GENETIC HYPER-COAGULATION PREDISPOSITION FOR MYOCARDIAL INFARCTION IN THE NEWFOUNDLAND POPULATION

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Genetic Hyper-coagulation Predisposition for Myocardial Infarction in the Newfoundland Population

by

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ABSTRACT

Studies associating prothrombin G20210A (FIIG20210A), Factor V Leiden (FVL), and Factor XIIIV34L (FXIII-A V34L), Factor VII R353Q (FVII R353Q), 5, 10-Methylenetetrahydrofolate reductase A1298C (MTHFR A1298C), and Interleukin-6 -174 G/C (Il-6 -174 G/C) with myocardial infarction (MI) have yielded conflicting results. Complicated gene-gene interactions, small sample sizes and heterogeneous genetic and environmental backgrounds may contribute to conflicting results. Simultaneous analysis of multiple gene variants in a large sample size from a genetically isolated population may overcome these weaknesses. Genotyping was performed in 500 MI patients and 500 controls from the genetically isolated Newfoundland population to determine the prevalence of these gene variants and association with MI. Gene-gene interactions were also analyzed. The prevalence of combined carriers of FXIII-A V34L and FIIG20210A alleles was 12-fold higher in MI patients compared with controls ($P = 0.002$) and with 92% penetrance. There was disequilibrium of FXIII-A V34L allele to MI patients carrying FIIG20210A as a genetic background.

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INTRODUCTION

1.1 Myocardial Infarction

Despite increased attention to cardiovascular disease, myocardial infarction (MI) remains the leading cause of morbidity and mortality in North America, Europe and much of Asia[1,2]. It is recognized as a multifactorial complex disease resulting from such components as lifestyle (activity levels, smoking and diet), atherosclerosis, thrombophilia and inflammation, as well as the genes which control these. The level at which an individual's genetics contribute to the development of an MI is still the subject of much debate, however, the role of genetic inheritance is strongly supported by the predictive value of a family history for premature ischemic disease[3,4,5]. Studies in twins indicate a greater genetic risk in monozygotic compared with dizygotic twins for ischemic heart disease[6], and susceptibility to death from coronary heart disease[7], and adoption studies have shown that much of the excess risk is genetic rather than environmental[6,7]. The relevance of genetic factors in determining the risk of MI is also supported by studying the incidence of disease in migrating populations. The Canadian Inuit, for example have maintained their low risk of MI in spite of adaptation to Western lifestyle[8], suggesting that genetic factors are indeed important to disease development. Thus far, when genetic risk for MI is identified by gender, it is greater in women than in men[7,9]. MI aggregates in families but is not consistent with Mendelian transmission which is compatible with its multifactorial pathogenesis[10]. Prevention of MI has long been focused on identification of traditional cardiovascular risk factors (such as smoking, hyperlipidemia, hypertension, and obesity) that are related to atherosclerosis. However, approximately 30%-50% of MIs

occur in patients without traditional cardiovascular risk factors[11] which has lead to extensive study further searching for novel genetic and environmental risk factors. Thrombosis is one of the most common pathological findings in MI[12,13]. Antithrombotic agents such as aspirin have been shown to be beneficial in primary and secondary prevention of arterial thrombosis[14]. Studies have recently attempted to identify genetic prothrombotic risk factors which associated with MI.

1.1.1 Clinical Features of Myocardial Infarction

A myocardial infarction occurs when myocardial tissue is deprived of oxygen resulting in rapid tissue necrosis[15] which causes a reduced stroke output from the cardiac muscle. This most commonly results from the narrowing and eventual occlusion of coronary arteries due to the development and rupture of atherosclerotic plaques and the ensuing thrombotic reactions. Chest pain is the most common symptom of an MI: typically, it is described as tightness, squeezing, pressure, aching or heaviness. The pain is located in the front and center of the chest and can radiate to the left arm, back, neck or jaw. Associated symptoms include shortness of breath, nausea, vomiting, and profuse sweating. The amount of tissue necrosis is dependant on the size and location of the infarction and the rapidity with which blood flow can be re-established by pharmacologic or mechanical modalities. After total occlusion myocardial necrosis is complete in 4-6 hours. Flow to an ischemic area must remain above 40% of pre-occlusion levels for that area to survive[15].

To confirm the diagnosis of an MI, an EKG (electrocardiogram) and blood tests

are performed. During an MI, the EKG goes through a series of abnormalities. The initial abnormality is called a hyperacute T wave. This is a T wave that is taller and more pointed than the normal T wave. The abnormality lasts for a very short time, and then elevation of the ST segment occurs. This is the hallmark abnormality of an acute MI[15]. It occurs when the heart muscle is being injured by a lack of blood flow and oxygen. This is followed by T wave inversions. Over time, when the heart muscle cells actually die, these abnormalities are replaced with Q waves. When a Q wave develops after an MI, it is called a Q-wave MI and usually corresponds to a transmural MI (entire thickness of the heart muscle wall has died). When a Q wave does not develop after an MI, it is called a non-Q-wave MI and usually corresponds to non transmural heart muscle death or a subendocardial MI (heart muscle just under the inner lining of the heart has died). An EKG can not only tell a physician if an MI is present but can also show the approximate location of the heart attack and often which artery is involved.

The majority of MI patients can be identified after the event by analysis of blood chemistry for the cardiac proteins. As heart muscle cells die, cellular proteins are released into the blood where they are not normally and found, or are normally present in extremely small quantities, and can be detected quite easily. This does not occur instantly and usually levels take several hours following an MI to reach their peak. These proteins include CPK (creatine phosphokinase), Myoglobin, and Cardiac Troponin I[15]. Some of these markers occur in other cells and can limit their usefulness in diagnosing an MI, and thus the majority of MI patients are in fact identified after the event by analysis of blood chemistry for Cardiac Troponin I. This is a contractile protein that is normally found in only trace amounts in serum and is released in greater quantities only when myonecrosis

occurs. Cardiac Troponin I levels become abnormally high in serum 3-6 hours after an MI, and remain elevated for up to14 days[15].

1.2 Mechanisms of Disease in Myocardial Infarction

As described previously, MI occurs when myocardial tissue is deprived of oxygen resulting in rapid tissue necrosis. This oxygen deprivation results when a blockage occurs in arteries leading to the myocardial muscle, preventing oxygen-rich blood from reaching the heart. This blockage is generally the result of atherosclerosis, or a combination of atherosclerosis and thrombosis.

1.2.1 Development of Atherosclerotic Plaques

An atherosclerotic lesion occurs when endothelial cells, activated by risk factors such as hyperlipoproteinemia, express adhesion and chemoattractant molecules that recruit inflammatory leukocytes such as monocytes and T-lymphocytes. The endothelium has a critical role in vascular haemostasis. It modulates regulation of the permeability of plasma lipoproteins, adhesion of leukocytes, release of prothrombotic and antithrombotic factors, growth factors and vasoactive substances[16]. Impairment of these functions is believed to play a central role in development of atherosclerosis[17]. Extracellular lipid and low-density lipoprotein (LDL) will begin to accumulate in the intima of the arterial wall

at this stage. If these responses continue unabated, they can thicken the artery wall (which compensates by gradual dilation) so that up to a point the lumen remains unaltered, a phenomenon known as "remodelling"[18]. LDL particles trapped in the artery can undergo progressive oxidation[19]. Monocytes recruited to the artery wall become macrophages and express scavenger receptors that bind lipids and oxidized LDL, and then internalize them. This internalization leads to the formation of lipid peroxides and facilitates the accumulation of cholesterol esters and the macrophages then become lipid-laden foam cells[20]. Leukocytes and resident vascular wall cells can secrete inflammatory cytokines and growth factors that amplify the leukocyte recruitment and cause smooth muscle cell migration and proliferation. As the lesion progresses inflammatory mediators cause expression of tissue factor (a potent procoagulent) and of matrix degrading proteinases that weaken the fibrous cap of the plaque.

1.2.2 Thrombosis

Evidence now supports the concept that the protective fibrous cap is not actually fixed or static, but instead actually undergoes continuous and dynamic remodelling and displays considerable metabolic activity[21]. If the fibrous cap ruptures at a point of weakening, coagulation factors in the blood can gain access to the thrombogenic, tissue factor containing lipid core, causing thrombosis on non-occlusive atherosclerotic plaques. If the balance between prothrombotic and fibrinolytic mechanisms prevailing at that particular region and at that particular time is unfavourable, occlusive thrombus causing

acute coronary syndromes may result. When the thrombus resorbs, products associated with thrombosis such as thrombin and mediators released from degranulating platelets (including platelet-derived growth factor and transforming growth factor-β) can cause a healing response, leading to increased collagen accumulation and smooth muscle cell growth. In this manner, the fibrofatty lesion can evolve into an advanced fibrous and often calcified plaque that may cause significant stenosis, and produce symptoms of stable angina pectoris. In some cases occlusive thrombi arise not from fracture of the fibrous cap but from superficial erosion of the endothelial layer. The resulting mural thrombus, again dependant on local prothrombotic and fibrinolytic balance, can cause acute myocardial infarction.

1.2.2.1 The Coagulation and Anti-Coagulation Systems

The haemostatic process is a carefully controlled balance of prothrombotic and anti-thrombotic factors within the vasculature. The prothrombotic mechanisms of coagulation activation and platelet adhesion/aggregation are balanced by naturally occurring anticoagulants, endothelial cells, and the fibrinolytic system. In the event of a blood vessel injury, the thrombotic process begins with injured endothelium or denuded vascular surfaces. When the subendothelium is disrupted, both the intrinsic and extrinsic blood coagulation pathways commence their actions. In the intrinsic pathway, von Willebrand factor (vWf) molecules are rapidly localized to the exposed collagen, tethering platelets to the wound[22]. Platelet glycoproteins then activate the platelets

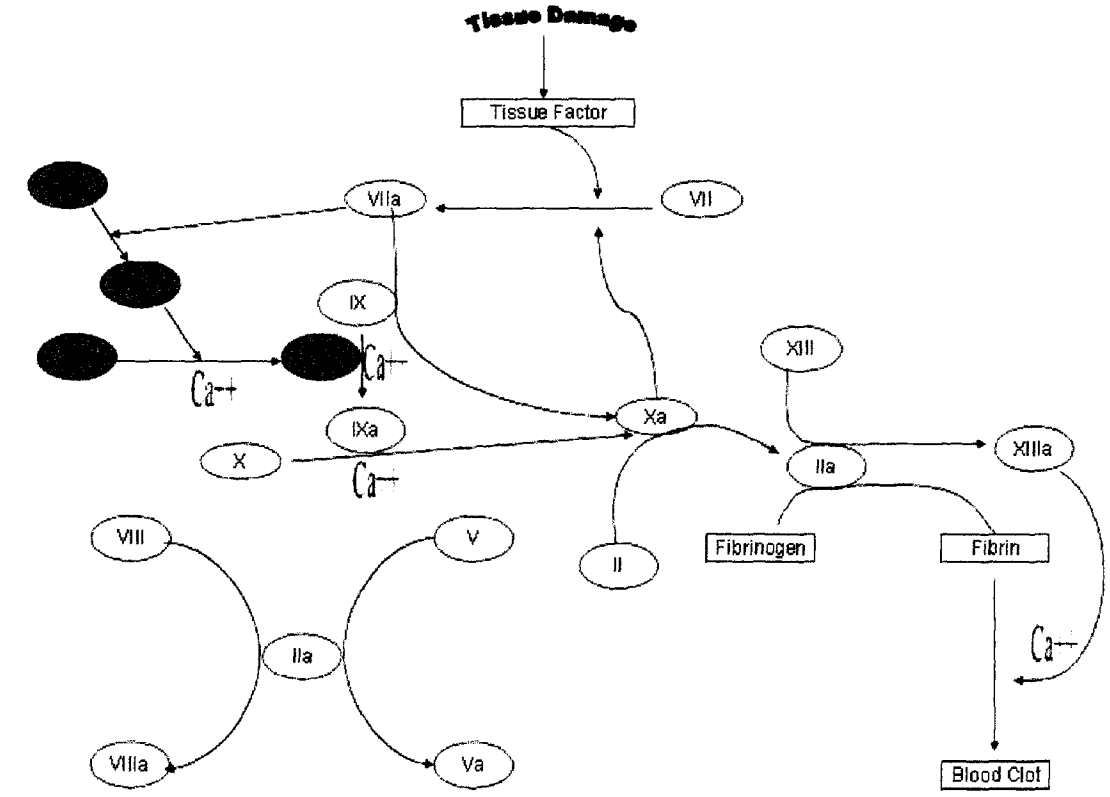
causing them to adhere to one another[23,24]. Lipopolysaccharides from platelets and cell surfaces then activate Factor XII which in turn activates Factor XI, which plays a role in the activation of Factor IX of the extrinsic pathway.

During this phase of platelet activation the extrinsic pathway of blood coagulation is activated. Initiation of the coagulation cascade is largely due to exposure of the various blood elements to non-vascular cell tissue factor. Tissue factor binds to activated factor VII, or FVIIa and the resulting enzyme complex activates (in the presence of Ca^{2+}) factors IX and X (converting both to FIXa and FXa) of the intrinsic and common coagulation pathways, respectively. FIXa in turn activates additional factor X. Once activated, FXa converts prothrombin to the serine protease thrombin (factor IIa) in a reaction that is accelerated by factor Va. Factor XIII is the last enzyme to be activated in the blood coagulation cascade. It is converted to FXIIIa, through a catalytic reaction with FIIa. In the final step of the coagulation pathway, thrombin cleaves fibrinogen to form fibrin monomers which then polymerize by factor XIIIA catalyzing the crosslinking between fibrin molecules to form a chemically stable clot rendering it less susceptible to proteolytic cleavage[25]. Thrombin then also further activates cofactors VIII and V thereby further serving to self-propagate the coagulation mechanism. Simultaneously platelets adhere to exposed extracellular matrix proteins, undergo activation by thrombin and other agonists, and aggregate to form a platelet-fibrin thrombus.

This prothrombotic process is regulated and balanced by the fibrinolytic system, consisting of natural anti-coagulants such as antithrombin, activated protein C (APC) and its cofactor (protein S), as well as tissue factor inhibitor. The fibrinolytic system is concomitantly activated by plasminogen activators released from endothelial cells,

leading to the production of the active plasmin and eventual degradation of the fibrin clot[26]. In the presence of endogenous heparin sulfate, the rate of inactivation is increased by a factor of several thousand. In the presence of thrombomodulin bound to endothelial cells, FIIa activates protein C, which in turn cleaves and inactivates FVIIIa and FVa. Endothelial cells normally have an antithrombotic effect, largely due to membrane-bound thrombomodulin, which is responsible for activating protein C[27]. Other antithrombotic endothelial functions include platelet inhibition due to release of prostacyclin and nitrous oxide. An outline of the blood coagulation system is shown in figure-1.

Figure -1: Outline of Blood Coagulation Pathway



= Extrinsic Pathway

= Intrinsic Pathway

1.2.2.2 Thrombotic Imbalance

Congenital and acquired hypercoagulable states arise when there is an imbalance between the anticoagulant and prothrombotic activities of plasma in which the prothrombotic activities dominate[28-30]. There are many recognized acquired prothrombotic risk factors such as age, obesity, oral contraceptives, surgery, trauma, paralysis. All of these factors can cause a tendency towards thrombosis resulting from such events as a decrease in blood flow, injury to the vessel wall, and a change in the systemic balance of the procoagulant and anticoagulant factors. Endothelial cells normally have an antithrombotic effect, largely due to membrane-bound thrombomodulin, which as previously stated, is responsible for activating protein C[27]. According to this scheme, one might predict that the loss of a circulating anticoagulant would cause a shift in the haemostatic balance and thereby promote a diffuse thrombotic diathesis: in fact, this prediction does not hold true[31]. Systemic alterations in the haemostatic mechanism typically give rise to local thrombotic lesions in discrete segments of the vascular tree. The pathophysiologic basis for this observation is poorly understood: the conventional wisdom is that the focal lesions are attributable to superimposed defects in the vascular wall or blood flow. In other words, the phenotypic fate of systemic hypercoagulable states rests on the ability of these two mechanisms to compensate for a uniform change in the haemostatic balance. As mechanistic studies have progressed, the pathophysiologic mechanism for thrombosis with many of the acquired risk factors has been associated with perturbations in procoagulant factors, the vasculature, or blood flow.

1.3 Candidate Genetic Thrombotic Risk Factors in Myocardial Infarction

Recognition of familial tendencies for thrombosis initially led to a search for genetic abnormalities in the coagulation system, and the central role of thrombosis in acute coronary ischemia has gained attention. The first mutations associated with thrombophilia were loss-of-function mutations in antithrombin[32], protein C[33], and protein S[34]. Heterozygous individuals have an increased risk for venous thromboembolism. These disorders are rare and show high allelic heterogeneity, thus making genetic testing difficult. For example, more than 70 different mutations in protein C have been described[35]. Of patients with thrombophilia, genetic deficiencies of the natural anticoagulants have been found in less than 20% of the cases. Deficiency of the profibrinolytic factor, plasminogen, has been associated with thrombophilia, but this association is less clear than that of the natural anticoagulants[36].

Endothelial causes for a thrombotic tendency could be genetic dysregulation of the antithrombotic mechanisms or endothelial denudation with exposure of prothrombotic plaque and extracellular proteins as occurs with atherosclerosis. Additionally, changes in blood flow may cause thrombosis due to stasis but may also effect endothelial gene expression[37].

The search to explain thrombophilia in familial patients for whom no cause could be found led to sequencing of the genes of many of the procoagulent proteins. This process in turn led to the description of relatively common single nucleotide polymorphisms (SNPs) in many of these genes, however, the functional implications of most of these SNPs were not always clear. Certain polymorphisms associated with

thrombosis have been found in many of the genes coding for proteins in the coagulation pathway including prothrombin or factor II, factor V, factor VII and factor XIII and others.

1.3.1 Factor V Leiden

The gene which codes for blood coagulation factor V is located on chromosome 1q21 – 1q25, has 25 exons and produces a 300-kd multidomain glycoprotein. Activated factor V (FVa) is a cofactor protein in the prothrombinase complex that, together with the serine protease factor Xa, is responsible for the conversion of prothrombin or FII to the active enzyme thrombin, or FIIa. Activated protein C (APC) down-regulates the functionality of this complex by proteolytic degradation of factor Va at two primary cleavage inactivation sites, amino acids Arg506, and Arg306. Patients resistant to the activity of APC were described by Dahlback *et. al* in1993[38] , and the molecular basis for this defect was shown by Bertina *et. al* to be a point mutation in the factor V gene – a G>A transition at nucleotide 1691 (G1691A), which they labelled factor V Leiden (FVL)[39]. The FVL mutation results in an arginine-glutamine substitution at amino acid 506 (Arg506Gln), the site of the first molecular cleavage of factor Va by APC. This substitution results in a diminished ability of APC to cleave factor Va and therefore causes continued formation of thrombin by the prothrombinase complex (i.e., APC resistance). Factor V Leiden is commonly accepted as the most common genetic factor associated with APC resistance; 95% of individuals with functional APC resistance carry

the FVL polymorphism[40]. The FVL polymorphism is common in the Caucasian population with a frequency ranging anywhere from 2% -15%[41-43], but averaging 5%. FVL frequency can vary a great deal among other ethnic populations, being extremely rare in African and Asian populations[41,42,44] to higher and very high in some populations of middle-eastern and Arabic descent[45-47]. The high prevalence of FVL did raise the question of whether the point mutation was the result of a founder effect or whether it is a frequent, recurring mutation in the factor V gene. An extensive haplotype analysis using six polymorphic sites in the factor V gene supported a single origin for FVL, and it was estimated that it occurred approximately 21000 years ago[48].

The FVL mutation is detected in up to 40% of patients with venous thrombosis[39], imparting an eightfold increased risk for heterozygotes and 80-fold increase in homozygotes[49]. FVL has been shown in clinical studies to be the most common risk factor for deep venous thrombosis (along with the prothrombin G20210A polymorphism)[50] and is also suggested to act a risk factor for pulmonary embolism, cerebral vein thrombosis, and superficial thrombophlebitis[51]. FVL has also been studied for an association with arterial thrombosis (such as MI), however the relationship is not yet clear. An association between FVL and arterial thrombotic disease was first suggested by Holm *et. al* who reported two young women with MI and homozygosity for the FVL mutation[52]. Subsequent association studies on arterial thrombosis, however, have provided mixed results. Several studies have shown no association between FVL and coronary artery disease or MI[53-61], even among patients who developed acute coronary syndromes at a young age[62,63]. In contrast, Rosendaal *et. al* reported an almost 2.5 times greater risk of non-fatal MI among female carriers of FVL versus the normal population,

and a great deal higher risk among smokers in the patient group (32-fold increased risk compared to non-smoking women without the FVL mutation)[64].

The association between FVL and Myocardial Infarction has been examined in at least three large case-control studies. Doggen *et. al* examined 560 middle-aged-to-elderly-Dutch-men and reported an increased risk of MI associated with heterozygosity for FVL that was most pronounced in men with other cardiovascular risk factors[65]. Masourati *et al* compared 3 groups of subjects: one group consisted of 107 patients with early onset myocardial infarction but no significant coronary artery stenosis; another group of 244 patients with myocardial infarction and significant coronary artery stenosis; a third group of 400 healthy controls. Factor V Leiden was found in 12.1% patients (13 of 107) who had a myocardial infarction without significant coronary artery stenosis, in 4.5% of the patients who had a myocardial infarction with significant coronary artery stenosis (11 of 244, p = 0.01) and in 5% of the controls (20 of 400, p = 0.01), giving respective odds ratios of 2.93 (CI95: 1.18-7.31) and 2.63 (CI95: 1.19-5.78) for MI associated with factor V Leiden when MI patients without significant coronary artery stenosis were compared to controls or to patients with significant coronary artery stenosis, respectively.[66]. Most recently, the Copenhagen City Heart Study reported an investigation of 20- to 95-year-old participants without cardiovascular disease (control population, n = 7907) or participants diagnosed with MI (n = 469), ischemic stroke (IS) (n = 231), or non-MI-ischemic heart disease (non-MI-IHD) (n = 365). In addition, 3 independent patient populations with MI (n = 493), IS (n = 231), or non-MI-IHD (n = 448) were included. They characterized FVL genotype; major cardiovascular risk factors; and MI, IS, and non-MI-IHD incidence and prevalence. Odds ratios and relative risks of MI in FVL carriers (heterozygotes +

homozygotes) versus noncarriers were found to be 1.24 and 0.83 in case-control and prospective studies, respectively. Corresponding odds ratio for IS were 0.92 (95% CI, 0.56-1.53) and 0.68 (0.45-1.04), and for non-MI-IHD 1.01 (95% CI, 0.71-1.44) and 0.97 (0.66-1.42) suggesting that FVL is not associated with MI, IS, or non-MI-IHD[67]. Obviously, with the large amount of conflicting data that has been presented, the relationship between the factor V Leiden mutation and myocardial infarction has not yet been fully elucidated.

1.3.2 Prothrombin 20210 G>A

The gene coding for factor II, or prothrombin is located on chromosome 11p11-q12. The gene is 21 kb long and contains 14 exons separated by 13 intervening sequences. The exons range in size from 25 to 315 basepairs, while the introns range from 84 to 9,447 basepairs. Intervening sequences constitute 90% of the gene[68], and after translation the gene product has a mass of 83kDa prior to cleavage[69]. As stated previously, thrombin, (the end product of the cleaving of prothrombin) plays a central role in haemostasis in that it converts fibrinogen to fibrin, but it also activates factors V, VIII, XIII, and protein C. Thrombin is also a potent activator of platelets. Knowing the central role of thrombin in the coagulation cascade, Poort *et. al* investigated the prothrombin gene as a as a candidate gene for venous thrombosis in patients from families with unexplained thrombophilia[70]. They identified a G>A transition at nucleotide 20210 in the 3' untranslated region of the prothrombin gene (prothrombin G20210A or

FII G20210A). The FII 20210A allele was present in 18% of unselected patients with a first, objectively confirmed episode of deep-vein thrombosis and a family history of the disease (n=28) compared with 2% of health controls (n=100) which gives a odds ratio of 2.8 (95% CI: 1.4 - 5.6), suggesting the presence of the FII G20210A mutation may increased the risk of deep-venous thrombosis. The FII 20210A allele has been found to be present at a frequency of 2% most Caucasian populations[71].Current estimates are that up to 25% of patients presenting with deep venous thrombosis carry either the FVL mutation, the prothrombin G20210A variant, or both[50]. Double heterozygotes for FVL and prothrombin G20210A are at greatly increased risk for deep venous thrombosis[63]: Emmerich *et. al* (2001)[50] reviewed and pooled the analysis of 8 case-control studies, comprising a total of 2310 venous thrombosis cases and 3204 healthy controls, in order to precisely estimate the risk of deep venous thrombosis in patients bearing both mutations (double heterozygotes). Odds ratios were 4.9 (95% CI; 4.1-5.9) for FVL carriers, 3.8 (95% CI; 3.0-4.9) for the prothrombin G20210A carriers, and 20.0 (95% CI; 11.1-36.1) for double heterozygotes. Twelve percent of patients heterozygous for factor V Leiden were also heterozygous for factor II G20210A and conversely 23% of patients heterozygous for the FII 20210A allele were also heterozygous for factor V Leiden[50].

The prothrombin G20210A mutation has been shown to be associated with a 50% increase in circulating prothrombin in carriers from several different ethnic populations[36, 70-73]. However, the mechanism by which this base pair substitution increases the plasma levels of prothrombin is not yet clear. Poort *et. al* hypothesized in their original paper that the 20210A allele may cause a higher translation efficiency or higher stability of the transcribed RNA. The G/A sequence variation is located at the last position of the 3'

untranslated region[70] at or near the cleavage site in the mRNA precursor to which the poly-A tail is added. Three conserved sequences in mRNA precursors located in the vicinity of this site, are required for cleavage and polyadenylation: The AAUAAA sequence, the nucleotide to which the poly-A tail is added, and the region downstream of this nucleotide[74,75]. Generally, the nucleotide to which the poly-A tail is added is an A[74-76], mostly preceded by a C[74,77]. As a consequence of the G to A transition at position 20210, a CA dinucleotide instead of GA has been introduced at or near the cleavage and polyadenylation site. However, in vitro experiments so far do not support a hypothesis in which this nucleotide substitution results in an increased efficiency of the 3' end formation.

As with factor V Leiden, the relationship between the FII G20210A mutation and risk for arterial thrombosis such as myocardial infarction is not completely clear. Several published studies have found no association between the 20210A allele and the risk of MI or coronary artery disease[78-87], while others have identified a correlation between the two[88-92]. However, the statistical power of many of these studies to determine an association is limited due to the low frequency of the FII 20210A allele within the general population. Interestingly, in a pooled analysis of 1115 patients and 1888 controls from five studies, the FII 20120A allele was significantly associated to patients with MI (OR = 2.5; 95% CI 1.5 – 4.3)[72]. In a large case-control study of 560 patients with MI (under age 70 years) and 646 healthy control subjects, the 20210A allele was associated with a mildly increased risk of MI (OR = 1.5; 95% CI 0.6 -3.8) that substantially increased to 3-6 fold in the presence of traditional cardiovascular risk factors such as smoking and hypertension[91]. In contrast to this conclusion, Boekholdt *et. al*[93] (2001) reviewed data

from thirteen previous studies[72,81,82,84-92]. The patients were re-examined using more stringent MI diagnostic criteria in order to better evaluate the conclusions reached. Nine of these studies were excluded because of low stringency in the diagnostic criteria for MI[86,87], or because of inadequate delineation of cases[72], or an inadequate spectrum of cases (meaning small sample size, or patients under age 55)[79,84,85,88,90,92]. A pooled analysis of the four remaining studies[81,83,86,91] included a total of 1434 patients and 2943 controls, and showed no association between the FI 20210A allele and MI (OR 0.89, 95% CI 0.59-1.35, p=0.6). Obviously, as with factor V Leiden, the role of the prothrombin G20210A mutation in the pathogenesis of myocardial infarction is not yet fully understood.

1.3.3 Factor XIII V34L

Factor XIII is a protein with transglutaminase activity consisting of two A subunits arranged as a dimer in association with two B subunits in a heterotetrameric structure (A2B2) of 320 kd[94,95]. The gene encoding the A subunit of human factor XIII is located on chromosome 6 and contains 15 exons and 14 introns spanning over 160 kb. Five distinct functional domains are encoded by separate exons[96]. The A subunits of FXIII are responsible for transglutaminase activity, and are activated following the cleavage of an N-terminal peptide by thrombin[97]. The B subunit is a glycoprotein and has no known enzymatic activity. It is thought that the B subunit forms a complex with the A subunit dimer and protects it from elimination[98-101]. Factor XIII is activated by the action

of thrombin cleaving an activation peptide of 37 amino acid residues from the N-terminal end to form the last enzyme generated in the blood coagulation cascade. It is the zymogen for fibrinoligase, a transglutaminase, which through an acyl-transfer reaction cross-links peptide bound glutamine and lysine residues through isopeptide bonds[102]. The main physiological task of FXIII is to form intramolecular γ-glutamyl-ε-lysine crosslinking between fibrin molecules, and also to cross-link α_2-plasmin inhibitor to fibrin. Crosslinking of fibrin stabilizes the blood clot and protects the newly formed fibrin from rapid elimination by the powerful fibrinolytic mechanism[103].

Previous studies of factor XIII have shown that the A subunit is genetically heterogenous and a number of polymorphisms have been identified in the protein sequence[94,96,103,104]. Several studies have shown that there is a large range of the A subunit transglutaminase activity in the normal population, and it is possible that the different levels of activity are related to the inheritance of different allelic variants[105-109]. A common G>T transversion in exon 2 of the A subunit results in the substitution of leucine for valine at amino acid residue 34[110]. This amino acid substitution is located three residues away from the thrombin cleavage activation site[104,111]. The 34L variant of factor XIII-A has been shown to have a great deal higher enzyme activity and earlier enzyme activation than the normal 34V enzyme in both recombinant DNA studies and in plasma from normal blood donors[112,113]. Recent studies have pointed towards a decreased risk of both venous thrombosis[114,115] and myocardial infarction[116-118] for carriers of the 34L allele. The most widely quoted of these studies compared the frequency of the factor XIII-A 34L polymorphism in 126 male MI patients and 344 male healthy controls from Finland[117]. The 34L allele was found to be present at a carrier frequency of 25% in

controls and 17% in MI patients (p=0.02, OR 0.59; 95% CI 0.38 – 0.93).

The mechanism of this suggested protection is not immediately obvious as the higher activity level of the 34-leucine variant should lead to an increase in resistance of fibrin clots to plasmin degradation. While several studies to date have reproduced data regarding this protective effect, only one has given data which fits the current understanding of the physiological coagulation system and associates the 34L allele of factor XIII with an increased risk of later onset of peripheral artery disease[119], however no association to an increased risk for MI has yet been reported.

1.3.4 Factor VII R353Q

Activated factor VII (FVII) is a vitamin K-dependant serine protease synthesized by the liver and secreted as a single chain 48 kDa glycoprotein that plays a central role in the extrinsic pathway of blood coagulation. The gene is located on chromosome 13q34, consists of 9 exons spanning approximately 12kb, and encodes a mature protein of 406 amino acids[120]. Upon endothelial injury, FVII is activated through cleavage by tissue factor (factor III) at residue 152 to form FVIIa, which then forms a complex with tissue factor, to initiate coagulation by activating both factor IX and factor X, and also autocatalytically further activates factor VII[121,122].

Circulating levels of factor VII vary greatly within the general population and are influenced by factors such as oral contraceptives and a high fat diet. FVII coagulant activity is associated with high triglyceride and cholesterol levels and has been shown to

increase with an increase in body fat[123]. Interest in the relationship between factor VII and cardiovascular disease was stimulated by the finding from the Northwick Park Heart Study that elevated levels of circulating FVII were related to fatal, but not to non-fatal MI. In this prospective study of white males (n=1511) aged 40 to 64 years, circulating FVII was found to be strongly associated with coronary risk (62% increased risk, $p<0.01$) with a 1 SD increase in FVII levels over the first 5 years of the study[124]. Further prospective studies, however, have not obtained similar results. Both the PROCAM (Prospective Cardiovascular Munster study)[125] and Edinburgh Artery Studies[126] failed to confirm FVII as an independent risk factor for coronary events. The ECTIM (Etude Cas-Temoins de l'Infarctus du Myocarde) study found that the control subjects had uniformly higher levels of FVII, which tended to mitigate against it having a major role as a cardiovascular risk marker[127]. It has been shown that genetic factors strongly influence factor VII circulating plasma levels and regulate the response to environmental stimuli[128].

An adenine to cytosine substitution polymorphism within the FVII gene identified by the restriction enzyme *Msp*I, resulted in a glutamine residue replacing an arginine residue at position 353[129]. The Arg353Gln (R353Q) site was noted initially to associate with a 20% to 30% variance below the group mean in factor VII levels in males and females and in different ethnic groups[130]. Many subsequent studies have confirmed that carriers of the allele coding for Gln353 have lower FVII levels[131-133]and Hunault *et al* have shown that FVII 353Q is secreted with a lower efficiency in vitro[134].

The association of the R353Q polymorphism with myocardial infarction is still highly controversial. While FVII 353Q was found to associate strongly to circulating factor VII levels in MI patients and controls from the ECTIM study, there was no

difference in genotype frequencies between the two groups. The largest published case-control study, SMILE (Survival of Myocardial Infarction Long-Term Evaluation) in the Netherlands found an association between the FVII 353Q polymorphism and circulating factor VII, however, it also found that FVII 353Q was associated with a decreased, rather than an increased risk for MI 560 patients vs 644 healthy controls OR 0.80, 95%CI 0.60 – 1.06) which was more pronounced in patients younger than 50 years (OR 0.49, CI:, 0.28 – 0.84)[135]. Most recently, Girelli et al. found that individuals carrying the FVII353Q polymorphism had a markedly decreased incidence of myocardial infarction despite severe, angiographically documented coronary atherosclerosis. The QQ genotype was associated with a 72% reduction of activated factor VII activity compared with the wild type genotype. Heterozygote carriers of the Q allele had a 0.47 risk of MI compared with patients with the wild type genotype (411 MI patients vs. 133 healthy controls: 95% CI: 0.27 to 0.81)[136]. Further investigation of the FVIII R353Q polymorphism will be required before conclusions can be effectively drawn as to its relationship to MI.

1.4 Non-coagulation Genetic Risk Factors in Myocardial Infarction

As MI is a multifactorial and polygenic disease involving several complex and as yet unknown interactions between genes and environment, a portion of this study was devoted to an investigation of genetic risk factors in MI beyond the blood coagulation system, encompassing genes in the inflammation and metabolic pathways.

1.4.1 Inflammation

A consensus has recently emerged that inflammation plays a decisive role in the pathophysiology of acute thrombotic events[137]. The inflammatory process which ultimately leads to an atherosclerotic plaque is triggered initially by endothelial dysfunction [138-140]. As described previously, an atherosclerotic lesion occurs when endothelial cells, activated by risk factors such as hyperlipoproteinemia, express adhesion and chemoattractant molecules that recruit inflammatory leukocytes such as monocytes and T-lymphocytes. Leukocytes and resident vascular wall cells can secrete inflammatory cytokines and growth factors that amplify the leukocyte recruitment and cause smooth muscle cell migration and proliferation. As the lesion progresses inflammatory mediators cause expression of tissue factor and of matrix degrading proteinases that weaken the fibrous cap of the plaque. Macrophage secretion of interferon gamma can inhibit *de novo* synthesis of interstitial collagen by smooth muscle cells, the major source of the extracellular matrix collagen[141]. In addition, inflammatory cytokines and *fas* ligand, which are overexpressed in atherosclerotic plaques, can trigger apoptosis and death of these surrounding smooth muscle cells, a critical source of extracellular matrix macromolecules in the artery wall[142]. Absence of smooth muscle cells jeopardizes the integrity of the fibrous cap because these cells repair and maintain the collagenous matrix of the fibrous cap: plaques that rupture have thin and friable fibrous caps because of the lack of collagen[143]. Sites of fatal thrombosis where plaques have ruptured typically have very few smooth muscle cells[144,145]. In addition to local effects of inflammation at the

level of the atherosclerotic lesion itself, systemic aspects of the inflammatory response may alter thrombotic risk. Inflammation upsets the prevailing homeostatic balance. Fibrinogen and plasminogen activator inhibitor circulate at higher concentrations in inflammatory states. A given plaque disruption could therefore have a greater chance to produce an occlusive thrombus under such conditions[137].

1.4.1.1 C-Reactive Protein

C-Reactive Protein (CRP) is a protein produced by the liver that is present in blood serum in relatively large amounts during acute inflammation. Levels of CRP in blood serum are otherwise very low (approximately 0.11 mg/dL). CRP is an acute phase protein which promotes inflammation, and activates the complement system to stimulate chemotaxis of phagocytes[146]. As a marker of inflammation, CRP is unique among the major plasma proteins in the level of increase that is observed, and that its levels appear to be unaffected by hormones and anti-inflammatory drugs, but instead are regulated by the proinflammatory cytokines, especially IL-1 and IL-6[147]. Prospective data from the Physicians Health Study indicates that among a population of apparently healthy men, baseline levels of CRP are predictive of a first myocardial infarction. Specifically, among men free of prior cardiovascular disease those with CRP levels in the highest quartile, a threefold increase in risk of developing future myocardial infarctions was observed when compared those individuals with CRP levels in the lowest quartile[148]. In addition to the findings of the Physicians Health Study, CRP levels have been identified as an

independent, prospective risk factor in the higher-risk middle-aged men of the MRFIT study[149], the healthy middle aged men of the MONICA-Ausburg cohort[150] and the healthy elderly men and women of the CHS and the Rural Health Promotion Project[151]. CRP levels are known to be related to smoking[152] and the MRFIT data indicated that although there was no confounding effect, there was an interaction of smoking with CRP: CRP better predicted events in smokers than in non-smokers, independently of smoking cessation[149]. Consistent with this finding, CRP levels were associated with lifetime exposure to cigarette smoke. Taken together these findings raise speculation that CRP, at least in some people may mark permanent underlying endothelial damage due in part to smoking. It is important to note however, that in the Physicians Health Study CRP predicted future events just as well in non-smokers as in smokers, raising the question as to whether or not CRP levels were indicative of a response to endothelial damage or are in fact an independent risk factor[148].

1.4.1.2 Interleukin-6

At the time this research was conducted, no data had been published on functional SNPs of the CRP gene, and therefore although CRP levels may be elevated in individuals without overt infections or trauma, there is currently no genetic explanation for persistently elevated CRP levels in some individuals. Interleukin-6 (IL-6) is a pro-inflammatory cytokine and a central mediator of the acute-phase response, which together

with IL-1 stimulates the liver to produce acute phase proteins[153]; indeed, Il-6 is a primary determinant of hepatic production of CRP[154]. The ability of IL-6 to promote transcription of CRP is mediated, in large part, by its ability to activate the transcription factor STAT3; via a tyrosine phosphorylation mediated by the IL-6 receptor complex)[155-157]. STAT3 binds directly to the promoter region of the CRP gene and induces transcription[158,159]. Fishman *et al.* (1998) investigated the 5' regulatory region of the IL-6 gene for polymorphisms which may influence expression, and identified a G/C polymorphism at position -174. This polymorphism showed a prevalence of 40.3% (95% CI 0.37-0.44) in a healthy population (383 men and women from north London, UK.) Individuals carrying the -174 C/C genotype showed 0.624-fold lower expression of IL-6 than individuals carrying the -174G/G genotype. This lower expression was also directly related to circulating IL-6 concentrations, as individuals homozygous for the GG genotype had approximately twice the plasma level of IL-6 than individuals homozygous for the CC genotype (*P=0.02)*[160]. This finding was confirmed in several subsequent studies[161-163]. Despite this lower level of Il-6 transcription and circulating Il-6 observed in the presence of this polymorphism, individuals carrying either of the GC or CC genotypes have been found consistently to have significantly higher base-line levels of CRP[164,165], although the mechanism by which this polymorphism increases base-line plasma CRP concentration is unknown. Some studies have shown that the IL-6 -174 G/C polymorphism has been associated with coronary heart disease and high systolic blood pressure[166], and other related conditions such as asymptomatic carotid artery atherosclerosis[167], cardiovascular mortality following small aneurisms[168], and peripheral artery occlusive disease[169]. The mechanism by which this polymorphism influences these conditions is unknown, but

believed to be due to its role in inflammation via the regulation of CRP[164-166]. Although expression of the CRP gene during the acute-phase response is regulated by IL-6, the relationship of IL-6 expression levels to baseline plasma CRP levels is mostly unknown. To date, only one previous study has attempted to relate the IL-6 -174 G/C polymorphism to myocardial infarction, and found no association[170]. Despite this result, there remains much evidence that IL-6 is related to other cardiovascular disease conditions, and thus remains an interesting investigative target as to its relationship with the onset of MI.

1.4.2 Hyperhomocysteinemia

Homocysteine is a sulfur-containing amino acid, an intermediate product formed during metathionine metabolism and is rapidly oxidized in plasma to the disulfides homocystine and cysteine-homocysteine. Plasma/serum total homocysteine, also termed homocyst(e)ine, is the sum of homocysteine in all 3 components[171]. Several studies published in the early 1990's showed that 12 - 14% of patients with coronary artery disease also had familial hyperhomocysteinemia[172,173], and also showed an association between high plasma homocysteine concentrations and low concentrations of folate and vitamin B6 (because of their role in homocysteine metabolism), as well as an increased risk of extracranial carotid artery stenosis in the elderly[174]. More than 20 cross-sectional and 3 prospective[175-180] studies in young and middle-aged patients have shown that high levels of homocysteine are associated with an increased risk of MI and stroke. The pathogenesis through which homocysteine actually causes vascular damage still remains

unclear[181].

Two major hypotheses have been proposed to explain how homocysteine induces its harmful effects: It can damage endothelial cells lining the vasculature, causing blood clotting and thus promoting atherosclerotic plaque formation[182,183]. Simultaneously, it interferes with the vasodilatory effect of endothelial derived nitric oxide[184]. Homocysteine has also been found to promote vascular smooth muscle cells hypertrophy; Tsai *et al* (1994)[185] performed an *in vitro* study examining the effect of homocysteine on the growth of both vascular smooth muscle cells and endothelial cells at concentrations similar to those observed clinically. The presence of as little as 0.1 mM homocysteine caused a 25% increase in DNA synthesis, and homocysteine at 1 mM increased DNA synthesis by 4.5-fold in rat aortic smooth muscle cells. In contrast, they found that homocysteine caused a dose-dependent decrease in DNA synthesis in human umbilical vein endothelial cells. It was also observed that homocysteine increased mRNA levels of cyclin D1 and cyclin A in aortic smooth muscle cells by 3- and 15-fold, respectively, indicating that homocysteine induced the mRNA of cyclins important for the re-entry of quiescent smooth muscle cells into the cell cycle[185]. The growth-promoting effect of homocysteine on vascular smooth muscle cells, together with its inhibitory effect on endothelial cell growth[186], may explain homocysteine-induced atherosclerosis.

1.4.2.1 5, 10- Methylenetetrahydrofolate Reductase

The enzyme 5, 10-Methylenetetrahydrofolate reductase (MTHFR) catalyzes the

reduction of 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the predominant circulatory form of folate and carbon donor for the re-methylation of homocysteine to methionine. A loss of function in this enzyme would result in an accumulation of homocysteine. Frosst et al (1995) identified a common polymorphism in MTHFR which alters a highly-conserved amino acid; the substitution (C677T) occurs at a frequency of approximately 38% of unselected chromosomes and occurs within the predicted catalytic domain of the MTHFR enzyme[187]. This gene variation in either heterozygotes or homozygotes was shown to be associated with reduced enzyme activity and increased thermolability in lymphocyte extracts; in vitro expression of the 677T allele confirmed its effect on thermolability of MTHFR. Individuals homozygous for the mutation were found to have significantly elevated plasma homocysteine levels. Several studies conducted since this polymorphism was identified have confirmed an association between MTHFR 677T and both premature cardiovascular disease[188,190] and coronary artery disease[191]. Klujitmans *et al.* found that homozygosity for the 677T allele was found in 9 of 60 (15%) cardiovascular patients and in only 6 of 111 (5%) control individuals from the Netherlands (OR 3.1, 95% CI: 1.0-9.2)[188]. Mager *et al.* showed that the prevalence of homozygosity for the 677T allele among Israeli patients with early CAD onset (aged < or = 45 years) was 28%, (n=67) which was significantly higher than that in patients with later onset (13%, n=102) and in control subjects (14%, n=313) (OR 2.4, 95% CI 1.24-4.69, p=0.006, and OR 2.7, 95% CI 1.15-6.42, p=0.01, respectively)[189]. Morita *et al.* observed that in 778 healthy male Japanese subjects, the frequency of the 677T allele was 0.33, whereas In 362 patients with CAD, the TT genotype was significantly more frequent than in control subjects (16% versus 10%, P=.0067). The

association of the TT genotype with CAD was further increased in patients with > or = 99% stenotic lesions (18%, P=.0010), whereas no significant association with the TT genotype was observed in patients without a stenosis. When the genotype frequency was compared among patients with different numbers of stenotic coronary arteries, the frequency of the TT genotype was significantly higher in patients with triple-vessel disease (26%) than in patients with single- or double-vessel disease (15% and 14%, respectively: $P<.0001$)[190]. Van Bockxmeer *et al.* did not, however, find such a relationship in their study of 555 white Western Australians with angiographically documented coronary artery disease and 143 unrelated controls[191].

Van der Put et al[192] identified another polymorphism of the MTHFR gene: This DNA sequence change, an A>C transition at nucleotide 1298 results in a glutamic acid-to-alanine substitution at amino acid residue 429 (E429A), and had an allele frequency of 0.33. The 1298 A-C transition is located in the presumed regulatory domain and is associated with decreased MTHFR activity, which was more pronounced in the homozygous than heterozygous state[192]. Despite the lower activity of the enzyme variant, neither the homozygous nor the heterozygous state was associated with higher plasma homocysteine or a lower plasma folate concentration - phenomena that are evident with homozygosity for the 677C-T mutation.

While the A1298C variant does not appear to raise homocysteine levels, some evidence does nonetheless indicate an association with coronary artery disease. Szczeklik et al (2001)[193] found that the prevalence of the 1298C allele was significantly higher in CAD patients (n=161 men) compared to control subjects with no history or evidence of CAD (n=211) (0.304 vs. 0.199, p=0.001) or from a random population sample or

residents of downtown Cracow, Poland (n=310) (0.304 vs. 0.235 p=0.003). The 1298C allele showed a significant association with early-onset CAD both in homozygotes and in heterozygous carriers despite the fact that there was no observed difference in fasting plasma homocysteine, folic acid and vitamin B12 levels between CAD patients and controls. The possibility of a founder effect explaining the two common MTHFR polymorphisms has been postulated due the short molecular distance of 2100 base pairs between the two polymorphisms, however this explanation has been discounted as no alleles sharing both the 677T and 1298C nucleotides have been identified[192,194]. The MTHFR A1298C polymorphism remains a valid potential risk factor for cardiovascular disease and MI.

1.5 Rationale and Goals for Current Study.

As described above, a large number of studies have been done on several different ethnic populations from different locations around the world attempting to correlate variations of the genes coding for proteins within the blood coagulation pathway to the clinical outcome of arterial thrombosis, including MI. These studies have frequently produced conflicting results, due to such features as relatively small sample sizes, a low frequency of the gene variant being investigated, and ethnic heterogeneity of the populations being investigated.

As MI is a multifactorial disorder, the genetic component in MI may be the result of a combined effect of a number of genes, with each playing only a small role. The

predisposition imparted by individual genes may act independently or interact with other genes to result in an additive effect and/or synergistic co-effect. One of the largest and most common challenges facing case control studies on possible gene-gene interactions is the ethnic heterogeneity of the investigated populations. Newfoundland presents an interesting and unique population structure which can help to overcome the heterogeneity frequently found in other studies.

The island portion of the Canadian province of Newfoundland and Labrador is located in the Atlantic Ocean, off the eastern coast of Canada. The population of the island of Newfoundland consists mainly of descendants of English and Irish settlers who arrived in the late 18th and early 19th centuries[195]. The settling of Newfoundland is somewhat unique when compared to colonization of other areas of the New World, as the majority of those who immigrated to Newfoundland can be traced almost exclusively back to small regions in southwest England (the neighbouring counties of Cornwall, Devon, Somerset, Dorset, and Hampshire) and southeast Ireland (roughly a 30 mile radius from the city of Waterford). These areas were the principal ports of the United Kingdom's fishing industry at the time[196]. The major migrations concluded in the mid-19th century at which point the population of the island was approximately 75, 000 individuals. From this limited number of founding individuals, the population grew almost exclusively through expansion as the geographical and social isolation of this island has ensured very little inward migration for several hundred years[197] and thus has lead to a small population (530 000 individuals; Statistics Canada 2001 – 50% of whom still live in communities smaller than 2500 individuals[197]) with a relatively homogenous genetic background ideal for the study of complex multifactorial disease such as MI.

In order to overcome the described weaknesses that are commonly faced, we simultaneously analyzed the frequency of FVL, FIIG20210A, FXIII-A V34L, FVII R353Q, MTHFR A1298C and Il-6 -174 G/C in 500 biochemically verified MI patients and 500 healthy individuals in the genetically isolated Newfoundland population in order to determine the individual and combined influence of these gene variants on the occurrence of MI.

2

MATERIALS AND METHODS

2.1 Ascertainment and Clinical Analysis of Myocardial Infarction Patients and Control Subjects

Whole blood samples were collected from 500 consecuitive MI patients and 500 healthy controls from the genetically isolated Newfoundland population. Patients categorized in the MI group represented those presenting to the emergency department or within one of the Health Care Corporation of St. John's hospitals with symptoms and biochemical evidence suggestive of MI. Only patients with cardiac Troponin I values that were greater than 2.0 μg/L (Axsym, Abbott Diagnostics)[198], or greater than 0.5 μg/L (Access II, Beckman-Coulter Corp)[199] were used in this group. Control subjects were selected from consecutive individuals without prior history of MI or thrombosis presenting to the emergency department for trauma, accidental injury, or other non-cardiac and non-thrombotic related events. Discarded blood samples collected for complete blood count were used for DNA extraction and analysis. Upon collection the sample was entered into a computerized database, for the purpose of holding such information as a patient's age and gender, as well as details of the biochemical evidence of their MI in the case of MI patient subjects. Each sample was then given a unique identifying number to correspond with this data and all further information regarding a patients' identity was then destroyed. All subjects, both MI patients and healthy controls were identified only by their unique case number for the entire duration of this project.

A protocol for clinical investigation for this study was granted ethical approval by the Human Investigations Committee of the Faculty of Medicine, Memorial University of

Newfoundland, and by the Health Care Corporation of St. John's.

2.2 DNA Extraction From Whole Blood

All DNA samples were extracted from white blood cells of venous blood which was collected from subjects in EDTA vacutainer tubes. DNA extraction was performed using a protocol based on a simple salting-out method described by Miller *et al.*[200] with the following modifications: 5ml of whole blood was first transferred to a 15ml centrifuge tube. 5 ml of TKM_1 buffer (10 mM Tris, 10 mM KCL, 10 mM $MgCl_2$, 2 mM EDTA) was then added to the blood, followed by 1.25 ml of a membrane-lysing agent, 10% Igepal (Sigma - a non-ionic detergent; Octylphenyl-polyethylene glycol). These components were then mixed by inversion several times, and centrifuged at 2200 rpm (1400 G) for ten minutes at room temperature. The supernatant was then poured off and the nuclear pellet was saved, washed with a further 10 ml of TKM_1 buffer, and centrifuged at 2200rpm for ten minutes at room temperature once more. Following this centrifugation, the supernatant was once again poured off, and the nuclear pellet was re-suspended in 0.8 ml of TKM_2 buffer (10 mM Tris, 10 mM KCL, 10 mM $MgCl_2$, 2 mM EDTA, 0.4 M NaCl). This mixture was then transferred to a 1.5 ml microfuge tube containing 50 µl of 10% SDS, mixed well, and incubated for 30 minutes in 50°C water bath. Following this incubation, 0.4 ml of 5M NaCl was added to the tube, mixed, and then centrifuged at 12000 rpm (30000 G) in a microfuge. The supernatant from this centrifugation was saved, transferred

to a clear 15 ml centrifuge tube to which 2.4ml of absolute ethanol was added. The tube was then closed and gently inverted several times while the DNA precipitated. A 9" glass pipette was melted into a hook and cooled prior to the beginning of the extraction procedure, and was then used to remove the DNA from the tube. The hook was then inverted and allowed to dry for one to two minutes and then re-dissolved in 100-200 µl of either TE buffer (10 mM Tris, 1 mM EDTA, pH 8) or ultra-pure deionized water.

2.3 Genotyping

All alleles were amplified via the polymerase chain reaction (PCR) using approximately 100 – 200 ng of template DNA from the acquired MI patients and healthy control subjects. All primers used were purchased from Integrated DNA Technologies Inc (Coralville, Iowa, USA). Amplification for was carried out using a standard 50 µl PCR reaction containing 2.5 µMol of each primer, as well as 125 µM dNTPs, GeneAmp 10X PCR Buffer consisting at final reaction concentrations of 15 mM $MgCl_2$ 10 mM KCl, 10 mM $(NH_4)_2S0_4$, 20 mM Tris-HCl, 2 mM $MgS0_4$, 0.1% Triton X-100, pH 8.8 @ 25°C, and 2.5 units of AmpliTaq DNA polymerase (All dNTPs, PCR buffer and polymerase were purchased from Applied Biosystems Foster City, California, USA). Thermocyclers used for all amplification reactions were the PerkinElmer Gene-Amp 9600 (PerkinElmer, Boston, Massachusetts, USA) and the Eppendorf MasterCycler (Eppendorf AG, Hamburg, Germany). All restriction endonucleases and buffers used were purchased from

New England Bio Labs, Beverly, Massachusetts, USA.

2.3.1 Factor V Leiden and Prothrombin G20210A

The Factor V and Prothrombin alleles were amplified simultaneously in a multiplex PCR reaction. The Factor V allele was amplified using the primers 5' - ACC CAC AGA AAA TGA TGC CCA - 3' (forward) and 5' – TGC CCC ATT ATT TAG CCA GGA - 3' (reverse) creating a 224bp DNA fragment. The Prothrombin allele was amplified using the primers 5' – TCT AGA AAC AGT TGC CTG GC - 3' and 5' - ATA GCA CTG GGA GCA TTG AGC - 3' creating a 345bp DNA fragment. All samples were amplified by using a denaturing of 5 minutes at 94°C, followed by 35 cycles of 40 seconds at 94°C, 40 seconds at 55°C, and 1 minute at 72°C, with a final extension of 5 minutes at 72°C. Following amplification of these alleles, all PCR products were digested with 10 units each of the restriction endonucleases *Hind*III and *Mnl*I in the restriction buffer NEB2 (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.9 @ 25°C) for four hours at 37°C. When present, the G>A transition at nucleotide 20210 of the Prothrombin gene creates a sequence of 5'-AGCTT-3' that is recognized by the *Hind*III enzyme. In the case of the G>A transition at nucleotide1691 of the Factor V Leiden polymorphism, a sequence of 5'-GAGG-3' which is recognized by the *Mnl*I enzyme is obliterated. The wild-type FII fragment contains two *Mnl* I sites but no *Hin*dIII sites, resulting in fragments of 15, 58, and 272 bp, whereas the 20210A (mutated) product contains two *Mnl* I sites and one *Hin*dIII site, resulting in fragments of 15, 23, 58, and

249 bp after double digestion. The FV fragment contains no *Hin*dIII sites, resulting in *Mnl* I fragments of 37, 67, and 116 bp for the wild-type allele and 67 and 153 bp for the mutated allele. Consequently, when both PCR products of a patient double heterozygote for FII and FV are digested, fragments of 272, 249, 153, and 116 bp are detected by performing gel electrophoresis on the digested PCR products by running them through 10% polyacrylamide for 220 minutes at 100v, and then staining with ethidium bromide in order to visualize the location of the DNA within the gels. The presence of bands of the appropriate length confirmed the presence of the polymorphism in both genes.

2.3.2 Factor XIII-A Val34Leu

Exon 2 of the Factor XIII-A allele was amplified using the primers 5'-CAT GCC TTT TCT GTT GTC TTC - 3' (forward) and 5'- TAC CTT GCA GGT TGA CGC CCC GGG GCACTA - 3' (reverse) creating a 192 bp DNA fragment. All samples were amplified by using a denaturing of 2 minutes at 94°C, followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 48°C, and 1 minute at 72°C, with a final extension of 7 minutes at 72°C. Following amplification for this allele, all samples were digested with 10 units of the restriction endonuclease *Dde*I in the restriction buffer NEB3 (100 mM NaCl, 50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.9 @ 25°C) for four hours at 37°C. When present, the G >T missense mutation in the 34^{th} amino acid residue of the Factor XIII-A subunit, creates a sequence of 5' – C^TNAG - 3' which is recognized by the *Dde*I enzyme resulting in two fragments of 161 bp and 31 bp in length. The presence or

absence of this polymorphism was then analyzed by performing gel electrophoresis on the digested PCR products by running them through 10% polyacrylamide for 220 minutes at 100v, and then staining with ethidium bromide.

2.3.3 Factor VII R353Q

The Factor VII allele was amplified using the primers 5'- TGA TGA CCC AGG ACT GCC T - 3' (forward) and 5' – GGG ATT TGG TGC CAG GAC A - 3' (reverse) creating a 372 bp DNA fragment. All samples were amplified by using a denaturing of 7 minutes at 94°C, followed by 35 cycles of 30 seconds at 92°C, 30 seconds at 60°C, and 45 seconds at 70°C, with a final extension of 7 minutes at 70°C. Following amplification for this allele, all samples were digested with 10 units of the restriction endonuclease *Msp*I in the restriction buffer NEB2 (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.9 @ 25°C) for four hours at 37°C. When present, the A>C transition in the 8^{th} exon of the Factor VII gene causes a substitution of a glutamine residue instead of the arginine which is normally found at 353^{rd} amino acid residue. This substitution creates a sequence of 5' – C^CGG - 3' that is recognized by the *Dde*I enzyme which then cleaves the PCR product resulting in two fragments of 227 bp and 145 bp in length. The presence or absence of this polymorphism was then analyzed by performing gel electrophoresis on the digested PCR products by running them through 2% agarose for 35 minutes at 200v, and then staining with ethidium bromide.

2.3.4 MTHFR A1298C

The A1298C polymorphism in exon 7 of the MTHFR gene was amplified using the primers 5'- CTT TGG GGA GCT GAA GGA CTA CTA C - 3' (forward) and 5' – CAC TTT GTG ACC ATT CCG GTT TG - 3' (reverse) creating a 163 bp DNA fragment. All samples were amplified by using a denaturing of 2 minutes at 92°C, followed by 35 cycles of 1 minute at 92°C, 1 minute at 51°C, and 30 seconds at 72°C, with a final extension of 7 minutes at 72°C. Following amplification for this allele, all samples were digested with 10 units of the restriction endonuclease *Mbo*II in the restriction buffer NEB2 (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.9 @ 25°C) for four hours at 37°C. When present, this A>C substitution causes a glutamine → alanine substitution at the 429th amino acid residue. This substitution creates a sequence of 5' – $GAAGA(N)_8$ - 3' that is recognized by the *Mbo*II enzyme which then cleaves the PCR product resulting in five fragments of 56, 31, 30, 28, and 18 bp if the mutation is present and four fragments of 84, 31, 30, and 18 bp in length if the mutation is absent. The presence or absence of this polymorphism was then analyzed by performing gel electrophoresis on the digested PCR products by running them through 12% polyacylramide for 75 minutes at 100v, and then staining with ethidium bromide.

2.3.5 Il-6 -174G/C

The -174 G/C polymorphism in the promoter region of the Il-6 gene was amplified using the primers 5'- TGC GAT GGA GTC AGA GGA AAC TCA - 3' (forward) and 5' –

AGC CTC AGA CAT CTC CAG TCC TAT - 3' (reverse) creating a 492 bp DNA fragment . All samples were amplified by using a denaturing of 5 minutes at 94°C, followed by 35 cycles of 45 seconds at 94°C, 30 seconds at 54°C, and 1 minute at 72°C, with a final extension of 7 minutes at 72°C . Following amplification for this allele, all samples were digested with 10 units of the restriction endonuclease *Nla*III in the restriction buffer NEB4 (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM dithiothreitol , pH 7.9 @ 25°C. Supplement with 100 µg/ml BSA) for four hours at 37°C). When present, this G>C substitution in the promoter creates a sequence of 5' –CAGT- 3' that is recognized by the *Nla*III enzyme which then cleaves the PCR product resulting in five fragments of 172, 122, 111, 58 and 29 bp if the mutation is present and four fragments of 233, 172, 58, and 29 bp in length if the mutation is absent. The presence or absence of this polymorphism was then analyzed by performing gel electrophoresis on the digested PCR products by running them through 3.5% agarose for 35 minutes at 200v, and then staining with ethidium bromide.

2.4 Statistical Analysis

The prevalence of each gene variant was calculated by counting the total carrier frequency including heterozygotes and homozygotes. The allele frequencies were determined by gene counting. Gene-gene interactions were determined by 1) comparing the prevalence of combined carrier for multiple gene variants in patients and controls; 2) analysis of the distribution of one chosen gene variant in sub-grouped patients and

controls who carry another gene variant as genetic background. Pearson Chi-square statistical analysis was performed to test the association between each genotype and the prevalence of MI. A multivariate analysis using logistic regression was also employed in the analysis in order examine the effect of several variables (genotypes) upon the outcome (presence or absence of a MI event). Odds ratios (OR) were calculated as a measure of the relative risk for MI and were given with 95% CIs. All statistical calculations were performed using SPSS v10.0 (SPSS Inc., Chicago, IL, USA).

3

AN EXTREMELY LOW PREVALENCE OF FACTOR V LEIDEN, FIIG20210A AND FXIIIV34L IN TAIWAN CHINESE POPULATION

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The gene variants, Factor V Leiden (FVL) and prothrombin G20210A (FIIG20210A) are the two most commonly recognized genetic prothrombotic risk factors for venous thrombosis[60,70,201,202]. In contrast, a gene variant of factor XIII, FXIIIV34L, has recently been reported to confer protection against arterial[116-118,] and venous thrombosis[114,115]. In most Caucasian populations, the prevalence of FVL and FIIG20210A approximates 6%, and 2%, respectively[70,201,202]. World wide heterogeneous geographic and ethnic distributions for FVL and FIIG20210A have been suggested by certain studies[41,43,47,203]. Although a low prevalence of these two gene variants has been reported in Asian populations by a few initial surveys, the small sample sizes from most of these studied populations have made it impossible to determine the prevalence of these gene variants. The FXIIIV34L shows a prevalence of 45% (allele frequency of 26%) among Caucasians[116,204], however the prevalence of this gene variant in Asian populations is unknown.

To determine the prevalence of FVL, FIIG20210A and FXIIIV34L in the Chinese population we analyzed DNA samples from 500 unrelated, healthy women from the Taiwan area of China. All of these blood samples were previously collected as normal controls for another research project. Genomic DNA was extracted using the standard salting out procedure[200]. Genotyping analyses of the FVL, FIIG20210A and FXIIIV34L variants were determined using previously described methods[116,205,206]. The allele frequency in different ethnic populations was compared using the Chi-square test.

The carrier frequencies, allele frequencies, and genotypes of FVL, FIIG20210A and FXIIIV34L from the 500 Chinese are given in Table 1. Data from 500 healthy,

unrelated individuals from the Newfoundland Caucasian population is also included in the Table. All subjects from the Newfoundland population were normal controls used in another on going project (Butt et al., in preparation). Newfoundland is geographically isolated from the rest of Canada with the majority of the population being of English and Irish descent. This has provided a relatively homogeneous genetic background.

Consistent with other Caucasian populations, the prevalence of FXIIIV34L and FVL in the Newfoundland population are 52.2% and 4.6%, which yields allele frequencies of *27.1%* and *2.3%*, respectively. However, the prevalence of 1.0% for FIIG20210A (giving a 0.5% allele frequency) among Newfoundlanders is lower than the 2% commonly reported for most Caucasian populations. Of great interest is the extremely low prevalence of all of these variants in Chinese. Among the 500 Chinese individuals, only one heterozygote for each of the three gene variants was detected. No homozygotes or combined heterozygotes were found. The prevalence and allele frequency were 0.2% and 0.1%, respectively, for all of the three variants in the Chinese population. This demonstrates a large and significant difference in the allele frequencies for the FVL ($P<0.001$) and FXIIIV34L ($P<0.001$) in the Chinese population compared with our Caucasian controls. The allele frequency of FIIG20210A was also lower but did not achieve statistical significance.

To the best of our knowledge, the present study is the first to establish the prevalence of FXIIIV34L in the Chinese population. These results also show a significant and highly heterogeneous and ethnic-dependent distribution of FVL, and FXIIIV34L variants between Chinese and Caucasian populations. The Chinese population has a 5-fold lower prevalence of FIIG20210A, a 23-fold lower prevalence of FVL and 240-fold lower

prevalence of FXIII34L compared with our Caucasian controls. Compared with the results of preliminary surveys on other Asian populations, our results suggest that the Chinese population have the lowest frequencies of these gene variants studied so far. The dramatically lower prevalence of FVL and FIIG20210A in the Chinese population suggests a less significant role in predisposing Chinese to venous thrombosis. Recognition of this in the North American Chinese population will be of clinical significance for proper clinical evaluation of thrombotic risk for ethnic Chinese patients. Furthermore, the dramatically lower prevalence of FXIIIV34L in Chinese suggests that this gene variant might not be a useful candidate allele for further study of arterial thrombosis in Chinese.

Table 3.1: Carrier frequencies, allele frequencies, and the frequencies for genotypes of FVL, FIIG20210A and FXIIIV34L in Chinese and Newfoundland Caucasians

	Genotype	Chinese (n = 500)	Newfoundlanders (n = 500)
	V/V	499 (99.8%)	261 (52.2%)
FXIIIV34L	V/L	1 (0.2%)	207 (41.4%)
	L/L	0 (0%)	32 (6.4%)
Carrier frequency		0.2%	47.8%
Allele frequency of *L*		0.1%	27.1%
	G/G	499 (99.8%)	495 (98.9%)
FIIG20210A	G/A	1 (0.2%)	5 (1.0%)
	A/A	0 (0%)	0 (0%)
Carrier frequency		0.2%	1.0%
Allele frequency of *A*		0.1%	0.5%
	G/G	499 (99.8%)	477 (95.4%)
FVL	G/A	1 (0.2%)	23 (4.6%)
	A/A	0 (0%)	0 (0%)
Carrier frequency		0.2%	4.6%
Allele frequency of *A*		0.1%	2.3%

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4

COMBINED CARRIER STATUS OF PROTHROMBIN G20210A AND FACTOR XIII-A LEU34 ALLELES AS A STRONG RISK FACTOR FOR MYOCARDIAL INFARCTION: EVIDENCE OF A GENE-GENE INTERACTION.

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Abstract

Studies associating prothrombin G20210A (FIIG20210A), Factor V Leiden (FVL), and Factor XIIIV34L (FXIII-A V34L) with myocardial infarction (MI) have yielded conflicting results. Complicated gene-gene interactions, small sample sizes and heterogeneous genetic and environmental backgrounds may contribute to conflicting results. Simultaneous analysis of multiple gene variants in a large sample size from a genetically isolated population may overcome these weaknesses. Genotyping was performed in 500 MI patients and 500 controls from the genetically isolated Newfoundland population to determine the prevalence of FIIG20210A, FVL and FXIII-A V34L variants and association with MI. Gene-gene interactions were also analyzed. The prevalence of FIIG20210A was higher in MI patients (3.2%) than in controls (1.0%; P=0.015). FIIG20210A was also 5.6 fold higher in MI patients younger than 51 years compared with age-matched controls (P=0.04). FVL showed 3.9-fold higher prevalence in young patients than older (>50 years) patients (P = 0.004), and 2.7-fold higher than age-matched controls (P =0.007). Furthermore, the prevalence of combined carriers of FXIII-A V34L and FIIG20210A alleles was 12-fold higher in MI patients compared with controls (P = 0.002) and with 92% penetrance. There was disequilibrium of FXIII-A V34L allele to MI patients carrying FIIG20210A as a genetic background. Based on our data, 1) FIIG20210A is a risk factor for MI, possibly important for early onset; 2) FVL may predispose for early onset MI; 3) FXIII-A V34L predisposes for MI in males only; however, 4) interaction between FIIG20210A and FXIII-A V34L form a synergistic co-effect which strongly predisposes for MI, placing combined carriers at high risk for MI.

Introduction

The pathogenesis of myocardial infarction (MI) involves an interaction between environmental influences and genetic predisposition. Genetic factors involving blood coagulation may contribute to the pathogenesis of atherosclerosis as well as play a role in the clinical progression to plaque rupture and localized occlusive thrombus formation[207]. The gene variants Factor V Leiden (FVL-R506Q) and prothrombin G20210A (FIIG20210A) are the two most commonly recognized genetic prothrombotic risk factors for venous thrombosis[60,70,201,202]. Based on the increased thrombotic tendency in venous thrombosis studies, these two gene variants have also been examined for possible association with arterial thrombosis in MI. Several studies have shown a higher prevalence of FIIG20210A in patients with MI compared to normal controls[72,89-91]. However, most of the results from these studies failed to achieve statistical significance, possibly because of the extremely low frequency of FIIG20210A in the studied population and the use of relatively small sample sizes. Nevertheless, a few studies have presented conflicting results[73,81]. Although FVL strongly correlates with deep venous thrombosis (DVT), the majority of the previous studies have failed to show a correlation between FVL and MI[54,61,84]. Recently, a few studies have suggested that FVL may associate with early onset MI[64,208] and MI with normal coronary angiography[66]. In contrast, a common gene variant, factor FXIII-A V34L (FXIIIV34L) has recently been suggested to confer a protective role against MI based on a lower prevalence of FXIII-A V34L in MI patients compared with controls[116-118]. However, conflicting results were also

reported[209-211]. Furthermore, results from function studies on the FXIII-A V34L allele do not support the hypothesis of a protective role against MI[112,204]. Therefore, the role of these three gene variants in the pathogenesis of MI remains unknown.

Being a multifactorial disorder, the genetic components in MI may be a combined effect of a number of genes with each playing only a small role. The predisposition imparted by individual genes may act independently or interact with other gene (genes) to result in an additive effect and/or synergistic co-effect. Common challenges facing case control studies on possible gene-gene interactions include relatively small sample sizes, a low frequency of gene variants, and ethnic heterogeneity of the investigated populations. To overcome these weaknesses, we simultaneously analyzed FVL, FIIG20210A, and FXIII-A V34L in 500 MI patients and 500 normal individuals of the genetically isolated Newfoundland population.

The island portion of the Canadian province of Newfoundland and Labrador is located in the Atlantic Ocean, off the eastern coast of Canada. The population of the island of Newfoundland consists mainly of descendants of English and Irish settlers who arrived in the 18^{th} and 19^{th} centuries[196]. The geographical and social isolation of this island has ensured very little inward migration for several hundred years[197] and thus has lead to a small population (530 000 individuals; Statistics Canada 2001) with a relatively homogenous genetic background ideal for the study of complex multifactorial disease such as MI.

Our analysis not only attempted to correlate MI with each of the three gene variants but also examined for possible gene-gene interactions among the three gene variants in MI. High allele frequency of FXIII-A V34L in both MI patients and normal populations permits us to determine the distribution of co-existence of the FXIII-A V34L and either FIIG20210A or FVL in both MI patient and control populations.

Materials and Methods

Subjects:

Blood samples were collected from 500 consecuitive MI patients (221 males and 279 females) and 500 normal controls (214 males and 286 females) of the genetically isolated Newfoundland population. Patients categorized in the MI group represented those presenting to the emergency department or within one of the Health Care Corporation of St. John's hospitals with symptoms and biochemical evidence suggestive of MI. Only patients with cardiac Troponin I values greater than 2.0 μg/L (Axsym, Abbott Diagnostics)[198] or greater than 0.5 μg/L (Access II, Beckman-Coulter Corp.)[199] were used in this group. Control subjects were selected from consecutive individuals without prior history of MI or thrombosis presenting to the emergency department for trauma, accidental injury, or other non-cardiac and non-thrombotic related events. Discarded blood samples collected for complete blood count were used for DNA extraction and analysis. Ethics approval for this study was granted by the Human Investigation Committee of Memorial University and by the Health Care Corporation of St. John's.

Genotyping of FII20210A, FVL and FXIII-A V34L:

Genomic DNA was isolated from the peripheral blood using standard methods[200]. Genotyping of the FVL, FIIG20210A and FXIII-A V34L were performed by PCR amplification of each of the target alleles from genomic DNA followed by restriction

digestion with each of corresponding enzymes *Mnl1*, *HindIII* and *DdeI,* respectively, as previously described[112,206]. The digested PCR products were separated by electrophoresis in 10% polyacrylamide gels and visualized by staining with ethidium bromide.

Prevalence determination and association study:

The prevalence of each gene variant was calculated by counting the total carrier frequency including heterozygotes and homozygotes. The allele frequencies were determined by gene counting. Pearson Chi Square statistical analysis was performed using SPSS v10.0 to test the association between genotypes and the prevalence of MI. Odds ratios (OR) were calculated as a measure of the relative risk for MI and were given with 95% CIs.

Analysis of gene-gene interaction:

Gene-gene interactions were determined by 1) comparing the prevalence of combined carrier for two of the three gene variants in patients and controls; 2) analysis of the distribution of one chosen gene variant in sub-grouped patients and controls who carry another gene variant as genetic background.

RESULTS

Genotyping FIIG20210A, FVL and FXIII-A V34L:

The genotype distributions, carrier frequencies, and allele frequencies of FIIG20210A, FVL, and FXIII-A V34L in both the MI patient and the control populations are given in Table 4.1. The FIIG20210A allele was detected in 3.2% of patients which was significantly higher than the 1% observed in controls (OR 3.2, 95% CI 2.6-4.0, P = 0.015). An identical prevalence of FVL was observed in both patient and control populations (4.6% vs. 4.6%). No homozygotes for either FIIG20210A or FVL allele were found in either population. In both patient and control populations, FXIII-A V34L had similar prevalence (47.0% vs. 47.8%) and allele frequency (27.7% vs. 27.1%). The prevalence of homozygous 34L was higher in patients compared with controls (8.4% vs. 6.4%), but the difference did not reach statistical significance.

Table 4.1: Distributions of genotypes, and carrier and allele frequencies of FIIG20210A, FVL and FXIII-A V34L in MI patient and healthy control (HC) populations.

	Genotype	MI (n = 500)	HC (n = 500)	OR	P value
	G/G	484 (96.8%)	495 (99.0%)		
FIIG20210A	G/A	16 (3.2%)	5 (1.0%)		
	A/A	0 (0%)	0 (0%)		
Carrier F.		3.2%	1.0%	3.2	0.015
Allele F.		1.6%	0.5%		
	R/R	477 (95.4%)	477 (95.4%)		
FVL(R506Q)	R/Q	23 (4.6%)	23 (4.6%)		
	Q/Q	0 (0%)	0 (0%)		
Carrier F.		4.6%	4.6%	1.00	NS
Allele F.		2.3%	2.3%		
	V/V	265 (53.0%)	261 (52.2%)		
FXIII-A V34L	V/L	193 (38.6%)	207 (41.4%)		
	L/L	42 (8.4%)	32 (6.4%)		
Carrier F.		47.0%	47.8%	0.97	NS
Allele F.		27.7%	27.1%		

The distribution of the three gene variations was further analyzed by sub-grouping patients and controls according to age (Table 4.2). MI patients were divided into those with an early age of onset (≤ 50 years) and those with a later age of onset (>50 years). The control population was also divided into the two corresponding age groups. Interestingly, a disequilibrium distribution of the FVL allele was observed in the early onset patient group. The FVL allele was detected in 13.0% of patients with early age of onset, which was significantly higher than the 3.8% in patients with a later age of onset (OR: 3.9, 95% CI 3.3-4.4; P = 0.004) and the 4.8% in the age matched controls (OR: 3.0, 95% CI 2.2-3.7; P= 0.007). The prevalence of FII 20210A was also significantly higher in the early onset group of MI (4.3%) compared to the controls under age 50 (0.8%). Although the prevalence of FII G20210A was slightly higher in the early onset group compared to the later onset group, this difference did not achieve statistical significance. The FXIII-A V34L allele showed a slight but statistically insignificant difference in prevalence between the patients with early and later onset age.

The distribution of the three gene variations was further analyzed by sub-grouping patients and controls based on gender (Table 4.3). The gender ratio in both the 500 MI patients and 500 controls were similar. The distribution of the three gene variations in controls showed no significant difference between males and females. However, there were significant differences in the distribution of FVL and FXIII-A V34L between sexes in MI patients. The prevalence of both FVL and FXIII-A V34L was significantly higher in males than in female patients. The prevalence of FIIG20210A was not significantly different between sexes among MI patients.

Table 4.2: Distribution of genotypes among MI patients with different onset ages and compared with age-matched healthy controls (HC).

	MI	**HC**	OR	P
FII 20210A Carriers	**16/500 (3.2%)**	**5/500 (1%)**	**3.3**	**0.015**
Age ≤ 50Y	2/46 (4.3%)	3/373 (0.8%)	5.6	0.04
Age > 50Y	14/454 (3.1%)	2/127 (1.6%)	2.0	NS
FVL Carriers	**23/500 (4.6%)**	**23/500 (4.6%)**	**1.0**	**NS**
Age ≤ 50Y	6/46 (13.0%)	18/373 (4.8%)	3.0	0.007
Age > 50Y	17/454 (3.8%)	5/127 (3.9%)	1.0	NS
FXIII 34LCarriers	**235/500 (47.0%)**	**239/500 (47.8%)**	**1.0**	NS
Age ≤ 50Y	19/46 (41.3%)	176/373 (47.2%)	0.8	NS
Age > 50Y	216/454 (47.6%)	63/127 (49.6%)	0.9	NS

Table 4.3: Comparison of genotypes distribution in different genders among MI patients and healthy controls

	Males	Females	**OR** (95% CI)	**P**
FII20210A				
MI	8/221 (3.6%)	8 /279 (2.9%)	1.3 (0.5, 3.5)	0.635
HC	3/214 (1.4%)	2/286 (0.7%)	2.0 (0.3, 12.2)	0.435
FVL				
MI	16/221 (7.2%)	7/279 (2.5%)	3.0 (1.2, 7.5)	0.012
HC	8/214 (3.7%)	15/286 (5.2%)	0.7 (0.3, 1.7)	0.426
FXIII-A V34L				
MI	140/221 (63.3%)	95/279 (34.1%)	3.3 (2.3, 4.8)	<0.001
HC	97/214 (45.3%)	110/286 (38.5%)	1.3 (0.9, 1.9)	0.123

Gene-gene Interactions:

FIIG20210A and FXIII-A V34L:

Interaction between FXIII-A V34L and FIIG20210A was first analyzed by comparing the prevalence of combined carriers (individuals carrying both FIIG20210A and FXIII-A V34L) in the total patient and control populations with their corresponding theoretical prevalence of combined carriers. Using carrier frequencies described in table 1 the theoretical prevalence for combined carriers of FIIG20210A and FXIII-A V34L is 1.5% (47.0% x 3.2%) in MI patients and 0.48% (47.8% x 1%) in controls (Figure-2). The observed prevalence of combined carriers in the MI patient population (2.4%; 12 of 500) was 1.6-fold higher than its theoretic prevalence (1.5%), and in normal control population (0.2%; 1 of 500) was 2.4-fold lower than its theoretic expected prevalence (0.48%). The observed prevalence of combined carriers was 12-fold higher in MI patient compared with the control population (P = 0.002).

The interaction between the FIIG20210A and the FXIII-A V34L was further examined by analysis of the distribution of FXIII-A V34L in sub-grouped patients and controls who carry FIIG20210A as a genetic background. Although the FXIII-A V34L showed an almost equal distribution in our MI patient and control populations, FXIII-A V34L alleles were detected in 75.0% (12 of 16) patients with a genetic background of FIIG20210A but only in 20.0% (1 of 5) of controls with the same genetic background (OR 3.7, 95% CI 2.4-5.1, P = 0.013) (Figure-3).

Of 13 combined carriers of FIIG20210A and FXIII-A V34L identified from the

studied population (500 patients and 500 controls), 12 subjects (92.3%), including 7 males and 5 females, belonged to the MI patient population but only 1 male (0.7%) from the control population. The co-existence of these two gene variants imparts a strong predisposition for MI with high penetrance.

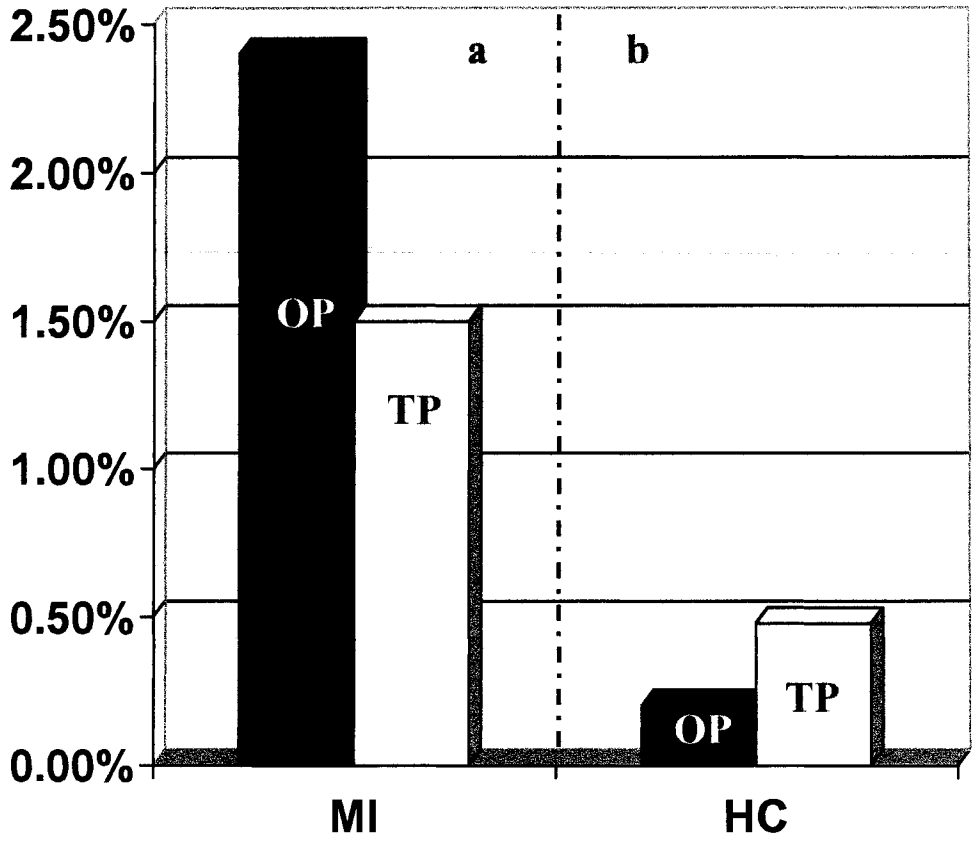
FVL and FXIII-A V34L:

The prevalence of combined carriers of FVL and FXIII-A V34L were similar in both MI patient (9 of 500, 1.8%) and control (12 of 500, 2.4%) groups and was consistent with their expected frequencies (2.16% in patients and 2.2% in controls). We further analyzed the prevalence of FXIII-A V34L in patients and controls who carry the FVL allele as a genetic background. The prevalence of FXIII-A V34L was slightly higher in the sub-grouped controls (12 of 23, 52.17%) than in the sub-grouped patients (9 of 23, 39.1%) but the difference was not statistically significant.

FIIG20210A and FVL:

There were no combined carriers of FIIG20210A and FVL in the MI patient or control populations. This is expected considering a calculated expected frequency of combined carriers of 0.13% in MI patients and 0.01% in normal controls, respectively.

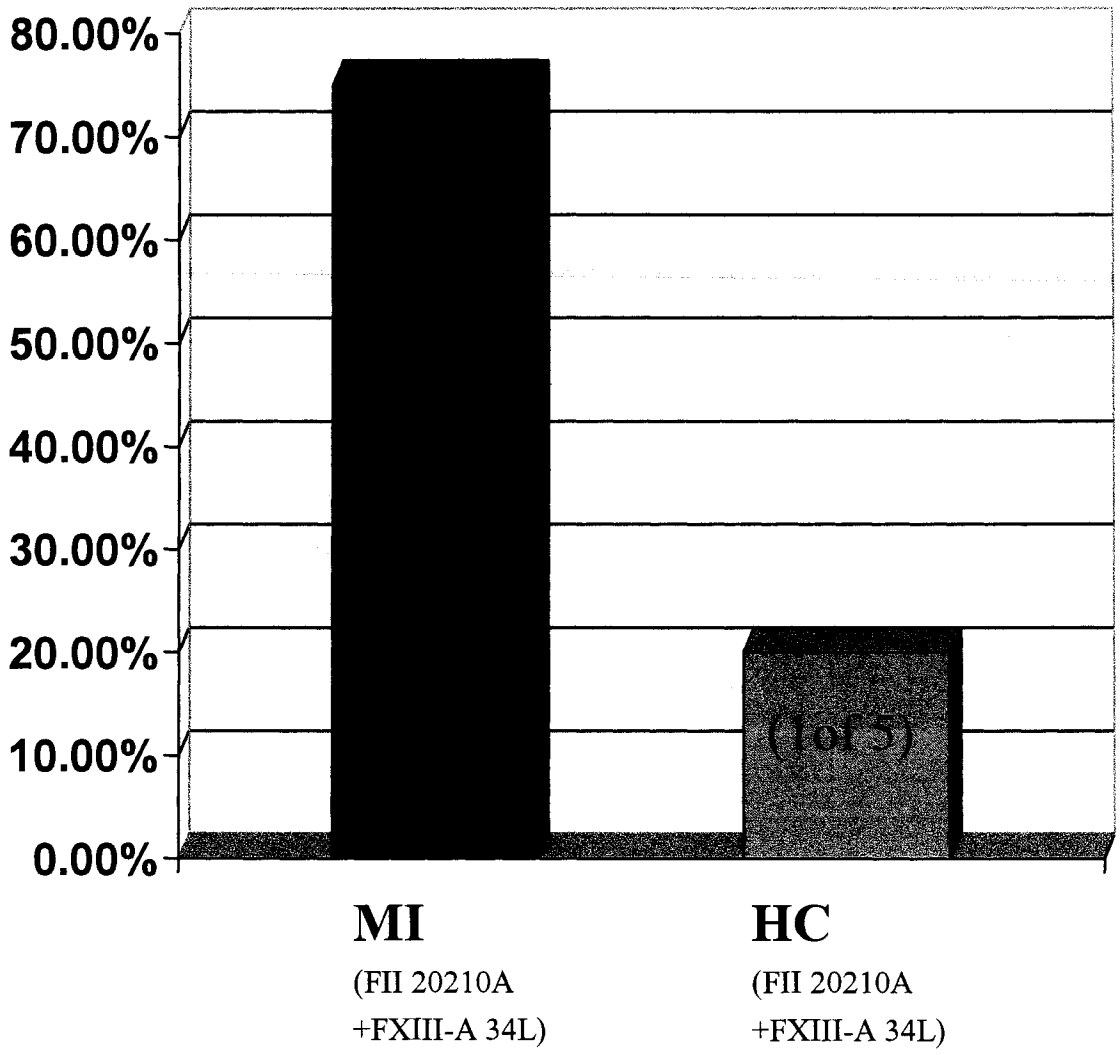
Figure-2: Comparison between the theoretic and observed prevalence of combined carriers of mutations in MI patients and healthy controls.



a) Comparison between the theoretic (TP) and observed (OP) prevalence of combined carrier for FII20210A + FXIII-A V34L in MI patients; and

b) Comparison between the expected and observed prevalence of combined carrier for FII20210A + FXIII-A V34L in healthy controls

Figure-3: Prevalence of FXIII34L in MI patients and Healthy Controls who carry the FII20210A allele



Discussion

Our results showed that the prevalence of FVL and FXIII-A V34L in the Newfoundland population are consistent with those reported from other Caucasian populations elsewhere. However, the 1% prevalence of FIIG20210A determined from the Newfoundland population was lower than that reported from most other Caucasian populations (2.0 – 2.5%)[71].

Although of lower prevalence in the Newfoundland population, the FII 20210A allele was still shown to be 3.3-fold higher in MI patients compared with the control population. The prevalence of FII 20210A is even higher among MI patients with early onset age (5.6-fold higher) compared with age-matched controls. This may suggest that FII 20210A is important for early age of onset of MI. The low frequency of the FII 20210A allele in Caucasians and extremely low frequencies in other populations[43,44,47,212] indicates that the FII 20210A allele may only contribute to the pathogenesis of MI in a small portion of MI patients.

FVL is commonly recognized as a strong risk factor in the pathogenesis of venous thrombosis; however the majority of studies have thus far failed to show an association between FVL and MI[52,61,213]. Recently, some studies have suggested that FVL may be associated with early onset MI[64,208] and MI with normal coronary angiography[66]. Although there was identical prevalence of FVL (4.6%) in both our patient and control

populations, the prevalence of FVL in patients with early onset age was 3.4-fold higher than patients with later onset age and 2.7-fold higher than age-matched controls. These data suggest a possible thrombotic predisposition of FVL for early onset of MI. Furthermore, the prevalence of FVL was higher in male MI patients than female MI patients by almost three fold. Nevertheless, the proportion of male MI patients with FVL was not significantly different from males in the control group ($p=0.110$). It is not possible with this data to suggest FVL as a gender specific risk factor for MI. As only a relatively small number of early onset MI patients have been studied, further efforts focusing on FVL in other early onset MI patients and on possible gender differences will be of value.

FXIII-A V34L has been proposed as a protective factor against MI because of the reduced prevalence observed in MI patients. We observed a slightly but non-significantly lower prevalence of FXIII-A V34L in MI patients (47.0%) compared with our control population (47.8%). However, homozygosity for the L allele was more frequently observed in MI patients than in controls (8.4% vs. 6.4%) and the gene frequency for FXIII-A V34L was similar in both patient and control populations. This data suggests that FXIII-A V34L does not confer a protective role against MI. Comparison of male MI patients to female MI patients showed an almost 2 fold higher prevalence of FXIII-A V34L in males. Comparison of male MI patients with FXIII-A V34L to males in the control group showed a significantly higher prevalence among MI patients ($p=0.012$). This data suggests that FXIII-A V34L may impart an increased risk for MI that is influenced by gender.

Genetic predisposition for MI can result from either an additive effect of several genes or a synergistic effect from interactions between multiple genes. Each of these genes may only make a small contribution to the global pathogenesis of MI. In our study, an interaction between FII 20210A and FXIII-A V34L was identified based on the distribution disequilibrium of combined carriers among MI patients. The prevalence of combined carriers in MI patients was higher than expected, but lower than expected in controls. FXIII-A V34L shows equal carrier frequency in both MI patients and controls and does not seem to independently predispose for MI. Furthermore, FII 20210A itself showed only a 3.2-fold increased risk for MI. However, combined carriers of FII 20210A and FXIII-A V34L showed a 12-fold increased risk for MI. These results indicate that the interaction of FII 20210A and FXIII-A V34L forms a synergistic rather than an additive effect in the pathogenesis of MI. In our study, penetrance of MI in individuals carrying both FIIG20210A and FXIII-A V34L alleles was 92.3%. We, therefore, conclude that co-existence of the FII 20210A allele with the FVIIIV34L allele is a strong predisposing factor for MI.

The possibility has been considered that since the Newfoundland population is descended from a relatively small number of founders that a closely linked gene rather than FXIII-A V34L may be the causative mutation. This, however, is a considerably less likely possibility for a number of reasons. Firstly there is a very close biochemical functional relationship between FXIII and FII. The two factors are very closely related sequentially in the clotting cascade. Activated FII (thrombin) is responsible for generation

of fibrin from fibrinogen. Fibrin monomers quickly polymerize into larger polymers. These are relatively loosely held together. Activated FII also activates FXIII which helps strengthen the clot by crosslinking fibrin chains. The reinforced clot is much more resistant to fibrinolysis by plasmin. Secondly, the FII 20210A allele is observed to correlate with elevated levels of prothrombin in plasma, which may enhance activation of FXIII leading to a greater tendency toward thrombosis. Thirdly, the FXIII-A V34L variant allele has been clearly shown to be much more prone to activation by thrombin than the wild type allele: FXIII-A V34L variant results in an amino acid substitution 4 residues from the thrombin cleavage site that enhances the rate of activation by thrombin[112]. Both heterozygotes and homozygotes for the FXIII-A V34L allele display increased sensitivity to activation by thrombin[112,205] while a more significant increase in FXIII activation occurs in homozygotes for FXIII-A V34L[112,205]. No doubt the higher levels of the FXIII-A V34L enzyme can create a greater risk for plasmin resistant clots. Our data suggests that the increased FXIII activity due to FXIII-A V34L alleles is not in itself sufficient to impart a significant effect on the development of MI. However, the co-existence of both FII 20210A and FXIII-A V34L alleles results in a synergistic enhancement of prothrombotic tendency through interaction of the two gene products at the biochemical level[112]. Specifically, higher tendency for clot formation by higher levels of FII with the FII 20210A allele coupled with a tendency for greater resistance to fibrinolysis due to greater FXIII activity by the FXIII-A V34L allele. This enhanced prothrombotic tendency could result in a greater risk for MI in individuals carrying both variants.

Typical of a later onset disease, 90% of our patients had their MI at age 50 or older. Only 25% of our controls were within this older age group. It is very possible that some of our controls under age of 50 will develop MI later in life. This may result in underestimation of the true risk associated with certain alleles. The positive associations described here would only be strengthened by such an underestimation. Other alleles found to impart no significant risk for MI may in fact be weak risk factors. We also recognize the small number of individuals in the control and patient group carrying the FII 20210A allele. Although, the difference in carrier frequency for this allele was significantly greater in the younger MI patient group, this observation would be strengthened further by examining a larger number of younger individuals with MI. Further studies examining more MI patients and age-matched controls in the Newfoundland as well as other populations will be of even greater value to more clearly establish the magnitude of risk imparted by presence of these genetic risk factors. These studies are currently underway.

In summary, we analyzed three gene variants, FII 20210A, FVL and FXIII V34L in 500 patients with MI and 500 normal controls from a genetically isolated Newfoundland population. The FII 20210A was associated with MI and possibly more important for early age of onset of MI. FVL was only associated with MI in patients with early onset age. A strong predisposition for MI results from interaction between FII 20210A and FXIII-A V34L alleles as was suggested by a 12-fold increased prevalence of combined carriers in MI patients compared with controls. There was also a very high penetrance (92%) of MI in combined carriers. Therefore we conclude: 1) FII 20210A is a

genetic risk factor for MI, possibly for early onset; 2) FVL predisposes for early onset of MI; 3) FXIII-A V34L may independently predispose to MI in males only; 4) interaction between FII 20210A and FXIII-A V34L results in a synergistic co-effect which strongly predisposes to MI. Combined carriers of both FII 20210A and FXIII-A V34L are at very high risk for MI.

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THE MTHFR A1298C POLYMORPHISM IS ASSOCIATED WITH MYOCARDIAL INFARCTION

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Myocardial infarction (MI) is known to be a complex multifactorial polygenic disease involving several complex and as yet unknown interactions between genes and environment. It has recently been demonstrated that several different SNPs may play a role in the pathogenesis of MI. In the blood coagulation pathway, the R353Q polymorphism of the coagulation factor VII gene (FVII R353Q) has been shown to confer a protective effect against MI[127,128,135,136], while in the pathway for the metabolism of homocysteine a SNP at nucleotide 1298 (A1298C) in the gene that codes for the enzyme 5, 10-Methylenetetrahydrofolate reductase (MTHFR) which catalyzes the reduction of 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate have been frequently associated with cardiovascular disease[188-190,193]. Also, high base-line levels of one of the most widely accepted markers for systemic inflammation, C - reactive protein (CRP), have been implicated in the occurrence of MI and cardiovascular disease[148-151]. Interleukin-6 (Il-6) is the primary determinant of hepatic production of CRP[154], and a known promoter polymorphism in the Il-6 gene located at nucleotide -174 upstream from the start of transcription (G>C) has been shown to increase circulating CRP. However, inconsistent results for each of these have also been reported by a number of different studies[124-126,170,191]. The role of these SNPs in the pathogenesis of MI remains unknown.

In order to further investigate the relationship between these polymorphisms and MI, we simultaneously analyzed the frequency of the MTHFR A1298C, FVII R353Q, and Il-6 -174 G/C polymorphisms in 500 biochemically verified MI patients and 500 healthy individuals in the genetically isolated Newfoundland population. The island portion of the Canadian province of Newfoundland and Labrador is located in the Atlantic Ocean, off the eastern coast of Canada. The population of the island of

Newfoundland consists mainly of descendants of English and Irish settlers who arrived in the 18th and 19th centuries[196]. The geographical and social isolation of this island has lead to very little inward migration for several hundred years[197] and the population remains genetically isolated. With a relatively homogenous genetic background, the population is an ideal subject for the study of complex multifactorial diseases such as MI. In order to determine the individual and combined influence of these gene variants on the occurrence of MI, we simultaneously analyzed the frequency of the FVII R353Q, MTHFR A1298C and Il-6 -174 G/C polymorphisms in 500 biochemically verified MI patients and 500 healthy individuals in the genetically isolated Newfoundland population.

Patients and controls were selected as described previously[214]. Genomic DNA was isolated from the peripheral blood using standard methods[200]. Genotyping of the FVII R353Q, MTHFR A1298C and Il-6 -174G/C were performed by PCR amplification of each of the target alleles from genomic DNA followed by restriction digestion with each of corresponding enzymes[134,192,160]. The prevalence of each gene variant was calculated by counting the total carrier frequency including heterozygotes and homozygotes. The allele frequencies were determined by gene counting. Pearson Chi Square statistical analysis was performed using SPSS v10.0 to test the association between genotypes and the prevalence of MI. Odds ratios (OR) were calculated as a measure of the relative risk for MI and were given with 95% CIs. The detailed results of the genotyping are given in Table 5.1.

Table-5.1: Distributions of genotypes, and carrier and allele frequencies of FVII R353Q, MTHFR A1298C and IL-6 -174 G/C in MI patient and healthy control (HC) populations.

	Genotype	MI (n = 500)	HC (n = 500)	OR	P value
	A/A	156 (31.2%)	237 (47.4%)	-	-
MTHFR A1298C	A/C	288 (57.6%)	215 (47.0%)	-	-
	C/C	56 (11.2%)	48 (9.6%)	-	-
Carrier F.		68.8%	52.6%	1.3	p<0.001
Allele F.		40.0%	31.1%	-	-
	R/R	416 (83.2%)	410 (82.0%)	-	-
FVII R353Q	R/Q	82 (16.4%)	86 (17.2%)	-	-
	Q/Q	2 (0.4%)	4 (0.8%)	-	-
Carrier F.		16.8%	18.0%	0.93	NS
Allele F.		8.6%	9.4%	-	-
	G/G	166 (31.2%)	157 (31.4%)	-	-
Il-6 -174 G/C	G/C	257 (51.4%)	255 (51.0%)	-	-
	C/C	77 (15.4%)	88 (17.6%)	-	-
Carrier F.		66.8%	68.4%	0.98	NS
Allele F.		41.1%	43.0%	-	-

The MTHFR 1298C allele shows a prevalence of 52.6% in the Newfoundland population which is higher compared with other reported Caucasian populations (%)[193,194]. the The MTHFR A1298C allele interestingly shows a significantly increased prevalence in our MI patients (68.8%) compared to our healthy controls subjects (52.6%) (p<0.001, OR 1.3, 95% CI 1.1 – 1.8).

Although the FVII 353Q allele shows a slightly higher prevalence in our healthy controls (18%) than compared to our MI patients (16.8%), the small difference does not give statistical significance. Our results therefore, do not support a protective role of FVII 353Q in MI, which has been suggested in previous studies[123-131].

The Il-6 -174 G/C allele shows a similar prevalence in both our MI patients and healthy controls (66.8% vs 68.4%). Therefore our data does not suggest an association between Il-6 -174 G/C and MI. However, at this moment, we cannot completely rule out CRP as a genetic risk factor for MI, as several other novel reported variations within the CRP gene or its regulators have yet to be studied. These markers are currently being examined in our laboratory as part of our ongoing study into genetic risk factors in MI.

A multivariate analysis using logistic regression was also employed in the analysis in order examine the effect of the presence of multiple polymorphisms upon the presence or absence of a MI. No association was found between any combination of two or more genes.

It is worth noting that with respect to the results obtained for the FVII R353Q, MTHFR A1298C, and Il-6 -174 G/C mutations, possible interactions with other nearby proteins in their respective systems were not examined. It is possible for instance, much like the gene-gene interaction between FII 20210A and FXII-A (reference), that FVII R353Q may interact with other polymorphic proteins such as tissue factor, FIX or FX to reduce the action of the coagulation cascade and actually function in the protective role others have attributed to it. Likewise, the MTHFR A1298C gene variant may be influenced by the presence of the C677T mutation or other polymorphic markers in the homocysteine metabolic pathway, and Il-6 may react with other cytokines in the inflammation pathway to influence the occurrence of an MI. The investigation of further gene polymorphisms in these pathways in the Newfoundland and other populations will be of tremendous benefit to clinicians world-wide in the diagnosis and counselling of patients abnormally

predisposed to suffer MI. The observations reported here would be further strengthened by examining an increased number of patients with MI. This effort is currently underway.

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CONCLUSIONS AND FUTURE RESEARCH

6.1 Conclusions

Myocardial Infarction is a complex multifactorial disease resulting from contributions from an individual's lifestyle (including smoking, activity levels and diet), as well as the development of atherosclerosis, thrombophilia and inflammation, and the genes which control these. The level at which genetics contribute to the development of an MI is still the subject of much debate. The results in our study into the role of genetic polymorphisms in the blood coagulation and metabolic systems indicate: **1)** There is a significant and highly heterogeneous and ethnic-dependent distribution of the FVL, and FXIIIV34L variants between Chinese and Newfoundland Caucasian populations. The Chinese population shows a 5-fold lower prevalence of FIIG20210A, a 23-fold lower prevalence of FVL and 240-fold lower prevalence of FXIII34L compared with our Newfoundland Caucasian controls. **2)** The prevalence of FVL and FXIII-A V34L in the Newfoundland population are consistent with those reported from other Caucasian populations elsewhere. However, the 1% prevalence of FIIG20210A determined from the Newfoundland population was lower than that reported from most other Caucasian populations (2.0 – 2.5%). **3)** FIIG20210A is a genetic risk factor for MI, possibly for early onset. **4)** FVL predisposes for early onset of MI. **5)** FXIII-A V34L may independently predispose to MI in males. **6)** Interaction between FIIG20210A and FXIII-A V34L results in a synergistic co-effect which strongly predisposes to MI. Combined carriers of both FIIG20210A and FXIII-A V34L are at very high risk for MI. **7)** There is no association between a MI and a protective role for the FVII R353Q gene variant in the Newfoundland population. **8)** The MTHFR A1298C polymorphism is associated with MI

in the Newfoundland population, and at a frequency of 47.4% this allele has a higher prevalence within the Newfoundland population than has been reported in other Caucasian populations (25 – 35%). **9)** The Il-6 -174G/C promoter polymorphism does not appear to play a role in the pathogenesis of MI. **10)** A multivariate analysis using logistic regression was also employed in the analysis in order examine the effect of the presence of multiple polymorphisms upon the presence or absence of a MI. No association was found between any combination of two or more genes other than between FII 20210A and FXIII-A 34L.

6.2 Future Research

It is worth noting that with respect to the results obtained for the FVII R353Q, MTHFR A1298C, and Il-6 -174 G/C mutations, possible interactions with other nearby proteins in their respective systems were not examined. It is possible for instance, much like the gene-gene interaction between FII 20210A and FXII-A 34L described in chapter 4 of this thesis, that FVII R353Q may interact with other polymorphic proteins such as tissue factor, FIX or FX to reduce the action of the coagulation cascade and actually function in the protective role others have attributed to it. Likewise, the MTHFR A1298C gene variant may be influenced by the presence of the C677T mutation in MTHFR or other polymorphic markers in the homocysteine metabolic pathway, and Il-6 may react with other cytokines in the inflammation pathway to influence the occurrence of an MI. The investigation of further gene polymorphisms in these pathways in the Newfoundland and other populations will be of tremendous benefit to clinicians world-wide in the diagnosis and counselling of patients abnormally predisposed to suffer MI.

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