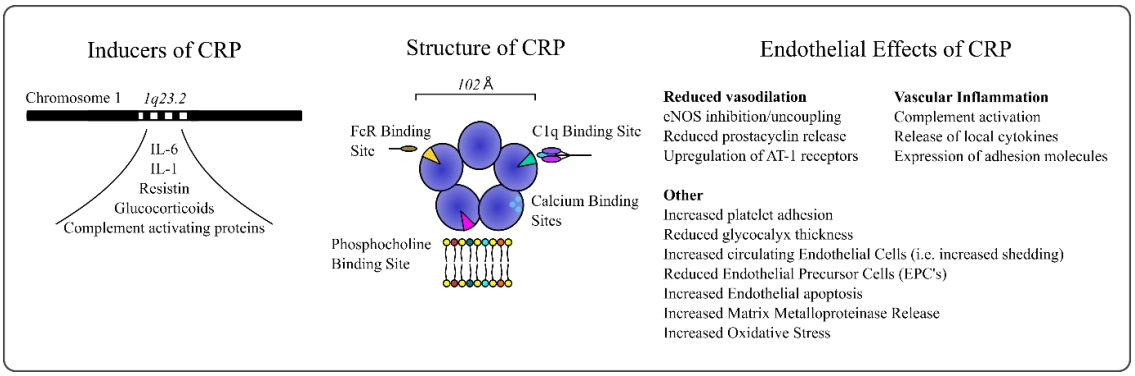


Figure 3.1: C-reactive Protein inducers, structure and effects on the endothelium.

CRP has multiple endogenous ligands including oxidised LDL and oxidised phosphatidylcholine in cell membranes[174]. It has been shown to bind to apoptotic and damaged cells to target them for immune destruction in a process termed opsonisation. CRP also has many deleterious effects on the normal functioning of endothelium. It has been shown to diminish the protective glycocalyx that covers endothelial cells and which is important in intercellular communication and in mechanotransduction[175]. Furthermore, it induces endothelial activation with increased expression of ICAM-1 and VCAM-1 with further local cytokine release[176]. It also increases platelet adherence to the endothelium, mostly through the upregulation of P-selectin while also upregulating angiotensin II type 1 receptor expression, which can cause vasoconstriction. Of further significance when one considers the putative pathogenesis of CSX, CRP downregulates endothelial Nitric Oxide Synthase (eNOS) transcription and reduces eNOS mRNA stability while also uncoupling eNOS activity

resulting in a reduction in available NO[177]. This prevents endothelial-derived flow-dependent vasodilation in the vascular tree[178]. CRP may even induce endothelial apoptosis[179]. Finally, CRP has also been shown to stimulate the release of matrix metalloproteinase 1 and 10, which may lead to plaque instability from stromal breakdown.

The cause of increased CRP in many vascular conditions such as atheromatous coronary artery disease is unknown but many infective and autoimmune causes have been investigated but none has been found to be a compelling inducer. It may just reflect tissue injury from traditional risk factors with a resultant inflammatory response.

Serum Amyloid A

Serum Amyloid A is a family of apolipoproteins that normally associates with HDL. When present in high proportions SAA has the ability to modify the biological activity of HDL by altering reverse cholesterol transport, reducing its anti-oxidant function and ultimately rendering the HDL pro-inflammatory[180]. Like CRP, SAA is also released in response to many various stimuli (such as IL-1, IL-6, TNFα) and can increase 1000-fold from its normal range of 1-5mg/ml in response to stimuli such as infection or tissue necrosis. Again like CRP, SAA correlates with increased CV mortality in large studies and as such is another non-traditional cardiac risk factor[181,182]. SAA activates receptors involved in pathogen identification, such as TLR 2 and 4, while also being able to bind to CD36, a scavenger receptor. It appears to preferentially increase neutrophil activity over monocyte/macrophage activity. It too has several pro-inflammatory effects on endothelium with reduced endothelial cell proliferation, increased adhesion molecule expression and endothelial activation, increased NFκB transcription and increased intracellular oxidative stress all being described[183,184]. It has also been shown to reduce

endothelium-dependent vasodilation and NO bioavailability[185,186]. SAA concentrations have never before been investigated in CSX.

3.1.2 Adhesion Molecules

In addition to evaluating markers of basal inflammation we will examine plasma markers of endothelial activation. Endothelial cells that are activated by certain stimuli (including pro-inflammatory cytokines) will express adhesion molecules. These molecules allow the endothelial cells to interact with circulating immune cells and thereby recruit them to cause affect a local inflammatory response. We intend to examine the degree of endothelial activation using serum concentrations of VCAM-1 and ICAM-1, the two most important endothelial adhesion molecules

Intercellular Adhesion Molecule (ICAM-1)

ICAM-1 (CD54) is a transmembrane molecule that is expressed on endothelial cells and on the surface of some leucocytes. It is constitutively expressed and its function is to allow the interaction between immune cells and endothelial cells. Its expression is increased by pro-inflammatory cytokines (such as IL-1 and TNFα) and CRP. ICAM-1 levels are also increased in diabetes mellitus, heart failure and ischaemic heart disease[187]. Elevated levels are associated with reduced myocardial perfusion reserve as assessed by MRI[188]. It binds to leucocyte integrin and Mac-1 and mediates the firm adhesion of leucocytes to activated endothelium and the transmigration of these cells across the endothelial barrier. It has been implicated in the pathogenesis of atherosclerosis and has been shown to be elevated in CSX[59,161,169].

Vascular Cell Adhesion Molecule (VCAM-1)

The *VCAM*-1 gene is a member of the Immunoglobulin-gene superfamily and is found on chromosome 1. Its transcription product, the protein VCAM-1 (CD106) is a type I transmembrane protein expressed exclusively on activated endothelial cells. It is upregulated in response to turbulent shear stress or oxidative stress from reactive oxygen species and may also be induced by inflammatory stimuli though the activation of NFκB transcription factors. Its main cytokine stimuli include TNFα and IL-1, although it is also induced by CRP and its mRNA is stabilised by IL-4. It is often co-expressed with ICAM-1 on tetraspanin-rich microdomains and this allows for the firm adhesion of leucocytes to endothelial cells through the co-operative action of the two adhesion molecules[189].

VCAM-1's main ligand is integrin, a protein expressed on the surface of many immune cells including lymphocytes, monocytes and eosinophils. VCAM-1 plays an integral role in vascular inflammation and allows for the recruitment of immune effector cells to the local perivascular space by causing cell flow slowing, rolling adhesion and finally firm adhesion to occur. VCAM-1 has been shown to be an early finding in areas predisposed to atheroma formation and VCAM-1 levels are an indicator of the degree of endothelial activation and as such are of great interest in microvascular angina[190]. Indeed, elevated VCAM-1 levels have been shown to be associated with reduced coronary flow reserve in CSX patients[191]. There have been conflicting results from studies into VCAM-1 in CSX with some showing no elevation of levels and others showing a near-significant increase in VCAM-1 concentrations in CSX populations[59,192].

Our contention is that CSX patients will have evidence of inflammation and endothelial activation and that these levels will likely correlate with the degree of disease severity. We therefore hope to establish the baseline inflammatory profile, including APRs and adhesion molecules, in Irish CSX patients by comparing them with healthy age- and sex-

matched controls. We will then longitudinally study changes in clinical status with changes in levels of these immune markers with the hope that improved clinical status is reflected in reduced levels of immune and endothelial activation

3.1.3 Chapter Objectives

1. Initially we aim to **verify that our CSX population reflects those seen in previous CSX studies, which have almost universally demonstrated low-grade inflammation**. We will examine baseline acute phase reactants as markers of overall inflammatory status. We have chosen high-sensitivity C-Reactive Protein (CRP), the most studied Acute Phase Reactant (APR), as our primary biomarker and Serum Amyloid A (SAA) as our confirmatory signal.
2. Having established that CSX patients have low-grade inflammation we will further investigate if they have **biomarker evidence of early endothelial activation** and dysfunction. Intracellular Adhesion Molecule-1 (sICAM-1) and vascular cell adhesion molecule-1 (sVCAM-1) are early markers of endothelial activation and we will evaluate if these markers are deranged in CSX and if they correlate with the acute phase reactants, investigating a possible interaction.
3. We will also evaluate the **interaction between the inflammatory and endothelial biomarkers with symptoms** in the form of SAQ results and EST parameters. If inflammation and endothelial dysfunction are causative in CSX one would expect an interaction.
4. Stress has been shown to be associated with increased inflammatory markers and upregulated HPA axis activity. We will investigate if there is an **interaction between our biomarkers and perceived or actual life stress.**
5. We aim to see if **changes in clinical status are reflected in changes in APRs and markers of endothelial dysfunction**. In those patients who improve, we want to show reduced endothelial activation and reduced general inflammation indicating that it is a state marker rather than a trait marker.

Methods

3.2 Participant Recruitment

Patient recruitment is as detailed in chapter 2.2. We enrolled a total of 17 patients with CSX, 7 with LCSX and 21 controls. Almost 1,850 patients undergoing coronary angiography for chest pain were screened to obtain this sample cohort of CSX patients. Every suitable patient was approached and all agreed to be enrolled in the study. The study protocol was approved by the local research ethics committee. CSX patients were followed-up at 2 further visits when blood and questionnaire investigations were repeated.

3.3 Investigations

At enrolment all subjects gave full-informed written consent and filled out a cardiac risk factor questionnaire. Venous blood samples were taken between 0900 and 1200 and were drawn into dipotassium EDTA tubes and immediately centrifuged at 112 RCF for 15 minutes. Plasma was then aliquoted into 2ml microtubes and frozen at -80˚C for later analysis. CSX patients also completed the Seattle Angina Questionnaire (SAQ), the list of threatening experiences questionnaire (LTE-Q) and the Perceived Stress Scale (PSS) questionnaire as detailed in 2.3.2. No symptomatic treatment was undertaken in CSX patients at diagnosis. The CSX patients were followed at two further visits, planned to occur around 6 months and 12 months after the angiogram, when the exercise stress test, blood sampling and questionnaires were repeated. Stress testing was performed as previously described (2.3.4).

3.4 Biomarker Detection

Plasma samples from all time points were later thawed and analysed together immediately using the MesoScale Discovery Human Vascular Injury II

Electrochemiluminescence kit (Meso Scale Discovery, Rockville, MD). This assay has a lower limit of detection (LLOD) of 0.1 ng/mL and a lower limit of quantification (LLOQ) of 0.7ng/mL for human CRP and the average co-efficient of variation (CV) was 2.5 (1.1 to 3.9) % between duplicated samples. It has an LLOD of 0.09ng/ml and LLOQ of 0.5ng/ml for SAA with an average CV of 3.4 (1.4 to 5.4 %). The VCAM-1 LLOQ was 0.07ng/ml and LLOQ was 0.7ng/ml with an average CV of 3.2 (0.0 to 7.4%). The ICAM-1 LLOD was 0.01ng/ml and the LLOQ was 0.09ng/ml with an average CV of 3.2 (1.0 to 4.5%). Any sample with a CV>20% was excluded from analysis. Inter-run CVs are shown in figure 3.2 below, which is reproduced from the manufacturer's product insert.

	Control	Average Conc. (pg/mL)	Average Intra-run %CV	Inter-run %CV	Inter-lot %CV
SAA	Control 1	42 586	4.7	9.6	10.6
	Control 2	4211	3.6	14.6	8.3
	Control 3	494	4.6	15.6	6.8
CRP	Control 1	22 730	4.1	6.7	7.1
	Control 2	5345	2.2	7.2	7.1
	Control 3	641	2.3	9.9	10.5
VCAM-1	Control 1	11 119	3.5	5.2	2.4
	Control 2	1208	2.2	5.8	2.5
	Control 3	152	3.7	8.4	2.6
ICAM-1	Control 1	12 341	5.3	9.1	12.6
	Control 2	1377	3.5	11.6	14.9
	Control 3	145	3.3	13.4	17.8

Figure 3.2: Average intra- and inter-run CV (co-efficient of variation) from the product insert of the MSD Vascular Injury II Electrochemiluminescence kit.

The exact protocol for the MSD Human Vascular Injury plate is available on the vendor's website but the basic procedure is as follows. The 96-well 4-spot plates are first blocked with Blocker A, a solution of proteins in phosphate buffer that is designed to prevent the binding of non-specific proteins to the plate. This allows for less background noise and enhances the sensitivity of the procedure. The plates are then incubated at room temperature with shaking for 1 hour before being washed with PBS-

T 0.05%. Calibrator blend was then diluted appropriately and added to give a standard curve while plasma samples were also added to the plates, which were then incubated and shaken for another 2 hours. After further washing, the Sulfo-Tag Detection antibody solution was added to each well and further incubated at room temperature with shaking for 2 hours. Finally, the plates were washed and read buffer was added before the plates were analysed with the SECTOR analyser, allowing for quantification of luminescence and hence concentrations of the substances under investigation.

3.5 Data Management

All data was analysed using SPSS Statistics for Windows v20.0 (Armonk, NY: IBM Corp.). Continuous variables are reported as mean ± SEM if normally distributed and as median (IQR) if non-normally distributed. Categorical variables are reported as the absolute number (percentage). The Mann-Whitney U or student t-test were used where appropriate to compare CRP concentrations between CSX and HC groups. Comparisons between multiple groups were achieved using the One-way ANOVA with Bonferroni post-hoc testing or Kruskal Wallis test where appropriate. Categorical variables were compared using the Chi-squared test or Fisher's exact test where appropriate. Correlations were examined using Spearman's rank correlation test. All reported p-values are two-tailed and reported confidence intervals are calculated to the 95% confidence level.

Results

3.6. Acute Phase Reactants

3.6.1 Baseline CRP

Baseline CRP was significantly higher in CSX patients when compared to healthy controls (1.57 [0.48 to 4.81 vs 0.77 [0.14 to 1.17] mg/L, U=281.0, p=0.002; see Fig 3.3 Panel A). Thirty-five percent of CSX patients had a CRP in the range of 1-3mg/L

(classified by the AHA as intermediate risk for cardiovascular disease) while a further 35% had a CRP>3mg/L (defined as high risk for cardiovascular disease). This compares with only 38% of healthy controls in the intermediate risk category and none in the high risk category (p=0.007 for difference). Baseline CRP correlated with EST markers of disease severity especially time to symptoms (r_s=-0.686, df=12, p=0.014) and time to ECG changes (r_s=-0.551, df=15, p=0.033) (see fig 3.3 Panel B). CRP did not correlate, however, with life stress scores or SAQ domain scores.

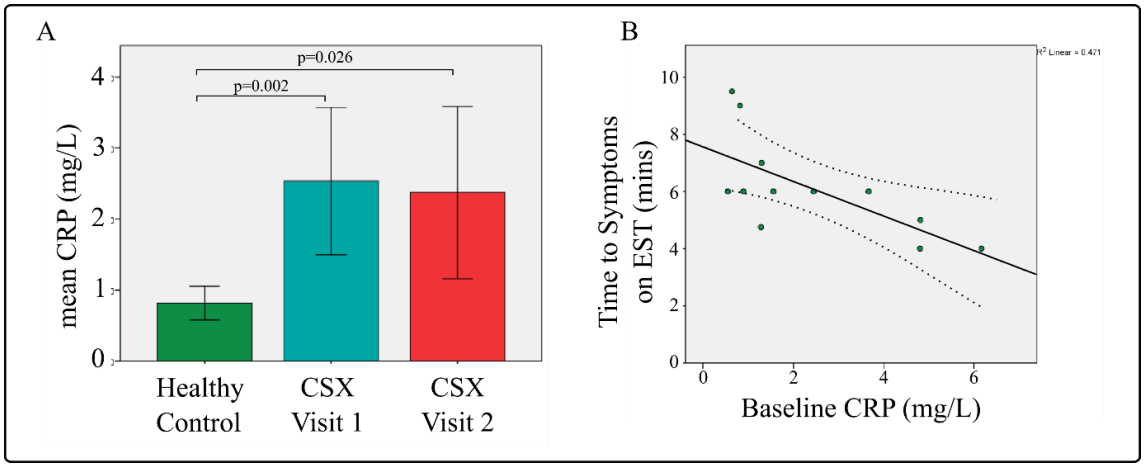


Figure 3.3 A: Differences in mean CRP concentration between HC, Baseline CSX and Follow-up CSX. **B.** Scatter plot showing the correlation between CRP and time to symptoms on EST.

3.6.2 Follow-up CRP

The CRP concentrations in CSX patients as a group remained higher at visit 2 when compared with the same control group CRP (2.38 ± 0.57 vs 0.82 ± 0.11mg/L, mean difference 1.56 ± 0.57, 95% CI 0.14 to 2.97, adjusted p=0.026; See Fig 3.3 Panel A). There was no statistical difference between CRP at visit 1 and visit 2 for the CSX patients (test statistic=67, p=0.653; Related-Samples Wilcoxon Signed Rank Test).

As mentioned in chapter 2, 10 CSX patients had improved in terms of their physical symptoms as assessed by their Physical Limitation Score from their Seattle Angina Questionnaire with the remaining 7 worsening. Interestingly, the patients whose symptoms had improved had an average drop in CRP of -0.51 (-1.47 to +0.11mg/L) while those whose symptoms worsened had an average increase in CRP of 0.28 (0.13 to 0.81mg/L); difference between the two groups, U=8, p=0.027. This is illustrated in fig 3.4 below. Similarly, the 9 patients who claimed to have had no angina in the previous month (angina frequency score of 100) had a lower CRP at follow-up compared with those patients who had experienced angina recently (1.23 ± 0.24 vs 2.53 ± 0.46mg/L, mean difference -1.30 ± 0.52, 95% CI -0.19 to -2.41mg/L, p=0.025).

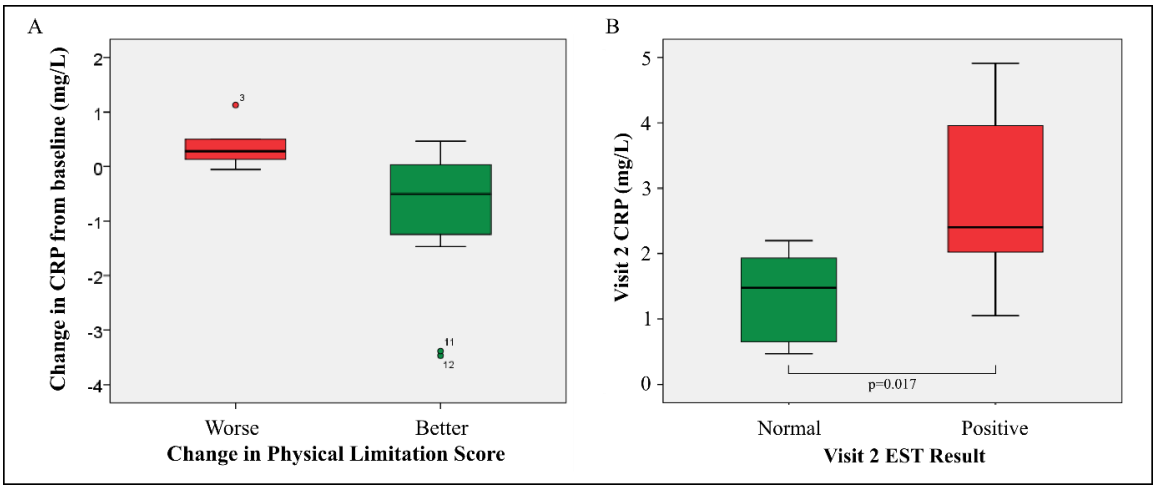


Figure 3.4 A. Net change in CRP in those patients whose physical limitation score worsened or in those who improved. **B.** CRP in patients with a second positive EST compared to those with a normal 2nd test.

Only 6 patients had electrically positive ESTs at follow-up with the remaining 9 patients who underwent follow-up EST having a normal test. Those patients with positive ESTs had higher CRP concentrations on average at follow-up than patients who now had normal stress test (mean difference of 1.48 ± 0.54mg/L, 95% CI 0.31 to 2.66, p=0.017; See Fig 3.4 Panel B above). CRP at visit 2 also strongly correlated with time to symptoms on the follow-up EST (r_s= -0.899, df=4, p=0.015)

The average CRP of the entire group at visit 3 had dropped to 1.47 [0.73 to 2.42mg/L], a point where it was no longer significantly different from healthy controls (adjusted p=0.102). Only 2 patients remained with a positive EST but their CRP was no different from that of the 9 patients with a normal EST (p=0.868). Again only 4 patients had no symptoms by PLS but their CRP was non-significantly lower than that of the remaining 11 symptomatic CSX patients (0.88 [0.40 to 1.13 mg/L] v 1.90 [0.29 to 3.00 mg/L], p=0.101).

3.6.3 Baseline SAA

Baseline SAA concentrations were markedly positively skewed (skewness of 1.38) with the concentration in CSX being 4.95 [1.98 to 7.95mg/L] and healthy controls having a median 2.36 [1.62 to 4.08mg/L]. A square-root transformation was performed to render the values normally distributed. The CSX SAA concentrations were significantly higher than healthy controls as shown below in fig 3.5 (t_{36}=2.114, p=0.045). No significant correlations were seen between baseline SAA and SAQ domain scores, PSS-10 results or EST parameters.

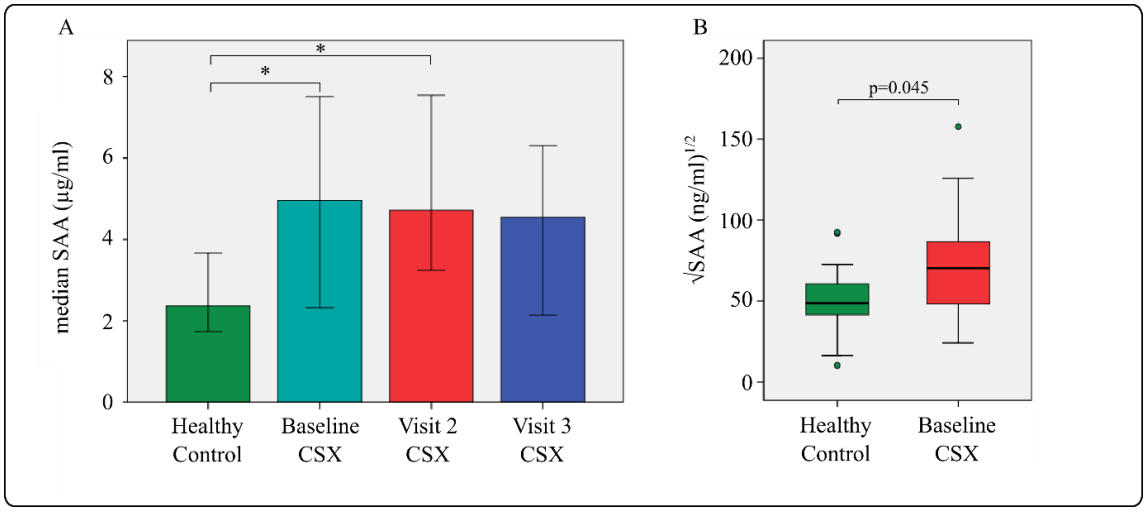


Figure 3.5 A. Bar chart demonstrating median SAA concentrations in the healthy controls compared with the CSX group at different time-points. **B**. Transformed baseline SAA showing a significant difference between SAA levels in CSX and controls.

3.6.4 Follow-up SAA

At follow-up, the SAA levels dropped slightly in CSX patients but they overall remained higher than that seen in healthy controls as shown in figure 3.5a above (adj. p=0.010). Unlike CRP, SAA did not differ significantly between patients who continued to have a positive EST and those who improved. Similarly, SAA did not distinguish patients with ongoing symptoms by SAQ at visit 2, although there was a borderline significant difference in levels by visit 3 with patients with ongoing PLS symptoms having higher SAA concentrations (p=0.06).

3.7 Markers of Vascular Inflammation

3.7.1 Baseline ICAM-1

Baseline ICAM-1 was significantly higher in CSX patients compared to healthy controls 353 ± 25 vs 284 ± 20 ng/ml (95%CI: 5 to 133ng/ml; t_{35}=2.192, p=0.035; see Fig 3.6 Panel A).

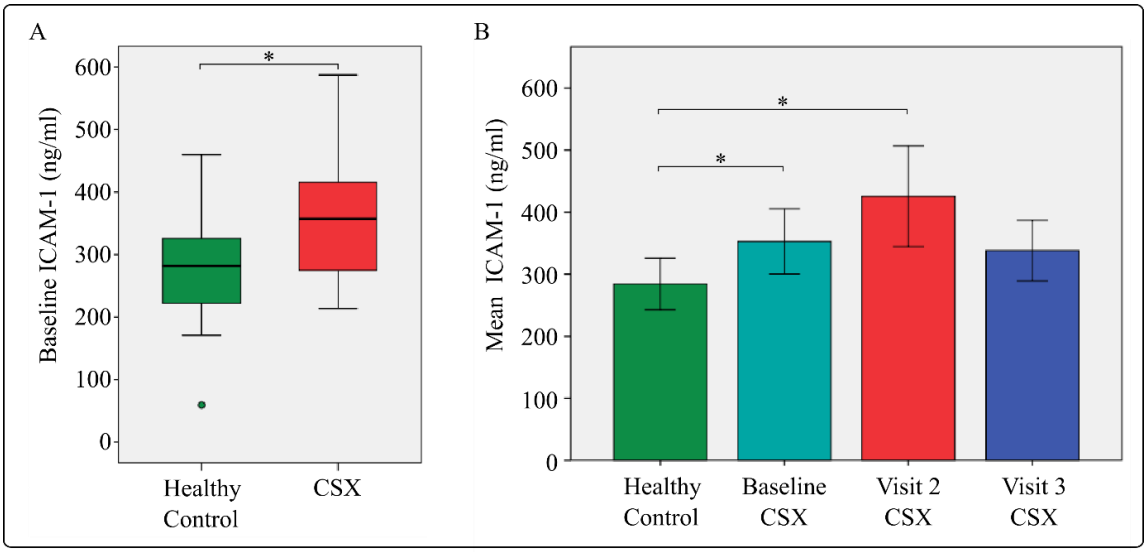


Figure 3.6 A. Comparison of ICAM-1 concentrations in healthy controls and CSX patients. **B.** Bar chart demonstrating the relative ICAM-1 concentrations over time in the CSX group with the healthy controls as a comparison.

3.7.2 Follow-up ICAM-1

ICAM-1 remained elevated in CSX patients during their early follow-up compared with healthy controls but by visit 3 was not statistically higher (p=0.09). Furthermore, ICAM did not show any relationship with clinical markers of disease severity either in the form of EST parameters or questionnaire results.

3.7.3 Baseline VCAM-1

Unfortunately, our study appears to have been underpowered to detect a significant difference in VCAM-1 levels in our populations. There was only a trend towards a higher VCAM-1 in CSX patients (433 ± 31 vs 373 ± 19; T_{36}=1.721, p=0.09). There was no correlation between VCAM-1 concentrations and symptom burden as defined by SAQ or EST.

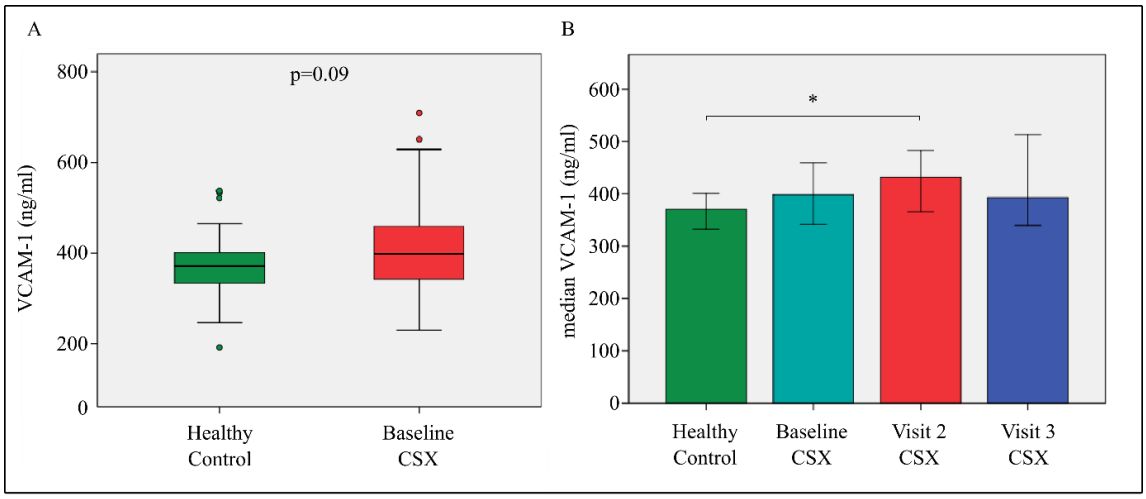


Figure 3.7: A. Trend towards higher VCAM-1 concentrations in CSX populations. **B**. Median VCAM-1 Concentration at differing time points.

3.7.4 Follow-up VCAM-1

VCAM-1 concentrations were higher in CSX patients at visit 2 compared to healthy controls (432 [361 to 504] vs 371 [329 to 418]ng/ml; U=246, p=0.048) although when

corrected for repeated comparisons it lost its significance (p=0.096). Unfortunately, VCAM-1 was not significantly different in populations defined by their EST or SAQ results.

3.8 Correlations

As one would expect, there was a moderate correlation amongst the two acute phase reactants at baseline in all participants (r_s=0.517, df=36, p=0.001). This was also true for the CSX group (r_s=0.640, df=15, p=0.006). There was also strong correlation between CRP and ICAM-1 (r_s=0.450, df=36, p=0.005) and SAA and ICAM-1 (r_s=0.437, df=35, p=0.007), while in the CSX group SAA correlated with ICAM-1 (r_s=0.522, df=14, p=0.038). VCAM-1 levels, however, did not correlate with APRs but did correlate with ICAM-1 levels at baseline (r_s=0.511, df=14, p=0.043) and at follow-up (r_s=0.566, df=14, p=0.018).

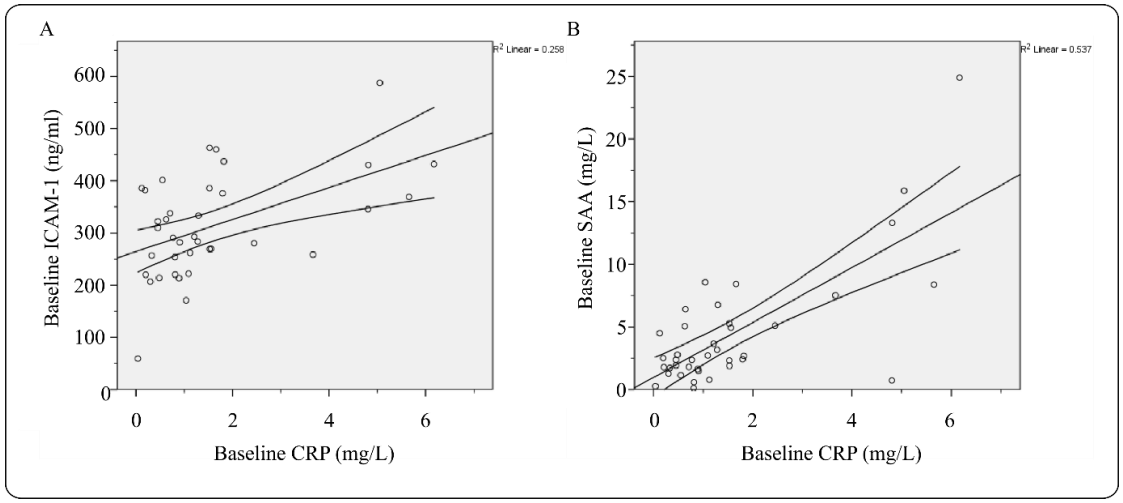


Figure 3.8 A. Correlation between CRP and ICAM-1 at baseline in the entire group. **B.** Correlation between both Acute Phase Reactants at baseline.

There were some correlations between markers of vascular inflammation and EST measures of disease severity. There was a moderate negative correlation between baseline CRP and time to symptoms (r_s= -0.690, df=10, p=0.013) and maximum Rate–Pressure Product (r_s = -0.636, df=8, p=0.048) on baseline EST while there was a trend towards a shorter time to ST-depression with increasing CRP (r_s= -0.502, df=13, p=0.056). SAA was found to correlate with time to ST-segment depression on EST (r_s=-0.538, df=12, p=0.047) while ICAM-1 correlated with time to symptoms on the EST (r_s=-0.610, df=10, p=0.035). CRP at visit 2 also strongly correlated with time to symptoms on the follow-up EST (r_s = -0.899, df = 4, p = 0.015) however there was no significant correlation with time to ECG changes at this visit.

3.9 LCSX

As mentioned in chapter 2, we followed 7 LCSX patients (those patient with typical angina, normal angiograms and normal exercise stress tests) to further investigate the utility of stress testing in the diagnosis of CSX. We found in chapter 2 that a normal EST at baseline heralded an excellent prognosis, with the majority of these patients recovering completely from their symptoms by their follow-up visit at visit 2, while the majority of CSX patients continued to suffer with symptoms.

Importantly we found that LCSX patients had significantly lower CRPs than CSX patients, where the EST is required to be positive. CRP was 0.64 [0.35 to 0.81mg/L] vs 1.57 [0.89 to 4.80mg/L]; Kruskal-Wallis Test Stat=15.1, adj. Sig=0.032. LCSX patients were no different to healthy controls in terms of CRP (adj. Sig=1.000). This data is illustrated in Figure 3.9.

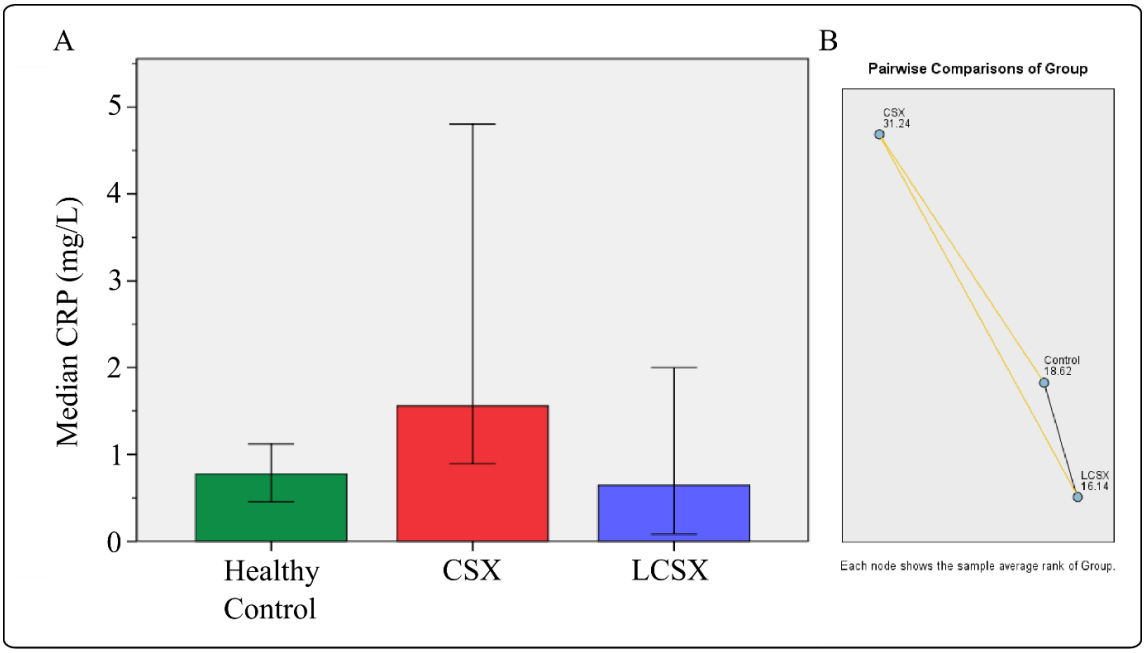


Figure 3.9: A. Median CRPs compared between controls, CSX and LCSX groups. **B.** Kruskal-Wallis pairwise comparison demonstrating the significant difference between CSX and both LCSX and Controls.

Similarly, SAA concentrations were also significantly lower at baseline in the LCSX group compared to CSX (1.42 [0.85 to 2.09mg/L] vs 4.95 [2.32 to 7.51mg/L]; p=0.05) while ICAM-1 levels trended lower by one-way ANOVA (255 ± 21 vs 353 ± 25ng/ml; p=0.065) being significantly lower by t-test (t_{21}=-2.4, p=0.025). Again, there was no difference in terms of inflammatory biomarkers between LCSX and healthy controls. Interestingly, there was no significant difference between LCSX and CSX in terms of VCAM-1.

In all, this hints that the EST, as well as being prognostically important, also selects patients with a distinct immune profile. This fact resembles the differences in CRP concentrations between CSX patients at visit 2 with positive versus negative EST's where the EST again defined a group that differed in CRP concentrations.

3.10 Regression

We examined the ability of baseline biomarkers and clinical parameters to predict those CSX patients who would have ongoing symptoms at follow-up using logistic regression analysis. All pretest assumptions were met. As shown in Chapter 2, baseline physical limitation score was an excellent predictor of follow-up EST positivity. Adding CRP to the model does not improve this predictive power (predicts only 87% of cases v 93% with PLS alone) but remains significant (Nagelkerke R-square of 0.743, p=0.003 for the model). The best model for predicting follow-up EST positivity is baseline Duke treadmill score (DTS) combined with baseline CRP, which predicts 100% of patients with positive EST at follow-up ($p<0.001$) compared to DTS alone, which only predicts 61% of positive cases. CRP is also reasonably good at predicting follow-up symptoms as determined by SAQ being able to predict 75% of people who will have had no or minimal symptoms (defined as an Angina Frequency Score of 100) at follow-up (Nagelkerke R-square of 0.334, p=0.03) and improves the predictive power of baseline PLS alone (together predicting 100% of asymptomatic cases at follow up, $p<0.001$).

3.11 ROC curves

We used Receiver Operating Characteristic curves to assess the ability of the various biomarkers to discriminate CSX patients from the overall cohort. Obviously, an elevated CRP is a non-specific finding but, as can be seen in Fig 3.10, we can select CSX subjects out from the study population quite well using CRP and reasonably well using SAA and ICAM-1. A cut-off concentration of 1mg/L of CRP gave a sensitivity of 71% and a specificity of 72% to detect CSX cases. A higher cut-off of 2mg/L gave near 100% specificity but the sensitivity dropped to under 50%. The overall area under the curve (AUC) for CRP was 0.794, which was better than SAA (0.689) and ICAM-1 (0.685).

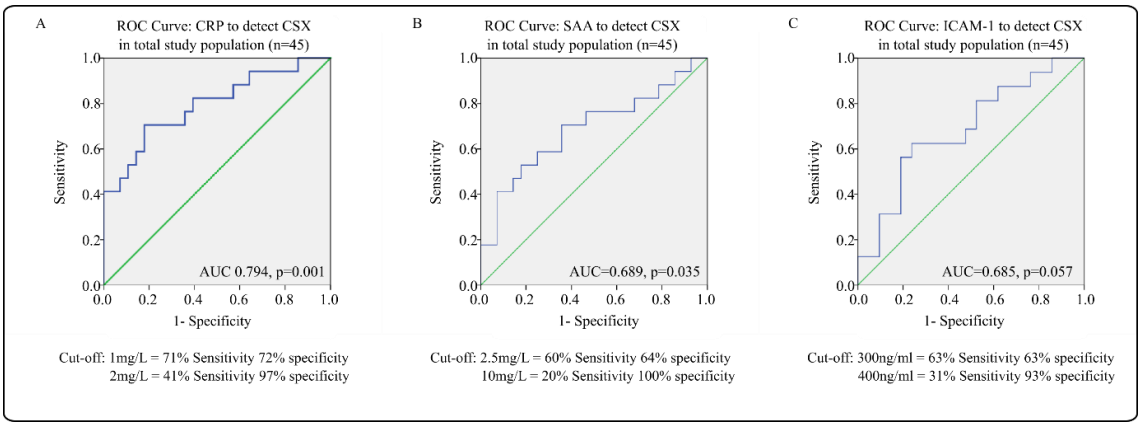


Figure 3.10 ROC curves and landmark cut-off values for CRP (A), SAA (B) and ICAM-1 (C) for the detection of CSX in our cohort.

CRP is even better at discriminating CSX from LCSX with an AUC of 0.815 (p=0.02) with a CRP of 1mg/L giving a 71% sensitivity and 86% specificity and 2mg/L giving 100% specificity and 40% sensitivity. Certainly an elevated CRP is not specific for CSX in a general population but it may have some utility in identifying CSX patients in a cohort of people with angina pectoris and normal coronary arteries.

3.12 Principal component analysis

PCA was performed on the 4 markers of vascular injury and predictably, a two-component solution was found. Bartlett's Test for Sphericity was significant ($p<0.001$), indicating that component analysis was appropriate. Both components met the Kaiser criterion (i.e. had Eigenvalues >1) and were above random data Eigenvalues as determined by parallel analysis. The oblimin rotation was used due to allow for correlation between the components. The solved structure and pattern matrices are shown below in Fig 3.11. Component one relates strongly to the two acute phase reactants while component 2 is focused on the markers of endothelial activation. Our 2 components explain 83% of the variance seen in our original 4 parameters.

Pattern Matrix[a]

	Component	
	1	2
SAA1	.976	
CRP VISIT 1	.867	
VCAM1		.984
ICAM1	.446	.592

Extraction Method: Principal Component Analysis.
Rotation Method: Oblimin with Kaiser Normalization.

a. Rotation converged in 5 iterations.

Structure Matrix

	Component	
	1	2
SAA1	.938	
CRP VISIT 1	.899	.397
VCAM1		.949
ICAM1	.656	.750

Extraction Method: Principal Component Analysis.
Rotation Method: Oblimin with Kaiser Normalization.

Component Score Coefficient Matrix

	Component	
	1	2
CRP VISIT 1	.452	.019
SAA1	.520	-.135
ICAM1	.202	.421
VCAM1	-.106	.746

Extraction Method: Principal Component Analysis.
Rotation Method: Oblimin with Kaiser Normalization.
Component Scores.

Figure 3.11: Output for Principal Component Analysis indicating a 2 component solution

$$FS_1 = 0.452(CRP) + 0.520(SAA) + 0.202(ICAM1) - 0.106(VCAM\text{-}1)$$

$$FS_2 = 0.019(CRP) - 0.135(SAA) + 0.421(ICAM1) + 0.746(VCAM\text{-}1)$$

Thus factor 1 describes their acute phase reactants and factor 2 their vascular inflammation. Factor 1 is significantly different (U=385, n=38, p=0.001) in between the groups while factor 2 just fails to reach significance (p=0.06).

Discussion

3.13 Acute Phase Reactants

To our knowledge this study is the first to prospectively examine a cohort of CSX patients in order to observe the relationship between changes in markers of inflammation and in symptoms. We have replicated the general finding that CRP is elevated in patients with CSX and have shown that this holds true even when patients are followed over time. We have bolstered the idea that CSX patients have chronic low-grade systemic inflammation by also showing for the first time that serum amyloid A is similarly elevated. Of the two acute phase reactants studied, CRP shows more promise in terms of being a state molecule in CSX and even a possible effector molecule in the pathogenesis of this condition.

We found that CRP measurements distinguished a subset of CSX patients whose ESTs became symptomatically and electrically negative at their first follow-up visit. These patients, by definition, could be considered to no longer have CSX and were shown to have developed significantly lower CRP concentrations than those patients who continued to have a positive EST. It is important to note that there was no significant difference between the groups in terms of CRP at initial diagnosis. Additionally, the CRP in the overall CSX cohort fell back towards levels comparable to healthy controls by the end of follow-up as most of the patients had improved in terms of EST parameters and symptoms. These findings would suggest that CRP is a state marker of Cardiac Syndrome X.

We also provided further evidence to suggest that CRP may be linked to the severity of symptoms. This was illustrated by the fact that CRP inversely correlated with time to symptoms on EST (a reproducible marker of symptom severity) at both baseline and at the second visit. CRP also tended to increase as time to ST-depression on EST (an objective measure of disease severity) decreased. Moreover, CRP concentrations fell on average in patients whose symptoms improved while the opposite occurred in patients whose symptoms worsened. It was also higher in patients who had more frequent angina at follow-up. Taken together with Cosin-Sales' data relating CRP to disease activity in patients with chest pain and normal coronary arteries, the case for CRP as a state marker in CSX becomes compelling[27]. This is tempered, however, by the fact that another moderately large study of CSX patients failed to find a correlation between CRP and symptoms in CSX[62].

The relative importance of a positive EST in the diagnosis of CSX has been unclear. In modern diagnostic criteria a positive EST is an imperative for a diagnosis of CSX as it provides objective evidence of possible myocardial ischaemia in patients with typical angina pectoris. By comparing our CSX cohort with a LCSX cohort (where the EST is

normal), we have shown that a baseline positive EST in patients with angina and angiographically normal coronary arteries selects out a population that have higher CRP concentrations and who, incidentally, are more likely to have ongoing symptoms at follow-up. Conversely, a higher baseline CRP had a reasonably good AUC (0.815) to select out CSX patients from LCSX without performing an EST and could be used to diagnose and prognosticate in terms of symptom burden going forward in patients presenting with chest pain and normal coronary arteries.

If CRP is indeed a state marker in CSX, the question becomes one of whether CRP is an innocent bystander or an active player in CSX disease activity. As noted in 3.1.1, CRP has the ability to affect vascular function. CRP certainly has the potential to directly induce endothelial dysfunction through the activation of LOX-1, an endothelial receptor that is also activated by oxidised-LDL and is thought to be involved in the development of atherosclerosis. LOX-1 activation can lead to NFκB activation, increased local inflammation and endothelial apoptosis with reduced endothelium-dependent vascular smooth muscle relaxation [193]. Moreover, CRP has been shown to inhibit endothelial prostacyclin synthesis, to reduce NO bioavailability and to be associated with increased coronary microvascular resistance [174]. (See: Fig 3.12 Panels B,C below).

Given then that CRP can induce the vasomotor dysfunction, a possible cause of symptoms in CSX, what causes the relatively elevated CRP in CSX patients? As noted in chapter 2, the prevalence of hyperlipidaemia in our CSX cohort was high and this is one of the most common causes of vascular inflammation via the formation of oxidised LDL and the activation of local effector cells (see figure 3.13 below). The prevalence of hyperlipidaemia was similar in the comparison control group, however, and the degree of inflammation in the CSX patients was still significantly higher.

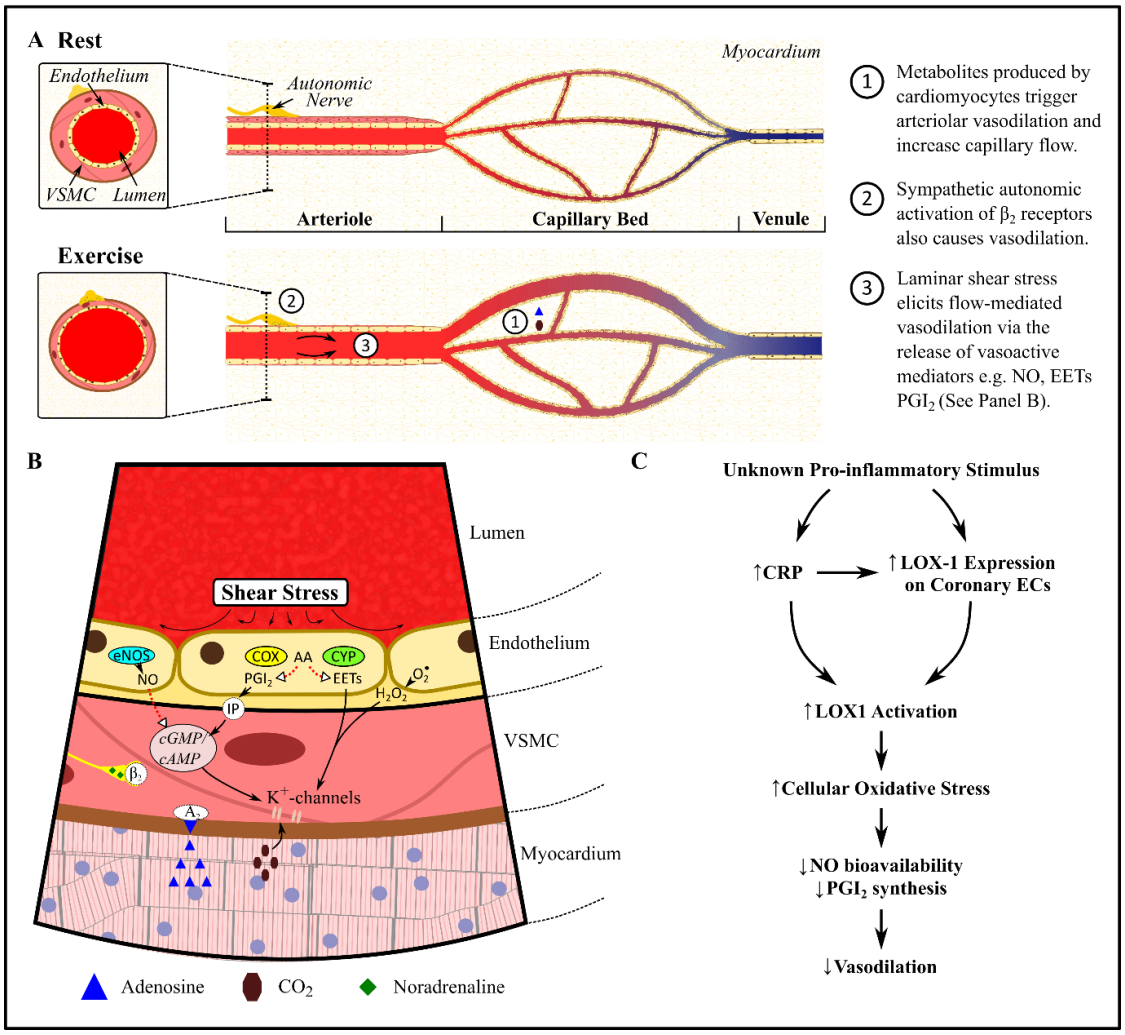


Figure 3.12 Coronary microvascular dilation in response to exercise (believed to be blunted in CSX) and the possible impact of CRP thereon. **A**. Longitudinal cross-section of the microvasculature detailing the arteriole, capillary bed and draining venule in the myocardium. Inset shows a cross-section of the arteriole. During exercise the vascular smooth muscle cells (VSMCs) in the arteriolar walls relax allowing increased luminal diameter and blood flow. **B**. Cross-section of the arteriolar wall showing pathways responsible for vasodilation. Steps that are affected by CRP are delineated by dotted lines with empty triangles. **C**. Diagram showing the possible role of CRP as an effector agent in microvascular dysfunction in CSX. A2- Adenosine receptor 2; AA-Arachidonic Acid; β2- β2 adrenoceptor; COX- Cyclo-oxygenase; CYP- Cytochrome P450; EC- Endothelial Cell; EET- Epoxyeicosatrienoic acid; eNOS-Endothelial Nitric Oxide Synthase; IP- Prostacyclin Receptor; LOX1- Lectin-type Oxidised LDL Receptor 1; PGI2- Prostacyclin.

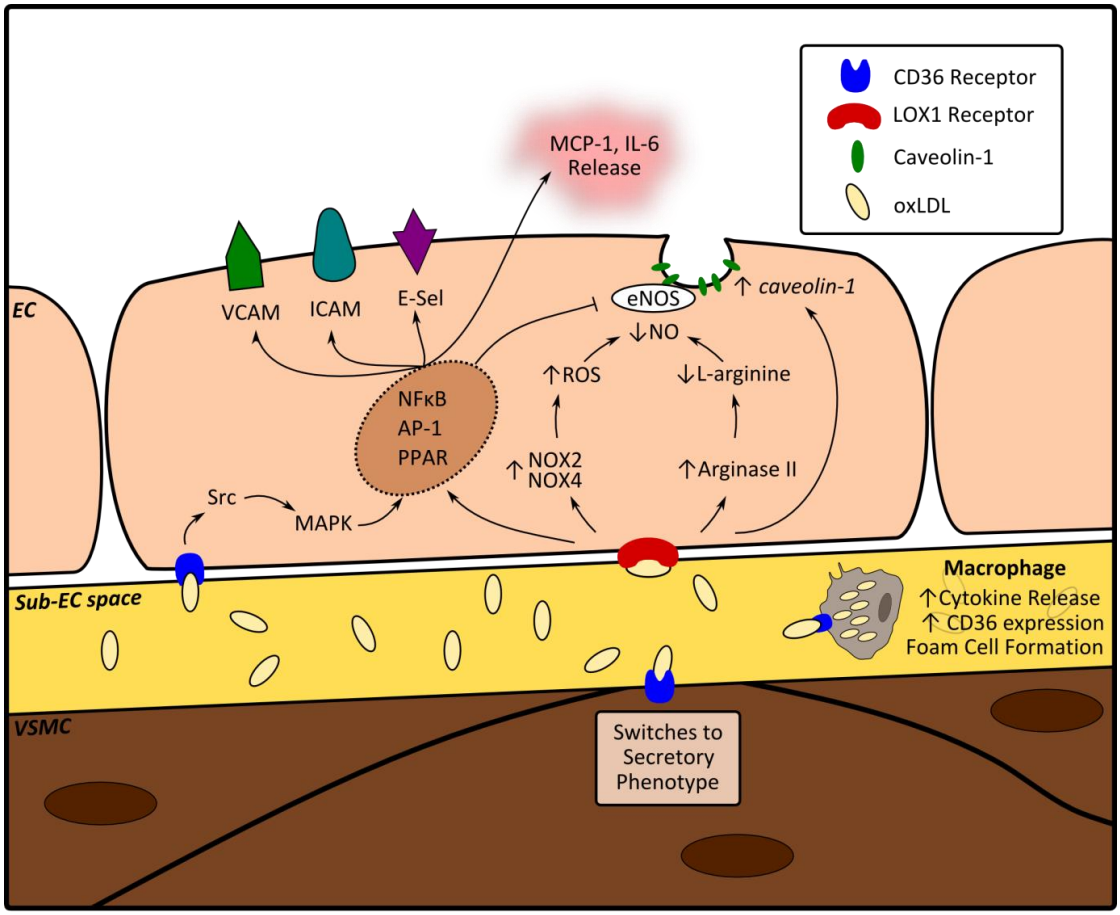


Figure 3.13: Effects of oxLDL on the vascular wall. When LDL becomes trapped in the subendothelial space it becomes oxidised by local chemicals. The oxLDL then binds to CD36 receptors on nearby cells. It triggers phenotype switching in VSMCs, increasing cytokine release and reducing contractile responsiveness. It activates local tissue macrophages, upregulating their CD36 expression, thereby increasing their uptake of oxLDL forming foam cells, an important source of pro-inflammatory cytokines. ECs are activated via CD36 and LOX1 pathways. Adhesion molecule expression, cytokine release and oxidative stress are all increased while NO bioavailability is reduced.

AP-1 -Activator Protein-1; EC- Endothelial Cell; eNOS- Endothelial Nitric Oxide Synthase; E-Sel- E-Selectin; IL- Interleukin; MAPK- Mitogen Activated Protein Kinase; MCP- Monocyte Chemoattractant Protein; NFκB -Nuclear Factor Kappa-light-chain-enhancer of Activated B Cells; NO- Nitric Oxide; NOX- NADPH Oxidase; ROS- Reactive Oxygen Species; Src- Src Family Kinase; VSMC- Vascular Smooth Muscle Cell.

It should also be noted that CRP can be elevated in purely stress-related conditions. Studies have attempted to identify possible infective pathogens as triggers for inflammation in CSX (such as H. Pylori) without success[62]. Diet, demographics and subclinical infections may also trigger minor elevations in CRP[194]. The ultimate cause remains elusive and it may be that several separate causes exist and trigger an

elevation of CRP with the eventual result of endothelial dysfunction and symptoms in susceptible individuals (as many patients with much higher CRP levels do not suffer from microvascular angina). It must also be stressed, however, that CSX patients only have minor elevations in CRP concentrations and indeed they have CRP concentration within the normal range, despite being significantly greater than that seen in healthy controls. Minor CRP elevations are commonly seen and may just represent tissue injury from multiple vascular insults rather than being a causative agent in the pathogenesis of CSX.

On balance, we believe that CRP fulfils several of the Bradford-Hill criteria for causal association with the symptoms of CSX. CRP is consistently elevated in CSX, there appears to be a dose-response relationship between symptoms and CRP, the condition reverses when CRP falls and there is a biologically plausible pathway for its action. Serum Amyloid A also has the potential to affect endothelial function and so the demonstration that it is elevated in CSX may have similar relevance to that of CRP. Unlike CRP, however, we could find no relationship in our study population between SAA levels and markers of disease activity other than the observation that as time passed the SAA levels in the overall improved CSX population fell back towards normal.

3.14 Endothelial Activation

As well as showing that our patients had general inflammation, we were able to illustrate that they had evidence of endothelial activation. Both ICAM-1 and VCAM-1 were elevated in our patients with CSX compared with healthy controls and this elevation was also seen through the period of follow-up. There was a correlation between acute phase reactants and adhesion molecules indicating that they may be responding to the same stimulus, that the endothelial activation is dependent on general inflammation or that the endothelial activation is driving general inflammation. Unfortunately, apart from demonstrating increased levels of markers of endothelial

activation in CSX, we were unable to demonstrate a close relationship between concentrations of VCAM or ICAM and markers of disease activity. This was probably due to the small size of our study population.

The presence of endothelial activation in itself is, however, a very important finding in CSX as it supports the notion of microvascular dysfunction being integral to the pathogenesis of the condition. Upregulation of adhesion molecule expression on the endothelium indicates an activated state. As described further in chapter 1.4.3, endothelial activation is usually in response to an extrinsic insult such as oxidative stress, hypertension, dyslipidaemia or an inflammatory stimulus. As well as increasing the recruitment of inflammatory cells to the local microenvironment and being pro-thrombotic, endothelial activation reduces the bioavailability of many endothelial-derived vasodilating substances such as prostacyclin and nitric oxide. It is known to be associated with the blunting of flow-mediated dilation of arteries and with diminished coronary flow reserve[191]. Impaired coronary microvascular reactivity due to endothelial activation is the most common theory in most papers regarding CSX pathogenesis and our study supports this hypothesis.

3.15 Limitations

The small number of patients in this study certainly under-powered it for some of the parameters and limited our ability to measure significant differences. Numbers were limited by the availability of patients at the recruitment centres during the recruitment period. Consecutive appropriate patients were enrolled but the overall incidence of CSX in Ireland was much lower than anticipated. Despite this, the fact that the study included a repeated-measures design allowed us to compare our sample with the healthy controls over several time points. The fact that the main significant findings were consistently found is reassuring. Also the stringent inclusion/exclusion criteria

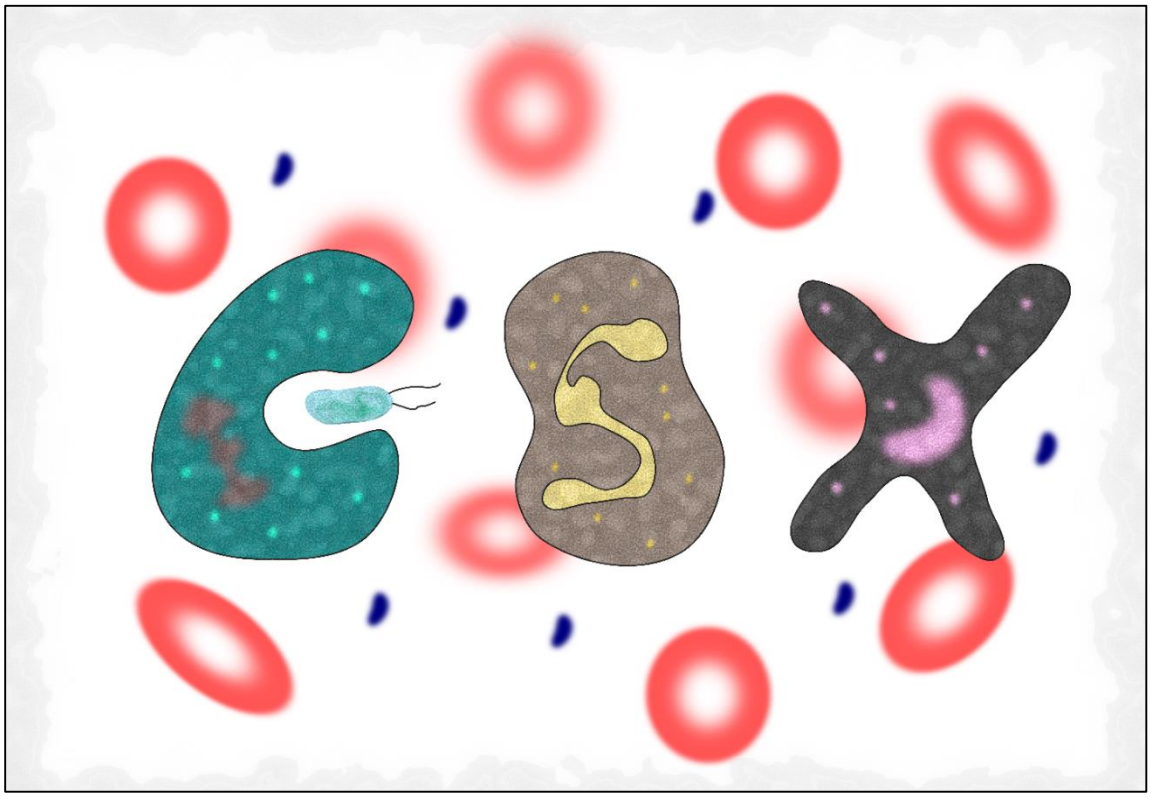
were designed to ensure that every patient meeting them had true CSX. A study with a larger sample group would help to confirm the findings of this study.

Exercise stress testing is an imperfect tool for detecting possible ischaemia with the possibility of recording false positive or negative results. Ideally this type of study could be performed using cardiac magnetic resonance imaging to more definitively demonstrate ischaemia. Changes in the degree of hypoperfusion over time as detected by CMR could be correlated with changes in inflammatory biomarkers. A further approach would be to diagnose the patients using coronary reactivity testing, an invasive approach but one likely to become the gold standard in diagnosing microvascular angina in the future. The advantages of EST, however, include its low cost, safety, wide availability and that it also allows the objective assessment of the patient's functional status.

It would have been interesting to have included a small cohort of patients with obstructive coronary artery disease in our study as this would have allowed us to compare CRP concentrations in more symptomatic patients with that of our CSX population. Similarly, it would have been useful to include another cohort of patients with atypical chest pain, a positive EST and normal coronary angiogram to compare so that we could see which of the diagnostic criteria are important in CSX. Certainly our data suggests that the EST is of great importance in the diagnosis of CSX as it defines an entirely distinct population in terms of markers of vascular injury and prognosis. The answer to the question, "how important is the nature of chest pain in the diagnosis of Cardiac Syndrome X?" has not been answered. It may be that the nature of the chest pain is not as critical. Unfortunately, time and funding constraints prevented the pursuit of an answer to this question.

Conclusions

Our small study demonstrates that CSX patients have mild systemic inflammation and evidence of endothelial activation. Both of these facts support the notion of microvascular endothelial dysfunction in CSX. This is the first time that SAA has been shown to be elevated in CSX. CRP is also identified as a feature of CSX and our study adds credence to the idea that CRP is an active mediator of symptoms in Cardiac Syndrome X. We demonstrate a novel finding that CRP remains significantly higher in CSX patients who remain symptomatic than in either healthy controls or CSX patients in whom symptoms abate over a short period of follow-up. In addition, we show that, in our patients, symptom severity as measured by EST performance correlates significantly with contemporaneous CRP concentrations. We also highlight the importance of an electrically positive stress test in the diagnosis of CSX. This test distinguishes CSX patients from other patients with angina and normal coronary arteries. These two populations are shown to be distinct in terms of acute phase reactants, markers of endothelial activity and prognosis. We also found that CSX patients with lower CRP concentrations, better physical limitation scores and better Duke Treadmill Scores are more likely to have early resolution of symptoms than those patients with more symptoms and a greater burden of inflammation. The basis for the pro-inflammatory state in CSX remains unknown.



Chapter 4: Cytokine Expression in CSX

Introduction

4.1 Chapter Overview

Having established that our CSX cohort has mild baseline elevation of acute phase reactants and markers of endothelial activation, thereby conforming with the results of the majority of reported CSX studies, the next step was to evaluate the potential mechanism of immune activation in CSX with particular interest in the cause for the persistently elevated acute phase reactants. APRs are just that, an acute response to an inflammatory stimulus. Their production is mainly regulated by cytokines and a handful of studies have demonstrated elevated cytokines, including TNFα, IL-6 and IL-10, in CSX populations[63,155,195]. Indeed, higher levels of these cytokines have been shown to associate with reduced myocardial perfusion on cardiac MRI[188]. There is some evidence that peripheral mononuclear cells (PMCs), a key source of pro-inflammatory cytokines, are activated in CSX and contribute to oxidative stress and endothelial dysfunction in CSX. The role of cytokines in the persistent inflammatory response seen in CSX has not been clearly elucidated. Given also that half of our population improved over time, the changes in cytokines in these patients would also be of interest as one may potentially infer pathogenesis from the differential cytokine profile displayed in these CSX patient sub-groups.

4.2 Cytokines

Cytokines are soluble polypeptides that play a major role in the intercellular communication that orchestrates the immune response. Many cell types, from lymphocytes to epithelial cells, are capable of releasing cytokines in order to communicate in a paracrine, autocrine or systemic humoral capacity. Each cell type has a particular repertoire of cytokines that they are capable of producing in response to a stimulus such as tissue injury, oxidative stress, ischaemia, shear stress and other cytokines. Indeed, cytokines frequently regulate the production of other cytokines in a complicated web of interactions. Cytokines display pleiotropy and redundancy,

meaning that each cytokine has varied effects and that several cytokines have the same effect. They may have local and systemic repercussions and cause these through interactions with receptors resulting in alterations in gene transcription.

4.2.1 Classification conventions

There is no standardised method for the classification of cytokines but they may usefully be classified by several different systems. For example, one may examine the cytokines that typify an innate immune response (mediated mainly through complement, physical barriers and phagocytes such as macrophages and neutrophils) or that are more typical of an adaptive immune response (i.e. one driven mainly by T and B-lymphocytes). Equally validly, one could examine the cytokines seen in an acute inflammatory response as opposed to a chronic one. The cell of origin could also be used to distinguish cytokines with lymphokines being produced by lymphocytes and monokines being produced by macrophages and monocytes. A well-used classification describes type 1 and type 2 cytokines with type 1 referring to cytokines mainly produced by T_h1 Helper T-lymphocytes to promote a cellular immune response against an extracellular pathogen and type 2 identifying T_h2-cell cytokines activating a humoral response against intracellular agents. These two pathways mutually inhibit each other. The simplest concept of classification involves the subdivision of cytokines into pro- and anti-inflammatory substances but this is rendered complex by the aforementioned pleiotropy of cytokines with some being pro-inflammatory in certain situations and anti-inflammatory in others. In short there is no perfect way to classify cytokines but for our purposes we broadly classify our cytokines into pro-inflammatory and anti-inflammatory with further reference to type 1 and type 2 cytokines. The various categories and the appropriate cytokines are shown below in table 4.1. As one may appreciate, there is considerable overlap between the classification systems.

Classification Parameter		Main cytokines
Timing	Acute inflammation	TNFα, IL-1, IL-5, IL-6, IL-8, IL-11, IL-17, GM-CSF
	Chronic Inflammation	*Humoral* IL-4, IL-5, IL-6, IL-7, IL-13; *Cellular* IL-1, IL-2, IL-3, IL-4, IL-7, IL-9, IL-10, IL-12, IFNγ, TNFα
System	Innate Immunity	TNFα, IL-1, IL-12, IFNα
	Adaptive Immunity	IL-2, IL-4, IL-5, IL-17, IFNγ
Cell of Origin	Monokine	IL-1, TNFα, IFNα/β
	Lymphokine	IL-2, IL-3, IL-4, IL-5, IL-6, IFNγ, GM-CSF
Response Type	Type 1	IL-2, IL-10, IFNγ, TNFβ
	Type 2	IL-4, IL-5, IL-6, IL-9, IL-10, IL-13
Effect Type	Anti-inflammatory	IL-4, (IL-6), IL-10, IL-13, TGFβ
	Pro-inflammatory	IL-1, IL-6, TNFα, IFNγ

Table 4.1: Classification systems for cytokines.

4.2.2 Principal Vascular Cytokines

There are many different cytokines but for the purposes of this chapter we shall limit our discussion to the cytokines with most relevance to vascular function and describe their cardiovascular impacts.

Tumour necrosis factor alpha ***(TNFα)*** is a mediator of the acute inflammatory response. It is primarily released by activated mononuclear phagocytes, principally monocytes and macrophages, but may also be released by neutrophils, vascular smooth muscle cells and even endothelial cells[196]. These cells may be activated by extrinsic pathogens or other cytokines such as interferon gamma (IFNγ). TNF α activates NFκB and upregulates the expression of the adhesion molecules VCAM-1 and ICAM-1. It also increases the production of reactive oxygen species (ROS) via increased NADPH oxidase

activity thereby increasing oxidative stress. Furthermore, TNF has been shown to uncouple the activity of eNOS and to enhance AT_1R receptor expression and endothelin production, thereby reducing vasorelaxation. Other effects include the induction of apoptosis in endothelial cells by high plasma concentrations while TNFα also promotes the production of acute phase reactants such as CRP and SAA from the liver. ***IL-1*** is functionally very similar to TNFα, may be produced by endothelial cells and induces similar vascular effects.

Interferon-gamma ***(IFNγ)*** is the typical cytokine produced by activated T_h1 Helper T-lymphocytes and NK cells. Its primary function is to activate macrophages, causing them to release cytokines and upregulating their ROS-producing machinery thereby equipping them to deal with extracellular pathogens. Apart from augmenting the local vascular inflammatory response, IFN has also been shown to upregulate endothelin activity in the vasculature and to assist TNF-induced expression of adhesion molecules on endothelial cells. It also stimulates the production of superoxide radicals in endothelial cells themselves[197]. ***IL-6*** is released by activated T-cells, macrophages, VSMCs and endothelial cells and is a potent stimulus for hepatic production of acute phase reactants. It is produced by adventitial monocytes as part of vascular inflammation in response to Angiotensin II, cytokines (such as IL-1 and TNFα), oxidative stress and vascular injury and, like hsCRP, has been shown to independently predict cardiovascular risk. It upregulates Angiotensin II Type-1 receptor (AT_1R) gene expression and is implicated in the regulation of oxidative stress in endothelial cells[198]. IL-6 levels have been seen to negatively correlate with endothelium-dependent vasodilation[199]. ***IL-8*** is the main chemokine responsible for the recruitment of phagocytes to the sub-endothelial space in atherosclerotic plaques and is released by macrophages and monocytes. ***IL-5*** is an important type 2 cytokine released by T_h2 CD4+ T-cells and mast cells. It stimulates eosinophil activation and the proliferation of B-cells.

Finally, ***IL-10*** is the other side of the coin. It is the main anti-inflammatory cytokine and is an inhibitor of activated macrophages. It generally opposes the actions of TNFα and reduces TNF release from macrophages. It may be released by monocytes, Lymphocytes and epithelial cells. It has been shown to reduce oxidative stress by reducing iNOS and cytokine release by T-cells while also playing a role in anergy induction. More interestingly it has been shown to inhibit the deleterious effects of angiotensin II, diabetes and ageing on vascular cells, preventing oxidative stress and vascular dysfunction[200-202]. It generally preserves normal vascular function through the inhibition of iNOS with the consequent reduction of oxidative stress.

4.2.3 Cytokines in Cardiovascular Disease

4.2.3.1 Ischaemic Heart Disease

Inflammation has been implicated in several important cardiovascular conditions. The best described of these is atherosclerosis, the process of blood vessel wall injury with the initiation of a local pro-inflammatory microenvironment followed by the alteration of local cytoarchitecture with resultant remodelling of the vessel wall itself. A primary driver of this inflammation is believed to be oxidised Low Density Lipoprotein (LDL), which provides a potent pro-inflammatory stimulus in susceptible areas of intima. These areas are generally regions of turbulent or disrupted blood flow where the usual laminar shear stress is perturbed. The endothelium responds to oscillatory shear stress by reducing Kruppel-like Factor-2 (KLF-2) activity and allowing the unfettered activation of NFκB, a transcription factor that activates several pro-inflammatory cascades. This switches the endothelium to its dysfunctional and activated phenotype, with the increased expression of cellular adhesion molecules as well as increased permeability and release of chemokines. This in turn promotes the recruitment of monocytes to the vessel wall where they interact with local LDL particles, which have also seeped through the disrupted endothelial barrier, by the activity of scavenger receptors. The monocytes become activated macrophages and release TNFα, IL-6 and IL-1 as well as increasing local reactive oxygen species production through the upregulation of NADPH

oxidase activity. These pro-inflammatory cytokines recruit T-cells and more macrophages to the locality as well as triggering phenotype switching in local VSMCs and further potentiating endothelial activation. T-cells attracted by the local inflammation are typically of the T_h1 subtype. These respond to oxLDL and heat shock proteins to release IFNγ and more TNFα, thereby further stimulating TNFα release from local monocytes and the vicious cycle continues with local inflammation, endothelial activation, VSMC apoptosis and matrix metalloproteinase production expression with tissue breakdown. The ultimate result of severe atherosclerosis is myocardial infarction and this has been repeatedly shown to be associated with large elevations in the pro-inflammatory cytokines TNFα, IL-6 and IL-1[203].

4.2.3.2 Vasospastic Conditions

Inflammation has also been observed in several disorders characterised by altered blood vessel tone with certain cytokines believed to predispose blood vessels to undergo spasm. The most obvious example of this is Prinzmetal angina, a condition in which the epicardial coronary arteries go into transient occlusive spasm with resulting typical angina pectoris and associated ST-elevation on ECG. Another extreme example of this is in Takotsubo cardiomyopathy where catecholamines cause such significant vasospasm that a myocardial infarction supervenes. Similarly, allergic immune activation may also lead to acute coronary syndrome in the relatively rare Kounis Syndrome where mast cell activation leads to coronary vasospasm[204]. In these conditions, pro-inflammatory cytokines such as TNF, IL-6 and IL-1β are associated with increased tendency towards acute epicardial coronary arterial spasm and may be described as spasmogenic. Inflammatory cytokines are known to sensitise blood vessels to vasoconstrictor substances through the upregulation of Angiotensin (AT_{II}) and endothelin receptor (ET-1) upregulation. Experimentally, predisposition to spasm may be induced through the application of IL-1β onto in vitro arteries[205]. CRP and IL-6 have also been shown to be elevated in patients with vasospastic angina and levels have appeared to correlate with disease severity[206,207].

4.2.3.3 Heart Failure

Cytokines are also elevated in both traditional heart failure (with a reduced ejection fraction) and the modern epidemic of heart failure with preserved ejection fraction (HFPEF). Studies have shown that HFPEF is associated with a pro-inflammatory phenotype with increased TNFα, IL-1β and IL-6[208]. Simple hypertension, the main cause of HFPEF, may also be associated with raised TNFα and IL-6, possibly through the effects of the angiotensin and sympathetic nervous systems[209].

4.2.3.4 Cardiac Syndrome X

There have been a handful of studies that have evaluated the role of cytokines in CSX and these have produced conflicting results. The most recent and largest of these studies showed an elevation of IL-6 and IL-10 in 111 CSX patients. Strangely there was no elevation of CRP seen in these patients and plasma levels of TNFα were lower than that seen in healthy controls[195]. Other smaller studies, however, have shown elevation of TNFα and IL-6 in CSX patients[63,155]. To date there is no evidence for IFNγ elevation but increased IFNγ receptor subunit expression has been observed in Peripheral Blood Mononuclear Cells (PBMCs) in CSX[210].

4.3 Chapter Objectives

The presence of inflammation in CSX is generally accepted. The ultimate cause of this inflammation, however, remains obscured. The analysis of the cytokine profile in CSX may allow one to infer the likely cell types responsible for the persistence of inflammation and therefore lead to possible explanation as to the causes of the immune activation ab initio. As we have seen in chapter 3, our CSX cohort has evidence of chronically raised APRs with mild resolution of inflammation over time. It will be interesting to see the concurrent changes in cytokine expression that follow these alterations in acute phase reactants over time. The aims of this chapter are as follows:

1. We aim to **investigate the baseline cytokine profile** in our CSX patients. We will attempt to identify a pattern in this profile and hypothesise as to the likely cells of origin for the expressed cytokines. For example, IFNγ may indicate the involvement of T_h1 cells, while TNFα may implicate mononuclear phagocytes etc.
2. Having established the nature and magnitude of the cytokine expression in CSX we will attempt to **correlate these with the degree of expression of acute phase proteins** and adhesion molecules as elucidated in chapter 3.
3. We will also **investigate if the observed symptoms are greater in patients with greater cytokine expression**. In essence we will try to correlate cytokine concentrations with symptoms and objective EST findings.
4. Knowing that some of our cohort improved symptomatically over time, we will investigate the **differential expression of cytokines** in these patients with the hope that we may see a discernible pattern that may implicate a particular cytokine in the causation of symptoms in CSX.
5. We finally hope to make appropriate inferences from the data provided to **characterise the inflammation** seen in CSX. Is it chronic in nature? Is the innate or adaptive immune system implicated? Is the immune response primarily cellular or humoral?

Methods

4.4 Participants

Participants from the original study cohort described in chapter 2 also comprised the study population for this chapter[211]. Seventeen symptomatic CSX patients were identified and enrolled from the catheterisation lab in Cork University Hospital while 21 age- and sex-matched controls were also identified. The 7 LCSX patients were again considered. Study participants were between 42-69 years of age and were predominantly female. Exclusion criteria included the use of anti-inflammatories (including NSAIDs, steroids etc.) in the month prior to enrolment or the presence of a

systemic inflammatory condition (such as known renal or liver disease, connective tissue disease or active infection). CSX and LCSX patients were followed at visit 2 (at 8.2 ± 0.7 months) and CSX patients were followed at a further visit 3 (at 16.8 ± 0.7 months). The study protocol was approved by the local research ethics committee and all participants gave full informed consent.

4.5 Investigations

Seattle angina questionnaires (SAQ), Cohen Perceived Stress Scale (PSS-10) and Brugha's List of Threatening Experiences (LTE-Q) were administered as outlined in Chapter 2.3. Also, Exercise Stress Testing (EST) and venesection were performed as previously outlined.

4.6 Measurement of Plasma Cytokines

Levels of IFNγ, IL-2, IL-4, IL-5, IL-10, IL-12p70 and IL-13 were assessed using the commercially available high-sensitivity Mesoscale Discovery (Rockville, MD, USA) Human T_h1/T_h2 7-plex Ultra-Sensitive Kit, which employs electrochemiluminescence to allow the detection of Type 1 and Type 2 Cytokines. A custom Mesoscale Discovery Human Pro-Inflammatory Panel Kit was also used to determine quantities of IFNγ, IL-1β, IL-6, IL-8 and TNFα. Both kits employed similar standard protocols. Samples were run in duplicate and included standard calibrator dilutions to establish standard curves. No sample dilutions were performed based on past experience with these particular plates. Results with a co-efficient of variation <25% were deemed acceptable for inclusion in analysis. The median lower limits of are shown in table 4.2 in section 4.8 below.

4.7 Data analysis

Data was analysed using SPSS v 20 for windows (IBM, Armonk, NY, USA). Data was expressed as mean ± SEM if it was normally distributed. Some data (such as IL-6 concentrations) were strongly positively skewed but were normalised with a simple reciprocal transformation. Some data (such as IL-10 concentrations) could not be normalised and are reported as median [IQR]. Means were compared using student t-tests or one-way ANOVA with Bonferroni correction where appropriate. Non-parametric data were compared using the Mann-Whitney U-test or Kruskal-Wallis test as appropriate. Repeated measures were compared using paired sample T-test or repeated measures ANOVA as specified for normally distributed data and Related Samples Friedman's Two-Way Analysis of Variance by rank for skewed data. Correlations were investigated using Spearman's Rank Correlation. Missing data was only imputed if >15% of sample data were missing and this only occurred for IL-10. All p values are two-tailed and calculated to 0.05 significance levels.

Results

4.8 Data Quality

Table 4.2 demonstrates the overall quality of the data obtained from the analyses. It becomes apparent that the MSD TH1/TH2 plates gave a poor overall quality of data. Only IL-5 and IL-10 gave useful data from these plates. The remaining cytokines appeared to be present in levels below the LLOD (see figure 4.1). The pro-inflammatory panel on the other hand, gave excellent data throughout.

Cytokine	Median LLOD (pg/ml)	Average CV observed (%)	Missing Cases (below LLOD)	Excluded Cases (CV>25%)	Total Cases Analysed
IFNγ[1]	0.55	33.7±4.7	20	8	**17**
IL-2	0.43	67±10.5	35	1	**9**
IL-4	0.16	None detected	45	0	**0**
IL-5	0.07	13.9±2.7	1	4	**40**
IL-10	0.51	14.9±2.5	6	10	**29**
IL-12p70	2.0	22.0±5.6	30	4	**11**
IL-13	1.3	28±7.3	34	4	**7**
IL-1β	0.04	7.3±1.2	0	2	**43**
IFNγ[2]	0.20	7.2±1.2	0	0	**45**
IL-6	0.06	8.1±2.4	0	1	**44**
IL-8	0.04	5.1±0.9	0	1	**44**
TNFα	0.04	6.2±0.6	0	0	**45**

Table 4.2 QC of Cytokine Measurement. CV- Co-efficient of Variation. LLOD- Lower Limit of Detection.

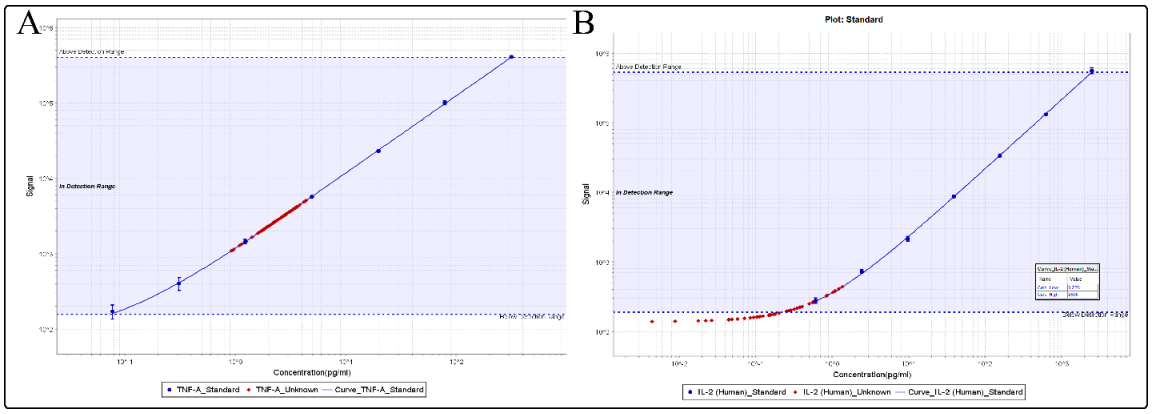


Figure 4.1: A. Plot showing the Standard Curve (blue) and sample signals (red dots) for TNFα on the Pro-Inflammatory Plate. Note that all of the samples fall on the linear part of the calibration curve in the detection range. **B.** Similar plot for IL-2 on the TH1/TH2 Plate. Note that most samples fall below the LLOD or do not fall on the linear part of the standard curve.

Only IL-5 and IL-10 data was salvageable from the T_h1/T_h2 plates. As can be seen in table 4.2, 89% of baseline IL-5 data and 66% of baseline IL-10 data was in the detection

range of the assay with a CV of <25% between duplicate samples. Between the 3 time points, 15.9% of all values were missing. Missing data was treated as missing at random following a missing value pattern analysis in SPSS (see figure 4.2. below). Missing values were imputed using multiple imputation (linear regression method).

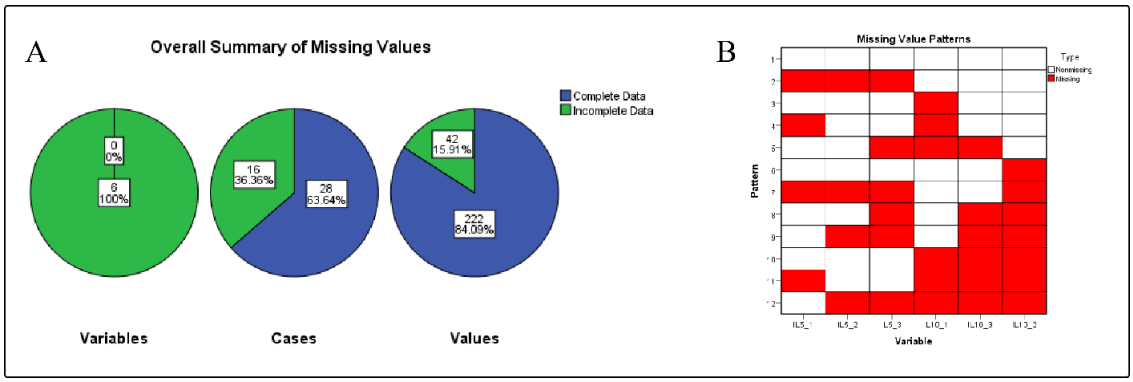


Figure 4.2: Missing Data Analysis prior to multiple imputation. A. Pie charts showing the number of cases with at least one missing value and also the overall number of missing values. B. Missing Value Pattern grid showing no monotonic pattern in our dataset.

4.9 Pro-inflammatory cytokines

4.9.1 Baseline

Baseline IFNγ was elevated in CSX patients (8.7 ± 1.3 vs. 5.8 ± 0.5; mean difference 3.0, 95% CI 0.39 to 5.6 pg/ml, p=0.04). Interestingly, IL-1β was no different between groups and if anything trended lower CSX patients (0.25 ± 0.02 vs. 0.19 ±0.03 pg/ml, MWU 121, p=0.15). IL-6 was significantly higher in the CSX population (1.50[1.01 to 2.05] vs. 0.60 [0.46 to 0.74]pg/ml). The reciprocal transformation gave a significant t-test value of t_{36}=-5.3, p<0.001. There was no significant difference in IL-8 levels at baseline (p=0.642). TNFα levels trended towards being significantly higher at baseline (2.49 ± 0.2 vs. 2.08 ± 0.11 pg/ml; t_{36}=1.9, p=0.06).

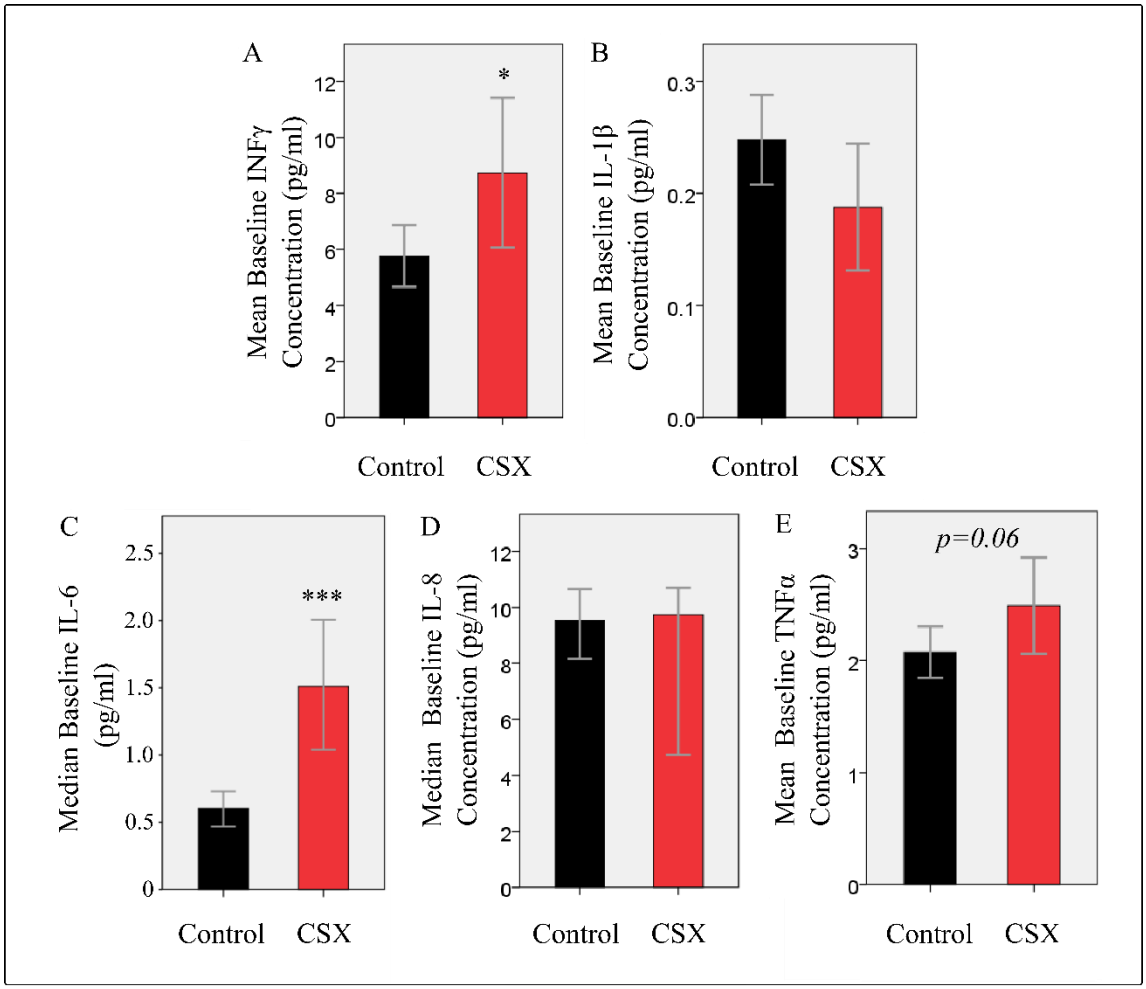


Figure 4.3: Baseline pro-inflammatory cytokines in healthy controls and CSX patients.

4.9.2 Follow-up

Broadly speaking, IFNγ concentrations were persistently elevated in the CSX group until the end of follow-up. It remained significantly higher at visit 2 (8.1 [4.0 to 13.0] vs. 5.0 [4.0 to 7.5] pg/ml; H=18.6, adjusted p=0.039) but only trended so at visit 3 (p=0.07). There was no significant difference between CSX patients and controls in terms of IL-1β at follow-up, although the concentration of this cytokine had increased significantly (paired sample t-test t_{16}=3.1, p=0.007) in the CSX cohort from visit 1 to visit 2 and this remained the case at visit 3 (W=117, p=0.01). Perhaps the most striking change in cytokines was the precipitous drop in IL-6 concentrations to control levels at the first follow-up visit (adj p=1.0 between controls and CSX group at visit 2 and 3; Related

Samples Friedman's Two-Way Analysis of Variance by rank Q=18.875, adjusted p<0.001 for CSX baseline to visit 2 and adjusted p=0.002 for Baseline CSX to visit 3). IL-8 concentrations were similar across all time periods.

Despite the cohort becoming less symptomatic, TNFα concentrations increased over time. A repeated measures ANOVA with a Greenhouse-Geisser correction determined that the mean concentration differed significantly between the 3 time points (F(1.53,22.98)=4.23, p=0.036) but post-hoc testing with Bonferroni correction showed only a trend towards a higher TNFα at visit 3 (p=0.08). Follow-up TNFα in the CSX group did differ from control TNFα at both time points, however (mean difference 0.69 ± 0.24pg/ml, p=0.042 at visit 2; mean difference 0.79 ± 0.25pg/ml, p=0.015 at visit 3).

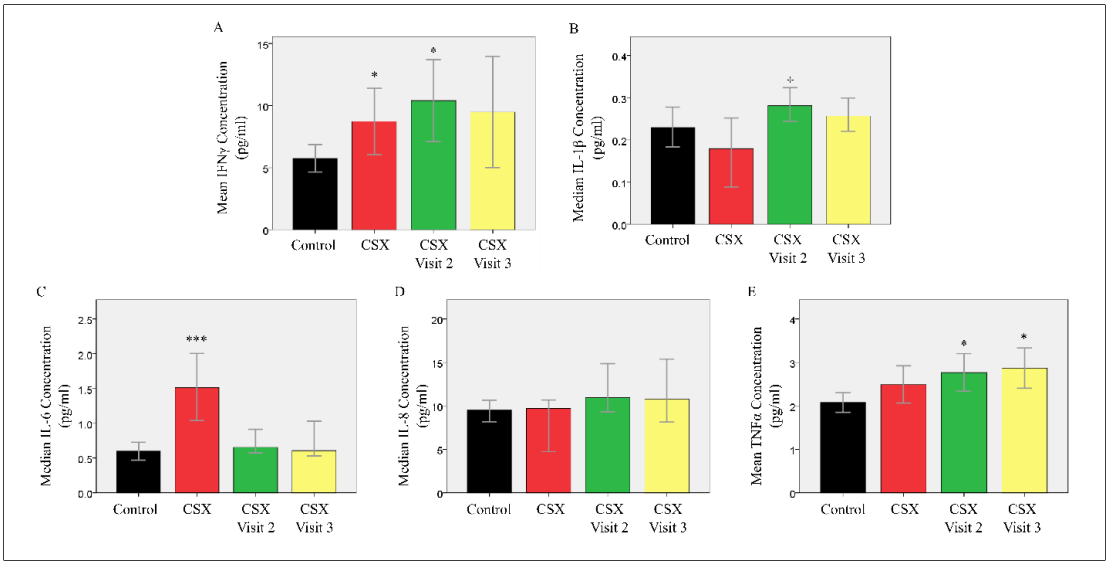


Figure 4.4: Pro-inflammatory cytokines at follow-up visits in the CSX cohort. Asterisks refer to significance versus healthy controls with correction for repeated measures. The cross refers to comparison with the baseline CSX value.

4.9.3 Cytokines by Clinical Outcome

Given that our sample size was small and that the overall changes in absolute cytokine concentrations were low, it is not surprising that there was no consistently significant

signal of cytokine change in patients who improved in terms of disease severity. There was a general trend towards greater drops in IL-6 concentrations in patients whose symptoms had improved but these did not reach statistical significance (p-values 0.08-0.12) and were influenced by an extreme outlier value as shown in figure 4.5 below.

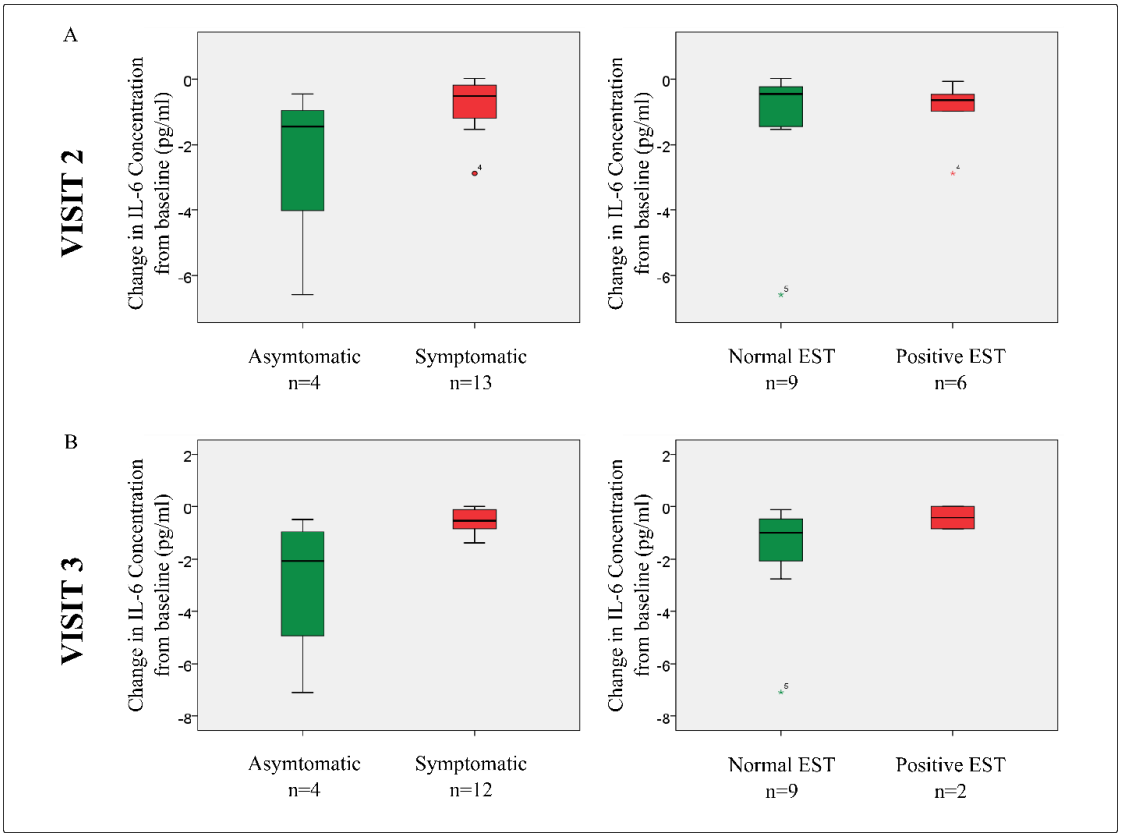


Figure 4.5: Changes in IL-6 concentration from baseline at Visit 2 (Panel A) and Visit 3 (Panel B) in patients who symptomatically improved and those whose EST normalised.

4.10 Type 2 Cytokines

4.10.1 Baseline IL-5 and IL-10

There was no significant difference in baseline IL-5 or IL-10 between the two groups (U=156, p=0.670; and U=147, p=0.497 respectively). Both datasets were strongly positively skewed and no simple transformation could normalise the data distribution. IL-5 concentrations were 1.11 [0.44 to 1.58 pg/ml] in the control group and 1.03 [0.75

to 3.1 pg/ml] in the CSX group while IL-10 concentrations were 2.23 [1.76 to 3.71 pg/ml] in the Control group and 2.97 [1.45 to 4.74 pg/ml] in the CSX group.

4.10.2 Follow-up IL-5 and IL-10

There was no significant change in IL-5 or IL-10 at follow-up. Median IL-10 had increased to 3.6 [2.6 to 5.8 pg/ml] but this was a non-significant change from baseline (p=0.13) while IL-5 also increased non-significantly to 1.6 [0.8 to 3.6] by the end of follow-up.

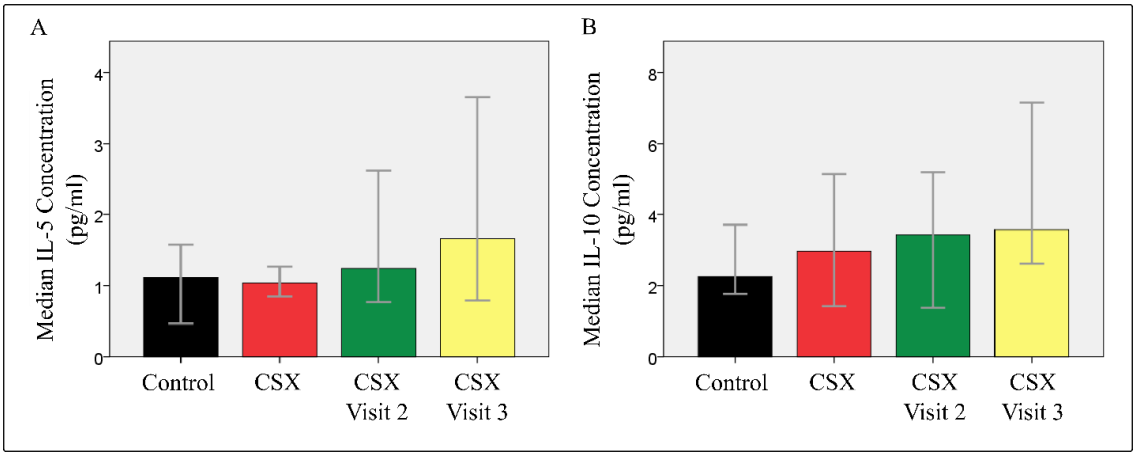


Figure 4.6: A. Bar chart depicting the longitudinal results of IL-5 concentrations in CSX patients compared with healthy controls. **B**. Bar chart illustrating IL-10 concentrations over time in the same populations.

4.10.3 IL-5, IL-10 and Clinical Outcomes

There were no significant differences in IL-5 or IL-10 concentrations in those CSX patients that improved in terms of symptoms or EST findings and those that did not.

4.10.4 IL-6:IL-10 ratio

The IL-6:IL-10 ratio dropped significantly as a result of the overall reduction in IL-6 concentrations and the mild trend towards IL-10 increases. The ratio improved more in patients whose symptoms resolved than in the symptomatic cohort at follow-up and

the absolute ratio was lower in asymptomatic patients at visit 3 (p=0.04, see figure 4.7 below).

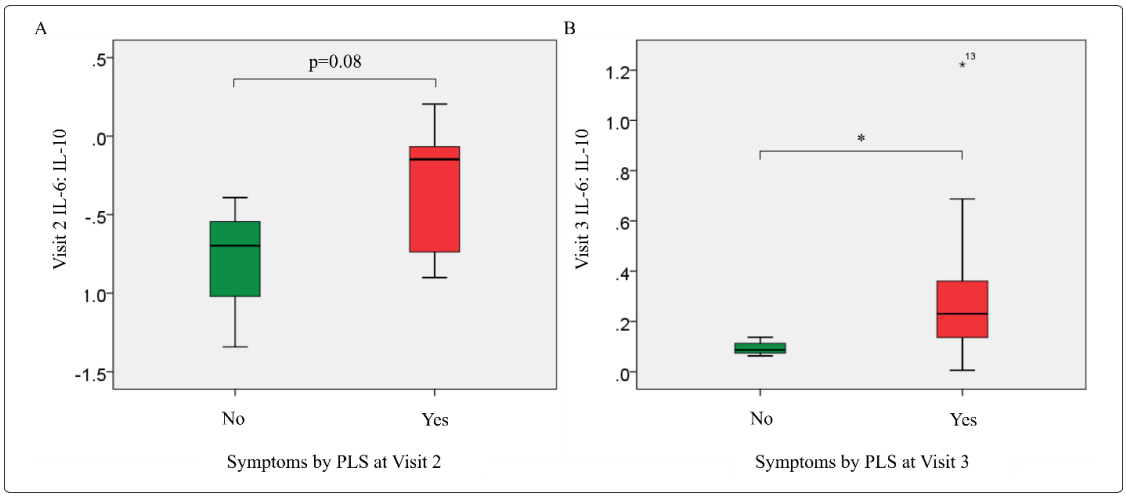


Figure 4.7: A. Box plot showing the relative change in IL-6:IL-10 ratio in CSX patients whose symptoms had resolved (n=4) at follow up and those who remained symptomatic (n=13). **B.** Absolute IL-6:IL-10 ratios in patients whose symptoms had resolved at visit 3 (n=4) and those who remained symptomatic (n=12).

4.11 Correlations

There was a strong correlation between baseline IFNγ and TNFα (r_s=0.538, df=36, p<0.001) in the overall group, which was also observed consistently in the CSX cohort at all time points (r_s=0.672, df=15, p=0.003 at baseline, r_s=0.654, df=15, p=0.004 for visit 2 and r_s=0.624, df=14, p=0.01) as shown in figure 4.8 Panels A and B below. Baseline IL-1 also strongly correlated with IL-8 concentrations in the overall cohort (r_s=0.549, df=36, p<0.001) and in the CSX group (r_s=0.821, df=15, p<0.001), which is expected as IL-1β is an inducer of IL-8 expression. Predictably, IL-6 concentrations correlated closely with TNFα levels (r_s=0.545, df=36, p<0.001; See Fig. 4.8 Panel C) but this did not reach significance for the CSX cohort on its own (p=0.09). IL-5 and IL-10 concentrations correlated strongly in the entire cohort (r_s=0.656, df=36, p<0.001) and at all time points in the CSX group (r_s=0.600, df=14, p=0.014 at baseline, r_s=0.770, df=8, p=0.009 at visit 2 and r_s=0.771, df=13, p=0.001 at visit 3) but these data are positively skewed. There was a moderate correlation between the IL-6 concentrations and CRP

and SAA concentrations (r_s=0.468, df=35, p=0.003 and r_s=0.432, df=35, p=0.008 respectively), although this was not seen in the smaller CSX population. There were no significant correlations between cytokines and age, BMI or cholesterol concentrations.

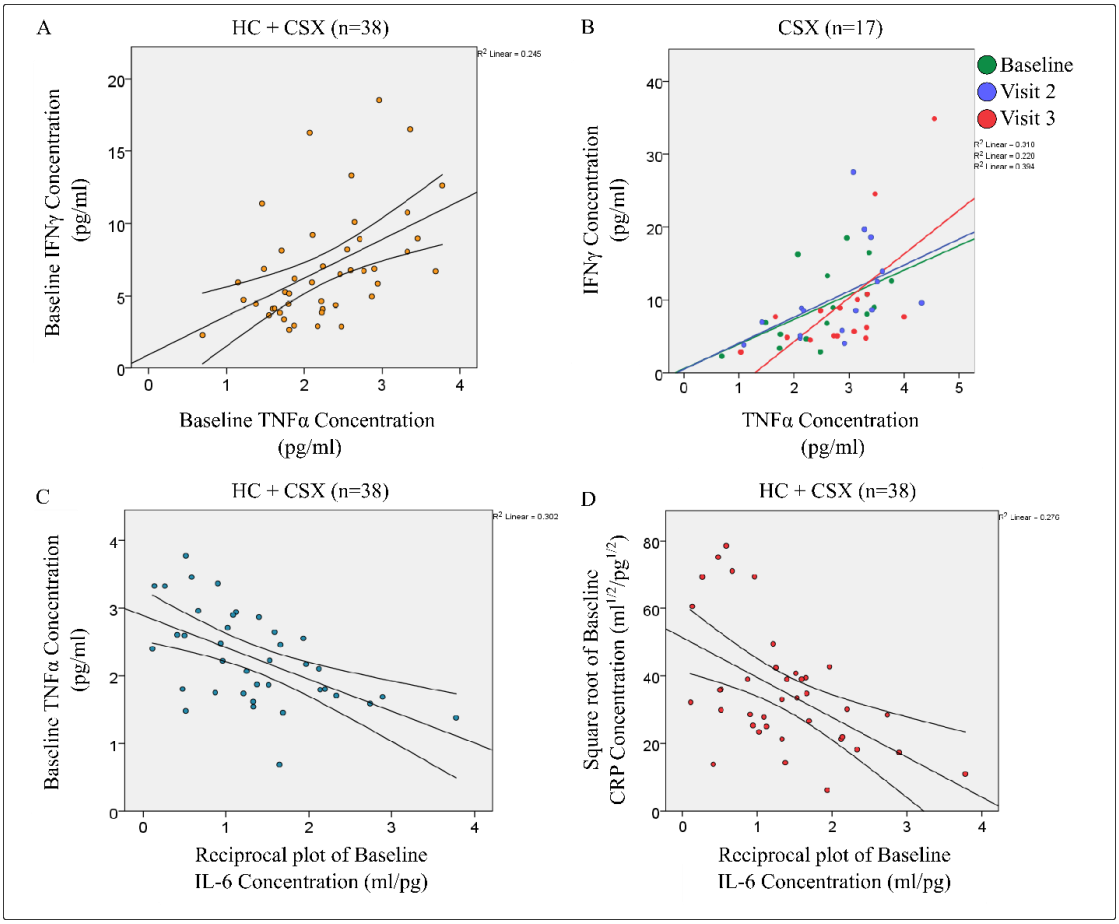


Figure 4.8: A. Scatter plot of IFN and TNF concentrations at baseline in all participants. **B.** Scatter plot showing IFN and TNF concentrations at all three time points in the CSX group. **C.** Scatter plot showing the correlation between baseline TNF and 1/IL6 in all participants. **D.** Scatter plot showing the correlation between the square root of the baseline CRP concentration and the reciprocally transformed baseline IL-6 concentrations in all comers.

4.12 LCSX

LCSX patients' plasma IFNγ concentrations did not differ significantly from healthy controls (mean difference 0.12, 95% CI -3.8 to 4.1pg/ml) or from the CSX group (mean difference -2.85; 95% CI -6.9 to 1.2pg/ml). Similarly, there was no difference in TNFα

concentrations between LCSX and healthy controls (mean difference 0.12, 95% CI -0.64 to 0.89pg/ml). There was a slight trend towards higher IL-6 in the LCSX patients (adj. significance 0.09) compared to the HC group. There was no significant difference in concentrations of IL-5 and IL-10 between the three groups.

4.13 Regression

Logistic regression analysis was performed to investigate the effects of baseline cytokines on clinical outcomes in our CSX patients. All pre-test assumptions including the absence of multicollinearity were met. While a model using baseline IL-6 and TNFα to predict symptomatic improvement (as assessed by the Seattle Angina Questionnaire PLS at the end of follow-up) was statistically significant ($\chi^2(2)=9.62$, p=0.008, explaining 73% (Nagelkerke R^2) of the variance and correctly predicting 87.5% of the outcomes), neither of the beta-coefficients in the variables output reached significance.

4.14 ROC

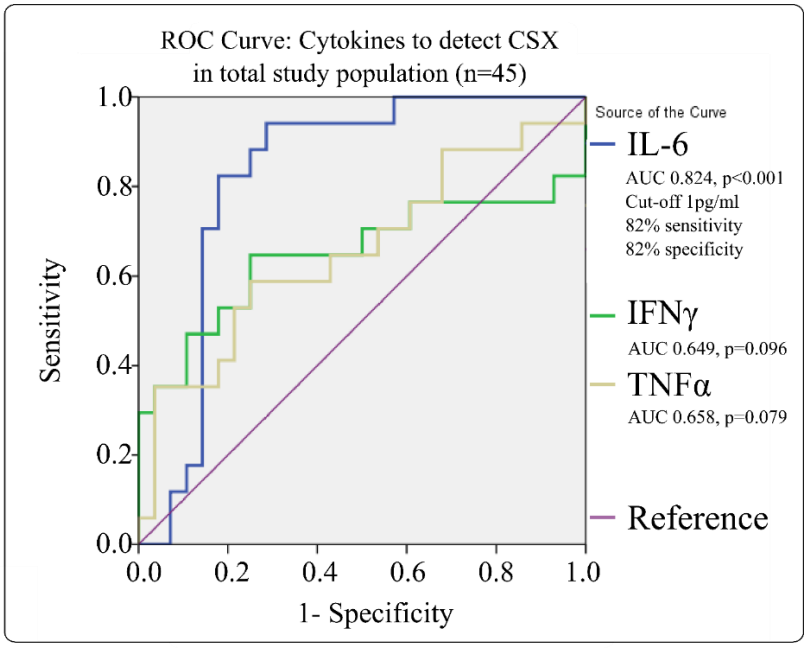


Figure 4.9: ROC curves for IL-6, IFN and TNFa to identify CSX patients from overall study population.

Receiver Operating Characteristic Curves were drawn for each of the significantly differentially expressed cytokines. Only IL-6 illustrated a significant ability to discriminate between CSX and non-CSX patients in the study cohort. Cytokine biomarkers were unable to discriminate between CSX patients and LCSX patients.

4.15 Principal Component Analysis

A PCA was performed including the maximum 9 predictor variables (√CRP, SAA, ICAM-1, PSS10, LTE-Q, IFNγ, TNFα and the reciprocal of IL-6) for 45 patients. The Kaiser-Meyer-Olkin measure was >0.6 and Bartlett's Test for Sphericity was significant at $p<0.001$, indicating that factor analysis was appropriate. Three components met the Kaiser criterion (i.e. had Eigenvalues >1 and were above the elbow in the Scree plot) and were extracted. These 3 components explained 70% of variance in the samples. The pattern and structure matrices are shown below.

Pattern Matrix[a]

	Component		
	1	2	3
IFNG1			-.758
TNF			-.838
SAA1	.751		
ICAM1	.877		
VCAM1	.677		
PSS10		.935	
LTEQ		.887	
invil6			.810
sqrtcrp	.764		

Extraction Method: Principal Component Analysis.
Rotation Method: Oblimin with Kaiser Normalization.
a. Rotation converged in 5 iterations.

Structure Matrix

	Component		
	1	2	3
IFNG1			-.746
TNF			-.874
SAA1	.786		
ICAM1	.849		
VCAM1	.657		
PSS10		.936	
LTEQ		.891	
invil6			.806
sqrtcrp	.815		

Extraction Method: Principal Component Analysis.
Rotation Method: Oblimin with Kaiser Normalization.

Figure 4.10: Matrices describing the 3 component output of principal component analysis on our study population. Both pattern and structure matrices are shown as we utilised oblimin rotation.

Component 1 relates closely to our markers of vascular injury (CRP, SAA, ICAM-1 and VCAM-1), Component 2 relates to markers of stress (PSS-10 and LTE-Q) and component

3 related to general inflammatory markers (IFNγ, IL-6 and TNFα). Component 1 differs significantly between CSX and the non-CSX group (t_{33}=2.8, p=0.007). Component 2 did not differ between the groups (p=0.133) while component 3 was different between the 2 groups (t_{33}=3.5, p=0.001).

Discussion

4.16 Cytokine expression in CSX

This chapter confirms the presence of chronic low-grade inflammation in CSX patients and shows for the first time an ongoing elevation of cytokines in this population. This inflammation is characterised by a persistent elevation of IFNγ and TNFα for the duration of follow-up regardless of symptom severity, indicating that elevation of these cytokines may be a trait finding in CSX. The finding of elevated baseline TNFα is in broad agreement with the few previously published studies into cytokines in CSX populations (the most recent study excepted). This is the first time, however, that IFNγ has been shown to be elevated in CSX. Our CSX patient cohort also resembled previously studied populations in that they demonstrated elevated plasma IL-6 concentrations but we have shown for the first time that this elevation is transient and appears to dissipate with time in tandem with waning symptoms. It may be that IL-6 is a state marker of CSX.

4.16.1 Cellular sources

The exact cell of origin for these cytokines is difficult to determine. The major cellular source of TNFα is the macrophage/monocytes population but several other possible sources include lymphocytes, secretory vascular smooth muscle cells and endothelial cells themselves. IFNγ is primarily produced by Th1-differentiated T-lymphocytes (although it may also be released by macrophages) and may be responsible for the maintenance of higher than normal basal TNFα release via the activation of

macrophages. Interleukin-6 is also released from activated macrophages in response to TNFα but similarly may be released from a plethora of cells including lymphocytes and endothelial cells (in an NFκB dependent fashion).

One possible pathway in CSX may therefore involve the activation of macrophages by various stimuli with the consequent release of TNFα and IL-6 from these cells with this response being augmented by IFNγ co-stimulation of the macrophages. Levels of TNFα and IL-6 cytokines in our cohort highly correlate, suggesting co-release from a common cellular source or else inter-dependent release. Similarly, IFNγ and TNFα levels were shown to tightly correlate suggesting that it may well be signals from Th1 cells that govern macrophage activity in CSX. Certainly, macrophages may play a central role in CSX as monocyte counts have been shown to be elevated in CSX, although this was not observed in our population[2]. Furthermore, plasma levels of Monocyte Chemoattractant Protein-1 are known to be raised in CSX, which is not surprising as CRP is known to induce MCP expression[157,212]. Similarly, T-lymphocytes are known to play an important role in atherosclerosis, so the IFNγ seen in CSX may be attributable to increased T_h1 cell activity[213].

Circulating neutrophils provide another possible source for cytokine production in CSX. As was noted in chapter 2.5.8, our patients had an elevated neutrophil to lymphocytes ratio (NLR) when compared with healthy controls, indicating a predominance of neutrophils in the plasma. This replicates previously described findings in CSX populations and is sometimes observed in chronic inflammatory conditions[214]. Like CRP and IL-6, the NLR has been shown to independently predict outcomes in coronary artery disease patients[215]. Interestingly, the NLR has been associated with measures of disease activity in CSX, with higher NLR being associated with reduced myocardial perfusion on coronary angiography and slower heart rate recovery after EST (a measure of cardiac autonomic activity)[216,217]. Furthermore, activated neutrophils are a

source of oxidative stress, which may drive further endothelial dysfunction in CSX, and neutrophils have been implicated in the induction of endothelial dysfunction[218]. Given that there is a predominance of neutrophils in our patients it is possible that they, either alone or in concert with macrophages, are responsible for the basal cytokine secretion.

4.16.2 Stimulus for cytokine expression

The stimulus for immune activation in CSX has eluded researchers for decades. Macrophage or neutrophil activation may be in response to a pathogen. One group investigated this possibility, studying serology for H. pylori, C. pneumoniae, Cytomegalovirus and Epstein Barr Virus infection in CSX patients but found no excess prevalence in the population[62]. Another study, however, did notice an increased prevalence of active H. pylori infection in 50% of their 30 patient CSX population using the urease breath test, although this population appeared to have been diagnosed with "atypical chest pain"[219]. A further study examined IgG for H. pylori and found that 95% of the CSX population studied had previous H. pylori infection as opposed to only 45% of controls[63]. The overall prevalence of H. pylori in the country of origin, Iran, is about 80-95%, however[220]. The notion of H. pylori being the causative agent in at least some CSX populations is interesting, especially as treatment with a proton pump inhibitor has been shown to be of benefit in some patients with possible CSX[143].

Other possible stimuli for immune activation in CSX include oxidised LDL from dyslipidaemia coupled with oxidative stress. Oxidised LDL is a potent stimulus for endothelial and macrophage activation. There is certainly a high prevalence of dyslipidaemia in our CSX cohort (82% v 42% in other patients with normal angiograms, p=0.009; see chapter 2.4.2) while previous studies have demonstrated increased oxidative stress in CSX[221,222]. It is clear that our CSX population have angiographically normal coronary arteries but this does not preclude the presence of atherosclerosis,

however, as the initial response to atherosclerosis is outward stretching of the vessel to preserve lumen diameter in a process termed positive remodelling, a process not readily apparent on angiography (see figure 4.11 below).

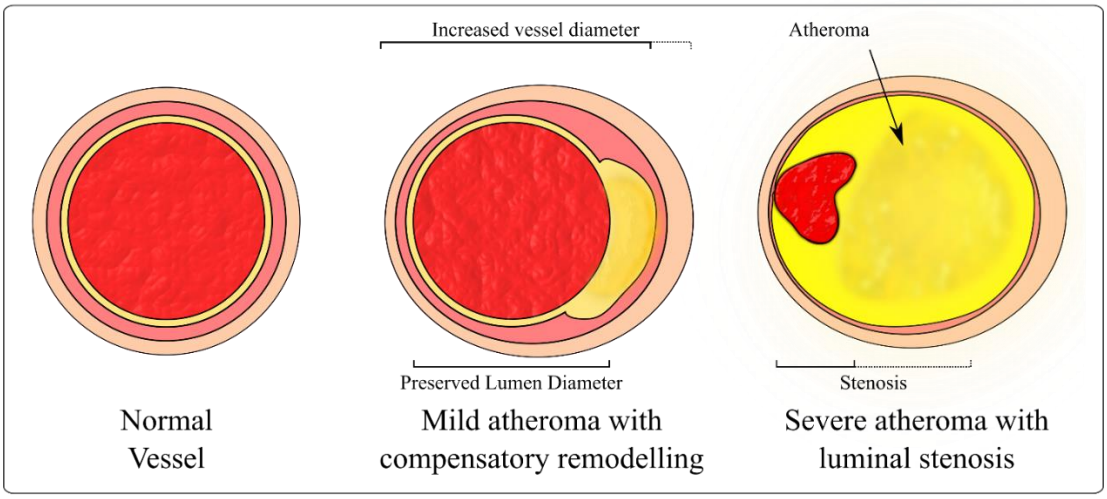


Figure 4.11: Positive remodelling of a coronary artery (the Glagov phenomenon) illustrated by the increased overall diameter of the blood vessel in response to mural atheroma (middle panel) to preserve luminal cross-sectional area. This is overcome in advanced atherosclerosis, where mural atheroma eventually compromises lumen size.

This remodelling may be more noticeable on coronary arterial CT or MR imaging and indeed CSX patients are known to have elevated coronary calcium scores, indicative of atherosclerosis, despite normal luminal diameters[68]. Alternatively, the CSX patients may have predominantly arteriolosclerosis or indeed have atherosclerosis in other vascular trees, as each vascular territory behaves differently and there is some histological evidence of arteriolosclerosis in CSX[67]. Certainly the observed Type 1 cytokine profile is in keeping with atherosclerosis.

Another possible source of increased basal cytokine expression is hypertension. Even pre-hypertension (systolic blood pressure between 120 and 139mmHg) has been associated with increased levels of TNFα, IL-6, MCP-1 and ICAM-1[223]. It is believed that hypertension may induce the same endothelial dysfunction as dyslipidaemia via NFκB induction, perhaps in an angiotensin-II-dependent manner. It is also possible that

inflammation induces hypertension in these patients due to alteration of blood vessel function as detailed in the following section. Our population of CSX patients had a similar incidence of mild hypertension as the matched healthy controls (35% vs. 43%) and did have evidence of mildly elevated left ventricular end-diastolic pressure (LVEDP) during angiography, which may be a consequence of mild hypertension.

4.16.3 Endothelial Sequelae of Increased Cytokine Expression

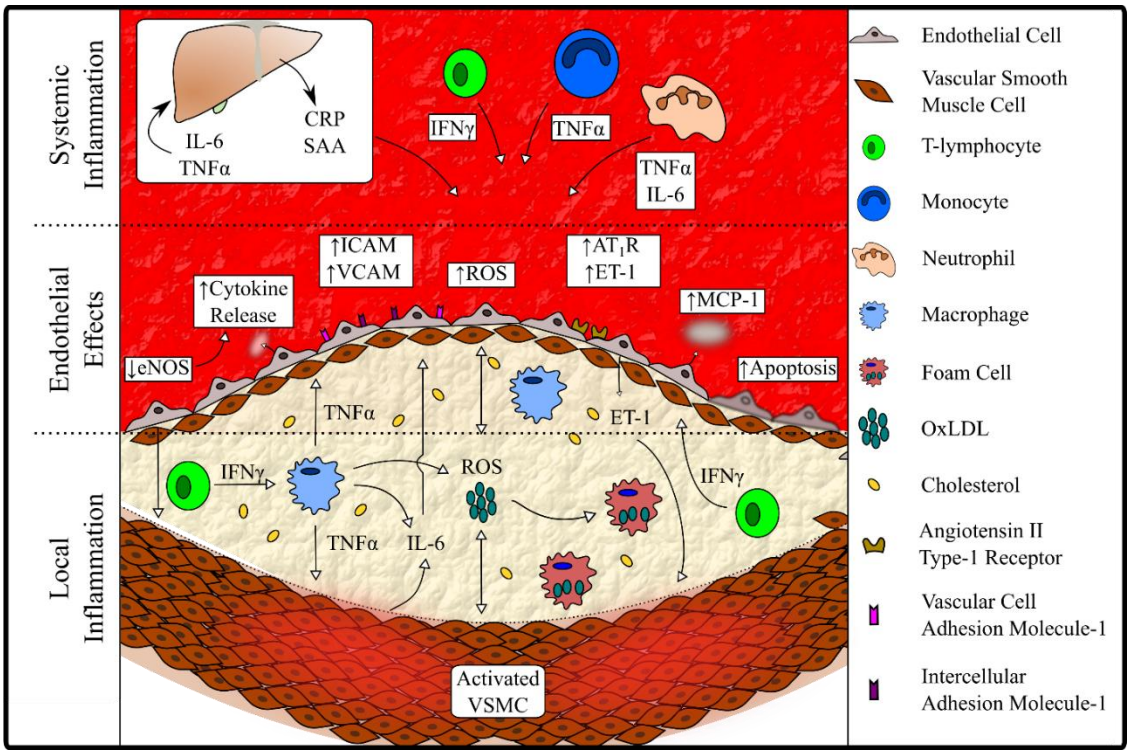


Figure 4.12: Potential effects of cytokines on endothelial function in CSX. Note that both circulating mediators and locally produced cytokines, acting in an autocrine or paracrine fashion, may influence endothelial and vascular smooth muscle behaviour. See text for abbreviations.

The persistently elevated TNFα and IFNγ may reflect an ongoing pro-inflammatory stimulus in CSX patients or else might point to a genetic "priming" of the immune systems in these patients. These cytokines remain elevated despite the resolution of clinical symptoms in many of the CSX patients and so may not be effector molecules in the pathogenesis even though TNFα is known to be a potent cause of endothelial

activation and dysfunction via NFκB upregulation. This results in the expression of adhesion molecules, reduced nitric oxide bioavailability and increased cellular oxidative stress (see Figure 4.12 above). It is possible that this basal pro-inflammatory state keeps the endothelium in a dysfunctional state with symptoms supervening in the presence of a provoking factor such as glycaemic loading, further immune activation or blood pressure elevation.

The general decrease in IL-6 concentrations over time at the same time as improvement in symptoms, on the other hand, may be a signal that IL-6 is a mediator of disease activity. Predictably, the acute phase reactants CRP and SAA correlated closely with IL-6 concentrations and we have shown in chapter 3 that CRP may be a state marker in CSX, meaning that it is only elevated in patients with active disease. This may also be the case with IL-6. IL-6 is known to have several deleterious effects on healthy endothelium. Perhaps most relevantly, IL-6 causes the upregulation of the angiotensin II type 1 receptor (AT_1R) expression in the vascular wall, increasing its responsiveness to circulating angiotensin II, itself an inducer of vascular IL-6 production. This causes increased production of reactive oxygen species (ROS) by the VSMCs, reduced endothelial dependent vasodilation and enhanced vasoconstriction, all known features of CSX[224]. IL-6 is also known to be a potent inducer of endothelial endothelin (ET-1) production[225]. ET-1 is the most potent endogenous vasoconstrictor which is also known to impair endothelial vasodilation and has previously been found to be elevated in CSX patients following glycaemic stress[192]. It is not surprising that IL-6 concentrations are linked with the degree of impairment of flow-mediated vasodilation in healthy men[199]. While endothelial cells appear to lack IL-6 receptors, IL-6 may induce endothelial activation via trans-signalling, where IL-6 binds to a soluble IL-6 receptor.

Most pro-inflammatory cytokines are capable of inducing oxidative stress. The vascular wall contains many possible sources of ROS including uncoupled eNOS activity,

increased NADPH oxidase activity, from mitochondrial sources and from local phagocytes undergoing IFNγ -induced oxidative burst. These ROS, when present in sufficient quantities, may potently induce endothelial dysfunction through direct chemical interaction with NO, forming peroxynitrite and reducing NO bioavailability[226]. The role of oxidative stress in CSX has been investigated. There is evidence of reduced serum antioxidant levels, increased malondialdehyde concentrations and increased myeloperoxidase activity in CSX, all signals of increased oxidative stress[221,222,227].

4.16.4 Cytokines and Clinical Improvement

As mentioned, clinical improvement occurred concurrently to a drop in serum IL-6 in patients. The reasons behind this reduction in IL-6 over time in our population, despite persistently elevated TNFα and increasing IL-1β, are not apparent in this study. It should also be noted that CRP was also found to fall across this time period, perhaps as a result of the reduced IL-6 levels. Our IL-10 levels showed a non-significant trend towards generally increasing during follow-up. One recent study into CSX demonstrated significantly elevated levels of IL-10 but their patients cohort was atypical in that they had normal CRP and low TNFα, in contradiction to most published studies on CSX[195]. The IL-6:IL-10 ratio is a well-studied measure of the pro-anti-inflammatory cytokine balance. In our study, CSX patients whose reported symptoms improved had lower IL-6:IL-10 ratios that patients who remained symptomatic at the end of follow-up. It may be that there is a slow increase in IL-10 over time in the CSX population and that this in turn switches off IL-6 production and hence alleviates the harmful endothelial effects inherent in higher IL-6 levels as well as counteracting the activated angiotensin system by reducing oxidative stress.

4.17 Limitations

The main limitation with respect to this chapter was the inability to glean valuable data from the TH1/TH2 plates due to technical reasons. The loss of IL-4, IL-12 and IL-13 data

in particular prevented further discussion regarding T_h1-T_h2 balance in CSX. It would also have been useful to examine IL-17 concentrations as this may be implicated in CSX through its ability to recruit monocytes and neutrophils to site of inflammation. Unfortunately, funding was not available to investigate this facet of cytokine expression in CSX. Again, our small sample size potentially deprived us of some power to detect differences in IL-10 levels.

Conclusions

Our CSX patient cohort had evidence of persistent low-grade inflammation that was driven primarily by elevated IFNγ and TNFα. This was present regardless of symptomatology indicating that mild baseline phagocyte activation may be a trait of CSX patients. Whether the responsible cells are macrophages or neutrophils is difficult to tell, although our patients had an elevated neutrophil to lymphocyte ratio. There was an initial pronounced elevation of IL-6 concentrations in symptomatic CSX patients, which did not persist through follow-up and there was some evidence that IL-6 may be a state marker in CSX as greater drops in this cytokine appeared to follow improved clinical status in tandem with an improved IL-6:IL-10 ratio. This may highlight a role for these cytokines in disease resolution. Pro-inflammatory cytokines may be the trigger for endothelial activation in CSX and may induce microvascular dysfunction through upregulation of angiotensin and endothelin pathways in the endothelium as well as being a trigger for oxidative stress. Modulation of vascular inflammation in CSX using statins and angiotensin receptor blockers has been shown to be effective in controlling symptoms, highlighting the importance of cytokines in the pathogenesis of this condition. The baseline stimulus for their release has not been elucidated but the main culprits likely include traditional cardiovascular risk factors such as dyslipidaemia and hypertension.



Chapter 5: Tryptophan Metabolism in CSX

Introduction

5.1 Chapter Overview

Tryptophan, an amino acid characterised by the inclusion of an indole ring in its central structure, is one of the nine essential amino acids in humans, this meaning that it cannot be produced endogenously. As such, it must be ingested from dietary sources such as cheese, red meat and eggs. Furthermore, tryptophan is typically only present in small quantities in the human body and is at risk of being rapidly depleted through utilisation. Apart from its primary role as a substrate for protein synthesis in the liver, tryptophan is also the precursor substrate in the biosynthesis of several important substances in human physiology, including serotonin, kynurenine and melatonin. These substances play a role in various body functions including mood, circadian rhythm, immune function and vascular function. Thus, tryptophan and its metabolites have been implicated in various diseases including depression, anxiety, somatisation, atherosclerosis and vascular inflammation. It is possible that deranged tryptophan metabolism may be implicated in the pathogenesis of CSX, given the prevalence of anxiety and vascular inflammation in this condition[64,92,138,228,229]. To date, research into the metabolism of tryptophan in CSX has not been published. In this chapter, the main bifurcation in the pathway of tryptophan metabolism is examined.

5.2 Overview of Tryptophan Metabolism

Tryptophan is not stored in large quantities in humans. Apart from its incorporation into proteins in the liver (its primary fate), tryptophan is metabolised through two main pathways in the body, the kynurenine pathway and the methoxyindole pathway. Being the minor pathway, only about 3-5% of plasma tryptophan is metabolised through the methoxyindole pathway but it remains one of great importance, being responsible for the formation of serotonin and melatonin. The initiating step in serotonin production is the hydroxylation of tryptophan. This mainly occurs in the gastrointestinal tract's enterochromaffin cells, where 90% of the body's serotonin is synthesised. The rest is

formed by neurones of the serotonergic system in the CNS. Circulating platelets take up and store large quantities of serotonin in dense granules and these are used to assist with local haemostasis by inducing vasoconstriction.

Melatonin is formed primarily in the pineal gland in the absence of ultraviolet radiation. The pineal gland responds to impulses from the retina, autonomic inputs and other CNS inputs that are channelled through the suprachiasmatic nucleus. Blue light essentially suppresses the production of melatonin in the pineal gland and this allows the body to have a biochemical chronometer. Daytime levels of melatonin are negligible due to pineal suppression, while there is a surge in its production during the night-time hours with a peak at about 0300. Melatonin concentrations, therefore, are believed to define the photoperiod for the body and control many chronobiotic effects. It is well documented that most adverse cardiovascular events have a peak in the hours just after dawn and this coincides with endothelial dysfunction and increased platelet reactivity at these times after the end of the scotophase and withdrawal of melatonin synthesis.

The predominant pathway, the kynurenine pathway, is initiated via the action of two important oxidoreductases, Tryptophan 2,3-dioxygenase (TDO) and Indoleamine 2,3 dioxygenase (IDO) (see figure 5.1 below). TDO and IDO divert the tryptophan down the kynurenine pathway and this results in the formation of many substances including kynurenine, several stable intermediates and ultimately nicotinamide adenine dinucleotide (NAD), an important co-enzyme for redox reactions. TDO is chiefly found in the liver while IDO is found in most tissues, including the endothelium. IDO is interesting in that it is primarily upregulated by active inflammation (IFNγ and cell-mediated immunity specifically), with the result that the rate of tryptophan metabolism into kynurenine is accelerated in inflammatory conditions. Thus, as inflammation supervenes, tryptophan stores are depleted and the Kynurenine pathway

is activated. The ratio of the kynurenine to tryptophan (KTR or KT ratio) is therefore a crude barometer of the activation of IDO and hence of cell-mediated immune activity and has been shown to be elevated in many inflammatory conditions such as rheumatoid arthritis[230].

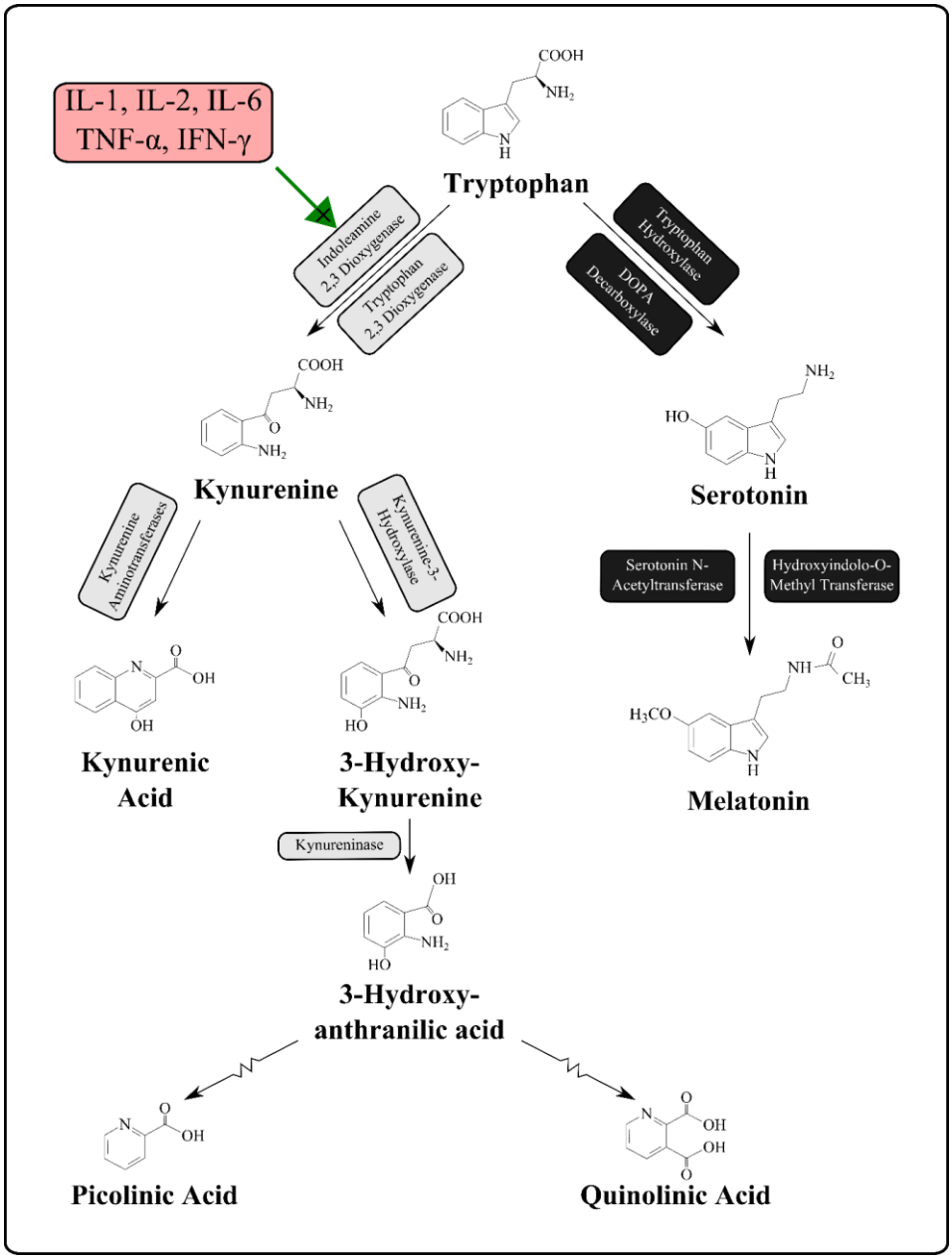


Figure 5.1 Tryptophan metabolism in humans. It bifurcates initially into the kynurenine pathway (left) and the methoxyindole pathway (right)

5.2.1 Indoleamine 2,3-dioxygenase

Indoleamine 2,3-dioxygenase (IDO) is an inducible enzyme that is found almost ubiquitously in human cells. It is coded for by the *IDO1* gene on chromosome 8. It has an iron-containing haeme group as its sole prosthetic group and relies upon this to affect the transfer of dioxygen or singlet oxygen from a suitable donor (often superoxide radicals) to tryptophan. Alongside Tryptophan 2,3 dioxygenase (TDO), it catalyses the rate limiting step in the kynurenine pathway of tryptophan metabolism. TDO is constitutively expressed in hepatocytes and is upregulated by the presence of tryptophan while IDO is found in most tissues around the body, but particularly in the lungs and lymphoid tissues, and relevantly may be induced in vascular smooth muscle cells and in the endothelium. The expression of the IDO gene is upregulated by inflammatory mediators. IFNγ is a particularly potent inducer of IDO but other stimuli such as IFNα, IFNβ, IL-2, IL-6, TNFα, oxidative stress and the presence of microbes or lipopolysaccharide may also upregulate IDO production[231,232]. Thus, activation of IDO predominantly occurs in the presence of an activated innate or adaptive immune response. Typically, IFNγ will be released from macrophages, NK cells and T-cells in response to a perceived threat and this switches on *IDO1* transcription.

The exact role of IDO in the immune response is uncertain but there are several competing theories and IDO is believed to provide a counter-regulatory feedback pathway to blunt the immune response. The first is termed the “Tryptophan Depletion Hypothesis” where the activated IDO is believed to deplete the local supplies of tryptophan by directing it down the kynurenine pathway and thereby preventing microbe metabolic activity. This is believed to be an early biostatic protective response to prevent microbial replication but it is now believed that most microbes can synthesise tryptophan through alternative means. This depletion effect also affects local immune cell function and may therefore have a role in the induction of immune system tolerance. IDO, for example, has been shown to be essential in preventing destruction of certain immunologically distinct tissues, such as a growing foetus in the

gravid uterus, by preventing immune cell function[233]. Indeed, some cancers take advantage of this immune subversion by producing IDO and thereby preventing attack by immune effector cells.

It is far from certain, however, that depletion of tryptophan is the main effector mechanism of IDO modulation of immune function. The "Tryptophan Utilisation Hypothesis" holds that it is the products of the kynurenine pathway more than the depletion of tryptophan that modulates immune cell function[234]. Kynurenine pathway metabolites have been shown to induce apoptosis in T_h1 cells and monocytes, thereby moving the immune phenotype preferentially to the T_h2 type. They have also been shown to have a role in endothelial dysfunction and oxidative stress. Regardless of the mechanism of tryptophan depletion, low levels of plasma tryptophan have been shown to predict increased cardiovascular mortality with patients in the lowest tryptophan quartile (<34μM) having an OR of 1.41 (1.05-1.89, p=0.02) for cardiovascular death over 10-years[235].

5.2.2 The Kynurenine :Tryptophan Ratio (KTR)

As IDO activation increases more tryptophan is actively converted into kynurenine. The relative concentrations of these two substances (i.e. the KTR) is therefore a measure of IDO activity and a possible signal of immune system activation. Researchers have studied this parameter in several vascular conditions. For example, IDO has been shown to be locally upregulated in the antigen presenting cells (APCs) of atherosclerotic plaques, implying that it plays a role in mediating the inflammatory component of atherogenesis[236]. The same group showed that IDO activity was also significantly correlated with the extent of overt atherosclerosis (such as the intima/media thickness of the carotid artery) as well as with plasma lipids and systolic blood pressure[237]. More specifically, the KTR has been shown to be increased in angiographically confirmed coronary artery disease patients than in healthy

controls[238,239]. It is known that IDO activity leads to nitric oxide synthase downregulation and significantly reduces microvascular reactivity in the setting of sepsis[240]. In this way, IDO is seemingly associated with endothelial dysfunction and atherosclerosis.

Furthermore, KTR has been linked with prognosis in coronary arterial disease. Researchers studied urinary KTR in over 3000 patients attending for coronary angiography and followed them for 55 months. They demonstrated that increased baseline urinary KTR was associated with an increased relative risk of major cardiac outcome, even after controlling for confounders. Indeed, they showed that each standard deviation increase in log-transformed urinary KTR led to a 43% relative increase in observed MACE. A similar but slightly weaker association (HR=1.28) was seen in angina patients using plasma KTR as a predictor for MACE[241].

Finally, the activation of IDO and increased KTR in CAD may be relevant to the increased prevalence of depression observed in IHD patients. Importantly, it has been demonstrated that the KTR actually correlates with the diagnosis of depression ($p=0.055$) and with scores on a depression scale ($P=0.002$) in patients with IHD[242]. Furthermore, the KTR is also noted to be higher in patients with somatization disorders and pain syndromes[243].

5.3 Chapter Objectives

Given the overview of tryptophan metabolism above it may be appreciated that immune-activation of IDO with the subsequent favouring of the kynurenine pathway over the methoxyindole pathway may lead to many deleterious vascular effects. Increased vascular activation, oxidative stress and vascular inflammation may be coupled with activation of endothelial apoptotic pathways leading to the overall

progression of generalised endothelial dysfunction. Furthermore, tryptophan and serotonin depletion may lead to depression, anxiety and somatisation with abnormalities in central pain processing. All of these features are entirely relevant to the putative pathophysiology of CSX. While it is known that CSX patients have an inflammatory phenotype, the activity of IDO and the consequent role of kynurenine pathway metabolites have not been assessed in this condition. In this study, we investigate the state of tryptophan metabolism and its relationship with markers of vascular inflammation and disease activity in CSX and theorise about its possible role in the pathogenesis and symptomatology of this condition. Our chapter aims are summarised below.

1. We will examine the plasma **ratio of kynurenine and tryptophan (KTR)** to estimate the activity of the enzyme indoleamine-2,3-dioxygenase (IDO). Our initial hypothesis is that patients with CSX will have altered tryptophan metabolism due to the induction of IDO, which is upregulated by inflammation. If IDO is upregulated, we would expect to see shunting of tryptophan down the kynurenine pathway with consequent increases in KTR coupled with tryptophan lack and kynurenine excess.
2. We will investigate the **association of IDO activation with the markers of vascular and general inflammation** that were shown to be altered in CSX patients (as investigated in chapters 3 and 4).
3. Additionally, we will look at the **KTR in relation to perceived disease severity and life stress** as a crude measure of the possible effects of altered tryptophan metabolism on the neuropsychiatric aspects of CSX.
4. Finally, we will examine the **longitudinal changes in tryptophan metabolism** in CSX patients and assess if any correlation with changes in symptom severity occurs.

Methods

5.4 Participants

The same patient cohorts as defined in chapter 2.2 were used. Again, our CSX cohort consisted of 17 patients while we also had 21 age- and sex-matched healthy controls and 7 patients with LCSX. Consecutive patients with CSX were recruited from the cardiac catheterisation laboratory of a tertiary cardiac referral centre. Every suitable patient who was approached consented to participating in the study. The study protocol was approved by the local research ethic committee. CSX patients were seen at follow-up visits, as previously detailed, where blood sampling and questionnaires were repeated. Baseline patient characteristics are shown in table 2.3. The CSX and healthy control groups were well matched in terms of gender, age, cardiovascular risks and medication use except for aspirin use, which predominated at baseline in the CSX group due to their presentation to the catheterisation lab where low dose aspirin is usually prescribed. Aspirin has been shown in vivo to reduce IDO activity in stimulated PBMC's[244]. No patients had used anti-depressants in the preceding year.

5.5 Investigations

Identical investigations were completed as outlined in chapter 2.3 including Exercise Stress test reports, cardiac risk factor questionnaires, the Seattle Angina Questionnaire (SAQ), List of Threatening Experiences questionnaire (LTE-Q) and the Perceived Stress Scale (PSS). All patients gave full informed consent at enrolment. Venous blood was drawn from the antecubital vein into a 10ml dipotassium EDTA tube before being centrifuged at 4°C for 15 minutes at 115 RFC. The plasma was then transferred to 2ml microtubes and immediately frozen at -80°C until analysis. All blood samples were taken between 0900 and 1100 and no patients were fasting for more than 3 hours before blood was drawn.

5.6 Measurement of tryptophan metabolism

Aliquots of plasma were thawed and the concentrations of tryptophan, kynurenine and kynurenic acid were determined using high performance liquid chromatography (HPLC) following established methods[245]. Briefly, plasma samples were spiked with 3-nitrostyrosine as an internal standard and were then deproteinised using 20µl of 4M perchloric acid. The samples were centrifuged for 15 minutes at 14000 RPM on a Hettich Mikro 22R centrifuge (AGB, Dublin, Ireland) and the supernatant was transferred to a HPLC vial for analysis. Suitable stock solutions of each standard were prepared using HPLC grade water and were acidified with 20 µL of 4M perchloric acid. The HPLC system consisted of a Waters 510 pump (Waters, Dublin, Ireland), a 717plus cooled Autosampler (Waters), a Hewlett Packard 1046A fluorescent Detector (Agilent, Dublin, Ireland), a Waters 486 tunable UV absorbance detector, a Waters bus SAT/IN module and a Croco-Cil column oven. All samples were injected onto a reversed phase Luna 3µm C18 (2) 150 x 2mm column (phenomenex) which was protected by Krudkatcher disposable pre-column filters (Phenomenex) and SecurityGuard cartridges (Phenomenex).

The mobile phase consisted of 50 mM acetic acid, 100 mM zinc acetate with 3% (v/v) acetonitrile and was filtered through Millipore 0.45 µm HV Durapore membrane filters (AGB) and vacuum degassed prior to use. Compounds were eluted over a 30-minute run time at a flow rate of 0.3mls/min after a 20µl injection. The column was maintained at a temperature of 30°C and samples/standards were kept at 8°C in the cooled auto-injector prior to injection. The fluorescent detector was set at an excitation wavelength of 254 nm and an emission wavelength of 404 nm. The UV detector was set to 330 nm. L-tryptophan and its metabolites were identified by their characteristic retention times as determined by standard injections which were run at regular intervals during the sample analysis. Concentrations were determined by

comparing the respective peak heights of analytes and internal standards. Results are expressed as µmol/L of plasma.

5.7 Data analysis

All data were analysed using SPSS v20 (Armonk, NY: IBM Corp.) and are reported as mean ± SEM. The Student t-test or Mann-Whitney U test were employed to compare groups as appropriate to the distribution of the given data. Furthermore, One-way ANOVA with Bonferroni post-hoc testing or the Kruskal Wallis test were employed when necessary. Repeated measures were assessed using paired t-tests or the Wilcoxon signed-rank test. Fisher's exact test was used to compare categorical data. Correlations were assessed using Spearman's rank-correlation test. Two-tailed p-values of <0.05 were considered significant in this study.

Results

5.8 Baseline tryptophan and its metabolites in CSX patients.

Tryptophan levels were significantly lower in patients with CSX when compared to healthy controls (50.0 ± 3.9 vs. 59.2 ± 2.4 µmol/L of plasma; U=83, n=38, p=0.004). Conversely, kynurenine concentrations tended to be higher in the CSX cohort (10.3 ± 3.4 vs. 3.2 ± 0.16 µmol/L, p=0.056). Consequently, the kynurenine: tryptophan ratio (KTR) was significantly elevated in CSX patients at baseline (0.202 ± 0.059 vs 0.056 ± 0.002; U=298, n=38, p<0.001). Transformation of this with a simple reciprocal transformation lead to a significant t-test results (t_{23}=-4.27, p<0.001) as shown in figure 5.2 below.

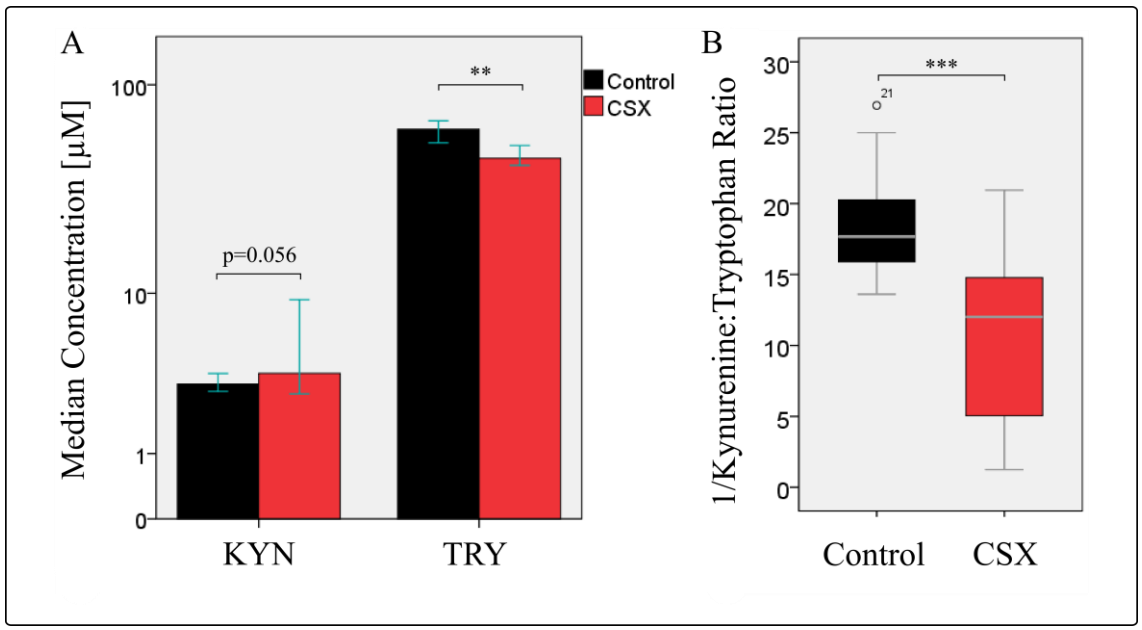


Figure 5.2 A. Bar chart showing the baseline kynurenine and tryptophan concentrations in Controls and CSX patients. Note logarithmic scale. **B.** Kynurenine Tryptophan Ratio transformed using a reciprocal transformation. Normal axis scale.

Analysis of kynurenic acid (KYNA) showed that levels were also significantly reduced in CSX patients (20.9 ± 1.6 vs 28.9 ± 1.9 nmol/L; t(24) = -2.465, p=0.021) as was the kynurenic acid:kynurenine ratio (0.0051 ± 0.0013 v 0.0083 ± 0.0006; t_{24} = -2.629, p=0.015). Importantly KYNA could only be quantified in 7 of 17 CSX patients. There were no significant differences in tryptophan or its metabolites between patients in terms of gender, age, aspirin use or statin use.

5.9 Follow-up Results

As a group, the CSX patients continued to have lower tryptophan at visit 2 (47.8 ± 2.3 µmol/L of plasma; H=19.8, adj. p=0.01) but by visit 3 it had drifted up towards control values (52.0 ± 2.6 µmol/L; H=12.0, adj. p=0.07). There was no significant difference in kynurenine or kynurenic acid concentrations at follow-up CSX visits compared with control levels. The CSX patients did, however, have a persistently elevated KTR at visit 2

(0.074 ± 0.004; H=-20.0, adj. p=0.02) and visit 3 (0.071 ± 0.002; H=-19.0, adj p=0.032) compared to controls. (see Fig 5.3 below)

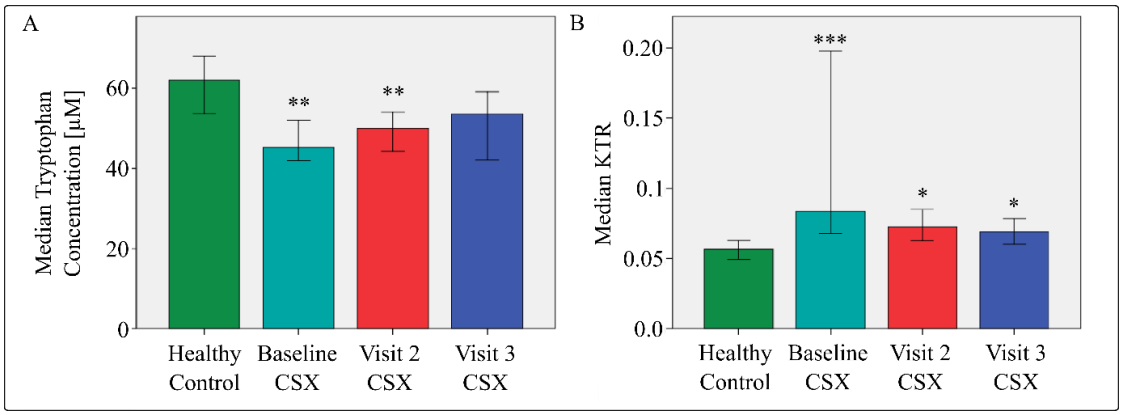


Figure 5.3: A. Bar chart of Tryptophan concentrations in CSX patients at the 3 time points compared to healthy control values. **B.** Kynurenine Tryptophan ratios in controls and CSX at all 3 time points.

At the second visit, 4 CSX patients reported complete resolution of symptoms as judged by a Physical Limitation domain score of 100 on their SAQ. These patients showed no significant difference in plasma tryptophan (p=0.177) when compared with healthy controls but still had a trend to higher KTR (p=0.052). Those with ongoing symptoms as measured by the SAQ continued to have significantly reduced tryptophan (mean difference 10.76, 95% CI 1.31 to 20.2 µmol/L, p=0.021) and elevated KTR (mean difference 0.018, 95% CI 0.003 to 0.0.029, p=0.012) when compared with the control population. Surprisingly, the reverse was true with the Exercise Stress Test results. Those patients with a normal EST had marginally higher KTRs than healthy controls (mean difference 0.016; 95% CI 0.002 to 0.031) and lower plasma tryptophan (mean difference 10.4, 95% CI 0.02 to 20.8µM), while those with positive ESTs showed no difference in KTR or Tryptophan (p=0.09 and p=0.18 respectively). These findings are illustrated in Figure 5.4 below.

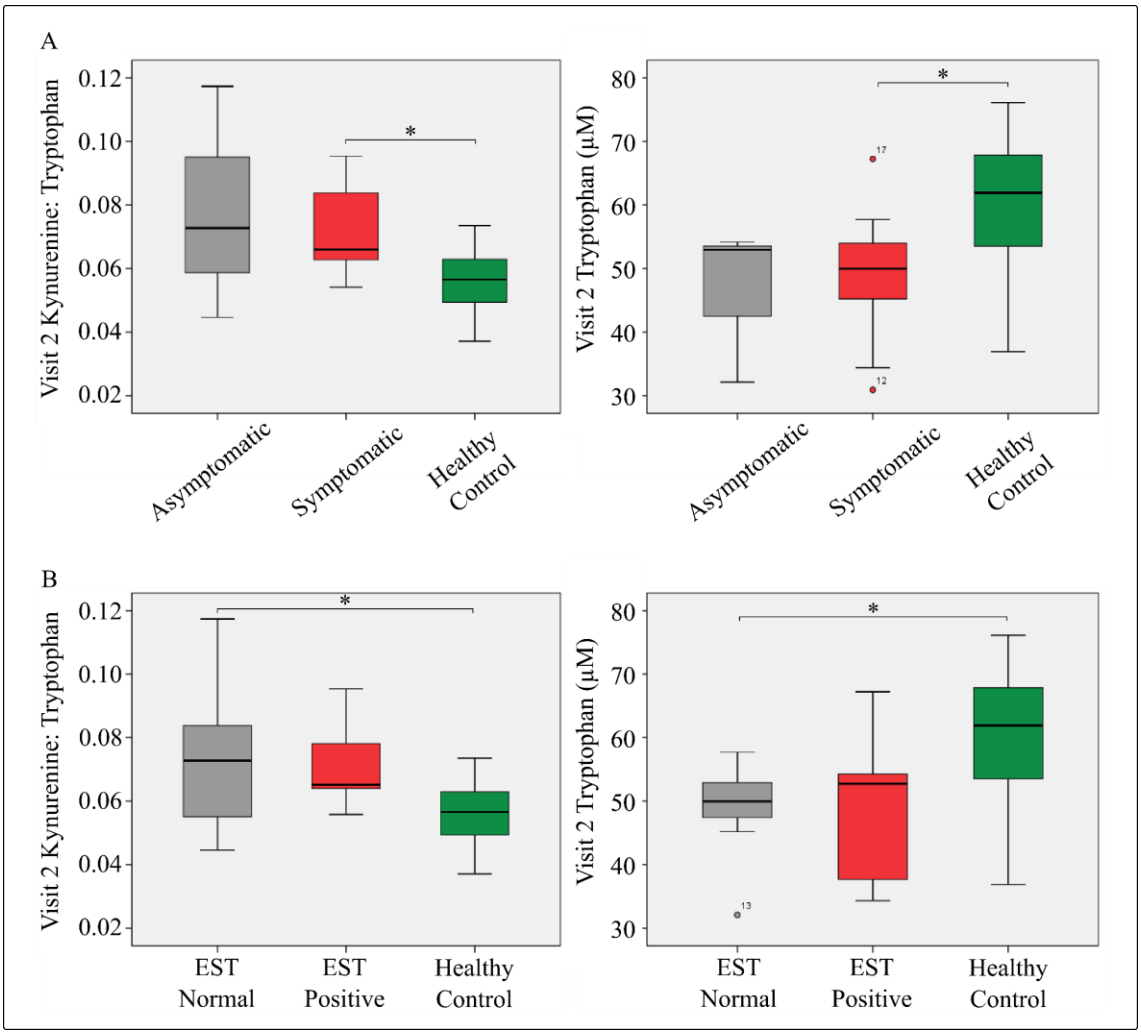


Figure 5.4: A. Comparing KTR and Tryptophan concentrations in CSX patients with and without self-reported symptoms (by PLS on the SAQ) and healthy controls. **B**. Comparing KTR and Tryptophan concentrations between healthy controls and CSX patients with and without electrically positive EST's at visit 2.

By the end of follow-up at visit 3, 4 patients were asymptomatic by SAQ results while 11 had ongoing symptoms. As at visit 2, the patients with ongoing symptoms had higher KTRs than healthy controls (H=14.3, n=36, p=0.001) while the asymptomatic patients did not (H=6.9, n=36, p=0.696). Again there was a recapitulation of the visit 2 results in terms of plasma tryptophan levels with symptomatic patients having lower levels of tryptophan than healthy controls (mean difference of 10.3µmol/L, 96% CI 0.56 to 20.0, p=0.035) while the asymptomatic patients had no difference to controls (p=0.557) see Fig. 5.5 Panel A.

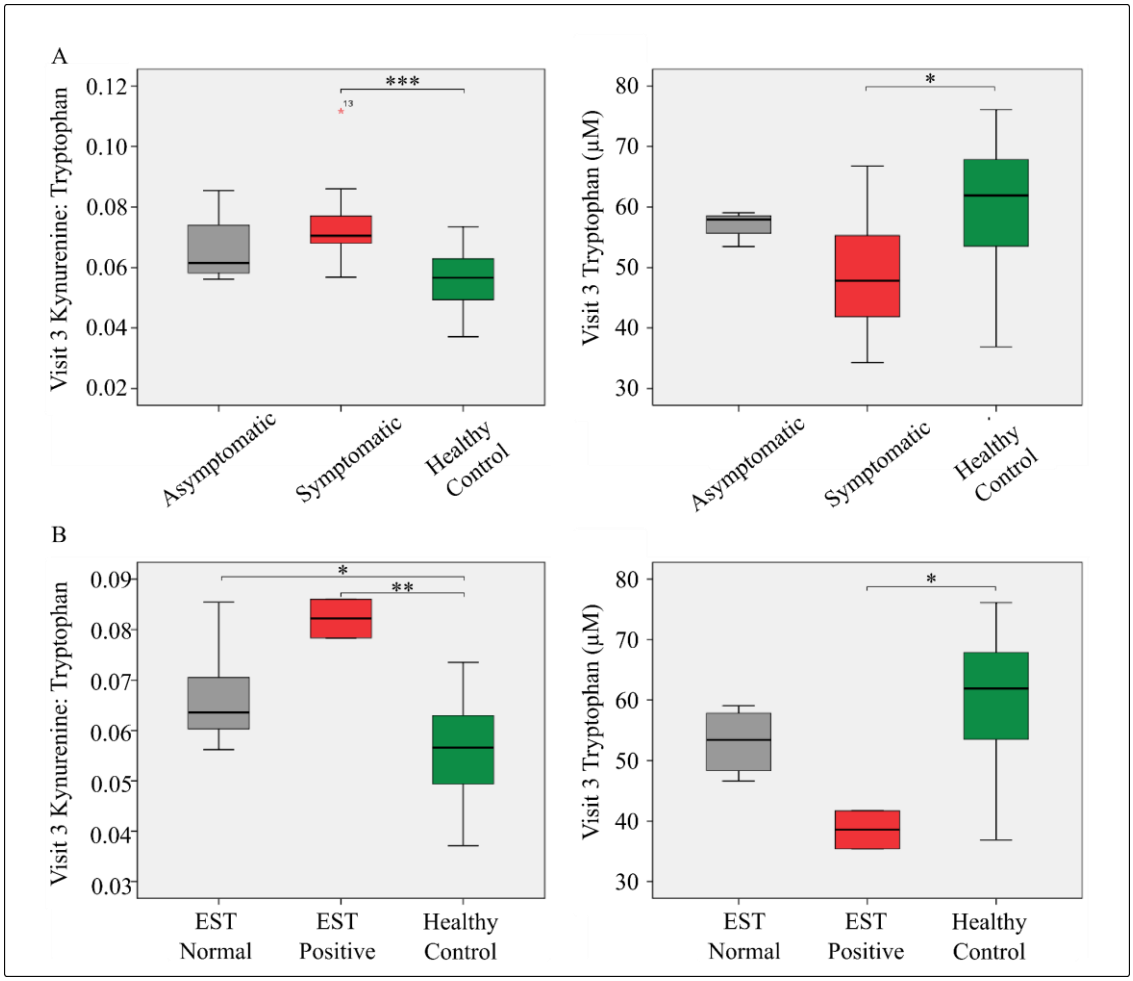


Figure 5.5 A. Comparing KTR and Tryptophan concentrations in CSX patients with and without self-reported symptoms (by PLS on the SAQ) and healthy controls at Visit 3. **B**. Comparing KTR and Tryptophan concentrations between healthy controls and CSX patients with and without electrically positive EST's at visit 3.

Only 2 patients had electrically positive stress tests at the end of follow-up. Unlike at visit 2, however, these patients had the highest KTR and this was significantly different from controls (mean difference 0.026, 95%CI 0.008 to 0.045, p=0.003). They also had the lowest levels of plasma tryptophan (mean difference 20.65, 95% CI -2.9 to -38.4µmol/L, p=0.019) as shown in Figure 5.5 Panel B above.

5.10 Correlations

Despite the differences between groups noted above, tryptophan and its metabolites did not significantly correlate with disease severity as assessed by SAQ parameters or EST parameters, nor did they correlate with perceived stress. When compared with the markers of vascular activation investigated in chapter 3, however, KTR was found to weakly correlate with both serum ICAM-1 and VCAM-1 at baseline across both groups (r_s=0.358, df=35, p=0.030 and r_s=0.318, df=36, p=0.052 respectively). There was no correlation noted between KTR and acute phase proteins. There was a significant correlation noted between KTR and IL-6 (r_s=0.478, df=36, p=0.002) but not with IL-1 or IFNγ (for methods of IL-6, IFNγ and IL-1 assessment see chapter 4).

5.11 LCSX

The LCSX patients did not differ from controls in terms of plasma tryptophan at baseline, although there was a slight trend towards a lower tryptophan (H=12.5, n=38, p=0.09) in LCSX patients. There was no significant difference in terms of Kynurenine levels or Kynurenic acid concentrations but interestingly LCSX patients still had significantly higher KTRs than healthy controls (H=18.0 n=38, p=0.005). At follow-up, however, the LCSX patients had normal Tryptophan concentrations and normal KTRs.

5.12 ROC

KTR was an excellent differentiating marker to separate CSX cases from healthy controls with an AUC of 0.835 and p<0.001. A value of 0.074 gave a 65% sensitivity and 100% specficity in our sample. It did not do a good job, however, of differentiating LCSX

from CSX patients meaning that its clinical utility in the differentiation of patient cohorts with chest pain and normal coronary arteries is poor.

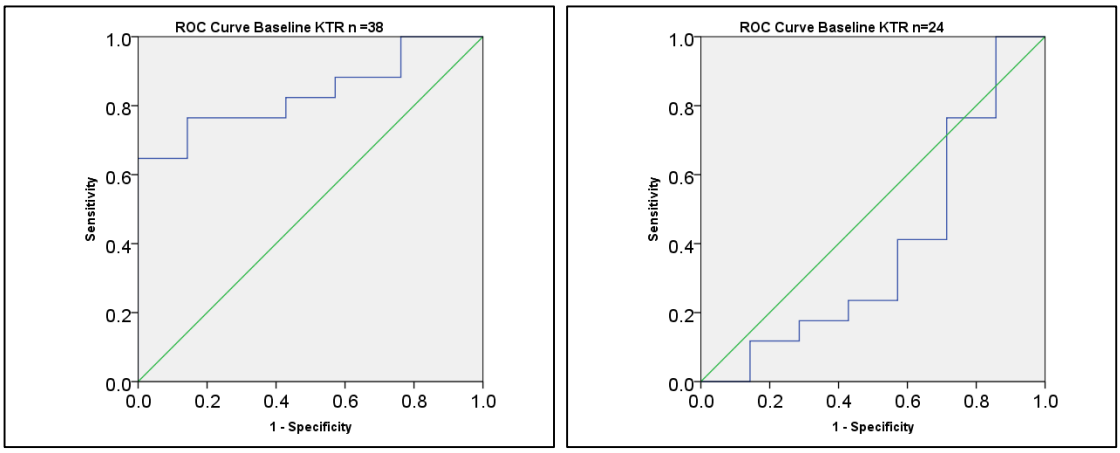


Figure 5.6: ROC curves using KTR to select out patients with CSX from the 38 patient cohort of CSX and healthy controls (left) and the 24 patient LCSX/CSX cohort (right).

Discussion

5.12 Tryptophan Metabolism in CSX

This chapter illustrates the novel finding of increased activity of the enzyme Indoleamine-2,3-dioxygenase in a CSX population. As has been shown in the previous 2 chapters, this CSX cohort has elevated levels of acute phase reactants, endothelial adhesion molecules and type 1 cytokines. The type 1 cytokines (IFNγ, TNFα and IL-6 in particular) are known to upregulate IDO activity and so, given this degree of active inflammation, it is not surprising to find increased IDO activity in CSX patients as evidenced by an increased KTR. Specifically, due to IDO activation our CSX study population had relatively low plasma tryptophan and high plasma kynurenine concentrations. This may help to explain two of the main features of CSX, viz. endothelial dysfunction and a high prevalence of psychological comorbidities.

It is possible that the increased IDO activity is just an innocent byproduct of inflammation in CSX and plays no role in disease pathogenesis. It is also conceivable that it is merely a feedback phenomenon attempting to restore inflammatory balance to the body as IDO has been shown to be necessary in developing immune tolerance and may reduce immune effector cell activity as discussed in the introduction. On the other hand, it may be possible that the products of the kynurenine pathway, upregulated by IDO induction, play a biologically active role in the pathogenesis of CSX. In this study, it appears that IDO activity may be related to disease activity as patients with resolution of angina at follow-up no longer had significantly elevated KTRs when compared with healthy controls, although this may be a function of sample size as the KTR does not correlate directly with symptom severity.

The prognostic implications of increased IDO activity is also established with increased plasma and urinary KTR being associated with adverse cardiovascular outcomes while also predicting future acute coronary events in older adults[246-248]. While early studies in CSX indicated a relatively benign prognosis, studies are increasingly showing a greater incidence of cardiovascular events in CSX with one large study showing a 7.9% five year annualized event rate[102]. The increased KTR seen in our patients may identify patients with increased medium-term cardiovascular risk but the duration of follow-up was insufficient to investigate this possibility.

The upregulation of IDO activity and consequent increased of KTR has two main implications in CSX. Firstly, the increased degradation of tryptophan, via its shuttling down the kynurenine pathway, reduces its bioavailability as a precursor for the production of serotonin and melatonin. Secondly, increased IDO activity increases the concentrations of kynurenine pathway metabolites, which may be biologically significantly active in CSX. We will deal with both of these aspects separately.

5.12.1 Reduced Methoxyindole Pathway Products (5-HT and melatonin)

Altered melatonin handling has been previously observed in CSX cohorts. A small study of only 5 CSX patients showed a significant reduction in both initial and peak melatonin levels in the CSX group compared with a healthy control group[249]. The authors believed this to be due to reduced pineal sensitivity to sympathetic inputs due to the tonic activation of the sympathetic nervous system and autonomic imbalance in CSX but increased IDO activity would also explain this finding. Melatonin has been shown to have several vascular effects being able to induce both vasodilation and vasoconstriction in various tissues. In general, the MT_1 receptor mediates vasoconstriction and MT_2 vasodilation. It seems to have a predominantly vasoconstrictor effect on coronary arteries but oral supplementation of sustained release melatonin compounds has demonstrated an ability to reduce peripheral blood pressure by as much as 6.1/3.5mmHg[250].

Further cardiovascular benefits of melatonin include its potent anti-oxidant effects. Melatonin can act as a scavenger for reactive oxygen species, including the hydroxyl radical (OH·) and the peroxynitrite moiety (ONOO·). It also upregulates superoxide dismutase, glutathione reductase and other anti-oxidants. This has been shown to reduce the amount of lipid peroxidation and has beneficial effects in reducing the oxidation of LDL, a substance implicated in atherogenesis, and in minimising the cardiotoxic side effects of anthracyclines. Furthermore it has been shown to reduce the overall total serum cholesterol and to increase HDL concentrations[251]. These effects are also believed to reduce the reperfusion damage and dysrhythmias seen after episodes of myocardial ischaemia[252].

Apart from its anti-oxidant effects, melatonin is also atheroprotective through its anti-inflammatory effect. It has been shown to reduce the expression of vascular adhesion molecules, reduce leucocyte rolling and adhesion, inhibit cyclooxygenase 2 (COX2) and

to inhibit NFκB[253-255]. Finally, melatonin has also been shown to be protective against endothelial cell senescence through the maintenance of sirtuin and eNOS activity[256]. In addition, it also prevents the opening of mitochondrial permeability transition pores, which are responsible for initiating the apoptotic cascade[257]. Thus, reduced plasma concentrations of melatonin may contribute in several diverse ways to the endothelial and microvascular dysfunction that is characteristic of CSX.

Similarly, reduced serotonin bioavailability (which could be reasonably inferred from reduced tryptophan and increased kynurenine levels) may also explain several of the psychological features of CSX. Our population have increased perceived life stress scores and disproportionately impaired quality of life while CSX patients in general have been shown to have severe burdens of anxiety and depression. Centrally, the serotonergic nervous system modulates mood and behaviour and is implicated in many neuropsychiatric conditions such as anxiety and depression. Indeed, depression has been linked to tryptophan depletion and reduced availability of serotonin. Notably, depression and heart disease often co-exist and are linked bidirectionally. That is to say that heart disease frequently causes depression while depression is known to increase a patient's risk of heart disease. The common link between these two disease states may be inflammation[258]. Similarly, anxiety states are also associated with the upregulation of the HPA axis and altered serotonin and noradrenaline metabolism.

In addition to its effects in the CNS, serotonin may also play a role in visceral hypersensitivity, a potential factor in CSX[259]. This has been explored with respect to Irritable Bowel Syndrome where 5-HT_3 and 5-HT_4 receptors have been implicated[260]. Use of 5-HT_3 antagonists in IBS appeared to reduce sensation to painful colonic stimuli through peripheral and central actions while tricyclic antidepressants have also been shown to reduce visceral hypersensitivity[261-263]. Finally it should be noted that serotonin is a free radical scavenger and as such may reduce endothelial oxidative

stress, although further studies show that its breakdown by MAOA may in fact lead to the formation of reactive oxygen species[264].

5.12.2 Biological activity of Kynurenine Pathway metabolites

It is possible that the products of the kynurenine pathway are themselves biologically important in the pathogenesis of CSX, specifically in the induction of oxidative stress and endothelial dysfunction as IDO activity has been demonstrated in endothelial cells themselves. Figure 5.7 below illustrates the possible roles of kynurenine pathway products in the induction of endothelial dysfunction in CSX.

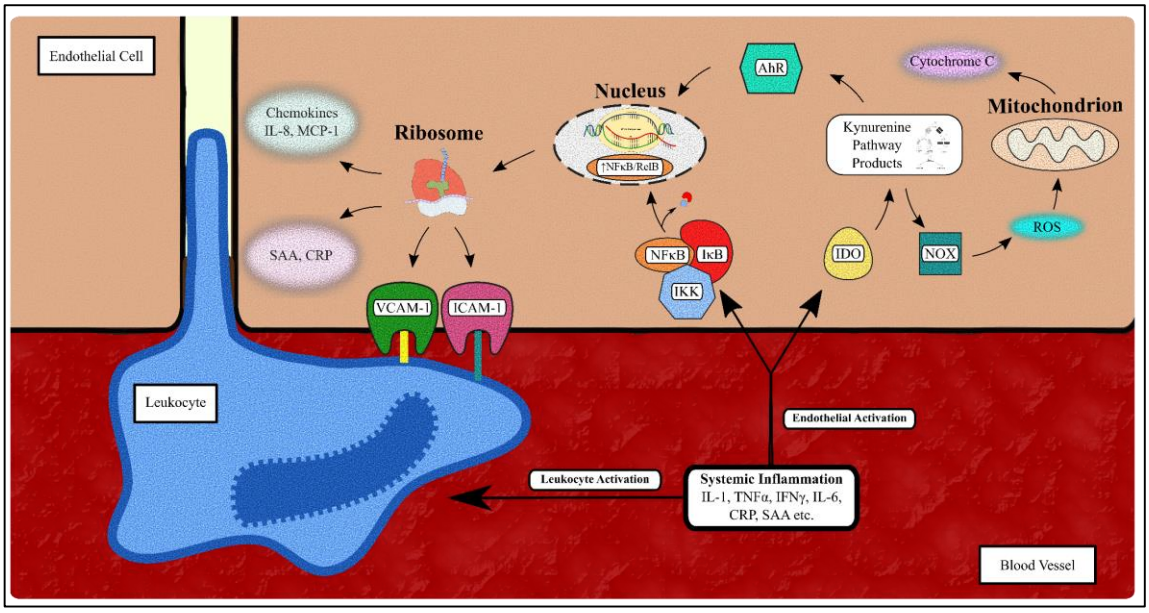


Figure 5.7 Cross-section of the blood-endothelial interface illustrating the possible role of endothelial IDO in the induction of endothelial activation and dysfunction. AhR- Aryl-Hydrocarbon Receptor. NOX- NADPH Oxidase. ROS- Reactive Oxygen Species.

Kynurenine, as well as being an endothelium-derived relaxing factor through the activation of Kv7 channels in the vascular smooth muscle cells, has been shown to be an endogenous agonist of the aryl hydrocarbon receptor (AhR) [265-267]. Activation of this receptor initiates an endothelial intracellular cascade involving NFκB and results in endothelial activation with a pro-inflammatory and pro-oxidant phenotype[267-269].

Kynurenic Acid (KYNA) is formed from KYN via the action of kynurenine aminotransferase and was found to be significantly reduced in our CSX population. KYNA appears to be a vascular protecting agent, being able to scavenge reactive oxygen species, thereby reducing the endothelial dysfunction caused by oxidative stress. It has also been demonstrated to minimise the deleterious effects of homocysteine-induced endothelial dysfunction, possibly through the inhibition of vascular NMDA receptors[270-272]. Thus, its reduced bioavailability may contribute to the pathogenesis of CSX.

The second intermediate on the kynurenine pathway, 3-hydroxykynurenine (3-HK), is produced locally in endothelial cells and strongly induces the production of reactive oxygen species through the upregulation of endothelial NADPH oxidase (NOX) activity[273]. These ROS can then cause cellular dysfunction with increased vascular inflammation and activation. Most importantly, 3-HK has been shown to be a potent trigger of endothelial apoptosis[273]. It achieves this by triggering the release of mitochondrial cytochrome c with a resultant cascade triggering apoptosis. We did not, however, measure plasma levels of this metabolite in our cohort.

Like methoxyindole products, several members of the kynurenine pathway have also been implicated in depression and anxiety. Many of these substances have important neurological effects. Specific products such as 3-hydroxykynurenine and quinolinic acid have been shown to be directly neurotoxic through N-methyl-D-aspartate (NMDA) receptor agonism and oxidative stress induction while kynurenic acid is neuroprotective, being an NMDA antagonist. Animal models show elevated KTR in anxiety/depression while human studies show similar results[274]. Increased kynurenine has also been implicated in post-natal depression while increased peripheral kynurenine concentrations has been shown to correlate directly with the magnitude of anxiety symptoms[275,276]. Somatization has also been linked to abnormalities of

tryptophan metabolism, correlating positively with the KTR and Kynurenine concentration and negatively with tryptophan[243]. Kynurenine metabolites have also been implicated in the pain transmission in migraine with KYNA having anti-nociceptive effects[277,278].

There are also several immunomodulatory effects of products of the kynurenine pathway. Some have been shown to shift the immune balance from a Type-1 cytokine profile to a type 2 profile via the attenuation of T_h1 cell function. Kynurenine is also known, for example, to increase regulatory T-cell (T_{reg}) formation by inducing FOXP3 expression in undifferentiated T-cells[279]. T_{reg} cells release IL-10 and are important in inducing immune tolerance. Although our CSX patients had elevated IL-5 and IL-10, the clearly elevated TNFα and IFNγ implicate a significant type-1 response despite IDO activity, hinting at an insufficient counter-regulatory effect by IDO induction.

The cause of the pro-inflammatory state in CSX is uncertain but is likely a combination of multiple features including conventional cardiovascular risk factors such as dyslipidaemia. Studies have also investigated the potential role of infective pathogens in CSX and there are mixed reports implicating gastric Helicobacter pylori as a possible agent[62,63,280].

5.13 Limitations

The main limitation of this study is its small sample size, with only 17 suitable CSX patients identified over the course of the recruitment period. This, however, is a result of the stringent application of appropriate exclusion criteria and the necessity for completely normal coronary arteries and convincing angina. Many studies have accepted patients with non-obstructive coronary stenoses (defined as <50%) and angina-like chest pain, a nebulous definition at best. Our approach has ensured a

homogeneous population with a reliable CSX diagnosis but does limit the external validity of the results. Reassuringly, despite this limitation the findings were consistent in a repeated-measures design.

Additionally, exercise stress testing as a tool for investigating ischaemia is sub-optimal as it is subject to inter-observer variability and does have an inherent deficit in sensitivity and specificity for myocardial ischaemia. We attempted to minimize some of these problems by ensuring that the ESTs were reviewed by two independent cardiologists. Ideally, patient recruitment into a study such as this should use state of the art techniques such as perfusion cardiac magnetic resonance imaging or patients could be further stratified according to results of invasive coronary reactivity testing to select patients with demonstrable microvascular dysfunction. Unfortunately, these techniques have a time and cost implication, are not without risk and are not widely available.

Similarly, an opportunity was missed by not taking a more comprehensive psychiatric history (such as the utilization of the structured clinical interview for DSM disorders) as it would have been useful to tie the increased IDO activity to increased prevalence of depressive symptoms etc. The SAQ treatment satisfaction and quality of life domain scores along with the perceived stress scale might give some indication as to the patient's mental wellbeing but a more direct assessment at baseline would have been useful. Similarly, it would have been useful also to measure further tryptophan metabolites, particularly 3-HK, melatonin and serotonin, to be more certain of their possible role in CSX rather than relying on indirect measures of their activity.

Conclusions

Our novel finding of increased IDO activity in CSX raises the possibility of a role for tryptophan metabolism in its pathogenesis. Melatonin lack and kynurenine product effects may contribute to the induction of endothelial dysfunction typical of CSX while the reduced production of serotonin may account for the psychological comorbidities commonly seen in these patients. The general finding at follow-up that symptomatic CSX patients rather than asymptomatic patients were more likely to have higher IDO activity than healthy controls may indicate that increased IDO activity is a state marker in CSX. This must be considered with the fact that there was no observed direct correlation between the degree of IDO activity and the severity of symptoms. The possibility of a role of kynurenine pathway products in the endothelial dysfunction seen in CSX merits further research while the role of tryptophan metabolism in nociception in CSX also warrants attention.



Chapter 6: The microRNA Transcriptome in Cardiac Syndrome X

Introduction

6.1 Introduction to microRNA

MicroRNAs (or miRNA) are short, non-coding ribonucleic acids between 21-23 nucleotides in length. They were initially identified in *Caenorhabditis elegans*, a nematode, in 1993 but it wasn't until 8 years later that a mammalian example of a miRNA was demonstrated. About 2555 distinct human miRNAs had been identified (as of September 2013) but recent studies have expanded this number greatly. These small molecules are transcribed from their own "genes". They post-transcriptionally modulate the function of 30% (but possibly up to 60%) of our genes by altering mRNA stability and transcription. Dysregulation of miRNA has been shown in many cardiovascular disease states. miRNAs have also been shown to be essential in cardiac development and 18 miRNAs constitute 90% of the myocardial miRNA population. The vascular endothelium is also a hotbed of miRNA activity and this is affected by local blood flow and shear stress and has many effects on endothelial function. To date, nothing is known of miRNA activity in CSX.

6.1.1 miRNA Biosynthesis

1. Transcription

Most miRNAs are transcribed in the nucleus from DNA by RNA polymerase II. The code for about 50% of miRNAs is found in so-called "non-coding" regions of DNA. A further 40% are coded by sequences found in the introns of genes coding for protein synthesis. These intronic miRNAs, also known as mirtrons, can be transcribed independently of the host gene through the action of different promoters. The transcription of the miRNAs is controlled by regular transcription factors (TF), which are upregulated in response to various cellular stimuli. Transcription is subject to many other regulatory processes including epigenetic phenomena such as hypermethylation and histone deacetylation. Additionally, it has also been demonstrated that miRNAs can initiate negative feedback against their own TFs as well as initiating positive feedback

pathways in other instances. This first step results in the formation of the **primary transcript** (pri-miRNA), complete with a 5' cap and a poly-A tail and may be thousands of nucleotides in length (c500-3000bp usually).

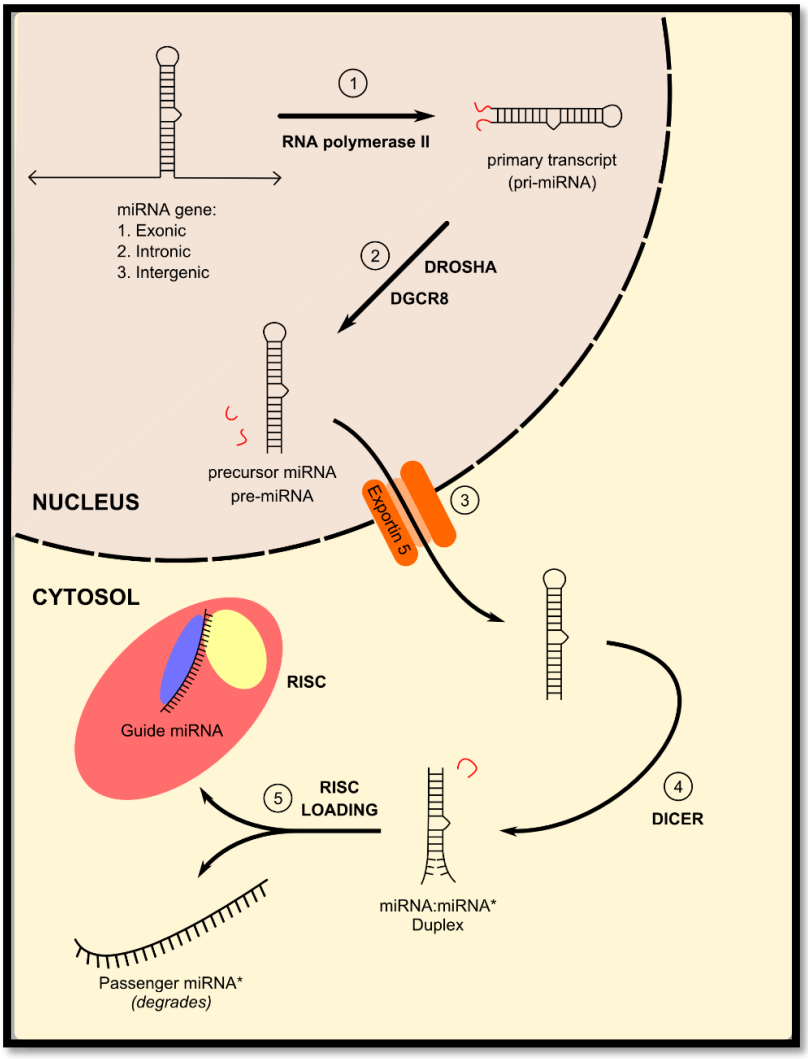


Figure 6.1: Schematic representation of miRNA biosynthesis

2. Intranuclear processing

The pri-miRNA is then cleaved by the DROSHA complex, which includes the DROSHA RNase III enzyme and DGCR8 amongst others. This process may occur directly following or even during transcription of the miRNA. DROSHA cleaves off a large portion of the pri-miRNA, creating a 60-100nt RNA strand that includes a hairpin and a 2nt 3'

overhang. This is known as **precursor miRNA** (pre-miRNA). The exact site of DROSHA action is important to produce viable pre-miRNA strands. The fidelity of this process is improved by the other proteins resident in the DROSHA complex, which help to direct DROSHA to the correct site. DROSHA itself is also subject to regulation by signal transducers such as Smads and p53.

3. Exiting the nucleus

The pre-miRNA is then transported from the nucleus out into the cytoplasm by the exportin 5 transporter, found associated with a GTPase Ran in the nuclear envelope. Studies have shown that this step may be the rate-limiting step in miRNA production.

4. Further cleavage

The final step in the formation of mature miRNA is further cleavage of the pre-miRNA by the Rnase type III Dicer. This occurs in the cytoplasm, possibly in direct association with the formation of a microRNA induced Silencing Complex (RISC, see below). Dicer cleaves off the hairpin loop of the pre-miRNA and leaves a ≈22nt miRNA double strand comprised of the guide miRNA, destined for incorporation into the RISC, and the passenger strand (or miRNA*) that is usually destined for degradation.

5. RISC loading

The selection of which strand becomes miRNA and which is miRNA* appears to be partly determined by their relative thermodynamic stability, with the miRNA being more stable and being preferentially loaded into the RISC. The mature miRNA-RISC complex is now ready to perform its function.

6.1.2 Functions of miRNA

miRNAs are involved in the fine tuning of the expression of some genes. They affect this by altering the stability and functionality of the mRNA produced by gene transcription, thereby regulating ultimate gene expression. The miRNAs include a seed

sequence at nucleotides 2-7, which confer a specificity of targeting to each miRNA. The seed sequence is complementary to a sequence on the target mRNA and allows miRNA:mRNA interaction through Watson-Crick base pairing. The target sequence is usually, but not exclusively, found in the 3' untranslated region (3'UTR) of the mRNA, although some are found in the 5'UTR and in some cases non-seed dependant miRNA:mRNA interaction has been shown to occur. Despite this seed-sequence specificity, a single miRNA can affect multiple mRNAs and multiple miRNAs can affect a single mRNA. This leads to a complex network of interactions. The miRNA:mRNA interaction takes place in association with the RNA-induced Silencing complex (RISC) and this regulates the effector phase of mRNA function.

***R**NA-**I**nduced **S**ilencing **C**omplex formation*

The RISC is found in the cytoplasm and incorporates the guide strand from the mature miRNA. Loading of the guide strand into the RISC can occur immediately following Dicer action on the pre-miRNA. Of note, RISCs can incorporate other types of non-coding RNAs such as siRNA, which all perform the same function, namely conferring target specificity. The simplest RISC capable of carrying out RNA degradation consists merely of the miRNA associated with an Argonaute protein. The Argonaute proteins are a family of proteins ubiquitous in plants and animals and are found in the nucleus and cytoplasm. Four argonaute genes are found in humans. Most RISCs, however, will also incorporate other proteins, which can modify RISC activity. The guide miRNA sits in a groove in the Argonaute protein and the target mRNA strand is first bound by the seed sequence of the miRNA, with the phosphate backbone then interacting with the Argonaute in a non-specific manner. This now forms a fully loaded RISC with miRNA:mRNA binding (see Fig 6.2).

RISC effector activity

The primed RISC usually then leads to a downregulation of the gene target by degrading mRNA though some RISCs have been shown to have an upregulatory effect

on gene function. The downregulation may be affected by preventing translation or by slicing/hydrolysing the mRNA. The latter requires significantly more extensive Watson-crick binding between miRNA and mRNA, thereby ensuring only the intended target mRNA is in fact sliced. Argonoaute 2 is capable of catalyzing this process and slices the target mRNA just distal to the portion bound to the seed sequence of the complementary miRNA (i.e. between bases 10 and 11, with the seed sequence bound from base 2-7.) The resultant sliced mRNA is further degraded by exonucleases found in the cell. Translational repression involves the prevention of the protein-protein interaction required by the ribosome to allow translation. Furthermore, the RISC containing the mRNA may localize to p-bodies found in the cytoplasm, thereby sequestering the mRNA and preventing translation. Note also that miRNA may prevent gene transcription ab initio in a process called RITS (RNA-induced Transcriptional Silencing), although like mRNA cleavage, this requires more sequence complementarity between the miRNA and the DNA and is more often carried out by siRNAs.

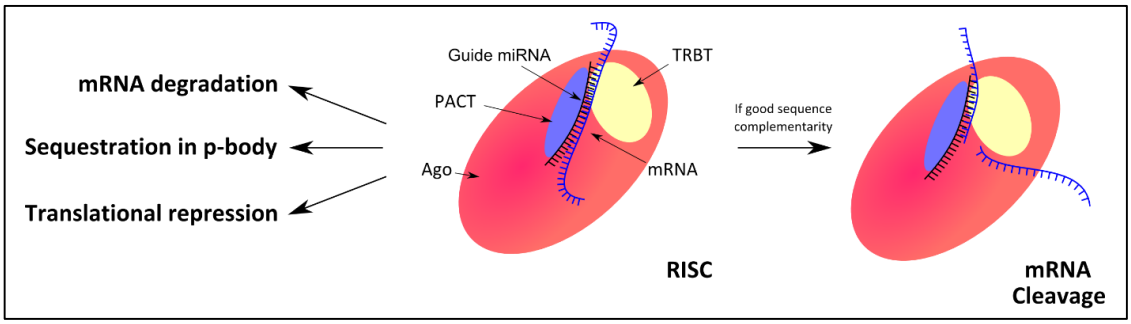


Figure 6.2: RNA-Induced Silencing Complex (RISC) Effects. Ago- Argonaute 2, PACT- Protein Kinase RNA activator, TRBP- Transactivation Response RNA Binding Protein

The overall effect of the miRNA depends upon the gene that has been regulated. miRNAs play an important role in the development and maintenance of a healthy cardiovascular system in particular and their role in the pathogenesis of many cardiovascular maladies is now being elucidated.

6.1.3 Release of miRNA from the cell

As described above, the miRNA is transcribed initially in the nucleus before being exported to the cytoplasm, where it associates with Argonaute proteins to become the RISC. miRNA, pre-miRNA and RISC can then be packaged inside multivesicular bodies. The exact means by which the majority of miRNAs enter the circulation is unknown but potential mechanisms are shown in figure 6.3 below.

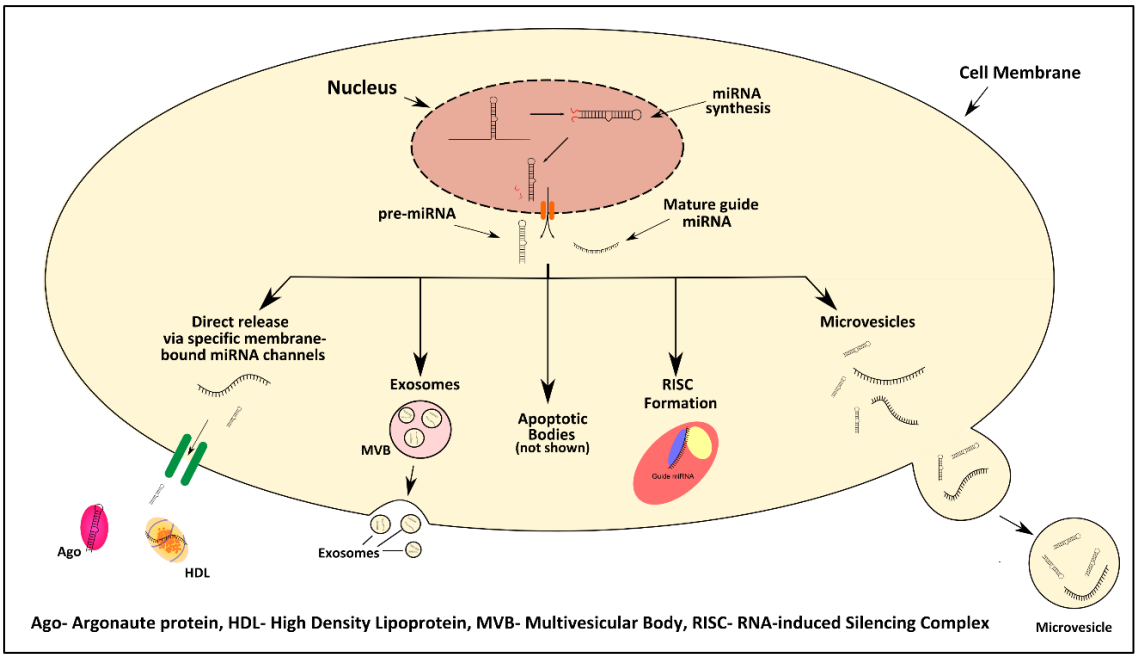


Figure 6.3: Fate of miRNAs and possible mechanisms for their cellular release

Kosaka et al demonstrated that release might be coupled to a ceramide-dependant process [281]. Despite the uncertainty as to the exact mechanism of cellular extrusion of miRNA, what does appear clear is that this process is regulated. Pigati et al showed that cells could selectively modify the miRNA content of exosomes [282]. They showed that about two thirds of miRNAs were found in exosomes at concentrations approximating their intracellular concentration, indicating that the majority of miRNA release is passive. In their model of breast cancer, however, they showed that the malignant cells secreted almost 90% of the miR-451 contained within the cell, while releasing only 2% of the most-abundant miRNA in the cells, miR-72. The method of this

control is uncertain but does show that cells can selectively release miRNAs in different quantities.

6.1.4 Circulating miRNA

miRNAs have been detected in many components of blood, viz. platelets, erythrocytes and plasma. Their presence in plasma is surprising as the circulating ribonuclease (RNase) enzyme is present in concentrations adequate to neutralize any unprotected RNA. What is even more surprising is just how robust plasma miRNA actually is, being stable even at the extremes of pH as well as when boiled or frozen. miRNAs do not have an innate resilience to RNase action. The protection is conferred to miRNA by the manner in which it is transported in plasma. Three main methods of transportation have been identified recently.

a. Microparticles

   miRNAs can be enclosed in microparticles released from cells. These particles vary in size from 50nm to 2μm. At the smaller end of the scale they are termed exosomes (from 50-100nm). Slightly larger particles are termed microvesicles (0.1-1.0μm) while the largest are apoptotic bodies (0.5-2.0μm). All of these microparticles retain surface markers of their cell of origin and are filled with cytoplasm that can contain cytokines, RNA, miRNA and other chemicals. In general, their contents are pro-inflammatory. They are capable of binding to cell-surface receptors of other cells and can then be internalized. In this way miRNA may be released from a particular cell, move through the circulation in a protected environment before subsequently being taken-up by a distant cell where the miRNA could perform its usual effects by binding to ubiquitous Argonaute proteins. Microparticles (MPs) appear to be an important mode of miRNA transport as Diehl et al showed that when plasma was depleted of MPs, the absolute numbers of miRNAs present in the plasma was reduced by over 50% [283].

b. Protein bound

miRNAs also associate with circulating proteins, which protect them from RNase action. Arroyo et al determined that up to 90% of plasma miRNA might not be found in vesicles, which is at variance with Diehl's data above [284]. They used size-exclusion chromatography (SEC) and differential centrifugation to first show that the majority of miRNA was likely not membrane-bound before demonstrating that the miRNA was susceptible to protease action on the plasma samples indicating that the miRNA was indeed protein bound. They went on to show using immunoprecipitation that the main protein associated with miRNA in plasma was Argonaute 2 (the main protein found in the RISC). This raises the possibility that cells secrete RISCs directly into the circulation. Finally, Arroyo et al found that certain miRNAs were exclusively found in vesicles and they hypothesized that the differential release of miRNA into vesicle bound and unbound populations represented a cell-type-specific method of miRNA expression.

c. Lipoprotein bound

Nucleic acids are known to bind to certain lipids. Phosphatidylcholine in particular can form stable ternary complexes with nucleic acids. This has been exploited as lipid-vehicles have been used to deliver nucleic acids to cells in vitro and in vivo. Vickers et al showed that human HDL contains populations of miRNAs in proportions that are altered in disease states[285]. They also found that LDL-associated miRNA more closely resembled exosome-associated miRNA than the HDL-miRNA did. Finally, they showed that the HDL-mediated miRNA delivery to other cells depended on cellular uptake by the SR-BI scavenger receptor.

Circulating miRNA is therefore protected from degradation by the membrane of microparticles, by their close association to proteins and by their propensity to bind with circulating lipoproteins. Plasma miRNA levels may be readily measured and their possible use as biomarkers in disease states is attractive.

6.2 miRNAs in endothelial cells

The endothelium is a cellular monolayer that lines all blood vessels in the body. It is responsible for regulating vascular tone, localizing blood constituents to the site of injury and inflammation and is a very active secretory tissue, being able to release NO and many other important biologically active substances. For a full review of endothelial cell (EC) function see chapter 1.4. ECs may produce an abundance of miRNAs, which can alter the local cellular behaviour. One important factor that may regulate the local production of miRNA is the local blood flow specifically the nature of endothelial stresses. As was described in 1.4.3, the endothelium may be exposed to laminar shear stress or oscillatory shear stress. The endothelium is nominally adapted to the former and the latter induces a pro-inflammatory phenotype locally through the downregulation of Kruppel-Like Factors (KLF) 2 and 4 with resultant activation of NFκB. Many miRNAs are modulated by local shear stress. A sample of these is listed in table 6.1 below.

miRNA	Effect
Induced by Laminar Stress	
miR-10a	↓ NFκB activation
miR-21	↑ eNOS, ↓ apoptosis
miR-126	↓ VCAM-1
miR-143	↓ VSMC activation
miR-155	↓ eNOS, ↓ EC activation
Induced by Oscillatory Stress	
miR-221/222	↓ eNOS
miR-92a	↓ KLF2 activity
miR-23b	↓ EC proliferation
miR-21	↑ VCAM and MCP1↓ SOD
miR-33	↓ ABCA1,↓ HDL formation
miR-217	↓ SIRT1 activity,↑ Senescence

ABCA- ATP-binding cassette transporter; **eNOS**- endothelial Nitric Oxide Synthase; **EC-** Endothelial Cell; **HDL-** High Density Lipoprotein; **KLF-** Krüppel-like Factor; **MCP**-Monocyte Chemoattractant Protein; **NFκB**- Nuclear Factor κ B; **SIRT**- silent mating type information regulation 2 homolog; **SOD-** Superoxide Dismutase; **VSMC-** Vascular Smooth Muscle Cell; **VCAM**-Vascular Cell Adhesion Molecule.

Table 6.1: miRNAs regulated by endothelial shear stress

6.2.1 miRNA and Endothelial Inflammation

As discussed in 1.4.3, the endothelium plays an important role in the inflammatory response. It recruits immune effector cells to local target tissues via the expression of surface molecules. These allow adherence and translocation of immune cells across the endothelium. It is also capable of secreting pro-inflammatory substances that can affect nearby cells such as VSMCs, which also have an important function in inflammation as they may de-differentiate from contractile cells into secretory cells. Importantly, ECs themselves may be the target of an inflammatory reaction and this is at the heart of many disease processes, such as atherosclerosis. Predictably, miRNAs have been shown to be important in endothelial inflammation.

NFκB regulation

Levels of miRNA-10a and -10b are reduced in atheroprone vascular regions and knock-in of miR-10a in mice leads to reduced expression of surface inflammatory molecules on ECs. It is believed that miR-10a targets two enzymes (MAP3K7 and βTRC) that are important in the activation of IKK, an enzyme that removes the IκB-inhibitory unit from NFκB, while 10b targets MAP3K7 only. By preventing the activation of NFκB in this way, miR-10 reduces the expression of many inflammatory substances in ECs (such as Selectins, ICAM, VCAM, MCP-1 and MMPs), thereby reducing atheroma formation. Laminar shear stress, predictably, has been shown to increase miR-10a expression. A recent study in apo-E deficient mice showed that systemic delivery of miRNA-181b inhibited NFκB activity through the inhibition of importin-α3, a protein required for the transport of NFκB into the nucleus in endothelial cells[286].

ETS regulation

E26 transformation-specific sequences (ETS) are a family of transcription factors that are heavily involved in vascular inflammation and are activated in response to a plethora of stimuli, including IL-1β, Angiotensin II and TNF-α. ETS can then in turn trigger many downstream targets such as VCAM-1, MCP-1 and MMP, thereby

contributing to the pro-inflammatory phenotype exhibited by ECs activated by pro-inflammatory stimuli. Interestingly, Ets-1 also triggers transcription of miR-126, which can inhibit translation of VCAM-1 mRNA.[287,288]. miR-126 levels are reduced in patients with established CAD and reduced levels are associated with increased leukocyte adherence to ECs with an increased expression of VCAM-1. Overexpression of miR-126, on the other hand, was shown to be atheroprotective in mice. Other regulators of Ets-1 are the miRNA-221/222 cluster, miR-200b and miR-155. These directly target Ets-1 and, as such, can control the EC response to stimuli such as Angiotensin II. In addition, miR-155 also targets the AT1R. Like with miR-126 above, circulating levels of miR-155 are reduced in patients with CAD.

Regulation of Inflammatory Cell Adhesion

miR-31 and miR-17-3p have been shown to reduce neutrophil adhesion to ECs[289]. They blunt the response of the ECs to TNFα by reducing the expression of E-selectin and VCAM-1 respectively. miR-663 was shown to increase monocyte adhesion in areas with increased oscillatory shear stress[290]. miR-21 has also been shown to be induced by Oscillatory stress and increases VCAM and ICAM expression by inhibiting PPARα with resultant derepression of Activator Protein 1[291]. Finally, Endothelin-1 increases the adherence of neutrophils to ECs and is targeted by miR-125a and -125b. The exact role of this pathway is yet to be characterised.

miRNA	Targets	Consequence
miR-10a	MAP3K7, BTRC } NFκB activation	Reduced IKK activity and NFκB activation. Reduced P-selectin, E-selectin, ICAM-1, VCAM-1 MCP-1, MMP, IL-6 and IL-8.
miR-221/222	ETS-1	Reduced Ets-1 activity. Reduced transcription of VCAM-1, MCP-1 and MMP
miR-155	ETS-1 AT1R	Reduced ETS-1 activity Reduced EC activation by Angiotensin II
miR-31	SELE	Reduced E-Selectin expression Reduced neutrophil adhesion
miR-126	VCAM-1	Reduced VCAM-1 expression Reduced Neutrophil adhesion
miR-17-3p	VCAM-1	Reduced VCAM-1 expression Reduced Neutrophil adhesion
miR-21	PPARα	Increased AP-1 activation Increased VCAM-1 and MCP1

AP1- Activator protein 1**; AT1R**- Angiotensin II Receptor Type 1; **BTRC**- β-Transducin Repeat Containing Gene; **ETS-1**- E26 Transformation-specific sequence; **ICAM-1**- Intercellular Adhesion Molecule 1; **MAP3K7-** Mitogen Activated Kinase Kinase Kinase 7; **MCP**-Monocyte Chemoattractant Protein; **MMP**- Matrix Metalloproteinase; **NFκB**- Nuclear Factor κ B; **PPARα-** Peroxisome Proliferator Activated Receptor **SELE-** Selectin E; **VCAM**-Vascular Cell Adhesion Molecule.

Table 6.2: miRNAs affecting endothelial inflammatory activation

6.2.2 miRNA and Krüppel-like Factors

KLFs are zinc-finger containing transcription factors that play an important role in linking mechanotransduction to EC genomic activity. Their nomenclature was derived from the German for “cripple” as the Krüppel factors were originally found in Drosophila and when absent led to severe deformation of the thorax. They are numbered chronologically following their discovery. KLF2 and 4 are abundantly expressed in EC and their activity regulates many important flow-dependant processes. KLF2 is typical of KLFs. Its production in stimulated by laminar shear stress, which activates a MAPK/ERK/MEF2 pathway, and by statins, which lead to increased transcription of KLF2. The KLF2 then acts in the nucleus and regulates transcription of substances involved in inflammation, thrombosis and vasodilatation. The overall result is the induction of endothelial activity that prevents local inflammation and thrombosis and which promotes vasodilatation. In essence, KLF2 activity is good for a healthy EC.

KLF4 acts in a similar fashion, but is induced by inflammation whereas KLF2 is inhibited by pro-inflammatory stimuli[292].

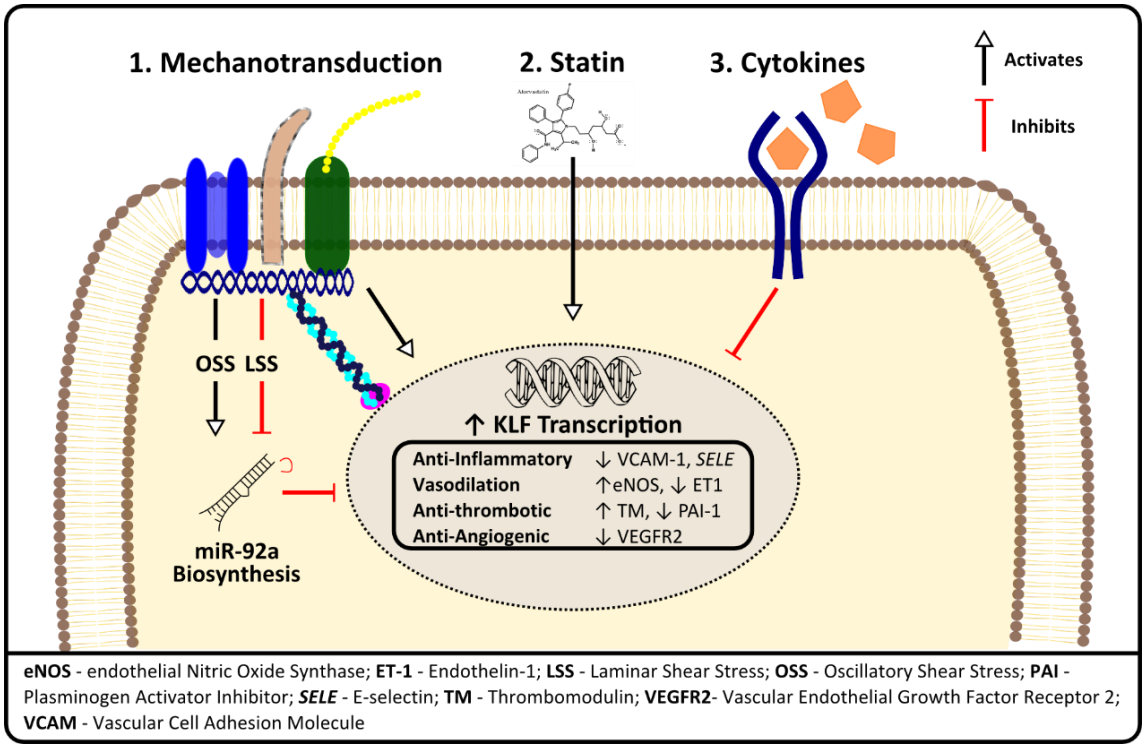


Figure 6.4: KLF Regulation and Effects

In addition to increasing KLF2 expression, laminar shear increases KLF2-mRNA stability in ECs. In an excellent study, Wu et al showed that miR-92a transcription is inhibited by laminar shear stress and that it varies reciprocally with KLF2 levels [293]. They went on to demonstrate that miRNA-92a has seed-region complementarity with a sequence in the 3'-UTR of KLF2-mRNA and that miR-92a forms a RISC that binds the KLF2-mRNA and destines it for degradation. During laminar shear, miR-92a is downregulated and KLF2-mRNA is not targeted and therefore KLF2-mRNA levels are maintained. Wu et al also demonstrated that turbulent flow and oscillatory shear stress upregulated miR-92a levels with a concomitant drop in KLF2 activity. It became clear that miR-92a expression in response to shear stress determined KLF2 activity with many downstream effects. When miR-92a levels were increased using plasmid-transfection,

KLF2-mRNA/protein, eNOS mRNA/protein and thrombomodulin mRNA/protein were all reduced, clearly delineating the pathway.

Inhibition of miR-92a in mice actually decreased atherosclerotic plaque dimensions and reduced macrophage and T-lymphocyte accumulation with an increased collagen component, indicating an increase in plaque stability [294]. These effects were mediated through increased KLF2 and 4 signalling. Similarly, the regulation of KLF4 transcription by miRNAs has also been described as miR-143/145 reduces KLF4 transcription and translation[295]. Finally, it should be noted that KLF2 is also regulated by factors other than miRNAs. TNFα can inhibit MEF2 in the above pathway, preventing KLF2 expression, by modulating NFκB and HDAC activity [296]. It is possible that patients with CSX have reduced KLF2 and/or KLF4 activity as they have reduced vasoreactivity and high levels of markers of vascular injury. The expression of miR-92a and mir-143/145 in CSX has not been described to date although TNFα levels are known to be increased.

6.2.3 miRNA and Oxidative Stress Responses

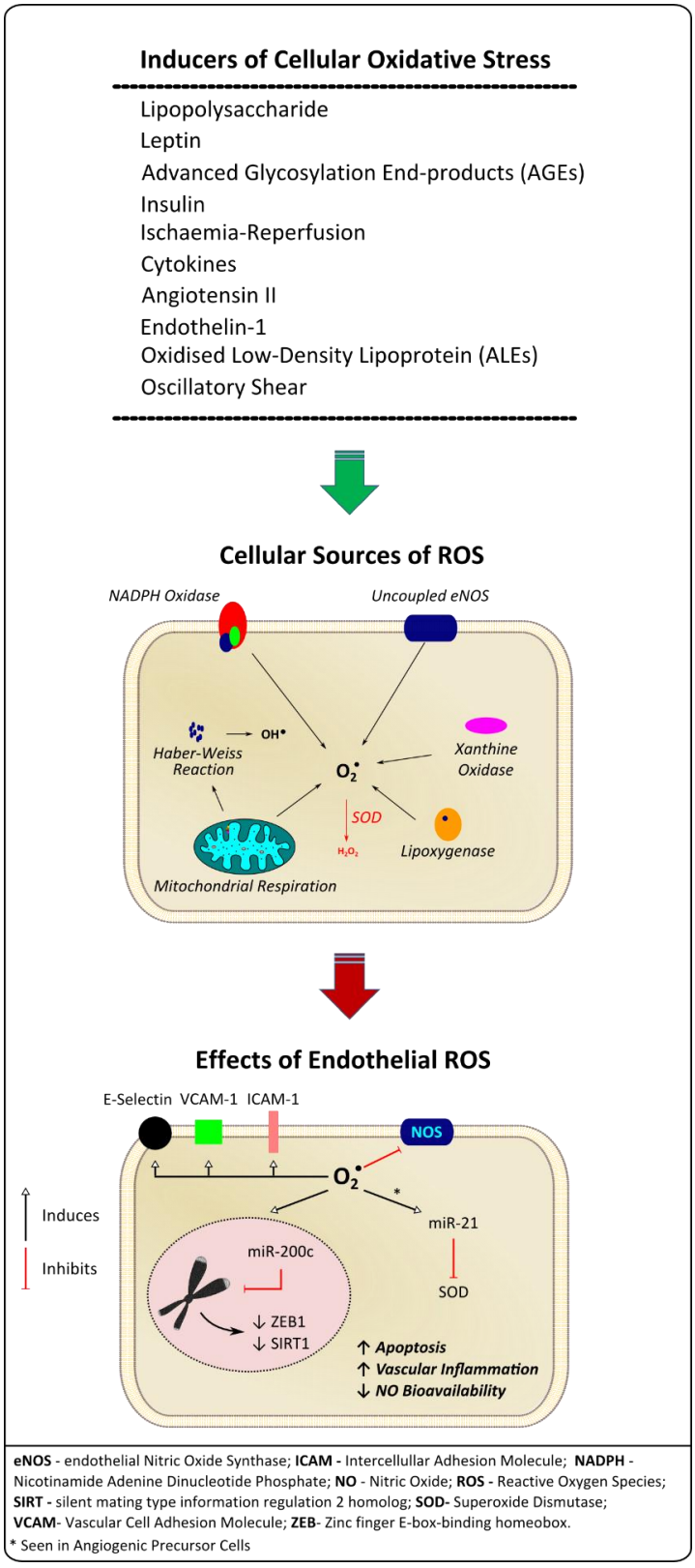


Figure 6.5: Endothelial Oxidative Stress

Reactive Oxygen Species (ROS) are produced as a by-product of normal cellular metabolism. In blood vessels, they may be produced by ECs, VSMCs or tissue macrophages. The main source in ECs is probably plasma membrane NADPH oxidase (NOX) activity, although some quantities may also be produced by the uncoupling of NOS (when tetrahydrobiopterin levels are low). ROS contain an unpaired electron in their outer orbital and are highly reactive. This allows them to damage many surrounding substances such as DNA and proteins. CSX patients are known to have increased markers of oxidative stress, such as maliondialdehyde (MDA) and serum superoxide, and decreased levels of protectors such as superoxide dismutase (SOD) [110,221,222]. Studies have corroborated this by showing that DNA damage is increased in the leucocytes of CSX patients in concert with an increase in oxidative stress [297]. Oxidative Stress leads to endothelial dysfunction and activation.

miRNAs are capable of regulating many processes involved in the EC's response to oxidative stress. Many of the factors involved in this response will be further mentioned in this chapter (ZEB1, SIRT1 etc.) Studies have looked at the alteration of miRNA levels in response to oxidative stress exposure and several miRNAs have been identified as being particularly important. Perhaps the most important are the miR-200 Family. This family consists of five members. miR-200a, miR-200b and miR-429 are located on chromosome 1 and miR-200c and miR-141 are found on chromosome 12. They are known to have a role in the metastasis of cancers, with reductions in their levels being associated with increased epithelial-mesenchymal transition of the malignant cells. The most relevant in the ECs is miR-200c, which is highly expressed in response to oxidative stress. miR-200c then induces cellular senescence or apoptosis through the inhibition of ZEB1, an anti-apoptotic gene.

miR-200a can also target SIRT1 and reduce its activity in response to oxidative stress. Other miRNAs involved in sirtuin regulation (see 6.2.4) may also mediate reduced SIRT1 activity in oxidative stress with resultant endothelial dysfunction. miR-217 and miR-199

might be of particular interest. Finally, miR-21 is induced by oxidative stress and has been shown to reduce SOD activity in angiogenic progenitor cells resulting in reduced NO availability in these cells [298]. It is also upregulated in atherosclerotic plaques.

6.2.4 miRNA and Sirtuin activity

Sirtuins are a family of 7 nicotinamide adenosine dinucleotide (NAD)-dependant deacetylases. They remove acetyl-groups from lysine in certain proteins, thereby altering protein charge and potentially conformation and function. They perform important roles including epigenetic regulation of gene transcription. They can deacetylate histones, increasing lysine positive charge and histone-DNA binding affinity, thereby limiting transcription factor access. Unlike HDACs, sirtuins can also deacetylate many non-histone proteins and have been shown to be important in cellular senescence, whole body metabolism and gene expression. They also happen to be highly expressed in endothelial cells. SIRT1, in particular, is known to have several important cardiovascular effects and is theorised to be the effector molecule for the beneficial effects of calorie restriction on cardiovascular parameters such as blood pressure, insulin sensitivity and lipid profiles. SIRT1's effects on many important cardiovascular pathways are shown below [299-303].

Pathway Affected	Targets	Consequence
Inflammation	Rel A/p65 subunit of NFκB	Reduced vascular inflammation Reduced TNFα, MCP-1 and VCAM-1 Reduced foam cell formation
Metabolic	FOXO1 LXR	Increased insulin sensitivity Improved fasting glucose Improved fasting cholesterol Reduced endothelial senescence
Endothelial Function	MEK5, MEF2 } KLF2 transcription	Increased KLF2 expression Increased eNOS activity Increased thrombomodulin Decreased NFκB transcription Reduced Endothelin-1 production
Nitric Oxide	eNOS K496 & K506	Increased eNOS activity Improved endothelium-dependant vasodilatation Increased SIRT1 expression (positive feedback)
Angiotensin II	Angiotensin receptor	Reduced Nox1 gene expression Reduced VSMC hypertrophy
Senescence	p53 FOXO3a/FOXO4	Prevents cell cycle arrest and endothelial senescence

eNOS- endothelial Nitric Oxide Synthase; **FOXO**- Forkhead Box Protein O; **KLF**- Kruppel-like Factor; **LXR**- Liver X Receptor; **MCP**-Monocyte Chemoattractant Protein; **TNFα**- Tumour Necrosis Factor alpha; **VCAM**-Vascular Cell Adhesion Molecule; **VSMC**- Vascular Smooth Muscle Cell.

Table 6.3: Selected SIRT1 targets with relevance to cardiovascular disease

miRNA regulation of SIRT1

In health, SIRT1 levels change dynamically according to nutritional status to help maintain an appropriate metabolic response. In diseases such as diabetes and obesity, SIRT1 remains suppressed, leading to many inappropriate metabolic behaviours such as reduced insulin sensitivity, increased foam cell formation and increased vascular inflammation, which can result in vascular pathology. Many miRNAs have seed-sequence targets in the 3'-UTR of SIRT1-mRNA and several have been shown to directly regulate SIRT1 expression. The most important of these with respect to endothelial SIRT1 are miR-34a, miR-210 and miR-217[304,305]. These can induce endothelial senescence and Menghini et al showed that miR-217 also reduced NO availablility and FoxO1 activity[306]. Furthermore, miRNAs can control SIRT1 activity by altering the availability of NAD^+ levels. Some miRNAs (e.g. miR-34a and miR-26b) target nicotinamide phospho-ribosyltransferase (NAMPT), an important enzyme in NAD biosynthesis[307]. Further vascular miRNAs capable of regulating SIRT1 include miR-

143/145, miR-199 and miR-200a [308-310]. Interestingly, as sirtuins can control gene expression, it is possible that they can modulate miRNA transcription. At least one such case has been described where SIRT1 regulated the brain-specific miRNA-134 that resulted in altered neuronal plasticity[311]. Given the important role of SIRT1 in endothelial cells, miRNA disruption of its function could play a key role in CSX.

6.2.5 miRNA and eNOS

Endothelial Nitric Oxide Synthase (eNOS or *NOS3*, 7q35-36) is an essential component of the endothelial-dependant vasodilation pathway. It oxidises L-arginine to the gaseous NO, which diffuses rapidly to nearby cells and mediates vasodilatation as well as promoting an anti-apoptotic and anti-proliferative environment around the endothelium. It mediates this effect via activation of a primary NO receptor or through nitrosation of certain iron-sulphur containing proteins such as caspase 3 [312]. Reduced bioavailability of NO is a feature of CSX. Factors and miRNAs confirmed to regulate eNOS activity are shown in Table 6.4 below.

A. Factors that modulate eNOS activity

Induces eNOS	Downregulates eNOS
Laminar Shear Stress	Hypoxia
Oestrogen	Lipopolysaccharide
Vascular Endothelial Growth Factor	Tumour Necrosis Factor Alpha
Statins	Oxidised Low-Density Lipoprotein
Bradykinin	
Cellular Metabolic Stress	
Sirtuin 1	

B. miRNAs that affect eNOS activity

miRNA	Stimulus	Effect on eNOS
NOS 3 Intron 4 mirtron	Co-transcribed	↓ NOS3 Transription
miR-155	TNFα, IL-1β and IFNγ	↓ eNOS. Binds 3'-UTR of eNOS-mRNA
miR-221 and -222	Uncertain	↓ eNOS protein. Unknown mechanism
miR-21	Shear Stress (x5.2 fold)	↑ eNOS activity through reduced PTEN
miR-133	Uncertain	↑ eNOS activity through ↓ caveolin-1
miR-199	Uncertain	↓ eNOS activity through ↓ SIRT1

Table 6.4: Factors influencing eNOS activity

6.2.6 miRNA and Endothelin-1

Endothelin-1 (ET-1) is a vasoactive 21-amino acid peptide that is released by endothelial cells and is a highly potent vasoconstrictor and mitogen. It mediates these effects by activating the endothelin-type A receptor (ET_aR). Dysregulation of ET1 can lead to pulmonary and systemic hypertension, vasospasm and myocardial and blood vessel fibrosis. Interestingly, the endothelin type B receptor (ET_bR) mediates the release of NO, prostacyclin and endothelial-derived relaxing factor and is found in almost all parts of the vascular tree. Usually, the predominant effect of ET-1 is vasoconstriction. Endothelin production is mainly regulated at the transcription level of the endothelin-1 gene (*EDN1*, 6p24.1). Figure 6.6 shows many of the factors involved in this transcriptional regulation. It should be noted that laminar shear stress induces a dose-dependent reduction in ET-1 release (as well as reducing the levels of ppET-1 mRNA and endothelin-converting enzyme 1 isoform) but upregulates ET_bR levels by a NO- and PKC- dependent mechanism[313]. KLF2 has also been shown to decrease *EDN1* expression.

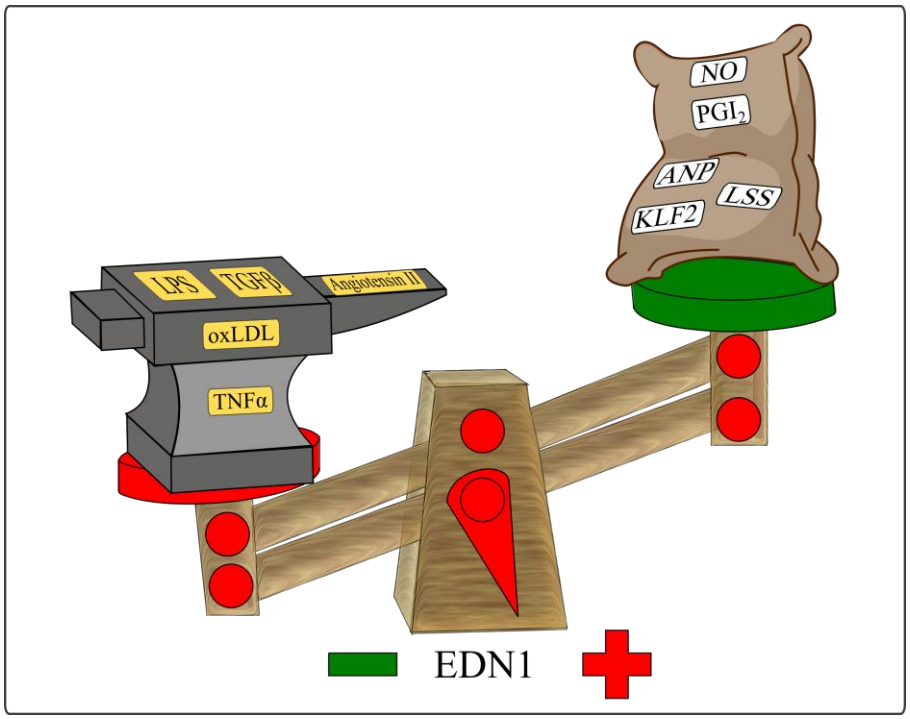


Figure 6.6: Endothelin-1 Gene Regulation: **AT2**- Angiotensin II; **ANP**- Atrial Natriuretic Peptide; **KLF2**- Krüppel-like Factor 2; **LPS**- Lipopolysaccharide; **LSS**- Laminar Shear Stress; **NO**- Nitric Oxide; **oxLDL**- oxidised Low Density Lipoprotein; **PGI_2**- Prostacyclin; **TGFβ**- Transforming Growth Factor; **TNFα**- Tumour Necrosis Factor

Post-transcriptional regulation of *EDN1* also occurs and *EDN1*-mRNA is quite unstable, having a relatively short half-life of 15 minutes [314]. The main reason for this is that the 3'-UTR constitutes over 50% of the *EDN1* transcript and contains multiple AU-rich elements (AREs), which target the mRNA for degradation. Studies have also shown that miRNAs are also involved in this post-transcriptional regulation. The *EDN1* 3'UTR has potential binding sites for up to 48 miRNAs and 4 of these have been shown to interact with the mRNA in vivo (miR-1, miR-125a and -125b and miR-199) with all of these reducing ET-1 production[315-319]. ET-1 plays an important role in vasomotor tone. It has also been implicated in microvascular dysfunction, causing vasoconstriction and endothelial dysfunction [320,321]. It is present in higher circulating concentrations in patients with CSX and appears to reduce the pain threshold in these patients[162,322,323]. Decreased miRNA-dependant regulation of *EDN1* might account for these findings.

6.2.7 miRNA and Endothelial Senescence

As described in chapter 1.4.3, endothelial senescence renders the endothelium dysfunctional with reduced endothelium-dependant vasodilatation and increased expression of markers of vascular inflammation, all hallmarks of CSX. The degree of endothelial cell senescence in CSX is not known as there are no particular plasma biomarkers that can be measured to reliably ascertain cellular status. miRNA profiling may be of interest, however, as miRNAs have been shown to have an important role in the regulation of senescence in ECs. Dicer, but not Drosha, knockout induced cellular senescence in mouse embryonic fibroblasts but the effects of Dicer knockdown in ECs is not known. miRNAs implicated in endothelial senescence are shown in table 6.5 below.

miRNA	Target	Alteration	Consequence
miRNAs Directly Regulating Senescence			
miR-217 miR-34a	SIRT1	Increased	Increased SA-β-gal-positive cells Reduced Telomerase activity Reduced eNOS activity p53 activated FOXO3a activity altered
miR-200c	ZEB1	Induced by Oxidative Stress	Stress-induced Senescence Apoptosis
miR-17-92 Cluster (miR-17, -19b, -20a, and -106a)	? p21 ? CDKN1A	Reduced	Increased ROS production Increased p21/CDKN1A activity
miR-146a	IRAK1 NOX4	Conflicting reports	Increased β-galactosidase ? Reduced Telomerase activity ? Reduced ROS production by NOX ? Reduced TLR signalling
miR-21a miR-214	PTEN PDCD4 eNOS (miR-21a)	Reduced	Apoptosis Reduced cell proliferation Reduced eNOS phophorylation
Other miRNAs Altered in Senescence			
miR-221 miR-222	eNOS (indirect)	Increased	Reduced eNOS levels
miR-133	Caveolin-1	Reduced	Increased Caveolin-1 Reduced eNOS activity
miR-126	VCAM-1	Reduced	Increased VCAM-1 Expression
miR-125b	Histone H3K9me3	Increased	Increased MCP-1 protein

CDKN1A- Cyclin-dependent Kinase Inhibitor 1A; **eNOS**- endothelial Nitric Oxide Synthase; **IRAK1**- Interleukin-1 Receptor-associated Kinase 1; **MCP** - Monocyte Chemoattractant Protein; **NOX4**- NADPH Oxidase 4; **PDCD4**- Programmed Cell Death Protein 4; **PTEN**- Phosphatase and Tensin Homolog; **ROS**- Reactive Oxidant Species; **SA-β-gal** - Senescence-associated β-galactosidase; **SIRT1**- Sirtuin 1; **TLR**- Toll-like Receptor; **VCAM-** Vascular Cell Adhesion Molecule; **ZEB1**- Zinc Finger E-box-binding Homeobox 1

Table 6.5: miRNAs affecting endothelial senescence

Given the fact that CSX patients have evidence of redox imbalance, it would not be surprising to find that they have some degree of stress-induced senescence. This senescence could explain the CSX phenotype quite well, given that many of the features of endothelial senescence are also typical of CSX. Studies in human aortic

endothelial cells (HAECs) show that the expression of many miRNAs are changed during endothelial senescence [324].

miRNAs may be involved in regulating the induction of senescence itself as well having a role in the pathways further downstream. miR-217 and miR-34a can both target SIRT1, a key player in cell longevity[304,306]. Downregulation of SIRT1 leads to increased acetylation and activation of p53, which is the main pathway involved in EC senescence induction. Likewise, targeting of anti-apoptotic protein ZEB1 by miR-200c may also induce senescence and even apoptosis in endothelial cells[325]. Increased PTEN activity due to reduced miR-21a or -214 expression could also induce cell cycle arrest. Alterations in levels of miRNAs can also alter many of the cellular functions that are changed in senescence such as eNOS activity and the expression of vascular adhesion molecules, chemokines and cytokines. In non-endothelial tissue, miR-199 has been noted to be significantly downregulated in senescent mesenchymal stem cells while miR-143 and miR-10b are upregulated in senescent fibroblasts[326,327].

6.3 miRNA in Cardiovascular Disease

Given their robustness in plasma and relative ease of quantification, miRNAs have been identified as promising biomarkers in many cardiovascular diseases. Cardiomyocytes may release miRNAs in response to a variety of stimuli and miRNA profiling has been performed in many disease states.

6.3.1 miRNAs in Atherosclerosis

Atherosclerosis is a focal inflammatory process that takes place in the intima of blood vessels and results in the accumulation of cells, oxidised lipids and fibrous elements in the vessel wall. Under normal pulsatile shear stress, the endothelium protects itself by producing anti-inflammatory and anti-thrombotic substances. When the endothelium becomes injured, from the effects of hypertension, dyslipidaemia or advanced

glycosylation end products (AGEs) for example, local endothelial changes occur causing upregulation of adhesion molecules with local recruitment of inflammatory cells. These cells then oxidise and ingest LDL particles with the formation of foam cells. Smooth muscle cells in the wall also dedifferentiate and become secretory cells. The local inflammatory process results in thickening of the intima that causes vascular remodelling and ultimately obstruction of blood flow with resultant ischaemia in tissues distal to the stenosis.

miRNAs are involved in many stages in the development of atheromatous plaques, from cholesterol synthesis and handling to vascular wall remodelling. Many miRNAs believed to be involved in this pathway are depicted in figure 6.7 overleaf. Patients with CSX have no macroscopic atherosclerosis and have no excess risk of CAD compared with a healthy population. Despite this, the endothelial dysfunction typified by the early stages of atherosclerosis is likely to be quite similar in CSX patients. The reason for the lack of macroscopic atheroma in CSX patients despite significant endothelial dysfunction is unknown. miRNA profiling in CSX patients to determine the levels of miRNAs associated with atherosclerosis will be of great interest.

6.3.2 miRNA, Myocardial Ischaemia and Preconditioning

Despite the presence of angina in CSX, there is conflicting evidence regarding the presence of myocardial ischaemia in these patients. Lactate studies examining the venous blood draining from the coronary sinus have given conflicting reports and early myocardial perfusion imaging techniques failed to show any myocardial ischaemia in CSX patients[8,328,329]. Modern imaging techniques, however, show the presence of sub-endocardial ischaemia and reduced coronary flow reserve in CSX[10]. CSX patients, therefore, would likely undergo many cycles of ischaemia and reperfusion.

Figure 6.7: Potential Roles of miRNAs in Atheromatous Plaque Formation

Ischaemic tissue becomes biochemically unstable. Many substances are released from ischaemic myocardium, such as bradykinin, adenosine, endothelin etc. Additionally, the respiratory chain is exhausted of oxygen and many of the complexes are in their most reduced state. Reperfusion results in a huge increase in the number of ROS present in the cell, increasing oxidative stress on the cell. In fact, reperfusion is also a time of marked inflammation in myocardial cells.

miRNA in Ischaemia-Reperfusion Injury (IRI)

Most of the data for miRNA in reperfusion injury focus on changes after myocardial infarction, which would obviously require a longer duration of ischaemia that is seen in CSX, where patients don't suffer infarction. Unfortunately, there seems to be a lot of contradictory information regarding the changes in expression of certain miRNAs after reperfusion. For example, miR-1 levels may increase or decrease, depending upon which study you read. Overexpression of miR-1 seems to reduce cell viability in oxidative stress yet reduces the rate of apoptosis in rat models of IRI. Despite this current lack of clarity, this new field is progressing rapidly. The microRNA changes seen after ischaemia are tabulated below.

miRNA	Change	Target	Comments
miR-1	↑/↓	Bcl-2	Conflicting data. May be an anti-apoptotic factor
miR-21	↑	PTEN	↓ in the core ischaemic region. ↑ in the penumbra. ↓ infarct size ↑ MMP
miR-133a	↓	Caspase 9	↑ Apoptosis
miR-320	↓	HSP20	↑ Apoptosis and infarct size
miR-494	↑	PTEN, ROCK1, FGFR2	Overall anti-apoptotic effect Short lived changes

Bcl- B-cell Lymphome; **FGFR-** Fibroblast Growth Factor Receptor; **HSP-** Heat Shock Protein; **MMP**- Matrix Metalloproteinase
PTEN- Phosphatase and tensin homolog; **ROCK**-Rho-associated protein kinase.

Table 6.6: miRNA changes after myocardial ischaemia

Repeated bouts of short-lived ischaemia induce many protective changes in myocardial cells and have been shown to limit subsequent infarct size in the case of vessel occlusion. This is known as ischaemic preconditioning.

miRNA in Ischaemic Preconditioning (IPC)

IPC has been shown to have early and prolonged effects (up to 96 hours) following ischaemia of even 5 minutes' duration. Early preconditioning is believed to be mediated by several substances that are released by the cells during the ischaemia. Adenosine appears to be a key player in early preconditioning by acting on the A1 receptor and Bradykinin has also been shown to reduce dysrhythmia in ischaemic hearts and reduces infarct size if given intravenously. These substances are believed to act via G-protein coupled receptors to mediate many changes in the myocytes with activation of Protein Kinase C as a common step. They appear to prevent cell death by preventing the opening of the mitochondrial permeability transition pore (MPTP) and hence preventing uncoupling.

More long term changes appear to be mediated by the modulation of mitochondrial ATP-dependant potassium channels (KATP) and through the activation of various kinases (such as ERK1/2, and PI3K-Akt preventing apoptosis) and iNOS, the inducible form of NOS. NO can also activate KATP channels in the mitochondria, limiting the cardiac action potential duration. Cyclo-oxygenase-2 is also upregulated in the pre-conditioned hearts of and may produce cardioprotective prostaglandins. Hypoxia-inducible factor-1 may play an important role in preconditioning by regulating mitochondrial respiration and is a known target of several miRNAs including miR-199.

miRNA	Effect
↑miR-1	↓ HSP60, HSP70 and bcl-2 ↓ apoptosis ? target
↑miR-21	↓ PDCD4,PTEN ↑ eNOS, HSP70 ↓ Apoptosis
↑miR-24	↓ bcl-2 ↓ Apoptosis
?↑ miR-17-92	↓ PTEN ↑ Proliferation
↓miR-199b	↑ HIF-1α ↑ SIRT1
↓miR-200a	↑ Ets-1 ↑ Angiogenesis

BCL- B-cell Lymphoma**; eNOS**- endothelial Nitric Oxide Synthase; **HIF-** Hypoxia Inducible Factor; **HSP**- Heat Shock Protein; **PDCD4**- Programmed cell death protein 4; **PTEN**- Phosphatase and tensin homolog; **SIRT**- Silent Mating-Type Information Regulation Homolog

Table 6.7 miRNAs affected by tissue hypoxia

A cocktail of miRNAs taken from an ischaemic preconditioned mouse can reduce infarct size in another animal when injected directly into the myocardium. This protection was associated with an increase in eNOS (without a change in iNOS levels), HSP70 and HIF-1[330]. The specific miRNA effectors in IPC are uncertain but many are shown to be increased in ischaemia. These are listed in table 5.6. As CSX patients have cycles of ischaemia and are likely to have some levels of ischaemic preconditioning it will be interesting to see if the profiles overlap.

6.3.3 miRNA in Cardiac Syndrome X

The simple fact is that nothing is known of miRNA profiles in CSX to date. As endothelial dysfunction plays a central role in CSX one might expect to see dysregulated miRNA expression in those miRNAs that modulate eNOS, KLF2, KLF4 and SIRT1 expression. Also, the pattern of miRNA expression might implicate a certain mechanism of endothelial dysfunction such as atherosclerosis or oxidative stress. It would also be interesting to see if there is any overlap in miRNA profile between CSX and IHD.

6.4 Chapter Objectives

Given the relative specificity of miRNAs and the fact that the miRNA profile has not been established in CSX, the potential to discover a particular miRNA signature in CSX and therefore provide some insight into the pathways that are dysregulated in CSX should be pursued. In this chapter we aim to:

1. Determine the **identity of differentially expressed microRNAs** in active CSX using Massively parallel (next-generation) sequencing with real-time qPCR validation.
2. **Identify specific targets of these differentially expressed miRNAs** and therefore pinpoint potential culprit pathways involved in endothelial dysfunction in CSX. Once differentially expressed miRNAs are identified we will examine miRBASE and other repositories to identify known and potential targets of these miRNAs. The relevance of these targets to CSX pathogenesis will be assessed.

Methods

6.5 Participants

The same patient cohorts that have been used in each of the chapters 2-5 were again utilised in this chapter. All study participants gave full informed consent to miRNA analysis. CSX patients fulfilled the strict criteria as discussed in chapter 1; the LCSX patients had typical angina, a normal exercise stress test and normal coronary arteries; while the healthy controls were age- and sex-matched to the CSX patients, suffered from no cardiac symptoms and fulfilled the same exclusion criteria as the CSX and LCSX patients (viz. no other cardiac disease, diabetes mellitus, chronic inflammatory condition or recent use of anti-inflammatory medications). As noted in table 2.3, there was no significant difference in medication use between CSX, LCSX and healthy control patients other than an increased use of aspirin at baseline for the CSX patients. Blood samples from patients for miRNA analysis were available for all 17 CSX patients, 6 LCSX patients and 15 healthy controls.

6.6 Sample Preparation and Quality Control

Samples consisting of 2.5mls of blood were drawn into PAXgene Blood RNA Tubes (PreAnalytiX GmbH, Hombrechtikon, Switzerland) from participants' antecubital fossa veins. Following inversion, the tubes were allowed to stand for 2 hours before freezing at -20^{o}C for 24 hours. Samples were then kept at -80^{o}C until needed for further analysis. Intercellular RNA remains stable for a minimum of 50 months at -20^{o}C in PAXgene tubes. Once all samples had been collected, we proceeded to RNA extraction using the PAXgene Blood miRNA Kit (PreAnalytiX GmbH, Switzerland) according to the manufacturer's instructions. This involves centrifuging and washing blood before adding proteinase and incubating. The resulting fluid is passed through a shredder tube and the supernatant is then passed through an RNA spin column before digestion of DNA using DNase followed by washing and elution of RNA using buffers. Using this protocol, all RNAs >18 nucleotides in length are purified.

Purity and concentrations of the isolated RNA was assessed initially using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, MA, USA). This system uses 1µl of sample and is capable of determining the concentration of ribonucleic acid samples up to 3000 mg/µl with a lower limit of detection of 2ng/µl and has a typical CV between duplicated samples of 2%. A ratio of absorbance at 260nm and 280nm is useful in assessing the purity of the extracted RNA. A ratio of ≥2.0 is generally accepted as "pure" for RNA. A lower ratio than this may be caused by impurities in the samples (such as protein contamination). The quality of the extracted RNA was further assessed using the Agilent 2100 Bioanalyser (Agilent Technologies Inc., CA, USA) to produce an RNA integrity Number (RIN), determined by the electrophoretic trace of the sample. The RIN scales from 1 to 10, with 10 indicating completely intact RNA. An RIN of >7 is adequate, but >8 is preferable in order to allow for accurate microRNA sequencing. Pure RNA was frozen at -80^{o}C until subsequent analysis.

6.7 Next Generation Sequencing

The initial step in analysis of the miRNA profile was to perform next-generation sequencing (NGS) of the miRNA. Samples from 4 CSX patients and 4 controls were sent for NGS. These samples were taken randomly from CSX patients who were symptomatic at the time of blood draw and were taking statins and randomly from healthy controls who were on statins. There was no statistically significant gender, medication use or age difference between the randomly selected groups. NGS was performed by Exiqon (Exiqon Inc., Vedbaek, Denmark) and followed the work plan detailed in figure 6.8 below. Exiqon performed quality control analysis of all the samples submitted, to ensure no degradation in transport, using an Agilent Bioanalyser 2100. They then performed a library preparation involving the conversion of RNA to cDNA followed by ligation with custom adapters and amplification. The RNA fraction of interest was highlighted by size selection. This fraction was then sequenced by synthesis using the Illumina platform with a minimum of 5 million reads. Exiqon also performed the statistical analyses to identify differentially expressed miRNAs and attempted to ascribe the observed results to a particular Gene Ontology pathway and give a biological interpretation.

6.8 Validation with Quantitative Polymerase Chain Reaction

In order to validate the NGS results and to investigate the results in the wider CSX cohort and control group, we assessed the differential expression of the miRNAs identified at NGS by performing quantitative real-time PCR using the miRCURY LNA Universal RT microRNA PCR system using SYBR Green (Exiqon Inc., Vedbaek, Denmark) and specific qPCR primers for the differentially expressed miRNAs identified at NGS. miR-423 was used as the reference RNA as this has been typically detected at medium to high levels in serum and plasma samples and is provided by Exiqon as a candidate reference gene. The primer mixes included UniSP6 RNA spike-in control primer mix v2 to ensure good internal validation.

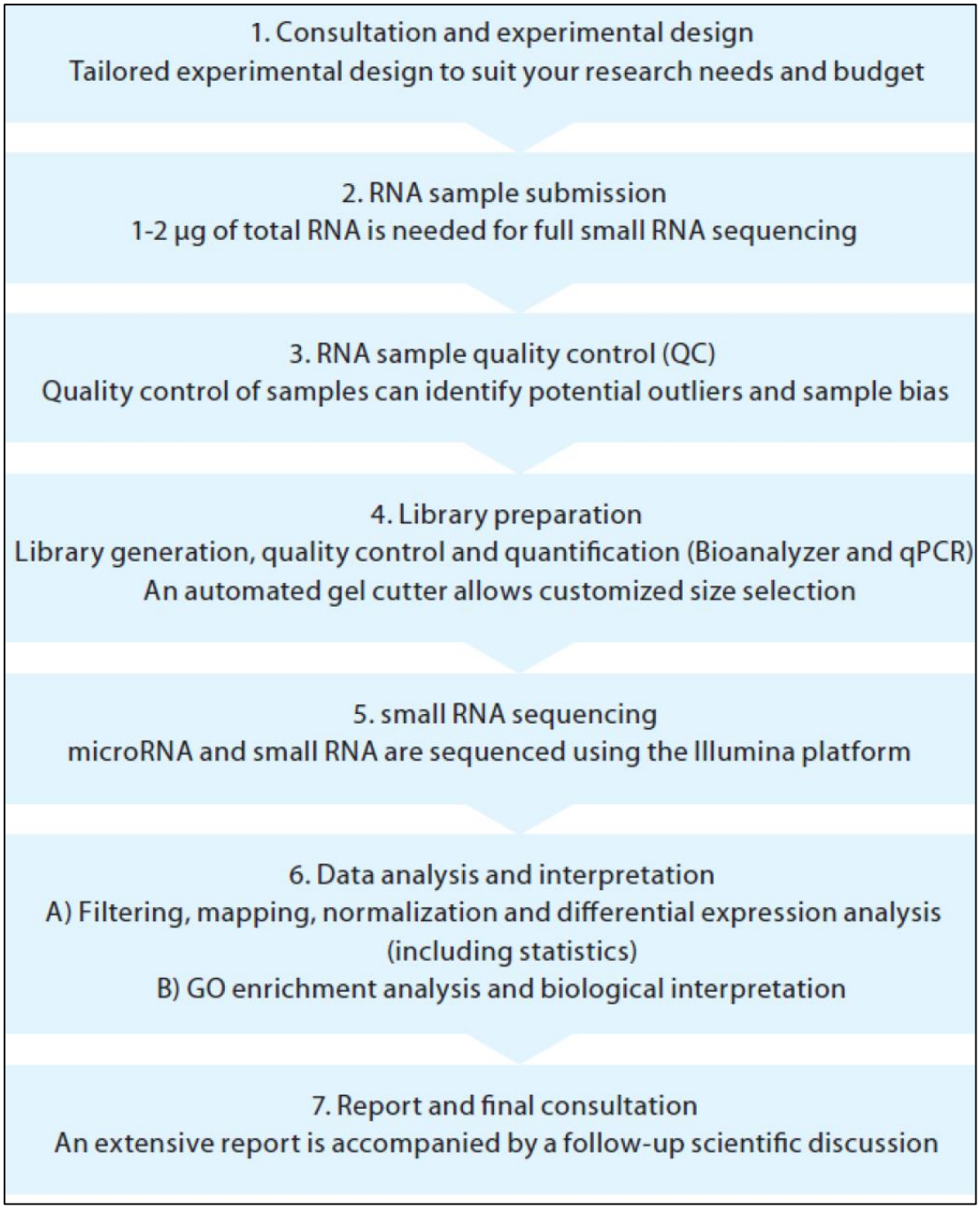


Figure 6.8: Work flow of Next-generation Sequencing by Exiqon Inc. (taken with permission from the Exiqon report)

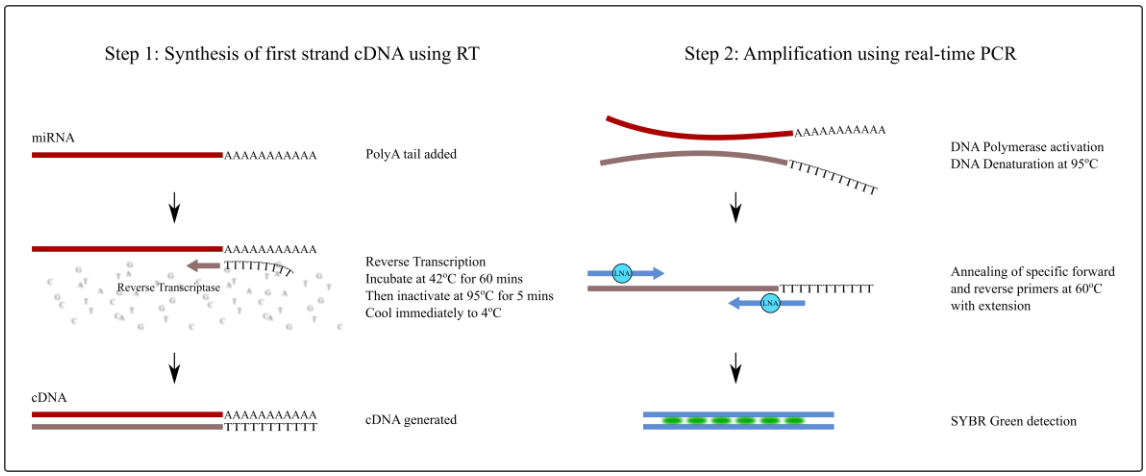


Figure 6.9 Illustration of the employed quantitative real-time polymerase chain reaction (PCR) processes. LNA- locked nucleic acid. RT- Reverse transcription.

The procedure is outlined in figure 6.9 above. The initial step was to perform one first-strand cDNA synthesis reaction using reverse transcription as per the manufacturer's instructions. This procedure adds a poly-A tail to the RNA sample (polyadenylation) before using a poly-T primer with a universal 5' tag to allow for cDNA synthesis for all RNA. A 1:40 dilution of the cDNA was then made using nuclease free water before proceeding to rtPCR. rtPCR was performed using a Lightcycler 480 (Roche Diagnostics, Basel, Switzerland) with 45 amplification cycles as per the standard conditions. Raw Cq (or Ct) values were obtained and normalised against the hsa-miR-423 reference gene. The reaction constituents and Lightcycler conditions are illustrated in figure 6.10 below.

A

Reagent	Volume (µl), RT reaction
5X Reaction Buffer	2
Nuclease Free Water	4.5
Enzyme Mix	1
RNA spike ins	0.5
Template RNA	2
Total Volume	10

B

Reagent	Volume (µl), PCR reaction
PCR Master mix	5
PCR Primer Mix	1
Diluted cDNA template	4
Total Volume	10

C

Process Step	LC480 Settings
Polymerase Activation/ cDNA Denaturation	95°C, 10 mins
Amplification	45 cycles, 95°C 10s, 60°C, 1 min Ramp rate 1.6°C/s Optical read
Melting Curve Analysis	Yes

Figure 6.10 A. Reagents used in the reverse transcription (RT) step. **B**. Reagents used for the qPCR step. **C**. Real Time PCR Cycle Conditions

6.9 Data management

Average values of continuous variables such as RNA concentrations are expressed as mean ± SEM if normally distributed otherwise they are reported as median (interquartile range). Next generation sequencing analysis produced several outputs including a Principal Component Analysis, Heatmap, Differential Expression Analysis and Gene Ontology Enrichment Analysis. The handling of data for each of these outputs is summarised in the relevant results sections. In the qPCR, the observed cycle threshold (C_T) at which the amplification curves crossed a computer-derived common threshold was noted. The deltaCt (ΔC_T) was calculated for each sample by subtracting the C_T for the reference gene (hsa-miR-423) from the C_T of the target gene (e.g. miR-143), thereby standardising the outputs across all samples. The average ΔC_T across all samples was then calculated. The delta-delta Ct ($\Delta\Delta C_T$) was calculated by subtracting the average ΔC_T from the ΔC_T of the individual samples. The fold-change difference in expression for each group versus the average was then assessed by raising 2 to the power of $-\Delta\Delta C_T$. The statistical significance of these changes were assessed using the Mann-Whitney U test. The confidence intervals for the relative difference in expression between the two groups were also obtained by performing a student t-test on the average ΔC_T values for each group. The 95% confidence intervals for the relative expression were then converted into fold-expression increase by raising 2 to the negative power of the confidence intervals. The foldchange in respective miRNAs was compared between LCSX and the other groups using the Independent Samples Kruskal-Wallis test. All p-values are two tailed and the confidence intervals are to the 95% limit.

Results

6.10 RNA Quality Control

The quality of the RNA was assessed both locally in UCC prior to being sent to Exiqon and again at multiple stages during the NGS process by Exiqon themselves.

6.10.1 Nanodrop Concentrations

The average total RNA concentration was 62.4±10.6 ng/µL for the healthy controls and 55.9 ± 8.0 for the CSX group. The median 260/280 was 2.09 [2.07 to 2.13] for the controls and 2.12 [2.06 to 2.16] for the CSX group. No sample had a 260/280 of less than 2, indicating that our RNA samples were pure.

6.10.2 Bioanalyser RNA integrity

The mean RIN for the healthy control patients was 8.4 ± 0.1 and was 8.6 ± 0.1 for the CSX population. All samples had an RIN >7, therefore making the samples suitable for sequencing.

6.10.3 Quality Control by Exiqon

The RNA samples were of good data quality and the vast majority had Q scores of >30, indicating a 99.9% accuracy in base identification. This is illustrated in figure 6.11 below.

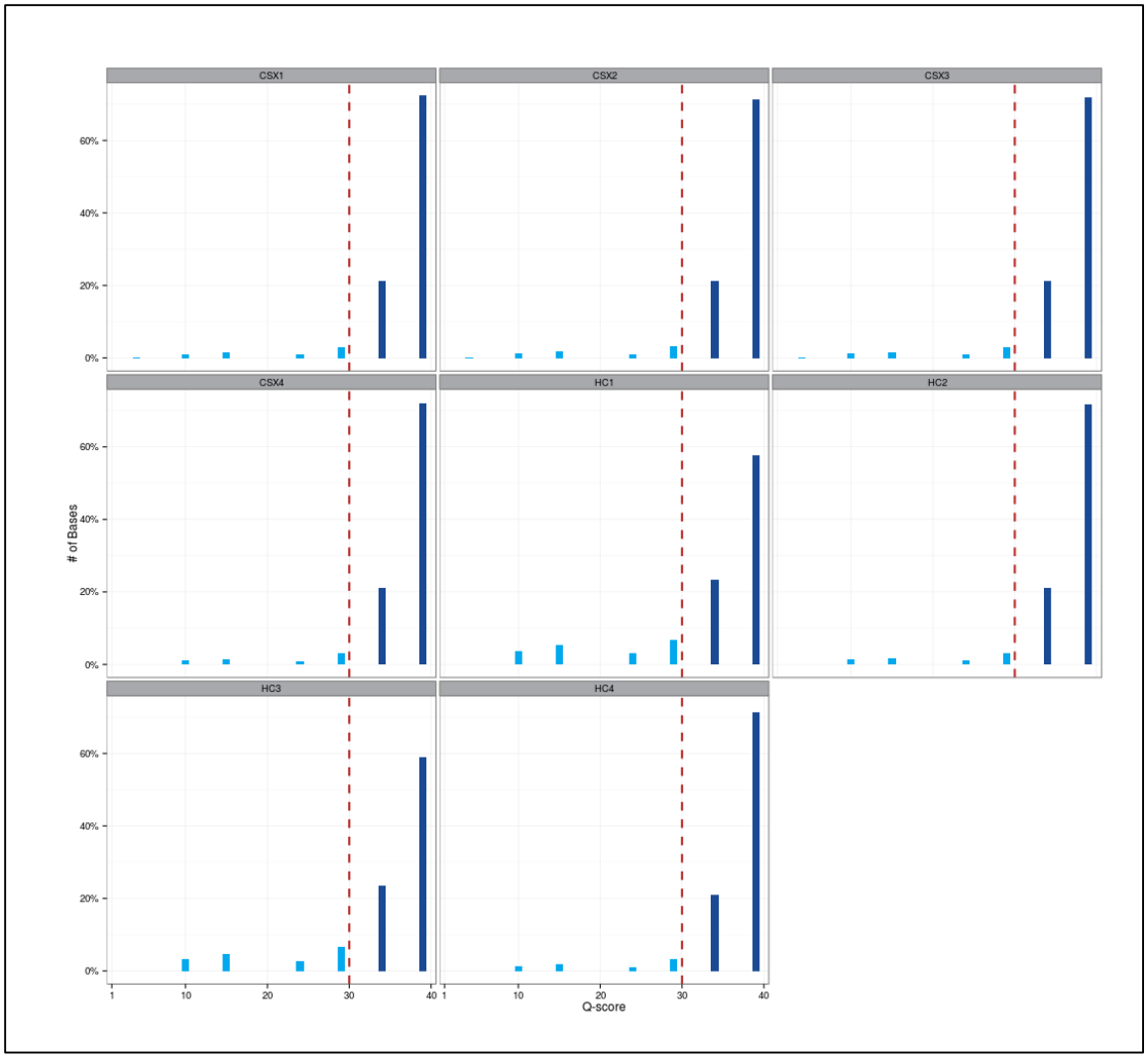


Figure 6.11: Q-scores of the various base samples in each subject. The vertical dotted lines delineate a Q-score of 30. The vast majority of data had a Q-score in excess of 30 indicating good quality data.

The next step in quality control was to identify the read length distribution of the identified RNAs. As shown in figure 6.12, each sample had a spike in the 18-22nt range (in keeping with miRNA) and smaller other spikes at greater lengths that correspond to other substances such as tRNA and mRNA fragments. This illustrates that miRNAs were selected appropriately during the NGS.

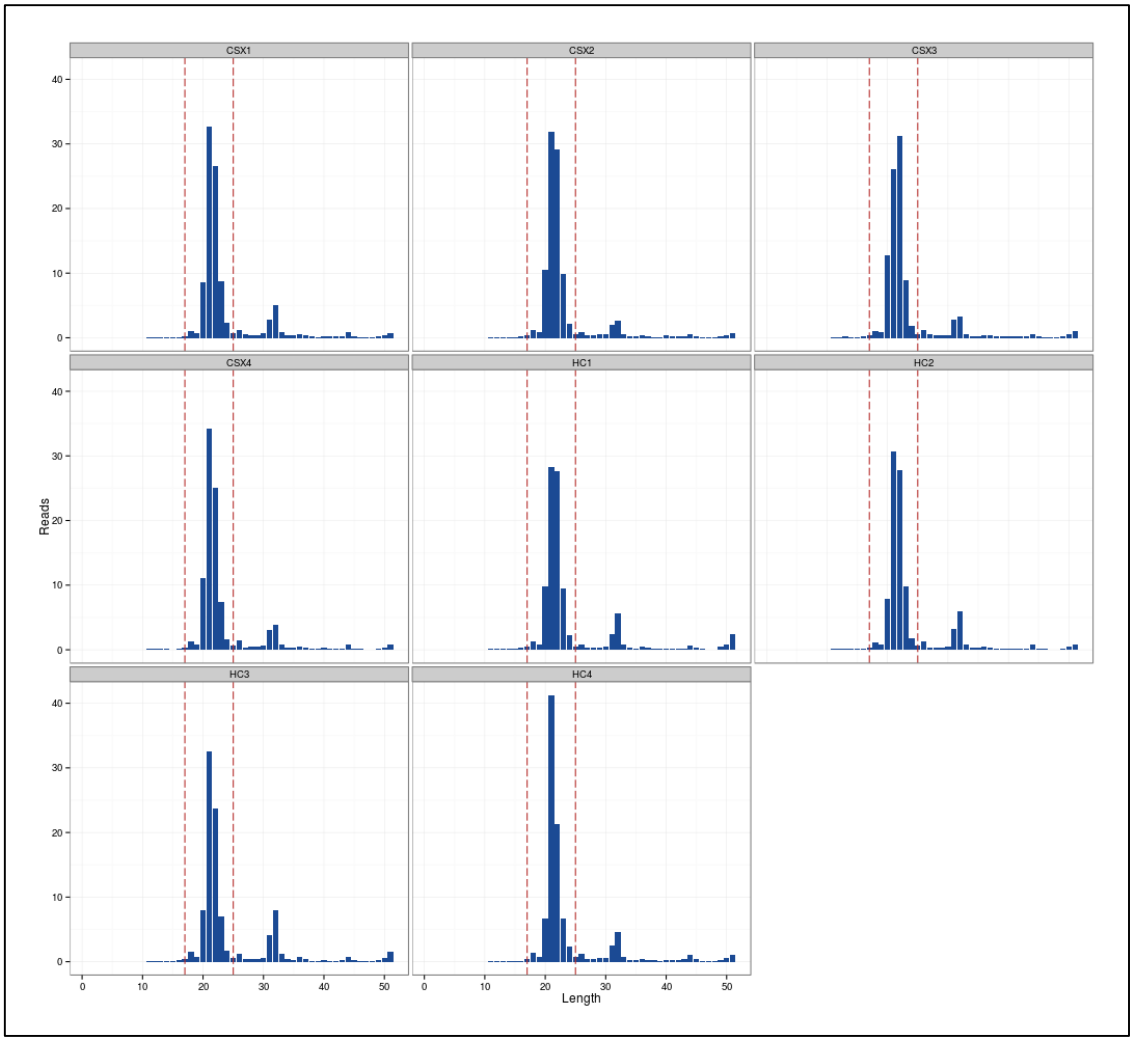


Figure 6.12 Read lengths. Note the spikes in the 18-22nt length, corresponding to detection of the miRNAs in the samples.

A further measure of quality control during the NGS protocol was to map the sequenced data. The reads could be mapped to miRNA or to the reference genome, or be outmapped (contaminants such as adapters, primers and rRNA) or unmapped (which do not map to any of the aforementioned categories). Typically, approximately 50% of the mapped reads will be from miRNA but this may be lower if the samples have degraded or are contaminated. As can be seen from Fig. 6.13, there was a consistent quantity of miRNA mapped from each subject in the experiment and overall 58.8% of all reads were mapped to identified miRNA.

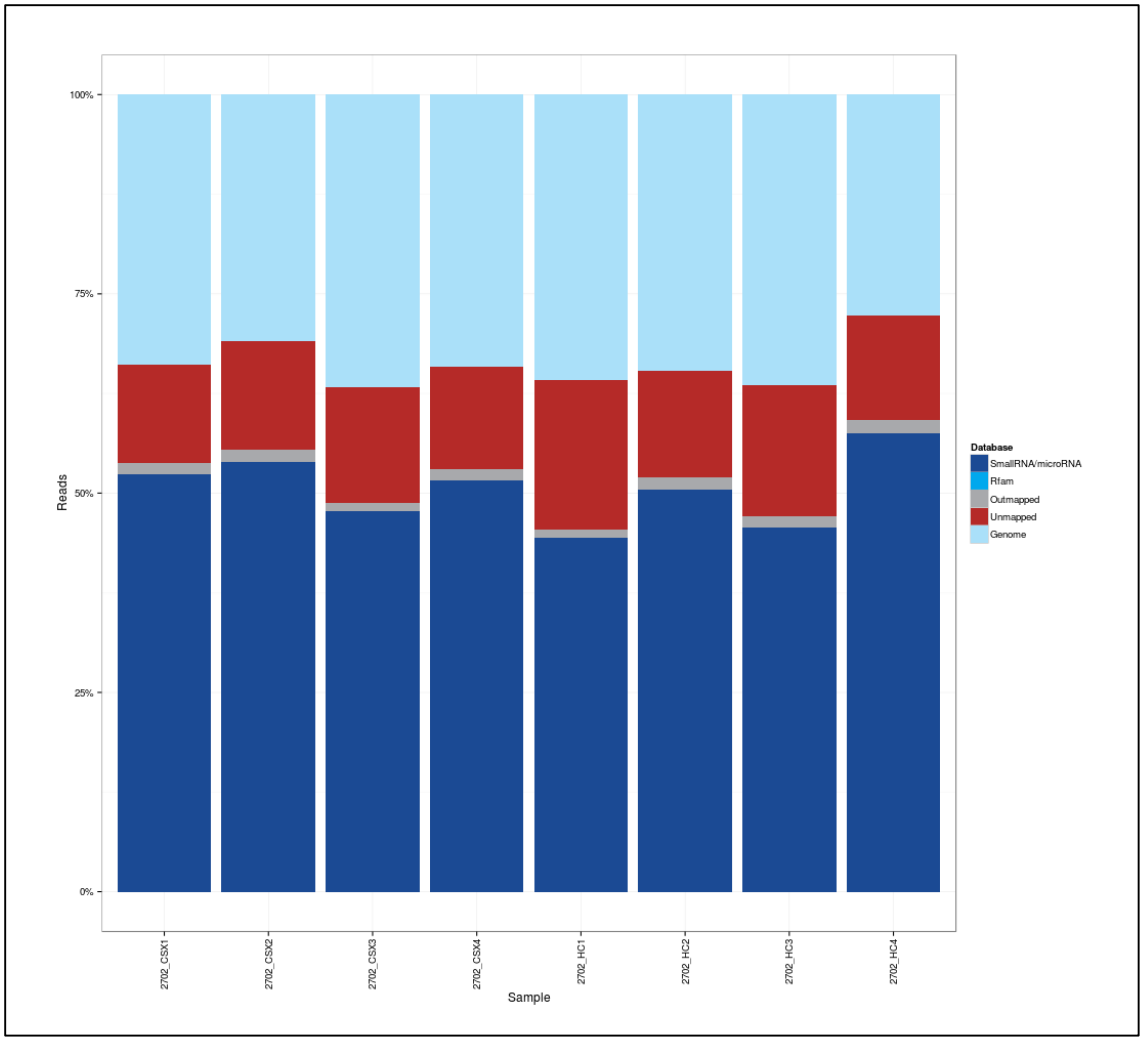


Figure 6.13 Mapping of NGS reads. Note the dark blue represents reads that map to miRNA's and accounts for approximately 50% of all reads.

6.11 miRNA Next-Generation Sequencing by Exiqon

NGS was successfully completed by Exiqon. On average, 10.8 million reads were obtained per sample. Overall, 195 RNAs with > 50 counts were identified in all samples. Five miRNAs were found to be differentially expressed between CSX patients and healthy controls.

6.11.1 Principal Component Analysis

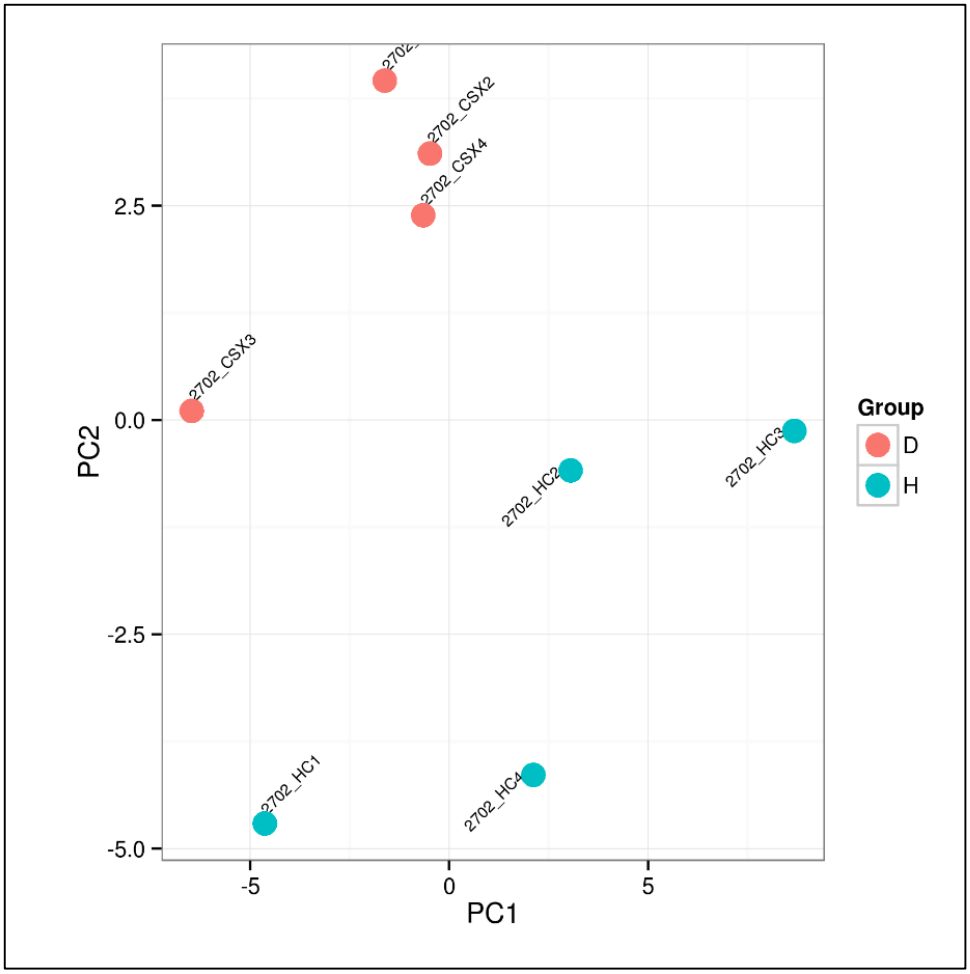


Figure 6.14: Principal Component Analysis plot with rescaling to be zero-centered and to have unit variance (i.e. the variance across samples is scaled to 1). D- Disease (CSX), H- Healthy. PC- Principal component.

By including the top 50 microRNAs that had the largest variation across all samples, an overview of how the participants clustered based on the variance in these data was obtained. The data was normalized with the tag per million (TPM) method and was converted to a log2 scale. If the biological differences between the samples were pronounced, they would form a primary component of the variation and this would lead to separation of samples in different regions of a PCA plot. If other factors, e.g. sample quality, inflict more variation on the samples, the samples would not cluster according to the biology. As can be seen in figure 6.14, the CSX patients separate clearly from the healthy control patients.

6.11.2 Heat map and unsupervised clustering

The heat map diagram in the figure below shows the result of the two-way hierarchical clustering of microRNAs and samples. The data was normalized with the tag per million (TPM) method and converted to a log2 scale. Then all features were filtered on "expressed in all samples" criteria and the 50 features with the highest the coefficient of variation (%CV) were selected for the analysis. Each row represents one microRNA, and each column represents one sample. The color of each point represents the relative expression level of a microRNA across all samples. The color scale is shown at the bottom right with red representing an expression level above the mean and green representing an expression level less than the mean.

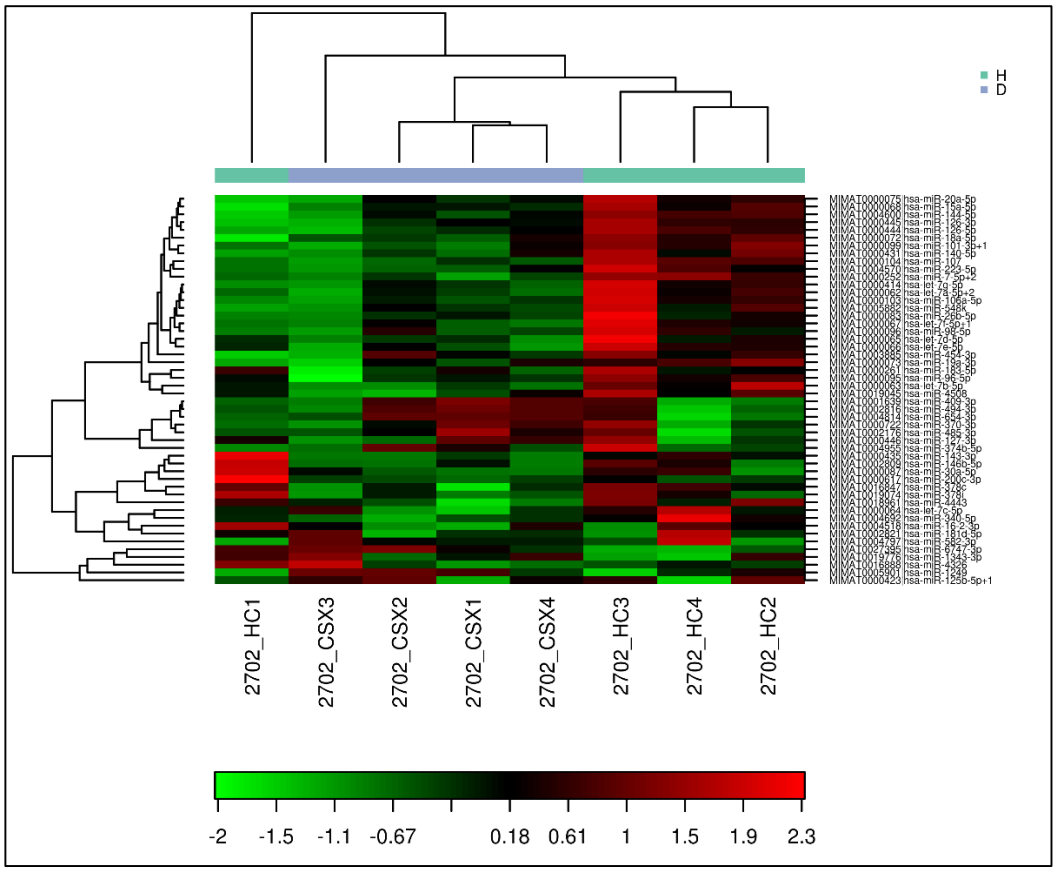


Figure 6.15: Heat Map and unsupervised hierarchical clustering by sample and microRNA. The clustering was performed on all samples, and on the top 50 microRNAs with highest %CV based on TPM normalized reads. There appears to be a clear differential expression of miRNAs between the CSX patients (2702_ CSX1-4) and the control participants (2702_HC1-4).

6.11.3 Differential Expression Analysis

Differential expression analysis attempts to distinguish biological variation from technical variation within the experiment, assuming that this varies amongst microRNAs. For normalisation Exiqon used the trimmed mean of M-values method based on log-fold and absolute gene-wise changes in expression levels between samples, as studies have shown it to be most effective for NGS experiments[331]. For two factor experiments, the count data was modelled by a negative binomial distribution. A common dispersion across all microRNAs, as well as tag-wise dispersions for specific microRNAs, was then estimated using a quantile-adjusted conditional maximum likelihood (qCML) estimator. P-values for significantly differentially expressed microRNAs were estimated by an exact test on the negative binomial distribution. For more general experiments containing multiple factors, the dispersion was estimated using the Cox-Reid (CR) profile adjusted likelihood method.

Name	LogFC	LogCPM	p-value	p-value (FDR)	Raw Mean H	Raw Mean D
hsa-miR-200a-3p	9.561976806	3.351089475	4.56E-06	0.002299603	145	0
hsa-miR-200b-3p	5.195233108	4.421003668	2.86E-05	0.007204057	319	4
hsa-miR-199b-3p	2.070198839	2.858008866	0.000194416	0.032661934	76	10
hsa-miR-143-3p	2.044082505	7.190570419	0.000260529	0.032826714	1680	242
hsa-miR-10b-5p	5.307331888	2.675462073	0.000418524	0.042187198	83	1
hsa-miR-378i	1.827420031	3.559851966	0.001409965	0.101766199	126	19
hsa-miR-4443	1.472440239	3.092614657	0.001413419	0.101766199	79	17
hsa-miR-499a-5p	2.284472649	1.414060523	0.001807083	0.107814641	22	3
hsa-miR-7-5p+2	1.569396137	8.92643264	0.001925261	0.107814641	4467	988
hsa-let-7b-5p	1.139386537	10.56931252	0.003632952	0.183100778	13588	4012
hsa-miR-1306-3p	1.600244183	1.115092055	0.004623666	0.20198086	16	3
hsa-miR-122-5p	1.797821501	3.214165026	0.004809068	0.20198086	81	16
hsa-miR-199a-3p+1	1.61975871	2.928238801	0.005271107	0.204356753	76	14
hsa-miR-378c	1.23780542	3.31490532	0.007966678	0.245131288	89	22
hsa-miR-550a-3p+2	-1.041005754	5.646948289	0.008642473	0.245131288	207	280
hsa-miR-210-3p	0.945112638	6.299190443	0.008658774	0.245131288	682	222
hsa-miR-4742-3p	-0.95645996	6.04012016	0.008697515	0.245131288	293	356
hsa-miR-200c-3p	1.688195374	5.054985967	0.009002656	0.245131288	381	62
hsa-miR-18b-5p	1.800754917	1.515334577	0.00924106	0.245131288	23	4
hsa-miR-96-5p	1.292973904	6.344531531	0.011352421	0.286081019	779	196

Figure 6.16: Table of the 20 most significantly differentially expressed microRNA names and annotation, with log fold change (logFC) between groups H (healthy control) and D (disease i.e. CSX) as well as Benjamini and Hochberg (FDR) corrected p-values.

A list of microRNAs predicted to be differentially expressed between the given experimental conditions is presented in Figure 6.16 above. Note that only the top 5 miRNAs show significant differences after B-H correction for multiple testing. These miRNAs were miR-200a, miR-200b, miR-199b, miR-143 and miR-10b and appeared to be relatively under-expressed in the CSX population.

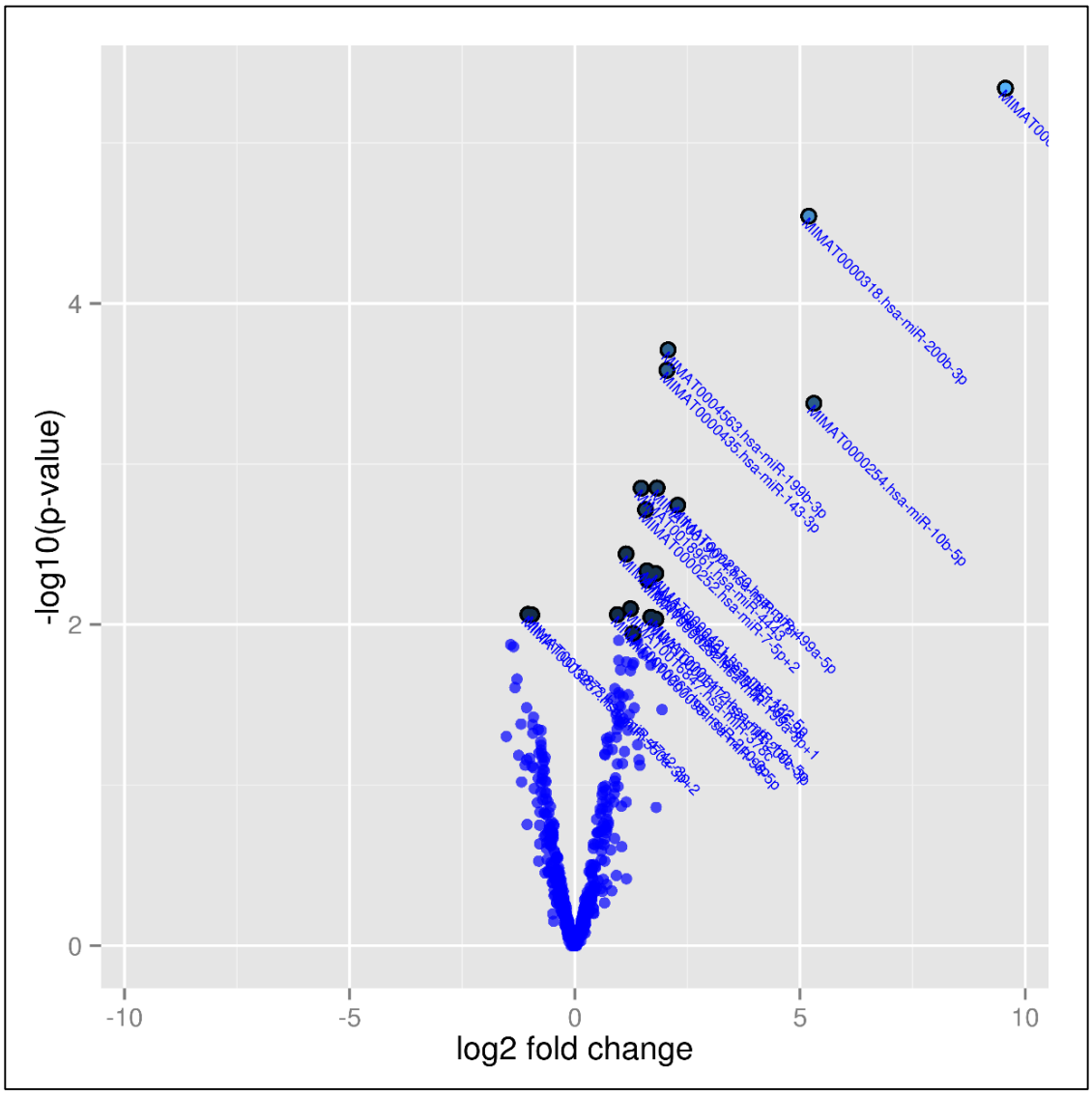


Figure 6.17: Volcano plot showing the relationship between the estimated p-value and the fold change in normalized expression of miRNAs between the two experimental groups. The most significant differentially expressed miRNAs are labelled.

The above volcano plot gives a graphical representation of the differential expression of miRNAs in the two groups. The higher a miRNA is on the y-axis the more statistically significant the difference between the cohorts. The more to the right on the plot, the more upregulated the expression is in the healthy group relative to the CSX population. As one can see, miRNAs were relatively upregulated in healthy controls compared to the CSX patients, indicating that reduced miRNA expression may be associated with the disease state in CSX. As miRNAs mostly act to silence mRNA expression, under-expression of miRNAs may allow the upregulation of certain biological processes.

6.12 qPCR confirmation

All miRNAs were differentially expressed between the two groups. Only miR-143 was validated by being underexpressed in the CSX group in both qPCR and NGS analyses. The remaining 4 miRNAs were found to be significantly overexpressed in the CSX group compared to the healthy controls by qPCR, in contradiction to the NGS results. As can be seen in table 6.8 below, the 4 miRNAs that did not validate when compared to the NGS results all had C_T values of >35, indicating that these miRNAs were very lowly represented in the cDNA, probably due to very low expression (as there is high amplification of the reference gene in the same conditions). It should be noted that these 4 miRNAs also had very low counts by NGS and miRNAs with counts <120 are known to be difficult to validate with qPCR. Figure 6.18 illustrates the relative fold-change in miRNA expression of the different groups versus the overall average.

Cardiac Syndrome X

	miR-423	*miR-10b*	*miR-143*	*miR-199b*	*miR-200a*	*miR-200b*
C_T	20.8±0.3	36.8±0.5	33.6±0.4	35.8±0.3	37.5±0.4	38.6±0.3
ΔC_T		16.0±0.5	12.8±0.5	15.0±0.5	16.4±0.5	17.7±0.4
$2^{-\Delta\Delta CT}$		0.70(0.12 to 1.9)	0.29(0.08 to 1.1)	0.92(0.33 to 1.87)	0.74(0.23 to 1.38)	0.53(0.16 to 1.04)
p		p=0.002	p=0.001	p=0.001	p=0.01	p=0.001

Healthy Control

	miR-423	*miR-10b*	*miR-143*	*miR-199b*	*miR-200a*	*miR-200b*
C_T	19.6±0.3	38.1±0.3	30.1±0.4	36.7±0.4	37.4±0.4	39.4±0.2
ΔC_T		18.6±0.4	10.4±0.4	17.0±0.4	18.5±0.5	19.8±0.3
$2^{-\Delta\Delta CT}$		0.11(0.04 to 0.21)	1.53(0.7 to2.6)	0.16(0.07 to 0.45)	0.22(0.08 to 0.39)	0.11(0.06 to 0.19)

	miR-10b	miR-143	miR-199b	miR-200a	miR-200b
95% CI for relative expression CSX:HC	**2.36 to 14.82 p<0.001**	**0.08 to 0.43 p<0.001**	**1.74 to 9.91 p=0.02**	**1.37 to 12.5 p=0.01**	**1.83 to 9.19 p=0.001**

Table 6.8: PCR results. Note the higher CT values in miR-10b, -199b, -200a and-200b. The relative expression of the target miRNAs in the CSX group versus the Healthy Control group is shown at the bottom of the table and is derived from the 95% CI of t-tests on the ΔC_T. P-values in the upper box refer to differences by Mann-Whitney U, p-values in the lowest section are the results of t-tests. See section 6.9.

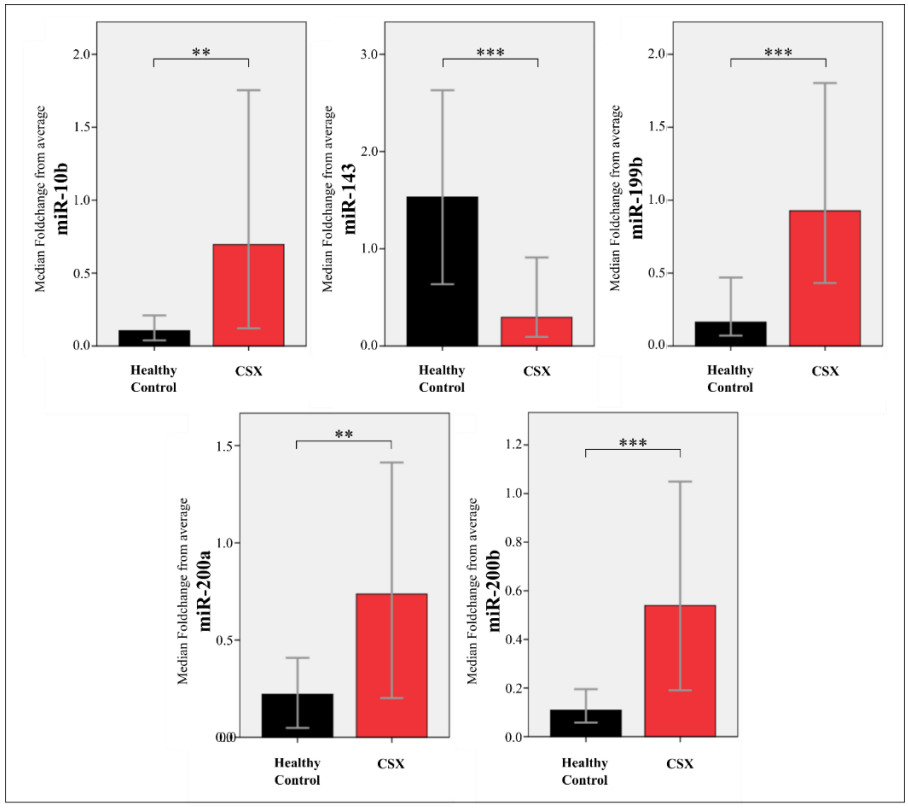


Figure 6.18: PCR Results. Bar charts of the median foldchange in expression when compared to the average expression level

6.13 Differentially Expressed miRNAs

In all, 5 distinct microRNAs were differentially expressed in our CSX cohort as determined by NGS and PCR. These were miR-10b, a miRNA associated with endothelial inflammation and Kruppel-like factor regulation; miR-143, which regulates VSMC phenotype switching; miR-199b, which appears to have a role in the response of tissue to hypoxia; and miR-200a and miR-200b, members of the miR-200 family that govern many elements of the endothelial response to oxidative stress. The miRSearch and miRBase databases were used to research experimentally proven and predicted gene targets for the individual microRNAs. It should be again noted that only miR-143 was validated as being significantly underexpressed in our CSX population with consistent results in both NGS and qPCR. The remaining miRNAs were differentially expressed, whether assessed by qPCR or NGS, but there was no agreement between the modalities as to whether they were under or over-expressed in the CSX cohort. This warrants further study.

6.13.1 microRNA-10b

miR10b is one member of the mir-10 microRNA precursor family. Like other members of this family, mir-10b is encoded by a gene in the Hox gene clusters, specifically just upstream of Hoxd4 on chromosome 2q31-2q37. It differs by only one base from closely related microRNA-10a and they are believed to have similar gene targets[332]. It may be induced by laminar shear stress or hypoxia and its promoter is hypermethylated in many cancers. Confirmed targets for miR-10b importantly include KLF4, HOXD10 and KLF11. It has been extensively investigated and labelled as a “metastomir” in many cancers, varying from breast cancer to melanoma. It seems as though mir-10b can initiate migration and invasion of gastrointestinal cancers through the downregulation of Hoxd10 and KLF4 with the initiation of epithelial-mesenchymal transition (EMT). Antagonizing mir-10b action upregulates NK cell activity against target cells through upregulation of the MICB gene, which regulates MHC expression on target cells[333].

More importantly for CSX, miR-10a is induced by laminar shear stress and is known to reduce NFκB in the endothelium through MAP3K7 and β-TRC downregulation. MiR-10b is predicted to similarly downregulate MAP3K7[334]. Reduced mir-10b, therefore, may allow for unfettered activation of NFκB in the endothelium, resulting in local inflammation. Also, it has been shown to control BCL2L11, an apoptosis inducer. In a study of renal transplant patients, the downregulation of mir-10b was associated with allograft rejection and glomerular endothelial apoptosis. Furthermore, the downregulation of mir-10b appeared to trigger a pro-inflammatory cascade of cytokines and promoted the recruitment of macrophages with endothelial release of TNFα, IL-6 and CCL2[335]. In other studies, it has been demonstrated that mir-10b levels are reduced by 83 ± 15% in athero-prone regions of the aortic arch. In these swine studies, mir-10b appeared to be largely confined to the endothelium[336].

Kruppel-like Factor 4 is a known target of miR-10b and is a key mediator of endothelial health, being responsible for regulation of endothelial anti-inflammatory, anti-thrombotic and anti-oxidant states. KLF4 is upregulated by laminar shear stress in an AMPK-dependent MEK5/ERK5/MEF2 pathway and is also upregulated by inflammation and vascular injury, being induced by TNFα and particularly by IFNγ[292,337]. It reduces endothelial activation, including VCAM and E-selectin expression, through the inhibition of NFκB activation[338]. It also affects VSMC differentiation and inhibits their proliferation. Several studies have suggested that KLF4 may lead to dedifferentiation and phenotype switching of the VSMC although others take the opposing view, that KLF promotes VSMC differentiation[295,339]. Similarly, KLF11 is a predicted target of miR-10b and also suppresses endothelial cell activation through the inhibition of NFκB[340].

Reduced miR-10b concentrations in CSX, therefore, would increase KLF4 and KLF11 concentrations and allow for an anti-inflammatory effect, perhaps as a negative feedback to the pro-inflammatory stimulus. Indeed, the inflammation-dependent induction of KLF4 may even be explained by an inflammation-dependent reduction of

miR-10b concentrations, thereby allowing the expression of KLF4 to supervene. The reduced miR-10b may also allow the increased activation of NK cells, a potential source for the IFNγ and TNFα seen in our CSX cohort, through upregulated expression of MICB MHC class I molecules. On the other hand, increased miR-10b levels in CSX would lead to reduced KLF4 activity, thereby switching off the anti-inflammatory apparatus in endothelial cells. The activity of KLF4 in CSX deserves further study.

6.13.2 microRNA-143

miR-143 is coded as part of the polycistronic miR-143/145 cluster on 5q33. It is the most highly expressed miRNA in VSMCs and is also produced locally by ECs. Exocytic transport of miR-143 and tunneling nanotubes allows communication between these two cellular populations. miR-143 expression is upregulated by as much as 7-20 fold in response to laminar shear stress and as such regulates EC response to local rheology. Expression is also increased by statins, BMP2 and particularly by KLF2 signaling while the Jag-1/NOTCH signalling pathway in VSMCs has also been shown to regulate miR-143 production. miR-143 expression is downregulated by TNFα, acute vascular injury and in chronic endothelial stress such as is seen in hypertension[341]. There are numerous genetic targets for miR-143 but most relevantly it regulates KLF4, KLF5, Elk-1, SIRT-1, COX2 and ACE activity. miR-143 strongly induces cell cycle arrest and increased quantities are seen in senescent cells. Conversely, reduced miR-143 levels have been seen in many cancers as its absence allows the proliferation of many cell lines. It is also atheroprotective and has been found to be reduced in patients with atherosclerosis and in damaged blood vessels[342].

Crucially, miR-143 appears to be significantly important in the maintenance of healthy VSMC differentiation and is key in the preservation of normal contractile function mainly through the downregulation of KLF4/5 and upregulation of myocardin[343]. VSMCs depleted of miR-143 de-differentiate and lose their contractile phenotype and instead become migratory, proliferative and secretory in nature. Dedifferentiated

VSMC usually secrete pro-inflammatory mediators such as TNFα and IL-6 and also activate local endothelium. miR-143 knockouts have dilated blood vessels with thin media and consequently suffer a drop in blood pressure. VSMC phenotype plasticity is crucial in vascular remodeling and in adults is seen mostly in response to vessel injury. Reduced miR-143 allows the contractile VSMCs to change function, proliferate and migrate, thereby facilitating alteration of the local vascular architecture.

Of further importance, miR-143 targets Angiotensin Converting Enzyme (ACE). miR-143 knockdown led to increased ACE mRNA concentrations[344]. Despite this increase in local ACE—an enzyme that increases the concentration of Angiotensin II, a potent inducer of vasoconstriction—vasodilation supervenes, possibly through a mechanism of tachyphylaxis or "angiotensin resistance" with increased local ATII saturating local receptors with consequent internalization of the receptors[345,346]. miR-143 has also been shown to downregulate SIRT1[308]. This deacetylase has many beneficial effects on the endothelium including anti-inflammatory effects and potentiation of eNOS activity. Finally, Cyclooxygenase 2 (COX2) also appears to be a target of miR-143. Downregulation of miR-143 leads to increased COX2 mRNA stability and prostaglandin E2 synthesis. PGE2 is known to cause pain, increased vascular inflammation and EC activation[347].

The validated reduction of miR-143 in our cohort could lead to VSMC dedifferentiation and upregulation of ACE and prostaglandin E_2 synthesis. Its reduction may be due to TNFα or else may be a result of downregulated KLF2 activity (as this is a primary driver of miR-143 release).

6.13.3 microRNA-199b

miR-199b is coded on chromosome 19p13.2 and its expression is controlled by the TWIST1 transcription factor (incidentally, the same factor that governs miR-10b

expression). Its expression is downregulated in hypoxia and in senescent cells. It has several relevant targets including sirtuin-1 (SIRT1), Hypoxia-Inducible factor-1 (HIF1), the Jagged-1/NOTCH pathway and the TRPV1 vanilloid receptor. Circulating levels have been demonstrated to be depressed in several conditions including hepatocellular carcinoma and dilated cardiomyopathy[348]. Elevated levels are seen in pathological myocardial hypertrophy as part of a feed-forward CN/NFAT signalling loop[349].

miR-199b is important in the differentiation of endothelial cells from stem cells and reduced miR-199b leads to reduced angiogenesis[350]. Reduced miR-199b also allows upregulation of hypoxia-inducible factor (HIF1α), which is responsible for 89% of the genes upregulated by hypoxia. Apart from inducing iNOS, VEGF and EPO in order to maximise blood supply to the hypoxic organ, HIF-1α also induces endothelin-1 via direct action on its promoter and can activate NFκB[351,352]. Reduced miR-199b also allows increased sirtuin-1 activity[326]. As shown in table 6.2, SIRT-1 has many benefits to endothelial function and its upregulation in CSX could represent an attempt to reverse the endothelial dysfunction present in this condition although cardiovascular SIRT1 is regulated more by miR-199a than 199b. Downregulation of miR-199b with consequent upregulation of the TRPV1 channel expression is seen in conditions with increased visceral pain such as IBS[353]. TRPV1 channels are also present in the nerves supplying the heart and blood vessels and are involved in the perception of cardiac pain. If these channels are upregulated in CSX it may allow heightened perception of angina pectoris. Increased visceral sensitivity is one of the putative causes of CSX.

Finally, reduced miR-199b has been implicated in upregulation of the ubiquitin-proteasome-system (UPS) as miR-199 targets ubiquitin ligases. The UPS is a cytoplasmic structure that is important in the breakdown of cellular proteins following their ubiquitination by ligases[354]. This is relevant to atheromatous coronary artery disease in that remodelling of the arterial wall depends on UPS function. The UPS system is also

implicated in endothelial oxidative stress and endothelial activation through ubiquitination of various cofactors involved in eNOS regulation[352].

6.13.4 microRNA-200 Family

The microRNA-200 family consists of 5 separate miRNAs that are encoded on 2 separate chromosomes. Mir-200a, -200b and -429 are all encoded from the same region on chromosome 1p36.3, while miR-200c and miR-141 are coded on chromosome 12p13.3. All of these miRNAs are richly expressed in epithelial tissues and regulate several aspects of the normal functioning of this tissue, being particularly implicated in the maintenance of cell polarity and the control of cellular senescence and migration. The expression of the miR-200 family is downregulated by hypoxia (40%) and specifically by HIF1α, which reduces miR-200b concentrations by 55%. Furthermore, inflammation characterised by elevated IL-6 also suppresses miR-200 expression. Inducers of miR-200 production include TGFβ, PDGF and, significantly, reactive oxygen species. As such, the miR-200 family play a key role in the endothelial dysfunction that is induced by oxidative stress. Validated and predicted targets of miR-200a and b include ZEB1, ZEB2, SIRT1, Endothelin-1, KLF11, MAPK (p38), ETS-1 and PTEN.

ZEB1 and ZEB2 are repressors of the release of the calcium-dependent adhesion molecule E-cadherin, a molecule responsible for the maintenance of endothelial integrity. By regulating these transcriptional repressors, miR-200 appears to exert important control over the phenotype of epithelium. Reduced miR-200 levels are associated with increased epithelial migration with so-called Epithelial-Mesenchyme Transition (EMT). Conversely, increased miR-200 leads to the opposite, mesenchyme-epithelial transition (MET). EMT is important in embryogenesis (Type 1 EMT), the induction of cancer metastasis (Type 3 EMT) and in vascular damage and tissue fibrosis in response to chronic injury (Type 2 EMT). This last EMT (also called Endothelial-

mesenchyme transition or EndMT) is believed to be implicated in the microangiopathy seen in pulmonary arterial hypertension and systemic sclerosis.

miR-200b is known to target the Ets-1 transcription factor, which is important in angiogenesis and endothelial cell migration. Increased Ets-1 leads to reduced endothelial differentiation, VSMC hypertrophy and increased release of vascular inflammatory mediators and endothelial dysfunction in an angiotensin-II-dependent fashion[355]. Furthermore, miR-200b but not MiR-200a appears to downregulate NFκB endothelial signalling in response to Toll-like Receptor (TLR) activity by targeting the MyD88 adaptor molecule and as such reduced TLR-induced activation of macrophages with the reduced release of cytokines such as IL-6 and TNFα[356].

6.14 Gene Ontology Enrichment Analysis

Gene ontology (GO) enrichment analysis attempts to identify GO terms that are significantly associated with differentially expressed microRNAs. Using Exiqon's miRSearch, we map the differentially expressed microRNAs identified above to their target genes and it then is possible to investigate whether specific GO terms are more likely to be associated with these microRNAs he (Gene Ontology Consortium, 2000). Two different statistical tests are used and compared. Firstly, a standard Fisher's test is used to investigate enrichment of terms between the two test groups. Secondly, the 'Elim' method takes a more conservative approach by incorporating the topology of the GO network to compensate for local dependencies between GO which can mask significant GO terms. Comparisons of the predictions from these two methods can highlight the truly relevant GO terms. The figure below shows a comparison of the results for the GO (Biological processes) terms associated with the significantly differentially expressed microRNAs that were identified between the groups. miRNAs were associated with the GO terms via their target genes.

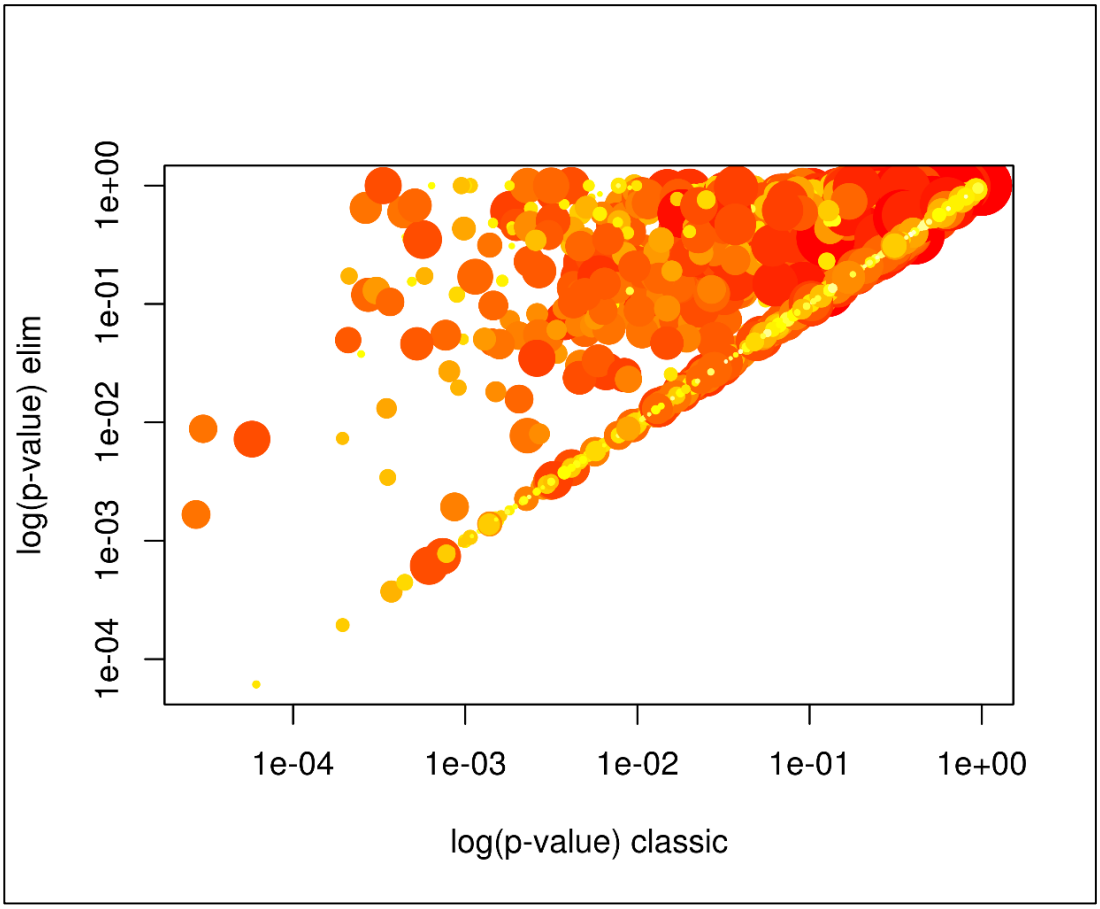


Figure 6.19: Scatter plot for significantly enriched GO terms predicted to be associated with differentially expressed miRNAs. The horizontal axis shows Fisher test results and the vertical axis shows the results by the Elim method. Values along the diagonal are consistent between both methods. The size of the dot is proportional to the number of genes mapping to that GO term and the colouring represents the number of significantly expressed genes corresponding to that term with dark red representing more terms and yellow representing fewer.

The top significant GO terms (biological processes) are given in table 6.9 below.

No	GO ID	Term	p-value
1	GO:0060218	hematopoietic stem cell differentiation	6.10E-05
2	GO:0035094	response to nicotine	0.00019
3	GO:0002460	adaptive immune response based on somati...	0.00037
4	GO:0060021	palate development	0.00045
5	GO:0021670	lateral ventricle development	0.00058

Table 6.9: Significant GO terms from the comparison between CSX patients and healthy controls.

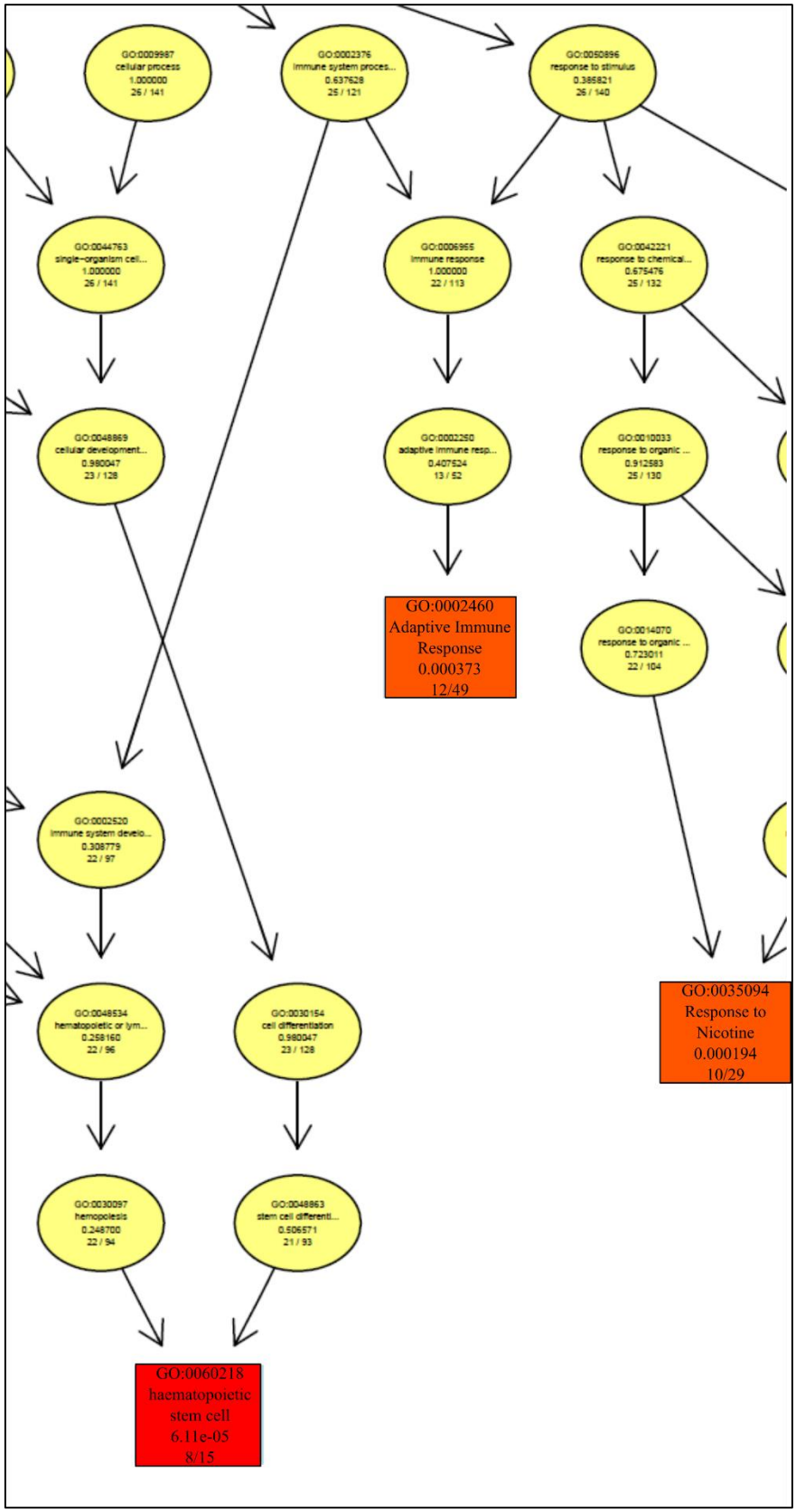


Figure 6.20: Gene Ontology network of Biological Processes

The GO network generated from our data shows the GO enrichment terms most highly associated with our dataset. The strongest supported nodes are red and nodes with no significant enrichment are yellow. As is demonstrated above, the adaptive immune response and haematopoietic stem cell responses are most highly affected in our enrichment analysis.

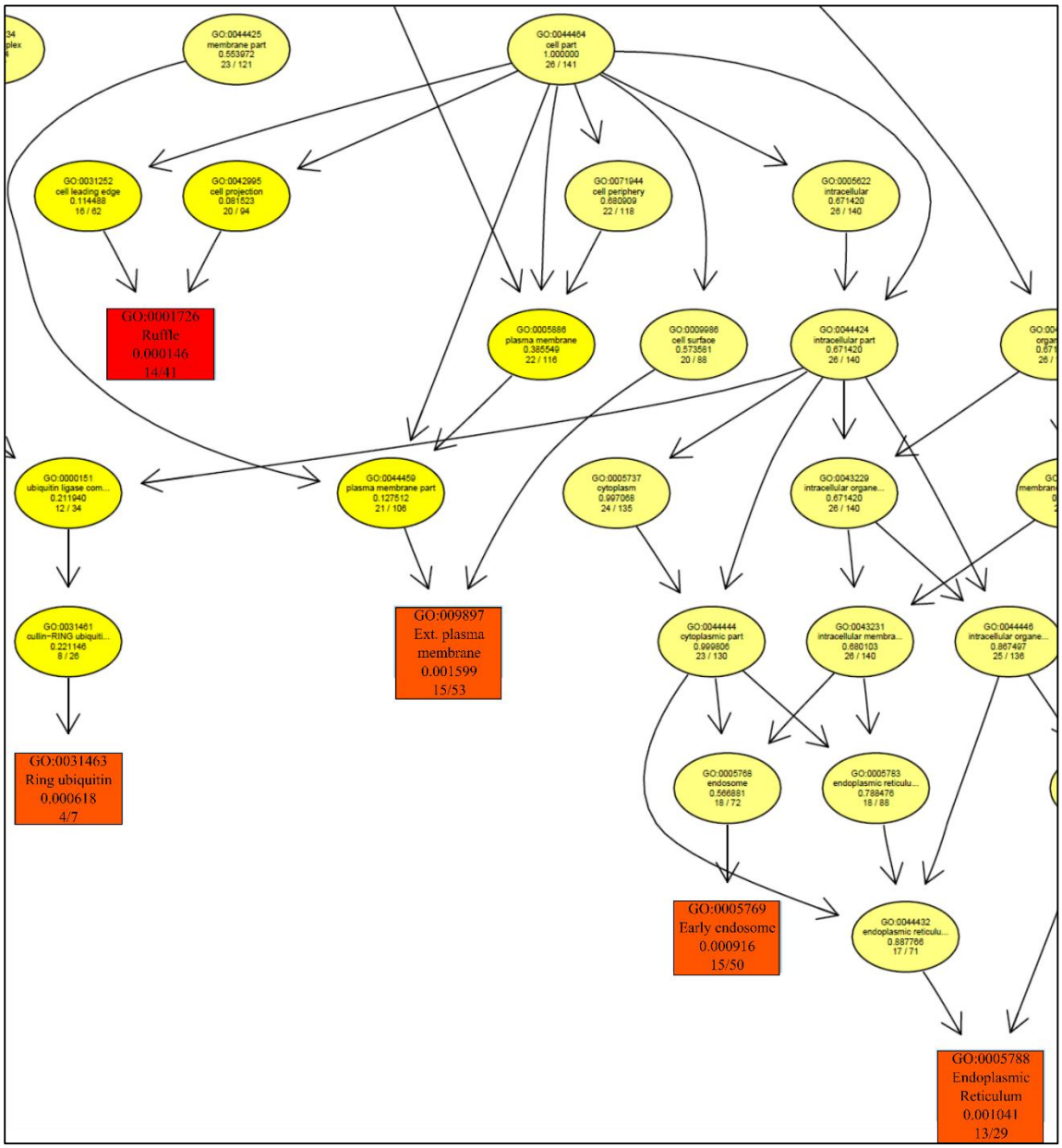


Figure 6.21 Cellular Component Gene Ontology Analysis.

Cellular component GO highlights the potential cellular locations that are affected by the biological effects of the genes regulated by the miRNAs in our analysis. Interestingly, membrane ruffling is the most strongly affected component. Membrane ruffling is seen in macrophages performing pinocytosis of oxLDL and is upregulated in EC in response to oxidative stress.

6.15 LCSX

Interestingly, the expression of all 5 miRNAs did not differ in the LCSX group when compared to the healthy controls (miR-10b, adj. p=0.192; miR-143, adj. p=0. 352; miR-199b, adj. p=0.631; miR-200a, adj. p=0.184; and miR-200b, adj. p=0.136).

Discussion

microRNAs provide a compelling and relatively discerning insight into the mechanisms at play in many disease states. As our understanding of the systems and genes that are influenced by miRNAs increases, we can begin to use this knowledge to decipher the pathogenesis of these conditions. It is reassuring that all five miRNAs that were significantly differentially expressed in our CSX population have known genetic targets with relevance to vascular function. The identity of these miRNAs directs our attention to specific aspects of vascular biology and implicates several pathways as being of particular importance in CSX. miRNAs are also attractive as they may provide a possible therapeutic option as modulation of their expression may allow one to intervene in disease by selectively modifying concentrations of the regulatory miRNAs.

In general, the NGS results showed that the CSX patients under-expressed key vascular miRNAs compared with the healthy control groups. Suppression of miR-199b, miR-200a and miR-200b is seen in tissue hypoxia while miR-143 is reduced by elevated levels of TNFα, as is seen in our population. Meanwhile, miR-10b is downregulated in atheroprone regions and in the absence of KLF2 activity. Downregulation of these

specific miRNAs can modulate several vascular processes that may be of importance in CSX. Chief among these is vascular inflammation, whereby the local pro-inflammatory machinery is activated by the upregulation of NFκB, amongst other transcription factors.

The qPCR results did not, however, concur with much of the NGS data. This was likely due to the very low concentrations of these miRNAs in the samples. Only miR-143 had a C_T less than 35 cycles. Indeed, the qPCR went so far as to show that the other miRNAs were significantly upregulated in our CSX population compared to our control group rather than being underexpressed as suggested by the NGS. The reliability of these results, given the high C_T values, is questionable. These results were, in a way, predicted by the extremely low counts in the NGS results for these miRNAs. Exiqon, however, claims that 85-90% of miRNAs found to be differentially expressed using NGS can be validated by Exiqon's qPCR system once >120 counts have been measured during NGS. Other factors should also be considered. Although our RNA samples were shown to be pure during our quality control steps, the purity of the cDNA was not assessed. This could affect the efficiency of replication and explain the high C_T values. The reference miRNA replicated with good efficiency, however, so this does not appear to be a likely cause. Another possible explanation for the discordant results would be if the primers were unable to anneal correctly. As Exiqon primers use locked nucleic acid (LNA) and we used the appropriate primers in the appropriate conditions, this appears unlikely. Finally, it may be that our reference miRNA was not stably expressed across our groups. This could be offset by running the samples with a panel of reference genes to improve standardisation across samples.

We can be reasonably certain, however, that miR-143 is suppressed in CSX patients while there is a strong suggestion that miR-10b, miR-199b, miR-200a and miR-200b are differentially expressed in CSX patients, although we cannot be sure if it is underexpressed or overexpressed.

6.16 miRNAs and Vascular inflammation in CSX

As endothelial dysfunction with inflammation seems to be a defining feature of CSX/MVA activity, knowledge of the involvement of miRNAs in this endothelial activation could allow for a viable therapeutic option. Our analysis of the miRNA profile in our CSX cohort has identified several likely pathways that may be dysregulated in our populations due to altered expression of regulatory miRNAs. NFκB was always likely to be implicated in endothelial activation and our miRNA analysis shows that this is surely the case in CSX as miR-10 is specifically implicated in the regulation of this transcription factor.

Low concentrations of miR-10b would allow NFκB activation through increased MAP3K7 availability for incorporation into the kinase complex with TRAF6, a complex that is critical for the release of NFκB from its inhibitors. NFκB is usually bound to its inhibitor IκB in the cytosol but the IKK complex phosphorylates IκB, thereby releasing NFκB to migrate to the nucleus and activate its gene transcription programme. Indeed, miR-10 may a key regulator of vascular inflammation in CSX through its control of the NFκB activation. The canonical NFκB pathway is initiated through its activation by TNFα or IL-1 signalling and its association with RelA (p65) complex. As noted in Chapter 4, our CSX patients have elevated TNFα concentrations further supporting this theory. Activated NFκB then induces adhesion molecules, chemokines (CXCL1 and CXCL10), cytokines (TNF, IL-6, IL-1), iNOS and COX2 in the endothelial cells causing a feed-forward pro-inflammatory cascade.

Another driving force for the perpetuation of vascular inflammation in CSX may be activation of the E26 Transformation-specific family of transcription factors. Ets-1 is targeted by miR-200b and if this miRNA is reduced in CSX it is reasonable to conclude that ets-1 activity is increased. Ets-1 upregulation is stimulated by angiotensin II, IL-1β and TNFα. Ets-1 appears to play a key role in the mediation of the vascular effects of

Angiotensin II on vessels. Ets-1 causes increased matrix metalloproteinase release with medial hypertrophy and increased VCAM and MCP-1, all known features of CSX.

Reduced 10b may lead to upregulated NK cell activity through the derepression of the MICB gene that encodes MHC class I proteins on endothelial cells in response to stress, which then interacts with NKG2D on NK cells thereby activating them. This may implicate NK cells rather than macrophages as the source of the elevated IFNγ and TNFα seen in our study population and by extension may indicate that a viral source may be at the root of the inflammation in CSX. Natural Killer cells are lymphocytes that play an important role in the innate immune system and are crucial in the host defence against viruses. An interesting study into CSX patients demonstrated possible myocardial viral infections on histological examination in 9/13 of the CSX patients studied[357]. NK cells are capable of targeting activated ECs through their interaction with the chemokine fractalkine (C-X3-C), a substance induced on ECs by TNFα. The close binding of NK cells to ECs allows the former to degranulate and cause direct cytolytic damage to the latter[358]. NK cells themselves have also been demonstrated to be atherogenic through their release of granzyme[359].

There may also be a role for increased Cyclooxygenase 2 (COX2) activity in our cohort as miR-143 targets COX2. COX2 is constitutively expressed in ECs and VSMCs and, as is discussed further in chapter 7, is responsible for the production of many vasoactive compounds, including prostaglandins and prostacyclin, but also isoprostanes that are a source of oxidative stress. It was initially thought that the upregulation of COX2 may compensate for reducing levels of available NO in endothelial dysfunction by increasing prostacyclin production, thereby attempting to rescue endothelial-dependent vasodilation. It has been noted that COX2 is upregulated in patients with CAD to potentiate bradykinin-induced vasodilation[360]. There is emerging evidence, however, that upregulation of COX2 occurs with age and may instead be responsible for the production of vasoconstrictor prostanoids as well as imposing an oxidative stress on

the endothelium, worsening endothelial dysfunction. COX2 inhibition has been shown to improve flow-mediated dilation in patients with CAD, highlighting the role of COX2 in endothelial dysfunction[361].

The role of oxidative stress in CSX has always been interesting as it is an inducer of endothelial dysfunction and vascular inflammation. There is clear evidence that these patients do have increased oxidative stress and it is believed that this may be at the centre of the endothelial dysfunction seen in CSX. The identification of the differential expression of the miR-200 family might provide evidence to implicate such oxidative stress in CSX. The miR-200 family has been shown to be potently induced by ROS and to induce endothelial apoptosis and senescence[362]. We are not certain if miR-200 is upregulated or downregulated in CSX. Hypoxia may reduce miR-200 levels even in the face of oxidative stress, with HIF1α signalling perhaps overriding the ROS stimulus for miR-200 expression. The upregulated SIRT1 and ZEB1 may also regulate oxidative stress, reducing its influence over miR-200 expression.

Finally, the role of Kruppel-like factors in the pathogenesis of CSX also bears mentioning. It is important to note that a reduction of miR-143 implies a relative lack of KLF2 activity as KLF2 is the most potent stimulus for miR-143 release. As discussed in 6.2.2 above, KLF2 is a critical anti-inflammatory transcription factor that abrogates the effects of NFκB in the endothelium and levels of this transcription factor are reduced by TNFα[296]. Interestingly, 2 of our differentially expressed miRNAs (miR-10b and miR-143) target KLF4 and as such one might expect that KLF4 levels are increased in CSX due to miR-143 downregulation. It should be noted that KLF4 is induced by inflammation but is itself anti-inflammatory in its behaviour. Upregulation of KLF4 may reduce MCP-1, IL-6 and CRP expression and it may be that activation of this transcription factor actually alleviates the symptoms in our cohort by downregulation of NFκB and is induced in a counter-regulatory capacity. Similarly, reduced miR-143,

miR-199 and miR-200a allow the upregulation of Sirtuin 1 (see 6.2.4) and it is likely that this is also an effort to preserve normal endothelial cell function in CSX.

In summary, miRNA profiling would suggest that upregulation of NFκB and Ets-1, perhaps ably supported by increased COX2 and NK cell activity, may be the major pathways involved in the induction of endothelial activation in CSX. Reduced KLF2 activity, implied by reduced miR-143 levels, may be compensated for by the likely upregulation of KLF4 and SIRT1, two transcription factors that attempt to downgrade endothelial inflammation. It may be that SIRT1 and KLF4 win out as CSX patients improve.

6.17 miRNA effects on VSMC in CSX

The pattern of miRNA downregulation in our cohort raises the possibility of VSMCs making a significant contribution to the phenotype seen in CSX. VSMCs normally differentiate into specialised contractile cells confined to the vessel tunica media. Indeed, VSMCs and the extracellular matrix (ECM) they release are the only components of the tunica media. They are in direct electrical communication with the overlapping ECs via gap junctions as well as via bidirectional signalling carried out by many chemical mediators. Their primary function is to modify vessel tone and luminal diameter and are particularly important in the resistance arterioles where they control the distribution of blood flow to different vascular territories.

Adult VSMCs are not terminally differentiated and demonstrate functional and phenotypic plasticity. At one end of the spectrum, quiescent, fully-differentiated VSMCs are said to be in the “contractile” state and express many state-specific contractile proteins such as SM α-actin, MHC, smoothelin and SM22α. These cells are capable of undergoing phenotype switching, where they de-differentiate back into non-contractile cells that are capable of migrating and proliferating with loss of their specialised contractile proteins. Additionally, these dedifferentiated SM-like cells are

capable of producing cytokines and pro-inflammatory mediators and are said to be in the “secretory” state.

Switching is seen in hypertension, where extensive remodelling of the entire vascular tree is observed with medial hypertrophy, microvascular rarefaction, hyalinisation and fibrosis. It is also an important feature of atherosclerosis where secretory VSMCs migrate to the intima and fuel the inflammatory cycle in atherosclerotic plaques by releasing cytokines. Their release of ECM and proteases also results in plaque enlargement, intimal thickening and luminal narrowing. Phenotypic switching is also activated in neovascularisation and vascular remodelling and may possibly be seen in coronary microvascular dysfunction in hypertrophic cardiomyopathy[363].

VSMC phenotypic switching may occur in response to vessel injury, mechanically transduced signals from local blood flow, cell-cell signalling or via inflammatory signalling. Oxidised LDL is a potent trigger of the switch through the activation of LOX-1 receptors on VSMCs. TNFα has been demonstrated to stimulate switching while Angiotensin II activation of AT_1Rs on VSMCs also switches the VSMCs to a secretory phenotype, upregulating VCAM-1, ICAM-1 and MCP-1 expression as well as leading to fibrosis of the vessel wall and ultimately increased arterial stiffness. Platelet-derived Growth Factor (PDGF) pushes the cells towards their secretory, proliferative state whereas TGFβ stimulates a stable contractile phenotype.

The genetic regulation of this switch appears to involve Serum Response Factor, in concert with its cofactor myocardin, acting via a CarG box to induce a contractile state. A parallel promoter of differentiation involves the activation of notch 3 by endothelial-expressed Jagged-1. Both pathways are believed to involve the increased expression of miR-143/145[364]. These miRNAs then target KLF4, KLF5 and Elk1, reducing their activity. Upregulated KLF4 and KLF5 are known to induce secretory states in VSMCs. KLF4, with Elk1, binds to and suppresses SMC contractile genes while it also modifies chromatin

structure in the VSMCs' genes[365]. Reduced miR-143/145, as seen in our cohort, would result therefore in increased KLF4 activity with subsequent induction of a secretory VSMC phenotype and all of the consequences thereof.

This problem may be compounded in CSX patients as these patients may also have increased numbers of SMC-type cells available for secretory activity (see Fig 6.22 below). If downregulated, miR-200 may allow increased Endothelial-Mesenchyme Transition (EndMT), where the endothelial cells themselves detach from the endothelial monolayer and migrate further into the intima and become SMC-like cells capable of becoming secretory cells thus adding to the pool of pro-inflammatory and pro-fibrotic cells in the vessel wall. EndMT is a subset of epithelial-mesenchyme transition (EMT), which is a process that has been implicated in the microangiopathy seen in Pulmonary Arterial Hypertension and Systemic Sclerosis as well as being an important step in the metastasis of many epithelial cancers.

ECs respond to an injurious stimulus such as chronic inflammation, oscillatory shear stress, mechanical injury or hypoxia by detaching themselves from each other through the breakdown of cadherin, desmoplakin and catenins. The newly freed cells then migrate through to the intima and media where they adopt mesenchymal or smooth-muscle like characteristics and induce local upregulation of ICAM-1, VCAM-1 and fibronectin. This has been shown to happen in the microvasculature when ECs are exposed to chronic inflammatory stimuli such as IL-1[366]. IFNγ and TNFα are also believed to initiate EndMT through the downregulation of FGFR1, a growth factor receptor. Endothelin-1 mediates EMT through activation of the ET-A receptor in a TGFβ-dependent fashion. Other triggers for EMT include TGFβ itself, EGF and HGF while the Jagged1/Notch interaction is again implicated in EMT. EndMT has been shown to be upregulated in MI, portal hypertension, PAH and graft failure.

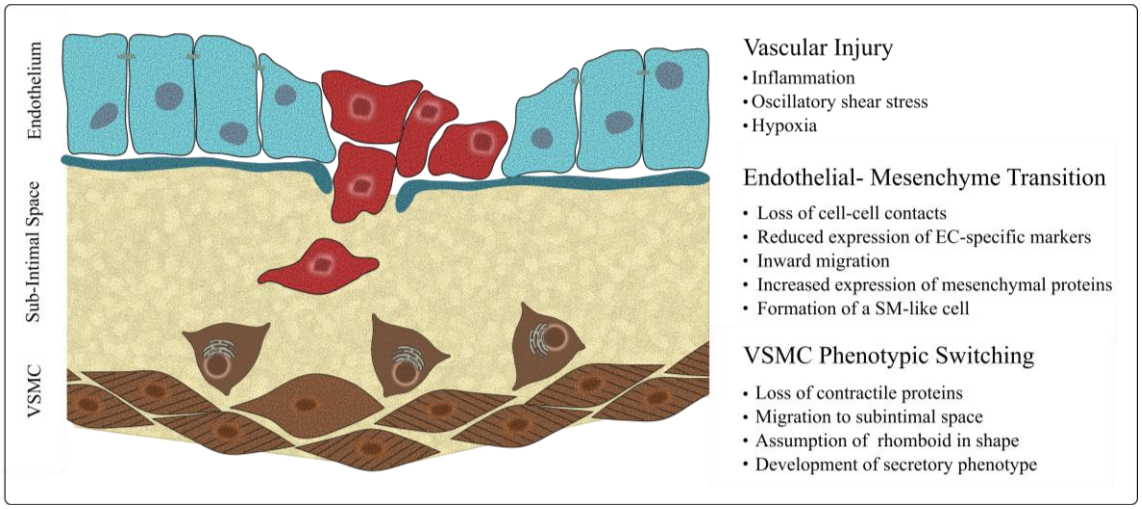


Figure 6.22: Possible role of Vascular Smooth Muscle Cells (VSMC) in the pathogenesis of CSX. miR-143 downregulation leads to VSMC phenotypic switching while miR-200 downregulation and chronic inflammation may lead to to Endothelial-Mesenchymal Transition. The resultant SM-like cells are a potent source of vascular inflammation and oxidative stress.

There is some evidence to support this EndMT and VSMC phenotype switching theory for CSX causation. Studies have demonstrated an increased number of circulating Endothelial Progenitor Cells (EPCs) in CSX[367]. EPCs are released from the bone marrow in response to vascular injury and hone to the injured endothelium to begin the repair of the damaged monolayer. EndMT would stimulate EPCs release but they are certainly not the only stimulus for CD133 cell release from the marrow as chronic inflammation is also a potent trigger. EPCs themselves may also translocate to the subintima and then become SM-like cells, adding even more to the reservoir of pro-inflammatory cells.

There is also evidence of increased VSMC secretory activity in CSX as histologically the microvessels of CSX patients demonstrate subendothelial hyalinisation, fibromuscular hyperplasia, medial hypertrophy, myointimal proliferation and perivascular fibrosis all indicating increased mesenchymal cell activity in CSX patients[67,368]. Increased fibrotic change in the tunica media is also reflected by increased medial stiffening of arteries. CSX patients are known to have greater carotid arterial stiffness than healthy controls[171]. If both EndMT and VSMC phenotype switching are present in CSX, then the

coronary microvascular dysfunction might be analogous to the angiopathy seen in the pulmonary circulation of Pulmonary Arterial Hypertension patients. One might implicate ET-1 or Angiotensin II as possible causes for this phenotype.

6.18 miRNA and Vasoactive Hormones

Angiotensin II (AngII) is converted from angiotensin I via the action of Angiotensin Converting Enzyme (ACE). miR-143 targets and downregulates ACE activity. The downregulation of miR-143 may therefore allow the increased formation of AngII through increased ACE activity. AngII has several important effects on the vasculature, acting on AT_1R to promote vasoconstriction, VSMC phenotype switching, EC apoptosis, NOX activity (oxidative stress), COX2 activity, LOX1 expression and vessel fibrosis. The benefit of ACE-inhibition in CSX has been demonstrated in many studies as it has been shown to partly reduce the endothelial dysfunction. The observation of reduced miR-143 levels adds support to the notion of Angiotensin playing an important role in CSX.

Endothelin-1 is also crucial in the modulation of normal endothelial function (as described in 6.2.6 above). miR-199 directly targets *END1* and as such its downregulation would allow increased endothelin production[319]. Endothelin is produced in endothelial cells and is the most potent endogenous inducer of vasoconstriction. It also has a plethora of other effects that are relevant in CSX. It, impairs endothelium-dependent vasodilation by reducing NO bioavailability, increases ROS formation through increased NOX activity causes VSMC proliferation and induces a pro-inflammatory phenotype including the stimulation of macrophages with the release TNFα, IL-1, IL-6 and IL-8[321]. Both a reduction of miR-199 and the implied reduction in KLF2 activity would both upregulate the endothelin system. There is evidence to support this in CSX as ET-1 levels increase during exercise and glycaemic loading in CSX compared to the opposite responses in healthy controls[192,369]. The use of endothelin antagonists has been shown to improve endothelial-dependent vasodilation in PAH subjects but has not been trailed in CSX patients.

6.19 TRPV1 and Visceral Hypersensitivity in CSX

As was laid out in 1.4.4, CSX patients exhibit abnormal pain processing both peripherally, with increased sensitivity to cardiac and cutaneous stimuli, and centrally, with altered insular activity on fMRI. Visceral hypersensitivity is described in patients with Irritable Bowel Syndrome (IBS) and has been tied to increased numbers of C and Aδ nerve fibres expressing transient receptor potential vanilloid type 1 (TRPV1). TRPV1 is found in many small and medium sized nerve fibres and is also expressed in peripheral mononuclear cells. It is a 6 transmembrane protein that forms a non-selective cationic channel with particularly marked permeability to Calcium. When activated by heat, lipids or capsaicin it imbues the patient with a sense of burning pain. The threshold for TRPV1 activation is reduced by a local drop in pH or local inflammation, particularly increased local prostanoids concentrations, and it is induced by the presence of reactive oxygen species. When stimulated the nerve endings also release Substance P and CRGP to initiate neurogenic inflammation.

miR-199 targets TRPV1 mRNA and miR-199 concentrations have been shown to correlate inversely with TRPV1 concentrations and visceral pain scores in patients with diarrhoea-type IBS, that is to say that patients with more GI pain have lower miR-199 concentrations and higher TRPV1 expression[353]. TRPV1 is believed to play a role in cardiac nociception and it may be that over-expression of this receptor, inferred by a reduction in miR-199, may lead to a type of visceral hypersensitivity in CSX analogous to the GI hypersensitivity seen in IBS. Quite apart from its role in cardiac nociception, TRPV1 has further relevance to the pathogenesis of CSX as it modulates innate immune function. As it is expressed on blood mononuclear cells, TRPV1 appears to be essential for normal monocyte integrity with reduced TRPV1 expression being associated with a failure of phagocytosis and oxidative burst and a failure to contain bacterial infections[370].

Finally, TRPV1 is important in the control of endothelial-dependent vasodilation. It is present in most arterioles and has varying effects depending on the vascular territory, being capable of causing vasodilation or vasoconstriction. Activation of TRPV1 by capsaicin leads to pronounced coronary vasoconstriction with increased vascular resistance and reduced coronary flow[371]. Antagonism of the receptor leads to vasodilation[372,373]. It appears that the TRPV1 activation allows increased cytosolic calcium concentrations in smooth muscle cells and ultimately leads to release of endothelin-1 from the ECs, with endothelin in turn potentiating TRPV1 activation.[374] Thus, reduced miR-199 could allow increased TRPV1 expression in CSX patients with repercussions on cardiac nociception and coronary vasomotion. This possibility certainly warrants further investigation.

6.20 Limitations

The main limitation of our analysis of miRNAs in CSX was the fact that we derived contradictory results from too well-executed methodologies. The qPCR results were not unexpected as we were aware of the low concentrations of the various differentially expressed miRNAs ab initio from our NGS analysis. Despite this, all 5 studied miRNAs were differentially expressed by both methodologies and the 4 unvalidated miRNAs require further analysis to reconcile the opposing results as they hold great potential to further explain the mechanisms at work in CSX.

Conclusions

This is the first study to examine the miRNA transcriptome in Cardiac Syndrome X. We have demonstrated that miR-143 activity is reduced in our CSX patients. This microRNA is the most highly expressed miRNA in VSMCs and plays a key role in the regulation of the VSMC phenotype. Downregulation of this miRNA raises the possibility of vascular smooth muscle phenotype switching as a possible contributing factor to the pathophysiology of CSX. Secretory VSMCs can promote local inflammation and vascular

remodelling with medial hypertrophy and hyalinisation. Apart from its effects on VSMCs, miR-143 is known to regulate endothelial Angiotensin Converting Enzyme activity, with knockdown of miR-143/145 leading to upregulation of ACE activity in the endothelium with all of the deleterious vascular effects this implies. This provides the rationale for the success of ACE inhibition in the symptoms of CSX (see 1.6.2).

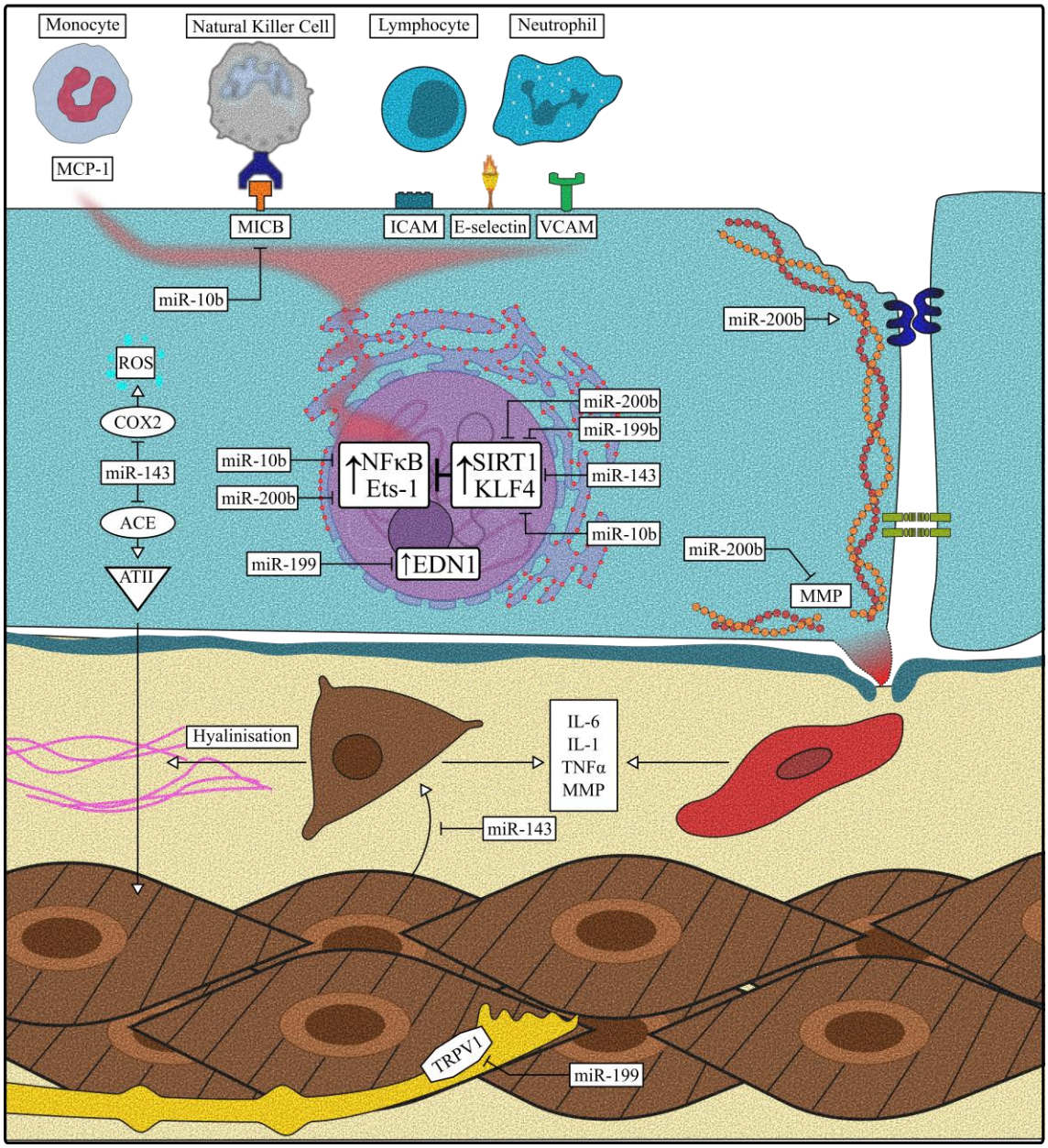
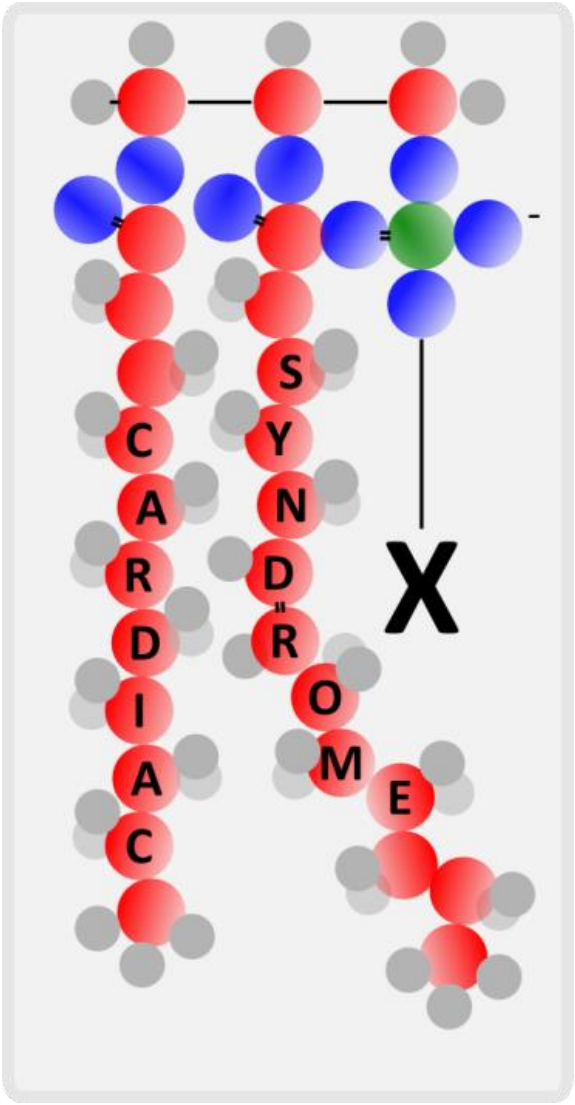


Figure 6.23: Overview of vascular processes affected by the miRNAs identified as being differentially expressed in CSX

The expression of miR-10b, miR-199b, miR-200a and miR-200b is also significantly altered in CSX. Unfortunately, the NGS and qPCR data did not agree on the nature of this alteration but these microRNAs are known to regulate NFkB, Endothelin-1, Ets-1 and Kruppel-like factors and thus it is reasonable to conclude that these factors may be involved in CSX. We also hypothesise about the possible contribution of endothelial-mesenchyme transition in CSX as this process is also regulated by miR-200. The abnormal nociception hypothesis is also supported by the notion of the possible alteration in expression of the TRPV1 receptor in the cardiac nociceptive neurons via the alteration of miR-199. Figure 6.23 above shows the many vascular pathways that are influenced by the miRNAs identified as being differentially expressed in CSX.

Going forward, it would be reasonable to perform repeat qPCR with multiple reference genes to allow us to control for any instability in the expression of any one control gene in an effort to clarify the alteration in the selected miRNAs' activity in CSX. Certainly the alteration of the above miRNAs warrants closer scrutiny. Investigation of Kruppel-like factors 2 and 4 activity as well as TRPV1 expression in CSX may also bear fruit.



Chapter 7: Plasma Fatty Acids in Cardiac Syndrome X

Introduction

7.1 Overview of Fatty Acids

Despite being united by their relative insolubility in water but solubility in organic solvents, lipids are a diverse group with many different physicochemical characteristics. Lipids may be divided in several ways. A widely used classification system is the LIPID MAPS system which is composed of eight different lipid categories (see figure 7.1). In this chapter we will concentrate on Fatty Acids and their derivatives, although SCFAs are outside the scope of this chapter.

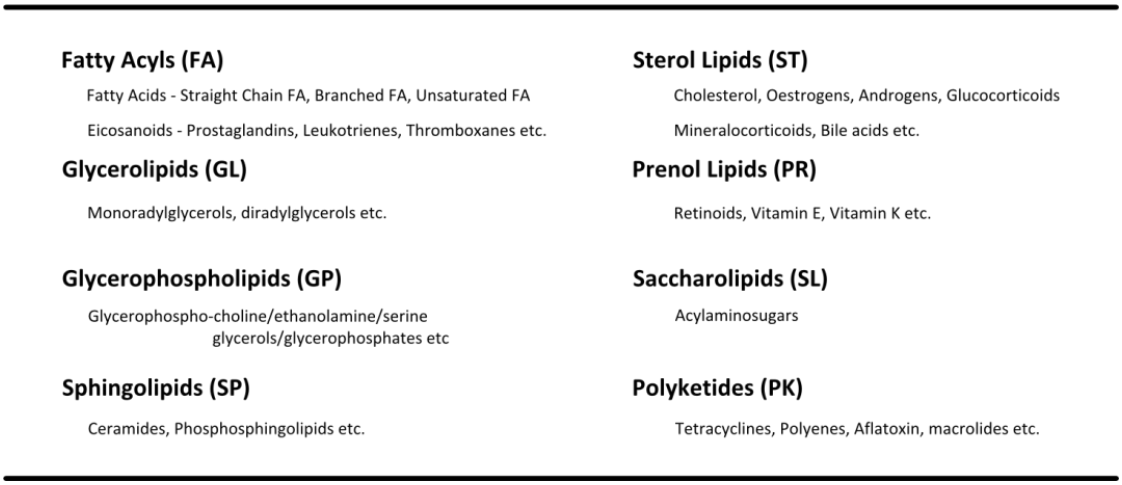


Figure 7.1: LIPID MAPS Lipid Classification System

7.1.1 Structure of Fatty Acids

Fatty acids are hydrocarbon chains with a methyl group at one end (termed the ω end) and a carboxyl group at the other (α end). There can be any number of carbon atoms in a fatty acid chain but it tends to be an even number in naturally occurring FA as their biosynthesis involves the iterative addition of 2 carbon atoms to a chain. The carbon atoms can be numbered from the carboxyl carbon (the α carbon, carboxyl reference system) or from the methyl group (ω-carbon, the omega reference system) and the carbons may be joined together by a single or a double bond. Figure 7.2 shows octanoic acid, an 8 carbon saturated fatty acid.

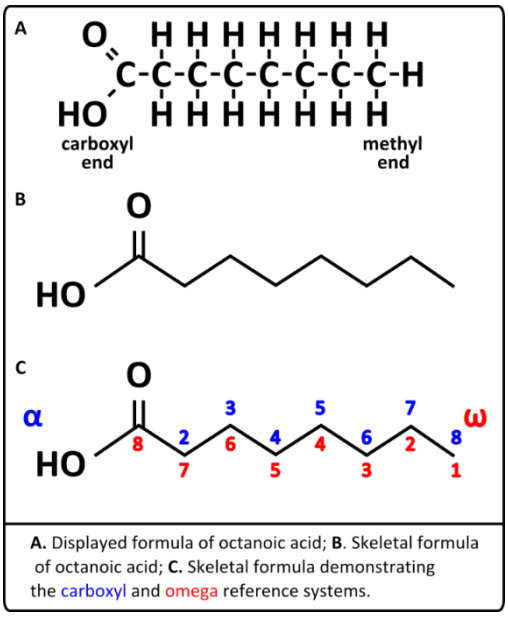
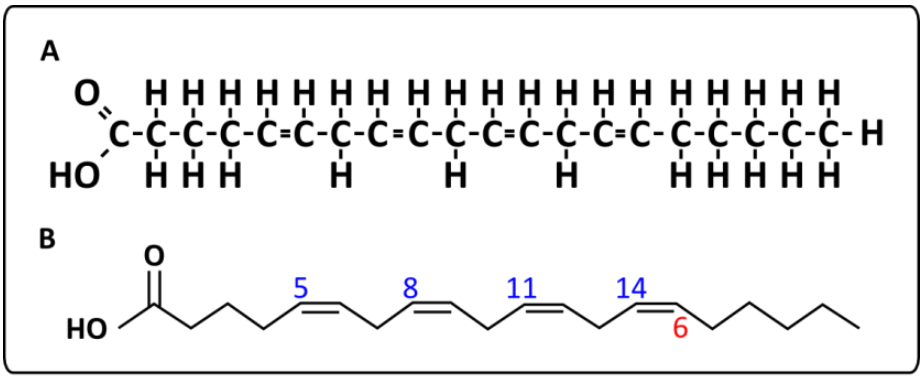


Figure 7.2: Basic Structure of Fatty Acids

FAs may be categorised by the length of their carbon chain and by the number of double bonds present. Short chain fatty acids (SCFAs) have less than 6 carbons, medium chain fatty acids (MCFAs) have 6-12 carbons and long-chain fatty acids (LCFA) contain 13-21 carbons. Very long chain fatty acids can contain 22 or more carbons. Saturated fatty acids (SFAs) do not contain any carbon=carbon double bonds and carbons are fully saturated with hydrogen atoms. The carbon chains of these FAs are straight. FAs that contain double-bonds are termed unsaturated fatty acids and may be mono-unsaturated (MUFA) if they only contain one double-bond or poly-unsaturated (PUFA) if they contain more. FAs are abbreviated with the convention C x:y $\Delta^{z1,z2\ldots}$, where x is the number of carbon atoms, y is the number of double bonds and z is the position of the double bonds as per the carboxyl reference system. It may also be described as its distance from the omega carbon (see Fig 7.3)



A. The structure of arachidonic acid (c20:4 $\Delta^{5,8,11,14}$ or c20:4, ω-6) **B.** The skeletal formula of arachidonic acid illustrating the numbering system.

Figure 7.3: A Polyunsaturated Fatty Acid

The orientation of the carbons around the double bond also offers a further method of FA sub-classification and is illustrated in figure 7.4. Most naturally occurring unsaturated fatty acids are *cis*- fatty acids, where the hydrogen atoms on the double-bonded carbons are found on the same side of the fatty acid chain (i.e. oriented in the same direction.) This creates a kink in the fatty acid chain. *Trans*- configuration FAs have the hydrogens on opposite sides of the chain and these FAs are mostly straight. Trans-fatty acids have been associated with an increased LDL and increased risk of coronary artery disease, cerebrovascular disease and diabetes.

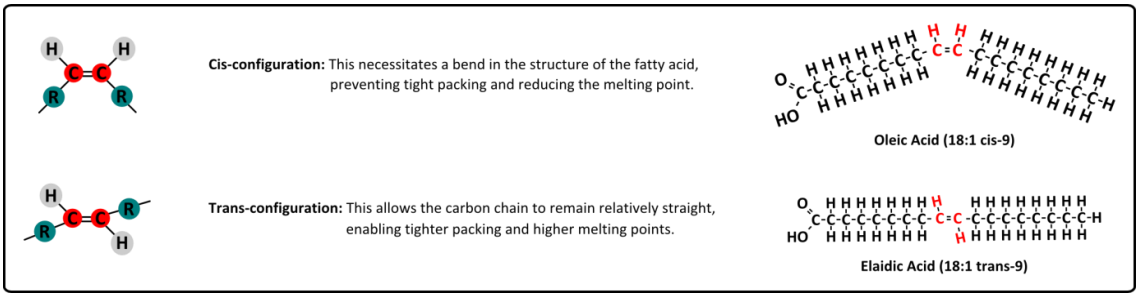


Figure 7.4: Cis- and Trans- isomers

7.2 Fatty Acids and Vascular Function

Fatty acids and their derivatives are of great importance in cardiology. Early efforts at modification of dietary risk factors for cardiovascular disease centred on saturated

fatty acids as these had been shown to increase LDL concentrations. As knowledge has progressed, dietary recommendations have changed to include the restriction of trans-fatty acids and the increase of MUFAs and PUFAs.

7.2.1 Saturated Fatty Acids

Early epidemiological observational studies noted a correlation between saturated fatty acid (SFA) intake, plasma LDL and long-term cardiovascular mortality. These results lend support to the lipid hypothesis, which propounds that atherosclerosis is due to elevated plasma lipids. Replacing SFA with MUFA or PUFA is of benefit with regards to LDL:HDL and so ideally we should reduce SFA intake while replacing it with MUFAs or PUFAs. It has been hypothesised too that not all SFAs have similar negative cardiovascular effects. It is possible that saturated SCFA and MCFA may be less dangerous that saturated LCFAs.

SFAs may have important impacts on EC function but the evidence is in broad disagreement on almost all points. Palmitic acid and stearic acid are weak agonists of PPARα but can also activate NFκB. Conflicting evidence exists regarding their effect on PGI_2 release, with some studies showing a reduction and others showing no change. Similarly, the impact of SFA on NO release in ECs is uncertain but some small studies show that oleic acid and palmitic acid reduce NO release from EC in animal cells and HUVECs [375,376]. Furthermore, SFAs have been shown to both increase and decrease the expression of adhesion molecules on the surface of ECs, further highlighting the lack of clarity surrounding the effects of SFAs in EC biology. Finally, a high SFA diet was not shown to worsen flow-mediated dilatation when compared with an SFA restricted, MUFA-supplemented diet [377].

7.2.2 Trans-fatty acids

Trans-fatty acids (TFA) contain at least one double bond in the trans-configuration (see figure 5.3) straightening the fatty acid carbon chain and allowing it to behave like a saturated fatty acid. Naturally occurring fatty acids are relatively uncommon in the human diet (accounting for <0.5% of total energy intake) and are produced by bacterial action in the intestines of ruminants. TFAs are produced industrially by the partial hydrogenation of vegetable oils and are incorporated into food by producers to allow customisation of food consistency and taste. Approximately 3% of our daily energy intake is composed of industrially produced TFAs and these are found in food such as pizza, french fries, breaded chicken etc. TFAs are particularly relevant as they are known to substantially increase cardiovascular risk. A 2% increase in energy intake in the form of TFAs is associated with a 23% increase in CHD [378].

TFAs increase LDL and triglyceride concentrations in blood while reducing HDL and LDL particle size, probably via the activation of Cholesterol Ester Transfer Protein (CETP). Furthermore, TFAs are pro-inflammatory and have been shown to be associated with increased IL-6 and TNFα in patients with dyslipidaemia [379]. TFAs may also induce endothelial activation and dysfunction and are known to increase ICAM-1, VCAM-1 and E-Selectin levels. It has been shown that TFAs reduce brachial artery flow-mediated dilatation when taken for 4 weeks [380]. The exact mechanism by which this occurs is unknown but it is believed that TFAs reduce PGI_2 production, possibly by reducing AA incorporation into the cell membrane, subsequently reducing its availability for PGI_2 formation.

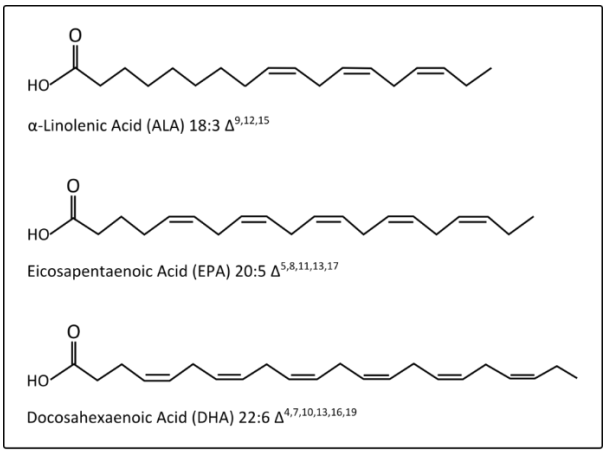
7.2.3 Mono-unsaturated Fatty Acids

Monounsaturated fatty acids (MUFAs) contain one double bond, usually in the cis-configuration. Oleic acid and palmitoleic acid are the two most common MUFAs in our diet, with oleic acid being the major component of olive oil. MUFA ingestion modestly

reduces plasma total cholesterol by reducing LDL while modestly increasing HDL levels. Also, LDLs containing MUFAs are less susceptible to oxidation than those containing PUFAs and as such may be less atherogenic (see lipid peroxidation below). Meta-analyses have also shown that diets high in MUFAs also reduce both systolic and diastolic blood pressures. The evidence, much like that for SFAs, is divided on long-term mortality outcomes with MUFA supplementation [381].

7.2.4 Polyunsaturated and Essential Fatty Acids

FAs that cannot be biosynthesised in humans are termed 'essential'. Only two fatty acids are known to be essential in humans; an omega-3 FA called alpha-linolenic acid (ALA, ω-3) and an omega-6 FA linoleic acid (LA, ω-6). The ultimate sources of these FAs are plants as they are readily synthesised there by Δ^{12} and Δ^{15} desaturase activity. As such, good dietary sources include plant seeds and fish oils (as the fish assimilate nutrients from ingested algae).



ω-3 fatty acids. Skeletal formula of the three most important examples ALA, EPA and DHA.

Figure 7.5 Omega-3 Fatty Acid Structure

Omega-3 Polyunsaturated Fatty Acids

α-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosohexaenoic acid (DHA) are the three important omega-3 polyunsaturated fatty acids. They share a common double bond between the 3rd and 4th carbons when counting from the methyl end. Skeleton diagrams for these FAs are shown in figure 7.5 above. They are found in chloroplasts and in certain nuts and seeds. These FAs play an important role in endothelial function and generally promote an anti-inflammatory, antithrombotic and anti-atherosclerotic phenotype and even improve vasodilatory responses. Importantly epidemiological studies show improved cardiovascular outcomes in terms of mortality and morbidity when patients take dietary omega-3 supplementation (up to a 50% reduction if in the upper tertile for PUFA ingestion). Interestingly, a recent methylation association study demonstrated that omega-3 PUFA ingestion can even alter DNA methylation patterns, indicating that these PUFAs may also play an important role in epigenetics [382]. The beneficial effects of omega-3 PUFAs are listed below and summarised in figure 7.6

Anti-inflammatory effects

Omega-3 fatty acids reduce vascular inflammation through several means [383]:

- Eicosanoid switching: They switch production of eicosanoids from the pro-inflammatory two- and four- series to the generally anti-inflammatory three- and five-series by directly competing with arachidonic acid for incorporation into membrane phospholipids, subsequent release by phospholipase A2 and ultimately eicosanoid production by cyclooxygenase and lipoxygenase action.
- Resolvins: They hasten the resolution of vascular inflammation by producing lipoxins, resolvins and protectins.

- Reduced NFκB activity: EPA is known to prevent IκB phosphorylation that in turn prevents NFκB activation [384]. NFκB is an important transcription factor involved in inflammation. Its inactivation reduces plasma TNFα in patients treated with EPA.

- Increased PPARα activation: PPARs are a family of nuclear receptors that mediate inflammatory pathways and can directly inhibit NFκB and also reduce expression of adhesion molecules and activation of endothelial cells. DHA in particular binds avidly to PPARs.

- Retinoic Acid Receptor (RXR) activation*:* RXRs are transcription factors and DHA has been shown to directly activate RXR. RXR has been shown to increase ABCA-1 (ATP-binding cassette transporter) and can improve HDL release and have cardio-protective effects.

- Toll-like Receptor 4 inhibition**:** Both EPA and DHA are capable of impairing the cytokine response to TLR4 activation in response to endotoxin in mice. Toll-like receptors are involved in innate immune responses. PUFAs have been shown to reduce plasma CRP levels.

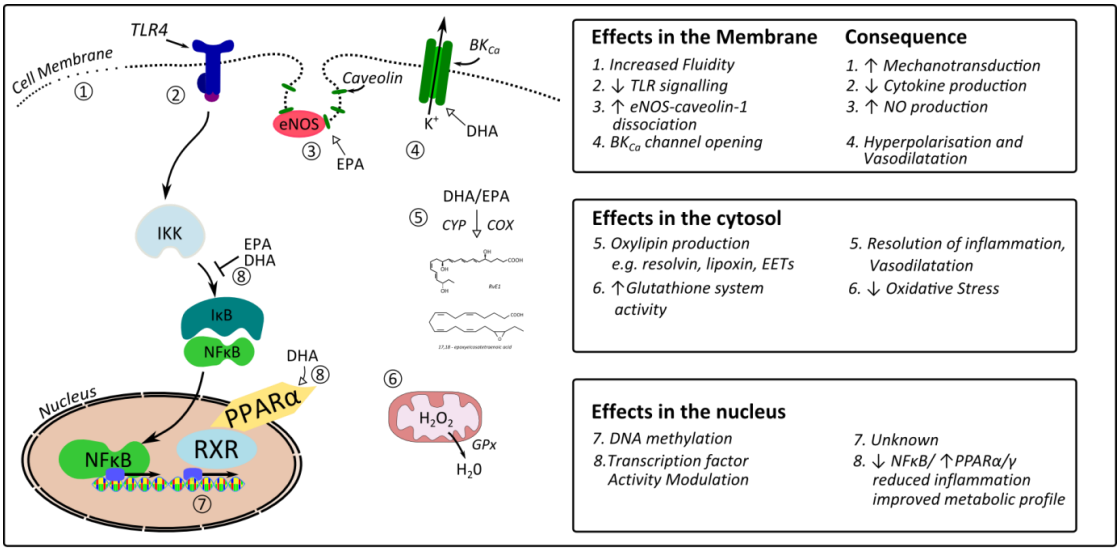
Anti-oxidant Effects

Omega-3 PUFAs are known to upregulate cellular anti-oxidant pathways such as glutathione peroxidase by upregulating mRNA transcription and translation, although this may have been in response to the formation of potentially harmful oxidation products of the omega-3 PUFAs [385].

Effects on Endothelial Function

Endothelial dysfunction is the hallmark of CSX and omega-3 fatty acids have several benefits in this area. DHA promotes vasodilatation by decreasing VSMC cytosolic

calcium thereby minimising activation of the contractile apparatus. The aforementioned eicosanoid switching is also important here and DHA may promote production of vasodilatory prostacyclin (PGI_2) instead of thromboxane, a vasoconstrictor. In addition, EPA has been shown to improve the dissociation of eNOS from caveolin in endothelial cells. Caveolin prevents eNOS activation in the caveolae and its removal allows migration of the eNOS to the cytosol with phosphorylation and activation then occurring with increased NO production [386]. This is borne out by evidence that dietary EPA supplementation leads to improved endothelial-dependant flow-mediated dilatation [387]. Finally, EPA and particularly DHA increase endothelial membrane fluidity and decreased membrane cholesterol (and possibly lipid raft) content, which improves mechanotransduction [388].



Potential Vascular Benefits of ω-3 PUFAs. BK_{Ca}- Large Conductance Calcium-Activated Potassium Channel; **COX**- Cyclooxygenase; **CYP**- Cytochrome P450; **DHA**-Docosahexaenoic Acid; **eNOS**- Endothelial Nitric Oxide Synthase; **EET**- Epoxyeicosatrienoic acid; **EPA**- Eicosapentaenoic Acid; **GPx**- Glutathione Peroxidase; **IκB**- Inhibitors of κB; **IKK**- IκB Kinase; **NFκB**- Nuclear Factor Kappa-light-chain-enhancer of Activated B-cells; **NO**- Nitric Oxide; **PPAR**-peroxisome proliferator-activated receptors; **RXR**- Retinoid X Receptor; **TLR**- Toll-like Receptor

Figure 7.6 Benefits of Omega-3 PUFAs

Omega-6 Polyunsaturated Fatty Acids

Linoleic acid (LA) is the archetypal omega-6 fatty acid and is the parent molecule for Arachidonic acid and has recently been shown to be an independent risk factor for adverse coronary events[389]. Prior to this it was believed that omega-6 PUFAs were

atheroprotective but recent studies have shown that this was due to the presence of omega-3 FAs as confounders. LA is found in nuts and vegetable oils and causes endothelial cell activation with increased expression of cellular adhesion markers and impairment of NO and prostacyclin production. It appears to affect these changes by increasing endothelial cell cAMP levels by inhibiting cAMP-hydrolysis by phosphodiesterase (PDE) and impairing endothelial Ca^{2+} responses and by upregulating NFκB activity. Despite being the parent molecule for Arachidonic Acid, LA reduces endothelial AA content and prostacyclin production in endothelial cells. This is likely due to competitive uptake of LA in lieu of AA into the cell membrane phospholipids coupled with the fact that endothelial cells have poor Δ-6 desaturase capability and thus minimally form AA from LA (indeed studies have shown that only 0.2% of dietary LA is actually converted to AA in vivo.) Arachidonic acid is itself an omega-6 PUFA of considerable importance. As well as being the precursor for many of the eicosanoids discussed below, it has also been shown to directly act as a vasodilator in human coronary arteries through the activation of TRPV4[390]. The omega-6 derived eicosanoids are generally more pro-inflammatory than their omega-3 counterparts.

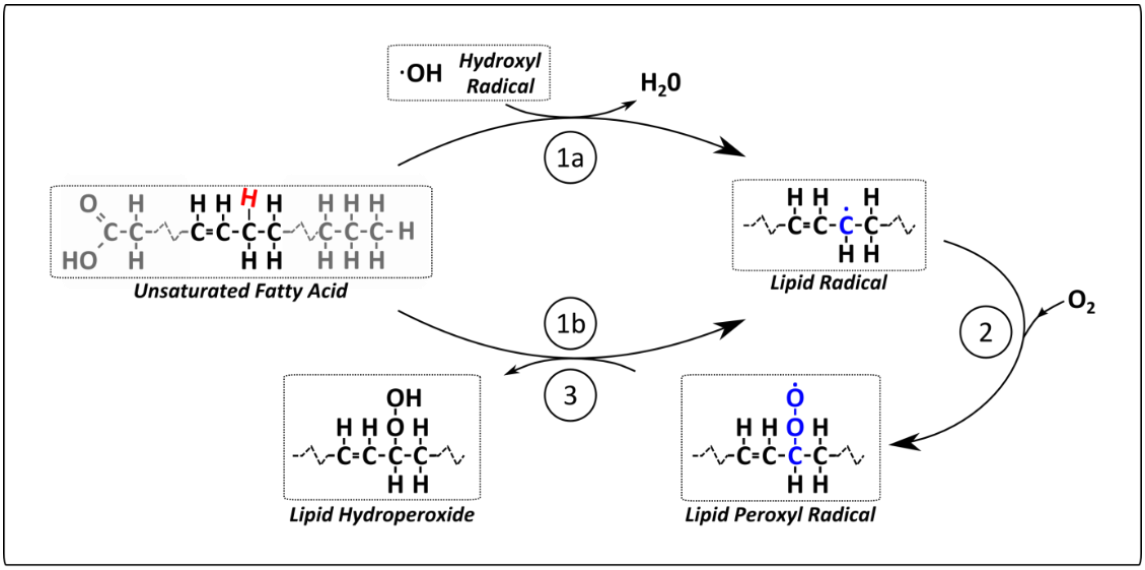
Dietary intake of ω-3 vs. ω-6 Fatty Acids

In general, ω-3 FAs are atheroprotective and ω-6 are atherogenic. It is believed that humans evolved with a diet that was relatively balanced in terms of ω-3 and ω-6 intake (≈1:1 ratio). A modern "western" diet has now tipped the scales heavily in favour of ω-6 intake with an estimated 16:1 split of ω-6 to ω-3 [391]. Interestingly, these two PUFA groups compete directly with each other for interaction with various important enzymes in vivo (e.g. cyclo-oxygenase and lipoxygenase and, to a lesser extent, elongases and Δ6 desaturase) with an excessive intake of one type of PUFA leading to its dominance in the various enzymatic pathways, diverting biosynthesis of eicosanoids along the dominant PUFA pathway. It has been shown, for example, that ingestion of ω-6 FAs in excess of ω-3 leads to increased production of series-2 prostaglandins and

series-4 leukotrienes, which have generally pro-inflammatory effects. The dietary ratio of ω-6 to ω-3 is probably of great importance in endothelial biology, although the exact ideal ratio remains unknown and medical guidelines recommending omega-3 supplementation are limited to only a few situations.

The Risks of PUFAs: Lipid Peroxidation

Lipids in the cell membrane are vulnerable to oxidative damage by reactive oxygen species and other free radicals. Unsaturated FAs are particularly vulnerable to peroxidation, a chain reaction that is initiated when hydrogen is removed (or abstracted) from a carbon chain. The carbon-hydrogen bonds are weakest in the carbons near to carbon-carbon double bonds in the unsaturated fatty acids and it is these hydrogens that are generally abstracted most easily. Removal of the hydrogen leaves a carbon-centred radical leading to a rearrangement of the double-bond distribution in the chain with the formation of a conjugated diene. This then reacts with oxygen to form a peroxyl radical and subsequently a hydroperoxide. This can then propagate and affect other fatty acids locally continuing the chain reaction. This only terminates when the radicals begin reacting amongst themselves (i.e. when they generally outnumber normal lipids locally) to form a non-radical compound or if local anti-oxidant species act to terminate it prematurely. The oxidised lipids form isoprostanes (a nonclassical eicosanoid) and are potently pro-inflammatory. PUFAs, while having more pronounced beneficial effects than MUFAs in terms of cardiovascular risk, are incorporated into LDLs and these LDLs are then more likely to be oxidised (due to the larger content of double bonds) forming a potent stimulus for atheroma formation.



Lipid Peroxidation. The chain reaction begins with an unsaturated fatty acid interacting with a reactive oxygen species. **Step 1a**: In this case an hydroxyl radical (containing an unpaired electron in electron shell 2) abstracts a loosely bound hydrogen (shown here in red) from a carbon in the methyl bridge beyond the double bond of the fatty acid forming water and a lipid radical. The lipid radical contains a carbon-centred radical (shown in blue). **Step 2:** The lipid radical reacts with local oxygen to form a lipid peroxyl radical. **Step 3**: This radical can then abstract a hydrogen from another source to form a non-radical lipid hydroperoxide. **Step 1b**: If the source of the hydrogen is another unsaturated fatty acid, the process self-amplifies as long as a supply of unsaturated fatty acids is maintained. In this way, an initially small oxidative insult can lead to severe local oxidative damage.

Figure 7.7 Lipid Peroxidation

7.2.6 Eicosanoids and other Oxylipins

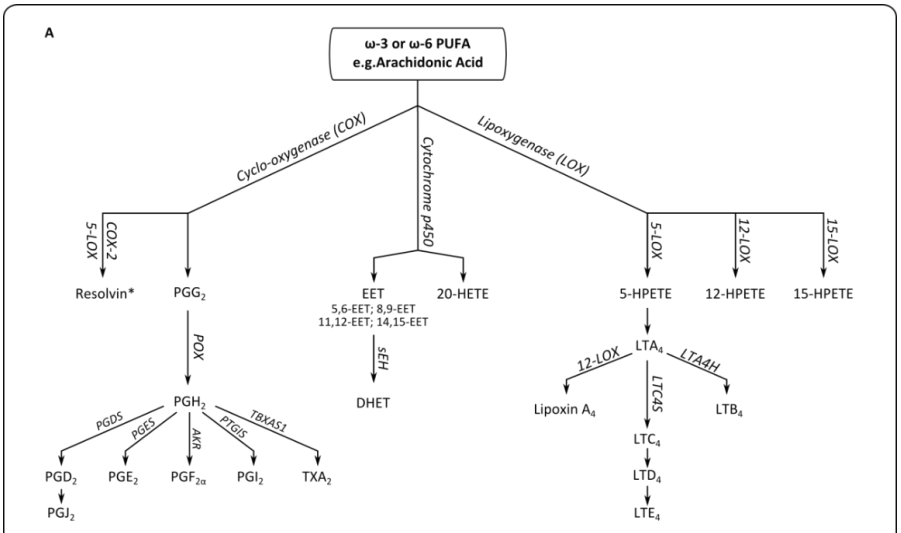
Eicosanoids are derivatives of certain 20-carbon polyunsaturated fatty acids, mainly ω-3 fatty acids such as Dihomo-gamma-linolenic acid (DGLA) and EPA and the ω-6 fatty acid AA. They play an important role in endothelial function and are mediators of inflammation, vasomotor function and cell signalling. Oxylipins are oxygenated fatty acids and include eicosanoids, docosanoids and others. They act locally but are also present in lipids that are housed in circulating lipoproteins. Eicosanoids are not stored in cells and are instead produced on an as-required basis. The nature of the precursor acid determines the structure and general function of the ensuing eicosanoid. For example, eicosanoids derived from ω-6 fatty acids are generally pro-inflammatory (this likely accounting for the negative effects of these acids on cardiovascular outcome) while eicosanoids produced from omega-3 FAs are less inflammatory. The arachidonic acid pathway is shown in the figure 7.8 below. In essence, AA is released from membrane PLs by Phospholipase A_2. The AA may then go down two main pathways

(cyclic or linear) to form various eicosanoids. This process is tightly regulated as it involves the oxidation of lipids which has the potential to damage nearby cell structures as described above. The cyclic pathway involves the action of Cyclooxygenase (COX) and produces the prostanoids, which include Thromboxane (TXA_2), Prostacyclin (PGI_2) and Prostaglandin D_2 and E_2, from the intermediate Prostaglandin H_2. This pathway is highly active in endothelial cells. Alternatively, the linear pathway produces the leukotrienes (e.g LTB_4) and lipoxins and occurs mainly in myeloid cells such as macrophages and neutrophils but these substances have potent effects on the endothelium. Similar pathways exist for the production of eicosanoids from ω -3 fatty acids (EPA in lieu of AA). It should be noted that the subscript number in the names of the various eicosanoids indicates the number of double bonds present in the substance. Omega-3 (i.e. EPA or DGLA) derived eicosanoids generally have 3 or 5 double bonds (e.g. PGI_3 and LTB_5) and tend to be less pro-inflammatory than their ω-6 counterparts (which have 2 or 4 double bonds). All of these substances are of importance in endothelial biology. The eicosanoids are then catabolised by sequential oxidation of the 15-hydroxyl group followed by β-oxidation.

EETs

The Cytochrome p450 (CYP) pathway deserves special mention. It is the third main pathway in eicosanoid production (alongside COX and LOX) and produces the non-classic eicosanoids, *epoxyeicosatrienoic acid*s (EETs). These are produced by ECs in response to shear stress and it should be noted that VSMC are not capable of producing EETs, leaving the endothelium as the only source of EETs in the vasculature. The CYP epoxygenases are capable of adding oxygen across any of the four double bonds found in AA resulting in 8 different possible EETs (as there are 2 stereoisomers possible for each double bond). CYP2C appears to be the most active form in coronary arterial endothelium and produces 14,15-EET. EETs have many biological effects including vasodilation, reduced VCAM expression, increased angiogenesis, increased

VSMC migration and reduced apoptosis. They are ultimately metabolised by ECs and VSMCs by esterification into lipids or else by hydration, terminating their effects quickly.



B

Eicosanoid	Source	Receptor	Relevant Effects
Prostanoids			
PGD_2	Mast Cell	DP1, DP2	Vasodilatation, anti-apoptotic in EC, variable effects on inflammation.
PGE_2	EC, immune cells	EP_{1-4}	Vasodilatation, ↑atrial contractility, hyperalgaesia and allodynia, ↑ permeability, ↑ vascular inflammation and EC activation.
$PGF_{2\alpha}$	Various	FP	Vasoconstriction and vasodilatation in various vascular territories.
PGH_2	EC	TP	Vasoconstriction
PGI_2	EC	IP	Vasodilatation, ↓ inflammation, ↓ EC activation, anti-proliferative, ↓ platelet activation.
TXA_2	Platelets	TP	Vasoconstriction, ↑ platelet activation and ↑ EC activation.
Leukotrienes			
LTB_4	Leucocytes	BLT_1	↑ EC activation, ↑ neutrophil transmigration, ↑ vascular permeability.
Cysteinyl leukotrienes	Leucocytes	$CysLT_1$	Vasodilatation, vasoconstriction (in some arterioles),↑vascular permeability mostly as a response to an allergic/anaphylactic trigger.
Others			
Lipoxin A_4	Platelets	ALX	↓ EC activation, ↓ angiogenesis and protects against oxidative stress.
EET	EC	Unknown	Vasodilatation, ↓ EC activation, ↓ EC apoptosis, ↑ angiogenesis.
DHET	EC	Unknown	Vasodilatation, COX inhibition.
HPETE	EC	Unknown	↑ Inflammation, vasodilatation.
HETE	EC	Unknown	Vasoconstriction, endothelial dysfunction, ↑ angiogenesis
RvE1	EC	Chem R23	↓ Neutrophil transmigration, ↓vascular inflammation, ↓ platelet activation

Eicosanoid Biosynthesis and Function. **A**. The biosynthetic pathways of the main eicosanoids. **B**. The relevant cellular source, specific receptor and function of the eicosanoids that can influence vascular function.
*E-series Resolvins are produced by COX-2 action on Eicosapentaenoic Acid (EPA) in ECs (especially in the presence of aspirin) followed by 5-lipoxygenase action in leukocytes. **AKR**- Aldo-keto Reductase; **ALX**- Lipoxin A_4 Receptor; **BLT**- Leukotriene B_4 Receptor; **CysLT**- Cysteinyl Leukotriene; **DHET**- Dihydroxy-eicosatrienoic Acid; **DP**- Prostaglandin D_2 Receptor **EC**- Endothelial Cell; **EET**- Epoxyeicosatrienoic Acid; **EP**- Prostaglandin E_2 Receptor; **FP**- Prostaglandin F Receptor; **HETE**- Hydroxyeicosatetraenoic Acid; **HPETE**- Hydroperoxyeicosatetraenoic Acid; **IP**- Prostacyclin Receptor; **LT_** - Leukotriene_ ; **LTA4H**- Leukotriene A_4 Hydrolase; **LTC4S**- Leukotriene C_4 Synthase; **PG_** - Prostaglandin_ ; **PGDS**- Prostaglandin D Synthase; **PGES**- Prostaglandin E Synthase; **POX**- Peroxidase; **PTGIS**- Prostacyclin Synthase; **RvE1**- Resolvin E1; **sEH**- Soluble Epoxide Hydrolase; **TBXAS1**- Thromboxane-A Synthase; **TP**- Prostanoid TP receptor; **TX**- Thromboxane.

Figure 7.8 Eicosanoids

Other Novel Eicosanoids and Related Compounds

In addition to the classic eicosanoids depicted above, several non-classic (or novel) eicosanoids can be produced. Non-enzymatic lipid peroxidation may lead to the production of *isoprostanes*, which are pro-phlogistic and can increase pain perception, which may be of particular relevance in CSX patients. The *docosanoids* (derivatives of DHA) such as D-series resolvins and protectins are the mediators of resolution of inflammation and should be considered along with eicosanoids. E-series Resolvins are derived from EPA.

Oxylipins and Vasomotor Function

Oxylipin mediators play an essential role in the control of vessel diameter in small resistance vessels in the microcirculation and are therefore likely to be of great importance in the inadequate vasomotor responses seen in CSX. The ECs themselves are capable of producing a great many of these mediators, which can then act on nearby VSMC causing vasodilation or vasoconstriction (See Fig. 7.9). As described in chapter 1, the endothelium is an active mediator of vascular tonal responses to shear stress. In addition to NO, the endothelium can produce oxylipins, particularly prostacyclin and EET, to mediate vasodilatation. Prostacyclin is perhaps the more important of these with respect to the larger coronary arteries. It is produced in large quantities by ECs through COX and prostacyclin synthase activity in response to shear stress [392]. It acts via the Prostaglandin I_2 GPCR and increases cAMP production, thereby activating PKA, which then inhibits Myosin Light Chain Kinase causing relaxation of the vascular smooth muscle and vasodilatation.

EETs are believed to be one of the Endothelium Derived Hyperpolarising Factors (EDHF). They appear to work as paracrine hormones, being released by ECs and activating large conductance calcium-sensitive Potassium channels (BK_{Ca}) in nearby

VSMCs via an unknown G-protein dependant process, resulting in K^+ efflux and VSMC hyperpolarisation. EETs can also activate Transient Receptor Potential (TRP) channels which also allow calcium influx and activation of K_{Ca} channels, similarly resulting in hyperpolarisation. The ability of EETs to activate TRPs may be of particular importance in the TRPV4 mediated flow-dependant vasodilatation of the coronary microcirculation. Activation of TRPV4 by shear stress (possibly mediated by EETs) results in the release of another EDHF, H_2O_2, from EC mitochondria with consequent vasodilatation. Finally, PGE_2 is a potent vasodilator via a c-GMP-dependant protein kinase.

Oxylipins are equally capable of causing vasoconstriction. The most important of these is platelet-derived thromboxane A_2, which causes potent vasoconstriction via the activation of the GPCR thromboxane receptor (a thromboxane prostanoid receptor, TP). TXA_2 mediated vasoconstriction appears to involve the GPCR-controlled activation of many kinases including Rho Kinase, MLCK and PKC. Other COX-dependant Endothelial-derived Contracting Factors (EDCFs) or vasoconstrictor prostanoids also exist and act via activation of the TP to similarly counteract NO, prostacyclin and EDHFs. These EDCFs include PGH_2 and PGI_2, although all prostanoids can activate the TP, albeit with different affinities.

Oxylipins and Endothelial Activation

TXA_2 is released by platelets and binds to TP receptors on endothelial cells. This activates the ECs with upregulated expression of ICAM-1, VCAM-1 and ELAM1 via Protein Kinase C modulated NFκB and Activator Protein-1 (AP-1) activation. Prostaglandin E_2 also activates Prostaglandin E Receptor 4 (EP4) and induces expression of ICAM-1 in endothelial cells. Finally, the cysteinyl leukotrienes (LTC_4, LTD_4 and LTE_4) also act via their GPCRs to promote an inflammatory endothelial phenotype as well as

to promote endothelial proliferation [393]. The protectins, resolvins and lipoxins all bring about the resolution of inflammation. Lipoxins activate the Lipoxin A4 receptor (ALX) and have been shown to inhibit inflammatory cell chemotaxis, increase prostacyclin production and reduce ROS production in endothelial cells. Aspirin modification of COX-2 results in the formation of epi-lipoxins, which also have potent anti-inflammatory capabilities. Resolvins also prevent phagocyte transmigration across the endothelium.

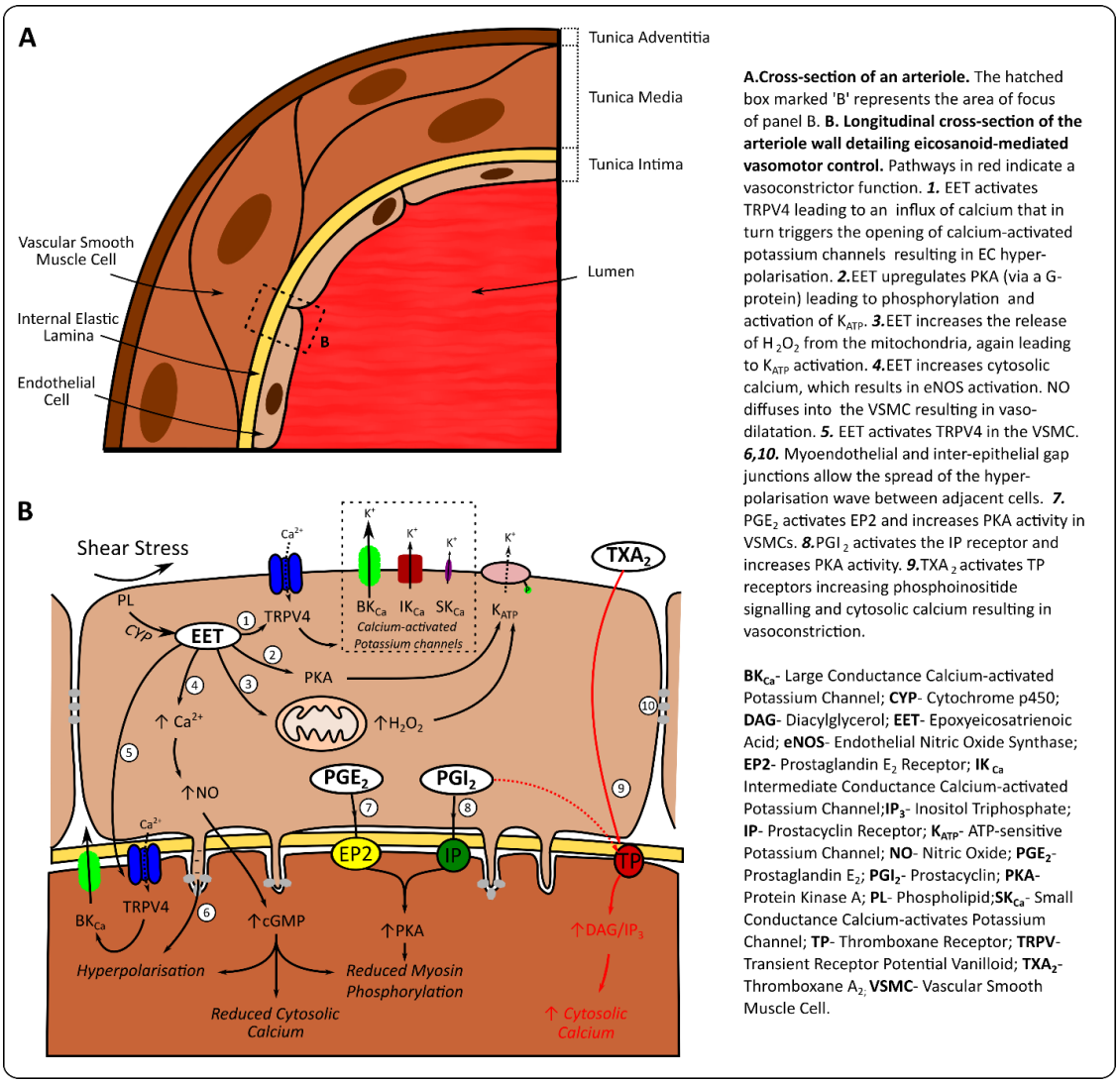


Figure 7.9: Eicosanoids and Vasomotor Function

7.3 Relevance of Fatty Acids in CSX

Overall fatty acids represent an important group of molecules in terms of cardiac and endothelial structure and function. Abnormal lipid metabolism might be responsible for many of the vascular abnormalities seen in CSX. Figure 7.10 highlights the most important of these impacts.

Parameter	Relevant Lipids	Nature of Effect	Relevance
Altered EC membrane fluidity	Cholesterol, SFA and TFA.	Direct incorporation into membrane or membrane PL, leads to tighter packing and decreased fluidity.	Reduces mechanotransduction. Reduces NO release. Alters β_2-adrenoreceptor function. Possible alteration in raft composition.
Cardiac Fatty Acid metabolism	FFA	Increased β-oxidation during ischaemia worsens aerobic efficiency and leads to accelerated depletion of oxygen.	Increases the metabolic impact of under-perfusion in cardiomyocytes.
Endothelial Cell activation	SFA, TFA, ω-6 PUFA, series 2 and 4 eicosanoids, LDL, TRL RLP, Lp(a) and isoprostanes.	Increased endothelial expression of adhesion molecules and secretion of chemotactic cytokines leads to heightened local inflammation, ROS generation etc.	Induces endothelial dysfunction with reduced NO bioavailablilty and increases the risk of local atherosclerosis.
	HDL, EET, EPA, DHA, SCFA, resolvins, lipoxins and protectins.	Induction of an anti-inflammatory phenotype in ECs and VSCMs	Allows normal endothelial function to prevail and enhances vasodilatory responses.
Endothelial vasodilatory function	TFA, TXA_2, series 2 eicosanoids, Lp(a), LDL, RLP and TRLs.	Reduced NO bioavailability and also direct promotion of vasoconstriction through receptor activation.	Impairs endothelium-dependent vasodilatation
	SCFA, PGI_2, EETs, HDL, EPA and DHA.	Increased NO bioavailability, phosphorylation of MLCK and hyperpolarisation of ECs and VSMCs.	Augments endothelium-dependent vasodilatation.

DHA-Docosahexaenoic Acid; **EC**- Endothelial Cell; **EET**-Epoxyeicosatrienoic Acid; **EPA**- Eicosapentaenoic Acid; **FFA**- Free Fatty Acid; **HDL**- High-Density Lipoprotein; **LDL**- Low-Density Lipoprotein; **Lp(a)**- Lipoprotein (a); **MLCK**- Myosin Light Chain Kinase; **NO**- Nitric Oxide; **PGI_2**- Prostacyclin; **PL**- Phospholipid; **RLP**- Remnant Lipoprotein; **ROS**- Reactive Oxygen Species; **SCFA**- Short Chain Fatty Acid; **SFA**- Saturated Fatty Acid; **TFA**- Trans-Fatty Acid; **TRL**- Triglyceride-Rich Lipoprotein; **TXA_2**- Thromboxane A_2; **VSMC**- Vascular Smooth Muscle Cell.

Figure 7.10: Summary of lipid effects with relevance to CSX

Studies have shown that fasting plasma triglycerides are within the normal range in CSX patients (see figure 7.11 below). It should be clear that diets rich in SFA and TFA as well as an excess of omega-6 PUFAs in lieu of omega-3 will lead to endothelial dysfunction and activation in vivo through effects on transcription factors governing inflammatory responses, eicosanoid-mediated effects and other receptor-mediated effects. There are very few interventional studies examining the effects of omega-3 treatment in CSX. Gaibazzi et al examined the effects of omega-3 supplementation in a small cohort of patients (n=9) with chest pain and normal coronary arteries with ST-depression occurring on stress echocardiography. They found that 1g of omega-3 PUFA given once daily for 4 months led to the resolution of ST-depression on subsequent stress echo,

suggesting remediation of endothelial dysfunction[394]. A slightly larger placebo-controlled, double-blind trial by Bozcali et al showed that 4/12 of 1440mg of omega-3 PUFA daily significantly improved flow-mediated dilatation of the brachial artery (from 47±48 to 104±23%, p<0.05) and reduced malondialdehyde, a plasma marker of oxidative stress, in CSX patients[395]. This is a proof of concept that dietary factors might modify the phenotype in CSX.

Author	Year	Cases	Controls	TC (mmol/L)	LDL (mmol/L)	HDL (mmol/L)	TG (mmol/L)	Lp(a) (mg/dl)	ApoB (mg/dl)
Langes et al	1995	34 (44%F)	15 Healthy age/sex/BMI	6.31 ↑22%	4.06 ↑28%	-	-	-	-
Tselepis et al	1998	31 (45%F)	32 Normal LHC (34%F)	6.08 ↑15%	3.49 ↑16%	1.24 NS	1.54 NS	13 ↑80%	146 ↑13%
Liu et al	2008	21 (32%F)	24 Healthy (59%F)	5.42 ↑16%	3.98 ↑54%	1.43 NS	1.45 NS	28 ↑75%	-

Lipid values are reported both as an absolute concentration and as a percentage change compared with values from the relevant control group.

Figure 7.11: Published studies of plasma triglycerides and lipoproteins in CSX.

7.4 Chapter Objectives

The potential role of dietary factors in the modulation or causation of Cardiac Syndrome X merits investigation. Given the myriad of functions governed by lipids in endothelial cells and also given the fact that diet can alter the types of lipids available to endothelial cells it stands to reason that dietary and pharmacological manipulation of lipids could be utilised to modulate endothelial function and ultimately disease pathophysiology in CSX. This chapter will examine the possibility of diet and plasma fatty acids playing a causative role in the chronic low grade inflammation seen in CSX. Fatty acids may be studied through the evaluation of plasma, red blood cell membrane content or in tissue itself. Plasma fatty acids give an estimation of the subject's diet over the previous 2 weeks while red blood corpuscle (RBC) membrane FA reflects the diet composition over the lifespan of the corpuscle, roughly 120 days. Plasma fatty acids correlate well with the lipid composition of circulating lipoproteins (see figure

7.12) while RBC FA appear to correlate more with tissue levels such as the myocardium. We chose to study plasma FA given the important role of lipoproteins in vascular function and given the fact that levels of FA in plasma reflects the patient's diet over the previous few weeks, when they were symptomatic. Also plasma fatty acid levels are well studied in cardiovascular outcome trials.

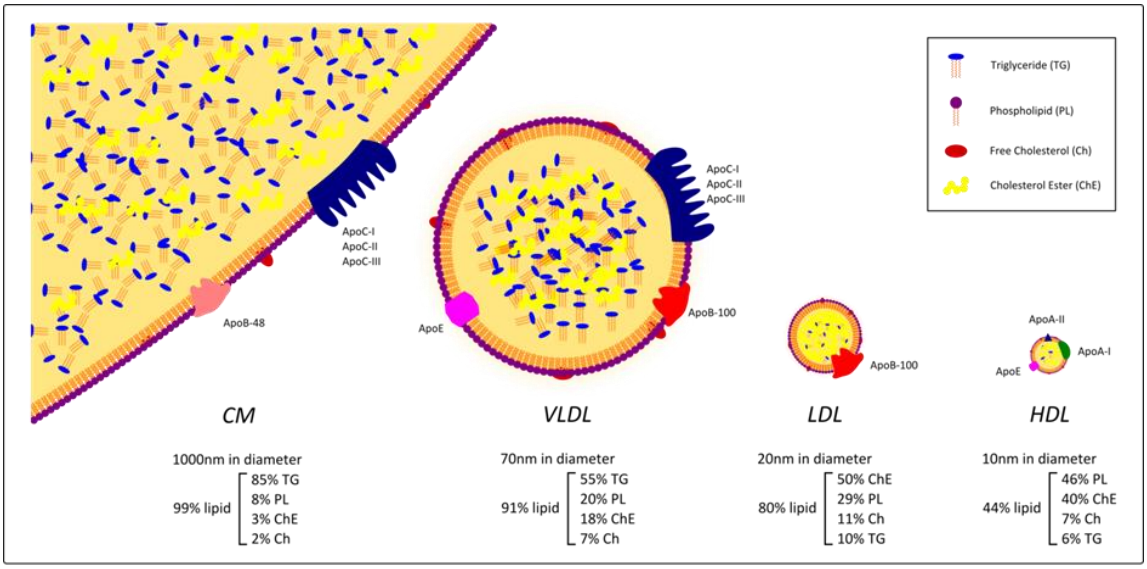


Diagram of the main Plasma Lipoproteins displaying their relative sizes and compositions. The larger the molecule, the higher its percentage lipid content. The ApoB-100 apolipoprotein allows interaction between the lipoprotein and the LDL-Receptor. Intermediate Density Lipoprotein, lipoprotein (a) and remnant lipoproteins are not shown.

Figure 7.12: Lipoprotein relative size and triglyceride content.

We will assess the percentage contribution of each identified fatty acid methyl ester in the plasma of our patients using the gas chromatography platform. If there is an excess of omega-6 fatty acids this may lead to the production of many pro-inflammatory oxylipins. It is hoped that it can be demonstrated that Irish CSX patients have abnormal plasma lipid profiles, have excessive dietary intake of SFA and TFAs and have a relative excess of omega-6 FAs compared to omega-3.

Methods

7.5 Participants

The same cohorts of participants that have been utilised for each of the preceding chapters in this thesis are again used here. Baseline samples are available for all 17 CSX patients and the 21 healthy controls. No samples from the follow-up visits were analysed. The patients had received no instruction to fast. All samples were taken between 0930 and 1130. Hospital laboratory data regarding the most recently obtained fasting lipid profile of these patients (14 samples in each group, taken at most 1 month prior to enrolment) were noted with permission.

7.6 Initial Investigations

A diet questionnaire was administered to the CSX patients (see appendix I). This self-completed questionnaire returns 4 domain scores including (a) The Prudent Diet Questionnaire score, (b) the Calorie Control Questionnaire, (c) the Fat Control Questionnaire and (d) the Sodium/Salt Control Questionnaire. The first 3 domains have 6 questions each with 4 possible answers. The salt control domain has only 5 questions. Each domain then gives a total score ranging from 6-24 (5-20 for salt), with 6-8 being excellent, 9-12 being good, 13-16 being fair, 17-20 being poor and 21-24 being very poor. Only 14/17 CSX patients returned this form. All patients completed the SAQ and PSS-10 as previously described in chapter 2. EST parameters were again recorded.

7.7 Assessment of Plasma Fatty Acids

Blood was taken from the ante-cubital fossa vein of participants. It was centrifuged at 1000 rpm for 15 minutes and the supernatant was aliquoted into microtubes and frozen at -80^{o}C until sent for further analysis. Lipids were then extracted from plasma

with chloroform: methanol (2:1 v/v) according to the method established in 1957 by Folch et al[396]. Fatty acid methyl esters (FAMEs) were prepared by first using 10 mls of 0.5 N NaOH in methanol for 10 min at 90^{o}C followed by 10 mls of 14% BF_3 in methanol (Sigma) for 10 min at 90^{o}C[397]. FAMEs were recovered with hexane. Before gas-liquid chromatography analysis, samples were dried over 0.5 g anhydrous sodium sulphate for 60 minutes and stored at 220^{o}C. FAMEs were separated by an Agilent 7890B gas chromatograph, equipped with a GC80 autosampler (Agilent Technologies, Little Island, Cork, Ireland) and flame ionisation detector. The column was a CP7420 Select FAME capillary column (100 m × 250µm I.D., 0.25 µm phase thickness) (Agilent Technologies, Little Island, Cork, Ireland). The injector was held at 250°C for the entire run and was operated in split mode using a split of 1/10. The inlet liner used was a split gooseneck liner (Part no: 8004-0164, Agilent Technologies, Little Island, Cork, Ireland). The column oven was held at 80°C for 8 min and raised to 200°C at 8.5 °C /min, this was held for 55 min. The total runtime was 77.118 min. The FID was operated at 300°C. The carrier gas was helium and was held at a constant flow of 1.0 ml/min. Results were processed using OpenLab CDS Chemstation edition software version Rev.C.01.05 (Agilent Technologies, Little Island, Cork, Ireland) and peaks were identified with reference to retention times of fatty acids in a standard mixture. The percentage of individual fatty acids was calculated according to the peak areas relative to the total area (total fatty acids were set at 100%). All fatty acid results are shown as g/100 g FAMEs

7.8 Data Management

All data was entered into SPSS v20 (IBM Corp, Armonk, NY). Measures of centrality and dispersion used with continuous variables were mean ± SEM if the data was normally distributed and median (IQR) if not normally distributed. Student t-test was used to compare the concentrations of normally distributed values while the Mann-Whitney U test was used for non-normally distributed data. Categorical data was compared using

Fisher's Exact Test. Correlations were calculated using the Spearman Rank Correlation Co-efficient. The EPA:AA ratio was calculated by dividing the % of eicosapentaenoic acid (EPA) present in the sample by the % of arachidonic acid (AA). The total omega-6 % was calculated by adding the % of Linoleic acid (LA), γ-linolenic acid, Dihomo-γ-linolenic acid (DGLA) and arachidonic acid. Similarly, the total omega-3 % was calculated by summing the individual percentages of α-linolenic acid (ALA), EPA, docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA). The omega-6:omega-3 was then calculated. All p-values are two tailed and calculated to a 0.05 significance level.

Results

7.9 Diet Questionnaire results

Domain	Score	Assessment
Prudent Diet	14 (11 to 15) /24	Fair
Calorie Control	10 (8.75 to 11) /24	Good
Fat/Cholesterol Control	11 (10.75 to 12) /24	Good
Sodium/Salt Control	7 (6 to 9) /20	Excellent

Table 7.1: Food Questionnaire Results for the CSX group (n=14)

As shown in table 7.1 above, the patients followed a relatively standard diet without evidence of salt, cholesterol or calorie excess. No patient had taken antibiotics in the previous 6 months. None were vegan or vegetarian.

7.10 Fasting Lipid Analysis

Group	Total Chol	LDL	HDL	Trig	VLDL
CSX	5.3 ± 0.2	3.2 ± 0.3	1.4 (1.3 to 1.7)	1.4 (1.2 to 1.7)	0.6 ± 0.1
Control	5.1 ± 0.2	2.8 ± 0.2	1.6 (1.4 to 1.9)	1.1 (0.9 to 1.5)	0.8 ± 0.2
p-value	0.44	0.24	0.18	0.08	0.26

Table 7.2: Baseline fasting lipid profiles. All values expressed as mmol/L. CSX n=14, HC n=14.

7.11 FAME Analysis

Fatty Acid	Control (n=21)	CSX (n=17)	p
Myristic Acid (C14:0)	1.6 (1.3 to 1.9)	1.4 (1.3 to 1.8)	0.31
Palmitic Acid (C16:0)	23.9 (23.1 to 24.9)	24.0 (22.9 to 25.1)	1.00
Palmitoleic Acid (C16:1)	2.6 (2.0 to 2.8)	2.6 (1.3 to 3.1)	0.52
Stearic Acid (C18:0)	9.7 (9.0 to 10.7)	9.6 (9.0 to 10.6)	1.00
Oleic Acid (C18:1c ω -9)	0.1	0	0.44
Elaidic Acid (C18:1t ω -9)	22.3 ± 0.7	21.3 v 0.4	0.88
Vaccenic Acid (C18:1t ω -7)	2.2 (1.9 to 2.6)	2.2 (2.1 to 2.7)	0.56
Linoleic Acid (C18:2 ω -6)	21.3 ± 0.9	23.5 ± 0.9	0.08
γ-Linolenic Acid (C18:3 ω -6)	0.2	0.3	0.50
Dihomo-γ- linolenic Acid (C20:3 ω -6)	2.0 (1.7 to 2.2)	2.4 (2.2 to 2.8)	0.00*
Arachidonic Acid (C20:4 ω-6)	7.8 ± 0.5	7.7 ± 0.4	0.83
α-Linolenic Acid (C18:3 ω-3)	0.4 ± 0.1	0.3 ± 0.1	0.36
Eicosenoic Acid (C20:1)	0.1	0	0.80
Eicosapentaenoic Acid (C20:5 ω-3)	1.6 (1.1 to 2.2)	1.5 (1.2 to 1.9)	0.62
Docosapentanoic Acid (C22:5 ω-3)	0.0 (0.0 to 1.1)	0.0 (0.0 to 0.0)	0.23
Docosahexaenoic Acid (C22:6 ω-3)	2.7 (2.2 to 3.5)	2.7 (2.1 to 3.0)	0.78
Total SFA	35.8 ± 0.6	35.4 ± 0.5	0.62
Total MUFA	27.3 ± 0.8	26.1 ± 0.6	0.25
Total PUFA	36.9 ± 1.2	38.5 ± 0.7	0.26
EPA:AA	0.19 (0.16 to 0.27)	0.22 (0.16 to 0.27)	0.82
Total ω-3	5.0 (3.8 to 5.9)	4.6 (3.6 to 5.1)	0.46
Total ω-6	31.2 ± 1.0	33.8 ± 0.8	0.05*
ω-6: ω-3	6.4 (5.4 to 8.3)	7.5 (6.3 to 9.3)	0.20
PUFA:SFA	1.04 ± 0.05	1.09 ± 0.03	0.39

Table 7.3: FAME results between groups given as % of total identified fatty acids (g/100g).

Table 7.3 above demonstrates the relative concentrations (expressed as g/100g) of the Fatty Acid Methyl Esters in both participant cohorts. Palmitic acid, linoleic acid and stearic acid together comprised over 50% of all measured fatty acids in our samples and the relative proportion of PUFAs to SFAs was favourable, being neither too low for the effects of excess SFA to occur nor too high to a point where lipid peroxidation would supervene (at approximately 1.5:1) [398]. The only significant differences between our CSX patients and the controls were slightly higher proportions of omega-6 PUFAs and DGLA in the CSX group. Fatty acid levels did not differ according to statin use and there was no significant difference in statin use between genders (FET, p=0.21).

Plasma omega-3 levels were, however, lower in women than men across the total study population [4.4 (2.4 to 5.8) vs 5.5 (5.1 to 9.1); U=37, n=37, p=0.019]. This was also reflected in a higher omega-6: omega-3 ratio in women [7.3 (6.1 to 9.0) vs 5.1 (3.2 to 7.0); U=143, n=36, p=0.023) and a lower EPA:AA (0.20 ± 0.02 vs 0.38 ± 0.08; t_{35}=-3.44, p=0.002, 95% CI -0.08 to -0.29). The total percentage of Omega-3 PUFAs also moderately inversely correlated with markers of vascular and general inflammation (IL-6, r_s=-0.371, df=37, p<0.01; CRP, r_s=-0.393, df=37, p=0.01; VCAM-1, r_s=-0.407, df=37, p=0.01). FAME parameters did not correlate with any parameters of symptoms as assessed by EST parameters, SAQ domain scores or PSS-10 scores.

7.12 Limitations

The lack of dietary questionnaires from the control group prevents a meaningful comparison between the groups in terms of diet. It may also have been more useful to investigate RBC cell membrane instead of plasma fatty acids as this might have given an insight into diet over a longer term. Again, the small numbers limited our statistical power but this was unavoidable.

Discussion

This is the first study to investigate the role of plasma fatty acids in CSX. As diet is one possible trigger for chronic low grade inflammation and dietary omega-3 supplementation has been shown to ameliorate endothelial function in CSX, we wished to evaluate the composition of the CSX plasma fatty acid profile as a measure of recent dietary fatty acid intake. Our hypothesis was that CSX patients would have a relative lack of omega-3 with a predominance of omega-6 PUFAs with the result that omega-6 derived pro-inflammatory oxylipins would predominate and potentially cause the general and vascular inflammation typical of CSX.

Surprisingly, we found that the plasma FA profile was strikingly similar in CSX patients and in the age- and sex-matched controls. It should be noted, however, that our controls did have a high prevalence of dyslipidaemia of over 80% (by design to match for statin use) and so do not represent true "normal" in terms of lipids. That notwithstanding, we did find a small but significant relative increase in omega-6 PUFAs in the plasma of the CSX cohort, perhaps supporting the notion of omega-6-derived eicosanoids resulting in inflammation. We did not, however, see a significant reduction of omega-3 or a significantly greater omega-6: omega-3 ratio. Indeed, the CSX omega-3 percentage is well within the normal range quoted in other studies[399]. It should also be considered, however, that several parameters suggest an unfavourable FA profile in our CSX patients. Firstly, the EPA:AA of the CSX group falls well below the preferred 0.75 level to prevent poor cardiac outcomes[400]. Secondly, the ratio of omega-6: omega-3 PUFAs is far higher than the putative ideal of 1:1 in both patient cohorts and also higher than the potentially beneficial levels quoted in studies. Indeed, a target of <4:1 is used for secondary prevention of coronary artery disease (where it was associated with a 70% relative risk reduction in mortality) while a ratio of 2-3:1 has been shown to suppress inflammation in RA[391]. This may provide the rationale for omega-3 use in CSX patients.

We also found a significantly greater % of DGLA in the plasma of CSX patients. This fatty acid has been shown to correlate with anxiety and depression symptomatology in patients with affective disorders which may have some relevance to our CSX patients, although DGLA levels did not correlate with perceived stress scores[401]. DGLA has also been shown to act as an anti-inflammatory mediator by reducing TNFα release from lymphocytes but again did not correlate with observed TNFα concentrations[402]. The DGLA-derived eicosanoids are also generally anti-inflammatory in nature but the significance of the increased DGLA is unknown.

It is also important to realise that, while we have calculated the relative proportions of plasma fatty acids in our patients, we did not calculate their absolute concentrations. It may be that the absolute quantity of FAs is higher in CSX than in health. This is relevant as high FA levels have been shown to induce endothelial dysfunction through NFκB activation with reduced endothelial-dependent vasodilation[403]. As shown in figures 7.11 and 7.12, the largest quantity of fatty acids is found in circulating triglycerides, chylomicrons and lipoproteins and the absolute concentration of total cholesterol and LDL is increased in CSX compared with healthy controls[404,405]. The patients in our study, however, had normal triglyceride levels

In all, this study does not provide much support to the notion that diet is a cause of the chronic low-grade inflammation seen in CSX. This may need to be confirmed in other studies, perhaps using a truly healthy control group without dyslipidaemia and a more detailed food frequency questionnaire. Other measures of the FA profile such as the RBC membrane evaluation could be employed to assess the impact of the longer-term diet. The role of lipids in CSX warrants further thought and investigation as these molecules are essential to normal endothelial function.

Perhaps focus should be turned to the actual FA derivatives directly relevant to endothelial function, the eicosanoids. These may be quantified using multiple methods such as gas chromatography-mass spectrometry (GC-MS), spectrophotometry or liquid chromatography-tandem mass spectrometry (LC-MS/MS)[406]. The levels of the various oxylipins in CSX would be of great interest. Another facet of the lipidome that may be of relevance to CSX is the SCFA fraction. These lipids have been shown to regulate GIT immune cell function (mediating cytokine release and eicosanoid production) and can also be quantified using GC-MS[407,408]. It is possible that our diet might influence our immune phenotype via the action of the GIT microbiome. Similarly, the phospholipid metabolite TMAO (produced by the action of the gut microbiome on choline-containing food) has been shown to be associated with worsened cardiovascular outcomes and to alter macrophage phenotype, a potential pathogenic mechanism in CSX[409].

Conclusion

In summary, we did not find convincing evidence that diet and plasma fatty acids play a role in the pathogenesis of CSX. The CSX patients did have an unfavourable lipid profile with a modestly elevated total cholesterol concentration and LDL level with normal HDL and triglyceride concentrations with the majority being on statin therapy. They have a relatively elevated level of omega-6 PUFAs, perhaps predisposing them to omega-6 derived pro-inflammatory eicosanoids, and a low EPA:AA ratio (albeit no different to that of our controls). The high omega-6: omega-3 ratio and low EPA:AA ratio provide a rationale for the use of omega-3 supplementation in these patients, as this has been shown to improve endothelial function, markers of oxidative stress and symptoms in a CSX population. The role of eicosanoids and SCFAs in CSX could be further investigated.



Chapter 8: General Discussion

8.1 Overview and summary

In this thesis we have identified an extremely well-defined and phenotyped cohort of Irish Cardiac Syndrome X patients and investigated the role of systemic low grade inflammation (LGI) in this condition. While LGI has been previously well documented in CSX, we have attempted to chart the course of this inflammation over the natural history of the condition, from first diagnosis through to disease quiescence. This is the first investigation to demonstrate that CRP and IL-6 appear to act as state markers of disease activity in CSX, being elevated when patients are symptomatic and returning to normal once symptoms and signs of disease abate. This is the first time also that these patients have been shown to demonstrate chronically elevated TNFα and IFNγ irrespective of symptom severity and disease activity, indicating that the immune system (and perhaps more specifically macrophages) of these patients may be tonically mildly active. The cause for this chronic activity merits further investigation but we have made some efforts here to investigate diet as a potential factor in this cohort. Additionally, we have made efforts to explain the pathophysiology behind the excess psychological comorbidity in CSX by examining the metabolism of tryptophan. Finally, we have provided evidence, through the analysis of the miRNA transcriptome, supporting the VSMC phenotype switching hypothesis that may indicate the presence of microvascular remodelling.

Despite the disagreement in the literature regarding the exact diagnostic criteria for and indeed cause of CSX, one constant finding has been the presence of mild systemic inflammation. Despite its near ubiquity, the exact nature of the inflammation in CSX has not been satisfactorily determined. Specifically, we do not know what the initiator of the inflammation is, which exact inflammatory pathways are involved and, perhaps more importantly, we do not know if the inflammation seen in CSX is a cause or an effect. Given that inflammation has the potential to dramatically impact the normal functioning of the endothelium and that this is a cornerstone of disease activity in CSX,

it does behove us to develop a greater understanding of the role of inflammation in this condition. The aim of this thesis was to further study the nature of inflammation in CSX by prospectively determining the values of various inflammatory biomarkers at different stages of disease activity in a well-defined cohort. We also pursued novel biomarkers in the CSX domain in an effort to further delineate the pathways and processes involved in CSX.

To this end, we needed to identify a local cohort of suitable patients. We enrolled, for the first time in Ireland, a cohort of CSX patients and attempted to thoroughly phenotype these patients in greater detail than has been attempted in previous studies. This is described in chapter 2, where we also describe our incidence study, performed as the incidence of CSX in Ireland was previously undetermined. We adhered to an extremely rigid and strict definition of CSX and included several questionnaires including an estimate of symptomatology (the Rose Angina Questionnaire and SAQ), life stresses (PSS and LTE-Q) and diet (Prudent Food Questionnaire). We also examined the data from their cardiac investigations, finding that they have an elevated left ventricular end-diastolic pressure; their routine blood tests, finding that they have normal autoimmune screens and an elevated neutrophil:lymphocyte ratio; and their urine tests, demonstrating that they do not have any evidence of microalbuminuia (a marker of glomerular endothelial dysfunction).

Having identified an Irish cohort of CSX patients, we first sought to establish that they too demonstrated systemic LGI before determining the dynamics of the inflammatory biomarkers during the natural history of the disease. We chose the method of electrochemiluminescence to determine plasma concentrations of acute phase reactants and showed that both CRP and SAA were elevated in our population (Chapter 3). Most interestingly, we showed the novel finding that there appeared to be an

association between CRP concentration and disease severity, with CRP levels falling as disease activity waned, indicating that CRP was a state marker in CSX. We also found that the endothelium of CSX patients was activated with increased expression of ICAM-1 and, described for the first time in the literature, VCAM-1. Thus, we had found that our patients did indeed exhibit chronic systemic LGI and had evidence of endothelial activation. Moreover, the inflammation appeared to mirror disease activity, at least in the case of CRP. This supported the notion of potential causality between inflammation and CSX.

With the presence of LGI established in our cohort, we then looked at the cytokines driving this inflammation to allow consideration as to the origin of this inflammatory drive (chapter 4). The cytokine profile hinted at macrophages potentially being the culprit cells as we found that IFNγ, IL-6 and TNFα were elevated in CSX. Intriguingly only IL-6 decreased as symptoms improved with both TNFα and IFNγ remaining elevated throughout the duration of follow-up. The improvement in symptoms was accompanied by a drop in the IL-6:IL-10 ratio, perhaps singling out IL-10 as the remediating factor in the disease process.

Another feature of CSX is the presence of extensive psychological comorbidity. This was borne out in our cohort, where they demonstrated increased perceived stress and poor disease-related quality of life. Why are these patients more stressed? Is that their baseline? Is it the rigours of having an active disease? In an effort to link this with their evident inflammation, we examined the role of tryptophan metabolism in CSX as studies in ischaemic heart disease have demonstrated increased activity of IDO in depressed and anxious patients post-MI. Using HPLC we demonstrated the original finding that our patients did indeed have upregulated IDO activity with consequent reductions in plasma tryptophan concentrations (chapter 5), which could impact on serotonin bioavailability. This could explain the psychological impact of CSX on the

patients and might explain the efficacy of imipramine in the treatment of this condition. Furthermore, many products of the IDO-regulated kynurenine pathway have the potential to dysregulate endothelial processes. Finally, the kynurenine: tryptophan ratio also acted as a state marker of disease activity, normalising at the end of follow-up in patients who improved.

Having seen the prominent role of inflammation in CSX, we then turned our attention to the microRNA transcriptome (chapter 6). We hoped that the analysis of the transcriptome might shed further light on the mechanisms at play in CSX pathophysiology. We discovered and validated that miR-143 is reduced in Cardiac Syndrome X. This microRNA is a critical regulator of VSMC phenotype with a reduction of miR-143 being associated with the adoption of a secretory phenotype in VSMCs. Secretory VSMCs play a key role in vascular wall remodelling, with medial hypertrophy and arteriolosclerosis. This would certainly explain many of the features of CSX (reduced coronary flow reserve, reduced vasomotor response to increased shear stress and endothelial activation). Furthermore, miR-143 targets endothelial Angiotensin Converting Enzyme and it is our contention that the angiotensin system also plays a crucial role in CSX pathogenesis.

Finally, we wished to attempt to explain the stimulus for the chronic inflammation seen in CSX. One possible trigger for LGI is diet and dyslipidaemia. We used gas chromatography to examine the plasma fatty acid profile of our patients in an attempt to determine the potential role of SFAs and omega-6 PUFAs in the pathogenesis of CSX (chapter 7). We found that our patients had low EPA:AA ratios and a relatively high omega-6 fraction in their plasma fatty acids, hinting that relative deficiency of omega-3 fatty acids may contribute to the pro-inflammatory phenotype that typifies the CSX patients. Our patients also had mildly elevated LDL and Total Cholesterol but no convincing dietary explanation for CSX emerged from our analysis.

8.2 Diagnosing and Treating Cardiac Syndrome X

Perhaps the greatest limitation of the research undertaken into Cardiac Syndrome X since its initial description in 1973 has been the lack of clarity regarding its diagnosis. As outlined in chapter 1, there are many possible diverse diagnoses that fall under the umbrella of chest pain with normal coronary arteries, with CSX representing a minority diagnosis. Physicians were initially disconcerted by the presence of classical anginal pain without any obvious disease on coronary angiography but quickly settled on the microvasculature as a probable cause. Despite this theory now being largely validated, it took 4 decades of contradictory results and inconsistent findings before the literature finally came to the consensus that Cardiac Syndrome X existed and was indeed likely caused by microvascular dysfunction. This has even led to a change in nomenclature, with CSX now also being termed microvascular angina. The main reason for the discordance in the reported results was that the definition of CSX had varied greatly between studies. Some studies demanded typical angina pectoris, some merely "chest pain", while the presence of a positive stress test was similarly occasionally optional. The modern definition of CSX now mandates a positive non-invasive test for myocardial ischaemia as well as angina pectoris in the presence of angiographically normal epicardial coronary arteries.

The Diagnostic Criteria

Our study provides interesting evidence in support of the necessity of a positive objective stress test in the diagnosis of CSX. By recruiting patients who had typical angina with angiographically normal coronary arteries but normal ESTs (the LCSX cohort) we were able to establish the ability of an EST to define two distinct populations. On one hand we had the CSX population, distinguished by the electrical positivity of their exercise stress tests, while on the other we have patients with angina and normal coronary arteries but without objective evidence of myocardial ischaemia (LCSX). Both groups were similar in terms of traditional cardiac risk factors,

demographics and baseline symptoms. The two groups differed clearly, however, in terms of the degree of baseline inflammation. In general, the LCSX group demonstrated less active LGI with lower CRP (p=0.06), SAA (p=0.05) and ICAM-1 (p=0.07) than the CSX group. They also had normal TNFα and IFNγ levels. Furthermore, their miR-143 levels were similar to those of healthy controls. Most importantly, the groups also differed in terms of prognosis as the LCSX patients were more likely to have resolution of their symptoms at follow-up than the CSX cohort. Thus, EST results in patients with CPNCA have implications for diagnosis and prognosis. The interrelationship between active LGI and EST positivity is further reflected in the follow-up results in our CSX patient cohort where CRP fell to normal levels in patients whose ESTs also normalised. This strongly implies that a positive EST in CSX does not simply represent a false positive result, as has been previously suggested.

We chose to be as strict as possible in our diagnosis of CSX so as to avoid the pitfalls suffered by so many previous forays into CSX research. We used the ESC definition of typical angina pectoris as a prerequisite for diagnosis as this pain is the most likely to be truly “cardiac” in origin. By so choosing, we excluded those patients with atypical chest symptoms (some of whom undoubtedly also had microvascular angina) and precluded the opportunity to determine if the nature of chest pain defined populations that differed in terms of inflammation and outcome as much as when defined by EST status. Our strict definition, however, certainly contributes to the relatively low incidence of CSX we observed in Ireland (1.3% of all angiograms for chest pain). Perhaps more importantly, we discovered that patients were not being diagnosed as having CSX despite meeting diagnostic criteria. This speaks to the lack of general awareness of the condition in Ireland. CSX should be suspected in younger post-menopausal women with dyslipidaemia presenting with angina and a positive EST and if the angiogram proves normal they should be diagnosed as CSX patients require active treatment and follow-up.

The use of baseline CRP as a biomarker in CSX to aid in its diagnosis also bears scrutiny. Our ROC curve analysis shows that CRP is excellent at separating CSX and LCSX patient populations with an AUC of 0.815 (p=0.02). A cut off of 1mg/L gave a 71% sensitivity and 86% specificity and 2mg/L gave 100% specificity and 40% sensitivity to distinguish between CSX and LCSX. Perhaps this might allow the use of CRP to distinguish patients with chest pain and normal coronary arteries who are more likely to have persistent symptoms and require treatment as they will likely have CSX. This could be more cost-effective and time-saving than EST, which requires the presence of a physician and cardiac technician for its duration.

Prognosis and Treatment in CSX

Our systematic review of the literature confirms that outcomes for CSX patients are generally favourable with low rates of MI and revascularisation but an increased incidence of CVA. This should alleviate some of the worry generated by studies in patients with demonstrable coronary microvascular dysfunction (as diagnosed by coronary reactivity testing), which demonstrated worse cardiovascular outcomes in patients with microvascular dysfunction[410]. It should be noted, however, that patients in these studies did not all have a positive stress test and some had obstructive CAD and so do not represent a true CSX/MVA population. Our review also showed that the majority of CSX patients (>70%) have symptoms at long-term follow-up and that a similar number require ongoing anti-anginal use. This was borne out over the course of our own study cohort, where we found that 73% of CSX patients were still symptomatic at the end of follow-up (almost a year and a half later).

We found that some baseline markers were predictive of a better symptomatic outcome. Although it appears obvious, it is satisfying to confirm that patients with milder symptoms (such as a higher DTS and better PLS) at baseline have a greater likelihood of symptom improvement at follow-up as confirmed by our logistic

regression analysis and ROC curve assessment. Therefore, the EST parameters and SAQ PLS domain score may also be used to prognosticate for patients. A PLS of >90 selected patients who would go on to be subjectively asymptomatic with 100% sensitivity and 77% specificity (AUC 0.949, p=0.02) while a cut off of >85 selected those who would have a negative EST at follow-up with 90% sensitivity and 100% specificity (AUC 0.907, p=0.01). Similarly, we showed that CRP, when combined with DTS, formed an excellent model to predict normalisation of the EST at follow-up and when combined with the PLS provided an extremely effective model for predicting 100% of patients with complete symptom resolution. Therefore, the routine measurement of hsCRP could be utilised alongside EST and questionnaires during the diagnostic process to help predict prognosis.

Quite apart from the physical symptoms, which are importunate, we also found that our patients suffered from excessive perceived life stress and endured a sizeable impact on their quality of life. These findings highlight the need to effectively diagnose CSX to allow for treatment. Such treatment should include agents that improve endothelial function (such as aspirin, statins and ACE inhibitors) and that minimise angina (e.g. beta-blockers and calcium channel antagonists) but some focus also needs to be applied to the psychological aspects of CSX, which also warrant therapy with exercise training and psychological interventions.

8.3 Inflammation in CSX

CSX appears to be intrinsically associated with systemic low grade inflammation and although this has been well documented its precise role remains poorly understood. The original objective of this thesis was to further evaluate the role of inflammation in CSX. In particular, we wanted to see if LGI was causal in CSX. We therefore wished to fully assess the nature of inflammation in CSX, the likely pathways and cells involved in its activity and the likely stimulus leading to its promotion. To that end we followed the

cohort of patients to look at their inflammation over time and have made several advances in the field of inflammation in CSX. We established that it is indeed chronic in nature, with TNFα, IFNγ and the neutrophil: lymphocyte ratio being persistently elevated in our patients. We have confirmed the presence of endothelial activation characterised by upregulated VCAM-1 and ICAM-1 expression. We have also seen that the IL-6/CRP axis appears to be important in disease activity in terms of both EST positivity and symptoms with both IL-6 and CRP normalising with symptom resolution. We have also demonstrated the novel finding that Indoleamine 2,3 dioxygenase activity, affected by inflammation, is upregulated in CSX.

8.3.1 Is inflammation causative in CSX?

Bradford- Hill Criteria	
Strength of Association	The size of the association as measured by appropriate statistical tests e.g. correlation strength, difference in mean etc.
Temporal Relationship	Cause must necessarily always precede an occurrence of the disease
Biological Gradient	An increasing amount of exposure increases the risk. There should be a direct effect on the risk factor (e.g. inflammation) and the disease (CSX)
Consistency	Results are replicated across multiple studies from different locations, at different times, using different methods
Theoretical Plausibility	There should be a biologically sound mechanism by which the risk could lead to the disease
Coherence	The association should be compatible with existing knowledge within the field
Specificity	When a single cause leads to a specific effect. This is not essential.
Experimental Evidence	The condition can be altered by an appropriate experimental intervention
Analogy	Has something similar been seen in other populations/systems?

Table 8.1: The Bradford-Hill guidelines for causation

The primary point of interest is whether or not inflammation is causative in CSX. Causal inference studies utilise the Bradford-Hill criteria, which was initially used to assess the role of smoking in lung cancer causation. These criteria are still used today and are shown in table 8.1 above. Recently, authors have attempted to condense the criteria into more general concepts. One particularly elegant solution was codified by Howick et al (see table 8.2 below) and we will use the resultant headings to structure the debate regarding the putative causal link between inflammation and CSX.

Howick's Revised Criteria[411]		**Original Criteria**
Direct Evidence	Evidence from studies (randomised or non-randomised) that show a probabilistic association between exposure and outcome that is likely to be causal and not spurious or due to confounding. This evidence should have appropriate temporal and spatial proximity with dose-responsiveness and reversibility.	Experiment *Strength* Temporality *Biological Gradient*
Mechanistic Evidence	A mechanism of action (biological, chemical or mechanical) that connects the intervention and outcome	Biological Plausibility
Parallel Evidence	Where the results replicate or are similar to those of previously published studies	*Coherence* Analogy

Table 8.2: The revised criteria for causal inference

Direct Evidence

There is a reasonable body of direct evidence supporting the notion of inflammation being causal in CSX. Our study design has attempted to minimise the role of confounding in our study. The lengthy list of exclusion criteria has attempted to deal with this by removing patients with known diseases that lead to LGI (chronic kidney disease, chronic liver disease, diabetes mellitus, connective tissue diseases, chronic infections etc.). In addition, we matched our patients and controls for age, gender and

hyperlipidaemia and there were also no differences in terms of BMI, anti-depressants, statins or ACE inhibitor use. Despite all of this matching, the CSX patients had consistently greater concentrations of biomarker measures of chronic low grade inflammation than the healthy controls. The possibility of an unmeasured confounder (such as exercise, adipokines, undiagnosed infectious disease etc) remains, however.

The strength of our findings also must be scrutinised. The measured concentrations of the various molecular biomarkers are only modestly elevated in the CSX group. Despite that, the findings are statistically significant. For example, the hsCRP levels seen in our CSX patients are still within the normal range on a population level but still represent a 100% relative increase when compared with the healthy controls. The case is similar with SAA concentrations. The ICAM-1 and VCAM-1 levels represent only a 25% and 16% relative increase respectively while IFNγ is increased by 50%, IL-6 by 150% and TNFα by 20%. Given the small effect sizes, it is easier for a potential confounder to influence the results. Additionally, as this is an observational study, our cohorts may be inadvertently influenced by a selection bias.

Perhaps the greatest addition we have made to the understanding of LGI in CSX is the demonstration of dose-responsiveness and reversibility in our patients. Our novel finding that the degree of inflammation correlated with the severity of symptoms (in terms of objective EST parameters) and the finding that an improvement in symptoms by both subjective (SAQ) and objective (EST positivity) measures was mirrored by a reduction in CRP and IL-6 concentrations certainly strengthen the causal link between inflammation and CSX. The potential use of baseline CRP as a prognostic tool also lends support to the dose-response criterion. Similarly, previous studies have shown that the greater the inflammation, the lower the CFR and FMD[412]. It should be realised, however, that many conditions with much greater activation of the immune system do not result in angina pectoris.

Temporality is a difficult criterion to meet given the relative infrequency of the diagnosis of CSX. One feasible approach would be to follow patients with known inflammatory disorders (such as chronic inflammatory rheumatoid conditions) to see if they develop microvascular angina. A recently published review reports that coronary microvascular dysfunction is prevalent in many chronic inflammatory rheumatoid conditions such as rheumatoid arthritis and systemic lupus erythematosus[413,414]. These patients also go on to have many adverse cardiovascular events and in such cases the inflammation certainly precedes the disease (microvascular dysfunction). This also demonstrates the criterion of Analogy, where inflammation in a non-CSX population has led to cardiovascular symptoms.

The final component of direct evidence linking LGI to CSX is the demonstration in some studies of the effectiveness of inflammation-reducing agents such as statins, exercise and ACE inhibitors in improving patients' symptoms and quality of life. Obviously these all have multiple effects other than a reduction in vascular inflammation and so only provide indirect evidence but their findings of benefit are consistent. No trials have been done in the use of steroids or drugs such as methotrexate in CSX. Interestingly, ongoing trials are currently examining the role of methotrexate (CIRT trial) and the anti-IL-1β canakinumab (CANTOS trial) in improving cardiovascular outcomes in patients with chronic atherosclerosis and MI. These agents both downregulate the IL-6/CRP inflammatory signalling pathway. Anakinra, another IL-1 antagonist, has been shown to improve CFR and endothelial function[415].

Mechanistic Evidence

Our causal hypothesis that LGI leads to endothelial dysfunction and angina is certainly coherent with the published literature. As was described in chapter 3, CRP itself has many direct effects on the endothelium while the ability of TNFα and IFNγ to activate the endothelium is also well-known. Endothelial dysfunction with resultant insufficient

microvascular dilation is almost certainly the primary mechanism in CSX. The aforementioned study detailing the remedial effects of IL-1 antagonism in restoring normal endothelial function solidifies this link. The control of inflammation in rheumatoid arthritis also has substantial cardiovascular benefits, again highlighting the importance of inflammation in vascular disease[416,417].

Parallel Evidence

The data we have presented in this thesis is certainly consistent with previously published results in the CSX population. While we have replicated other data we have also contributed many novel findings to the previously published research. It is important that similar findings have been found in many different CSX populations (in Ireland, Japan, Italy etc.) using different laboratory techniques. We have also shown that inflammation is persistent in the CSX population, meaning that LGI is a consistent finding in active CSX.

Conclusion

It must certainly be conceded that systemic low grade inflammation is not specific to microvascular angina and is more prominent in many other conditions. It is also true that not everyone with inflammation gets resultant angina pectoris and as such LGI must not be the only factor involved in CSX. Undoubtedly, however, inflammation plays an important role in CSX and meets 5 out of 9 criteria for causation. The control of inflammation in CSX is a therapeutic intervention that has not yet been specifically pursued.

8.3.2 What pathways and cells are involved in the inflammation seen in CSX?

Our research has suggested that several pathways may be involved in the pathogenesis of the inflammation of CSX. There appears to be a basal cytokine drive in these patients, with tonic elevation of TNFα and IFNγ. This seems to be punctuated by an activation of the IL-6/CRP axis, which may mediate symptomatic periods for patients. TNFα is a chief inducer of endothelial activation, upregulating many NFκB-dependent inflammatory processes and this process is augmented by IFNγ. Furthermore, IFNγ upregulates TGFβ and ET-1 production in the endothelium and may trigger EndMT. Thus, the presence of elevated concentrations of these cytokines may lead to an endothelium that is activated and vulnerable to further dysfunction.

IL-6 is released from immune cells and from endothelial cells in response to TNFα, oxidative stress, vascular injury and Angiotensin II (Ang II) in an NFκB-mediated pathway and is a marker of vascular inflammation. IL-6 then induces the expression of CRP from the hepatocytes and from endothelial cells themselves. IL-6 also has more local effects in the vasculature, mediating monocyte recruitment (via MCP-1) and activation in conjunction with increased AngII activity[418]. It also has the effect of upregulating AT_1 receptors on the nearby VSMCs, thereby allowing increased sensitivity of the VSMCs to circulating AngII, thereby increasing ROS production, vasoconstriction and vascular remodelling. As was outlined in section 3.1.1, CRP also shows a great ability to alter endothelial biology. As well as activating endothelium it has been shown to further upregulate the AT_1 receptor, uncouple eNOS activity, reduce *NOS3* mRNA levels and even induce endothelial apoptosis. It may also blunt the normal mechanotransduction necessary for flow-mediated vasodilation by damaging the glycocalyx.

The transcriptome analysis in our patients also revealed some possible endothelial pathways that might also be implicated in CSX. Although some of the miRNAs identified

as being differentially expressed by NGS were not validated by qPCR because of their scarcity, the fact that they were differentially expressed by both methods of detection hints that they may be dysregulated in CSX. miR-10b regulates endothelial NFκB and may be reduced in CSX, while the E26 transcription factor Ets-1 is also modulated by miR-200b. Both of these transcription factors regulate endothelial activation. The only validated miRNA from our study, miR-143, targets Kruppel-like factor 4, ACE and COX2 and each of these can modulate endothelial inflammatory activation. Our research is the first to confirm the upregulation of the enzyme Indoleamine-2,3-dioxygenase and the subsequent activation of the Kynurenine Pathway. This inflammation-responsive pathway may be implicated in some of the affective consequences of the CSX disease and will be discussed further in section 8.4.

The cellular origin of the cytokines is also difficult to determine with any great certainty. It is difficult to look beyond the obvious that there is a relative excess of neutrophils in our CSX patients, which may represent a response to stress, either physical or psychological, or may be a signal of neutrophilic involvement in CSX. A raised NLR has also been noted in other studies in CSX. Although our population had normal monocyte levels, other studies into CSX have demonstrated an increased number of circulating monocytes in patients with active disease. The idea that CSX is a monocyte/macrophage driven disease is attractive as M1 and M4 macrophages are known to be stimulated by the contents of atheromatous plaques and are a potent source of TNFα and IL-6[419]. Certainly, the basal IFNγ elevation would prime macrophages for activation. This begs the question as to the source of the IFNγ. Activated T_{h1} cells and NK cells are the primary sources of IFNγ but macrophages may also produce this important cytokine. Of course, the cells of the vascular wall (VSMCs and endothelial cells) may also be potent sources of these pro-inflammatory cytokines under suitable conditions and if they are the source this may explain the predominantly vascular effects of the LGI in CSX. Cells in adipose tissue are also implicated in the sustenance of LGI and represent another potential cellular source.

8.3.3 What is the stimulus for inflammation in CSX?

Cause	Examples	Effects
Sleep disturbance	Chronic and acute sleep deprivation	Increased IL-6, IL-1 and TNFα Sympathetic activation
Stress and trauma	Early childhood (abuse, social isolation, economic) Chronic stressors	Increased IL-1 and IL-6, apoptosis and Th1 response Reduced CC16 (uteroglobulin)
Immune Gene Polymorphisms	"Inflammaging" HLA haplotype	Polymorphisms/SNPs in TLRs, IFN, CRP and TNF may lead to LGI
Diet	Prudent diet vs Western diet	Whole grains, fish and legumes reduce CRP and IL-6 High glycaemic load food with red meat causes the opposite. FA intake also mediates inflammation (Omega-3, Omega-6, SFA)
Gut Microbiome	TLR4 activation via LPS/ bacteria translocation across leaky GI membranes	Leads to increased IgM and IgA against LPS Increased NFκB activation Oxidative and Nitrosative Stress with NADPH oxidase activation
Lack of Exercise	Sarcopenia Sedentary Lifestyle	Exercise transiently increases Myokines (IL-6) but healthy habituation occurs with time Exercise reduces leptin and adipokines
Obesity	"Metaflammation" Adipokines	Increases circulating leptin, increases Hypothalamic-pituitary-adrenal axis activity, IL-6 and ROS production
Smoking	Passive and active smoking	Cigarettes contain LPS and induce oxidative and nitrosative stress, IL-6, TNFα and CRP production
Atopic Disorders	Allergic rhinitis	Increased IgE and Th2 response (IL-4, IL-5, IL-13 and TNFα)
Periodontal diseases	Dental Caries Gingivitis	High prevalence (47% in US adults) Macrophage activation with increased IL-6, IL-8 and CRP
Vitamin D	Deficiency	Supplements reduce TNFα and IL-6 levels as well as reducing oxidative stress
Environmental Pollution	Particulate Matter	Increases CRP, COX2 activity, IL-1β release and endothelial activation
Chronic infections	H. Pylori Mycoplasma	Increased CRP and homocysteine

Table 8.3: Selected possible causes of chronic systemic low grade inflammation

The identification of the ultimate cause of the chronic LGI seen in CSX is crucial as this may allow one to address the problem of successful therapeutic intervention in CSX. Systemic LGI has many potential causes, some of which are shown in table 8.3 above. Several of these have already been investigated in published studies. As mentioned before, a high prevalence of Helicobacter pylori colonisation has been found in CSX patients and this is known to be associated with increased serum hsCRP and homocysteine levels[420]. Obesity and adipokines may also play a role in the initiation and maintenance of chronic LGI in a process dubbed metaflammation. Our CSX population has a moderately raised BMI (almost 28 kg/m^2 on average) but no more so than our healthy controls. It is certain, however, that BMI is not an accurate measure of adiposity with sarcopenic obesity and the distribution of fat also playing an important role on immune activity[421]. It is interesting, therefore, that visceral associated fat is increased in CSX[422]. Other markers of increased adiposity, such as leptin, are also increased in CSX, although it must be stated that leptin is upregulated by inflammation[161]. Interestingly, insulin resistance has also been demonstrated in CSX populations furthering the notion of metaflammation in these patients[423].

It is also possible that stress may be the cause of LGI in CSX. We have demonstrated that our patients have significantly elevated perceived stress scores when compared to the control group while many studies have demonstrated the psychological co-morbidity suffered by CSX patients. Psychosocial stressors have been shown to elicit inflammatory responses in humans including increased IL-6 and IL-1[424,425]. Early childhood trauma such as parental loss, social isolation and economic deprivation may also play a role as these have been shown to have lifelong immune repercussions[426,427]. Further evidence for the role of stress in CSX is the finding of upregulated sympathetic autonomic activity in this population in the form of ECG changes and abnormal cardiac adrenergic nerve function on MIBG scans. To date, the status of the HPA axis in CSX has not been investigated.

The gut microbiome could also potentially play a role in the chronic inflammation in CSX. The Firmicutes/Bacteroidetes ratio appears to be important in oxidative stress and inflammation. Similarly, intestinal gram-negative bacteria express LPS on their outer membrane and high fat diets have been shown to increase LPS production and translocation into the blood. This leads to a chronic low grade metabolic endotoxaemia, which can lead to TLR4 activation with resultant macrophage activity and increased IL-6 and TNFα release[428]. Intestinal permeability is increased by stressful stimuli that lead to increased HPA axis and sympathetic activation. It is therefore possible that increased stress in CSX patients leads to a metabolic endotoxaemia via increased intestinal permeability[429].

We attempted to examine diet and fatty acids as potential causative agents for the LGI. Our patients scored reasonably well on the Fat/Cholesterol control questionnaire but were only fair on the Prudent Diet questionnaire. They had elevated LDL and total cholesterol but had a normal HDL concentration. We did identify a mild excess of omega-6 PUFAs in our patient cohort and they also exhibited a suboptimal EPA:AA and omega-6:omega:3 ratios. Dietary interventions into CSX patients may warrant further study, although omega-3 and vitamin D supplementation have already shown promise.

Recent studies of CSX patients revealed no differences in terms of genotype distributions for the common inflammatory mediators IL-6, TNFα and IL-10 so genetic polymorphisms do not appear to play an important role in CSX[195,210]. Despite this, one study has shown that, although IFNγ gene transcriptional activity is not different, IFNγ receptor subunit gene expression was higher in CSX than in controls indicating increased immune cell sensitivity to IFNγ mediated stimuli[21].

It may be that inflammation is a self-perpetuating cycle in CSX as subclinical atherosclerosis is undoubtedly found in CSX (see 4.16.2). While atherosclerosis is initiated by abnormal local rheology, it then forms a nidus of inflammation from which a sustained systemic inflammatory signal can be sent. It may simply be that these patients are exhibiting a form fruste of coronary atherosclerosis, whereby we observe the sub-clinical inflammation without the objective coronary angiographic changes. Lifestyle and environmental factors are beyond the scope of this thesis but there we observed no excess alcohol intake in our cohort (median 0.0 [0.0 to 5.0 units per week]) and no CSX patients were actively smoking. Patients' sleeping habits and exercise regimes were not evaluated while exposure to environmental pollution was also not assessed, although 41% of the CSX population lived in a rural setting. Doubtless, there is scope to further investigate the potential instigators of inflammation in CSX and such research may reveal the key to understanding the pathophysiology of this important condition.

8.4 Novel Pathogenic Mechanisms in CSX

One of the main purposes of this thesis was to examine biomarkers of CSX disease activity and then theorise as to possible mechanisms responsible for the observed biomarker profile, perhaps giving a new insight into the underlying disease process. In this thesis we have corroborated old theories and have also expounded new ones based on our original data. We will not recap our evidence that inflammation may act as a mediator of disease activity as this is outlined more explicitly in 8.3 above. Instead we shall focus on two other novel facets of CSX disease activity.

8.4.1 Indoleamine-2,3-dioxygenase activity

We are the first to demonstrate that IDO activity is increased in CSX patients. This was not an unexpected finding given that IDO is potently induced by IFNγ and that we have also demonstrated increased IFNγ levels for the first time in CSX patients. It remains,

however, a significant finding as IDO has frequently been implicated as the link between inflammatory conditions and affective disorders such as in the bidirectional link observed between depression and coronary artery disease[430]. IDO upregulation starves the methoxyindole pathway of tryptophan and without this substrate serotonin cannot be synthesised. Reduced serotonin bioavailability could be part of the explanation for the high incidence of panic disorders, somatisation and depression found in CSX patients[228,431]. Certainly, serotonergic neurons modulate anxiety responses and dysregulation of these circuits can lead to anxiety while reduced plasma trypophan and serotonin is associated with depression, anxiety and obsessive symptoms[432,433]. It may also explain the increased perceived stress in our patient cohort as well as the disproportionately impaired disease-related quality of life despite no significant excess of actual non-disease related life stressors.

Equally, altered serotonin levels may play a role in the abnormalities of pain perception in CSX patients, who demonstrate increased peripheral pain sensitivity, reduced habituation to painful stimuli and abnormal cortical pain processing. Patients with depression also exhibit reduced pain tolerance and greater perceived pain[434]. The role of serotonin in nociception is quite complex, however. Serotonin modulates central pain processing, possibly through the action of opioid-releasing neurons as well as direct effects[435]. It may also, however, sensitise peripheral neurons to painful stimuli[436]. Furthermore, tryptophan supplementation has been shown to increase pain tolerance levels, implying that a relative lack of this amino acid might reduce pain tolerance[437]. CSX patients are also known to be deficient in melatonin, the other product of the methoxyindole pathway. This vasoactive compound has potent anti-oxidant and atheroprotective properties and reduced tryptophan bioavailability would also prevent its production.

Another facet of IDO upregulation is the activation of the kynurenine pathway and increased concentrations of its products. While we only measured kynurenine and kynurenic acid, it stands to reason that kynurenine pathway intermediates such as 3-hydroxykynurenine are increased in CSX. Several of the metabolites on this pathway have the potential to induce endothelial dysfunction, with kynurenine being capable of indirectly activating NFκB and 3-HK exacerbating endothelial oxidative stress. COX2 inhibitors can be used to inhibit IDO activity thereby switching off the kynurenine pathway and have been shown to improve endothelial function in IHD but they are generally not advised for patients with risk factors for cerebrovascular disease and as such should not be used in CSX. The role of anti-depressants in the modulation of pain in CSX is proven, highlighting the relevance of the serotonergic system in this disease. Further research into the potential role of tryptophan metabolites (including melatonin) in CSX should be undertaken.

8.4.2 Microvascular Remodelling

The term vascular remodelling describes the physical changes that occur in the wall of the blood vessels, typically in the tunica media, in response to injurious stimuli such as vessel trauma, oxidative stress and inflammation. It results in altered cellular populations in the vessel wall and an increase in extracellular matrix (ECM) deposition. The characteristics of the blood vessel are also altered in terms of elasticity, effective diameter and responsiveness to rheological stimuli. It is typically affected by the VSMCs, with input coming from the local endothelial cells and circulating compounds with Angiotensin II being a particularly potent stimulus.

VSMCs are not terminally differentiated and retain a plasticity that allows them to change from a contractile phenotype, typified by the expression of α-smooth muscle actin (α-SMC) and smooth muscle myosin heavy chain (SM-MHC) among other specialised contractile proteins, to a secretory phenotype when needed. VSMCs are

maintained in a healthy contractile phenotype by the interaction of serum response factor (SRF) and its myocardin cofactor with CArG boxes on promoters for the contractile genes. The VSMCs are also maintained in a differentiated state through Notch-dependent signals from the nearby endothelial cells. Additionally, microRNAs have also been noted to regulate VSMC phenotype with miR-143/145 being the most important via its downregulation of KLF4 and upregulation of myocardin activity through repression of Elk-1.

VSMCs respond to stress by dedifferentiating into secretory cells, thereby losing their contractile elements. The cells become larger and produce ECM consisting of collagen and fibronectin. They also release matrix metalloproteinases to allow for their migration further into the media and even to the sub-intimal space. This leads to vascular remodelling with medial hypertrophy and hyalinisation. Such vessels lose their ability to adequately vasodilate in response to stimuli and also develop smaller calibre lumina. Angiotensin II binds to AT_1 receptors and stimulates VSMC growth and ECM production via ROS signalling pathways[438]. As stated above, IL-6 and CRP increase VSMC AT_1 receptor expression, sensitising them to circulating Angiotensin and potentiating its effect on the VSMC phenotype. Predictably, angiotensin receptor blockers have been shown to reverse vascular medial hypertrophy when given to diabetic patients.

VSMCs may not the only source of activated mesenchymal cells in the vessel walls. Endothelial cells may similarly renege on their dedicated endothelial commitment and instead lose cell-cell adhesion and migrate into the media, acquiring a smooth muscle-like phenotype in a process termed Endothelial-Mesenchyme Transition (EndMT). This process also leads to vascular remodelling and is promoted by IFNγ activity through upregulation of Endothelin-1 and TGFβ[439]. Interestingly, reduced miR-200 (as was seen in our NGS analysis) has been implicated in the initiation of EndMT.

We contend that the main structural pathophysiological change responsible for Cardiac Syndrome X is microvascular remodelling brought about as a result of VSMC plasticity and possible EndMT. Reduced miR-143 leads to derepression of Angiotensin Converting Enzyme (with consequent Angiotensin II upregulation) and Kruppel-Like Factor 4, both of which are potent stimuli for VSMC phenotype switching. It also leads to increased levels of Elk-1, which competes with SRF to bind with myocardin, thereby inhibiting the VSMC stimulus to remain differentiated in its contractile state. As a result of all this, VSMCs in the microvasculature of CSX patients may de-differentiate into a more secretory state typified by their proliferation, migration and production of extracellular matrix (ECM), a process also stimulated by IFNγ. This is also fuelled by increased Angiotensin-II signalling through the upregulation of AT_1 receptors. This results in medial hypertrophy with narrowing of the vessel lumen and a resultant reduction in coronary flow reserve and increased vessel stiffness. Coronary microvascular dysfunction in diabetes mellitus is characterised by VSMC switching and arteriolosclerosis/microvascular remodelling and a similar process may occur in CSX.

There is histological evidence to substantiate this theory. Most importantly, microvessels in CSX patients demonstrate obvious subendothelial hyalinisation, perivascular fibrosis, myointimal proliferation and medial hypertrophy. There is also evidence of dermal capillary rarefaction in CSX, which may imply vessel destruction or functional rarefaction through remodelling[440,441]. Additionally, the functional impairment resulting from this remodelling can also be noted by the observation of increased arterial stiffness and reduced carotid artery distensibility in CSX patients[171]. Importantly, EndMT and VSMC phenotype switching are dynamic and reversible processes. It is, therefore, essential to aggressively treat CSX patients with ACE inhibitors or angiotensin receptor blockers and statins to maintain the VSMCs in a differentiated, contractile phenotype.

8.5 Limitations

The limitations specific to each chapter are expressed in their respective chapters but we recount the main limitations here. The overriding limitation is that our study population numbers were relatively low and this had a persistent effect throughout the entire study in terms of statistical power. As explained in chapter 2, this scenario was inevitable given the circumstances of the low incidence in Ireland, the lengthy follow-up period required and the limited time available to this investigator. Part of the problem was the long list of exclusion criteria and exacting standards for diagnosis, which limited the study population. There was certainly a trade-off to be made between diagnostic integrity and external validity but this was an essential compromise, however, as we enrolled a homogenous representative population with a robust and reproducible diagnosis that could be reliably made in most clinical settings worldwide. Despite this limitation we did detect many significant differences in our cohort but a larger sample size would possibly have been of benefit in the analyses with a borderline p-value and in investigating correlations between biomarkers and clinical parameters. Similarly, the lack of follow-up data for our healthy controls limited the statistical tests we could use in the analysis of data from CSX follow-up visits.

A further limitation, in retrospect, was the failure to obtain a thorough psychiatric history, perhaps using the Structured Clinical Interview for DSM disorders (SCID) as this would have informed our discussion on the psychological impact of CSX more clearly. Similarly, all of our patients had their ischaemia diagnosed by a single modality (namely the EST). It might have been useful to include patients with positive stress perfusion imaging (ECHO, SPECT, CMR or PET) but our recruitment policy merely reflects the current diagnostic paradigm used in the Cork cardiology departments. Finally, it is a pity that we did not concomitantly recruit patients with atypical chest pain, positive EST and angiographically normal arteries as this would have answered the question of the relevance of the type of chest pain experienced in CSX, which remains a mystery.

8.6 Recommended Areas of Interest for Future Research

Although we have made several important steps in furthering the understanding of coronary microvascular disease in general and CSX in particular, research efforts in this area have been hampered by the lack of specific animal models of the condition. This is compounded by the fact that research into the coronary circulation is restricted to functional assessment rather than anatomic. Many questions have been generated from this thesis and several warrant further investigation, not least because behind all of this research is a large body of patients with intractable symptoms who require new and effective therapies.

Firstly, we should establish the external validity of these findings. Our diagnosis of CSX is highly specific and the question of whether these findings can be replicated in similar populations is important. The state of the art in CSX is advancing rapidly and soon non-invasive estimation of coronary flow reserve and microvascular function will be more reliable and accurate thus enabling the clinical diagnosis of microvascular angina to be made safely. At present, invasive assessment of CFR is the gold-standard but carries several procedural risks and has only a IIb recommendation in the latest ESC guidelines, indicating that it may be considered in special cases. It will be necessary to demonstrate that inflammation is a cornerstone of disease activity in that population too. Furthermore, the generalisability of our findings to patients with possible CSX but with atypical chest pain is also uncertain and could be confirmed easily.

In terms of further assessing the nature of LGI in CSX, especially given that the neutrophil: lymphocyte ratio is elevated, one could employ flow cytometry to assess the neutrophilic functional responses in CSX. Specifically, it would be interesting to determine if the neutrophils of CSX patients are primed. It is likely that this will be the case given the elevation of IL-6 and TNFα in our population. Primed neutrophils change shape and become stiffer due to actin cytoskeletal rearrangement. This and other

markers of neutrophil priming such as NADPH oxidase activity and CD-11b expression can be assessed by flow cytometry and ancillary procedures[442,443]. Primed neutrophils can be a potent source of oxidative stress and vascular damage. The other cells of interest would be the cells of the monocyte-macrophage population. Many of the cytokines we see in CSX are released by the M_1 subtype macrophages. Unfortunately, we would need tissue to allow flow cytometric analysis of macrophage subtype. It might be feasible, however, to examine the peripheral monocytes to assess the various subset populations. The Intermediate monocytes sub-population is pro-inflammatory and has high CD14, CD16, CD64 and CX_3CR1 expression. Over-representation of this monocyte subset would be an important finding in a CSX population.

The stimulus for LGI in CSX is of critical importance. As noted in 8.3.3, there are many potential sources for this chronic inflammation. Given that perceived stress was elevated in our CSX population and sympathetic nervous system dysregulation is seen in many CSX patients, the evaluation of the Hypothalamic-Pituitary-Adrenal (HPA) axis in CSX patients might yield some valuable information. The cortisol awakening response using salivary cortisol assessment is a well validated tool for the assessment of HPA-axis functioning in CSX. Of course, chronic inflammation may also lead to HPA dysfunction. Patients should be evaluated for early life stressors as a potential trigger for lifelong immune dysregulation.

The gastrointestinal tract as a potential source of low-grade endotoxaemia would also be an attractive area of research in CSX. Furthermore, the microbiome also appears to play a role in visceral pain hypersensitivity. The composition of the microbiome in CSX patients could be assessed using high throughput sequencing techniques in an effort to identify dysbiosis as this has been associated with LGI[444]. Plasma lipopolysaccharide levels could also be interrogated alongside the assessment of intestinal permeability (which is known to be impaired in stress) to support the notion of translocation of

gram-negative bacteria and low-grade endotoxaemia being the cause of the LGI in CSX[445]. Assessment of Peripheral Blood Mononuclear cells (PBMCs) sensitivity to Toll-like Receptor 4 (TLR4) stimulation would also help to interrogate this hypothesis.

Lifestyle patterns should be assessed in patients using simple questionnaires and diaries to assess exercise volume and sleep quantity. The role of diet in CSX could be further clarified using red blood corpuscle membrane fatty acid analysis rather than plasma analysis as the former reflects longer term dietary fatty acid intake. A more detailed dietary questionnaire could be administered and special attention should be paid to the assessment of choline intake, given the recent findings that trimethylamine N-oxide (TMAO), a choline metabolite, can affect macrophage function in the vasculature[409].

New treatment avenues could also be explored. Given the reported success of Vitamin D supplementation in CSX, a simple study of blood vitamin D levels could provide the rationale for vitamin D supplementation in these patients. Similarly, the role for omega-3 supplements could be solidified if changes in EPA:AA could be demonstrated to correlate with improved functional and clinical measures. Research into other potential, albeit potentially harmful, therapies in CSX could include methotrexate and tocilizumab to downregulate IL-6 and CRP and hopefully alleviate symptoms.

The further interrogation of the VSMC phenotype switching and endothelial-mesenchyme transition hypotheses is the most compelling area of interest for me personally going forward. Unfortunately, these hypotheses may prove difficult to confirm as we have no viable animal model of the condition. Transcatheter myocardial biopsies typically only provide ≈2mm of vessels, which tend not to be the resistance microvessels and appear only in patches within the biopsy[446]. It is possible to look at

tissue removed during cardiac surgery (such as the left atrial appendage taken during coronary artery bypass) but these procedures will obviously not be done in CSX patients as they have otherwise healthy hearts. Another possible approach would be to examine the myocardin mRNA in circulating PBMCs to assess expression of this pro-contractile VSCM transcription factor[447]. Finally, a re-evaluation of miR-10b, miR-199b, miR-200a and miR-200b in a larger cohort of CSX patients is indicated to attempt to validate our NGS results. Given what we know so far of the vascular biology in CSX, there is a logical role for these miRNAs in CSX pathogenesis.

8.7 Conclusions

We set out to thoroughly investigate the immune phenotype in CSX patients and to monitor its changes over time. As well as confirming the chronic nature of inflammation in CSX in the form of elevated TNFα and IFNγ, we have narrowed the focus down to the IL-6/CRP axis as a potential mediator of acute vascular dysfunction and symptoms in patients as it was levels of these biomarkers that altered along with changes in symptoms and signs of disease activity. Indeed, we have demonstrated that hsCRP may be employed as a biomarker to facilitate the diagnosis and prognosis assessment in CSX patients. Unfortunately, we have not been able to adequately determine the exact stimulus for this chronic LGI but we have suggested several avenues of enquiry that could be pursued. The concept of VSMCs leading to microvascular remodelling and dysfunction in CSX is a compelling one that warrants further consideration.

Finally, CSX appears to be an importunate condition that impacts on patients' day-to-day life and predisposes patients to psychopathology, being associated with increased life stress and reduced quality of life. A positive EST is an essential component of its diagnostic work-up and unfortunately this condition has been heretofore underdiagnosed in Ireland, thereby depriving these patients of suitable therapy.

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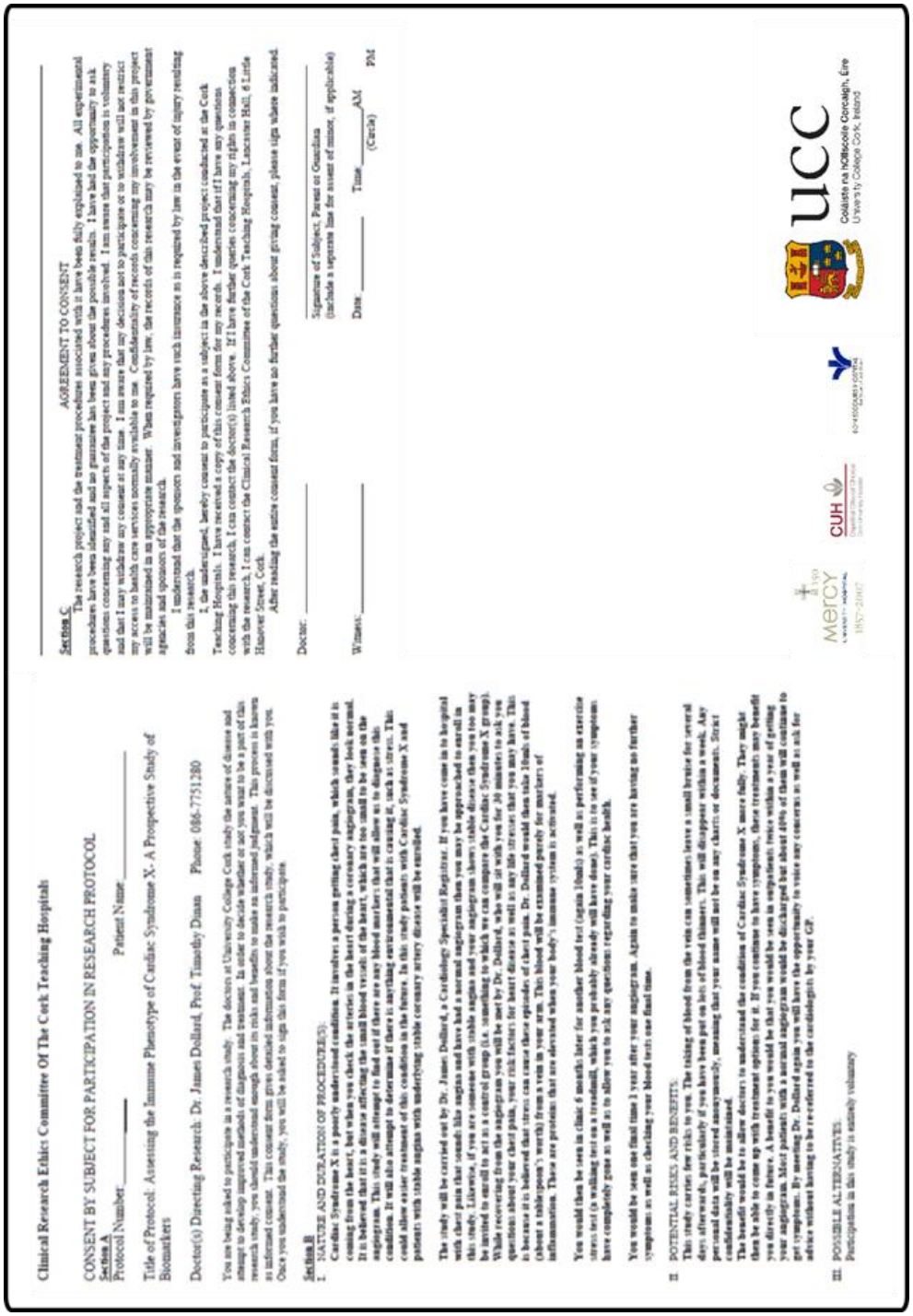
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Appendix I



Clinical Research Ethics Committee Of The Cork Teaching Hospitals

CONSENT BY SUBJECT FOR PARTICIPATION IN RESEARCH PROTOCOL

Section A

Protocol Number _____ Patient Name: _____

Title of Protocol: Assessing the Immune Phenotype of Cardiac Syndrome X- A Prospective Study of Biomarkers

Doctor(s) Directing Research: Dr. James Dollard, Prof. Timothy Dunn Phone: 086-7751280

You are being asked to participate in a research study. The doctors at University College Cork study the nature of disease and attempt to develop improved methods of diagnosis and treatment. In order to decide whether or not you want to be a part of this research study, you should understand enough about its risks and benefits to make an informed judgment. This process is known as informed consent. This consent form gives detailed information about the research study, which will be discussed with you. Once you understand the study, you will be asked to sign this form if you wish to participate.

Section B

I NATURE AND DURATION OF PROCEDURE(S):

Cardiac Syndrome X is a poorly understood condition. It involves a person getting chest pain, which sounds like it is coming from the heart, but when you check the arteries in the heart during a coronary angiogram, they look normal. It is believed that it is a disease affecting the small blood vessels of the heart, which are too small to be seen on the angiogram. This study will attempt to find out if there are any blood markers that will allow us to diagnose this condition. It will also attempt to determine if there is anything environmental that is causing it, such as stress. This could allow easier treatment of this condition in the future. In this study patients with Cardiac Syndrome X and patients with stable angina with underlying stable coronary artery disease will be enrolled.

The study will be carried out by Dr. James Dollard, a Cardiology Specialist Registrar. If you have come to hospital with chest pain that sounds like angina and have had a normal angiogram then you may be approached to enroll in this study. Likewise, if you are someone with stable angina and your angiogram shows stable disease then you too may be invited to enroll to act as a control group (i.e. something to which we can compare the Cardiac Syndrome X group). While recovering from the angiogram you will be met by Dr. Dollard, who will sit with you for 20 minutes to ask you questions about your chest pain, your risk factors for heart disease as well as any life stresses that you may have. This is because it is believed that stress can cause these episodes of chest pain. Dr. Dollard would then take 10mls of blood (about a tablespoon's worth) from a vein in your arm. This blood will be examined purely for markers of inflammation. These are proteins that are elevated when your body's immune system is activated.

You would then be seen in clinic 6 months later for another blood test (again 10mls) as well as performing an exercise stress test (a walking test on a treadmill, which you probably already will have done). This is to see if your symptoms have completely gone as well as to allow you to ask any questions regarding your cardiac health.

You would be seen one final time 1 year after your angiogram. Again to make sure that you are having no further symptoms as well as checking your blood tests one final time.

II POTENTIAL RISKS AND BENEFITS:

This study carries few risks to you. The taking of blood from the vein can sometimes leave a small bruise for several days afterwards, particularly if you have been put on lots of blood thinners. This will disappear within a week. Any personal data will be stored anonymously, meaning that your name will not be on any charts or documents. Strict confidentiality will be maintained.

The benefit would be to allow doctors to understand the condition of Cardiac Syndrome X more fully. They might then be able to come up with treatment options for it. If you continue to have symptoms, these treatments may benefit you directly in future. A benefit to you would be that you would be seen in outpatients twice within a year of getting your angiogram. Most patients with a normal angiogram would be discharged but about 40% of them will continue to get symptoms. By meeting Dr. Dollard again you will have the opportunity to voice any concerns as well as ask for advice without having to be re-referred to the cardiologists by your GP.

III POSSIBLE ALTERNATIVES:

Participation in this study is entirely voluntary

AGREEMENT TO CONSENT

Section C

The research project and the treatment procedures associated with it have been fully explained to me. All experimental procedures have been identified and no guarantees has been given about the possible results. I have had the opportunity to ask questions concerning any and all aspects of the project and my procedures involved. I am aware that participation is voluntary and that I may withdraw my consent at any time. I am aware that my decision not to participate or to withdraw will not restrict my access to health care services normally available to me. Confidentiality of records concerning my involvement in this project will be maintained in an appropriate manner. When required by law, the records of this research may be reviewed by government agencies and sponsors of the research.

I understand that the sponsors and investigators have such insurance as is required by law in the event of injury resulting from this research.

I, the undersigned, hereby consent to participate as a subject in the above described project conducted at the Cork Teaching Hospitals. I have received a copy of this consent form for my records. I understand that if I have any questions concerning this research, I can contact the doctor(s) listed above. If I have further queries concerning my rights in connection with the research, I can contact the Clinical Research Ethics Committee of the Cork Teaching Hospitals, Lancaster Hall, 6 Little Hanover Street, Cork.

After reading the entire consent form, if you have no further questions about giving consent, please sign where indicated.

Doctor: _____

Witness: _____ Date _____ Time _____AM PM (Circle)

_____

Signature of Subject, Parent or Guardian

(include a separate line for assent of minor, if applicable)

Mercy University Hospital 1857-2007

CUH

UCC

Coláiste na hOllscoile Corcaigh, Éire

University College Cork, Ireland

Figure I.1: Consent form as approved by the Clinical Research Ethics Committee for the Cork Teaching Hospitals

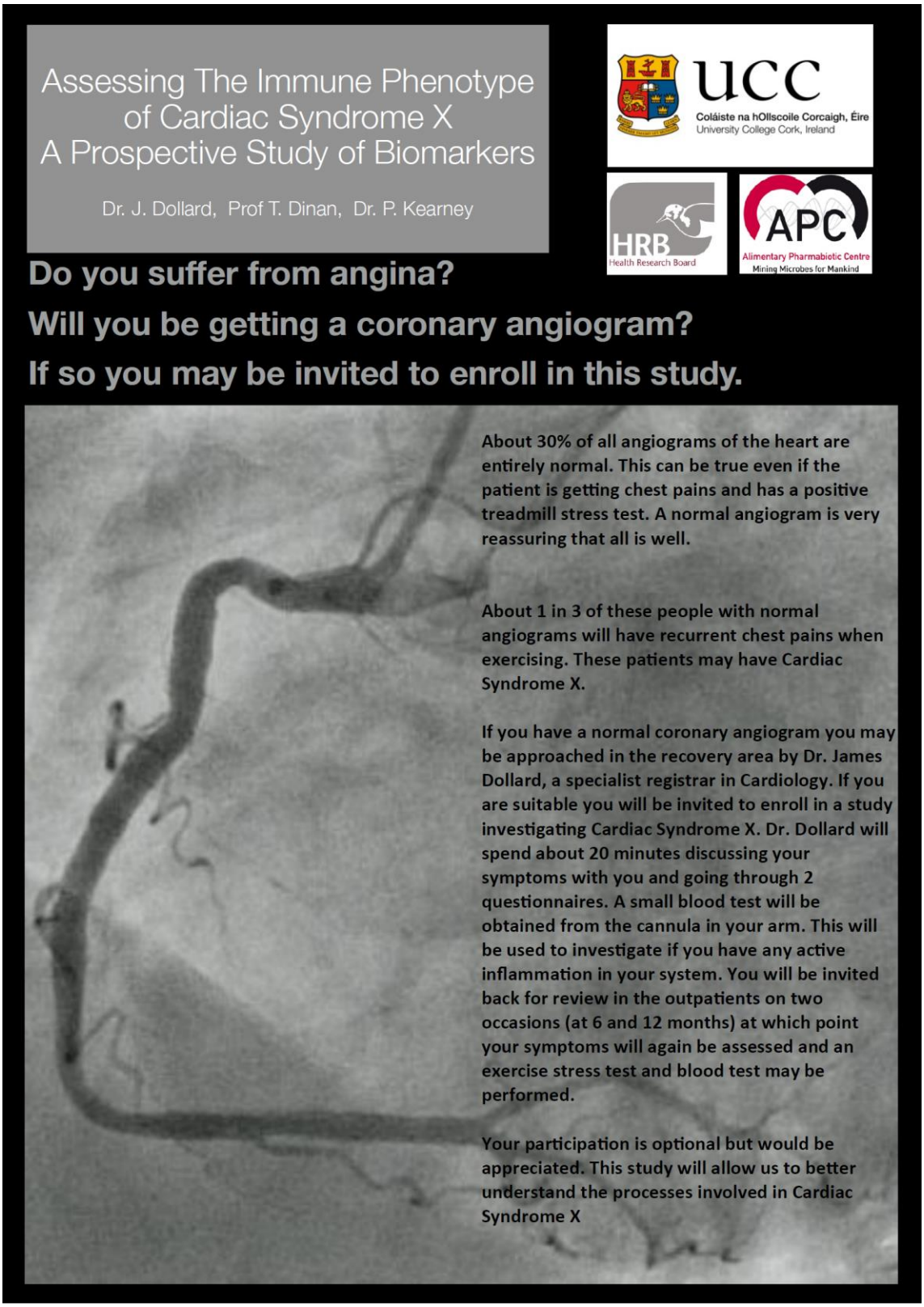


Figure I.2: Patient information sheet displayed in waiting rooms and outpatient departments

Assessing The Immune Phenotype in Cardiac Syndrome X- A Prospective Study of Biomarkers

Dr. J Dollard, Prof. T. Dinan, Dr. P Kearney

Background:

Cardiac Syndrome X: MRI (below) and coronary flow reserve studies have shown that sub-endocardial perfusion is impaired during angina attacks in those with CSX. This is despite normal epicardial coronary arteries. Endothelial dysfunction in the microvasculature is the likely cause. The pathophysiology of this is unknown but inflammation is a possible aetiology. This study will attempt to elucidate the inflammatory processes involved in CSX. It will also attempt to formalise diagnostic criteria, define the population at risk and study the natural history of CSX.

Methods: Patients will be approached in recovery after an angiogram and asked to participate. Full informed consent will be obtained. History, exam and chart review will be undertaken. Patients will complete standardised angina and life stresses questionnaires. 10mls of venous blood will be obtained and centrifuged. The plasma will be tested for unstimulated cytokines using ELISA. Patients will be asked to return at 6 and 12 months in a clinic setting at which point I will repeat the questionnaires and blood tests and perform a repeat EST. The same will be repeated at 12 months.

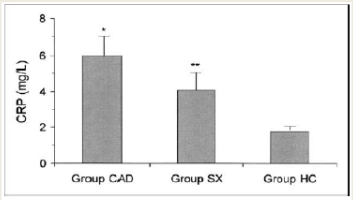
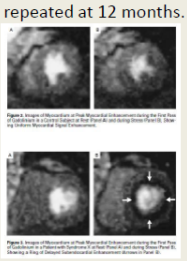


FIGURE 1. Mean ± SEM of serum levels of CRP in the 3 groups of subjects. *p = 0.013 versus the SX group and p <0.0001 versus controls, **p = 0.008 versus controls. HC = healthy con-



Inclusion Criteria:

1. **Typical Angina Pectoris**: all of the following features- central non-pleuritic chest pain, exacerbated by exercise, relieved by rest/nitrates.
2. **Normal Coronary Arteries** on diagnostic Coronary Angiography.
3. **Absence of a likely alternative diagnosis** by history
   Note: Patients with a positive Exercise Stress Test (symptomatically and electrically) or Myoview will meet the "strict" diagnosis for CSX and will enter subgroup analysis.

Exclusion Criteria:

1. **Other significant cardiac disease** (e.g. valvular HD, Cardiomyopathy)
2. **Active Systemic disease:** (e.g. connective tissue disease, IBS, CKD, active infection)
3. **Current use of NSAIDS, corticosteroids, high dose aspirin or other immunomodulators**
   Note: statin therapy is not a contraindication to enrolment.
4. **Age>70**
5. **Pregnancy**

Your help with recruitment would be appreciated.

UCC
Coláiste na hOllscoile Corcaigh, Éire
University College Cork, Ireland

APC
Alimentary Pharmabiotic Centre
Mining Microbes for Mankind

HRB
Health Research Board

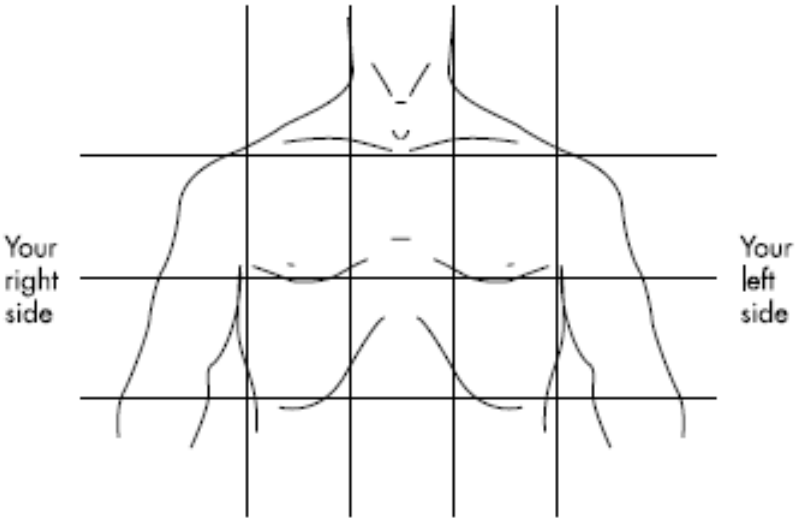
CUH
Ospidéal Ollscoil Chorcaí
Cork University Hospital

Figure I.3: The poster detailing the inclusion and exclusion criteria, which was displayed in the back of the catheterisation laboratories.

1 Do you ever have any pain or discomfort in your chest?

Yes/No

2 Where do you get this pain or discomfort?
Please mark **X** on the appropriate places



3 When you walk at an ordinary pace on the level does this produce the pain?

Yes/No/Unable

4 When you walk uphill or hurry does this produce the pain?

Yes/No/Unable

5 When you get any pain or discomfort in your chest on walking, what do you do?

Stop Slow down Continue at same pace Not applicable

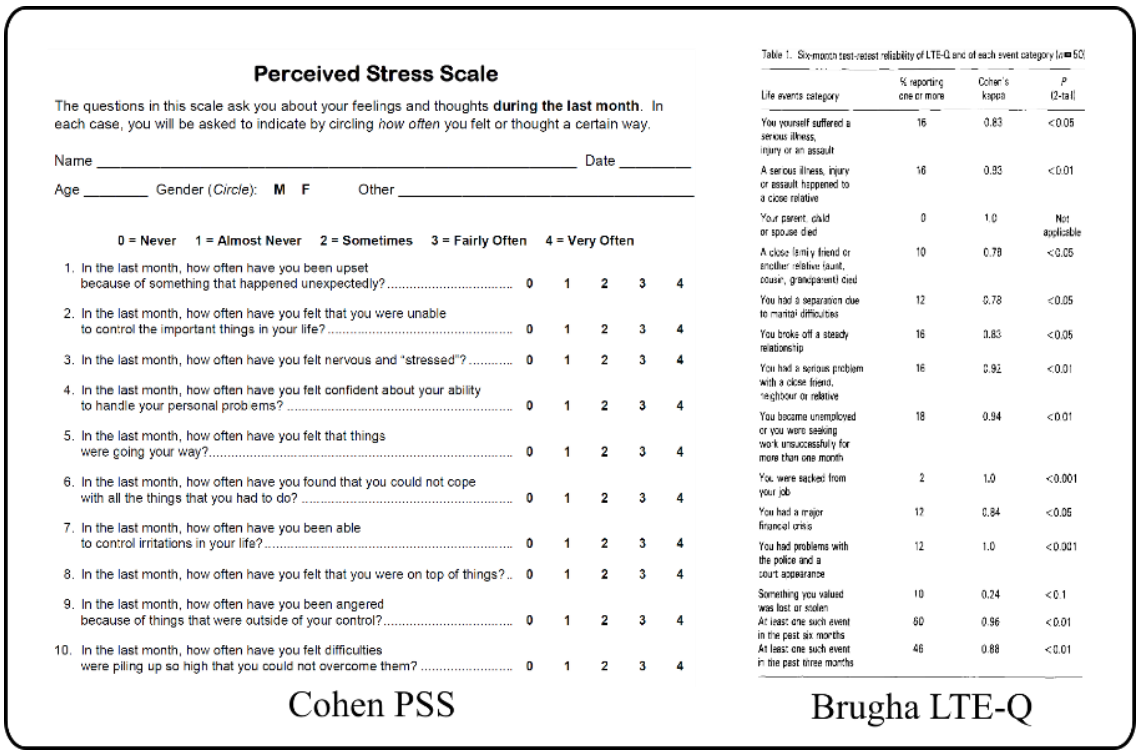
6 Does the pain or discomfort in your chest go away if you stand still?

Yes/No

7 How long does it take to go away?

10 minutes or less
more than 10 minutes

Figure I.4: WHO Rose Angina Questionnaire



Perceived Stress Scale

The questions in this scale ask you about your feelings and thoughts **during the last month**. In each case, you will be asked to indicate by circling *how often* you felt or thought a certain way.

Name _____ Date _____

Age _____ Gender (*Circle*): **M F** Other _____

0 = Never 1 = Almost Never 2 = Sometimes 3 = Fairly Often 4 = Very Often

1. In the last month, how often have you been upset because of something that happened unexpectedly? **0 1 2 3 4**
2. In the last month, how often have you felt that you were unable to control the important things in your life? **0 1 2 3 4**
3. In the last month, how often have you felt nervous and "stressed"? **0 1 2 3 4**
4. In the last month, how often have you felt confident about your ability to handle your personal problems? **0 1 2 3 4**
5. In the last month, how often have you felt that things were going your way? **0 1 2 3 4**
6. In the last month, how often have you found that you could not cope with all the things that you had to do? **0 1 2 3 4**
7. In the last month, how often have you been able to control irritations in your life? **0 1 2 3 4**
8. In the last month, how often have you felt that you were on top of things? .. **0 1 2 3 4**
9. In the last month, how often have you been angered because of things that were outside of your control? **0 1 2 3 4**
10. In the last month, how often have you felt difficulties were piling up so high that you could not overcome them? **0 1 2 3 4**

Cohen PSS

Table 1. Six-month test-retest reliability of LTE-Q and of each event category (n = 50)

Life events category	% reporting one or more	Cohen's kappa	P (2-tail)
You yourself suffered a serious illness, injury or an assault	16	0.83	<0.05
A serious illness, injury or assault happened to a close relative	16	0.93	<0.01
Your parent, child or spouse died	0	1.0	Not applicable
A close family friend or another relative (aunt, cousin, grandparent) died	10	0.78	<0.05
You had a separation due to marital difficulties	12	0.78	<0.05
You broke off a steady relationship	16	0.83	<0.05
You had a serious problem with a close friend, neighbour or relative	16	0.92	<0.01
You became unemployed or you were seeking work unsuccessfully for more than one month	18	0.94	<0.01
You were sacked from your job	2	1.0	<0.001
You had a major financial crisis	12	0.84	<0.05
You had problems with the police and a court appearance	12	1.0	<0.001
Something you valued was lost or stolen	10	0.24	<0.1
At least one such event in the past six months	60	0.96	<0.01
At least one such event in the past three months	46	0.88	<0.01

Brugha LTE-Q

Figure I.5: Cohen's Perceived Stress Scale (PSS) and Brugha's List of Threatening Experiences Questionnaire.

The Seattle Angina Questionnaire

1. The following is a list of activities that people often do during a normal week. Although for some people with several medical problems it is difficult to determine what it is that limits them, please go over the activities listed below and indicate how much limitation you have had **due to chest pain, chest tightness, or anginal attacks** **over the past 4 weeks**:

Place an x in one box on each line

Activity	Extremely Limited	Quite a bit Limited	Moderately Limited	Slightly Limited	Not Limited at all	Limited for other reasons or did not do the activity
Dressing yourself	☐	☐	☐	☐	☐	☐
Walking indoors on level ground	☐	☐	☐	☐	☐	☐
Showering or bathing	☐	☐	☐	☐	☐	☐
Climbing a hill or a flight of stairs without stopping	☐	☐	☐	☐	☐	☐
Gardening, vacuuming, or carrying groceries	☐	☐	☐	☐	☐	☐
Walking more than a hundred yards at a brisk pace	☐	☐	☐	☐	☐	☐
Running or jogging	☐	☐	☐	☐	☐	☐
Lifting or moving heavy objects such as furniture, or lifting children	☐	☐	☐	☐	☐	☐
Participating in strenuous sports (e.g. swimming, tennis)	☐	☐	☐	☐	☐	☐

Figure I.6: Sample page of the Seattle Angina Questionnaire

Prudent Diet Questionnaire

_____ How much low fat or skim milk, yogurt, and cheese do you consume in a week?

1. Consume at least 16 ounces milk or yogurt, or 3 ounces cheese per week.
2. 8 ounces milk/yogurt or 1 ounce cheese per week.
3. Only use it in cereal or consume it occasionally.
4. Do not consume milk/yogurt/cheese at all.

_____ How often do you choose to eat potato chips, corn chips, taco chips, olives, nuts, or similar foods as snacks or with a meal?

1. none or rarely
2. occasionally 1-2 times per week
3. 3-4 times per week
4. 5 or more times per week

_____ How many times do you eat fruit per day?

1. 7 or more
2. 4-6 times
3. 1-3 times
4. none

_____ How many whole grain breads and cereals, raw fruits and vegetables, and bran products do you eat each day?

1. 4 or more
2. 3-4 servings
3. 1-2 servings
4. none

_____ Which describes your consumption of vegetables?

1. Snack on raw vegetables and eat vegetables/salads with most meals.
2. Eat salads and vegetables at one meal a day.
3. Eat vegetables 2-3 times per week.
4. Rarely eat vegetables.

_____ How many glasses of water do you drink in a day?

1. 8 or more glasses
2. 5-8 glasses
3. 2-4 glasses
4. one glass or none

_____**Total Prudent Diet Questionnaire**

Figure I.7: Prudent Diet Questionnaire

Appendix II

Ir J Med Sci
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ORIGINAL ARTICLE

Cardiac syndrome X in Ireland: incidence and phenotype

J. Dollard[1,3] · P. Kearney[1] · T. G. Dinan[2,3]

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Abstract

Background Cardiac syndrome X (CSX) is typical angina pectoris with objective signs of myocardial ischaemia despite a normal coronary angiogram and may be due to microvascular dysfunction. The incidence of CSX has not been greatly investigated worldwide and its incidence in Ireland is unknown.

Aims We aimed to determine the incidence of CSX in Cork University Hospital (CUH) and to establish the phenotype of the typical Irish CSX patient.

Methods All patients undergoing coronary angiography in CUH during regular working hours over a 3-month period were investigated. CSX was diagnosed using standard criteria. An extended recruitment period of 14 months allowed enrolment of a sufficient number of CSX patients to enable phenotyping.

Results Only 5 of 372 (1.3 %) patients undergoing angiography to investigate chest pain met the diagnostic criteria for CSX. None were given a discharge diagnosis of CSX or received cardiology follow-up. Irish CSX patients were predominantly female (88 %) with a mean age of 59.2 ± 6.6 years. Although they were significantly less functionally limited than patients with obstructive CAD, they had an equally substantial impairment in quality of life.

Conclusions CSX is relatively uncommon in Ireland and is most frequently seen in middle-aged women with hyperlipidaemia. It has significant impacts on patients' quality of life. None of the CSX patients were diagnosed as such, highlighting the lack of awareness or acceptance of this condition in Ireland. These patients require diagnosis and active cardiology follow-up to effectively manage their symptoms.

Keywords Angina pectoris · Normal coronary arteries · Microvascular angina · Epidemiology

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Introduction

Cardiac syndrome X (CSX) is a condition with which many practising doctors are not familiar. It should not be confused with metabolic syndrome X (the coincident diagnoses of hypertension, dyslipidaemia, insulin resistance, and abdominal obesity) which is a separate entity. In essence, patients with CSX suffer from angina pectoris in the absence of demonstrable macrovascular coronary artery disease on coronary angiography, this despite initial findings on non-invasive testing suggestive of ischaemia. In the majority of cases it is believed to be due to inadequate coronary microvascular vasodilation during times of increased cardiac workload leading to insufficient oxygen delivery to the cardiomyocytes and this lends CSX its alternative (and more informative) title, microvascular angina [1]. Figure 1 shows the difference between ischaemia caused by obstructive coronary artery disease and by microvascular endothelial and vascular dysfunction. The exact cause of this vascular dysfunction is debated but inflammation has been implicated [2, 3]. Despite having a benign prognosis in terms of mortality, over half of patients

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A prospective study of C-reactive protein as a state marker in Cardiac Syndrome X

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ABSTRACT

Cardiac Syndrome X (CSX), the presence of angina pectoris despite normal epicardial coronary arteries seen on invasive angiography, is known to be associated with an elevation of several inflammatory biomarkers, suggesting a possible role for inflammation in its pathogenesis. We sought to establish if C-reactive protein (CRP) levels varied with disease severity and so whether it is a state or trait marker. We studied 16 CSX patients with typical angina pectoris, normal coronary arteries and an electrically positive exercise stress test (EST) and 13 age- and sex-matched healthy controls (HC). CSX patients were followed up at a subsequent visit with repeated exercise stress testing and CRP measurement. We found that CRP levels were significantly higher in the CSX group compared to the HC (1.5 [0.8–4.5] v 0.8 [0.4–1.4] mg/L, $p = 0.02$). This elevation in CRP persisted throughout the study length. CRP correlated with time to symptoms on EST at enrolment and at the second visit ($r = -0.690$, $df = 10$, $p = 0.013$ and $r = -0.899$, $df = 4$, $p = 0.015$, respectively). At the follow-up visit, 50% of CSX patients developed electrically and symptomatically negative ESTs. The mean CRP of this group was significantly lower than that of the CSX patients with ongoing symptoms and positive ESTs (1.2 ± 0.2 v 2.8 ± 0.6 mg/L, $p = 0.018$) and did not differ significantly from that of healthy controls. CRP levels also dropped in patients whose symptoms improved while they increased in patients who became more symptomatic ($p = 0.027$). We conclude that the results of this small study support the concept of CSX being an inflammatory-mediated condition with CRP levels prospectively varying with functional measures of disease severity. This indicates that CRP is a state marker in CSX.

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1. Introduction

1.1. Defining Cardiac Syndrome X

Angina pectoris is a particularly emotive symptom as it is one of the few that can invoke fear of immediate death in patients. Thus, patients and physicians both correctly consider angina to be an extremely important complaint requiring urgent evaluation. Exercise stress testing is the usual non-invasive first step with exercise-induced symptoms and ST-depression being indicators of possible coronary artery disease. If positive, this is followed by an invasive coronary angiogram to assess the epicardial coronary arteries. Between 20% and 30% of angiograms performed to investigate chest pain are normal, forming the diagnosis termed "chest pain with normal coronary arteries" or CPNCA. This term encompasses a heterogeneous group of patients who may have non-cardiac sources of chest pain (e.g. gastro-oesophageal disease) or cardiac causes other than epicardial coronary artery disease (such as valvular heart disease, myocardial disease, dysrhythmias and microvascular disorders). CSX patients represent a subset of CPNCA.

CSX is defined as the presence of typical angina pectoris and exercise-induced ST-depression on EST, but with normal coronary arteries on angiography and without contributory heart disease (Lanza, 2007). It is the presence of an objective test indicating probable myocardial ischaemia that mainly separates CSX patients from other patients with CPNCA. It has proven to be difficult to characterise and to treat but remains an important condition as CSX patients, despite having a favourable cardiovascular prognosis, have a significantly impaired quality of life and can remain markedly symptomatic even years after diagnosis (Lamendola et al.,

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