Novel markers of Plaque Rupture in Patients with Chest Pain.

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I declare that this thesis, which I submit to RCSI for examination in consideration of the award of a higher degree my own personal effort. Where any of the content presented is the result of input or data from a related collaborative research programme this is duly acknowledged in the text such that it is possible to ascertain how much of the work is my own. I have not already obtained a degree in RCSI or elsewhere on the basis of this work. Furthermore, I took reasonable care to ensure that the work is original, and, to the best of my knowledge, does not breach copyright law, and has not been taken from other sources except where such work has been cited and acknowledged within the text.

Signed

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Abbreviations

ACS- Acute Coronary Syndrome
ApoB- Apolipoprotein B
ApoE- Apolipoprotein E
CMRI- Cardiac Magnetic Resonance Imaging
CML- Chemokine motif ligand
CRP- C-reactive protein
CTCA- Cardiac CT Angiography
DTK- Developmental Tyrosine Kinase
ECG- Electrocardiogram
ECM- Extracellular matrix
ELISA- Enzyme linked immunofluorescent analysis.
ET-1- Endothelin 1
HF7- Human Frizzled Homolog 7
IL-2- Interleukin 2
IL15Rα- Human Interlukin-15 Receptor subunit alpha
IL-6- Interleukin 6
IL-15- Interleukin 15
IL-17- Interleukin 17
IFN-γ- Interferon gamma
ICAM-1- Intercellular adhesion molecule-1
IVUS- Intravascular ultrasound
LR- Likelihood ratio
MACE- Major adverse cardiac event
MCP-1-Monocyte chemoattractant protein 1
MI – Myocardial Infarction
NSTEMI- Non ST segment elevation myocardial infarction
NADH -Nicotinamide adenine dinucleotide
NADPH- Nicotinamide adenine dinucleotide phosphate
NO- Nitric oxide
OCT- Optical Coherence Tomography
oxLDL- Oxidative modifications of LDL
RCF- Relative centrifugal force
SMC- Smooth muscle cell
SPECT- Single Photon Emission Computed Tomography
STEMI- ST segment elevation myocardial infarction
TCFA-Thin-cap fibroatheroma
TGFIIβR-Transforming growth factor beta II receptor
TH1- T helper cells type 1
TLR 2- Toll-like receptor-2
TTE-Transthoracic Echocardiography
TXB2- Thromboxane B2
Treg- Regulatory T cells
TNF- Tumor necrosis factor
UA – Unstable Angina
VCAM-1 –Vascular cell adhesion molecule 1
VEGF- Vascular endothelial growth factor
VSMC- Vascular smooth muscle cells
Summary

Chest pain presentations to the accident and emergency department account for approximately 20% of all admissions. Identifying those patients at high risk of plaque rupture, the consequence of which is ischemia and myocardial necrosis, remains a significant challenge. Current clinical pathways aim to identify those who have undergone plaque rupture as early as possible. We currently use assays of markers of myocardial necrosis and screening electrocardiograms to confirm plaque rupture after myocardial injury has occurred.

We previously identified several candidate proteins in an Apolipoprotein E (ApoE) mouse model, that were present at significantly higher or lower levels in serum or plasma, prior to coronary plaque rupture. Thus they have the potential to predict atherosclerotic plaque rupture in mice. This MD thesis evaluates the clinical utility of these markers in a chest pain cohort. Patients (n=203) presenting to either the accident and emergency department or cardiac catheterization suite at St James’s Hospital, Dublin was recruited.

Gal-3 and IL-15Rα were significantly elevated in patients with cardiac chest pain (stable angina or an ACS). Notably both proteins were significantly elevated in patients with unstable angina who were troponin negative. IL-15Rα correlated significantly with Gal-3 in patients with ACS and specifically in those with unstable angina, potentially identifying unstable plaque. Furthermore, both markers were increased significantly in patients with cardiac chest pain who had ongoing symptoms.

Levels of TGFIIβR were significantly elevated in patients with stable angina, however were decreased in those with unstable symptoms and did not correlate with duration of chest pain or time from last episode of chest pain. Of the other markers assayed, DTK and HF7 were not significantly elevated in patients with cardiac chest pain compared to those with non-cardiac chest pain and did not correlate with coronary plaque burden.
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And to the supreme God who permeates all things.
Chapter 1

Introduction
1.1 Introduction

1.1.1 Background

Coronary artery disease (CAD) and myocardial infarction (MI) remain a significant cause of mortality and morbidity in the western world. Cardiovascular disease remains the most common cause of death in Ireland being responsible of one third of all deaths. Chest pain, the most common symptom of CAD, is a presenting complaint in five percent of all patients who attend the accident and emergency department and forty percent of patients admitted to acute medical units [9] [10]. The term, acute coronary syndrome (ACS) encompasses a composite of cardiovascular diagnoses that include ST segment elevation myocardial infarction (STEMI), non-ST segment elevation myocardial infarction (NSTEMI) and unstable angina (UA). Rapid identification and triage of patients with an ACS versus low risk patients who can be safely discharged and evaluated in the outpatient setting, remains a major clinical challenge. Identification of sensitive and specific biomarkers that facilitate early risk stratification and patient management has the potential to greatly improve patient care.

Current practice follows a standardized process that includes clinical evaluation, serial Troponin (Tn) assay and screening electrocardiograms (ECG) with progression to non-invasive testing as indicated and if available. Despite this screening process, patients continue to suffer cardiac morbidity and mortality following discharge[11]. In the case of STEMI a diagnosis may be made quickly. However STEMI patients represent only a small percentage of all chest pain presentations and up to 60% of patients with an ACS may present with a non-diagnostic ECG [12].

Pleural and pericardial irritation, gastrointestinal reflux, pulmonary embolism, hyperventilation, musculoskeletal pain and cholecystitis may all mimic ACS thus making a cardiac chest pain diagnosis more difficult [13]. In one-study, 6% of
emergency admissions over a one-year period were due to chest pain and only 30% of these were attributable to a cardiac cause [14]. Furthermore delays in excluding ACS delay detection of other medical conditions and contribute to emergency department overcrowding [15].

Thus assays that facilitate rapid identification of patients with high-risk cardiac chest pain and that also exclude those at low risk will greatly improve clinical care. Various approaches to screening patients who attend the emergency department with chest pain have been tested but have proven to be both time consuming and costly. Medication and chest pain treatment are also costly and therefore a simple and cheap test that allows one to target treatment is required.

Groake et al. showed that 61% of chest pain admissions to a tertiary referral Irish hospital were non-cardiac in nature. Furthermore, the average cost of non-specific chest pain admission to hospital was 3,729 euro per admission patients had an average length of stay of 3.7 days [16]. When extrapolated the national cumulative cost was 71 million euros and 73,000 bed days. Similarly Mourad G et al. reported an annual cost of non-cardiac chest pain admissions of 11,000 euro per admission, a cost comparable to that of admission with an acute myocardial [17].

Current cardiac biomarkers focus on the detection of serum or plasma proteins released during myocardial necrosis or cell death. There has been some progress in this area, however the extent of myocardial injury sustained by the time diagnosis is confirmed correlates directly with patient mortality. Reduced cardiac ejection fraction after an Acute Myocardial Infraction (AMI) determined by transthoracic echocardiography remains a robust predictor of cardiovascular mortality [18].

As the prevalence of CAD increases the economic burden continues to rise [19]. Delayed patient presentation despite national education programmes continues to contribute to this cost [20, 21]. Early identification of vulnerable coronary plaque that not only predict ACS but also allow early introduction of therapies that prevent disease progression should be the aim. A biomarker or panel of biomarkers could then be assayed not only in an accident and emergency setting but also in primary care.
facilities. The next section reviews current application and limitations of cardiac biomarkers to screen patient with a possible ACS.
Biomarkers in coronary artery disease

Biomarkers are measurable and quantifiable biological parameters that serve as indices for health and physiology assessment [22]. A good biomarker is something that is easily measured and can be used as a surrogate marker for disease and its severity. A good biomarker will diagnose or predict risk accurately (that is, high specificity and sensitivity), promptly provide affordable but meaningful results, and should provide this incrementally over existing markers or clinical characteristics. Cardiac biomarkers are of greatest value in patients with chest pain without overt changes on clinical examination or ECG. Thus biomarkers should complement clinical assessment and the ECG in the diagnosis, risk stratification, triage, and management of patients with suspected ACS.

Current mainstream biomarkers in chest pain focus on the detection of myocardial injury by measuring proteins released into the bloodstream after myocardial cell injury and cell death. Whilst there has been and continues to be extensive research into biomarkers that detect unstable plaque, the precursor of ischemia and myocardial cell death, the results have been mixed with no single marker in routine clinical use. Figure 1.1 summarizes the general concepts involved in plaque instability and ischemia that are explored in detail in subsequent sections.
Figure 1.1 - Summary of pathophysiological implications of different biomarkers in the cardiovascular continuum and where our biomarkers may be additive.

(Cys-c Cystatin C, BNP-Brain natriuretic peptides, ANP-Atrial natriuretic peptides, IL6-Interleukin 6, IL18-Interleukin 18, CRP-C reactive protein, HsT- High sensitivity troponin, Heart-Type Fatty Acid Binding Protein -H-FABP, Tn- Troponin, IL15Rα-Interleukin 15 receptor alpha, DTK-Developmental tyrosine kinase, HF7-Homolog frizzled 7, Gal-3- Galectin 3)
1.1.3

Biomarkers of myocardial ischemia and necrosis

Cardiac Troponin

Myocardial necrosis is accompanied by the release of structural proteins and other intracellular macromolecules into the cardiac interstitium as a consequence of compromise of the integrity of cellular membranes. Serum and plasma biomarkers that detect myocardial injury are pivotal to diagnostic and management chest pain algorithms and are centered on the use of Troponin (Tn) and more recently high sensitivity Troponin (HsT) [23].

Troponin, the gold standard for biochemical diagnosis of AMI was first commercialized in 1989 [24]. Applied in the appropriate clinical context it’s specificity approaches 100%. Troponins are protein complexes contained within muscle cells that modulate the contraction and relaxation of striated muscle. They are contained in a heterotrimeric complex consisting of 3 troponin molecules, whose names are derived from their functions: calcium-binding troponin (TnC); inhibitory troponin (TnI) and tropomyosin-binding troponin T (TnT). The complex attaches to the thin filaments of muscle and regulate muscle contraction. Muscle contraction is produced when the thick and thin muscle fibers slide past each other [25].

Troponin isoforms expressed in muscle tissue differ based on the tissue’s role, with the cardiac myocyte uniquely expressing the cardiac troponin TnI and TnT isoforms. Tissue specificity facilitates the use of cardiac troponins as biomarkers of cardiac injury. Approximately 7% of cardiac TnT and 3.5% of TnI exist freely in the cardiac myocyte cytoplasm. The rest is bound to the sarcomere [26].

Detection of Tn in peripheral blood indicates cardiomyocyte damage and Tn quantity correlates with extent of injury. A major limitation of conventional Tn assays is their low sensitivity among patients who present early with an ACS. Due to its slow release from the damaged myocyte and circulation, reliable troponin detection requires serial
sampling for 6–9 h in a significant number of patients [27]. HsT assays differ from conventional Tn assays. HsT can be detected in 50-90% of the normal population, therefore a value above the 99th centile is considered as pathological with a coefficient of variability of <10% [22].

HsT has been adopted as the point of care assay to detect myocardial injury in patients presenting with chest pain by the ESC [23]. Data from large multicenter studies have shown that sensitive and high-sensitivity cardiac troponin assays increase diagnostic accuracy for ACS at the time of chest pain presentation when compared with standard troponin assays. They are particularly useful when used in patients who present early with chest pain to rapidly rule in or out an ACS [28].

HsT assay facilitates rapid rule-out strategies by increasing diagnostic accuracy at time of presentation, reducing the ‘troponin-blind’ interval and allowing the time interval to the second measurement of HsT to be shortened. This results in reduced time to clinical decision and/or diagnosis [23]. The ESC recommends an immediate HsT measurement for patients presenting with chest pain with a repeat measurement at one to three hours after presentation to rule out ACS. A diagnosis of NSTEMI can be made if the clinical presentation is compatible with cardiac ischemia and the HsT concentration is greater than the 99th percentile for the healthy population [23].

The ESC standardized approach for detecting myocardial injury in patients with chest pain is summarized in Figure 1.2. All patients receive an HsT measurement at presentation and at one or three hours after presentation as illustrated.
Figure 1.2 - Flow chart using the European Society of Cardiology (ESC) algorithm for ruling out NSTEMI. (UML- upper limit of normal, GRACE score- See section 1.8)

In attempt to reduce time to discharge and clarify the diagnosis of NSTEMI earlier Neumann et al. compared the application of a 1 hour and 3 hour HsT algorithm in patients with suspected ACS and found that both the one-hour and three hour approaches yielded similar one year mortality outcomes [29]. However, in their study they considered an initial HsT measurement of <6ng/ml as being negative, much lower than the 99th percentile for their assay, with change of >12 ng/ml on repeat measurement being considered diagnostic of a NSTEMI. Patients that did not undergo this delta change i.e >12 ng/ml on second measurement but had a small rise in HsT were considered as ‘grey zone’ and assigned to observation versus treatment. This observed “grey zone” cohort experienced higher mortality when compared to patients who were treated earlier for NSTEMI [29]. Herein lies one of the challenges with current HsT assay. Variability in what is considered the upper limit of normal continues to evolve for the different assays.

Patients left in this ‘grey zone’ constitute at least 20% of the chest pain population. Pilot
studies suggested that further HsT measurements, functional stress imaging and coronary angiography are the appropriate next steps [30]. However evidence to support these contrasting approaches is limited.

Whilst HsT has progressed early detection of AMI it is not a marker of plaque instability or rupture but an indicator of myocardial damage and myocardial necrosis. Additionally not all patients who have ruled out for NSTEMI with a negative HsT should be discharged. Patients with unstable angina benefit from an early invasive management strategy [31-33]. Furthermore, late increases in HsT have been described in small (1%) numbers of patients [23]. Thus, serial HsT testing beyond 3 hours may be required if clinical suspicion remains high or if patients develop further chest pain. Finally, some challenging patient groups were either underrepresented or excluded from previous studies. These include patients on hemodialysis and those that presented more than 12h after chest pain onset [22].

In our study we assayed HsT in the majority of enrolled patients. Some Irish hospitals have not adopted HsT and continue to use the Tn assay. Indeed many larger centers in the United States of America do not use HsT due to the absence of its inclusion in their national guidelines [34].

In conclusion, whilst HsT has been extensively evaluated for detection of myocardial injury as a result of coronary plaque rupture, HsT elevation occurs after the index event.
**Copeptin**

Copeptin is a stable surrogate of arginine vasopressin, with well-known effects on osmoregulation and cardiovascular homeostasis [35]. Copeptin is released in stoichiometric proportion to vasopressin and is stable and easily assayed. Copeptin reflects endogenous stress, which is already present at the onset of AMI and is able to identify AMI even when Tn is negative [36, 37].

Post AMI, vasopressin is thought to increase peripheral vasoconstrictor activity thus increasing afterload and ventricular stress. Resulting increases in protein synthesis in myocytes leads to hypertrophy and increased vasoconstriction of coronary arteries. These effects are mediated via the V1 receptor. Effects on the V2 receptor mediate water retention in the renal tubules [38-40].

In its most recent guidelines the ESC provides for use of serum Copeptin to achieve early rule-out of AMI whenever sensitive Tn or HsT cardiac troponin assays are not available. They also state that Copeptin may have some added value over high-sensitivity cardiac troponin in the early rule out of AMI [23]. The concept in which Copeptin seems to have the greatest appeal to clinicians is its use within a dual-marker strategy for very early rule-out of AMI. In a landmark study Mockel et al. compared the standard treatment pathway of serial HsT measurements in patients at low to intermediate cardiovascular risk with HsT and Copeptin measurements in a similar risk profile group. Patients were discharged based on an initial negative HsT and Copeptin with no excess major adverse cardiac event (MACE) at 30 days when compared to standard practice [15].

Further randomized studies of Copeptin have produced promising yet mixed results. In a follow up study this year by Vafaie et al. there was no added benefit of Copeptin measurements in patients with undetectable HsT [41]. Additionally, whilst Copeptin is elevated after 30 minutes in patients with MI, similar to HsT its detection implies plaque rupture may have already taken place.
**B-type Natriuretic Peptide (BNP)**

BNP is one of the best-known biomarkers of biomechanical stress. Secreted by the ventricles in response to cardiomyocyte tension, BNP binds and activates receptors causing reduction in systemic vascular resistance, central venous pressure and promotes natriuresis [42]. BNP has been studied extensively and provides prognostic information following an MI [43]. It has a short half-life but when released the N-terminal portion of the pro-BNP peptide (NTproBNP), a peptide much more stable in serum, can be measured easily [44].

On its own, BNP is at least as good as the GRACE score at predicting in-hospital mortality post AMI. It also improves prognostic accuracy when added to the GRACE score. In NSTEMI BNP predicts in-hospital and 180 day death or heart failure [45, 46]. BNP however is not an acute marker of plaque rupture and much of its main clinical application relates to heart failure.

**Atrial Natriuretic Peptide (ANP)**

Like BNP, ANP has similar neurohormonal effects and has a similar secretory profile post AMI. Prior studies have attempted to accurately measure levels of ANP and N-ANP, with limited success. N-ANP has been associated with late mortality following AMI [47]. Such early N-ANP assays were often affected by interferences and instability of the analyte. Thus ANP was thought to provide limited prognostic information. However, the discovery of the novel Mid-Regional pro-Atrial Natriuretic Peptide (MRproANP) fragment provided a substantially more stable peptide compared to N-ANP and ANP. MRproANP is at least as good at predicting death and heart failure as NTproBNP [48]. Again like BNP, ANP and MRproANP do not detect acute plaque instability. Most of the research to date indicates a utility in predicting longer-term outcomes post AMI [49].
1.1.4

General concept of our research thesis

Many individual protein biomarkers have been shown to correlate with cardiovascular risk and patient prognosis. However, when considered individually their clinical application has been disappointing. For this reason, recent studies have looked for panels of biomarkers with greater predictive power.

We initially performed serum quantification of our novel markers in an Apo lipoprotein (ApoE) mouse model of plaque rupture (discussed further in section 1.9). On the foot of this, a human study was undertaken the results of which form the body of work for this thesis. The unifying aim has been to determine the utility of these markers in identifying plaque instability and rupture in an all comer group of patients presenting with chest pain. As summarized in Fig. 1.1 and discussed in detail in the following sections, inflammation plays a central role in the development and rupture of plaque. Therefore it would seem intuitive that systemic markers of inflammation could be applied to detect plaque instability and rupture.

Traditional risk assessment algorithms and risk scores are useful in identifying individuals at low or high risk for atherothrombotic cardiovascular events. These algorithms however poorly serve the large group of individuals at intermediate risk. There is an opportunity for other markers to reclassify this cohort into low and high risk.

In the next sections I will first review the development and rupture of plaque and include current imaging methods of detecting unstable coronary plaque, before introducing our biomarkers.
1.2

Pathophysiology of Plaque Rupture

Atherosclerosis is the process of accumulation of plaque within the arteries. This disease has a venerable history having been identified in Egyptian mummies. With economic development and urbanization came high fat diets, reduced physical activity and an epidemic of atherogenesis that is now endemic in western lifestyles. Atherosclerotic disease continues to be the leading cause of death and a major source of morbidity in developed countries [50]. Typically symptoms do not develop until later in life but plaque can be identified in children as young as twelve years old [51]. The vast majority of acute ischaemic vascular events occur due to underlying atherosclerotic plaque. Coronary plaque rupture is responsible for 60–70% of ACS, while plaque erosion is responsible for most of the remainder [52].

1.2.1

Normal Arterial Anatomy

Tunica Intima

The normal artery has a well-shaped tri-laminar structure. The innermost layer is known as the tunica intima and has a complex structure. The endothelial monolayer resides on a basement membrane containing non-fibrillar collagen types, such as type IV collagen, laminin, fibronectin and other extracellular matrix molecules. The complexity of the tunica intima changes with age as the intima develops more smooth muscle cells and type 1 and 3 collagen. These changes result in diffuse intimal thickening that is often described by pathologists [53]. The tunica intima is bounded by the internal elastic membrane, which serves as the border with the tunica media.
**Tunica Media**

The tunica media, which lies under the intima, has well-developed concentric layers of smooth muscle cells with layers of elastic rich extracellular matrix. The media is well adapted to store the kinetic energy of left ventricular systole. The lamellar structure contributes to the structural integrity of the artery. The extracellular matrix neither accumulates nor atrophies and rates of arterial accumulation and dissolution normally balance each other.

**Tunica Adventitia**

The adventitia is the outermost layer of the arterial wall. It contains collagen fibrils in a looser array than in the intima. Recent studies show that the adventitia functions as a dynamic compartment for cell trafficking into and out of the artery wall, it participates in growth and repair of the vessel wall, and it mediates communication between vascular endothelial cells and smooth muscle cells (SMCs) and their local tissue environment [54].

The adventitia contains lymphatic vessels and autonomic nerves, and it plays a critical role in the control of lumen size by regulating medial smooth muscle tone and control of inward (negative) and outward (positive) wall remodeling responses. Moreover, the adventitia contains resident populations of macrophages, T-cells, B-cells, mast cells, and dendritic cells that carry out important surveillance and innate immune functions in response to foreign antigens [54].
1.3

Atherosclerosis Initiation

Atherosclerosis underlies most heart attacks and strokes. Atherosclerosis is a complex process with a variety of interplay between local factors such as vascular shear stress patterns and endothelial injury in addition to systemic mediators such as circulating lipoproteins, hyperglycemia, environmental and genetic predisposition [55]. In the following sections we will review the process of plaque initiation and formation.

1.3.1

Extracellular Lipid Accumulation

Extracellular lipid accumulation is the earliest process in plaque formation. Accumulation of small low-density lipoprotein (LDL) particles within the arterial intima has been postulated as the initial insult. These lipoprotein particles appear to decorate the proteoglycan of the arterial intima and coalesce into aggregates. Detailed kinetic studies in rabbits have shown that prolonged residence of these lipoprotein particles characterizes sites of early lesion formation [56]. The binding of lipoproteins to the proteoglycan in the intima accounts for their prolonged residence time. Lipoprotein molecules bound to the proteoglycan are more susceptible to oxidative stress and chemical modification. These processes are considered to be important in the pathogenesis of early atherosclerosis [57]. Some studies suggest that permeability of the endothelial monolayer increases at sites of lesion predilection to LDL. Potential contributors to oxidative stress in the atheroma include nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH), oxidases expressed by the vascular cells and lipoxygenases expressed by infiltrating leukocytes or the enzyme myeloperoxidase [58].
1.3.2

**Leukocyte recruitment**

Leukocyte recruitment and accumulation plays a pivotal role in atherosclerotic lesion generation. The normal endothelial cell is able to resist adhesive interactions with leukocytes. Within the vessel wall, crystallization of cholesterol and oxidation of low-density lipoprotein (LDL) result in a cell-activating pro-inflammatory signal, which stimulate both innate and adaptive chronic inflammatory responses in the arterial intima [59]. The innate immune system initiates the recruitment of monocytes from blood and their differentiation to macrophages in the vessel wall. The adaptive immune response enhances and regulates inflammation in mature lesions. In inflamed tissue most recruitment of leukocytes occurs in the post-capillary venules and not within the arteries [59]. However after the binding of lipoprotein particles within the intima leukocytes can adhere to the endothelium and accumulate between endothelial cell junctions. They subsequently enter the intima where they accumulate and form foam cells [58]. Additionally T lymphocytes begin to accumulate as the expression of certain leukocyte adhesion molecules on the surface of the endothelial cell regulates the adherence of monocytes and T cells to the endothelium [60].

Activated T cells were detected in the human atherosclerotic plaque in 1986. This provided the first indication that the adaptive immune system is involved in atherosclerosis [61]. Notably, T cells are the second largest leukocyte population in the atherosclerotic aorta after monocytes and produce cytokines including interferon gamma (IFN-γ), interleukin (IL-2), and interleukin (IL-17) that mediate local inflammation [62]. The majority of T cells in atherosclerotic lesions are activated CD4+ effector and memory T cells. The type 1 T helper (Th1) subset of CD4+ T cells, which preferentially produces IFN-γ and tumor necrosis factor (TNF), is both the most pro-atherogenic and abundant T cell population in human atherosclerotic plaques [63]. Inhibition of IFN-γ either by deleting IFN-γ or its receptor decreases atherosclerosis and alters plaque antigen-specific immune responses while injection of recombinant IFN-γ increases lesion size [64].
Regulatory T cells (Tregs) act to suppress the potentially deleterious activities of Th cells. They play a protective role in the progression of atherosclerosis. Studies strongly suggest that the Treg mediated immune tolerance is hampered in atherosclerosis [65]. Patients with coronary artery disease have reduced numbers of Tregs in peripheral blood with reduced immune-suppressive capacity in vitro [66].

B-lymphocytes are found in plaque but at a much lower level than T cells and their role has been debated. Early splenectomy studies in mice suggested a protective role of B cells [67]. Splenectomized mice showed exacerbated atherosclerosis and adoptive transfer of splenic B cells from non-splenectomized mice rescued these mice from the pro-atherogenic effect. In addition, adoptive transfer of B cells from mice attenuated atherosclerosis in non-splenectomized mice [68]. Consistent with these findings mice transplanted with bone marrow from B cell deficient mice showed increased atherosclerosis [68].

B cells can be divided into two lineages, B-1 and B-2. B-1 cells secrete natural antibodies that are predominantly immunoglobulin M (IgM) and immunoglobulin A (IgA). The atheroprotective role of B cells may be related to observations that some natural antibodies are atheroprotective. In a mouse model lacking soluble IgM, these mice developed larger atherosclerotic lesions. B-2 cells produce IgG antibodies in a CD4 T cell-dependent manner after isotype switching and affinity maturation. B-2 cells may exacerbate atherosclerosis by producing pathogenic IgG antibodies. IgG antibodies reactive to oxidation specific epitopes have been detected in both in the plasma and vascular lesions of patients with CAD and animal models of atherosclerosis [69].

Oxidative modifications of LDL (oxLDL) induce inflammatory responses and are key to initiation of plaque formation and perpetuate chronic vascular inflammation. Besides their moderate pro-inflammatory effects modified lipoproteins induce cell-mediated and humoral responses. Both IgG and IgM autoantibodies to oxLDL can be readily detected in both humans and animal models of atherosclerosis, and in some studies antibody titers correlated with disease severity. Clinically, the concentration of IgM reactive to oxLDL has an inverse relation to carotid artery atherosclerosis [70]. A possible mechanism might be that oxLDL specific autoantibodies bind to oxLDL,
preventing it from being taken up by macrophages and consequently preventing foam cell formation [71].

The expression of certain leukocyte adhesion molecules on the surface of endothelial cells regulates the adherence of T lymphocytes and monocytes to the endothelium. Two broad categories of leukocyte adhesion molecules exist. The first of these is vascular cell adhesion molecule 1 (VCAM-1), a member of the immunoglobulin superfamily. It has been found to interact with receptor proteins like integrin, which is characteristically expressed by those classes of leukocytes that accumulate in atheroma. Studies in rabbits have shown expression of VCAM-1 on endothelial cells overlying very early atheromatous lesions. Selectins constitute the other broad category of leukocyte adhesion molecules. Specifically P selectin is expressed on the surface of endothelial cells overlying atheroma and has been shown to promote leukocyte recruitment and modulate thrombotic responses in vivo [72]. Additionally in two models of experimental atherosclerosis Woollard et al. showed that raising plasma concentrations of P-selectin to pathophysiological levels had an effect on plaque phenotype with reduced collagen content and increased inflammatory cellular content, typical of vulnerable plaque [72].

1.3.3

Lymphocyte Homing in Atherosclerosis

Once leukocytes are adherent to the endothelium they must receive a signal to then penetrate the arterial wall. Various concepts have been proposed. The current concept of direct migration involves the action of protein molecules known as chemokines. Certain groups of chemokines are key to recruiting the mononuclear cells characteristic of early atheroma. Monocyte chemoattractant protein 1 (MCP-1) is produced by the endothelium in response to oxidized lipoprotein. MCP-1 and selectively promotes directed migration or chemotaxis of monocytes. Studies conducted with genetically modified mice lacking MCP-1 or its receptor demonstrate delayed and attenuated
atheroma formation. Human atherosclerotic lesions express increased levels of MCP-1 as compared to uninvolved vessels [73].

B cells and T cells are present in normal (non-atherosclerotic) aortas, suggesting that constitutive homing mechanisms must exist to allow lymphocytes to traffic into the aortic wall. L-selectin a cell adhesion molecule found on the surface of lymphocytes was found to play a role in recruitment of lymphocytes to both normal and atherosclerotic aortas. To determine the role of L-selectin in lymphocyte homing into atherosclerotic aortas, L-selectin-/- lymphocytes were transferred to ApoE knockout mice. L-selectin-/- B cells displayed a 57% reduction in migration into atherosclerotic aortas of recipient mice in comparison with migration of wild type B lymphocytes. L-selectin-/- T cells demonstrated a similar 50% reduction in homing to atherosclerotic aortas. The partial reduction of lymphocyte homing in the absence of L-selectin suggests that migration of T and B-lymphocytes into atherosclerotic aorta is regulated by L-selectin and other adhesion molecules[74].

Other chemokines which play a role in T cell homing includes CCR7, a chemokine receptor expressed on activated naive T and B cells, central memory T cells, and some T regs [75]. It is known to regulate T cell homing to lymph nodes and Peyer’s patches. Both CCR7 and its two ligands, chemokine motif ligand CCL19 and CCL21, have been identified in mouse and human atherosclerotic lesions, suggesting a pathological role of CCR7 in atherosclerosis [76].

CXCR3 a chemokine receptor expressed on activated Th1 cells, B cells, natural killer cells, and endothelial cells is expressed in high levels in in human atheroma. The importance of CXCR3 for Th1 cell differentiation is highlighted by the discovery that CXCR3 is required for optimal generation of Th1 cells in vivo [77]. Recent studies with a CXCR3 antagonist significantly inhibited atherosclerotic lesion formation in the aortic valve leaflet area and the entire aorta in a mouse model [77].

Macrophage migration inhibitory factor (MIF) is an atypical chemokine involved in the pathogenesis of atherosclerosis [78]. Upon stimulation by oxLDL, endothelial cells and smooth muscle cells macrophages express MIF. The expression of MIF has been shown
to correlate with increased intima-media thickening and lipid deposition in the aorta of mice and in advanced human carotid artery plaques [78].

In summary, lymphocytes play a vital role in the pathogenesis of atherosclerosis. Chemokines, chemokine receptors and other factors (L-selectin, MIF) are critical for lymphocyte homing to the atherosclerotic aorta.

1.3.4

Foam Cell and fatty streak formation

Monocytes play a central role in atherogenesis and are rapidly attracted to sites of disturbed flow characterized by low-grade inflammation. The monocyte cells recruited to the arterial intima assimilate lipid and becomes foam cells or lipid-laden macrophages [79]. Disturbed flow promotes endothelial cell activation in part through repression of major anti adhesive and antithrombotic transcription factors. Disturbed flow and other proatherogenic factors down regulate the endothelial expression of negative guidance cues and facilitate chemokine-directed migration of monocytes and their infiltration within the intima.

Brief exposure of monocytes to oxLDL increases trimethylation of lysine 4 at histone 3 in promoter regions of several proinflammatory mediators and scavenger receptors leading to foam cell formation and long-term proinflammatory cytokine production [80]. Once macrophages have taken up residence in the intima and become foam cells they replicate. Recent findings suggest that cardiovascular risk factors other than elevated plasma cholesterol levels may significantly modulate macrophage foam cell formation. For example, the endogenous nucleoside adenosine, which is released extracellularly under stress conditions, profoundly affects foam cell formation through G-protein–coupled receptor A2A–dependent regulation of reverse cholesterol transport [81]. Also, increased expression of xanthine oxidoreductase, a key enzyme in the uric acid production pathway, is up regulated in many CVD settings and localizes to macrophages. Overexpression of xanthine oxidoreductase promotes foam cell
formation through up regulation of LDL and scavenger receptors [82]. Figure 1.3 further illustrates the initiation of the atherosclerotic lesion and fatty streak formation.

**Figure 1.3** - The Role of Lipids and Lipoproteins in Atherosclerosis Davies SS et al.[1]. Reproduced with permission.

1. The fatty streak phase of atherosclerosis begins with dysfunctional endothelial cells and the retention of Apolipoprotein B (Apo B) -containing lipoproteins in the subendothelial space.

2. Activated endothelial cells have increased expression of monocyte interaction/adhesion molecules (Selectins, VCAM-1) and chemoattractants (MCP-1) leading to attachment and transmigration of monocytes into the intimal space.

3. Activated endothelial cells promote the recruitment of mast cells, Treg cells and Th1 cells. The monocytes differentiate into macrophages and express receptors that mediate the internalization of VLDL, ApoE remnants, and modified LDL to become foam cells. Fatty streaks do not result in clinical complications and can even undergo regression. However, once smooth muscle cells infiltrate, and the lesions become more advanced, regression is less likely to occur [83].
1.3.5

**Smooth Muscle Cell Migration and Proliferation**

Atheroma initiation involves primarily endothelial dysfunction and recruitment and accumulation of leukocytes mediated by both the humoral and innate immune system as described previously. The evolution of atheroma into more complex plaques is in part a consequence of smooth muscle cell (SMC) migration and proliferation. Smooth muscle cells, which form non-striated muscle, are responsible for involuntary contraction but have an expanded and varied role in the context of plaque development.

Some smooth muscle cells are present within the intima in early life but those that accumulate in advancing atheroma arise from cells attracted to the intima from the media. These areas, known as intimal thickenings can be either eccentric or diffuse although these two types are often contiguous and can be difficult to distinguish from each other [84]. Eccentric intimal thickenings tend to be focal and involve up to half of the circumference of the arterial wall. They are found in conserved locations, including branch points and areas of turbulent blood flow. Eccentric intimal thickenings have been identified as early as 36 weeks’ gestation and are present in nearly all humans by 1 year of age [85]. Most interestingly regions of eccentric intimal thickening correlate with the locations at which advanced atherosclerotic lesions are later observed [86].

Unlike eccentric thickenings, diffuse intimal thickenings occur throughout the vasculature, particularly in older patients, suggesting that it may be part of the normal aging process [86]. Both types of intimal thickening consist almost exclusively of SMCs and the proteoglycans that they produce [86].

Smooth muscle cells in the normal arterial tunica media differ considerably from those in the intima of evolving plaque [87]. Whereas human medial smooth muscle cells predominantly express proteins involved in the contractile function of the cell such as smooth muscle myosin heavy chain or smooth muscle actin, smooth muscle cells found in the intima express lower levels of these proteins, have a higher proliferative index, and have a greater synthetic capacity for extracellular matrix, proteases, and cytokines.
These smooth muscle cells have a greater capacity to proliferate and can synthesize 25 to 46 times more collagen than regular smooth muscle cells [89]. In addition, they express a greater proportion of VLDL, LDL, and scavenger receptors allowing more efficient lipid uptake and foam cell formation.

Although the majority of foam cells in the atherosclerotic lesion are thought to be derived from macrophages, SMCs also give rise to a significant number of lipid laden cells [86]. Additionally although endothelial cells are thought to be the major cell type responsible for interacting with macrophages, SMCs are also capable of doing so. Electron microscopic and immunohistochemical analysis of human atherosclerotic plaques have shown that SMCs and macrophages are in direct contact [86]. This process is mediated by the expression of a variety of adhesion molecules on endothelial cells and smooth muscle cells, including intercellular adhesion molecule-1 (ICAM-1) and VCAM-1.

The role of the smooth muscle cell in plaque development also extends to its role in cytokine production. Cytokines attract and activate leukocytes, induce proliferation of SMCs, promote endothelial cell dysfunction and stimulate production of extracellular matrix components. Some of the more important cytokines include platelet derived growth factor, macrophage inhibitory factor, interferon gamma and MCP-1 [90]. SMCs are pivotal in the production of the extracellular matrix, which accumulates over the course of lesion progression.

SMCs are known to be the major producers of connective tissue both in the healthy and atherosclerotic vessel. Whereas most of the ECM within a healthy artery is type I and type III fibrillar collagen, atherosclerotic lesions tend to contain mostly proteoglycans with scattered type I collagen fibrils and fibronectin [91]. This transition can alter not only the architecture of the vessel, but also the lipid content and the proliferative index.

In summary smooth muscle cells are pivotal in pathological plaque development. Their role extends to being major producers of extracellular matrix within the vessel wall which affects the lipid content of the developing plaque and the proliferative index of the cells that are adherent to it. SMCs are also capable of functions typically attributed
to other cell types. Like macrophages, SMCs can express a variety of receptors for lipid uptake and can form foam-like cells, thereby participating in the early accumulation of plaque lipid. Like endothelial cells, SMCs express a variety of adhesion molecules to which monocytes and lymphocytes can adhere and migrate into the vessel wall. All contribute to the initiation and propagation of plaque formation.

1.3.6

Lesion Formation

In the preceding sections we have summarized mechanisms involved in plaque initiation and the histological and immunological basis for plaque formation. The focal nature of lesion formation has however provoked much debate with several theories proposed.

Blood borne risk factors such as lipoproteins bathe the endothelium yet atheroma forms focally. The location of sites of plaque formation at proximal portions of arteries after branch points suggest a hemodynamic basis for early lesion development [92]. Local factors play a major role in the regional localization of plaque formation. The flow of blood, by virtue of its viscosity, generates a frictional force per unit area on the vessel wall and endothelial surface known as shear stress [1]. Endothelial shear stress is the tangential stress derived from the friction of the flowing blood on the endothelial surface of the arterial wall and is expressed in units of force / unit area (dyne/cm$^2$). The magnitude of shear stress in the vasculature can be estimated by Poiseuille law, which states that shear stress is proportional to the flow velocity and inversely proportional to the third power of the internal radius [93]. Shear stress has been shown to vary from 1-6 dyne/cm$^2$ in the venous system to 10-70 dyne/cm$^2$ the arterial system.

Figure 1.4 shows the pattern of flow within a vessel. Fluid flow may be either laminar (Fig.1.4 A) or turbulent (Fig. 1.4B). Laminar flow maybe divided into undisturbed laminar flow or disturbed characterized by areas with reversed flow (i.e., flow separation, recirculation, and reattachment to forward flow) or circumferential swirling
In turbulent flow the velocity at any given point varies continuously over time, even though the overall flow is steady (Fig. 3 C). For a given geometry, whether the flow will be laminar or turbulent is determined by its Reynolds number (Re). The Re number is used to predict the flow in a given vessel taking into account both inertial forces and viscous forces. For low Re values, flow is laminar, whereas for high Re values (typically, above 2,000), flow is turbulent [95].

![Flow patterns in an artery](image)

**Figure 1.4** - Flow patterns in an artery- reproduced with permission from Chatzizisis, Y. S. *et al.* [3].

The pulsatile nature of the arterial blood flow in combination with the complex geometric configuration of the coronaries determines endothelial shear stress (ESS) patterns, which are characterized by direction and magnitude [96]. In relatively straight arterial segments, ESS is pulsatile and unidirectional with a magnitude that varies within a range of 15 to 70 dyne/cm² over the cardiac cycle. In contrast, in geometrically irregular regions, where disturbed laminar flow occurs, pulsatile flow generates low and/or oscillatory endothelial shear stress [3].

Low ESS refers to endothelial shear stress that is unidirectional at any given point but has a periodically fluctuating magnitude. The magnitude of local low ESS is critically
associated with the severity of atherosclerotic plaque characteristics [3]. Low ESS typically occurs at inner areas of curvature as well as upstream of a stenosis [3]. Oscillatory endothelial shear stress occurs primarily downstream of a stenosis, at the lateral walls of bifurcations, and in the vicinity of branch points.

1.3.7

**Role of low endothelial shear stress in atherosclerosis.**

The role of low endothelial shear stress in promotion of atherosclerosis is multifactorial as illustrated in figure 1.5 below. The endothelial surfaces are equipped with numerous mechanoreceptors which detect and respond to endothelial shear stress stimuli [97]. After activation of mechanoreceptors, a complex network of intracellular pathways is triggered, a process known as mechano-transduction. These pathways are activated simultaneously and cross-talk with each other; lead to phosphorylation of several transcription factors, which bind positive or negative shear stress responsive elements at promoters of mechanosensitive genes, inducing or suppressing their expression and modulating cellular function and morphology [98].

In arterial regions with non-disturbed flow, where endothelial shear stress varies, endothelial cells express various atheroprotective genes and suppress pro-atherogenic ones, leading eventually to stability in that region. In contrast, in regions with low and disturbed flow where low shear stress occurs, atheroprotective genes are suppressed and pro-atherogenic genes are upregulated thereby promoting atherosclerosis [99].

Low ESS promotes LDL uptake, synthesis, and permeability [100]. Low ESS causes a sustained endothelial activation of sterol regulatory elements binding proteins; a family of endoplasmic reticulum-bound transcription factors that upregulate the expression of genes encoding LDL receptor, cholesterol synthase, and fatty acid synthase. In the context of systemic hyperlipidemia, this effect results in an increased engagement and synthesis of LDL by the endothelial cells, ultimately promoting the subendothelial accumulation of LDL [101]. Once LDL particles are engulfed in the subendothelial layer, they are associated with intimal proteoglycans, become entrapped, and undergo
oxidative modification as described previously. Low ESS promotes production of reactive oxygen species into the intima and, eventually, oxidation of LDL, by enhancing gene expression and post-transcriptional activity of the major oxidative enzymes (nicotinamide adenine dinucleotide phosphate NADPH] oxidase and xanthine oxidase) at endothelial cell membranes [102]

The recruitment of circulating inflammatory cells (monocytes, T-lymphocytes, mast cells, eosinophils, dendritic cells) into the intima to scavenge oxidized LDL constitutes a major pathogenic component in the atherosclerotic process [103]. Low ESS plays a key role in the localized attachment and infiltration of these cells into the arterial wall through activation of certain transcription factors, notably nuclear factor-kappa and subsequent translocation to the nucleus [104]. In addition, a negative feedback mechanism occurs between nuclear factor kappa and NO. This reduces eNOS expression and subsequent NO production in low ESS regions and increases nuclear factor kappa activity [105].

Low ESS promotes vascular smooth muscle cell (VSMC) migration, differentiation, and proliferation. Low ESS also promotes endothelial gene and protein expression of potent VSMC mitogens, such as platelet-derived growth factor (PDGF)-A and -B isoforms and vascular endothelial growth factor (VEGF) as shown in figure 1.5.

Furthermore low and disturbed flow decreases the endothelial expression of plasminogen activator inhibitor (PAI)-1, an inhibitor of VSMC migration [106]. Ultimately, low ESS mediates over-expression of growth promoters and under-expression of growth inhibitors by endothelial cells and stimulates VSMCs to migrate from media to intima through a disrupted internal elastic lamina [107].

Within the intima VSMCs acquire a “synthetic” phenotype, producing collagen and other extracellular matrix proteins. Over time, VSMCs along with the fibroblasts create a fibrous cap around the lipid core isolating the thrombogenic lipid material from the circulating platelets. The fibrous cap along with the lipid core constitutes the early atherosclerotic plaque [108].
Extracellular degradation of the vascular wall and fibrous plaque is promoted by low shear stress. The extracellular matrix of the vascular wall and fibrous cap is composed of a complex mixture of collagen and elastin fibers embedded in a matrix of proteoglycans and glycosaminoglycans. Animal experiments have demonstrated that low ESS upregulates gene expression and activity of matrix metalloproteinases, which are the major proteases, associated with extracellular matrix degradation in the atherosclerotic plaques [109, 110]. Other major stimuli for the release of matrix metalloproteinases from endothelial cells and macrophages are pro-inflammatory cytokines like TNF and IL-1 [110]. In summary low ESS promotes plaque formation via several complex interrelated inflammatory signaling pathways.

1.3.8

Summary of plaque initiation

The initiation of plaque within arteries is a complex process with a delicate interplay of numerous factors, which begins in early life. Genetic and environmental factors determine the rate of progression and extent of plaque proliferation. Plaque development may be summarized by the accumulation of low-density lipoprotein within the intima, oxidation of LDL, recruitment of monocytes-macrophages, uptake of oxidized LDL by macrophage scavenger receptors and transformation of macrophages into foam cells, and formation of a fibrous cap containing smooth muscle cells, which permits stabilization of the plaque.

Low endothelial shear stress is pivotal in plaque development by inducing endothelial dysfunction by reducing nitric oxide and increasing endothelin production, provoking endothelial cell apoptosis and conformational changes of endothelial cells, inducing subendothelial accumulation of low-density lipoprotein and modulating the oxidative transformation of low-density lipoprotein cholesterol by stimulating the production of reactive oxygen species.
Figure 1.5 - Multifactorial role of Low Endothelial Stress on Atherosclerosis
Reproduced with permission from Chatzizisis et al. [3]
1.4

1.4.1

The evolution of atheroma

In the previous section we discussed the initiation of atheroma, its underlying etiology and role of innate and adaptive immunity in plaque development. In this section we will elaborate the mechanism of plaque accumulation, plaque rupture and erosion.

After the initial insult to the arterial wall and invasion of monocytes, formation of foam cells and the development of the fatty streak, a response is generated in the arterial wall. This is mediated by matrix protein synthesis and breakdown, which maintains the integrity of the arterial wall. The main mediators are the matrix-producing cells, primarily vascular smooth muscle cells and fibroblasts, and the matrix degrading proteases. Vascular smooth muscle cell function is regulated by equilibrium between growth promoting factors like endothelin 1 (ET-1) and vascular endothelial growth factor (VEGF) and growth inhibiting molecules like nitric oxide (NO) [111].

However low ESS has been shown to alter this homeostasis as low and disturbed flow attenuates extracellular matrix synthesis. Interferon gamma, a pro-inflammatory cytokine derived by the activated T-lymphocytes in response to low ESS, constitutes a potent inhibitor of collagen synthesis by VSMCs and promotes vascular smooth muscle cell degeneration [112]. The end result is arterial wall remodeling and formation of early fibroatheroma.

The vascular response to this early fibroatheroma determines the plaque’s evolution as illustrated in figure 1.6. If there is local compensatory expansive remodeling, then ESS normalizes, the hemodynamic stimulus for further plaque progression resolves and the early lesion evolves to a quiescent plaque with limited inflammation.

However, in the presence of certain local, systemic, and genetic factors, the local vascular wall might undergo excessive expansive remodeling. In this context the local
low ESS environment persists, promoting further plaque progression and vessel expansion. A self-perpetuating vicious cycle is established comprised of local low ESS, excessive expansive remodeling, and plaque inflammation that transform the early fibroatheroma to a thin cap fibroatheroma.

**Figure 1.6** - Effect of low endothelial shear stress on arterial remodeling Reproduced with permission from Chatzizisis et al. [2].

The artery responds to arterial plaque by expansive remodeling in an attempt to preserve lumen patency. This has been shown in various animal models and human studies [113, 114]. In a pioneering study of histologic sections of postmortem human left main coronary arteries, Glagov et al. detected a correlation between the lumen area and the area of the lesion. They demonstrated as plaque burden increased lumen area did as well for <40% stenosis. When the lumen area was plotted against the percent stenosis of the lumen area a biphasic relation was present. For lesions with <40% stenosis, there is no relation between lumen area and percent stenosis, whereas when
there is >40% stenosis, the lumen area decreases markedly as the stenosis increases [114].

Why the artery undergoes expansive remodeling and whether this becomes excessive or contained is not known. However, low ESS plays a significant role. In the setting of very low ESS, local lipid accumulation, inflammation, and oxidative stress are enhanced, thereby promoting intensive extracellular matrix degradation, culminating in excessive vascular wall expansion, which perpetuates the local low ESS environment [115].

Low ESS leads to the development of focal plaque and in the setting of a continued low ESS environment, the wall beneath the plaque becomes inflamed and acquires the enzymatic products that shift the extracellular matrix balance toward degradation. The internal elastic lamina undergoes severe fragmentation and there is extension of the atherosclerotic process to the media degrading the collagen and elastin fibers thereby promoting arterial expansion [111, 115].

When fibroproliferative processes predominate against inflammation and subsequent matrix breakdown, the atherosclerotic wall constricts and remolds inward leading to luminal narrowing. Approximately 20% of even minimally diseased human coronary arteries exhibit constrictive remodeling, suggesting that vascular constriction might occur as a direct response to plaque growth [115]. The plaque develops a fibrous cap in its early development and this becomes the dominant lesion. Fibrous cap formation develops from the migration and proliferation of vascular smooth muscle cells and from matrix deposition [116].

Initially, both high-risk and low-risk groups develop fatty streaks, but shortly after the start of development, fibrous plaques become dominant and progressively expand to cover about 20% to 46% of the coronary arterial surface. Together these plaques can reach a total extent of about 20% to 60% by age 60 years [4].

Advancing atheroma can occur in any age group. Thin-cap fibroatheroma (TCFA) develops when proteolytic enzyme activity continues unchecked and dissolves the fibrous tissue. This thin cap is susceptible to rupture, which exposes the thrombogenic
interior arterial wall and produces a thrombus that extends into the arterial lumen. This lesion usually is labeled a vulnerable plaque because of the risk of rupture and thrombosis [4].

These lesions can appear at any age but peak between 55 to 65 years, just before the peak incidences of myocardial infarction and stroke [117]. Distribution of TCFAs and ruptured plaques within coronary arteries is highly focal [118]. This contrasts with the extensive distribution of all earlier grades of atherosclerotic lesion in coronary arteries. Thin-cap atheroma and ruptured plaques, respectively, involve means of 1.6% and 1.2% of the epicardial portions of coronary arteries. Most of these lesions are limited to the proximal portions of major coronary arteries, and 92% cluster within adjacent 20-mm artery segments [119].

As lesions continue to develop one acellular, lipid-rich material accumulates in the intima [120]. Smaller lipid pools are initially seen beneath the layers of foam cells without gross disruption of the normal structure of the intima. In some lesions, isolated lipid pools grow into confluent necrotic cores (or synonymously lipid-rich core) through macrophage invasion [120]. This process irreversibly disrupts the normal structure of the intima. When a necrotic core is present the lesion is a fibroatheroma (Fig 5).

It is unclear why necrosis occurs in some but not all lesions. The causal factors are at least partly dissociated from those that cause xanthomas. For instance, men and women develop similar amounts of coronary xanthomas early in life, but men have many more progressive atherosclerotic lesions by the age of 30 [121].

Simultaneously angiogenesis occurs within plaques in conjunction with smooth muscle cell proliferation, endothelial cell migration and replication. This microcirculation most likely form in response to the overexpression of angiogenic peptides in the atheroma. Angiogenesis factors include basic fibroblast growth factors, vascular endothelial growth factor and placental growth factor [122]. Abundant microvessels in plaque provide a large surface area for leukocyte trafficking. The microvascular endothelium displays the mononuclear selective adhesion molecules such as VCAM-1 much more
prominently than the macrovascular endothelium overlying the plaque [122]. Plaque microvascularization allows plaque growth to overcome the diffusion limitations on oxygen and nutrient supply. Plaque microvessels can also become friable and prone to rupture, which promotes local accumulation of smooth muscle cells and further matrix accumulation [123].

Finally, plaque mineralization is pivotal in plaque evolution. Atherosclerotic calcification is an organized, regulated process similar to bone formation that occurs only when other aspects of atherosclerosis are present [116]. Proteins like osteocalcin, which are actively involved in the transport of calcium out of vessel walls, are suspected to have key roles in the pathogenesis of coronary calcification. Osteopontin and its mRNA, known to be involved in bone mineralization, have been identified in calcified atherosclerotic lesions. Calcified human atherosclerotic plaque also contains mRNA for bone morphogenetic protein-2a, a potent factor for osteoblastic differentiation, and cells that are capable of osteoblastic differentiation. These cells may be those from which vascular calcifying cells are derived [124].
1.4.2

Complex Lesion formation and Classification

The initiation and evolution of atheroma as described in the previous sections lasts many years, during which the patient has no symptoms. Once plaque burden exceeds the capacity of the artery to remodel outward, the arterial lumen is encroached upon. As this process progresses and partially occludes blood flow patients begin to experience symptoms. Generally this occurs when plaque accumulation has narrowed the lumen by greater than 60%.

During the chronic asymptomatic phase growth probably occurs discontinuously with periods of relative quiescence. The net result is formation of complex lesions. Many thin fibrous cap ruptures are clinically silent. The plaques heal by forming fibrous tissue matrices of cells, collagen fibers, and extracellular space but may rupture again with thrombus formation [125]. These cyclic changes of rupture, thrombosis, and healing may recur resulting in multiple layers of healed tissue [125]. Table 1 below classifies lesion formation by histopathological appearance and clinical consequence whilst figure 1.7 illustrates the histological changes of plaque development.
**Table 1** - Histopathological classification of lesion formation (Adapted with permission from Insul W et al. [4]).

<table>
<thead>
<tr>
<th>Lesion Name</th>
<th>Description by Histopathology</th>
<th>Thrombosis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nonatherosclerotic intimal Lesions</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Intimal thickening</td>
<td>The normal accumulation of SMCs in the intima with the absence of lipid or macrophage foam cells</td>
<td>No</td>
</tr>
<tr>
<td>Thrombus is absent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Intimal xanthoma or fatty Streaks</td>
<td>Subendothelial accumulation of foam cells in intima without necrotic core or fibrous cap</td>
<td>No</td>
</tr>
<tr>
<td><strong>Progressive atherosclerotic lesions</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1a. Pathologic intimal Thickening</td>
<td>SMCs in a proteoglycan-rich matrix with areas of extracellular lipid accumulation without necrosis</td>
<td>No</td>
</tr>
<tr>
<td>1b. With erosion</td>
<td>Luminal thrombosis</td>
<td>Yes, mural</td>
</tr>
<tr>
<td>2a. Fibrous cap atheroma</td>
<td>Well-formed necrotic core with overlying fibrous cap</td>
<td>No</td>
</tr>
<tr>
<td>2b. With erosion</td>
<td>Luminal thrombosis; plaque same as above, no communication of thrombus with necrotic core</td>
<td>Thrombus most often mural and not occlusive</td>
</tr>
<tr>
<td>3. TCFA</td>
<td>A thin fibrous cap infiltrated with macrophages with intraplaque lymphocytes, rare SMCs, and an underlying necrotic core</td>
<td>No</td>
</tr>
<tr>
<td>a. With rupture</td>
<td>Fibroatheroma with cap disruption; luminal thrombus communicates with underlying necrotic core</td>
<td>Yes</td>
</tr>
<tr>
<td>4. Calcified nodule</td>
<td>Eruptive nodular calcification with underlying fibrocalcific plaque.</td>
<td>No</td>
</tr>
<tr>
<td>5. Fibrocalcific plaque</td>
<td>Collagen-rich plaque usually with significant stenosis; contains large areas of calcification with few inflammatory cells; necrotic core may be present</td>
<td>NO</td>
</tr>
</tbody>
</table>
Figure 1.7 - Lesion types of atherosclerosis and a proposed sequence of their development. Adapted with permission from Insul W et al. [4].

A. Adaptive intimal thickening characterized by smooth muscle cell accumulation within the intima.
B. Intimal xanthoma corresponding to the accumulation of foam cell macrophages within the intima.
C. Pathological intimal thickening denotes the accumulation of extracellular lipid pools in the absence of apparent necrosis.
D. Fibroatheroma indicating the presence of a necrotic core. The necrotic core and surrounding tissue may eventually be calcified, which forms fibrocalcific plaque.
E. Fibrocalcific plaque
1.5

Plaque Vulnerability

Atherosclerotic plaque rupture and subsequent thrombosis remains the cause of the majority of ACS and sudden coronary death. Patients with ACS present with unstable angina, AMI with or without ST elevation, and sudden coronary death. Most ACS are believed to result from luminal thrombosis, which from post-mortem studies have been described to arise from three distinct morphologic entities: rupture, erosion, and calcified nodules [120]. The underlying mechanism of sudden coronary death from thrombi occurs most frequently due to plaque rupture (55–65%), then erosion (30–35%), and least frequent due to calcified nodules (2–7%) [120].

1.5.1

Plaque rupture

Atherosclerotic plaque lies dormant for many years and may never result in clinical consequence. Damage to the plaque surface however can result in thrombotic occlusion of the artery. Key histopathological findings associated with regions of fatal disruption include a thin fibrous cap (<50–60 micrometers), increased signs of inflammatory activity and high amounts of proteolytic enzymes [126]. In eccentric plaque, the weakest spot is often the cap margin or shoulder region, and only extremely thin fibrous caps are at risk of rupture [127].

Assessed by microscopic examination in an autopsy study of sudden cardiac death, the average thickness of ruptured cap was found to be only 23μm and 95% of ruptured fibrous caps were below 65μm. Thinning of the fibrous cap occurs due to various mechanisms. The first is gradual loss of smooth muscle cells from the fibrous cap. Ruptured caps contain fewer smooth muscle cells and less collagen than intact caps [128]. Inflammatory stimuli such as local immune reactions might activate macrophages, mast cells and T cells to release cytokines that inhibit cap formation and
proteases that digest fibrous components of the cap. Matrix metalloproteinases and cysteine proteases in the plaque have the ability to disrupt collagen within culprit plaques [129]. Postmortem examination of ruptured caps have shown that they are heavily infiltrated with macrophages, foam cells which secrete various proteolytic enzymes such as plasminogen activators, cathepsins, and matrix metalloproteinases [130]. Matrix metalloproteinases are capable of degrading virtually all components of the extracellular matrix.

Other destabilizing factors include immune cytokines (Fig. 1.8). Interferon-C, a pro-inflammatory, macrophage-activating cytokine produced by Th1 cells and NK cells, inhibits collagen fiber formation, causing plaques to adopt a vulnerable phenotype with reduced collagen content [131]. This is due to the action of interferon-gamma, as it both inhibits smooth muscle differentiation, procollagen-I gene expression and the collagen crosslinking enzyme, lysyl oxidase [132].

Additionally smooth muscle cells are usually absent at the fissures and erosions that trigger thrombosis by exposing thrombogenic material inside the plaque, such as phospholipids, tissue factor and matrix molecules, to platelets and coagulation factors [133]. Platelet aggregates precipitating on these exposed surfaces are stabilized by fibrin networks. Tissue factor, expressed by macrophages and by vascular smooth muscle cells in the atherosclerotic plaque, can initiate the blood coagulation cascade that leads to fibrin formation [134].

We know that atheroma is present in coronary arteries from the early adult years. However these plaques are much less likely to rupture early in life, which suggest that progression to plaque rupture is a slow smoldering process but in some cases, a temporary increase in emotional or physical stress provides the final trigger [135]. Recognized triggers include physical and sexual activity, anger, anxiety, work stress, earthquakes, war and terror attacks, temperature changes, infections, and cocaine use [135]. Also simple daily activities or the circadian rhythm of biological pathways may determine the onset of ACS, which are most frequent in the morning [136]. Sympathetic nervous system activation with increased heart rate and blood pressure may trigger
plaque rupture or increased coagulability and platelet reactivity accentuating the thrombotic response [137].

Mechanisms that limit extent of plaque disruption and instability are summarized in Fig. 1.8. The action of Th1 cells is counterbalanced by Treg cells producing TGF-β [138]. This cytokine has a direct fibrogenic action on smooth muscle cells and fibroblasts. In addition, it inhibits Th1 and macrophage activity, leading to reduced plaque inflammation. Treg also enhances the catabolism of very low-density lipoproteins, resulting in reduced plasma lipid levels.

A type of T cell, the Th17 cell type, is involved in wound healing and exerts powerful fibrogenic activity [139]. Th17 cells activated in the context of atherosclerosis promote the formation of thick collagen fibers that can withstand the mechanical assault on the plaque exerted by hemodynamic forces [140]. This is due to the capacity of the cytokine secreted by Th17 cells i.e. IL-17A, to promote procollagen expression [140].

In summary specific features of plaque morphology are associated with high risk of rupture. These include the presence of thrombus, a large necrotic core, thin fibrous cap covering the necrotic core, presence of intraplaque hemorrhage, adventitial or perivascular inflammation and spotty calcification.
1. Activated macrophages and Th1 cells produce metalloproteinases and cytokines that hamper the tensile strength of the collagen cap. Several pro-inflammatory cytokines and tumour necrosis factor (TNF), as well as CD40/CD40L cell surface receptors of the TNF superfamily promote an inflammatory state that enhance cell death and prothrombotic activity in the plaque.

2. When the cap can no longer withstand the mechanical force of the blood pressure, superficial fissures are formed in the plaque, which exposes the core to thrombogenic material. This triggers platelet activation and thrombus formation.

3. Counteracting all these pro-inflammatory and tissue-destructive signals, subsets of macrophages and T cells produce anti-inflammatory molecules. Amongst them are transforming growth factor-β (TGF-β) and interleukin-10 (IL-10) that inhibit inflammation. TGF-β has fibrogenic properties that it shares with IL-17 produced by Th17 cells.
1.5.2

Plaque Erosion

Plaque erosion is the initiating insult in myocardial infarction in approximately 30% of cases [120]. Thrombus formation without overt fibrous cap rupture was described by van der Wal et al. in 1994 [126] and the group of Virmani et al. detailed loss of the endothelial layer underlying such thrombi [141]. Durand et al. confirmed that endothelial loss could be sufficient to precipitate thrombosis by inducing apoptosis with intravascular Staurosporine and in doing so also implicated apoptosis of endothelial cells as a potential cause [142].

Despite extensive research thrombus formation without rupture is an unresolved question in atherosclerosis research. In postmortem studies the surface endothelium under the thrombus of plaque erosion is usually absent. Plaques are often scarcely calcified, often associated with negative remodeling, and less inflamed than ruptured plaques [126]. There have been some reports of focused inflammation immediately below the superimposed thrombus but this has only been reported in a few studies and not consistently [120].

Vasospasm has been suggested as a cause of endothelial damage and subsequent thrombosis [6]. Plaque erosion lesions typically show intact internal and external elastic lamina and a well-developed media with contractile smooth muscle cells unlike lesions of plaque rupture where the intact internal lamina is often disrupted and the underlying media thin and disorganized [143].

Plaques that erode are morphologically different to those that rupture. Eroded plaques are more common in single compared to double vessel disease (56 % vs 26 %), are more fibrous, have an intact plaque cap and either absent or deeply seated necrotic cores [144]. In contrast to ruptured plaques, which are typified by high numbers of activated macrophages and T cells, there is a predominance of smooth muscle cells and these plaques are proteoglycan rich [144].
More recent studies implicate innate immunity in this process (Fig.1.9). Endothelial cells overlying atherosclerotic lesions abundantly express the pattern recognition receptor, toll-like receptor-2 (TLR2) [145]. Ligation of this receptor results in endothelial apoptosis in a process accelerated by polymorphonuclear leucocytes, a cell type found at sites of fatal plaque erosion [146]. TLR2 ligands include the extracellular molecule hyaluronan as well as components of Gram-positive bacteria; therefore, endogenous as well as infectious factors may operate to promote atherothrombosis through this mechanism [146]. Figure 1.9 summarizes the mechanism of plaque erosion.

**Figure 1.9-** Mechanism of Plaque Erosion. Reproduced with permission from G. K. Hansson *et al*. [5].

(PMN- Polymorphonuclear cell, PAD4, Peptide arginine deaminase-4- component of NET, TLR- Toll like receptor, G+ - Gram positive toxins, SM- Smooth muscle, E- Endothelium, MPO- Myeloperxidase)

1. Endothelial cells of atherosclerotic plaques commonly express Toll-like receptor _2_ (TLR2) that can ligate both Gram-positive toxins (G+) of bacterial pathogens and hyaluronan released from the extracellular matrix. TLR2 ligation can trigger endothelial dysfunction.

2. Neutrophils attack the endothelium. As a result, endothelial cells may detach, exposing the subendothelial matrix with its thrombogenic components.

3. Activated neutrophils contribute to a prothrombotic state by releasing a set of proteases including neutrophil elastase and by forming neutrophil extracellular traps (NETs) that can damage endothelial cells, trap leucocytes and enhance thrombosis.
1.5.3

**Calcified nodule**

Calcified nodule, the least frequent cause of coronary thrombi, is a lesion that occurs in highly calcified arteries. It consists of areas of fragmented calcified sheets that form small-calcified nodules that are surrounded by fibrin and have a small luminal thrombus [4]. The eruptive calcified nodules are usually eccentric, protruding into the lumen, and there is an absence of endothelium and collagen above the nodules of calcium with a platelet-rich thrombus which is usually non-occlusive [120].

The mechanisms of nodular calcification remain unknown. Histologically fibrin is often present between the calcified spicules, along with rare osteoclasts and inflammatory cells, indicating that at site of calcified sheet cracking and breaks in the calcified region with intraplaque haemorrhage and cell transformation may be involved [120]. Lesions with nodular calcification are more common in highly calcified tortuous arteries, in older individuals, in males, and are preferentially found in the middle right coronary or the left anterior descending coronary arteries [55].
1.6

**Summary of plaque development and rupture.**

Chest pain presentations to the accident and emergency department continue to increase, both nationally and internationally. A biomarker or panel of biomarkers that identifies patients at higher risk thereby expediting their evaluation and treatment would be of significant benefit. Plaque accumulation and rupture are the predominant mechanism by which ACS and AMI occur.

Figure 1.10 summarizes the main concepts in atherosclerosis. Atherosclerosis is a complex process, which arises from, the interplay of local factors, such as vascular shear stress patterns and endothelial injury: in addition to systemic mediators, that include circulating lipoproteins, hyperglycemia, environmental exposures and genetic predispositions. Fundamentally, it is a chronic inflammatory disorder governed by cellular and humoral components of the immune system. Its process can be subdivided into overlapping stages, which may remain clinically silent, or progress to the development of acute or chronic symptoms.

By early adulthood, there is near universal development of adaptive intimal thickening whereby vascular smooth muscle cells accumulate within the superficial layers of the vessel wall at sites of low endothelial shear stress. Concomitantly these regions retain LDL that bind to subendothelial proteoglycans. Enzymatic reactions induce oxidization of LDL and drive endothelial and VSMC expression of cellular adhesion molecules that promote migration and differentiation of circulating monocytes.

The resultant scavenger macrophages phagocytose lipids and become foam cells within the developing fatty streak or xanthoma. Many of these lesions remain dormant or regress while others develop an acellular lipid pool i.e. pathological intimal thickening. In some cases a fibroatheroma forms as persistent apoptosis and necrosis of macrophages and VSMCs generates a necrotic core overlain with a collagen-rich surface layer. Atherosclerotic progression reflects the relative balance of certain cellular
subtypes of the innate and adaptive immune systems, predominantly macrophages and T helper cells, which act via secreted cytokines, macrophages and Th1 lymphocytes to promote chronic inflammation.

The majority of lesions display a stable phenotype as plaque becomes calcific and forms an overlying fibrous layer. However ongoing expansion of the necrotic core and degradation of collagen may result in development of high-risk atheroma. Plaque rupture is characterized by thin cap fibroadenoma, large plaque volume and necrotic core, positive remodeling, peripheral neovascularization, microcalcification and intraplaque haemorrhage. Plaque erosion accounts for 20-30% of acute myocardial infarctions and is more common in single compared to double vessel disease. These lesions are more fibrous, have an intact plaque cap, deeply-seated necrotic cores and a predominance of smooth muscle cells. Calcified nodules are the least frequent cause of thrombosis and occur in older individuals with heavily calcified and tortious arteries.
Figure 1.10- Flow chart of the general concepts of the development of atherosclerosis.

Adapted with permission from Virmani R et al. (103)
1.7

Measuring total plaque burden

The GENSINI score is formed by visually estimating plaque stenosis in different coronary arteries, which is given a number 1-32 dependent on the degree of stenosis (Fig 1.11). This is then multiplied by a factor dependent on plaque location to generate a total score. The Syntax score was developed in 2005 and is an estimate of disease severity. The score is the sum of the points assigned to each individual lesion identified in the coronary tree with >50% diameter narrowing in vessels >1.5mm in diameter. Bifurcation lesions, long lesions, total occlusions and aorto-ostial lesions are weighted differently.

Fig. 1.11 – How to calculate total GENSINI score- Reproduced with permission from Sullivan R et al. American Heart Journal 1990:119:1262
Detection of unstable coronary plaque

Chest pain assessment starts with a history and examination. Invasive and noninvasive imaging is employed to confirm ischemia and identifying features of plaque instability.

Coronary angiography depicts arteries as a planar silhouette of the contrast-filled lumen. It is limited in its ability to quantify the extent or distribution of atherosclerosis and changes that occur in the vessel wall with time. Whilst there is a correlation between the degree of stenosis at angiography and risk of future cardiac events, it is the composition of the plaque that determines the risk of future instability [147]. Intravascular ultrasonography (IVUS) and Optical Coherence Tomography (OCT) are now standard adjunctive invasive tools in catheterization suite to assess plaque. Thermography, spectroscopy and intravascular MRI all experimental techniques in development.

1.7.1

Intravascular Ultrasound (IVUS)

IVUS is a catheter-based imaging modality that provides high resolution cross-sectional images of the coronary arterial walls [148]. It consists of a coronary catheter incorporating a miniaturized ultrasound probe at the distal end and a console into which the proximal end attaches. High ultrasound frequencies are used to image the arteries typically in the region of 20-50Hz. In non-diseased arteries IVUS differentiates the vessel wall into three components, the intima composed of endothelial cells, sub adjacent smooth muscle cells and the extracellular matrix that is 150-200μm in diameter and partitioned from the media by the internal elastic media [149]. The media composed of collagen and smooth muscle cells is 100 to 350μm in diameter and the adventitia which contain fibrous tissue and is 300-500μm in diameter. The differences in acoustic impedance generate a three layered appearance of the vessel wall Fig. 1.11 below.
Figure 1.11 - Normal arterial anatomy adapted with permission from Nissen et al: Intravascular Ultrasound - Novel Pathophysiological Insights and Current Clinical Applications.

IVUS assessment of plaque has shown certain features that are associated with ACS. Firstly positive remodeling of the vessel has been associated with ACS and angiographically complex lesions [150]. Stable plaques are more calcified than unstable plaques [151].

Plaque morphology by ultrasound is often characterized by the intensity of the signal. Soft echoes (hypoechoic), very high-intensity reflectors that create distal shadowing, and echoes of intermediate intensity (high echoic) are features that correspond to tissue, calcium, and fibrosis, respectively. Echolucent or signal-free zones have been found to represent lipidic tissue accumulation. In human cadaver coronary arteries, plaque with ultrasound attenuation contained fibrolipid tissue and necrotic core features of unstable plaque. Other IVUS features associated with plaque vulnerability may include eccentric pattern, presence of an echolucent zone, and presence of thrombi [152]. Stone et al. performed a seminal analysis of IVUS characteristics of plaques associated with recurrent cardiac events and found that TCFA, mean luminal area
<4mm² and plaque burden >70% were predictive of further cardiac events [153]. At three year follow up 20% of patients had recurrent ischemic events of which 50% were in lesions characterized as having mild degrees of stenosis. This exemplifies the limitations of conventional angiography in determining which plaque is unstable.

The major limitation of IVUS is its spatial resolution, at frequencies in the 20–40-MHz range IVUS has an axial resolution of 100–200μm and a lateral resolution of 250μm [7]. Although it can visualize deep structures, IVUS is not a suitable imaging modality for detecting the very thin fibrous cap that is one of the main components of vulnerable plaques. Additionally IVUS is unable to accurately differentiate plaque composition since atherosclerotic plaque can comprise complex lipids, fibrotic tissue, calcified nodules and extracellular matrix.

1.7.2

**Optical Coherence Tomography (OCT)**

OCT uses near-infrared light to generate cross sectional images of the arterial wall. It does this by measuring the echo time delay and intensity of light that is reflected or backscattered from the arterial wall [148]. Compared to IVUS, OCT utilizes much higher bandwidths resulting in much increased resolution. The resolution is as high as 10μm that is twice the size of a red blood cell [8].

Similar to IVUS it utilizes a specially designed coronary catheter attached to a console. Unlike IVUS however near infrared light is unable to penetrate red blood cells so images are acquired after a rapid injection of contrast. OCT measures the intensity of light returning from within a tissue. Tissue with a higher optical index of refraction show stronger optical scattering and therefore a stronger OCT signal [148]. Normal intima is represented by the bright signal of collagen fibers. The media gives a dark, homogeneous signal because it has less fiber and more smooth muscle cells with the adventitia also represented by the bright signal of collagen fiber [8].
Like IVUS, OCT studies have identified that TCFA are more common in patients with acute and unstable lesions than those with stable angina [154, 155]. In patients who have undergone plaque rupture OCT characterization is excellent. Coronary thrombus can be visualized along with the thin fibrous cap.

As discussed in previous sections vulnerable plaques are typified by a thin fibrous cap, large lipid core (more than 40% of the overall plaque volume) and increased infiltration of macrophages into the plaque cap [156]. OCT is the only modality that allows for visualization of the thin fibrous cap, which correlate very well with histological findings in patients presenting with ACS [157] (Fig 1.13).

Patients with ACS are more likely to have secondary non-culprit plaque rupture identified by OCT [158]. This pan coronary vulnerability identifies patients at higher risk of future events and the need for intensified treatment and close follow up. Plaque erosion and calcified nodule can also be readily identified by OCT. In a cohort of patients with STEMI (n =30), plaque erosions were more often found by OCT than by IVUS (23% vs. 3%). Jia et al. in a retrospective review of their registry found that in plaque erosion there was a lower frequency of lipid plaque, thicker fibrous cap and smaller lipid arc than plaque rupture [159]. Patients with plaque erosion were younger, had less severe stenosis, and less frequently presented with STEMI than those with plaque rupture. Calcified nodule was the least frequent cause of ACS in this study but was also easily identified with OCT.
Figure 1.12- Appearance of normal artery using OCT from Intravascular OCT: basics and applications, Abtahian et al.– reproduced with permission [160].

OCT catheter (yellow arrow) within a coronary artery reveals the normal three layers of the coronary artery: intima (white arrow), media (red arrow) and adventitia (blue arrow).

OCT does have its limitations. OCT is unable to detect vessel remodeling, a principal feature of plaque vulnerability, due to its poor tissue penetrance. Additionally lipidic tissue appears as low signal intensity regions with diffuse borders because of the strong optical absorption of lipids at light wavelengths around 1,000 nm [161]. Thrombus has a similar appearance.

Both OCT and IVUS are complementary imaging techniques which have added significantly to imaging of vulnerable plaque. However, both techniques are invasive, time consuming and costly. A biomarker or panel of biomarkers assayed at the time of presentation with chest pain would that identified vulnerable plaque, would complement these invasive imaging techniques.
Cardiac coronary CT (CTCA)

CCTA is now widely used as a less invasive imaging modality to diagnose CAD [162]. Furthermore, CCTA allows evaluation of coronary plaque composition [163]. Classification of coronary plaque by CCTA has important clinical implications and may be associated with myocardial ischemia and predict of adverse cardiac events [164].

The ESC guidelines recommend CTCA to evaluate chest pain patients with low pre test likelihood of obstructive CAD [165]. CTCA is performed using contrast-enhanced imaging and can offer detailed visualization and characterization of coronary
atherosclerotic plaque, with good agreement between CTCA and IVUS [166]. CTCA gives a total calcium score measured by the Agatston score. The total calcium score (CAC) is a surrogate marker of total plaque burden. A CAC score >300 Agatston units is associated with a 4-fold higher risk of cardiovascular events than a CAC score of zero, which is itself associated with an excellent prognosis [167]. Indeed, in patients with a score of zero the annual mortality rate for up to 15 years in asymptomatic and otherwise low- or intermediate-risk patients is <1% [168].

CTCA demonstrates excellent diagnostic accuracy for the detection of obstructive luminal stenosis compared with invasive coronary angiography. A recent meta-analysis described a sensitivity of 93% and specificity of 96% for CTCA on a per-segment basis to detect stenosis of >50%. Additionally CTCA has demonstrated its ability to identify a range of adverse plaque characteristics, including positive remodeling, necrotic core, napkin ring sign, and spotty calcification [169].

CTCA as a non-invasive modality in the assessment of chest pain is mostly confined to low risk patients. The SCOT-HEART investigators extended this to low and intermediate risk patients referred with stable angina. They compared standard clinical care to use of CTCA and found that at 6 weeks, CTCA reclassified the diagnosis of coronary heart disease in 558 (27%) patients and the diagnosis of angina due to coronary heart disease in 481 (23%) patients (standard care 22 [1%] and 23 [1%]; p<0.0001). This changed planned investigations and treatments but did not affect 6-week symptom severity or subsequent hospital chest pain admission. After 1.7 years, CTCA was associated with a 38% reduction in fatal and non-fatal myocardial infarction but this was not significant when compared to standard clinical care [170].

Hoffman et al. randomized all comers with suspected ACS and no diagnostic ECG or HsT troponin changes to CTCA or standard care. They found that this improved the efficiency of clinical decision making when compared to standard evaluation in the emergency department, but resulted in an increase in downstream testing and radiation exposure with no decrease in overall cost of care [171]. Although these and numerous other studies have confirmed the utility of CTCA in the assessment of low risk chest pain, in patients with a high pretest probability of obstructive CAD, CTCA is
not recommended. Ongoing research with the addition of routine stress perfusion imaging to CTCA may change its clinical utility.

1.7.4

**Positron emission tomography CT (PETCT)**

This nuclear medicine method of plaque assessment detects gamma rays emitted directly by a radionuclide tracer administered at the time of testing. It attempts to overcome the limitations of anatomical imaging by employing molecular imaging techniques. Molecular markers for atheromatous plaque can provide information regarding plaque vulnerability and progression.

Several biomolecules have been proposed as targets for atherosclerotic plaque such as F-18 fluorodeoxyglucose (FDG) and more recently 18F-sodium fluoride (18F). FDG is a well-known marker for glucose metabolism. Because of macrophage accumulation in the atheromatous plaque, plaque has high FDG uptake, especially if is vulnerable [172].

On the other hand 18F is attracted to areas of microcalcification within plaque. Studies have shown that microcalcification in the thin fibrous cap increases risk of plaque rupture, and subsequent stress-related microfractures result in acute thrombosis [173]. Calcification in patients with atheroma is a bi-phasic reaction. The early phase is not visible on conventional imaging, but is associated with plaque instability. Joshi et al. in their landmark study compared 18F uptake to FDG uptake in patients presenting with ACS and stable angina. The highest uptake of 18F was seen in culprit plaque whereas FDG uptake was commonly obscured by myocardial uptake and there were no differences between culprit and non-culprit plaques [174]. Use of PETCT in ACS will continue to evolve and ongoing research will determine its ultimate role in ischemic chest pain presentations.
1.8

Assessment of the patient with chest pain.

1.8.1

Patient history

The approach to evaluating a patient presenting acutely to the emergency department or electively in an outpatient setting with possible cardiac chest pain commences with a detailed history and targeted physical examination. Varying patient groups present with different symptoms but the majority of patients will have similar stories albeit told in varying manners. Elective ambulatory encounters allow more time for the development of a comprehensive assessment whereas the interaction in an emergency setting is targeted to achieve a differential diagnosis in timely fashion.

The history is an invaluable source of information and often will provide clues linking disparate aspects of the patient’s presentation. It offers an insight into their intelligence, comprehension, motivation, fear and prejudices. The interview can reveal genetic influences and the impact of other medical conditions on their current presentation.

Chest pain is the most frequent symptom in patients presenting with ischaemic heart disease. The first decision point for most physicians is whether or not the chest pain is caused by coronary ischaemia [175]. Questioning should address symptom location, onset, time course, character, severity, radiation, alleviating and exacerbating factors, history of similar episodes, and associated symptoms, including diaphoresis, shortness of breath, and nausea or vomiting. Angina has been described as deep, poorly localized chest or arm discomfort (pain or pressure) that is reproducibly associated with physical exertion or emotional stress and is relieved promptly with rest or sublingual nitroglycerin [176].

Unstable angina is defined as angina at rest, new-onset angina, or angina that has become more severe or longer in duration [177]. However not all patients presenting
with ACS have chest pain and predictability may be influenced by patient description of their symptoms. Patients often do not use the term pain to describe their symptoms, but frequently use other terms like discomfort, tightness, squeezing, or indigestion [177].

Fanaroff et al. performed a meta-analysis analyzing factors in history and examination which were most likely to be predictive of underlying CAD. When they evaluated risk factors for CAD a family history of CAD, history of tobacco use and obesity were not strong predictors of an ACS diagnosis [178]. Findings that support an ACS diagnosis (likelihood ratio (LR) +2.0) were history of abnormal prior stress test (specificity 96%; LR, 3.1) and peripheral arterial disease (specificity 97%; LR, 2.7). Some chest pain characteristics decrease the likelihood of cardiac chest pain, for example pleuritic chest pain, sharp or stabbing pain, pain reproduced by palpation and response to nitroglycerin all showed LR of < 1 [178].

Certain characteristics of chest pain have been shown to increase the likelihood of ACS in patients with acute chest pain and include pain that radiates to both arms (specificity of 96% and LR 2.6), pain similar to prior ischaemia (specificity 79%; LR, 2.2, and change in pain pattern over the prior 24 hours (specificity 86%; LR, 2.0) [178].

Whilst chest pain in the most common presenting complaint in patients with cardiac ischaemia other angina equivalents warrant attention. Indigestion, dyspnoea, belching and syncope command the clinician’s attention when ACS is being considered. Dyspnoea may occur with exertion, recumbence (orthopnea) or even with standing (platypnoea). Paroxysmal nocturnal dyspnoea of cardiac origin usually occurs greater than two hours into sleep and compels the patient to sit upright to relieve their symptoms, which usually occur over a few minutes.

Syncope although an infrequent presentation of cardiac ischaemia is well described with varying mechanisms proposed. Severe ischaemia rarely produces the depressed cardiac output and hypotension required to result in syncope. More likely, however, ischaemia may cause syncope indirectly through the production of arrhythmias or activation of certain reflexes [179]. Inferior wall myocardial ischemia may trigger the
Bezold–Jarisch reflex, resulting in hypotension and bradycardia, which could precipitate syncope. However, such a high-grade right coronary artery occlusion would be rare in the absence of typical ischaemic symptoms. Another plausible pathophysiological link between ischaemia and syncope may be related to ischaemia-triggered ventricular arrhythmias or atrioventricular block.

Additionally atypical presentations of ACS are more prevalent in certain patient populations such as the elderly, women and patients with diabetes. Peculiarly, the characteristic expressions of ACS seen in younger persons are less typical and may even become non-existent in the elderly. Thus, in the elderly, ACS may go unnoticed, unless the patient is aware that sudden shortness of breath, sudden fatigue, discomfort that may be confined to the abdomen more than to the chest, profound sweating, irregular heartbeat, or even fainting (syncope) may herald the development of ACS. Savonitto et al. demonstrated that an early aggressive approach (coronary angiography, and when indicated, revascularization within 72 hours) resulted in a significant reduction in the primary endpoint (the composite of death, MI, disabling stroke, and repeated hospital stay for cardiovascular causes or severe bleeding within 1 year) in elderly patients with non-ST-segment elevation ACS [180].

The presenting symptoms of a diabetic with cardiac ischemia warrant special mention as in patients with diabetes mellitus, cardiovascular disease is the principal cause of mortality. Diabetics may have a diminished awareness of ischemic chest pain, which could result in an uncharacteristic presentation and delays in diagnosis and treatment. Possible explanations for the dissimilar symptoms in patients with diabetes mellitus comprise central mechanisms such as altered thresholds of pain sensitivity, beta-endorphin levels, in addition to autonomic neuropathy resulting in sensory denervation [181]. Diabetes mellitus also affects vascular endothelium, causing endothelial dysfunction increasing the prevalence of silent MI in the group.

In summary history is the cornerstone of a patient’s initial assessment on presentation to an emergency department or outpatients clinic. Variation in a patient’s appreciation of their symptoms, pain thresholds and atypical symptoms in certain subgroups
continue to add to the diagnostic dilemma. A biomarker array would certainly complement current care pathways and increase diagnostic accuracy.

1.8.2

Clinical Examination

The physical examination performed within the context of a detailed history can help to establish or narrow the diagnosis before further biochemical testing and imaging evaluation. It allows for the evaluation of disease severity and progression or can identify patients at early stages of a disease.

As for any other systematic examination we start with a general appearance of a patient including his or her age, posture and general health. Often one can garner numerous indicators of underlying disease severity. For example diaphoresis an autonomic response is an involuntary response and portends a diagnosis of serious disease. Respiratory pattern, pursing of the lips, increased anteroposterior chest diameter, cyanosis, jaundice, pallor, weight, temperature, blood pressure and oxygen saturations can all be assessed on general inspection and dictates the pace and scope of the evaluation and provide initial clues to the presence of a cardiovascular disorder.

The skin and general appearance gives additional clues as to any underlying syndromes for example Marfan’s, Holt-Oram or Turner’s are but a few syndromes with underlying cardiac abnormalities. Central and peripheral cyanosis of the fingers, toes, ears and nose is characteristic of reduced blood flow in severe heart failure, shock or severe peripheral vascular disease. Ecchymoses are often present in patients on antiplatelet or anticoagulation therapy. Various lipid disorders can manifest with tendon xanthomas and xanthelasma around the eyes.

Head and neck examination demonstrate dentition an index of general hygiene; periodontitis is a known risk factor for cardiovascular disease. Premature arcus senelis
and prominent earlobe creases may be associated with hyperlipidemia and underlying CAD. The jugular venous pressure (JVP) is the most important measure of haemodynamic measurement to ascertain volume status at the bedside. Both the external jugular vein and the internal jugular vein may be utilized. The internal jugular vein is preferred as the external jugular is not directly in line with the superior vena cava and the right atrium. The venous pressure is measured as the vertical distance between the top of the pulsation and the angle of Louis. A distance of > 3cm is considered as abnormal. However the measurement is fraught with difficulty especially in obese patients. In 160 patients receiving outpatient coronary CT scans the median vertical distance was ranged considerably for different body angles with values of 8.0, 9.7 and 9.8cm for body angles of 30, 45 and 60 degrees [182]. An elevated JVP in suspected ischemia and heart failure is a non specific finding and needs to be interpreted in the context of clinical presentation and other examination findings [183].

Accurate inspection and auscultation of the chest, the heart and lungs is paramount in the cardiovascular examination. The apical heartbeat may be visible at approximately the fifth intercostal space in the thin chested adult. Visible pulsations anywhere other than the ventricular apex are abnormal. The left anterior chest may heave in patients with enlarged and hyperdynamic left ventricles. A left parasternal heave indicates right ventricular pressure or volume overload. A proximal pulsation over the third intercostal space may indicate pulmonary hypertension.

Palpation of the heart begins with the search for the apex beat with patient in the supine position but sometimes if not palpable the patient may be examined in the left lateral position with the arm above the head. The point of maximal impulse is normally over the left ventricular apex beat and should be located in the mid clavicular line in the 5th intercostal space. The apex beat represents ventricular systole and is created by counterclockwise rotation of the heart as it twists and shortens along its long axis. Left ventricular enlargement or cardiomegaly which may be attributable to cardiac ischaemia and ischaemic cardiomyopathy is an advanced sign of underlying ischaemia and manifests as a downward and leftward displacement of the apex beat. A sustained apex beat is a sign of left ventricular pressure overload and may be related to other
mimics of cardiac ischaemia such as aortic stenosis or hypertensive heart disease both of which may present as angina in the unsuspecting patient.

A parasternal heave occurs with right ventricular pressure overload and signs of pulmonary hypertension should be sought out in clinical examination. Rarely patients with an acute coronary syndrome can have a myocardial complication such as acute mitral regurgitation, which can manifest as prominent left parasternal impulse because of systolic expansion of the left atrium and forward displacement of the heart within the thorax.

Auscultation of the heart and lungs is crucial in making an informed differential diagnosis for the patient with chest pain. Ventricular systole represents the interval between the first and second heart sounds. The first heart sound indicates the closure of the mitral and tricuspid valves, which are best, heard at the lower left sternal border for the tricuspid valve and over the apex for the mitral valve. The intensity and timing of the first heart sound varies for varying mechanical and electrical reasons with right bundle branch block, long PR interval, obstructive lung disease, mitral stenosis or regurgitation, pneumothorax and pericardial effusion but a few explanations.

The second heart sound comprises the aortic and pulmonary valve closure with normal physiological splitting, ie closure of the aortic valve just before the pulmonary more prominent with inspiration and narrows with exhalation. The second heart sound is best heard at second left interspace for the pulmonary valve and second right interspace for the aortic valve. The evaluation of the second heart sound is most prescriptive for a variety of reasons. The interval between the aortic and pulmonary valve closure widens with right bundle branch block and severe mitral valve regurgitation (MR). Fixed splitting of the second heart sound may imply an ostium secundum atrial defect and reverse splitting a consequence of left bundle branch block, severe aortic stenosis, hypertrophic obstructive cardiomyopathy (HOCM) and myocardial ischemia.

Murmurs represent audible variations caused by increase turbulence across a valve. The magnitude, dynamic variability and duration of pressure difference between two
cardiac chambers or between the chambers and representative great arteries dictate the frequency, intensity and configuration of the murmur. Not all murmurs are indicative of valvular or structural heart disease but in the patient presenting with chest pain a murmur is can be very descriptive and prescriptive in formulating a management strategy.

Systolic murmurs are early, mid, late or holosystolic in timing. Mitral regurgitation results in an early systolic murmur with acute severe MR often related to acute myocardial infarction or papillary muscle rupture. Mid systolic murmurs begin after the first heart sound and end before the second heart sound. Aortic stenosis is the most common cause of a systolic murmur in an adult patient. Other possible aetiologies include HOCM, pulmonary stenosis and an atrial septal defect. A late systolic murmur implies mitral valve prolapse but can also be heard transient during an episode of myocardial ischaemia. A ventricular septal defect a possible acute complication of cardiac ischaemia or myocardial rupture can be heard as a holosystolic murmur.

Diastolic murmurs when present often indicate significant cardiac disease. In the acute patient with chest pain the murmur of aortic regurgitation may represent an acute aortic dissection with valvular extension. Mitral stenosis is the classic diastolic murmur but often is chronic and not ischaemic in nature.

In summary both the clinical history and the examination of the patient with chest pain will continue to form the skeletal structure by which we assess our patients. However as shown in the meta-analysis by Bruyninckx et al. there exist no uniform symptom or sign which can reliably differentiate the patient with ischaemic cardiac chest pain from the patient with non-cardiac chest pain. In his review it was not possible to define an important role for signs and symptoms in the diagnosis of acute MI or ACS. Only chest-wall tenderness on palpation largely ruled out AMI or ACS in low-prevalence settings [184].
1.8.3

**Chest Pain Scoring Tools**

The management of patients with chest pain is a common and challenging clinical problem. Chest pain is one of the few disease processes in which patients may initially appear to be well but in fact have an underlying life-threatening condition. The clinician must distinguish between those who require urgent management of a serious problem such as an ACS and those with more benign entities who do not require admission. Clinical judgment continues to be paramount in meeting this challenge. However the symptoms and signs of cardiac disease in a patient with chest pain are variable making accurate decision-making sometimes impossible. Myocardial infarction can be misdiagnosed for a number of reasons. Misinterpretation of findings on ECG occurs in 23% to 40% of misdiagnosed ACS. Younger age, physician inexperience and atypical presentations are more common in these patients [185].

Numerous scoring systems have hence been developed and internationally validated to better assist the physician in making informed clinical decisions in the investigation and management of chest pain patients. The goal of the initial evaluation of a patient who presents to an outpatient setting with potential ACS has changed from diagnosis to risk stratification.

### 1.8.3.1

**HEART score**

The HEART score developed in 2008 looked specifically at patients presenting to hospital with chest pain. It is composed of both clinical and biochemical factors i.e. history, ECG changes, age, risk factors and troponin levels. As detailed in table 2 below each component is graded 0-2 with a cumulative total maximum score of 10. The composition of the HEART score was not based on multivariate regression analysis but on the decision making clinical factors according to expert opinion [186].
Table 1.2- Composition of the HEART score for chest pain patients in the emergency room.

<table>
<thead>
<tr>
<th>HEART score for chest pain</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>History</td>
<td></td>
</tr>
<tr>
<td>Highly suspicious</td>
<td>2</td>
</tr>
<tr>
<td>Moderately suspicious</td>
<td>1</td>
</tr>
<tr>
<td>Slightly</td>
<td>0</td>
</tr>
<tr>
<td>ECG</td>
<td></td>
</tr>
<tr>
<td>Significant ST depression</td>
<td>2</td>
</tr>
<tr>
<td>Nonspecific Repolarization disturbance</td>
<td>1</td>
</tr>
<tr>
<td>Normal</td>
<td>0</td>
</tr>
<tr>
<td>Age</td>
<td></td>
</tr>
<tr>
<td>≥ 65</td>
<td>2</td>
</tr>
<tr>
<td>45-65</td>
<td>1</td>
</tr>
<tr>
<td>&lt; 45</td>
<td>0</td>
</tr>
<tr>
<td>Risk Factors</td>
<td></td>
</tr>
<tr>
<td>≥ risk factors or history of atherosclerotic disease</td>
<td>2</td>
</tr>
<tr>
<td>1 or 2 risk factors</td>
<td>1</td>
</tr>
<tr>
<td>No risk factors known</td>
<td>0</td>
</tr>
<tr>
<td>Troponin</td>
<td></td>
</tr>
<tr>
<td>&gt; 2× normal limit</td>
<td>2</td>
</tr>
<tr>
<td>1-2× normal limit</td>
<td>1</td>
</tr>
<tr>
<td>≤ normal limit</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>0-10</td>
</tr>
</tbody>
</table>

The HEART score divides patients into low (0-3), intermediate (4-6) or high-risk groups (7-10), with mean risks of MACE of 0.9%, 12% and 65%, respectively. Consequently, an evidence-based decision may be made to discharge the patient from the ED or to admit for clinical observation or immediate aggressive therapies.

In their trial Backus et al. showed that the HEART score had a c statistic of 0.83 indicating a good to excellent ability to discriminate all cause chest pain patients at the emergency department for their risk of MACE [186]. Low HEART scores (0-3), occurring in one third of the patients in their prospective trial, excluded short-term
MACE with >98% certainty. In patients with high HEART scores (7–10) the high risk of MACE indicates the need for more aggressive management.

1.8.3.2

**Thrombosis in Myocardial Infarction (TIMI) Score**

The TIMI risk score is used to determine the likelihood of ischaemic events or mortality in patients presenting with symptoms of unstable angina or non-STEMI. The TIMI risk score (2000) is derived from the Thrombolysis in Myocardial Infarction (TIMI)-11B trial, a multinational, randomized clinical trial, comparing unfractionated heparin to enoxaparin, which included all patients with confirmed ACS [187]. Data from 1,957 patients enrolled in the unfractionated heparin group were used to identify twelve elements of typical ACS findings by use of multivariate regression analysis. Seven of these elements remained statistically significant in a multivariate analysis.

Together these seven elements comprise the TIMI score for unstable angina/NSTEMI: age > 65 years, 3 classical risk factors for coronary artery disease (CAD), known CAD, use of Aspirin in the past 7 days, severe angina in the past 24 hours, elevated cardiac markers and ST-deviation. Table 1.3 details how the TIMI score is calculated.
<table>
<thead>
<tr>
<th>Variable</th>
<th>Score if Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age &gt; 65</td>
<td>1</td>
</tr>
<tr>
<td>≥ 3 CAD Risk Factors</td>
<td>1</td>
</tr>
<tr>
<td>CAD (Known &gt; 50% stenosis)</td>
<td>1</td>
</tr>
<tr>
<td>ASA Use in Past 7 days</td>
<td>1</td>
</tr>
<tr>
<td>Severe Angina in past 24h</td>
<td>1</td>
</tr>
<tr>
<td>Elevated cardiac markers</td>
<td>1</td>
</tr>
<tr>
<td>ST Deviation on ECG</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>0-7</strong></td>
</tr>
</tbody>
</table>

**Table 1.3** – TIMI score parameters that are added to give a total score.

The TIMI score predicts the risk of all-cause mortality, MI and severe recurrent ischemia requiring urgent revascularization within 14 days. Event rates increased significantly at 14 days with increasing TIMI scores. The c statistic for the model in the initial TIMI study was 0.65 and has subsequently been shown to 0.8 as a rule out score for patients presenting without ACS to the emergency department.

Whilst initially validated in the NSTEMI and unstable angina population it was subsequently validated in STEMI patients as well. The TIMI risk score for STEMI was found to reliably identify patients at very high risk while maintaining good discriminatory capacity in the low-risk range. Used as an epidemiologic tool, the TIMI risk score provided a convenient method to identify baseline differences in risk profile and offers a framework for analyses stratified by risk group at presentation [188].
1.8.3.3

The Global Registry of Acute Coronary Events (GRACE) Score

Known as the GRACE Risk Score, this model was originally developed to estimate the risk of in-hospital mortality for patients presenting to hospital with a suspected ACS. GRACE was a multicenter prospective registry of patients presenting with ACS. Inclusion criteria for the registry included age ≥18 years old, being alive at hospital presentation, and presumed ACS diagnosis. Patients who were already hospitalized at the time of development of ACS and those with a significant acute comorbidity such as trauma were excluded.

Patients were categorized as having STEMI, NSTEMI, or unstable angina. GRACE enrolled patients from 1999 to 2007. The model was further developed to include prediction of mortality six months post discharge.

Currently there are eight clinical measurements involved in calculating the risk for death, and death or myocardial infarction from admission to hospital to 6 months after discharge. These include age, heart rate, systolic blood pressure (mmHg) creatinine (mg/dL or μmol/L), congestive heart failure (Killip), cardiac arrest at admission, ST-segment deviation and elevated cardiac enzymes/biomarker. The European society of Cardiology guidelines recommend using this score to identify patients who will benefit from an invasive management strategy.

The GRACE score is scored from 1-372 with score of 1-108 being low risk, 109-140 intermediate and 141-372 being high risk. This equates to an in-hospital mortality of <1%, 1-3% and >3% respectively for patients presenting with NSTEMI. Similarly in STEMI patients low, intermediate or high-risk mortality varies between <2% for low risk and > 5% for high-risk patients [189].

The GRACE score has been validated in many studies not only for in-hospital and 6 month mortality and morbidity, but for predicting mortality for >1 year. Littnerova et al. evaluated six scoring systems for patients presenting with STEMI evaluating their
ability to predict one year, two year and three year mortality. The best predictive values for long-term mortality were obtained by GRACE [190]. Additionally they found that risk scoring systems established to predict short-term outcomes (not longer than one-year mortality) were also useful for the estimation of three-year mortality. One possible explanation being that the overall mortality after STEMI is mainly influenced by higher mortality rates during the first six months after discharge from hospital. Mortality in subsequent periods (2–3 years after STEMI) corresponds with that of patients with stable CAD [190].

1.8.3.4

Summary

Cardiac risk scores estimate a patient’s risk of future cardiac events or death. They have been developed to inform treatment decisions of patients diagnosed with non-specific chest pain, stable and unstable angina, NSTEMI and STEMI. Despite recommending their use in guidelines and evidence of their prognostic value, there continues to be variability among physicians in their usage. Health care practitioners disagree on the importance of cardiac risk scores used to decide on the management of unstable angina or non-ST-elevation myocardial infarction patients. Researchers found intrinsic motivators such as a uniform approach in admission and treatment practices, and education of less experienced physicians as the main reasons for using scoring systems. In examining the reasons for lack of routine implementation of scoring systems doctors cited administrative burden, absence of clinical consequence and frequent rotation of staff as the main reasons. In addition, practitioners who felt forced to use cardiac risk scores were less likely to take into account the cardiac risk score in their treatment decisions [191]. Additionally scoring systems validated for predicting risk at six weeks and six months are less robust for in estimating risk of MACE at 48-72 hours.

With variability in usage of scoring systems the addition of a validated biomarker of unstable plaque to the model could significantly increase adoption and diagnostic accuracy.
1.9 Introduction to our novel biomarkers

1.9.1 Background

Previous work in our laboratory performed by Dr. Marian Brennan in collaboration with Professor Christopher Jackson (Bristol University) identified a number of novel murine serum biomarkers for pre and post plaque rupture (Table 1.4). Serum was collected from ApoE−/− mice fed a high fat diet and mice were classified as either pre- or post-rupture based on histological analysis of their brachiocephalic artery plaques [192].

The results are the average from 15 controls, 6 pre-rupture and 4 post-rupture mice. Plaque rupture was determined by histological analysis of the brachiocephalic artery. In mice, CRP is not a marker of inflammation, and in the mice samples as expected, this marker was not increased. Pentraxin 3 is a marker of inflammation in mice and this was elevated more than 3-fold in both pre and post rupture samples ($P<0.001$). Six proteins were present at significantly higher or lower levels in the serum prior to plaque rupture and thus may be predict risk of rupture in mice. These proteins have been investigated as putative human biomarkers in our study. Our mouse model data is currently unpublished.
<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Control</th>
<th>Pre-Rupture</th>
<th>Post-Rupture</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-reactive protein (CRP)</td>
<td>2.8 ± 1.0</td>
<td>1.9 ± 0.6 (NS)</td>
<td>2.3 ± 1.5 (NS)</td>
</tr>
<tr>
<td>Pentraxin 3</td>
<td>0.56 ± 0.13</td>
<td>1.60 ± 0.61 (P&lt;0.001)</td>
<td>1.87 ± 0.20 (P&lt;0.001)</td>
</tr>
<tr>
<td>Transferrin</td>
<td>2.54 ± 0.35</td>
<td>1.43 ± 0.49 (P&lt;0.01)</td>
<td>1.3 ± 0.42 (P&lt;0.01)</td>
</tr>
<tr>
<td>Interleukin 15 receptor alpha (IL-15Rα)</td>
<td>0.03 ± 0.01</td>
<td>0.07 ± 0.01 (P&lt;0.001)</td>
<td>0.06 ± 0.04 (P&lt;0.05)</td>
</tr>
<tr>
<td>Homolog Frizzled 7 (HF7)</td>
<td>0.05 ± 0.02</td>
<td>0.05 ± 0.02</td>
<td>0.03 ± 0.00 (P&lt;0.05)</td>
</tr>
<tr>
<td>Galectin 3 (Gal-3)</td>
<td>1.6 ± 0.4</td>
<td>15.9 ± 2.1 (P&lt;0.001)</td>
<td>23.8 ± 3.1 (P&lt;0.001)</td>
</tr>
<tr>
<td>Transforming growth factor beta (TGFβR)</td>
<td>0.004 ± 0.004</td>
<td>0.65 ± 0.05 (P&lt;0.001)</td>
<td>0.93 ± 0.37 (P&lt;0.001)</td>
</tr>
<tr>
<td>Developmental Tyrosine Kinase (DTK)</td>
<td>0.00 ± 0.02</td>
<td>1.76 ± 0.78 (P&lt;0.01)</td>
<td>1.85 ± 0.18 (P&lt;0.01)</td>
</tr>
</tbody>
</table>

**Table 1.4:** Presentation of results for biomarkers identified in ApoE knockout mouse model. (*P*-values are presented in brackets.)
1.9.2

**C-reactive protein (CRP)**

CRP, named for its capacity to precipitate the somatic C-polysaccharide of *Streptococcus pneumoniae*, was the first acute-phase protein to be described and is an exquisitely sensitive systemic marker of inflammation and tissue damage. The acute-phase response comprises the nonspecific physiological and biochemical responses to most forms of tissue damage, infection, inflammation, and malignant neoplasia. CRP is rapidly up regulated principally in hepatocytes. CRP concentration is thus a useful nonspecific biochemical marker of inflammation [193].

Inflammatory mechanisms play a central role in all phases of atherosclerosis, from the initial recruitment of circulating leukocytes to the arterial wall to the rupture of unstable plaques. CRP may be involved in each of these stages by directly influencing processes like complement activation, apoptosis, vascular cell activation, monocyte recruitment, lipid accumulation and thrombosis [194].

CRP is one of the substances present in the atherosclerotic lesion, more specifically in the vascular intima, where it co-localizes with monocytes, monocyte-derived macrophages and lipoproteins. This localization makes a direct contribution to the atherosclerotic process possible.

CRP activates the classical pathway of the complement system and in so doing directly amplifies and facilitates innate immunity [195]. For example CRP co-localizes with C5–C9, the membrane attack complex of complement. Activation of this membrane attack complex is initiated by direct binding of CRP to C1q, also present in the atherosclerotic lesion [195, 196]. CRP binds to several receptors on human monocytes increasing phagocytosis and the release of inflammatory cytokines [197]. CRP also contributes to an arterial pro-inflammatory and atherosclerotic phenotype by directly upregulating adhesion molecules and chemoattractant chemokines in endothelial cells, vascular SMCs and monocytic cells [198].
CRP is directly involved in the process of apoptosis. It binds to apoptotic cells in a calcium dependent manner and augments the classical pathway of complement activation. Furthermore, CRP enhances opsonization and phagocytosis of apoptotic cells by macrophages associated with the expression of various anti-inflammatory cytokines [199].

We assayed serum CRP concentration as a marker of acute inflammation in our cohort correlating the total detectable CRP level to levels of our novel biomarkers.
1.9.3

**Developmental Tyrosine Kinase (DTK)**

Receptor tyrosine kinases are essential components of signal transduction pathways that mediate cell-to-cell communication. These single-pass transmembrane receptors play key roles in processes such as cellular growth, differentiation, metabolism and motility [200].

Antigen presenting cells (APCs) like macrophages and dendritic cells play a critical role in presenting antigens to B and T cells that generate an appropriate immune response. This process is closely regulated and DTK (also known as Tyro3), Merkt and Axl receptors belong to a family of protein tyrosine kinase receptors that function as homeostatic regulators [201]. These were first identified in 1991 with two ligands for these receptors, GAS-6 and Protein-S subsequently identified [202].

Various groups have demonstrated that DTK, Mertk and Axl are predominantly expressed by monocytes and their derivatives. All three receptors are defined by a shared sequence in their amino acid code within their kinase domain [203]. DTK is predominately expressed in developing brain, adult kidney, ovary and testis. Axl appears to be the most widely distributed member of the family, due to its expression in cell lines of epithelial, mesenchymal and hematopoietic origins as well as non-transformed cells. Mertk is expressed in monocytes, epithelial cells and reproductive tissue [204].

All three receptors have been implicated in megakaryocytopoiesis and platelet aggregation. Blockade of the DTK receptor has been shown to inhibit thrombosis without affecting bleeding. The Axl receptor is also implicated in natural killer cell differentiation and vascular remodeling [205]. All three tyrosine kinase receptors are receptors for GAS-6 yet only DTK is a receptor for Protein-S.

The GAS-6-tyrosine kinase receptor has been implicated in atheroma development in various animal models. The expression of GAS-6 has been shown to increase after
mechanical damage to the vessel wall [206]. Inversely in Gas-6 knockout mice there was decreased neointima formation and less severe plaque restenosis [207].

Other studies have shown a role of different components of the GAS-6-tyrosine receptor in platelet function, inflammation and efferocytosis of apoptotic cells, all of which are implicated in atheroma development [208]. Apoptosis of macrophages have been shown to correlate with features of plaque instability like thin fibrous cap and plaque necrosis (Chapter 1). In one study of atherosclerotic carotid plaque there was a down regulation of DTK and Axl in plaque with features of instability. Simultaneously there was an increased concentration of GAS-6 and Mertk receptor suggesting potential markers of unstable plaque [209].

DTK in our mouse model was elevated pre plaque rupture and peaked post rupture. We evaluated the utility of the serum concentration of DTK in a cohort of patients with chest pain aiming to detect differences in concentration for different clinical presentations.
1.9.4

Galectin-3 (Gal-3)

Galectins are a group of lectins characterized by a galactose-specific carbohydrate recognition domain with affinity for beta-galactosides. Galectins recognize specific oligosaccharide structures or ligand glycoproteins on the membranes of neighbouring cells or in the extracellular matrix and thus provide information to cells as to their position and cell physiologic state respective to each other [210].

Presently 15 Galectin members are identified and classified into 3 subgroups based on protein structure and the number of carbohydrate recognition domains within the polypeptide chain [211]. Based on the number and on the organization of carbohydrate recognition domains, members of Galectin family have been classified into three subtypes: the prototype group, the chimera group and the tandem repeat group [212].

Gal-3 was initially identified as a 29- to 35-kDa protein cell surface antigen expressed on murine thioglycollate-elicited peritoneal macrophages [213]. In adults, Gal-3 is ubiquitously expressed. Although it was found in many normal tissues, Gal-3 expression in adults, similarly to its expression during embryogenesis, is mainly related to the epithelial cells and myeloid/amoeboid cells. Gal-3 has been found in epithelial cells in kidney, lung, thymus, breast, prostate and myocardium [214].

Gal-3 has been found to be involved in many biological processes, such as cell–cell and cell–extracellular matrix adhesion, cell growth and differentiation, cell cycle, signaling and apoptosis, as well as in angiogenesis. Gal-3 is mainly produced by macrophages, and is implicated in a variety of biologic events, such as inflammation and angiogenesis [214].

To date Gal-3 in the myocardium has mainly been implicated for its role in cardiac fibrosis and as a prognostic marker of heart failure. Gal-3 is a shared factor in fibrosis formation in different organs. When secreted, Gal-3 acts on fibroblasts and initiates a pro-fibrotic program [214]. Calvier et al. showed that Gal-3 was activated in
aldosterone induced cardiac fibrosis. Specifically, *in vivo*, Gal-3 is associated with fibroblast to myofibroblast differentiation and collagen synthesis [215].

Additionally there is a growing body of evidence that Gal-3 expression in cardiac tissue after myocardial infarction portends a poorer prognosis. Tsi *et al.* showed that elevation of Gal-3 levels 6 hours post myocardial infarction predicted 30 day MACE [216]. In a trial consisting of 263 STEMI patients who underwent primary PCI, Gal-3 was an independent predictor of left ventricular ejection fraction at 4 months [217].

The role of Gal-3 in atherogenesis continues to evolve. Up regulation of Gal-3 is well described in rodent and human models of atherosclerotic disease [218]. One mechanism proposed is a role in the transformation of macrophages into foam cells. Gal-3 expression in highly up regulated when monocytes differentiate into macrophages that follows after monocyte recruitment from the artery wall [219].

Gal-3 is also secreted by foam cells, like activated macrophages, a potent chemoattractant for monocytes and macrophages and thus continues to attract these cells to sites of vascular injury [220]. Gal-3 stimulated endocytotic uptake of modified lipoproteins resulting in intracellular cholesterol accumulation and binding to lipopolysaccharides help in explaining its role in atherogenesis [221-223].

In one study Gal-3 and Gal-3 positive cells were increased in atherosclerotic lesions that were rich in foam cells. Fibrotic lesions on the other hand had lower Gal-3 levels and fewer Gal-3 positive cells. Gal-3 positive cells were also close to a lipid core, or to areas with fibrosis, hemorrhage, or thrombosis in the atherosclerotic lesions [218]. In another study, in the absence of Gal-3 expression, the incidence of atheromatous plaques was lower and Gal-3 was strongly expressed in foam cells of the atheromatous plaques [224]. These findings support the hypothesis that Gal-3 may be involved in the active phase of vulnerable atherosclerotic plaque evolution.

In our mouse model Gal-3 was elevated pre plaque rupture and peaked post rupture. We hence assayed Gal-3 in our cohort and evaluated its utility as a marker of plaque instability and rupture.
1.9.5

**Transforming growth factor beta II receptor (TGFβIR)**

TGF-β receptors are a family of cytokine receptors that are ubiquitous, multifunctional and essential to survival. They play important roles in growth and development, inflammation and repair and host immunity [225]. There are three mammalian isoforms, TGF-β1, TGF-β2, and TGF-β3, of which TGF-β1 represents the predominant isoform and the prototype member of the TGF-β superfamily of multifunctional cytokines [226]. TGF-β signals through two receptors TGF-βI and TGF-βII which are serine-threonine trans membrane receptors [227].

When activated the TGF-β1 and TGF-βII receptor come together and the β1 receptor is phosphorylated by βII. This then propagates the signal through phosphorylation of a group of proteins known as Smad proteins. The activated Smad complexes are translocated into nucleus and in conjunction with other nuclear cofactors regulate the transcription of target genes [227]. TGF signaling generally has a negative effect on cell growth; inactivation of this pathway contributes to tumorigenesis. The TGF-βII receptor has been shown to be inactivated by various mutations in a variety of human gastrointestinal cancers [228].

A growing body of evidence suggest that TGF-β is a major regulator of immune function, acting both by suppressive and stimulatory mechanisms on leukocytes to achieve a balanced immune response [229]. Leeven *et al.* demonstrated that disruption of TGF-ΙΙΙβR gene in a mice model led to uncontrollable inflammation and a lethal inflammatory disorder [230].

Whilst the role of TGF-β in plaque rupture remains to be elucidated it has been implicated in cardiac fibrosis. Cardiac fibrosis is characterized by the excessive production and deposition of scar tissue and is often a result of conditions such as hypertension and diabetes mellitus. The cells ultimately responsible for the development of scar tissue are called myofibroblasts. These are mesenchymal cells, which are resident within connective tissue and possess the highly contractile protein
α-smooth muscle actin. This acts to remodel the extracellular matrix after cell death. TGF-β1 is up regulated in cardiac fibrosis and normal tissue repair within the myocardium [231]. Fig.1.14 demonstrates the complex interplay of factors involved in cardiac fibrosis to which TGF-β is central.

**Fig – 1.14** A series of direct and indirect interactions among growth factors and proteins drive fibrogenesis. Ang-II represents angiotensin II; ALK5, activin-linked kinase 5; AT1, angiotensin 1 receptor; ET-1, endothelin-1; MMP, matrix metalloproteinase; PDGF, platelet-derived growth factor; PDGFR, platelet-derived growth factor receptor; SMA, smooth muscle actin; TGF-β, transforming growth factor-β; and trkA, tyrosine receptor kinase A.

In the border zone of healing infarcts, the TGF-β/Smad pathway is activated and has been shown to induce negative remodeling [232]. In a mouse model of myocardial infarction ALK5 inhibitors were shown to decrease TGF-β activity and reduce both systolic and diastolic dysfunction [233]. However blocking of TGF-β receptor with antibodies in a mouse model did not produce the desired result. While it reduced collagen production and increased matrix metalloproteinase expression it worsened vascular remodeling and resulted in increased mortality [228].

In the catalog of events in myocardial infarction there exist a marked inflammatory response that can be divided into the inflammatory phase, proliferative phase and
maturation phase. TGF-β may play an important role in monocyte recruitment in the healing infarct and promoting granulation tissue formation. Activation of TGF-β signaling pathways may be important in suppressing expression of pro-inflammatory cytokines and chemokines in the infarcted myocardium resulting in resolution of the inflammatory infiltrate.

In our mouse model we noted an increase of serum concentration of TGF-β2 receptor pre plaque rupture that peaked post rupture. We aimed therefore to determine utility of TGF-IIβR receptor as a biomarker of coronary plaque rupture and plaque instability in our population.
1.9.6

**Interleukin 15 receptor alpha (IL-15Rα)**

Interleukins (ILs) are a group of cytokines (secreted proteins and signal molecules) expressed by white blood cells. The majority of interleukins are synthesized by helper CD4 T lymphocytes. ILs modulate growth, differentiation, and activation during an immune response. ILs act in a paracrine or autocrine fashion, rather than as an endocrine signal, which is more common with steroidal and amino acid-derived hormones. The response of a particular cell to these cytokines depends on the ligands involved, specific receptors expressed on the cell surface, and the particular signaling cascades that are activated [234, 235]. ILs mediate their effects through the Janus kinase-signal transducers and activators of transcription (JAK-STAT) pathway [236].

Interleukin 15 (IL-15) acts through its specific receptor, interleukin 15-receptor alpha (IL-15Rα) that is expressed on antigen-presenting dendritic cells, monocytes and macrophages [235]. The human IL-15 gene maps to chromosome 4 and is transcriptionally active in several normal cell types including epithelial cells, monocytes, macrophages, dendritic cells, fibroblasts and some tumor cells [237]. The heterotrimeric IL-15 receptor is composed of a beta subunit (IL-2R/15Rβ) that is shared with the IL-2 receptor, a common gamma subunit shared with IL-2, IL-4, IL-7, IL-9 and IL-21, and a unique alpha subunit (IL-15Rα) that confers receptor specificity to IL-15 [238].

IL-15 was initially identified for its ability to stimulate T cell proliferation in an IL-2-like manner through common receptor components and signaling through JAK1/ JAK3 and STAT3/STAT5. Like IL-2, IL-15 has been shown to stimulate proliferation of activated CD4-CD8-, CD4+CD8+, CD4+ and CD8+ T cells as well as facilitate the induction of cytotoxic T-lymphocytes, and the generation, proliferation, and activation of NK cells [239].

In addition to the effects on T and NK cells, IL-15 also has several effects on other components of the immune system. IL-15 protects neutrophils from apoptosis,
modulates phagocytosis and stimulates the secretion of IL-8. In mast cells, IL-15 can act as a growth factor and an inhibitor of apoptosis. IL-15 also induces B lymphocyte proliferation and differentiation, and increases immunoglobulin secretion. Monocytes, macrophages and dendritic cells effectively transcribe and translate IL-15. They also respond to IL-15 stimulation. Macrophages respond by increasing phagocytosis, inducing IL-8, IL-12 and MCP-1 expression, and secreting IL-6, IL-8 and TNF α [240, 241].

The role of cytokines in atherosclerotic plaque propagation is varied. Some cytokines are known to be protective or anti-inflammatory and others have been shown to have an incendiary role. Pro-atherogenic cytokines such as TNF-α, IL-1, and IL-6 are secreted by macrophages, lymphocytes, natural killer cells, and vascular smooth muscle cells [236]. TNF-α and IL-1 signaling affects almost all cells involved in atherogenesis by promoting the expression of cytokines, adhesion molecules, and the migration and mitogenesis of vascular smooth muscle and endothelial cells [236]. IL-6 is one of the most widely studied cytokines in CAD. Biasucci et al. measured IL-6 and IL-1Rα in UA patients showing that a fall in levels 48 hours after presentation to hospital was associated with improved outcome [242].

IL-15 levels are elevated in patients with CAD. High levels of IL-15 mRNA and its protein were found to be expressed by the majority of macrophages in both lipid-rich and fibrolipid plaques but there was hardly any IL-15 expression in fibrous plaques and normal vessels suggesting a role for IL-15 in plaque destabilization [243]. Gokkusu et al. provided further evidence by demonstrating that in both patients with ACS and stable chronic CAD there was an increased concentration of IL-15 compared to control patients and this was also influenced by genetic variants of IL-15 gene [244].

Three different functional forms of IL-15 have been identified. The soluble cytokine IL-15, IL-15R-independent membrane-bound IL-15 and membrane-IL-15 anchored through IL-15Rα [245, 246]. IL-15Rα may be secreted as a functional soluble molecule and could behave as an agonist by forming a complex with IL-15, which is 100-fold more efficient than the non-complex soluble cytokine or as an antagonist for IL-15 [247]. IL-15Rα has been shown to correlate with increased epicardial fat.
thickness in patients undergoing CABG but there are no studies utilizing IL-15Rα as a marker of acute plaque rupture.

Given its immunological role in plaque formation we aimed to determine the utility of IL-15Rα as a plasma biomarker of acute plaque rupture and its correlation with CAD.
1.9.7

**Homolog Frizzled 7**

Homology frizzled 7 (HF7) belongs to a family of transmembrane spanning receptors. The receptor contains a cysteine rich domain that binds its ligands, which are proteins of the Wingless (Wnt) family of lipoglycoproteins [248]. The Wingless (Wnt) gene was first identified in a random mutagenesis screen in the Drosophila melanogaster fly [249]. There are ten isoforms of the mammalian frizzled receptors many of which are expressed in the cardiovascular system. Three main signaling pathways are activated by agonist-activated HF-7. Fig. 1.15 highlights the main mechanisms involved in activation of the HF7-Wnt pathway.

The Wnt proteins constitute a family of cysteine-rich glycosylated proteins involved in a variety of modeling and remodeling processes which include cell proliferation, differentiation, apoptosis and control of cell orientation [250]. There are 16 Wnt proteins that bind to frizzled receptors. Based on functional classification the Wnt receptors can be divided into the Wnt1 class and the Wnt5a class. The Wnt proteins from the Wnt1 class signal through the β-catenin referred to as the canonical pathway (Fig. 4.41) whereas the proteins from the Wnt5a class signal via the non-canonical pathway [251].

The Wnt- Frizzled pathway has been implicated in a variety of cardiac disorders. In a mouse model Frizzled 2 was up regulated in myofibroblasts during migration and proliferation after myocardial infarction [252]. The expression of Frizzled was confined to the migratory phase of myofibroblasts. That suggests that Frizzled was involved in the alignment of myofibroblasts. When this hypothesis was tested in a mouse model lacking the disheveled (dvl) gene there was increased levels of myocardial rupture post infarct suggesting that the Wnt- frizzled pathway was required for infarct healing [253].
Proteins form the Wnt family can bind to frizzled receptors. This causes an activation of the signal transduction molecule dishevelled (dvl), which in turn inhibits the enzyme glycogen synthase kinase 3- β (GSK3-β). This enzyme is responsible for the phosphorylation of α-catenin, a protein that can act as a second messenger in this cascade. β-Catenin is phosphorylated in a complex with, among other components, Axin and adenomatous polyposis coli (APC) protein, which is a first step in its degradation by the ubiquitin proteasome pathway. β-Catenin can activate transcription factors from the T-cell factor /lymphocyte enhancer factor (TCF/LEF) family, and can form a complex with α-catenin and members cadherin family at the plasma membrane. Soluble frizzled-related.

β-Catenin can activate transcription factors from the T-cell factor /lymphocyte enhancer factor (TCF/LEF) family, and can form a complex with α-catenin and members cadherin family at the plasma membrane. Soluble frizzled-related proteins (sFRPs) share the Wnt binding domain with frizzled receptors but lack the transmembrane domain. sFRPs can bind Wnt proteins, thereby preventing the interaction with frizzled receptors.
The Wnt-frizzled pathway is implicated in angiogenesis as over expression of the Wnt-1 in a primary endothelial cell culture resulted in the proliferation of these cells [255]. In an in vivo study in neovascularization after myocardial infarction, β-catenin was translocated from the plasma membrane to the cytoplasm during the phase of neovascularization of the infarct area. Simultaneous expression of Dvl in these cells provides evidence that the Wnt-frizzled pathway is closely involved [253].

The Wnt-Frizzled pathway is implicated in various fibrotic diseases. In cardiac fibrosis the Wnt-Frizzled pathway is upregulated. In normal adult cardiomyocytes, Wnt-Frizzled signaling is quiescent. However, the pathway becomes reactivated in disease states that include hypertrophy [253].

The inflammatory response after myocardial infarction plays a crucial role in the healing process [256]. There is accumulating evidence that the Wnt-Frizzled pathway may play a distinct role in inflammation. sFRP was able to block leukocyte activation and cytokine production, and sFRP impaired the loop of cytokine amplification and decreased neutrophil activation and recruitment into the scar [257]. Sklepiewicz et al. found that cardiac fibroblasts lacking endogenous secreted sFRP showed increased α-smooth muscle actin expression, higher cell proliferation rates, and increased collagen production in sFRP knockout mice [258]. These data indicate that sFRP might be a novel anti-inflammatory factor protecting the heart from damage after myocardial infarction because sFRP is known to be an inhibitor of Wnt signaling.

The role of HF7-Wnt in plaque formation is not fully understood. Endothelial activation is crucial for the initiation and progression of inflammation. Wnt signaling has been proposed as a possible regulatory process in endothelial dysfunction associated with inflammation [259]. There are two types of endothelial activation involved in inflammatory regulation, type I activation for immediate response, and type II activation that promotes inflammatory gene expression. Wnt5a-HF binding increases type 1 endothelial activation whereby through up regulation of cyclooxygenase 2
(COX2) expression it acts as an inflammatory mediator to promote type 2 endothelial activation [6].

Additionally the pathological role of Wnt5a is supported by the fact that it is expressed in inflamed atheromatous plaques and co-localises with toll-like receptor 4 in macrophage-rich regions of atherosclerotic plaque [260]. Stimulation of macrophages by lipopolysaccharides and activation of TLR4 leads to increased expression of Wnt5A suggesting a putative functional interaction between these two molecules. Blumenthal et al. showed that stimulation of human macrophages with lipopolysaccharides induces Wnt5A expression [261].

In our mouse model we showed that HF7 was elevated pre-plaque rupture and decreased post-rupture suggesting its utility as a biomarker of unstable plaque. On the foot of this evidence we assayed its concentration in serum in our human study.
Chapter 2

Materials and Methods
2.1

Study Design

This was a prospective observational cohort study whereby patients presenting with chest pain to St James's Hospital, Dublin, Ireland were eligible for inclusion. Patients admitted to the cardiology service at St James's Hospital via the Accident and Emergency department or at presentation to the catheterization suite were recruited. Inclusion criteria comprised chest pain presentation with the ability to give informed consent and aged over eighteen. Baseline demographic data collected (see appendix) included age, sex, duration of chest pain, time after the last chest pain episode or if ongoing pain, previous diagnosis of CAD, ECG findings, TIMI score, GRACE score and HEART score and results of stress tests, echocardiography and angiography if available. Biochemical data recorded included urea and electrolytes, full blood count, HsT or Tn levels and CRP if available.

Patients were classified initially into two groups; non-cardiac chest pain and cardiac chest pain. Cardiac chest pain included stable angina and ACS (UA, NSTEMI, STEMI). The definitions of which were adapted from the American Heart Association [262]. A final diagnosis was made by the admitting consultant cardiologist at the time of discharge.

Stable angina was defined as pain, tightness or heaviness brought on by exertion, with or without radiation down the ulnar surface of the arm. A typical episode of angina pectoris was considered as gradually beginning and reaching maximal intensity over a few minutes before dissipating with patients resting, sitting or stopping what they are doing with symptoms relieved within minutes [262].

Unstable angina was defined as angina pectoris with no elevation of HsT or Tn. The characteristics of the chest pain had at least one of the following: Pain occurring at rest with at least 20 minutes of chest pain (if not interrupted by nitrate administration), being severe and described as frank pain, and of new onset (within 1 month) or
occurring with a crescendo pattern (i.e. more severe, prolonged or more frequent than previously) [34].

ACS was composed of UA, NSTEMI and STEMI. Patients with NSTEMI and STEMI were defined as having undergone acute myocardial infarction (AMI). AMI was diagnosed as an increase in Hst or Tn, with at least one of the following:

1. Symptoms of ischaemia.
2. New or presumed new significant ST-T wave changes or left bundle branch block on 12-lead ECG.
3. Imaging evidence of new or presumed new loss of viable myocardium or regional wall motion abnormality.
4. Intracoronary thrombus detected on angiography or autopsy [263]

Non-cardiac chest pain was defined as non-exertional chest pain with no ischemic abnormalities on serial ECG’s and troponin measurements or any cardiac specific investigations these patients underwent.

Patients were recruited at all time points after the last chest pain episode with no specific time frame chosen. For presentation of results we have grouped time after last the chest pain episode into cohorts of <3 hours and <12 hours after the last pain episode. After recruitment patients were followed until discharge. Cardiac specific investigations were recorded and included results of angiography, echocardiogram and stress testing if performed. Total plaque burden and coronary lesion complexity were determined using the GENSINI and Syntax scores.
2.2

Sample size

To calculate the sample size for the novel biomarker arm of this study we started with Gal-3 from the mouse study where control mice had a mean level of 1.6 ng/ml and the pre rupture animals had a mean of 15.9 ng/ml. With an alpha value of 0.01 a sample size of 20 will give a power of 0.96. Based on an incidence of myocardial infarction of 3.6% in an all comers with chest pain presenting to accident and emergency we would need to recruit 555 chest pain patients to achieve 20 patients who develop an MI. This was a pilot study and thus the primary goal was to determine the baseline characteristics of the population to allow a power analysis for a larger
study. It was also designed to determine if it was possible to get samples of blood from pre-rupture patients. Thus, rather than being designed to confirm the hypothesis it was designed to optimize the study design for a future study if warranted.

2.3

**Ethical Approval**

Full consideration was given to the ethical implications of this study. Ethical review was performed by the Joint Research Ethics committee of St. James’s Hospital and Tallaght Hospital, Dublin. This was for a study on blood samples from patients with chest pain presenting to accident and emergency or for angiography either coronary CT (CTCA) or to the catheterization laboratory. The title of the approved study was “Novel Markers of Plaque Rupture in patients with Chest Pain”.

2.4

**Patient consent**

All patients approached for enrollment into our protocol underwent informed consent. Patients were consented by named study investigators only. The protocol was explained in detail, study literature provided and written consent obtained prior to any sample collection (see appendix). Patients were also aware that they could withdraw their consent at any point.

In total 205 patients were approached for informed consent of whom two declined. Study recruitment was undertaken between the hours of 800-1900 Monday to Friday. Dr Amrit Bajrangee recruited all patients.
2.5

Sample collection

Samples were taken and collected by Dr Bajrangee. Venous blood was collected using 3ml Vacutainers from *BD Lifescience Inc*. Blood for serum was phlebotomized directly into Vacutainers containing serum clot activators (silicone coated plastic). For plasma collection we utilized Vacutainers with ethylenediaminetetraacetic acid (EDTA). Both serum and plasma Vacutainers were subsequently placed on a heating block for one hour at 37°C with subsequent centrifugation at 1500 rcf (relative centrifugal force) for 10 minutes with a deceleration of 9/5, a moderate break to ensure no pellet disruption. The supernatant was then aliquot into 2 ml eppendorphs and stored at -80°C for ELISA analysis.

Urine was collected as a mid-stream sample with 5ml being pipetted into an Eppendorph, which was subsequently centrifuged at 1000 rcf for 5 minutes to remove impurities. The aliquot obtained was pipetted and stored at -80°C for subsequent ELISA performance.

2.6

Statistical Analysis

We utilized Graph pad from *GraphPad Software Inc.* (California, USA) and SPSS version 22 from *IBM Inc.* to perform all statistical analysis. Data are presented as means and standard deviations for normal populations and medians for non-normally distributed populations. We have utilized t-tests to compare population means between two groups and ANOVA for more than two groups for normally distributed data. For non-normal distributed data the Mann Whitney test was used to compare the difference between two means and the Kruskall-Wallis test for >2 means. Univariate and multivariate regression analyses, receiver operated curves and odds ratios have also been presented for certain data.
2.7.1

Enzyme Linked Immunofluorescent Analysis (ELISA)

2.7.1.1

Introduction

ELISA is a plate-based assay where we detect and quantify substances such as proteins, antibodies, peptides and hormones [264]. In the first step the antigen to be detected is coated onto the plate where small quantities are absorbed onto the plate. The free antigen is then washed away and the plate blocked with a non-specific protein to prevent further non-specific binding later. After an incubation period the plate is washed again and the test substance/antibody to be detected is then added to the plate. This is normally covalently coupled to an enzyme such as peridoxase. After a repeat wash the addition of a chromogen is acted upon by the peridoxase to produce a color change with the reaction being stopped after a fixed time period. The final step is to determine the optical density of the color change, which is a surrogate marker of protein/antibody concentration.

There are different forms of ELISA. In our protocol we have utilized indirect and sandwich ELISA. In indirect ELISA, antigen is coated on the microplate and serum or plasma containing the primary antibody is added and allowed to react. Any free primary antibody is washed away and the bound antibody/antigen complex is detected by adding an enzyme conjugated secondary antibody that binds to the primary antibody. Unbound secondary antibody is then washed away and a specific substrate for the enzyme is added. Enzyme hydrolyzes the substrate to form colored products and absorbance is then determined.
In sandwich ELISA antibody is coated on to the microplate. A sample-containing antigen is added to the microplate and allowed to react with the antibody attached to the well, forming antigen-antibody complex. After the well is washed, a second enzyme-linked antibody specific for a different epitope on the antigen is added and allowed to react with the bound antigen. There after unbound secondary antibody is removed by washing. Finally substrate is added to the microplate that is hydrolyzed by enzyme to form colored products and the reaction read using a microplate reader.
2.7.1.2

ELISA methods

Generic materials and reagents utilized

<table>
<thead>
<tr>
<th>Material</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pipettes</td>
<td>Gilson Inc. USA</td>
</tr>
<tr>
<td>Pipette tips</td>
<td>SIGMA Aldrich - USA</td>
</tr>
<tr>
<td>Glassware</td>
<td>5ml -500ml beakers</td>
</tr>
<tr>
<td>Plate sealers</td>
<td>Rand D Systems Inc</td>
</tr>
<tr>
<td>Phosphate Buffered Saline</td>
<td>SIGMA Aldrich- USA</td>
</tr>
<tr>
<td>Bovine Serum Albumin</td>
<td>SIGMA Aldrich- USA</td>
</tr>
<tr>
<td>0.05% Tween</td>
<td>SIGMA Aldrich- USA</td>
</tr>
<tr>
<td>Microplate reader</td>
<td>BioTek Inc.</td>
</tr>
<tr>
<td>Microplate shaker</td>
<td>BioTek Inc.</td>
</tr>
</tbody>
</table>

2.7.1.3

C Reactive Protein

C-Reactive Protein (CRP) is a non-glycosylated protein that functions as a sensor and activator of the innate immune response [265]. It is a major acute-phase protein with its circulating concentration dramatically elevated at the onset of inflammation. We determined the concentration of CRP in all patients in our protocol. We utilized a 4.5 hour quantitative sandwich ELISA from RandD systems Inc. (catalogue no. DCRP00).
Materials utilized

CRP ELISA was performed according to the manufacturers (R&D Systems Inc) instructions. Briefly 500 μl of plasma (1/100 dilution) or standard and 100 μl of diluent were added to wells for 2hrs at room temperature. Wells were then washed four times and 200 μl of CRP-horse radish peroxidase conjugate were added to each well and incubated for 2 hrs. Wells were then washed 4 times and 200 μl of substrate (mixture of x M tetramethylbenzidine and stabilized hydrogen peroxide) then added for 30 mins at room temperature. The reaction was then stopped by the addition of 50 μl of stopping solution and absorbance read at 450 nm utilizing a mutliplate reader from PerkinsElmer inc.

Table 2.2 – Materials utilized in performing CRP ELISA.

<table>
<thead>
<tr>
<th>Material</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRP microplate</td>
<td>96 well pre coated plate with a mouse monoclonal antibody against CRP</td>
</tr>
<tr>
<td>CRP conjugate</td>
<td>21 mL vial of monoclonal antibody against CRP conjugated to horseradish peroxidase.</td>
</tr>
<tr>
<td>CRP standard</td>
<td>Six vials of 50 ng/vial of recombinant human CRP in a buffered protein base.</td>
</tr>
<tr>
<td>Assay diluent</td>
<td>6 mL vial of a buffered protein base with preservatives.</td>
</tr>
<tr>
<td>Calibrator diluent concentrate</td>
<td>21 mL vial of a concentrated buffered protein base.</td>
</tr>
<tr>
<td>Wash buffer concentrate</td>
<td>21 mL vial of a 25-fold concentrated solution of buffered surfactant.</td>
</tr>
<tr>
<td>Color reagent A</td>
<td>12 mL vial of stabilized hydrogen peroxide</td>
</tr>
<tr>
<td>Color reagent B</td>
<td>12 mL vial of stabilized chromogen (tetramethylbenzidine)</td>
</tr>
<tr>
<td>Stop solution</td>
<td>6 mL vial of 2 N sulfuric acid</td>
</tr>
</tbody>
</table>
2.7.1.4

Development Tyrosine Kinase (DTK)

We utilized a sandwich ELISA from RandD Systems Inc (catalogue no. DY859) to determine the concentration of DTK in our patient serum samples.

Materials Utilized

DTK ELISA was performed according to the manufacturers (R&D Systems Inc) instructions. Briefly 100 μl of serum or standard and 100 μl of diluent were added to wells for 2hrs at room temperature. Wells were then washed four times and 200 μl of CRP-horse radish peroxidase conjugate were added to each well and incubated for 2 hrs. Wells were then washed 4 times and 200 ul of substrate (mixture of x M tetramethylbenzidine and stabilized hydrogen peroxide) then added for 30 mins at room temperature. The reaction was then stopped by the addition of 50 μl of stopping solution and absorbance read at 450 nm nm utilizing a multiplate reader from PerkinsElmer inc.

| Table 2.3 – Materials utilized in performing DTK ELISA. |
|----------------------|---------------------------------------------------------|
| **Material**         | **Description**                                         |
| Human Dtk capture antibody | 1 vial which is reconstituted with 1 ml of PBS to a stock concentration of 360μg/ml |
| Human Dtk detection antibody | 1 vial which is reconstituted with 1 ml of PBS to a stock concentration of 36μg/ml |
| Human Dtk standard   | 3 vials provided which is reconstituted as directed below |
| Streptavidin-HRP     | 1 vial which contains 1.0ml of streptavidin conjugated to horse radish peridoxiase. |
| Phosphate buffered saline (PBS) | See preparation below |
| Reagent diluent      | 1% Bovine serum albumin (BSA) in PBS |
| Substrate solution   | Substrate Solution: 1:1 mixture of Color Reagent A (H2O2) and Color Reagent B (Tetramethylbenzidine) |
| Wash Buffer          | 0.05% Tween in PBS- see preparation below |
| Stop solution        | 2 N H2SO4 |

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2.7.1.5

**Transforming growth factor beta II receptor (TGFIIβR) ELISA**

The TGF-β receptors are a family of cytokines receptors that are ubiquitous, multifunctional and essential to survival. They play important roles in growth and development, inflammation and repair and host immunity [225]. We measured the concentration in serum of TGFIIβR utilizing an ELISA kit from *RandD Systems Inc* (catalogue no. DY241).

**Materials Utilized**

TGFIIβR ELISA was performed according to the manufacturers (*R&D Systems Inc*) instructions. After blocking plates with capture body overnight we added 100 μl of serum or standard and 100 μl of diluent, which were incubated for 2hrs at room temperature. Wells were then washed four times, detection antibody added and plates incubated for two hours. After a repeat wash Streptavidin-horse radish peroxidase conjugate were added to each well and incubated for 2 hours. Wells were then washed 4 times and 100 μl of substrate (mixture of x M tetramethylbenzidine and stabilized hydrogen peroxide) then added for 30 minutes at room temperature. The reaction was then stopped by the addition of 50 μl of stopping solution and absorbance read at 450 nm utilizing a mutliplate reader from PerkinsElmer inc.
Table 2.4: Materials utilized in performing TGFIIβR ELISA

<table>
<thead>
<tr>
<th>Material</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human TGFIIβR capture antibody</td>
<td>1 vial which is reconstituted with 1ml of PBS to produce a stock concentration of 144μg/ml</td>
</tr>
<tr>
<td>Human TGFIIβR detection antibody</td>
<td>1 vial which is reconstituted with 1 ml of reagent diluent to a stock concentration of 36μg/ml</td>
</tr>
<tr>
<td>Human TGFIIβR standards</td>
<td>3 vials supplied, see reconstitution below</td>
</tr>
<tr>
<td>Streptavidin HRP</td>
<td>1 vial which is diluted with reagent diluent 1:200</td>
</tr>
<tr>
<td>PBS</td>
<td>See reagent preparation for instructions</td>
</tr>
<tr>
<td>0.05% Tween in PBS</td>
<td>See reagent preparation for instructions</td>
</tr>
<tr>
<td>Reagent diluent: 1% BSA in PBS</td>
<td>See reagent preparation for instructions</td>
</tr>
<tr>
<td>Substrate solution</td>
<td>1:1 mixture of Color Reagent A (H2O2) and Color Reagent B (Tetramethylbenzidine)</td>
</tr>
<tr>
<td>Stop solution</td>
<td>2 N H2SO4</td>
</tr>
</tbody>
</table>
2.7.1.6

**Galectin 3 (Gal-3)**

Galectins are a family of carbohydrate-binding proteins with specificity for N-acetyl-lactosamine-containing glycoproteins [266]. Galectin-3 as a biomarker appears to be actively involved in both the inflammatory and fibrotic pathways whose expression is associated with inflammatory cells including macrophages, neutrophils, and mast cells. We utilized an ELISA kit from *RandD Systems Inc.* (catalogue no. DGAL 30) to determine the concentration of Galectin 3 in serum samples. This assay employed a quantitative sandwich enzyme immunoassay technique.

**Materials Utilized**

Gal-3 ELISA was performed according to the manufacturers (*R&D Systems Inc*) instructions. After blocking plates with capture body overnight we added 50 μl of serum or standard and 100 μl of diluent, which were incubated for 2hrs at room temperature. Wells were then washed four times, Gal-3 conjugate added and plates incubated for two hours. After a repeat wash Streptavidin-horse radish peroxidase conjugate were added to each well and incubated for 2 hours. Wells were then washed 4 times and 100 μl of substrate (mixture of x M tetramethylbenzidine and stabilized hydrogen peroxide) then added for 30 minutes at room temperature. The reaction was then stopped by the addition of 50 μl of stopping solution and absorbance read at 450 nm utilizing a multiplate reader from PerkinsElmer inc.
Table 2.5 - Materials for performing Galectin 3 EILSA

<table>
<thead>
<tr>
<th>Material</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human Galectin-3 Microplate</td>
<td>96 well polystyrene microplate coated with a monoclonal antibody specific for human Galectin</td>
</tr>
<tr>
<td>Calibrator Diluent</td>
<td>21 mL vial of animal serum with preservatives.</td>
</tr>
<tr>
<td>Human Galectin-3 Conjugate</td>
<td>21 mL vial of a polyclonal antibody specific for human Galectin-3 conjugated to horseradish peroxidase.</td>
</tr>
<tr>
<td>Human Galectin-3 Standard</td>
<td>Recombinant human Galectin-3 in a buffered protein base reconstituted as stated below.</td>
</tr>
<tr>
<td>Assay Diluent</td>
<td>11 mL vial of a buffered protein.</td>
</tr>
<tr>
<td>Wash Buffer Concentrate</td>
<td>21 mL vial of a 25-fold concentrated solution of buffered surfactant with preservative. Diluted as instructed below.</td>
</tr>
<tr>
<td>Color Reagent A</td>
<td>12 mL vial of stabilized hydrogen peroxide</td>
</tr>
<tr>
<td>Color Reagent B</td>
<td>12 mL vial of stabilized chromogen (tetramethylbenzidine)</td>
</tr>
<tr>
<td>Stop Solution</td>
<td>6 mL vial of 2 N sulfuric acid.</td>
</tr>
</tbody>
</table>
Human Frizzled Homolog 7 (HF7)

Frizzled 7 belong to a family of G-protein couple receptors that serve as receptors in the Wnt pathway and play a key role in cell differentiation and development [267]. Utilizing a quantitative sandwich ELISA kit from *MyBioSource Inc.* (catalogue no. MBS9302973) we quantified the concentrations of frizzled seven in serum samples of our patients.

**Materials Utilized**

HF-7 ELISA was performed according to the manufacturers (*R&D Systems Inc.*) instructions. After blocking plates with capture body overnight we added 50 μl of serum or standard and 100 μl of diluent, which were incubated for 2hrs at room temperature. Wells were then washed four times, HF-7 conjugate added and plates incubated for two hours. After a repeat wash Streptavidin-horse radish peroxidase conjugate were added to each well and incubated for 2 hours. Wells were then washed 4 times and 100 ul of substrate (mixture of x M tetramethylbenzidine and stabilized hydrogen peroxide) then added for 30 mins at room temperature. The reaction was then stopped by the addition of 50 ul of stopping solution and absorbance read at 450 nm utilizing a mutliplate reader from PerkinsElmer inc.

**Table 2.6** Materials for performing HF7 ELISA

<table>
<thead>
<tr>
<th>Material</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample diluent</td>
<td>6 mls provided with kit.</td>
</tr>
<tr>
<td>Standards</td>
<td>6 different sample concentrations pre mixed.</td>
</tr>
<tr>
<td>HRP- conjugate reagent</td>
<td>10 mls provided.</td>
</tr>
<tr>
<td>Wash solution</td>
<td>25 mls provided and requires dilution.</td>
</tr>
<tr>
<td>Substrate solution A and B</td>
<td>6 mls of each solution provided with kit.</td>
</tr>
<tr>
<td>Stop Solution</td>
<td>6 mls provided with kit.</td>
</tr>
</tbody>
</table>
2.7.1.8

Human Interlukin-15 Receptor subunit alpha (IL15Rα)

IL15Rα specifically binds IL15 with very high affinity and is capable of binding IL-15 independently of other subunits. IL15 is a cytokine produced by mononuclear phagocytes and enhances the proliferation of natural killer cells. We utilized a sandwich ELISA kit from MyBioSource Inc. (catalogue no. MBS284419) to quantitate IL15Rα plasma concentrations in our patients.

Materials utilized

IL15Rα was performed according to the manufacturers instructions (MyBioSource Inc.). After blocking plates with capture body overnight we added 100 ul of plasma and incubated it for 2hrs at room temperature. Wells were then washed four times, biotin conjugate added and plates incubated for two hours. After a repeat wash Streptavidin-horse radish peroxidase conjugate were added to each well and incubated for 2 hours. Wells were then washed 4 times and 100 μl of substrate (mixture of x M tetramethylbenzidine and stabilized hydrogen peroxide) then added for 30 mins at room temperature. The reaction was then stopped by the addition of 50 μl of stopping solution and absorbance read at 450 nm utilizing a mutliplate reader from PerkinsElmer inc.

Table 2.7- Materials for performing IL15Rα ELISA

<table>
<thead>
<tr>
<th>Material</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assay plate</td>
<td>Pre coated 96 well plate</td>
</tr>
<tr>
<td>Sample Diluent</td>
<td>One 20ml vial is provided</td>
</tr>
<tr>
<td>Biotin conjugate</td>
<td>One 20 ml via is provided</td>
</tr>
<tr>
<td>Biotin conjugate diluent</td>
<td>Provided with kit</td>
</tr>
<tr>
<td>Streptavidin-HRP</td>
<td>One 20 ml vial is provided</td>
</tr>
<tr>
<td>Streptavidin- HRP diluent</td>
<td>Provided with kit</td>
</tr>
<tr>
<td>Stop solution</td>
<td>10mls supplied with kit</td>
</tr>
</tbody>
</table>
Chapter 3

Results
3.1

Baseline Demographic Data

3.1.1

Age

Between September 2014 - March 2016 we recruited patients (n=203). The mean and standard deviation of ages for the entire cohort was 61.6 ± 11.6 years. There were 136 males (67%) and 67 females (33%). The mean age for males was 62.5 ± 1 year and for females was 59.7 ± 1.4 years with no significant differences in ages between groups (Fig. 3.1).

![Box plot of mean ages of males and females](image)

**Mean age of males and females**

**Figure 3.1** - Box plot of mean ages of males and females analyzed using an unpaired t test.
Patients n=106 (52%) presented with cardiac chest pain i.e. stable angina and ACS and n=97 (48%) with non-cardiac chest pain. There was a significant difference in ages between both groups. Patients presenting with chest pain of cardiac etiology were on average older with a mean age of 66.8 ± 11.2 years versus 56.1 ± 9.3 years for the NCCP cohort (Fig.3.2). There were significantly more male patients in the cardiac chest pain cohort with 77% being male versus 23% being females (p=0.0004). There was no significant sex difference in the NCCP cohort with 55% females and 45% males (p=ns).

![Box plot](image)

**Age distribution for cardiac versus non cardiac patients**

**Figure 3.2** – Box plot created using an unpaired t test comparing ages of patients with cardiac (n= 106) and non-cardiac chest pain (n = 97).
In the cardiac chest pain cohort there were 51 of 106 (48%) patients with stable angina and 55 of 106 (52%) with ACS. The average age for patients presenting with stable angina was $66 \pm 11.2$ years and $67 \pm 11.6$ years for ACS patients (Fig 3.3). Figure 3.4 summarises the total number of patients recruited into each grouping.

Fig 3.3 - Age comparison for patients presenting with stable angina and ACS. Age comparison utilizing an unpaired t-test of patients with stable angina ($n = 51$) and ACS ($n = 55$).
**Fig. 3.4** - Flow chart summarizing numbers of patients recruited into each group.

(NCCP-non-cardiac chest pain, ACS-acute coronary syndrome, AMI- acute myocardial infarction, UA-unstable angina, NSTEMI-non ST elevation myocardial infarction, STEMI-ST segment myocardial infarction).

There were a higher proportion of patients presenting with cardiac chest pain than would be expected for an all-comer population with chest pain. This illustrates the fact that many patients were recruited at the time of presentation to the cathlab for angiography.
3.1.2

**Cardiovascular Risk Factors**

We documented traditional cardiovascular risk factors that included a family history of premature CAD, diabetes, hypertension, hypercholesterolaemia and a smoking history. Figure 3.5 below highlights the incidence of risk factors in both groups. Patients in the cardiac chest pain cohort had on average 2.8 risk factors versus 1.9 for those presenting with NCCP.

![Cardiac risk factors for each group i.e. non-cardiac and cardiac chest pain. The x-axis displays individual risk factors and y-axis percentage of patients in each group with that risk factor.](image)

**Fig 3.5** – Cardiac risk factors for each group i.e. non-cardiac and cardiac chest pain. The x-axis displays individual risk factors and y-axis percentage of patients in each group with that risk factor.
3.1.3

Chest pain scores

HEART Score

The HEART score was significantly higher for patients presenting with cardiac chest pain compared to NCCP. The average HEART score for ACS was 5.1 ± 2.1, 4.3 ± 1.4 for stable angina and 2.3 ± 1.9 for NCCP as shown in Fig. 3.6.

![HEART score for all groups](chart)

**Fig. 3.6** – Comparison of HEART score for all patient groups. This was analyzed using a one-way ANOVA.

TIMI and GRACE score

The TIMI score and GRACE score were utilized in patients presenting with ACS. The mean TIMI score was 3.8 ± 1.9 (range 0-7). The mean GRACE score was 149 ± 45.7 (range 1-372) which meant the average patient was in the higher range for death or
repeat infarction within six months (see chapter 1). Figure 3.7 below demonstrates the degree of correlation between the GRACE and TIMI score for ACS patients. There was only a weak degree of correlation between the scores.

![Correlation between TIMI and GRACE score in ACS](image)

*Figure 3.7 – The degree of correlation between GRACE and TIMI scores in patients presenting with ACS.*

3.1.4

**Duration of symptoms**

We recorded both the duration of chest pain symptoms and the last incidence of chest pain prior to sample acquisition. Patients in the NCCP had a significantly longer duration of symptoms compared to those presenting with cardiac chest pain (Fig. 3.8). The mean length of symptoms for NCCP was 143 ± 400 days versus 64 ± 100 days in the cardiac cohort.
NCCP group had a significantly longer duration from the last chest pain episode to time of recruitment demonstrating the varied nature of their chest pain symptoms. The mean duration since the last episode of chest pain in the cardiac cohort was $50.65 \pm 13.51$ hours versus $146.8 \pm 37.47$ hours in those with NCCP (Fig. 3.8).

**Fig. 3.8**- Comparison of duration of chest pain symptoms in cardiac and non-cardiac chest pain cohorts analyzed using an unpaired t test.
When we look at duration of chest pain symptoms and time after the last chest pain episode for NCCP, stable angina and ACS we find that patients with AMI (NSTEMI and STEMI) were most likely to have ongoing chest pain at the time or recruitment into the study (Fig. 3.10).

Fig. 3.9 - Difference in time after last chest pain to recruitment analyzed using an unpaired t test.
Fig. 3.10 - Time after last chest pain to recruitment for non-cardiac chest pain, stable angina and acute myocardial infarction. This analysis was performed using a one-way ANOVA.

3.1.5

Ischemic ECG changes

In all patients who were recruited a key component to their investigation and management was the presence of ischaemic ECG changes. We considered ST segment depression or elevation, new LBBB or RBBB, new T wave inversion and QT prolongation >500ms as markers of possible ischaemia. We found that in patients with ACS the ECG was ischaemic in 72% of patients with ACS and in 33% of those with stable angina (p<0.0001). No patients with NCCP had ischemic ECG changes but 17% did have an abnormal ECG.
3.1.6

Exercise ECG (stress testing)

For the entire cohort 68 of 203 patients had an exercise ECG evaluation. Of those 53 were performed in the evaluation of NCCP. A positive stress test was reported in 29% (20 of 68), which prompted further investigations for the detection of ischaemia.

3.1.7

Transthoracic Echocardiography (TTE).

One of the key predictors of mortality in cardiovascular disease is reduced ejection fraction. In patients with NCCP 81% had TTE performed with 82% of patients with cardiac chest pain having TTE performed. The vast majority of patients had a normal ejection fraction with a reduced ejection fraction reported in 9.8% of patients (20/203), 19 of who were patients presenting with ACS.

3.1.8

Coronary Angiography

The majority of patients underwent angiography either invasive or noninvasive. In total 87.6% (178/203) had coronary angiography performed. Of these 19.7% (35/178) had CTCA performed to determine total plaque burden.

All patients presenting with cardiac chest pain i.e. ACS and stable angina, had invasive coronary angiography. For patients with NCCP 70% (68/97) had invasive coronary angiography and 15% (15/97) had CTCA performed.

As a marker of plaque burden at angiography we calculated the GENSINI and Syntax scores, which have been discussed in the methods. The mean GENSINI score for the
entire population was $19.6 \pm 23.6$ and mean Syntax score was $5.6 \pm 7.7$. Fig. 3.11 and 3.12 shows the difference for both scores when grouped into NCCP, stable angina and ACS. As anticipated patients with higher plaque scores were more likely to have cardiac symptoms and present with ACS. The mean GENSINI score was highest for ACS presentations at $37 \pm 25$, for stable angina it was $25.6 \pm 23$ and $5.48 \pm 10.2$ for NCCP.

![Comparison of GENSINI score](image)

**Fig. 3.11** - Comparison of the GENSINI score for all groups analyzed using a one-way ANOVA.

Similarly when we analyzed the Syntax score for all three groups there was a similar dichotomy with the highest score in patients presenting with ACS and lowest in NCCP (Fig. 3.11). The mean scores for each group were $0.56 \pm 2.1$ in NCCP, $8.1 \pm 6.5$ in stable angina and $12 \pm 8.6$ in ACS patients ($p<0.0001$). Both scores indicate that this was a cohort of patients with intermediate plaque burden.
Finally we looked at the degree of correlation between the GENSINI and Syntax scores and found it to have a high degree of correlation.

Fig 3.12 – Syntax score comparisons between all three groups utilizing a one-way ANOVA analysis.

Fig 3.13- Correlation between Syntax and GENSINI scores for the entire patient cohort.
3.1.9

Biochemical Data

All patients (n=203) had baseline laboratory data recorded. This included full blood counts (FBC), urea and electrolytes (U/e) and HsT or Tn. Fifty-seven percent (115/203) of patients had C-reactive protein (CRP) and fasting risks (triglycerides, high density lipoprotein, low density lipoprotein and fasting glucose) performed.

The mean hemoglobin for the entire population was 13.6 ± 1.6g/dl with no significant differences between groups as demonstrated in Fig. 3.14.

![Fig. 3.14 – Hemoglobin for each group.](image)

The total white cell count (WCC) in patients with ACS was significantly higher than in patients with NCCP and stable angina. The mean total WCC for patients with NCCP was 6.8 ± 2.4 cells/l, 7.3 ± 2.2 cell/l for stable angina and 8.5 ± 2.9 cells/l for ACS. (Fig. 3.15)
Of the 203 patients 37/203 or 18% had impaired renal function. In those with impaired function the majority, 81% (30/37) fell into stage 1 and 2 chronic kidney disease classification.

Cardiac HsT was elevated in 31/203 patients with a mean of 957 ± 2067ng/ml (normal <14ng/ml). Only patients with NSTEMI and STEMI had elevated troponins. All patients with diagnosed with non-cardiac chest pain, stable angina and unstable angina had normal troponin values. There was a significant correlation noted between Tnt and total WCC with r=0.23(p<0.001).

Finally in patients whom had their total fasting cholesterol checked it was highest in the NCCP group at 4.58 ± 1.2g/dl, 3.8 ± 1.13g/dl for stable angina and 3.4 ± 1.4g/dl in patients presenting with ACS. However 47 % of patients with stable angina and AMI had already been prescribed cholesterol reduction medication on admission.
3.2

3.2.1

C Reactive protein

CRP quantification was performed in 200 of the 203 patients in the cohort. The mean concentration of CRP in patients with NCCP was $22 \pm 24$ pg/ml compared to $42 \pm 36$ pg/ml in patients with cardiac chest pain ($p < 0.0001$) (Fig. 3.16).

![CRP NCCP vs Cardiac Chest Pain](image.png)

**p < 0.0001**

**Fig. 3.16** – Comparison of CRP concentration using an unpaired t-test in patients with NCCP and cardiac chest pain (n= 200). CRP was significantly higher in cardiac chest pain patients.

In the cardiac chest pain sub groups the mean CRP concentration for STEMI was $57 \pm 37$ pg/ml, $48 \pm 41$ pg/ml for NSTEMI, $38 \pm 37$ pg/ml for UA and $37 \pm 34$ pg/ml for stable angina ($p = 0.34$) (Fig.3.16). There was significant correlation between CRP concentration and time after last chest pain for the entire cohort (Fig. 3.18). There was
no significant correlation between total duration of symptoms (days) and CRP concentration ($r = 0.1$, $p = 0.4$).

\[
p = 0.34
\]

Fig. 3.17 – One-Way ANOVA comparing CRP concentrations in different cardiac chest pain groups. This was not statistically significantly different.

\[
r = 0.2
\]
\[
p = 0.01
\]

Fig. 3.18 – Correlation between CRP and time after last chest pain episode for the entire patient cohort. There was a weak level of correlation.
When we look at correlations between markers of total plaque burden like the GENSINI and Syntax score we note that there is a significant correlation between CRP and the GENSINI score but not the Syntax score (Fig. 3.19 and 3.20).

**Fig. 3.19** – Correlation between CRP and GENSINI score for the entire patient cohort. There was a weak but statistically significant correlation.

\[ r = 0.2 \]
\[ p = 0.01 \]

**Fig. 3.20** – Correlation between CRP and Syntax score for the entire cohort. This was not statistically significant.

\[ r = 0.13 \]
\[ p = 0.09 \]
Similarly when the extent of correlation between total CRP concentrations, clinical scoring systems and biochemical data are analyzed we find varying degrees of correlation as presented in Table 3.1.

**Table 3.1** – Correlation between CRP and various clinical score and biochemical markers. (LDL- low-density lipoprotein, HDL- high-density lipoprotein).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Pearson's Correlation Coefficient</th>
<th>( p ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEART score</td>
<td>0.1</td>
<td>0.09</td>
</tr>
<tr>
<td>TIMI Score</td>
<td>0.3</td>
<td>0.04</td>
</tr>
<tr>
<td>GRACE score</td>
<td>0.2</td>
<td>0.08</td>
</tr>
<tr>
<td>Total WCC</td>
<td>0.4</td>
<td>0.04</td>
</tr>
<tr>
<td>Troponin</td>
<td>0.01</td>
<td>0.7</td>
</tr>
<tr>
<td>Creatinine</td>
<td>0.3</td>
<td>0.05</td>
</tr>
<tr>
<td>LDL</td>
<td>0.1</td>
<td>0.5</td>
</tr>
<tr>
<td>HDL</td>
<td>0.004</td>
<td>0.8</td>
</tr>
</tbody>
</table>
3.2.2

Developmental Tyrosine Kinase (DTK)

We enrolled 203 patients into our study of which 195 had DTK ELISA analysis performed to determine the total serum DTK concentration (range 0-10ng/ml). The mean age for the DTK cohort was 61.5 ± 11.5 years, with 116 (59.5%) being male patients and 79 (40.5%) female patients. 100 of these patients were diagnosed with cardiac chest pain and 95 diagnosed with NCCP. For patients with cardiac chest pain 50 patients presented with symptoms of stable angina and 50 with ACS.

Fig. 3.21 – Comparisons of ages for DTK groups. This analysis was performed using a one-way ANOVA and shows that patients with ACS and Stable angina were older than those with NCCP.

The oldest group was for those patients presenting with ACS with a mean age of 68 ± 11 years.
Fig 3.22 highlights the distribution of serum concentrations of DTK in all patients. The mean concentration for all DTK samples was \(2.53 \pm 2.1\) ng/ml. When divided into quartiles the 25\(^{th}\) quartile was \(0.97\) ng/ml, median \(1.83\) ng/ml and 75\(^{th}\) quartile \(3.79\) ng/ml.

![Fig. 3.22 – Scatter plot of DTK concentrations for all patients.](image)

When we compare the ranges in non-cardiac and cardiac chest pain we find that the mean DTK level is lower in the cardiac cohort at \(2.3 \pm 2\) ng/ml versus \(2.8 \pm 2.2\) ng/ml in NCCP (Fig.3.23).

![Fig 3.23- Comparison of DTK concentrations in NCCP and cardiac chest pain patients.](image)

We utilized an unpaired t-test for this analysis.
After subdivision into individual groupings as shown in Fig.3.24, there are no significant differences in serum DTK concentration between all three groups. The mean for patients presenting with stable angina was 2.2 ± 1.7 ng/ml which was less than in NCCP but slightly less than patients presenting with ACS with a mean of 2.37 ± 2.2 ng/ml.

![Graph showing DTK levels in NCCP, Stable Angina, and ACS patients.](image)

**Fig 3.24** - DTK levels showed no difference in NCCP, stable angina and ACS patients.

Further subgroup analysis revealed that in patients presenting with troponin positive ACS (n=44) the mean DTK serum concentration was 2.83 ± 2.4 ng/ml. This was higher than in patients with stable angina 2.2 ± 1.7 ng/ml but similar to those with NCCP 2.8 ± 2.2 ng/ml. However these differences were not significant Fig 3.25 below highlights the spread of data for these three cohorts.

Troponin positive patients were further divided into patients presenting with NSTEMI and STEMI. There were no significant differences between groups with the mean DTK concentration in STEMI patients of 2.9 ± 0.7 ng/ml versus 2.8 ± 0.5 ng/ml in patients with NSTEMI (Fig. 3.25).
**Fig 3.25** - DTK levels showed no difference in Troponin positive patients compared to NCCP and Stable Angina

**Fig 3.26** – DTK in STEMI compared to NSTEMI patients.
We also determined if there was any correlation of serum DTK to total plaque burden. There was a weak degree of correlation for total Syntax score and DTK (Fig. 3.27). However this was not significant.

![Fig 3.27 - Correlation between DTK and Syntax score. There was no significant correlation.](image)

Similarly when we determined the degree of correlation between total GENSINI score and DTK concentrations there was no significant correlation (Fig. 3.28).

![Fig 3.28 - Correlation between DTK and GENSINI score for the entire cohort. There was no significant correlation.](image)
In an attempt to better understand the nature of DTK in a population with coronary artery disease we performed a stepwise multivariate regression analysis with age, sex, creatinine, hemoglobin, troponin and total WCC as independent variables in our equation. As presented in table 3.2 below the only significant association was serum creatinine levels.

**Table 3.2-** Multiple stepwise linear regression results for DTK. (r = Pearson’s correlation coefficient, Hb- hemoglobin, WCC- total white cell count)

<table>
<thead>
<tr>
<th>Variable</th>
<th>r</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creatinine</td>
<td>0.15</td>
<td>0.04</td>
</tr>
<tr>
<td>Age</td>
<td>0.082</td>
<td>0.274</td>
</tr>
<tr>
<td>Sex</td>
<td>0.080</td>
<td>0.277</td>
</tr>
<tr>
<td>Troponin</td>
<td>0.045</td>
<td>0.549</td>
</tr>
<tr>
<td>Hb</td>
<td>0.014</td>
<td>0.846</td>
</tr>
<tr>
<td>WCC</td>
<td>-0.035</td>
<td>0.628</td>
</tr>
</tbody>
</table>

Finally we determined whether DTK level correlated with the HEART score, TIMI score or GRACE score. As demonstrated in Table 3.3 there was no significant correlation.

**Table 3.3-** Correlation between DTK and chest pain scoring tools. (r= Pearson’s correlation coefficient)

<table>
<thead>
<tr>
<th>Variable</th>
<th>R</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEART score</td>
<td>0.05</td>
<td>0.5</td>
</tr>
<tr>
<td>TIMI score</td>
<td>0.04</td>
<td>0.6</td>
</tr>
<tr>
<td>GRACE score</td>
<td>0.06</td>
<td>0.9</td>
</tr>
</tbody>
</table>
3.2.2

Transforming growth factor II beta

Of the 203 patients recruited we performed serum quantification of TGFIIβR in 193. The mean age of TGFIIβR group was 61.6 ± 1.5 years. There were 139 (69%) males and 54 (31%) females. Males were slightly older than females with a mean age of 62 ± 11 years compared to 60 ± 11 years for females (p=NS). When divided into NCCP (93/193), stable angina (50/193) and ACS (50/193), patients with stable angina and ACS were significantly older than those with NCCP. (Fig. 3.29)

![Ages distribution for TGFIIβR in each cohort. The mean age was highest for patients with stable angina and ACS.](image)

The mean concentration of TGFIIβR for the entire cohort was 1290pg/ml (15.6-2000pg/ml). The 25th percentile was 148.7pg/ml, median 323.3pg/ml and 75th percentile 613.3pg/ml.
The mean concentration of TGFIIβR for patients with NCCP was 955 ± 2620pg/ml, 2208 ± 5891pg/ml for stable angina and 1095 ± 3440 for ACS patients (p<0.0001) (Fig 3.31).

**Fig. 3.30**- Scatter plot of concentrations for TGFIIβR for the entire cohort.

**Fig. 3.31**- TGFIIβR concentration in each cohort with the highest concentrations in those with stable angina.
Whilst the overall mean concentration of TGFIIβR in ACS patients was 1095pg/ml there was a trend noted with the highest TGFIIβR concentration in STEMI patients when compared to NSTEMI and UA patients (Fig. 3.32). When compared TGFIIβR concentration in patients with UA versus NCCP there was no significant difference (p=0.9).

As discussed previously CRP used as a control in our study and confirmed the CRP concentration was highest in patients with ACS when compared to patients with stable angina and NCCP.

When we analyzed the extent of correlation between CRP and TGFIIβR in all groups, CRP levels did not correlate well with TGFIIβR, r= -0.03, p=ns (Fig. 3.33).
To determine the utility of TGFIIβR as a marker of plaque instability in the accident and emergency department we performed a correlation analysis between TGFIIβR and both the HEART and TIMI scores. The degree of correlation was not statistically significant for both scores as demonstrated in Fig. 3.34 and Fig. 3.35.

**Fig. 3.33** - TGFIIβR correlation with CRP for the entire cohort. There was no significant correlation.

**Fig. 3.34** – Correlation between HEART score and TGFIIβR concentration for all patients. This was not significant.
Fig. 3.35 - Correlation between the TIMI score and TGFIIβR concentration for the entire cohort. This was not significant.

Additionally there was no demonstrable correlation between TGFIIβR and total GENSINI or Syntax scores (p=ns for both).

Multiple linear regression was used to determine the associations between a range of biochemical analytes and serum TGFIIβR concentration. Table 3.4 presents the results. There was no significant association between TGFIIβR and hemoglobin levels, total WCC, platelet count, creatinine or LDL cholesterol.
Table 3.4 – Correlations between biochemical factors and TGFIIβR concentration. (LDL- low-density lipoprotein, WCC- white cell counts)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Pearson's correlation coefficient</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemoglobin</td>
<td>r = -0.07</td>
<td>0.3</td>
</tr>
<tr>
<td>WCC</td>
<td>r = -0.06</td>
<td>0.4</td>
</tr>
<tr>
<td>Total platelet count</td>
<td>r = -0.05</td>
<td>0.4</td>
</tr>
<tr>
<td>Creatinine</td>
<td>r = -0.06</td>
<td>0.4</td>
</tr>
<tr>
<td>LDL</td>
<td>r = 0.01</td>
<td>0.9</td>
</tr>
</tbody>
</table>

Finally we determined if there was any association between TGFIIβR and ejection fraction. In our population of 193 patients 20 patients had reduced left ventricular function on echocardiography. The mean ejection fraction for the normal population was 60 ± 7 and 45 ± 8 for patients with reduced function. However we did not detect any significant difference in TGFIIβR concentration between group means (p=0.2).
3.2.3

Galectin-3 (Gal-3)

We measured the serum concentration of Gal-3 in 191 (94%) of patients in our study. The mean age for the population was 61 ± 11 years with 126 (65%) male patients. There were 96 (50%) patients with NCCP and 95 patients (50%) with cardiac chest pain. In those with cardiac chest pain 47 presented with stable angina and 48 with ACS. Again patients in the ACS cohort were the oldest with a mean age of 69 ± 11 years compared to 67 ± 12 for stable angina and 56 ± 9 for NCCP (p<0.0001).

The mean concentration of serum Gal-3 for the entire cohort was 8.9 ± 2.7 ng/ml. The 25th percentile was 7.1 ng/ml, median 9 ng/ml and 75th percentile 10.6 ng/ml. Fig 3.36 shows the scatter plot for all data points.

![Scatter plot of Gal-3 concentration for all patients.](image)

Taking into account the ages of all patients we found a positive correlation between older age and increasing Gal-3 concentration as demonstrated in Fig. 3.37. There was
no significant difference in Gal-3 concentration between males and females (p= 0.9). Mean concentration in males was 8.9 ± 0.22 ng/ml and 9 ± 0.4 ng/ml in females.

![Graph](image)

**Fig. 3.37** - Correlation between Gal-3 concentration and age for the entire patient cohort. There was a significant correlation with increased concentration and increasing age.

There was a significant difference in concentration of Gal-3 between NCCP and cardiac chest pain patients. The mean Gal-3 concentration for those with NCCP was 8.4 ± 0.3 ng/ml compared to 9.3 ± 0.3 ng/ml for patients with cardiac chest pain (p=0.02). Fig 3.38 below illustrates the spread of data.
Fig. 3.38 - Box plots of Gal-3 concentration in NCCP and cardiac chest pain demonstrating a significant increase in patients with cardiac chest pain.

The concentration on Gal-3 was highest in patients with ACS presentations with a mean of 9.8 ± 2.6 ng/ml, for those with stable angina it was 9 ± 2.7 ng/ml and 8.4 ± 2.6 ng/ml in NCCP (p=0.2). Fig 3.39 below illustrates the spread of data for each group.

Further when the ACS cohort was divided into UA (21), NSTEMI (14) and STEMI (13) the highest concentration of serum Gal-3 was in those patients presenting with NSTEMI (p=ns). The mean Gal-3 concentration for NSTEMI was 10.15 ± 2.6ng/ml compared to 9.8 ± 1.3ng/ml for STEMI and 9.9 ± 2.9ng/ml in UA (Fig 3.40)
**Fig. 3.39** Gal-3 concentration in chest pain cohorts showing that there was a significant elevation in those with ACS.

**Fig. 3.40**- Gal-3 concentration in each ACS cohort with no significant differences between groups.
There was no significant correlation for Gal-3 and CRP for the entire cohort as shown in Fig. 3.41 below.

Fig. 3.41 - Correlation between CRP and Gal-3 for all patients. This was not significant.

When we examine the correlation between CRP and Gal-3 in patients with cardiac chest pain i.e. stable angina and ACS there was a significant degree of correlation, \( r = 0.2, p = 0.05 \) (Fig. 3.42).

Fig. 3.42 – Correlation between CRP and Gal-3 in patients with cardiac chest pain.
The degree of correlation is even stronger in patients with ACS between Gal-3 and CRP, \( r = 0.43, p= 0.004 \) as shown in Fig. 3.43 below.

![Graph showing correlation between Gal-3 and CRP](image)

**Fig. 3.43** – Degree of correlation between Gal-3 and CRP in ACS patients. There was a significant degree of correlation observed.

To determine if Gal-3 could be utilized as an acute marker of unstable plaque/plaque rupture we firstly compared the concentration in patients with ongoing chest pain or if their last chest pain was within three hours to those with whose symptoms were greater than three hours previously, in all groups. For pain <3h (n= 75) the mean concentration of Gal-3 was 9.6 ± 0.3 ng/ml compared to pain >3h (n= 116) which was 8.6 ± 0.2 ng/ml (p = 0.01)(Fig. 3.44).

The difference in serum concentration of Gal-3 in patients with their last chest pain <12h compared to > 12 h was examined and found to be statistically significant. For patients with >12 h since their last pain (n= 90) the mean concentration of Gal-3 was 8.5 ± 0.3 ng/ml versus 9.4 ± 0.3 ng/ml in patients with last pain episode <12h (n=101) (p=0.02) (Fig.3.45).
**Fig. 3.44** – Box plot of Gal-3 concentration in patients with pain <3h and > 3h showing that there was a significant elevation if symptoms were < 3h old or ongoing.

**Fig. 3.45** – Box plot of Gal-3 concentration in patients with pain <12h previously and >12h. In those with pain <12h there was a significant elevation of Gal-3 compared to patients who had symptoms >12h previously.
At 24 hours after chest pain there was no significant difference in concentration of Gal-3 between groups. Mean concentration of Gal-3 in patients with their last pain > 24 hours (n= 88) was 8.6 ± 0.3 ng/ml versus 9.1 ± 0.3 ng/ml in those with their last chest pain symptoms <24 h (n= 103) (p = 0.2).

Whilst we confirmed that there was a change in concentration in Gal-3 levels with respect to time after the last chest pain this was in the entire cohort of our study. In an attempt to examine the relationship more closely we analyzed the patient cohort with chest pain symptoms <12h. There were 101(53%) of patients presenting with symptoms of chest pain in the preceding 12 hours.

This was composed of 50 patients with NCCP and 51 patients with cardiac chest pain. As expected the mean Gal-3 concentration in the cardiac cohort was significantly elevated at 10.1 ± 0.4ng/ml versus 8.7 ± 0.3ng/ml in NCCP (p = 0.01) (Fig. 3.46).

Fig. 3.46 – Gal-3 concentrations in cardiac and non-cardiac chest pain for patients with pain <12hours. There was a significant elevation in Gal-3 concentration for patients with cardiac chest pain and symptoms <12h old.
As discussed Gal-3 has been found to be significantly elevated in patients with left ventricular dysfunction and is a marker of myocardial fibrosis. In our cohort 19 (10%) had a reduction in left ventricular function with mean ejection fraction of 39 ± 7.8. The mean Gal-3 concentration patients with normal LV function were 9.4 ± 0.25ng/ml and 9 ± 0.6ng/ml in patients with reduced LV function (p=0.6).

Serum Gal-3 concentrations are known to correlate with creatinine level. We observed a significant increase in Gal-3 levels in patients with all degrees of renal impairment as shown in Fig. 3.47. However only 5 patients with ACS had mild to moderate renal impairment and serum Gal-3 concentrations did not differ significantly between those with ACS and renal impairment and those without renal impairment (p=ns).

**Fig. 3.47** – Gal 3 in normal versus abnormal renal function. Patients with impaired renal function had a significant increase in Gal-3 concentration compared to those with normal function.
Finally we examined the extent of correlation for the entire cohort between Gal-3 and Heart score, TIMI score, GRACE score, Gensini score, Syntax score, total WCC, hemoglobin, cardiac troponin levels, creatinine, LDL and triglycerides. These results are presented in table 3.5. There were significant correlations between Gal-3 concentration and the GRACE score, serum creatinine and total hemoglobin concentration.

**Table 3.5**: Correlation between Gal-3 clinical and angiographic scores and biochemical data for entire cohort. (WCC-total white cell count, LDL – low density lipoprotein)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Pearson’s Correlation Coefficient</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEART score</td>
<td>0.09</td>
<td>0.2</td>
</tr>
<tr>
<td>TIMI score</td>
<td>0.02</td>
<td>0.9</td>
</tr>
<tr>
<td>GRACE score</td>
<td>0.4</td>
<td>0.01</td>
</tr>
<tr>
<td>GENSINI score</td>
<td>0.08</td>
<td>0.3</td>
</tr>
<tr>
<td>Syntax score</td>
<td>0.1</td>
<td>0.2</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>-0.17</td>
<td>0.001</td>
</tr>
<tr>
<td>WCC</td>
<td>0.12</td>
<td>0.1</td>
</tr>
<tr>
<td>Platelets</td>
<td>0.03</td>
<td>0.7</td>
</tr>
<tr>
<td>Troponin</td>
<td>0.09</td>
<td>0.2</td>
</tr>
<tr>
<td>LDL</td>
<td>-0.08</td>
<td>0.4</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>0.06</td>
<td>0.4</td>
</tr>
<tr>
<td>Creatinine</td>
<td>0.2</td>
<td>0.006</td>
</tr>
</tbody>
</table>

We then evaluated the degree of correlation of Gal-3 concentration and various clinical, angiographic and biochemical results in the entire cardiac chest pain cohort and in then in ACS patients only. The results are presented 3.6 and 3.7. The statistically significant correlations were between Gal-3 and the GRACE score, creatinine level and hemoglobin level, similar to that found in entire patient group.
Table 3.6- Correlation between Gal-3 and clinical, angiographic and biochemical data in cardiac chest pain patients. (WCC-total white cell count, LDL – low-density lipoprotein).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Pearson’s Correlation Coefficient</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEART score</td>
<td>0.05</td>
<td>0.6</td>
</tr>
<tr>
<td>TIMI score</td>
<td>-0.04</td>
<td>0.7</td>
</tr>
<tr>
<td>GRACE score</td>
<td>0.3</td>
<td>0.03</td>
</tr>
<tr>
<td>Syntax score</td>
<td>0.001</td>
<td>0.9</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>-0.3</td>
<td>0.004</td>
</tr>
<tr>
<td>WCC</td>
<td>0.1</td>
<td>0.2</td>
</tr>
<tr>
<td>Platelets</td>
<td>0.1</td>
<td>0.2</td>
</tr>
<tr>
<td>Troponin</td>
<td>0.5</td>
<td>0.4</td>
</tr>
<tr>
<td>Creatinine</td>
<td>0.3</td>
<td>0.005</td>
</tr>
<tr>
<td>LDL</td>
<td>-0.1</td>
<td>0.3</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>0.02</td>
<td>0.6</td>
</tr>
</tbody>
</table>

Table 3.7- Correlation between Gal-3 in ACS patients and clinical, angiographic and biochemical data. (WCC-total white cell count, LDL – low density lipoprotein).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Pearson’s Correlation Coefficient</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEART score</td>
<td>-0.11</td>
<td>0.5</td>
</tr>
<tr>
<td>TIMI score</td>
<td>0.1</td>
<td>0.5</td>
</tr>
<tr>
<td>GRACE score</td>
<td>0.4</td>
<td>0.01</td>
</tr>
<tr>
<td>GENSINI score</td>
<td>-0.17</td>
<td>0.2</td>
</tr>
<tr>
<td>Syntax score</td>
<td>-0.06</td>
<td>0.7</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>-0.4</td>
<td>0.003</td>
</tr>
<tr>
<td>WCC</td>
<td>-0.03</td>
<td>0.8</td>
</tr>
<tr>
<td>Troponin</td>
<td>0.006</td>
<td>0.9</td>
</tr>
<tr>
<td>Creatinine</td>
<td>0.24</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Table 3.8 – Multivariate regression to determine if there were any risk factors that were significant predictors of total Gal-3 levels in the entire cohort.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Pearson’s Correlation Coefficient</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypertension</td>
<td>0.084</td>
<td>0.4</td>
</tr>
<tr>
<td>Smoker</td>
<td>0.29</td>
<td>0.054</td>
</tr>
<tr>
<td>Family history</td>
<td>-0.19</td>
<td>0.084</td>
</tr>
<tr>
<td>Diabetes</td>
<td>0.18</td>
<td>0.95</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>0.109</td>
<td>0.9</td>
</tr>
</tbody>
</table>
3.2.4

**Homolog Frizzled 7 (HF7)**

In total we performed serum quantification of HF7 concentration in 195 (96%) of all patients recruited. The mean age was $62 \pm 12$ years with 131 (67%) males. The mean concentration of HF7 for the entire cohort was $120.8 \pm 93.8$ng/ml. Fig. 3.48 shows a scatter plot of HF7 concentrations for all patients.

![Fig. 3.48- Scatter plot for HF7 concentration in all patients.](image)

There were 101(52%) with cardiac chest pain with a mean concentration of $124 \pm 93$ng/ml and 94(48%) patients with NCCP with a mean concentration of $117.1 \pm 70$ng/ml. As shown in Fig. 3.49 there were no significant differences between groups.
There were 50 (26%) patients with stable angina and 51 (26.1%) patients with ACS. The mean concentration in patients with NCCP was 117.1 ± 70 ng/ml, in stable angina was 115 ± 95 ng/ml and 124.8 ± 83 ng/ml in patients with ACS (p=ns) as illustrated in Fig. 3.50 below.
Patients presenting with ACS were further subdivided into UA, NSTEMI and STEMI. The mean HF7 concentration was not significantly different for all three groups when compared to the control group with NCCP.

The mean for those with UA was 107 ± 73ng/ml, for NSTEMI it was 143 ± 90ng/ml and 124 ± 89ng/ml for those with STEMI (p = 0.6)(Fig. 3.51).

**Fig. 3.50**- HF7 data spread in NCCP, Stable angina and ACS. There were no significant differences observed.
To determine if there was an association between HF7 concentration and time after presentation with chest pain or the total duration of chest pain symptoms we performed a correlation analysis as shown in Fig.3.52 and 3.53. There was no significant correlation between HF7 concentration and either total duration of chest pain or time since the last episode of chest pain.
Fig. 3.53 - Correlation between HF7 and time after last chest pain episode. There was no significant correlation noted.

Whilst there was no significant correlation between HF7 and CRP for the entire cohort we further analyzed the degree of correlation by patient subgroup. These results are presented in table 3.9 below. There was a significant positive correlation between HF7 and CRP in STEMI with $r = 0.63$ ($p=0.02$).

Table 3.9 - Pearson’s Correlation coefficient between CRP and HF7 for different patient presentations.

<table>
<thead>
<tr>
<th>Group</th>
<th>Pearson’s Correlation Coefficient</th>
<th>$p$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stable angina</td>
<td>0.3</td>
<td>0.07</td>
</tr>
<tr>
<td>STEMI</td>
<td>0.63</td>
<td>0.02</td>
</tr>
<tr>
<td>UA</td>
<td>0.06</td>
<td>0.8</td>
</tr>
<tr>
<td>NSTEMI</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>NCCP</td>
<td>0.04</td>
<td>0.6</td>
</tr>
</tbody>
</table>
To determine if there was any association between clinical chest pain scoring tools a correlation analysis between HF7 and the HEART, TIMI and GRACE score was performed. The results are presented in Table 3.10.

Table 3.10- Correlation between HF7 and clinical chest pain scoring tools

<table>
<thead>
<tr>
<th>Score</th>
<th>Pearson's Correlation Coefficient</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEART score</td>
<td>0.12</td>
<td>0.1</td>
</tr>
<tr>
<td>TIMI score</td>
<td>0.23</td>
<td>0.04</td>
</tr>
<tr>
<td>GRACE score</td>
<td>-0.16</td>
<td>0.3</td>
</tr>
</tbody>
</table>

We also analyzed our data looking for any significant association between HF7 and total coronary plaque burden measured using the GENSINI score and Syntax score. We did not find a significant correlation for either. Pearson’s correlation coefficient for HF7 and GENSINI was 0.01 (p = 0.6). For HF7 and Syntax score the Pearson’s correlation coefficient was 0.001 (p = 0.9).
3.2.6

**Interleukin 15 receptor alpha (IL-15Rα)**

We determined the plasma concentration of IL-15Rα in 184 (91%) of the total patient cohort. The mean age was 61 ± 12 years with 101 (55%) male patients. The mean concentration of IL-15Rα for the entire cohort was 609 ± 513 pg/ml (31.25-2000pg/ml). Fig. 3.54 demonstrates the scatter plot plasma concentration of IL-15Rα for all patients.

![Scatter plot of IL-15Rα concentrations in all patients.](image)

There were 101 (55%) patients with cardiac chest pain and 83 (45%) of patients with NCCP. The mean concentration of IL-15Rα in patients with cardiac chest pain was 686 ± 52 pg/ml and was significantly higher than the value of 515 ± 54 pg/ml in patients with NCCP (Fig. 3.55).

In the patients with ACS the mean IL-15Rα concentration of 728 ± 74 pg/ml, which was higher than the mean of 629 ± 76 pg/ml in patients with stable angina (p=0.4) (Fig. 3.56).
**Fig. 3.55**- Box plots comparing IL-15Rα in NCCP and cardiac chest pain. Patients with cardiac chest pain had significant elevations of IL-15 Rα.

\[ p = 0.03 \]

**Fig. 3.56**- Box plots of IL-15Rα concentration in ACS and stable angina. There was no significant difference noted.

\[ p = 0.4 \]
When ACS patients were compared to patients with NCCP we note a significant difference in IL-15Rα concentration (\( p = 0.02 \)) (Fig. 3.57).

\[ p = 0.02 \]

**Fig. 3.57** - Box plots of comparison in IL-15Rα concentration in NCCP and ACS patients. ACS patients had a significantly elevated concentration of IL-15Rα.

ACS patients were divided into the subgroups NSTEMI, UA and STEMI and analyzed for any significant differences between mean concentrations of IL-15Rα (Fig. 3.58).

\[ p = 0.8 \]

**Fig. 3.58** - Spread of data for IL-15Rα concentration in ACS groups with no significant difference between groupings.
The mean concentration of IL-15Rα for NSTEMI was 698 ± 465 pg/ml, for STEMI it was 688.3 ± 546.3 pg/ml and for UA was 819 ± 608 pg/ml (p= 0.8).

The concentration of IL-15Rα in patients with UA was significantly higher than in patients with NCCP (Fig. 3.59)

![Box plot comparing IL-15Rα concentration in NCCP and UA. There was a significant elevation in IL-15Rα for unstable patients.](image)

**Fig. 3.59** - Box plot comparing IL-15Rα concentration in NCCP and UA. There was a significant elevation in IL-15Rα for unstable patients.

Whilst we have demonstrated that patients with UA had the highest concentration of IL-15Rα we analyzed our data to evaluate if there was any association with IL-15Rα concentration and total duration of symptoms in the entire cohort. As demonstrated in Fig. 3.60 below there was a significant correlation between increasing length of chest pain symptoms and IL-15Rα concentration. However there was no significant correlation for IL-15Rα concentration and time after the last episode of chest pain in the whole cohort (Fig. 3.61).
Fig. 3.60 - Correlation between duration of chest pain and IL-15Rα concentration.

\[
\begin{array}{c}
r = 0.3 \\
p = 0.0004
\end{array}
\]

Fig. 3.61 - Correlation between time after last chest pain episode and IL-15Rα concentrations.

\[
\begin{array}{c}
r = -0.01 \\
p = 0.9
\end{array}
\]
Patients presenting with cardiac chest pain had a higher concentration of IL-15Rα than those with non-cardiac chest pain as shown earlier. Additionally when we looked at time differences in the cardiac chest pain cohort we noted that those with chest pain within the preceding 12 hours had a higher concentration of IL-15Rα than those whose last chest pain was > 12 hours previously (Fig. 3.62). The mean concentration of IL-15Rα was 605 ± 70 pg/ml if chest pain had occurred > 12 hours previously compared to 765 ± 76 pg/ml if chest pain symptoms have occurred in the previous 12 hours.

\[ p = 0.1 \]

**Fig. 3.62** – Box plot demonstrating differences in IL-15Rα concentration in cardiac chest pain if pain was >12 hours or <12 hours previously.

When we extended the time frame to 24 hours after the last chest pain episode there was no significant difference in IL-15Rα concentration. The mean concentration if a patient’s chest pain was >24 hours previously was 600 ± 57 pg/ml compared to 668 ± 105 pg/ml for < 24 hours (p = 0.4).
If IL-15Rα were to be utilized as a marker of impending plaque rupture, levels would have to be highest in those with possible unstable plaque. We selected ACS patients who had symptoms of ischaemic chest pain in the preceding 12 hours. There were 33 such patients of whom 12 were STEMI, 10 UA and 11 NSTEMI. Fig. 3.63 below graphically represents the spread of this data.

We noted that the highest concentration of IL-15Rα was in those presenting with UA. The mean concentration in this cohort was 957 ± 619pg/ml compared to 758 ± 478pg/ml in NSTEMI and 688 ± 546pg/ml in STEMI patients (p = 0.5).

![Image of box plots](image-url)

**Fig. 3.63**- Box plots of IL15Rα in patients with ACS and chest pain in preceding 12 hours. There was no significant difference between groups.

Our data were then analyzed to determine if there was any significant correlation between IL-15Rα concentration and total plaque burden measured by the GENSINI and Syntax scores but there was no significant correlation noted (p=ns).
Similarly we determined if there was significant correlation between the clinical scoring tools like the HEART, TIMI and GRACE score and IL-15Rα concentration. Whilst there was no significant correlation with either the TIMI or GRACE score there was a significant correlation with the total HEART score (Fig. 3.72).

![Graph](image)

**r = 0.2  
*p = 0.03**

**Fig. 3.64** Correlation between the IL-15Rα concentration and the total HEART score.

Finally we analyzed our data to determine if there were any significant correlations between biochemical parameters and IL-15Rα concentrations. The results are presented in Table 3.12 below.
Table 3.11 – Correlation coefficients for IL-15Rα and various biochemical indices.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Pearson’s Correlation Coefficient</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin</td>
<td>0.1</td>
<td>0.4</td>
</tr>
<tr>
<td>Total WCC</td>
<td>0.2</td>
<td>0.06</td>
</tr>
<tr>
<td>Creatinine</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>HDL</td>
<td>-0.08</td>
<td>0.4</td>
</tr>
<tr>
<td>LDL</td>
<td>-0.13</td>
<td>0.4</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>0.09</td>
<td>0.3</td>
</tr>
<tr>
<td>Troponin</td>
<td>-0.08</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Table 3.12 – Multivariate regression analyzing whether any cardiac risk factors in the total cohort were predictors of IL-15Rα levels.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Pearson’s Correlation Coefficient</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypertension</td>
<td>0.1</td>
<td>0.4</td>
</tr>
<tr>
<td>Diabetes</td>
<td>0.11</td>
<td>0.38</td>
</tr>
<tr>
<td>Family history</td>
<td>0.14</td>
<td>0.24</td>
</tr>
<tr>
<td>Smoking</td>
<td>0.063</td>
<td>0.06</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>0.061</td>
<td>0.07</td>
</tr>
</tbody>
</table>
3.3

Correlation between biomarkers

We evaluated the degree of correlation between the different markers as shown in table 3.13. For markers that were found to be statistically significantly correlated further subgroup analysis was performed and presented in the preceding figures.

Table 3.13 - Correlation between biomarkers for the entire population.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>DTK</th>
<th>TGFIIβR</th>
<th>Gal-3</th>
<th>IL-15Rα</th>
<th>HF7</th>
</tr>
</thead>
<tbody>
<tr>
<td>DTK</td>
<td>NA</td>
<td>r = 0.33</td>
<td>p &lt; 0.0001</td>
<td>r = 0.01</td>
<td>r = 0.05</td>
</tr>
<tr>
<td>TGFIIβR</td>
<td>r = 0.33</td>
<td>NA</td>
<td>r = -0.01</td>
<td>r = 0.05</td>
<td>r = 0.2</td>
</tr>
<tr>
<td>Gal-3</td>
<td>r = -0.01</td>
<td>p = ns</td>
<td>p = ns</td>
<td>NA</td>
<td>p = ns</td>
</tr>
<tr>
<td>IL-15Rα</td>
<td>r = 0.05</td>
<td>r = 0.05</td>
<td>r = 0.4</td>
<td>NA</td>
<td>p = 0.01</td>
</tr>
<tr>
<td>HF7</td>
<td>r = 0.01</td>
<td>r = 0.01</td>
<td>r = 0.1</td>
<td>r = -0.4</td>
<td>NA</td>
</tr>
</tbody>
</table>

Fig. 3.65 – Correlation between DTK and TGFIIβR for all patients

Fig 3.65 and 3.66 present the degree of correlations between DTK and TGFIIβR in ACS and stable angina cohorts.
Fig. 3.66 – Correlation between DTK and TGFIIβR in ACS. This was a significant correlation.

\[ r = 0.3 \]
\[ p = 0.08 \]

Fig. 3.67 – Correlation between DTK and TGFIIβ in stable angina. There was significant correlation in this cohort.

\[ r = 0.6 \]
\[ p < 0.0001 \]
Fig. 3.68 - Correlation between IL15Rα and HF7 in ACS. This was a significant correlation.

Fig. 3.69 – Correlation between Gal-3 and IL-15Rα in ACS. This was a significant correlation.
Fig. 3.70 - Summary of all biomarker levels in NCCP, Stable angina and ACS cohorts.
(Gal-3- Galectin 3, DTK-Developmental tyrosine kinase, TGFIIβR-Transforming growth factor beta two receptor, HF7- Homolog frizzled 7, IL-15Rα- Interleukin 15 receptor alpha)
Chapter 4

Discussion
4.1

Introduction

Atherosclerosis is the leading cause of occlusive arterial disease and atherosclerosis complicated by local thrombosis is the initiating event leading to acute ischaemic syndromes such as unstable angina, acute myocardial infarction, and many cases of sudden cardiac death. Thrombosis superimposed on atherosclerotic plaque is most often due to rupture of the fibrous cap of the plaque with exposure of thrombogenic material in the plaque to circulating blood, triggering platelet accumulation, clotting cascade activation, and fibrin deposition. A smaller proportion of arterial thrombi occur without plaque rupture and result from superficial endothelial denudation (plaque erosion) coupled with a local or systemic pro-thrombotic state.

Plaque inflammation is believed to play a key role in plaque vulnerability and eventually plaque rupture and thrombosis by creating a local milieu that promotes collagen degradation in the fibrous cap and inhibits collagen synthesis by promoting smooth muscle cell death (Fig. 5.1).

![Diagram of plaque vulnerability determinants]

**Fig. 4.1**- Determinants of plaque vulnerability.
In view of the central role of inflammation and immune cell activation in determining plaque vulnerability to rupture and thrombosis, systemic markers of inflammation have been evaluated in asymptomatic subjects as well as in acute coronary syndrome patients. However the majority of validated markers currently available confirm that plaque rupture has occurred and are not predictive of its occurrence.

We sought to evaluate whether our novel biomarkers initially identified in our ApoE knockout mouse model, would be predictive of plaque instability in a human population. Whilst one biomarker of plaque rupture with high diagnostic certainty would be the gold standard we hypothesized that a panel of biomarkers predictive of plaque rupture would increase diagnostic accuracy. It was also envisioned that this panel would be additive to current clinical chest pain scoring tools which are utilized to identify higher risk patients presenting to the accident and emergency department. The individual markers we measured are discussed in the upcoming section.
4.2

4.2.1

Developmental tyrosine kinase (DTK)

Defective phagocytic clearance of dying cells is linked to the progression of atherosclerotic plaque. During atherogenesis, apoptotic cell turnover in the vascular wall is counterbalanced by neighbouring phagocytes thereby limiting cellularity and maintaining lesion integrity. However, as lesions mature, phagocytic removal of apoptotic cells (efferocytosis) becomes defective, leading to secondary necrosis, expansion of plaque necrotic cores and susceptibility to rupture.

Apoptotic cell recognition by mononuclear phagocytes activates an intracellular signaling cascade leading to efficient efferocytosis. The tyrosine kinase receptors play an important role in this process. The essential role of the receptors in apoptotic cell uptake by mononuclear phagocytes likely results because they can perform two crucial functions simultaneously i.e. providing assembly points for a broad range of signaling intermediaries and activating multiple molecules crucial for actin cytoskeleton restructuring [203].

DTK in our mouse model was expressed in serum pre-plaque rupture and peaked post rupture. In our human study we confirmed that DTK was present at varying concentrations and confirmed that it was easily assayed. We found that DTK in patients with NCCP was higher than those with cardiac chest pain. This was mainly driven by the down regulation of DTK in patients with stable angina with lower serum concentrations than in patients with NCCP. This finding is supported by findings of Huratdo et al. who showed that DTK and AXL are down regulated in advanced carotid atherosclerotic plaque along with up regulation of the Mertk receptor [209].

In patients presenting with ACS and specifically STEMI DTK concentration did not change significantly when compared to the control group with NCCP. DTK in ACS patients divided into STEMI and NSTEMI did not differ significantly. Additionally there
was no significant correlation between DTK concentration and total plaque burden measured by the GENSINI or Syntax scores. There was also no significant correlation between clinical chest pain scoring tools and serum DTK levels.

DTK in our study was not a marker of acute plaque rupture or coronary plaque burden. Firstly as discussed previously the DTK receptor is normally found in brain and adult kidney tissue whereas the other tyrosine kinase receptors are found in endothelial tissue. This may explain some of our findings. Secondly DTK may be down regulated in atherosclerotic plaque due to the affinity of GAS-6 for the other tyrosine kinase receptors specifically Mertk [268]. GAS-6 has been shown to be associated with unstable plaque but its interaction in coronary plaque is mostly with the Mertk receptor and not DTK [208].

Wan et al. demonstrated that Mertk one of the tyrosine kinase receptors was not elevated in non-infarcted hearts in a mouse model but was significantly elevated post infarction. Mertk deficient mice also had increased infarct size and reduced cardiac function [269]. Importantly the DTK receptor is known to be more prevalent in neural, testicular and ovarian tissue. It is known that heterodimerization of the tyrosine kinase receptors exist and it is possible that DTK acts with Mertk in unstable plaque to enhance efficient apoptosis. One future direction would be to simultaneously measure Mertk in serum and correlate it to DTK levels.

GAS-6 a ligand for the DTK receptor is present in higher concentrations in unstable plaque when compared to stable fibrous plaque [208]. Indeed, in a mouse model of atherosclerosis, loss of Gas-6 promoted the formation of more fibrotic atherosclerotic lesions and attenuated persistent inflammation, although the number and size of initial and advanced plaques and the systemic cholesterol levels were unaffected [208]. It would be interesting to measure GAS-6 in a chest pain population and correlate it to both DTK and Mertk.

In conclusion DTK was not a marker of atherosclerotic plaque burden or unstable plaque in our cohort.
4.2.2

**Transforming growth factor beta 2 receptor (TGFIIβR)**

TGF-β is a major modulator of the fibroproliferative response to tissue damage. In the early stages of repair, TGF-β is released from platelets and activated from matrix reservoirs; it then stimulates the chemotaxis of repair cells, modulates immunity and inflammation and induces matrix production. At later stages, it negatively regulates fibrosis through its strong antiproliferative and apoptotic effects on fibrotic cells [270].

We have shown that TGFIIβR is elevated in patients with ACS and stable angina when compared to patients presenting with NCCP. The highest concentrations in the ACS group were in those presenting with either STEMI or NSTEMI, i.e. patients with likely plaque rupture or erosion. These findings are consistent with those presented by Deten et al. who showed significant elevation of both TGF-β1 and TGF-β2 in a rat model 12 hours after ligation of the anterior descending artery reaching a maximum on day three and four after an infarct [271].

In the context of acute myocardial infarction TGF-β plays a significant role with different effects depending on the vascular cell being stimulated. Lymphocytes that proliferate in unstable plaque and plaque rupture are suppressed by TGF-β [272]. Additionally TGF-β induces apoptosis in both B and T cells and this may be why serum concentration is increased in ACS patients. At sites of vascular injury, TGF-β1 is released from degranulating platelets in mural thrombus [273].

Additionally attraction of macrophages to the site of vascular injury and thrombosis results in increased levels of TGF-β and activation of both TGFβ1R and TGFβ2R. This acts to modulate the response of the vessel wall to injury as demonstrated in a mouse model previously [274].

However the reason the levels of TGFIIβR are increased in patients presenting with stable angina remains to be determined. It is likely that in patients with stable angina there is an up regulation of the receptor in response to the underlying chronic
inflammatory process. TGF-β exerts potent and diverse actions on each of the cell types involved in vascular disease [275]. Endothelial cells tend to be strongly inhibited by TGF-β with respect to their migration and proliferation [276]. Smooth muscle cells that proliferate in the atherosclerotic process are inhibited by TGF-β. Immunohistochemical staining of carotid and coronary arteries demonstrated that TGF-β levels were higher in stable plaques compared to unstable plaques possibly explaining our findings [277].

With time and fibrotic plaque development there is a reduction in the TGFIβR with the majority of advanced fibrous lesions expressing low and variable levels of the TGFIβR, but generally much lower levels of the TGFIβR [278]. In our population of UA we recorded a lower concentration of TGFIβR when compared to a cohort of patients presenting with stable angina. This is consistent with previously described findings of low TGF-β levels in patients with UA [278]. We must however recognize that our population of patients with UA was small and these findings were not statistically significant.

Whilst TGF-β and TGF-βR play a crucial role in plaque development we were unable to correlate it to total plaque burden using either the GENSINI or Syntax score. This may be explained by the fact that most areas of plaque at any one time are stable and progress indolently, with periods of acute or sub-acute thrombosis and recruitment of inflammatory cells with subsequent increased expression of TGF-βR. Intravascular imaging such as OCT may have allowed us to determine features of ‘active’ from ‘inactive plaque’ to correlate to TGFIβR serum concentrations, however these imaging tools are used in a minority of cardiac procedures in Ireland.

Prior experiments indicate that TGFIβR is expressed in the blood stream 3-5 hours after myocardial infarction [295]. Lefer et al. first suggested a protective effect of exogenous TGF-β in the ischaemic heart. They demonstrated that TGF-β injection reduced ischaemic myocardial injury presumably by attenuating the effects of pro-inflammatory cytokines [296]. Further evidence suggests an important role for TGF-β signaling in resolution of inflammation and repression of cytokine and chemokine gene synthesis. Its effects on macrophages are primarily deactivating, suppressing
chemokine and pro-inflammatory cytokine synthesis. In addition, TGF-β inhibits chemokine synthesis by cytokine-stimulated endothelial cells [297].

TGFβ is shown to have an important role in the healing heart by promoting matrix deposition by inducing extracellular matrix protein expression by fibroblasts and by inhibiting matrix degradation [298]. TGFIIβR inhibition after the inflammatory phase of infarct healing resulted in decreased fibrous tissue deposition in the infarcted area. Although anti-TGFβ treatment markedly decreased collagen deposition in the infarct, it also altered the qualitative characteristics of the wound, increasing the cellular content and the density of myofibroblasts [299].

TGFβ both propagates inflammation and has a protective role in the post infarction heart. This delicate balance is borne out in various experiments. Anti TGFβ gene therapy within twenty-four hours following infarction enhanced cytokine and chemokine synthesis and increased neutrophil infiltration resulting in increased left ventricular dysfunction and increased mortality. In contrast, late TGF-β inhibition attenuated cardiac hypertrophy and decreased interstitial fibrosis in the remodeling heart reducing left ventricular dilatation and dysfunction [299, 300].

In summary we have demonstrated that TGFIIβR is elevated in both ACS and stable angina patients in keeping with previously published work in animal studies. Further evaluation in unstable angina patients is required to determine its utility in aiding the prediction of coronary plaque rupture.

4.2.3

**Galectin 3 (Gal-3)**

Gal-3 was significantly elevated in patients presenting with cardiac chest pain when compared to those with non-cardiac chest pain. In patients with cardiac chest pain the Gal-3 levels were significantly elevated in both ACS and stable angina. In ACS patients the concentration of Gal-3 was significantly elevated in patients with UA who were less
likely to have undergone plaque rupture given their negative troponin assays. In troponin positive patients presenting with either NSTEMI or STEMI we noted a significant increase in concentration of Gal-3 but not significantly different from patients presenting with UA.

Various studies have evaluated Gal-3 in patients presenting with confirmed myocardial infarction. Ipek et al. found that Gal-3 was elevated in patients presenting with STEMI compared to a normal control group [279]. In a study by Arar et al. Gal-3 gene expression was evaluated in animal models by inducing experimental atherosclerosis. The researchers observed activated expression of the Gal-3 gene in the smooth muscle cells of the hypercholesterolemic and artificially injured aorta, suggesting the involvement of the Gal-3 in atherogenesis [280].

The activity of Gal-3 in unstable plaque has also previously been postulated but clinical studies are sparse. Gal-3 levels were increased in atherosclerotic lesions that were rich in foam cells, whereas fibrotic atherosclerotic lesions had lower Gal-3 levels. Gal-3 positive cells were close to a lipid core, or to the areas with fibrosis, hemorrhage, or thrombosis in atherosclerotic lesions [218].

Our results demonstrate that Gal-3 levels were similar in patients with UA and STEMI or NSTEMI. This could imply that the elevation may not be related to actual plaque rupture and thrombus formation but the cascade of inflammatory events leading to the development of unstable plaque. Monocyte chemotaxis and differentiation into macrophages is pivotal to plaque destabilization. Degradation of the plaque cap results leading to the thin cap fibroadenoma that is a precursor of plaque rupture (Chapter 1). Gal-3 has been shown to dramatically increase in concentration when monocytes differentiate to macrophages possibly explaining the increase in Gal-3 in ACS [281]. We also noted an increase in Gal-3 levels in patients presenting with symptoms of stable angina. This likely reflects the indolent chronic inflammatory process that occurs within the arterial walls in stable angina and the recruitment of macrophages that occurs.

There were significant correlations between Gal-3 and the GRACE score, renal function and hemoglobin level. The GRACE score as described in chapter 1 is used to predict post
MI in-patient and six week mortality. Gal-3 is shown to be a predictor of long-term cardiovascular mortality with the highest rates in patients in the highest Gal-3 tertiles [282]. Certainly this would suggest that early measurement of Gal-3 concentration at presentation could complement standard models of adverse cardiac risk prediction. Patients with elevated Gal-3 at time of hospitalization with STEMI were more prone to develop new-onset atrial fibrillation and new-onset heart failure [283].

Special mention must be made of the association of Gal-3 levels and renal function. In our cohort increasing Gal-3 concentration was associated with increasing serum creatinine. This mirrors previous findings by Gopal et al. who demonstrated that declining renal function was associated with increasing serum Gal-3 [284]. As renal impairment and end stage renal function is directly related to cardiovascular and all-cause mortality it has been proposed that the increased mortality observed in patients with Gal-3 may in part be explained by underlying renal fibrosis and chronic kidney disease.

Studies have shown a positive correlation with the GENSINI score as a measure of total plaque burden and Gal-3 levels [279]. We were unable to replicate this finding. Additionally there was no significant correlation with the Syntax score. Overall our population was composed of mostly first time presenters with chest pain who had moderate plaque burden that may have contributed to the lack of correlation.

In summary Gal-3 shows promise as a marker of unstable coronary plaque in patients presenting with chest pain. Further larger studies are required to confirm this.

4.2.4

**Homolog frizzled 7 (HF7)**

HF7 concentration in our mouse model was higher pre-plaque rupture with a decrease in concentration post-plaque rupture. In our human cohort we did not find a statistically significant difference in HF7 concentration between patients with NCCP
and cardiac chest pain. The mean concentration of HF7 in patients presenting with cardiac chest pain was only marginally higher.

In STEMI patients we noted an increase in the mean concentration of HF7 suggesting that the Frizzled 7 receptor is being activated. Both Wnt5a and Dvl are expressed in inflamed atherosclerotic plaques that require binding to the HF7 transmembrane receptor as the first step [285].

The endothelium plays a crucial role in the initiation and progression of inflammation. In STEMI endothelial activation and subsequent dysfunction occurs. Wnt5a is a component of the endothelial inflammatory response. Kim et al. demonstrated that pulse-like endothelial stimulation with Wnt5a leads to significantly higher stimulation of proinflammatory pathways in vascular endothelial cells [259].

In NSTEMI patients the mean serum concentration of Hf7 was higher than in both patients with NCCP and STEMI. Patients presenting with STEMI were recruited immediately whilst in the midst of an acute myocardial infarction with ongoing chest pain, compared to those with NSTEMI who presented on average >24 hours after their last chest pain episode. This may have contributed to increased concentration of HF7 when compared to STEMI patients since there would have been further upregulation of the receptor as the infarct evolved. Upregulation of both Frizzled 1 and Frizzled 2 receptors has previously been demonstrated post-myocardial infarction [286].

Wnt signaling plays a pathophysiological role in myocardial infarction and its antagonism may be important for cell protection. In one study the frizzled receptors were identified as novel therapeutic targets in MI [53]. Laeremans et al. demonstrated that blockade of Frizzled receptors with a peptide fragment of Wnt5A resulted in the reduction of infarct expansion and prevented the development of heart failure after MI [286].

In our cohort of patients with NCCP we found that HF7 in serum was elevated. This was also above the recognised range of detection for the ELISA kit. Since we used many kits from the same company it would seem the results are valid. The exact reason for these
results is uncertain and possibly we needed to perform serial dilutions of serum samples. However the manufactures instruct that serum samples should be added neat to the microplate.

There is increasing evidence that Frizzled-Wnt complex plays in myocardial infarction and repair. We were unable to show a statistically significant increase in HF7 concentration or its utility as a marker of plaque rupture.

4.2.5

Interleukin 15 receptor alpha (IL-15Rα)

We have demonstrated that IL-15Rα is elevated in patients presenting with cardiac chest pain when compared to those with NCCP. In cardiac chest pain presentations, the highest concentration was in patients with UA when compared to stable angina, NSTEMI and STEMI. This elevation appears to occur in a time dependent manner with significant elevation for those patients presenting within 12 hours of their last chest pain.

IL-15 through its receptor has important effector functions. IL-15 is known to induce the proliferation of mature T cells, generation of cytotoxic T cells, and stimulation of cytokine production [287]. Furthermore, IL-15 acts as a chemoattractant for T cells and induces monocytes to secrete proinflammatory and chemotactic cytokines [288]. This activation of T cells and macrophages generate the production of plaque destabilization mediators such as IFNγ, TNF-α and matrix metalloproteinase [289]. Our observation of increased plasma concentration of IL-15Rα in patients with UA may reflect these factors. Furthermore the expression of IL-15 RNA has been demonstrated in unstable lipid-rich and fibrolipid plaque but not in stable fibrous plaques and normal vessels [243].

We have also demonstrated that there was an elevation in IL-15Rα plasma concentration in patients with stable angina symptoms compared to NCCP. This would be in keeping with previous findings by Gokkusu et al. who demonstrated that IL-15
was significantly elevated in patients presenting with both acute and chronic CAD which was also influenced by genetic variants of the IL-15 gene [244]. Whilst there was an elevation in IL-15Rα concentration in both stable and UA the increased levels in UA compared to stable angina lends additional weight to the utility of IL-15Rα as a possible marker of plaque instability.

In the published literature there are many studies that confirm significant correlation between total WCC, myocardial injury and long-term mortality from acute cardiac events [290]. In our cohort admission WCC was associated with six-week mortality post myocardial infarction (see appendix 1). In the correlation analysis we presented there was a significant correlation, albeit weak, between total WCC and IL-15Rα in patients presenting with chest pain suggesting an association between myocardial injury and IL-15Rα plasma concentration.

Perhaps the most well validated pathological cytokine in CAD is IL-6. IL-6 signaling is mediated by IL-6R and transduced via the JAK-1 transducer in a manner that is similar to IL-15Rα. Clinical studies have further revealed that IL-6 serum levels are increased in unstable angina patients and are considered an independent risk factor for CAD [62, 242]. It is likely that the inflammatory response in acute plaque instability is mediated by numerous cytokines. Whilst not validated in coronary disease there has been prior research showing upregulation of IL-6 and correlation with IL-15Rα in patients with rheumatoid arthritis and osteoarthritis [291].

Finally in attempting to understand the role of IL-15Rα in ACS, we note that the level of the IL-15Rα was lower in NSTEMI and STEMI patients than in UA. This mirrors the findings in our mouse model of plaque rupture with a decrease in concentration post plaque rupture. It is likely that there is an increase in concentration of IL-15 at time of plaque rupture leading to binding of IL-15 to its IL-15R and hence levels detected in plasma are lower. It would therefore be useful to measure IL-15 concentration in our samples to determine the association with the IL-15Rα.

In summary IL-15Rα shows promise as a marker of unstable coronary plaque and further investigation is warranted.
4.3

Translating a mouse model to a human population

In this thesis we found varying significance of our biomarkers in our human population when compared to ApoE knockout mouse model. Whilst a good animal model would reproduce the biochemistry of the disease it does not necessarily produce an identical morphology. For example cholesterol-fed rabbits have been used in atherosclerotic research for over 60 years and this model has provided highly relevant clues as to how lesions develop in humans. This research has been criticised because rabbits are herbivorous and handle cholesterol in a way very unlike humans.

The general method of choosing an animal model of atherosclerosis has been to use a top-down approach, whereby disease features known to be present in humans are required to be present in the animal model. However human lesions are extremely heterogeneous, even at similar anatomical sites. Most human atherosclerosis is the result of a lifetime of multiple risk factors such as tobacco smoking, elevated cholesterol levels, elevated blood pressure, unhealthy diet, age and genetic susceptibility. It is not possible to replicate all of these in a mouse model of atherosclerosis, but that does mean that these models should be ignored.

Apo E is a lipoprotein that plays a key protective role in atherosclerosis. ApoE also has antiatherogenic effects not involved directly in lipid metabolism which include inhibition of LDL oxidation, platelet aggregation, smooth muscle cell proliferation, endothelial cell proliferation and inhibition of T-lymphocyte activation and proliferation [292]. The exact effect of these changes in the cascade of events in plaque rupture is unknown and may account for some of the variability in biomarker levels between mouse and human populations. We also know that some biomarkers quantifiable in human populations may not exist in a mouse model, the example of CRP is one such, whereby mouse do not express CRP but do express Pentraxin as surrogate marker of inflammation. This may also explain some of the variability we have observed in our human study.
4.4

Final Conclusions:

There remains an unmet clinical need in diagnosing unstable coronary plaque thus facilitating intervention prior to myocardial injury. Many systemic markers are proposed. Traditional risk assessment algorithms such as the HEART risk score are useful means of discriminating individuals at low or high risk for atherothrombotic cardiovascular events but classify a large group of individuals as intermediate risk. Additionally there continues to be an over reliance on coronary angiography as demonstrated in our cohort where the majority of patients had either invasive or non invasive angiography. This finding reflects the fact the NCCP cohort had a mean HEART score of 2.3 and the availability of non invasive methods of ischemia detection are over subscribed in our hospital.

Circulating biomarkers that mirror the critical role of inflammation/oxidative stress in the pathophysiology of atherothrombosis have been tested in a number of clinical studies. Although statistically significant associations with risk have been identified for a number of such markers, the overall strength of these relationships has been rather weak making them of little clinical use. We measured the serum concentration of our five markers in a cohort of patients presenting with chest pain aiming to identify if these markers were predictive of plaque instability.

In our atherosclerotic mouse model we noted that DTK, a tyrosine kinase receptor was significantly elevated in pre rupture mice and peaked in mice that had histological confirmation of plaque rupture. The tyrosine kinase receptors have several signaling functions and are known to be located on platelets and mediate thrombosis and platelet stabilization. Much of this effect is mediated by activation of the tyrosine kinase receptor via its ligand GAS-6 which supports leukocyte extravasation and inflammation and adhesion of platelets to endothelial cells as discussed previously.

It would seem logical that DTK would be activated early in the process of plaque maturation and instability given the pivotal role of macrophages in the process of
thinning of the fibrous cap of plaque, prior to rupture. However our results did not demonstrate a significant change in DTK concentration in either patients with ACS or stable angina when compared to NCCP.

In our hands TGFβIIIR was significantly elevated in both patients with stable angina and among those with ACS at presentation. These findings mirror those of our mouse model. In mice we detected significant elevations in both pre rupture and post rupture assays that peaked post rupture. TGF-β plays a major role in the regulation of immune function and suppression of inflammation.

The cascade of events leading to plaque development is propagated by an underlying chronic inflammatory response. TGF-β via the TGFβI and TGFβII receptor is pivotal to regulation of this inflammatory process. Patients presenting with stable angina had moderate plaque burden as measure by both the GENsini and Syntax scores. As plaque proliferates there is activation of endothelial cells and proliferation of smooth muscle cells (Chapter 1). These mechanisms may explain the increase level of TGFβIIIR we observed in our stable angina population. In ACS patients we detected an increase in TGFβIIIR levels compared to NCCP. In unstable angina we noted a decrease and this is consistent with previous findings where TGFβIIR was up regulated in patients with early fatty plaque but suppressed in more advanced unstable plaques. Additionally with plaque maturation it has been observed that plaque is less responsive to the effects of TGFβ and hence provides a reason for the down regulation of the TGFβIIIR.

Myocardial infarction triggers an inflammatory response that ultimately leads to healing and scar formation. As discussed previously the repair process can be divided into the inflammatory phase, the proliferative phase and the maturation phase. Knowing that TGFβ plays a key role in this process the finding of elevated levels in ACS patients is not unexpected. However in determining the utility of TGFIIβR as a marker of unstable plaque an understanding of the time course of peak serum concentration of TGFβII receptor is essential.

Gal-3 serum concentration has gained momentum in the scientific literature as a marker of unstable plaque. Our findings may support this. Gal-3 is secreted by
macrophages and associated with fibroblast differentiation and collagen synthesis. It is involved in many biological processes, such as cell–cell and cell–extracellular matrix adhesion, cell growth and differentiation, cell-cycle signaling and apoptosis and in angiogenesis.

We noted a significant increase in Gal-3 levels in cardiac chest pain with highest levels in patients presenting with ACS. Highest levels were present if symptoms of chest pain were present in the preceding 12 hours. Given its role in macrophage activation this may explain the significant elevation of Gal-3 in ACS and patients presenting with stable angina. Additionally Gal-3 is implicated in the binding and internalization of modified lipoproteins resulting in intracellular cholesterol accumulation and binding to lipopolysaccharides, key pathological processes in the development of coronary plaque.

There have been conflicting reports in the literature as to the utility of Gal-3 in acute myocardial infarction and our work adds to the body of knowledge. In our cohort we demonstrated a significant elevation of Gal-3 in all ACS groups. Our finding of a direct correlation of Gal-3 levels to chest pain symptoms is novel and supports the hypothesis that Gal-3 may be a marker of plaque instability.

We noted a significant correlation between Gal-3 and IL-15Rα in population with coronary artery disease. This association was only statistically significant in patients with ACS. Gal-3 is a multifunctional molecule involved in diverse molecular and cellular events. It has been implicated in immune activation, allergic asthma, angiogenesis, obesity and idiopathic pulmonary fibrosis. Many of these effects are mediated by interleukins. The association of Gal-3 and IL-15Rα has not been directly studied. However given that both IL-15Rα and Gal-3 are increased in acute inflammation this may explain the association.

IL-15Rα showed encouraging results as a marker of plaque rupture. IL-15Rα was significantly elevated in our patients with cardiac chest pain and highest values were present among those with unstable angina. Why there should be increased IL-15Rα in patients with unstable plaque is explained by the attraction of macrophages and T cells to the site of an acute infarction. Elevation of interleukins in acute myocardial infarction
is well documented. However these have not always been evaluated as a marker of unstable plaque. What has not been determined specifically is the time course that IL-15/IL-15Rα complex stays elevated in blood. Specifically if we are to utilize IL-15Rα as a marker of unstable plaque we would have to determine the time after symptoms that it peaks and what length of time IL-15Rα stays elevated in serum and plasma.

In the further refinement of our potential biomarkers we would envision two main modifications to our protocol. Firstly serial measurements of our markers in the same patient would provide a better understanding of biomarker kinetics and the temporal relationship to symptoms. Secondly whilst we determined the concentrations of our markers in varying subgroups of chest pain we were unable to say definitively that patients had unstable plaque. The addition of intravascular characterization of unstable plaque using optical coherence tomography or intravascular ultrasound at the time of angiography would address this deficit.

In conclusion this thesis identified:

- Galectin 3 was significantly elevated in patients with cardiac chest pain, stable angina and ACS.
- In ACS patients Galectin 3 was significantly elevated prior to plaque rupture and correlated with the duration of symptoms.
- IL-15Rα was significantly elevated in patients presenting both with stable angina and ACS.
- IL-15Rα was elevated in patients with unstable angina who were troponin negative and possibly had unstable plaque.
- IL-15Rα correlated significantly with Gal-3 in patients with ACS, specifically in those with unstable angina.
- TGFIIβR was significantly elevated in patients with stable angina and decreased in those with unstable symptoms.
- DTK was not elevated in patients presenting with cardiac chest pain compared to those with NCCP. DTK was not elevated in ACS presentations.
- HF7 was a weak biomarker of acute inflammation in myocardial infarction and may play a role in remodeling post infarction.
**Future directions**

Inflammation plays a key role in atherosclerotic plaque formation, plaque destabilization, plaque disruption, and in ACS. Thus plaque rupture invariably precedes cardiomyocyte damage by minutes to potentially hours making biomarkers of plaque instability logical candidates for early ACS detection[315].

Relative risk for CAD is higher in people with more inflammatory biomarkers [tumor necrosis factor (TNF); CRP and IL-6] (one marker: RR, 1.17; two markers: RR, 1.22; three markers: RR, 2.13) [316]. A panel of three inflammatory biomarkers [IL-6; Serum A Amyloid and CRP] has been proposed to predict adverse events and death in middle-aged women with symptoms suggestive of myocardial ischemia. Patients without abnormal markers have fewer events (11.6%) compared to those with one (18.4%), two (20.9%), or three (37%) abnormal markers. This panel of inflammatory biomarkers added to the Framingham risk score [317].

Despite these individual successes a biomarker or panel of biomarkers the addition of imaging assessments in patients with chest pain have far out performed any inflammatory biomarker.

Firstly the use of CTCA to quantify total plaque burden and facilitate early discharge in the low to intermediate risk patient presenting with chest pain is safe and effective. The results of PROMISE trial presented in 2015 showed that CTCA was not however superior to functional testing in detecting cardiovascular outcomes at a median follow up of two years [318]. What CTCA does is quantify the degree of coronary stenosis, which is shown to predict future cardiac events. The addition of ischaemia testing in the form of PETCT will likely add incremental benefit in patients found to have >50% stenosis at CTCA. The presence of coronary ischaemia is directly related to increased rates of AMI as demonstrated in trials like COURAGE and FAME 2. Additionally CTCA features of unstable plaque like low attenuation plaque and PVR (external vessel wall diameter of ≥110% compared with normal proximal or distal segment) have been
shown to predict a higher risk of plaque rupture with further research currently ongoing [319].

Quantifying plaque inflammation is attractive as a method for detecting plaque instability. Non-invasive methods such as PET with high spatial resolution using FDG and recently 18F-sodium fluoride offers an attractive method of quantifying unstable plaque and requires further development and research. Along with imaging modalities novel biomarkers like MicroRNAs and individual proteomic signatures offers promise for earlier detection of unstable CAD. Micro RNAs function as gene regulators acting on mRNAs translation, with inhibition of protein synthesis. In the cardiovascular system, mRNAs fine-tune complex molecular signaling networks by acting on key target proteins involved in a variety of pathways and cellular processes many of which are involved in plaque maturation and instability.

With one quarter of global deaths attributed to cardiovascular disease the search for earlier detection and prevention of myocardial cell death will continue [320]. The panacea of a single maker to detect unstable plaque is unlikely. The development of integrated pathways utilizing non-invasive imaging and biomarkers of plaque inflammation probably offer the best approach of detecting plaque instability.
Appendix

Grant Support Awarded

2015- Royal Baggot Street Hospital, Dublin, Ireland
   Sum of 65,000 euro awarded for proteomic protocol
2014- Royal Baggot Street Hospital, Dublin, Ireland
   Sum of 100,000 euros awarded for research into
   “Novel Markers of Plaque Rupture” study.
2014- Irish Heart Foundation Research Bursary
   Sum of 5,000 euros awarded for “Novel Markers of Plaque
   Rupture” study.

Abstracts submitted to date from this work

A Bajrangee, S Mahabir, L Arkins, A Lopez, M Brennan, D Cox, AO Maree
Galectin-3 as a Novel Biomarker of Unstable Coronary Plaque
Circulation. November 2016;134:A18948

A Bajrangee, S Mahabir, A Tierney, R Flood, JJ Coughlan, C Hickie, FA Murray, B Gorna, P
Srinivas, I Ullah, V Sullivan, AO Maree
37 Neutrophil to lymphocyte ratio as a predictor of outcomes and plaque burden in ST
segment elevation myocardial infarction (STEMI)
Heart Oct 2016, 102 (Suppl 9) A20-A21; DOI: 10.1136/heartjnl-2016-310523.37

A Bajrangee, S Mahabir, R Flood, A Tierney, JJ Coughlan, I Ullah, V Sullivan, AO Maree
Does the HEART score predict outcomes after chest pain admissions and can it facilitate
a safe early discharge?
Heart Oct 2016, 102 (Suppl 9) A16; DOI: 10.1136/heartjnl-2016-310523.30
Role of each investigator

Dr Amrit Bajrangee wrote the initial ethics applications and all grant proposals. I also wrote the patient information leaflet and consent form. All patients were recruited by myself and I performed the initial bloodletting. All data recorded until discharge was undertaken by myself. I also performed initial sample preparation at St James’s Hospital, which involved centrifuge, pipetting the aliquot and storage of samples.

Raw materials for ELISA analysis were also bought by myself with grant support being administered by St James’s Foundation. Subsequently I performed 70% of the ELISA experiments and all interpretation at the department of Molecular and Cellular therapeutics at RCSI. Analysis of raw data including the statistical analysis was undertaken by myself. Finally preparation of this thesis and all abstracts submitted to date were prepared by myself.

Dr Andrew Maree in his role of clinical supervisor was involved in the initial study design. Dr Maree reviewed and edited the ethics and grant applications prior to submission. Additionally he has reviewed in detail the completed thesis and all published material from the work to date. His support and guidance has been invaluable.

Dr Dermot Cox and Dr Marian Brennan based at RCSI undertook the initial assessment of the serum from the ApoE knockout mice and developed the novel biomarker strategy. They were pivotal in reviewing all ethical and grant applications. Additionally they imparted the scientific knowledge and methods required for me to perform sample collection and analysis. They also review all drafts of this thesis.
Protocol

April 2014 Version 1

Novel Markers of Plaque Rupture in Patients with Chest Pain

MD Student
Dr Amrit Bajrangee

Supervisors
Dr Andrew Maree
Professor Dermot Cox
1. Background

Despite extensive research cardiovascular disease remains the leading cause of death in developed countries. Thus, its primary and secondary prevention remain a public health priority. Non-traumatic chest pain presentation to the accident and emergency department accounts for between 6-10% of patients’ seen and management of these patients is challenging. A recent study in a Spanish Centre found that over a three-month period 1518 patients presented with non-traumatic chest pain, representing 6% of the patients attending A&E. An ECG was performed in 1342 patients (88.4%), the troponin-T level was measured in 656 (43.2%), chest radiography was performed in 831 (54.7%), and 385 (25.4%) were evaluated by the cardiologist-on-call. Overall, 230 (15.2%) were admitted to hospital; of these, 99 (6.5%) had an acute myocardial infarction (AMI) and seven (0.5%) died during admission. Among patients discharged from the emergency department, the most frequent diagnoses were atypical chest pain (59%) and respiratory infection (12%). Although most do not have a life-threatening condition, the clinician must distinguish between those who require urgent management of a serious problem such as an acute coronary syndrome (ACS) and those with a more benign cause.

A small, but significant percentage of patients discharged from emergency departments with what is considered “non-cardiac” chest pain are ultimately diagnosed with ACS. Patients with acute MI are mistakenly discharged in 2% to 8% of cases. Management of acute chest pain also has significant economic implications. In Ireland patients make approximately 1.5 million visits to A & E each year. We previously published that the annual national cost of chest pain presentation to A & E is approximately €71 million and consumes 73,000 bed days nationally.

The main challenge is detecting patients with ACS who may be about to have an MI prior to them sustaining an injury. Certainly coronary angiography helps to discriminate the two processes but is invasive, expensive, and only available in a few hospitals and in most cases not on a 24 hr basis. The current approach to acute chest pain management therefore comprises, a history and clinical examination, screening with an ECG, Chest X ray and blood tests. Biochemical study typically involves serum
assay of either troponin and Mb fraction of creatine kinase. The problem with these assays is that there is a delay of at least six hours after onset of chest pain before they are detected in serum and when they are elevated myocardial injury has already occurred. Due to the risk of morbidity and mortality from myocardial infarction these patients are kept in a medical assessment unit until a cardiac cause can be ruled out. Furthermore, once a positive assay result is obtained it means that an acute myocardial injury has occurred and the likelihood of permanent impairment and development of long-term disability with coronary ischemia, congestive cardiac failure or conduction system disease is increased.

An assay that detects patients with chest pain who are at risk of plaque rupture and in whom coronary thrombosis and myocardial injury have not yet occurred would provide for pre-emptive treatment and thereby also diminish the risk of progression to chronic cardiovascular disease would be extremely useful. This is the basis of our novel serum biomarker study. We will evaluate patients who present with chest pain and screen them for novel serum biomarkers of coronary plaque instability. We will also develop assays for measuring these proteins and ultimately develop a blood test that will identify patients at high risk of MI. The aim is that when a patient presents to A&E with chest pain a series of point of care serum marker assay will facilitate rapid stratification as cardiac or non-cardiac chest pain. This will allow for early treatment or discharge.

We have been working with collaborators in Bristol who have developed a mouse model of atherosclerosis (Research Frontiers Programme funded by SFI). Using this ApoE mouse model of atherosclerosis and a protein array we have already identified a number of serum proteins (biomarkers) where changes in their levels predict plaque rupture. We collected serum from mice both before and after plaque rupture and analysed the levels of different proteins in the serum of these mice. Plaque rupture was determined by histological analysis of the brachiocephalic artery. We identified a number of novel proteins that were present at significantly high levels in the serum prior to plaque rupture and may be predictive of higher risk of rupture. A number of these proteins have never been shown to be associated with plaque rupture previously.
Although we provided evidence that these serum proteins have the potential to act as biomarkers of plaque rupture we did so in an artificial animal model of plaque rupture and therefore needed to confirm that the same protein pattern occurred in humans. We therefore conducted a preliminary pilot study of 40 patients who presented with chest pain. We identified similar markers of plaque rupture in patients presenting with unstable angina. While these biomarker proteins were clearly expressed in mice we needed to confirm that the same proteins were expressed in humans. To achieve this we enrolled a small number of patients at Waterford Regional Hospital in 2013 and repeated the same protein biomarker assays confirming that these markers were present in human serum and detectable by ELISA.

2. Clinical Study

We now propose a clinical study of candidate serum protein biomarkers of coronary plaque inflammation that may precede plaque rupture. We will identify 500 patients with chest pain who attended the Accident and Emergency department, for coronary CT scans or those attending the catheterization laboratory for angiograms at St James’s Hospital, Dublin. A blood sample will be taken and stored for further analysis.

Since we will not know their diagnosis at the time of phlebotomy we cannot determine the exact number of patients required. Patients will be characterized as Non cardiac chest pain, stable angina or Acute Coronary syndrome dependent on clinical history and coronary specific investigations. We will measure the levels of the potential biomarkers by ELISA and also use a cytokine antibody array. Samples will be analyzed initially in the Clinical Research Facility in St James’s Hospital in collaboration with the department of Molecular and Cellular Therapeutics at Royal College of Surgeons in Ireland, with ELISA being performed at RCSI.
3. Methods

3.1 Study Design

This is a prospective observational cohort study whereby patients presenting with chest pain to St James’s Hospital, Dublin, Ireland will be eligible for inclusion. Inclusion criteria comprise chest pain presentation with the ability to give informed consent and aged over eighteen. Baseline demographic data collected will include age, sex, duration of chest pain, time after the last chest pain episode or if ongoing pain, previous diagnosis of coronary artery disease, electrocardiogram findings, TIMI score, GRACE score and HEART score and results of stress tests, echocardiography and angiography if available. Biochemical data recorded included urea and electrolytes, full blood count, HsT or Tn levels and CRP if available.

Patients will be classified initially into two groups; non-cardiac chest pain and cardiac chest pain. Cardiac chest pain will include stable angina and ACS (UA, NSTEMI, STEMI). The definitions of which are adapted from the American Heart Association A final diagnosis will be made by the admitting consultant cardiologist at the time of discharge.

Stable angina is defined as pain, tightness or heaviness brought on by exertion, with or without radiation down the ulnar surface of the arm. A typical episode of angina pectoris is considered as gradually beginning and reaching maximal intensity over a few minutes before dissipating with patients resting, sitting or stopping what they are doing with symptoms relieved within minutes.

Unstable angina is defined as angina pectoris with no elevation of cardiac troponin. The characteristics of the chest pain will have to have at least one of the following. Pain occurring at rest with at least 20 minutes of chest pain (if not interrupted by nitrate administration), being severe and described as frank pain, and of new onset (within 1 month) or occurring with a crescendo pattern (i.e. more severe, prolonged or more frequent than previously) [34].
ACS will be composed of UA, NSTEMI and STEMI. Patients with NSTEMI and STEMI will be defined as having undergone acute myocardial infarction (AMI). AMI will be diagnosed as an increase in Hst or Tn, with at least one of the following:

1. Symptoms of ischaemia.
2. New or presumed new significant ST-T wave changes or left bundle branch block on 12-lead ECG.
3. Imaging evidence of new or presumed new loss of viable myocardium or regional wall motion abnormality.
4. Intracoronary thrombus detected on angiography or autopsy [263]

Non-cardiac chest pain was defined as non-exertional chest pain with no abnormalities on serial ECG’s and troponin measurements or any cardiac specific investigations these patients underwent.

Patients will be recruited at all time points after the last chest pain episode with no specific time frame chosen. After recruitment patients were followed until discharge. Cardiac specific investigations will include results of angiography, echocardiogram and stress testing if performed. Total plaque burden and coronary lesion complexity will be determined using the GENSINI and Syntax scores.

3.2 Optimizing the Assay

Pairs of antibodies to the human proteins will be used in the ELISA assays. Aliquots of serum from the patients that are anticipated to contain the highest levels of these proteins will be used to optimize the assays. This will include determining the ideal concentration of antibodies and dilution factors of the serum.
3.3 Biomarker validation

Using the optimized ELISA assays we will measure the levels of the proteins identified in the human samples. We hope to confirm the changes in their assay levels as seen in the mouse study and human pilot study. We will then identify a panel of biomarkers (minimum number of proteins) that best identify the different patient groups.

3.4 Identification of novel biomarkers

As we will have samples from different groups of patients we will have the opportunity to probe these samples for novel biomarkers not identified in the mouse study. We will use the strategy that proved successful with the mouse model. Antibody arrays to human serum proteins will be probed with serum samples from the different patient groups and any proteins that change will be identified and the changes confirmed by ELISA.

3.5 Outcomes

Collection of serum samples from the relevant different patient groups
Develop ELISA assays for the mouse panel of serum proteins
Identify potential biomarkers from the mouse panel of proteins in the human samples
Identify novel potential biomarkers from the human samples
Identify potential development partners for the potential biomarkers

4. Statistical analysis

We will utilize Graph pad from GraphPad Software Inc. (California, USA) and SPSS version 22 from IBM Inc.to perform all statistical analysis. Data are presented as means and standard deviations for normal populations and medians for non-normally distributed populations. We have utilized t-tests to compare population means between two groups and ANOVA for more than two groups for normally distributed data. For non-normal distributed data the Mann Whitney test was used to compare the difference between two means and the Kruskall-Wallis test for >2 means. Univariate and
multivariate regression analyses, receiver operated curves and odds ratios have also been presented for certain data.

**4.1 Sample size**

To calculate the sample size for the novel biomarker arm of this study we started with a biomarker from the mouse study where control mice had a mean level of 0.37ng/ml and the pre rupture animals had a mean of 13.3ng/ml. With an alpha value of 0.01 a sample size of 20 will give a power of 0.96. Based on an incidence of myocardial infarction of 3.6% in chest pain patients we would need to recruit 555 chest pain patients to achieve 20 patients who develop an MI. This was a pilot study and thus the primary goal was to determine the baseline characteristics of the population to allow a power analysis for a larger study. It was also designed to determine if it was possible to get samples of blood from pre-rupture patients. Thus, rather than being designed to confirm the hypothesis it was designed to optimize the study design for a future study if warranted.

**5. Notifications**

This study has been notified to the Ethics research boards of St James’s Hospital, Dublin, Ireland.

**6. Ethical aspects**

The study is conducted in accordance with the protocol, applicable regulatory requirements and the ethical principles of the Declaration of Helsinki as adopted by the 18th World Medical Assembly in Helsinki, Finland in 1964 and subsequent versions.

**6.2 Patient information and informed consent**

The patient is included in the study after a signed informed consent form is obtained according to the guidelines given by the local scientific medical ethics committee. Before signing the informed consent form, eligible patients are provided with full and
adequate verbal and written information by the professionals responsible for the study. This information is given when a patient is admitted to the department of cardiology. Eligible patients will on request be provided with sufficient time for consideration after initial oral information, where the written information is handed out. If the patient wishes so, study participation can be discussed with a third person; as well a third person can be present during the process of information. The investigator and assistants will help arrange that a third person can be present if requested.

6.3 Withdrawal

A patient can be withdrawn from the study at any time if it is the wish of the patient or if it is medically indicated. In any circumstance, every effort should be made to document patient outcome, if possible.

6.4 Biological material

All materials will be stored within the department of Molecular and Cellular therapeutics at the Royal College of Surgeons and destroyed after two years.
St James Hospital

Patient Information Leaflet

Title of Study: Novel Markers of Plaque Rupture in Patients with Chest Pain

Dear Sir/Madam,

Introduction

We are inviting you to participate in a research study. Patients like you can have different causes of chest pain. One possible cause is cardiac (heart) chest pain but blood tests, which detect heart damage, only become positive after a heart attack has occurred.

We are currently investigating newer methods of predicting who may have a heart attack, hence allowing for earlier intervention. One such method is looking at various markers of plaque rupture, which occurs when patients have a heart attack. Plaque is a build-up of cholesterol and other products in the blood vessels of your heart, which can rupture causing a heart attack. If there was a method to predict a plaque rupture before it occurred we could intervene earlier, reducing damage caused by a heart attack.

Procedures

If you are willing to participate, this will involve donating a 20 ml blood and 5 ml urine sample (if you are having a cardiac CT) whilst you are in hospital and further samples at your outpatient review. These samples will be frozen and analysed here in St James Hospital and at University College Dublin and the Royal College of Surgeons in Ireland on St Stephens Green and stored for up to three years. Your medical records will be reviewed to determine your prior history and the outcome of your hospital admission.

Benefits:

By volunteering to participate you will be enrolled in a clinical trial and be closely monitored throughout your admission. Your participation will allow for the development of a test, which could infer a heart attack is imminent so allowing earlier intervention before damage has occurred.

Risks:

We will require blood samples from you. Taking blood can be associated with some discomfort at the site but we will minimize this by taking an extra vile of blood along with your other planned blood tests.
**Alternative treatment:**

You do not have to be a part of this study to be treated.

**Confidentiality:**

Your identity will remain confidential. Your name will not be published and will not be disclosed to anyone not involved in this study.

**Compensation:**

Your doctors are covered by standard medical malpractice insurance. Nothing in this document restricts or curtails your rights.

**Voluntary Participation:**

You have volunteered to participate in this study. You may quit at any time. If you decide not to participate, or if you quit, you will not be penalised and will not give up any benefits, which you had before entering the study.

**Stopping the study:**

You understand that your doctor or the sponsoring company may stop your participation in the study at any time without your consent.

**Permission:**

This study has been reviewed and approved by the Ethics Review Board of St James Hospital.

**Further information:**

You can get more information or answers to your questions about the study, your participation in the study, and your rights, from Dr Amrit Bajrangee who can be telephoned at 014103000. If your doctor learns of important new information that might affect your desire to remain in the study, he or she will tell you.

Thank you,

Dr Andrew Maree
Consultant Cardiologist and Principal Investigator
Title of research study: Novel Markers of Plaque Rupture in Patients with Chest Pain

This study and this consent form have been explained to me. My doctor has answered all my questions to my satisfaction. I believe I understand what will happen if I agree to be part of this study. I have read, or had read to me, this consent form. I have had the opportunity to ask questions and all my questions have been answered to my satisfaction. I freely and voluntarily agree to be part of this research study, though without prejudice to my legal and ethical rights. I have received a copy of this agreement and I understand that, if there is a sponsoring company, a signed copy will be sent to that sponsor.

Name of sponsor:

PARTICIPANT’S NAME:

PARTICIPANT’S SIGNATURE:

Date:

Date on which the participant was first furnished with this form:

Statement of investigator’s responsibility: I have explained the nature, purpose, procedures, benefits, risks of, or alternatives to, this research study. I have offered to answer any questions and fully answered such questions. I believe that the participant understands my explanation and has freely given informed consent.

Physician’s signature:

Date:

(Keep the original of this form in the participant’s medical record, give one copy to the participant, keep one copy in the investigator’s records, and send one copy to the sponsor (if there is a sponsor).
### Novel Markers of Plaque Rupture
#### Patient Data Sheet

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<th>F</th>
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<td>Time after last chest pain</td>
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<td>Other Relevant Diagnosis</td>
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Standard curves
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