# STUDY OF GENETIC RISK FACTORS FOR MYOCARDIAL INFARCTION IN THE NEWFOUNDLAND POPULATION

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**Study of genetic risk factors for myocardial infarction**

**in the Newfoundland population**

by

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Abstract

Myocardial infarction (MI) is a leading cause of death in developed countries. Genetic association studies have been applied to search for associations between MI and candidate genes. In the present study, genetic variants for two MI candidate genes, thrombospondins-4 (THBS4) (29926C>G) and methylenetetrahydrofolate reductase (MTHFR) (677C>T and 1298A>C) were genotyped using a large cohort from the genetically homogenous Newfoundland population. This study focused on determining the distribution of each individual gene variants, and possible intragenic and intergenic allelic interactions. Our results suggest that: 1) homozygosity for the THBS4 29926C variant is associated with MI, especially in older females; 2) the MTHFR 677T is a weak protective factor against MI but its effect is silenced when it is in cis with 1298C; 3) the MTHFR 1298C is a risk factor for later onset MI and its effect is reduced when the trans configuration with 677T.

Keywords:

myocardial infarction, risk factors, association study, thrombospondin-4, Newfoundland population, MTHFR 677C>T, MTHFR 1298A>C, Haplotype

Abbreviations

| | |
|---|---|
| ACE | Angiotensin I converting enzyme 1 |
| AGT | Angiotensinogen |
| AGTR1 | Angiotensin II type 1 receptor |
| AMI | Acute myocardial infarction |
| ANP | Atrial natriuretic peptide |
| APOB | Apolipoprotein B |
| APOE | Apolipoprotein E |
| BNP | Brain natriuretic peptide |
| CCR5 | Chemokine (C-C motif) receptor 5 |
| CAD | Coronary artery disease |
| CD14 | CD14 antigen |
| CHD | Coronary heart disease |
| CI | Confidence interval |
| CNP | C-type natriuretic peptide |
| COL1A1 | Collagen type I alpha-1 |
| CRP | C-reactive protein |
| CVD | Cardiovascular disease |
| dTMP | Deoxythymidine monophosphate (deoxythymidylate) |
| Dump | Deoxyuridine monophosphate (deoxyuridylate) |
| EM | Expectation Maximization |

| | |
|---|---|
| FGB | Fibrinogen chain |
| FIBB | Fibrinogen |
| FVII | Factor VII |
| HSP70A1 | Heat shock protein 70-1 |
| IL6 | Interleukin 6 |
| LDLR | LDL receptor |
| LPL | Lipoprotein lipase |
| Lp(a) | Lipoprotein (a) |
| MI | Myocardial infarction |
| MMP3 | Matrix metalloproteinase 3 |
| MS | Methionine synthase |
| MTHFR | Methylenetetrahydrofolate reductase |
| MZ | Monozygotic |
| NC | Normal control |
| THBD | Thrombomodulin |
| THBS4 | Thrombospondin-4 |
| THcy | Total plasma homocysteine |
| THF | Tetrahydrofolate |
| OR | ODDS RATIO |
| PAI1 | Platlet activator 1 |
| PECAM1 | Platelet-endothelial cell adhesion molecule-1 |
| PON1 | Paraoxonase 1 |

| | |
|---|---|
| SELP | Selectin P |
| SNP | Single nucleotide polymorphism |
| TS | Thymidylate synthase |

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# Chapter 1

## Review of Literature

## 1.1 Myocardial Infarction (MI)

The clinical manifestations of coronary heart disease (CHD) are due to chronic arterial obstructions or acute arterial occlusions in various vascular territories, which ultimately leads to an inadequate supply of blood to the heart muscle causing ischemia. In some cases ischemia may become so severe that it causes a MI. This may result from atherosclerosis within the coronary arteries, or the formation of a blood clot. The most likely is a combination of both which ultimately leads to necrosis of cardiac muscle cells (Ulbricht and Southgate 1991).

Atherosclerosis, can start to develop in childhood and progresses undetected throughout life. In a large atherosclerotic plaque, the outermost shell can burst causing slight bleeding that can lead to the formation of a thrombus, which can block the vessel (Steinberg et al. 2002). Atherosclerosis with arterial wall inflammation involves diapedesis of monocytes across the endothelial barrier, and release of a variety of cytokines and chemokines (Stratford et al. 1986; Poole and Florey 1958; Lusis 2000; Libby 2002). The genetic basis of the process has not yet been deciphered.

MI remains a leading cause of morbidity and mortality in North America, Europe and Asia (Breslow 1997; Braunwald 1997). Moreover, acute myocardial infarction (AMI) is now the leading cause of death in Canada, especially among elderly patients (Boucher et al. 2001). Therefore, prevention of this disease is an important public health goal. One

approach to preventing myocardial infarction is to use observational genetic studies to identify disease-susceptibility genes, which will further deeper our understanding of the etiological pathways of MI and thus indicate new potential targets for intervention (Tobin et al. 2004).

MI is a complex multifactorial and polygenic disorder. The incidence of MI increases as the number of conventional risk factors present, including smoking, high blood pressure, high cholesterol, physical inactivity, obesity and diabetes (Psaty et al. 2001; Esteghamati et al. 2006; Tonstad and Andrew Johnston 2006). These traditional risk factors have only a limited sensitivity among subjects with lows-to-moderate levels of risk, which suggests the contribution of uncharacterized genetic components in susceptibility to MI.

## 1.2 Genetic risk factors

The level at which an individual's genotype contributes to the development of MI is still the subject of much debate. The role of inheritance is strongly supported by the predictive value of a family history for premature ischemic disease (Roncaglioni et al. 1992; Brenn and Njolstad 1998; Becker et al. 1998). A family history of cardiovascular disease (CVD) is one of the strongest risk markers for MI, with relative risk being as high as 3.4 for men with two or more affected parents or siblings (Leander et al. 2001). Twin studies indicate that the heritability of MI is 0.26 in males and 0.60 in females (Wienke et al. 2001; Zdravkovic et al. 2002; Nordlie et al. 2005). The mechanisms by which genetic variants

contribute to the strong familiarity of CHD are not fully understood, but it is likely the disease is influenced by a large number of genetic variants (Tang and Tracy 2001). These studies support the hypothesis that genetic factors contribute to the development of coronary artery disease (CAD) and MI. A high death rate, late-onset characteristics, and complications of phenocopy in families also present major challenges to the genetic dissection of this important disease (Sing et al. 2003).

Genetic studies of CAD and MI are lagging behind those of other cardiovascular disorders. The major reason for the limited success in the field of CAD/MI genetics is that they are complex multifactorial diseases. Genetic association studies have been applied to search for relationships associations between MI and various genes whose biological function are known to associate with vascular biological pathways. The pathways include blood coagulation, inflammation reaction, vascular function, and homocysteine metabolism (Wu and Tsongalis 2001; Marcucci et al. 2005).

Gene mutations in several metabolic pathways (lipoprotein metabolism, coagulation and inflammation) may lead to overproduction or underproduction of key proteins and hence upset the delicate balance of homeostasis. Intermediate phenotypes such as hypertension, diabetes, and obesity interact to modulate the risk for CHD. Figure 1 summarizes the role of some of the key candidate genes and intermediate traits involved in CHD (Stephens and Humphries 2003).

Outcomes of most association studies are highly contradictory and controversial, which reflects the complicated nature of genetic involvement in MI. Genetic predisposition in MI results from the co-effect of multiple genes, gene-gene and/or gene-environment interactions. These collectively produce an additive or synergistic effect which affects risk for disease. Individual genetic changes may produce small or insufficient effects to impart significant pathogenic risk. Gene-gene interaction as a significant factor in genetic predisposition to MI has been clearly demonstrated in previous studies, and genetic predisposition for CAD from co-effect of intragenic allelic interaction has also been previously reported for other genes (Butt et al. 2003, Xie et al. 2003).

## 1.3. Association studies

Association studies aimed to test whether single-locus alleles, genotype frequencies or haplotype frequencies are different between 2 groups (cases and controls). Genetic association studies are to determine whether a genetic variant is associated with a disease or trait. When a particular allele, genotype or haplotype of a polymorphism or polymorphism(s) was seen more often than expected in an individual with the disease, the allele or genotype is associated with increased risk of disease. The usual conclusion of such studies is that the gene variants being tested either affect risk of disease directly or are markers for some nearby genetic variants that affect risk of disease.

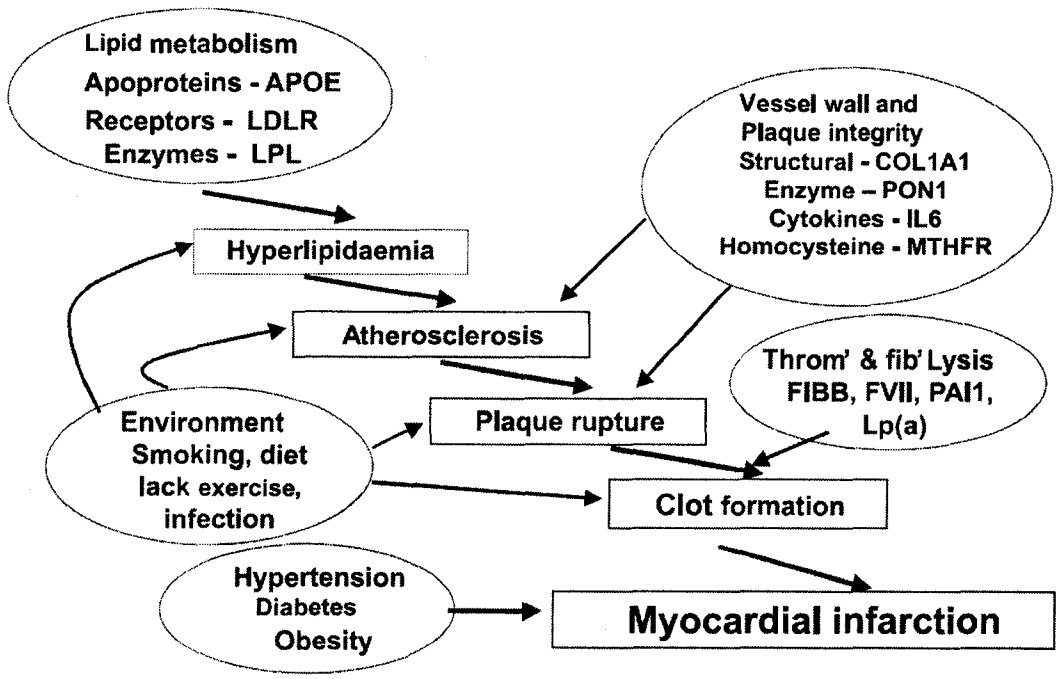


Figure 1. Pathophysiology, intermediate traits and candidate genes implicated in CHD (Stephens and Humphries 2003).

Genetic association studies essentially look for correlations between phenotype and genotype, aiming to detect association between one or more genetic polymorphism and a trait, which might be some quantitative characteristic, discrete attribute or disease. It has been realized that genetic susceptibility to common complex disorders probably involves many genes, most of which have small effects. This fact, together with the identification of large numbers of single nucleotide polymorphisms (SNPs) throughout the genome and the rapidly falling genotyping costs, has led to the importance of association studies in genetic epidemiology (Risch and Merikangas 1996). An association can be found either with functional genetic variants that have biological consequences related to a disease or with other variants that are in linkage disequilibrium with these variants. Although association studies provide important indirect evidence for genetic effects of a particular gene, it is clear from the literature that such studies have serious limitations. Thus, one must use caution when drawing conclusions from association studies (Almasy and MacCluer 2002; Gambaro et al. 2000). There are now explicit guidelines for publishing genetic association studies of complex diseases (Editorial 1999; Cooper et al. 2002).

SNPs in candidate genes have been tested for genes associated with myocardial infarction. Results from these studies have, in general, not been replicated or indicate only a modest risk of myocardial infarction. Case-control association studies have identified several genes with variants that are associated with either an increased risk of myocardial infarction or a protective effect (Topol et al. 2001; Butt et al. 2003; Wang and

Chen 2003; Xie et al. 2003; Shen et al. 2004). Most case-control association studies have focused on variants in genes related to known risk factors for coronary heart disease. Methodologically, this is the easiest approach by which a candidate gene is selected based on its potential involvement in CAD/MI. Some genes associated with MI were listed in the Table 1 (Gibbons et al. 2004).

### 1.3.1 SNP

A SNP is the most common type of stable genetic variation in the population. It is a DNA sequence variation occurring when a single nucleotide in the genome differs between members of a species or between paired chromosomes in an individual. SNPs are found throughout the genome (e.g. in exons, introns, intergenic regions, promoters, enhancers, etc). A SNP occurs in approximately every 1000 base pairs (Brookes 1999) and has a minor allele frequency of greater than 1% in at least one population (Risch, 2000). The total number of SNPs in the human genome is estimated to be more than ten million (Kruglyak and Nickerson 2001; Botstein and Risch 2003). The high frequency with which SNPs are found within the genome allows us to use SNPs as candidate polymorphisms to be tested directly as the functional or causal mutations for a disease or trait. To avoid doing genotypings that fail to identify disease-causing polymorphisms when conducting association studies, it is common to actively select for SNPs that are likely to have a direct impact on the disease or other phenotype of interest (Chanock 2001).

Table 1. Selected Genes Associated With MI (Adapted from Gibbons et al. 2004)

| Gene Symbol | Gene Name | Reference |
|---|---|---|
| MMP3 | Matrix metalloproteinase 3 | Terashima et al. 1999 |
| LPL | Lipoprotein lipase protein | Holmer et al. 2000 |
| CCR5 | Chemokine (C-C motif) receptor | Gonzalez et al. 2001 |
| APOB | Apolipoprotein B | Tybjaerg-Hansen et al. 1991 |
| APOE | Apolipoprotein E | Batalla et al. 2000 |
| AGTR1 | Angiotensin II type 1 receptor | Batalla et al. 2000 |
| AGT | Angiotensinogen | Batalla et al. 2000 |
| ACE | Angiotensin I converting enzyme 1 | Batalla et al. 2000 |
| CRP | C-reactive protein | Margaglione et al. 2000 |
| FGB | Fibrinogen _ chain | Zito et al. 1997 |
| THBD | Thrombomodulin | Ireland et al. 1997 |
| PECAM1 | Platelet-endothelial cell adhesion molecule-1 | Sasaoka et al. 2001 |
| ANP | Atrial natriuretic peptide | Cho et al. 1999 |
| BNP | Brain natriuretic peptide | Cho et al. 1999 |
| CNP | C-type natriuretic peptide | Cho et al. 1999 |
| HSP70A1 | Heat shock protein 70-1 | Gonzalez et al. 2001 |
| SELP | Selectin P | Tregouet et al. 2002 |
| CD14 | CD14 | Unkelbach et al. 1999 |

### 1.3.2 Haplotype

Because new SNP alleles arise as mutations at different loci and at different points in time, and because they occur with such great abundance in the genome, groups of neighboring SNPs may have alleles that show distinctive patterns of linkage disequilibrium (LD). As such, they may create a haplotypic diversity that can be exploited in both genetic linkage and direct association studies (Nickerson 1992).

Haplotype-based analysis could prove a more powerful approach to dissecting the genetic architecture of complex diseases. Haplotypes capture information about regions descended from ancestral chromosomes. They are essential for many genetic studies, especially for association based gene mapping, because they can be much more informative than single markers, and they give higher power for assigning a phenotype to a genetic region. Being able to use haplotypes is particularly important for SNP markers, which individually are relatively uninformative (Cardon and Bell 2001). It is demonstrated that only a few SNPs are needed to characterize the most common haplotypes occurring within each haplotype block. These are termed tag-SNPs. However, tag-SNPs have been discovered in only a small number of genes. In the future, because of the application of the haplotype map to delineate these tag-SNPs, haplotype-based association analysis are likely to replace single SNP-based analysis (The International HapMap Consortium 2003).

The HapMap project is currently defining these haplotype blocks in the human genome. The aim is to clarify how the human genome is organized into haplotype blocks by analyzing the block patterns in blood samples taken from people in Japan, Nigeria, and China, as well as from people of northern and western European ancestry in the United States. The biggest advantage of this approach is that if common haplotypes capture most of the genetic variation across sizable regions, they can be tested with the use of a few tag SNPs (Gabriel et al. 2002), potentially providing a powerful shortcut to identifying genes for complex traits, including CAD.

Current practical laboratory techniques provide unphased genotype information for diploids, i.e., an unordered pair of alleles for each marker. Reconstruction of haplotypes from genotype data is then a crucial step in the analysis process. There are two approaches to the problem. One is based on trios: haplotypes are determined from the genotypes of a subject's parents. This involves significant additional genotyping costs and potential problems in recruiting the parents. Furthermore, in the case of SNPs, on average up to 12% of the alleles can still remain ambiguous. The second approach is to use the available population sample of genotype data alone, and to apply computational or statistical inference to find the most likely haplotype configuration that is consistent with the observed genotype data. A number of approaches have been proposed to the haplotype inference problem, but currently none of them are targeted at sparse marker maps (Lauri et al. 2004).

Haplotype analysis has become increasingly common in genetic studies of human disease. Much of the attention was aimed at finding the haplotype phase. It is usually crucial to estimate correctly the haplotype frequencies in the population and not necessarily to phase the individual genotypes. There are, however, a few EM-based (Expectation Maximization) algorithms that directly estimate the haplotype frequencies (Long et al. 1995; Excoffier and Slatkin 1995; Kuhner et al. 2000; Fallin and Schork 2000). Those methods try to find a haplotype distribution that maximizes the probability of observing genotypes in a given sample, under the assumption of Hardy-Weinberg equilibrium. One of the main drawbacks in these methods is that there is no guarantee that the algorithm converges to a global maximum, or that the algorithm converges in polynomial time. The starting point of the algorithm heavily affected both the convergence of the EM algorithm to a global optimum and its running time. However, even in the worst case, individual haplotype frequency estimates via the EM algorithm do not deviate much beyond 5% of their true value among sample individual for sample size bigger than 100 (Fallin and Schork 2000).

### 1.3.3 Limitations of candidate gene association studies

Like all experimental designs and model systems, genetic association studies in human samples have strengths and limitations (Schork et al. 2000; Schork et al. 2001). These statistics make clear the importance of elucidating the genetic basis of CAD, but as mentioned previously, this is a difficult area to study. In many cases, genetic associations

found in one study are not supported by subsequent studies, which makes the search for definitive conclusions problematic.

The results from the candidate gene association studies should be interpreted with caution as many of these studies are compounded by the selection bias of cases and controls, population admixture, phenotyping errors, and small sample sizes in some studies. Numerous case-control association studies were carried out and variants in many genes have been implicated in increasing or decreasing the susceptibility to CAD or MI (Shen et al. 2004; Wang and Chen 2003; Butt et al. 2003; Xie et al. 2003). Publication and time-lag biases are also important considerations. Medical journals and authors both tend to select positive, rather than negative, findings for publication. Whereas negative studies often take longer time to be published than positive studies (Ioannidis et al. 2001; Winkelmann et al. 2000).

Little is known of gene-gene and gene-environment interactions in CAD, but it would be surprising if they were not important (Sing et al. 2003). There have been hundreds of association studies for CAD and MI, but most have been underpowered and probably represent type 1 errors. A few have been convincingly confirmed (Lusis 2000). Some large-scale association studies have been performed with many candidate genes. For example, a study of patients from the Cleveland Clinic Foundation examined 62 vascular biology candidate genes and observed the significant associations with SNPs of three

unlinked genes, all of which are the members of the thrombospondin family (Topol et al. 2001).

The slow process of gene identification in complex traits is most likely caused by several factors, such as locus and allelic heterogeneity, epistasis, difficulties in assessing the complex phenotypes, unknown modes of inheritance, phenocopies, reduced penetrance, and a high frequency of disease-predisposing alleles (Lusis et al. 2004).

Etiologic and genetic heterogeneity underlying the affected status of cases or the quantitative trait of interest can obscure the identification of an association, especially in small samples, and thus increasing the probability of a false-negative result. 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 unusual population structure, which can help to overcome the heterogeneity frequently found in other studies. The island of Newfoundland 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 $17^{th}$ and $18^{th}$ centuries (Bear et al. 1987; Martin et al. 2000). The geographical and social isolation of this island has ensured very little inward migration for several hundred years and has led to a small population (530 000 individuals; Statistics Canada 2001) with a relatively homogenous genetic background, which is ideal for the study of complex multifactorial disease such as MI.

## 1.4 Thrombospondins-4 (THBS4)

THBS4 is one of five members of the THBS family of multidomain extracellular matrix proteins with related sequences but diverse tissue distribution. All are structurally related and at least four of them (THBS-1, -2, -4 and –5) have been detected in vascular tissue (Wight et al. 1985; Lawler et al. 1993; Stenina et al. 2003). All are related to a wide range of processes involving the vessel wall, including smooth muscle cell proliferation (Majack et al. 1986) and migration (Taraboletti et al. 1990), and associated with various extracellular matrix proteins (Narouz-Ott et al. 2000). THBS-4 appears to be more specific to cardiac and skeletal muscle (Lawler et al. 1993). The variant THBS4 P387 is predicted to affect folding and secretion of the protein and disruption of the calcium binding site (Bornstein 1992; Geourjon et al. 1994), and may affect the cellular dynamics of the vessel wall (Stenina et al. 2003). The inhibitory effects of THBS4 P387 on endothelial cell (EC) repair functions, coupled with its stimulatory effects on smooth muscle cell (SMCs), may predispose the vasculature to the initiation and development of atherosclerotic lesions (Stenina et al. 2003).

Topol et al. (Topol et al. 2001) studied SNPs in 62 vascular biology genes in more than 350 families, in which CAD or MI had occurred before age 45 in males and 50 in females. They determined that certain variants of thrombospondin genes (thrombospondin is a platelet surface protein involved in blood clotting) were associated

with either an increased or decreased risk of MI. The 387P variant of THBS4 was most strongly associated with an increased risk of MI. Carriers of the P allele had an adjusted odds ratio for MI of 1.89 (P = 0.002). However, Topol's group noted that they were unable to replicate these findings in two smaller studies (Topol et al. 2001). This result was replicated by using a larger number of patients (Wessel et al. 2004).

The THBS4 A387P variant also shows ethnic distribution differences. The THBS4 387P allele showed a dramatically lower prevalence in Asian Chinese populations compared with Caucasians (3.8% vs 19.6-23.2%) and failed to associate with CAD or MI in the studied populations (Zhou et al. 2004). The THBS4 387P allele was associated in men (p=0.013) in Japanese population (Yamada et al. 2002). Interestingly, in a more recent investigation, the THBS4 variant 387P allele was significantly associated with reduced risk, rather than increased risk, for premature MI (OR=0.43, 0.22 to 0.85) in Netherlands Caucasians (Boekholdt et al. 2002). McCarthy et al. (2004) analyzed 210 polymorphisms in 111 candidate genes in 352 white subjects with familial premature coronary heart disease and 418 white controls, and found a strongest association with an A387P variant in THBS4 (p = 0.002) (McCarthy et al. 2004). Therefore, the reality of THBS4 A387P in MI remains unknown. We believe that the contradictory results may be due to either population genetic mixture or the effects of different modifiers for the THBS4 387P allele among the different ethnic populations.

## 1.5 Methylenetetrahydrofolate reductase (MTHFR)

MTHFR is a flavoprotein that catalyzes the reduction of 5,10-methylenetetrahydrofolate (5,10-CH2-H4 folate) to 5-methyltetrahydrofolate (5-CH3-H4 folate), the major circulatory form of folate and methyl donor for homocysteine remethylation, thereby regulating the fasting plasma homocysteine level (Figure 2). Hyperhomocysteinemia is an established risk factor for CAD and is associated with increased mortality in patients with confirmed CAD (Gallagher et al. 1996; Ma et al. 1996; Refsum et al. 1998). Thus, total plasma homocysteine is an independent and significant risk factor for CAD. A common polymorphism in the MTHFR gene is the underlying cause of moderate hyperhomocysteinemia in about half of the cases (Kang et al. 1993; Ma et al. 1996).

### 1.5.1 Two common polymorphisms in MTHFR

In 1995, a common polymorphism was reported, MTHFR 677C>T (rs1801133) (Frosst et al. 1995). The change induces thermolability of the enzyme, and is in the catalytic region. In a study with site directed mutagenesis, it was found that enzyme activity of the MTHFR carrying the 677T is reduced to 45 % and 677T homozygotes have higher level of homocysteine (Weisberg et al. 2001).

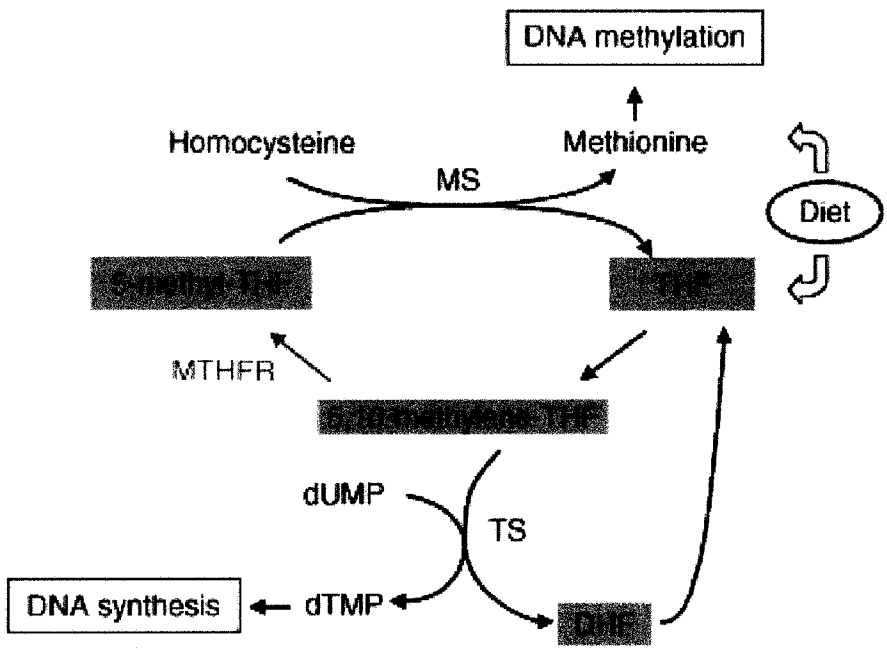


Figure 2. Schematic presentation of folate metabolism in relation to DNA methylation and thymidylate synthesis (Kono and Chen 2005).

The second common polymorphism, MTHFR 1298A>C (rs1801131) does not induce thermolability (van der Put et al. 1998). This polymorphism is as common as the 677C>T polymorphism with approximately 10 % homozygotes reported. In a study with site directed mutagenesis, enzyme activity of the MTHFR carrying 1298C (having a glutamate to alanine substitution) is reduced to 68 %. However, the effects of the 1298C and 677T variants are not additive. When this amino acid substitution was introduced into an enzyme that already carried the 677T (the alanine to valine substitution) the activity was reduced only from 45 % to 41 % (Weisberg et al. 2001). These *in vitro* findings support the observation that plasma homocysteine levels are hardly affected in subjects carrying the 1298C variant (van der Put et al. 1998; Weisberg et al. 1998; Weisberg et al. 2001).

The prevalence of homozygotes for the MTHFR 677T allele in newborns varies between different populations: from 2.7 to 32 % (Figure 3) (Wilcken et al. 2003). The prevalence of homozygotes for 1298C does not differ so much in different populations, 9 % in the Dutch (van der Put et al. 1998) and Canadian reports (Weisberg et al. 1998), 12.5 % in a French study (Chango et al. 2000), and 14.2 % in an Irish population (McCarthy et al. 2004).

The MTHFR 677C>T and 1298A>C mutation are in trans configuration (two mutation on the different chromosome) (van der Put et al. 1998). Only a few studies have reported

that MTHFR 677C>T and 1298A>C mutation occur in cis (co-location of two mutation on the same chromosome) (Isotalo et al. 2000).

### 1.5.2 MTHFR polymorphisms and MI

Two variants in the coding region of the MTHFR gene, 677C>T and 1298A>C, have been reported to be prevalent in North America, European and Asian populations. The 677C>T was suggested to moderately reduce the enzyme activity and increase the level of plasma homocycteine (Brattstrom and Wilcken 2000). In contrast, 1298A>C alone was reported not to significantly affect plasma homocysteine, but may do so only when combined with the 677T allele (Weisberg et al. 2001). The correlation between risk of CAD including MI and the two common variants, 677 C>T and 1298A>C has been investigated by a number of studies using a variety of different populations.

A meta-analysis by Brattström et al. (Brattström et al. 1998) looked at 23 studies that investigated an association between MTHFR genotype and CVD. They concluded that the 677T variant is commonly associated with mild hyperhomocysteinemia, while it does not increase the risk of CHD (Brattström et al. 1998).

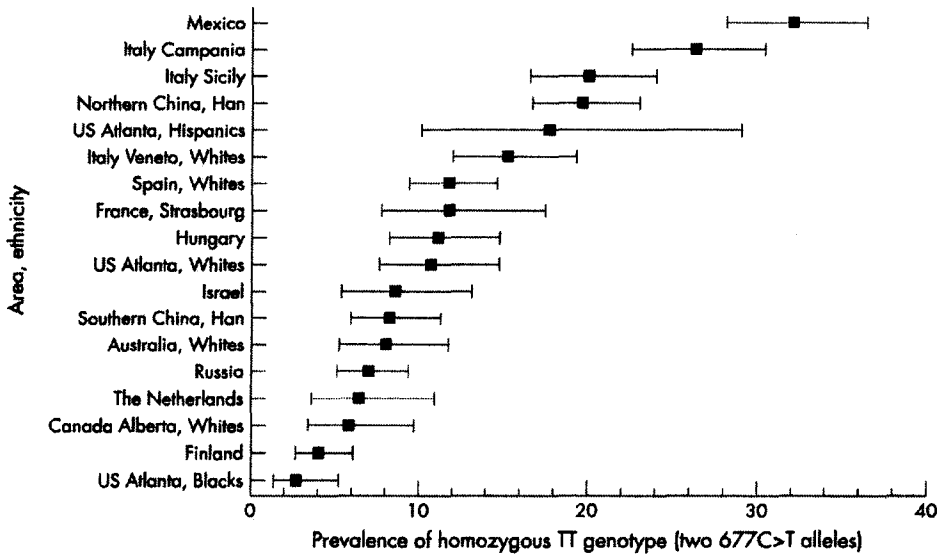


Figure 3 Prevalence of homozygous TT genotype among newborns (Wilcken et al 2003).

However, a meta-analysis of the MTHFR 677C>T polymorphism examined 40 observational studies, involving a total of 11,162 cases and 12,758 controls, found that the 677TT genotype was associated with an increased risk of CHD (odds ratio 1.16, 1.05– 1.28) (Klerk et al. 2002). The CHD risks in European and North American populations were determined separately. Europeans with the 677TT genotype had a significantly increased risk of CHD (odds ratio 1.14, 1.01– 1.28), while no increase in risk was noted for North Americans with this genotype (odds ratio 0.87, 0.73–1.05). There was a significant heterogeneity between the results obtained in European populations, compared with those in the North American populations, which might largely be explained by interaction between the MTHFR 677 C>T polymorphism and folate status (Klerk et al. 2002).

Another meta-analysis examined 72 retrospective studies in which the MTHFR 677C>T genotype was analysed in cases and controls, and 20 prospective studies in which total plasma homocysteine (tHcy) was determined (Wald et al. 2002). In the prospective studies, tHcy was associated with an increase in risk of ischemic heart disease and stroke. Similar results were seen for MTHFR genotypes. A meta-analysis including only prospective studies on cardiovascular diseases reported 14 studies with an average relative risk of 1.49 (95% CI: 1.31-1.70) for cardiac events (Bautista et al. 2002).

Recently, a meta-analysis investigated the association between the MTHFR 677C>T polymorphism and CHD (Lewis et al. 2005). No strong evidence was discovered to

support an association of the MTHFR 677 C>T polymorphism and CHD in Europe, North America, or Australia. A PubMed search reveals that only one study has verified an association between elevated homocysteine concentration and the 1298C allele (Haviv et al 2002), while there are more negative studies of reasonable size (Abu-Amero et al. 2003). The importance of the 1298A>C polymorphism has also been questioned from a biochemical point of view. Compatible with the negative studies, Yamada et al. found that purified 1298C-encoded MTHFR showed no difference from wild type either in its kinetic or stabilizing properties, regardless of if in *trans* with 677C or 677T (Yamad et al. 2001).

The results of numerous studies (Adams et al. 1996; van Bockxmeer et al. 1997; Morita et al. 1997; Schwartz et al. 1997; Friedman et al. 1999; Szczeklik et al. 2001; Kolling et al. 2004; Wald et al. 2002; Klerk et al. 2002; Ray et al. 2002; Haviv et al 2002; Abu-Amero et al. 2003; Lewis et al. 2005) have failed to resolve the controversy in this area. Therefore, the relation between the two common variants and the risk of CAD has not been established and continues to be the subject of much debate.

# Chapter 2

# Objectives of the study

The development of a first myocardial infarction is associated with a large number of contributing factors. Age, male sex, hypertension, smoking, diabetes, body mass index and hypercholesterolemia are considered established risk factors. The primary aim of the present dissertation was to evaluate whether specific biomarkers could improve the prediction of subjects at risk for myocardial infarction. The two SNPs in the MTHFR gene and one SNP in the THBS4 gene were suggested to associate with increased risk of myocardial infarction with contradictory results. To investigate the possible association between these polymorphisms and MI, we study 1032 patients with MI and 1014 normal controls in the Newfoundland population.

The aims of the study conducted for this thesis were:

1. To test the hypothesis that the THBS4 gene variant is a risk factor for MI.
2. To confirm the results of our initial study that the 1186 G>C (A387P) variant in the THBS4 gene is associated with MI.
3. To investigate the genetic effects of the 677C>T and 1298A>C in MI, to analyze the distribution of combined genotypes and to estimate haplotype frequencies.

# Chapter 3

# Materials and Methods

## 3.1 Subjects

Blood samples were collected from 1032 consecuitive MI patients (640 males and 392 females) and 1014 normal controls (477 males and 537 females) from the genetically isolated Newfoundland population. Patients categorized in the MI group represented those presenting to the emergency department 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) or greater than 0.5 μg/L (Access II, Beckman-Coulter Corp.) were used in this group (Uettwiller-Geiger et al. 2002). 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. The unmatched controls for age are used, for the patients are mostly in older groups, but there were not enough older control. The younger control sample may dilute the association power. Subgrouping according gender and age will often more precisely estimate the association. Ethics approval for this study was granted by the Human Investigations Committee of Memorial University and by the Health Care Corporation of St. John's.

3.2 Salt extraction-isolation of human genomic DNA from whole blood

Whole blood was collected from subjects in a lavender vacutainer (EDTA). 5ml of blood was then transfered into a 15ml centrifuge tube and 5ml of TKM1 buffer (10mM Tris HCl, 10mM KCl, 10mM $MgCl_2$, 2mM EDTA pH7.6)was added. Following this, 1.25 ml of 10% IGEPAL CA_630 was added, mixed by inversion several times, and centrifuged at 2200 rpm for 10 minutes at room temperature. The supernatant was slowly poured off to save the nuclear pellet. The pellet was then washed twice in 10ml of TKM1 buffer, gently resuspended in 0.8ml of TKM2 buffer (10mM Tris HCl, 10mM KCl, 10mM $MgCl_2$, 4M NaCl, 2mM EDTA pH7.6 0.) and transfered into a microfuge tube. 50 µl of 10% SDS was then added, and the whole suspension was mixed thoroughly by pipetting back and forth several times and incubated for 20 to 30 minutes at 50°C. 0.40ml of 5M NaCl was added in the tube, vortexed well and centrifuged at 14000rpm for 20 minutes. The supernatant was then transferred into a 15 ml polypropylene tube. 2.4ml of absolute ethanol at room temperature was added and the tubes inverted several times until the DNA precipitated. The precipitated DNA strands were removed with a plastic spatula or pipette, transferred to a 1.5 ml microcentrifuge tube containing 100-200 µl TE buffer (10 mM Tris-HCl, 0.2 mM $Na_2EDTA$, pH 7.5) and incubated at 65°C for several hours. The concentration of DNA was measured by taking A260 and A280 (Miller et al. 1988).

## 3.3 Genotyping of the THBS4 A387P polymorphism

Genotyping of the THBS-4 1186G>C variant was conducted using Taq Man SNP genotyping technology on real-time PCR (ABI Prism® 7000 Sequence Detection System) which gives call rate of 99.996%. Primers and probes were designed using the Primer Express 2.0 software according to Applied Biosystems' guidelines (Foster city, CA, USA). The primers used were: forward: GCACTAGGTCTGCACTGACATTG; and reverse: CCGGTTCTGCTTTGATAACAACA. The probes used were: 387A allele: VIC-AAATGGACCGTGCGTT-TAMRA; 387P allele: FAM-AAATGGAGCGTGCGTT-TAMRA. PCR reactions were carried out in 96-well optical reaction plates and each reaction consisted of 1.5µl genomic DNA (10 ng/µL) as template, 7.5µl of TaqMan Universal PCR Master Mix, 700 nM (each) primer and 200 nM (each) probe in a total volume of 15µl. After activation of UNG (2 min; 50°C) and AmpliTaq Gold (10 min; 95°C), 42 cycles of denaturation (15 s; 95°C) and elongation (1 min; 58°C) were used for two-step PCR. The fluorescent signals of the two reporter dyes were directly determined after PCR. The four distinct clusters were manually categorized as 387GG(VIC), 387CC(FAM), 387GC (VIC and FAM) and no amplification control (NTC) based on the VIC to FAM ratio (Figure 5).

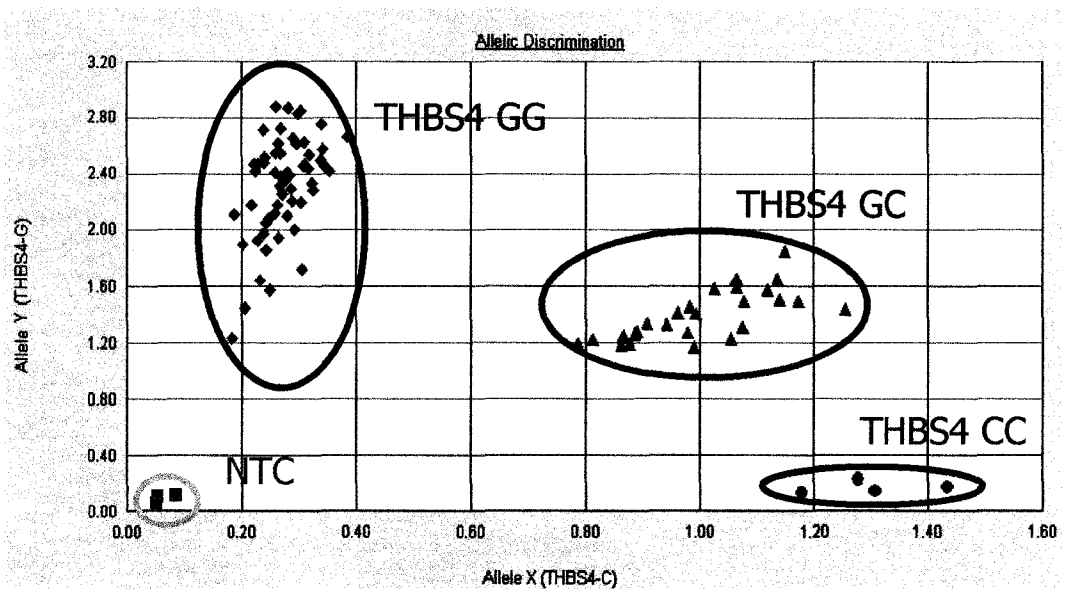


Figure 5 Example of distribution of THBS4 genotypes when assayed with realtime-PCR.

3.4 Genotyping of the MTHFR 677C>T and 1298A>C polymorphisms

Genotyping of the 677C>T and 1298A>C was conducted using Taq Man SNP genotyping technology on real-time PCR (ABI Prism® 7000 sequence Detection System). The primers and probes for 677C>T and were obtained from the Validated TaqMan SNP genotyping kit supplied by Applied Biosystems (ABI; Foster City, CA). The primers for 1298A>C were: forward: GGAGGAGCTGCTGAAGATGTG; and reverse: CCCGAGAGGTAAAGAACAAAGACTT. The probes used were: 1298A allele: VIC-ACCAGTGAAGAAAGTGT-TAMRA, 1298C allele: FAM-CAGTGAAGCAAGTGT-TAMRA. PCR reactions were carried out in 96-well optical reaction plates and each reaction consisted of 0.3μl genomic DNA (100 ng/μl) as template, 2.5μl of TaqMan Universal PCR Master Mix, 700 nM (each) primer and 200 nM (each) probe in a total volume of 5μl. After activation of UNG (2 min; 50°C) and AmpliTaq Gold (10 min; 95°C), 40 cycles of denaturation (15 s; 95°C) and elongation (1 min; 60°C) were used for two-step PCR. The fluorescent signals of the two reporter dyes were directly determined after PCR. The four distinct clusters were manually categorized as 1298A or 677C (VIC), 1298C or 677T (FAM), 1298A/C or 677C/T (VIC and FAM) and no amplification control (NTC) based on the VIC to FAM ratio.

### 3.5 Prevalence determination and 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. Tests of Hardy–Weinberg equilibrium (HWE) were carried out for all loci among MI patients and controls separately by the Chi-square test. 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. Genetic power was calculated using the method for case-control studies of discrete traits posted on the web page of the Psychiatric and Neurodevelopmental Genetics Unit (PNGU) at Harvard Medical School (http://pngu.mgh.harvard.edu/~purcell/cgi-bin/cc2.cgi).

### 3.6 Haplotype frequency estimate

The haplotype frequencies were estimated via the method of maximum likelihood from genotype data through the use of the expectation-maximization (EM) algorithm under the assumption of HWE (Fallin and Schork 2000). Haplotype frequencies for various marker combinations were estimated for MI patients and controls separately. Linkage disequilibrium between the 677C>T and 1298A>C variants was calculated as D', which ranges from 0 (no linkage disequilibrium) to 1 or –1 (complete linkage disequilibrium) (Lewontin 1988).

Chapter 4

## Gender Dependent Association of THBS4 A387P Polymorphism With MI

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Arterioscler Thromb Vasc Biol. 2004 Nov;24(11):e183-4.

A recently identified novel missense variant of THBS4, A389P (29926 G>C) was correlated with premature myocardial infarction (MI) by an exploratory genetic association study on 184 MI patients from the American population (Topol et al. 2001). In this study, the THBS4 387P allele showed the strongest association, with an adjusted odds ratio for MI of 1.89 (P=0.002 adjusted for covariates) for individuals carrying the 387P allele. This was replicated by the same group using a larger number of patients patients (Wessel et al. 2004). An in vitro functional study of the THBS4 A387P substitution provided further evidence in support of a proatherogenic effect. The THBS4 A387P substitution was shown to have a gain of function effect leading to suppression of endothelial cell adhesion and proliferation (Stenina et al. 2003). The THBS4 A387P variant also shows ethnic distribution differences. The THBS4 387P allele showed a dramatically lower prevalence in Asian Chinese populations compared with whites (3.8% versus 19.6 to 23.2%) and failed to associate with coronary artery disease (CAD) or MI in the studied populations (Zhou et al. 2004). Interestingly, in a more recent investigation, the THBS4 variant 387P allele was significantly associated with reduced risk, rather than increased risk, for premature MI (OR=0.43, 0.22 to 0.85) in Netherlands whites (Boekholdt et al. 2002). Therefore, the true effect of THBS4 A387P in MI remains unknown. We believe that the contradictory results may be caused by either population genetic mixture or the effects of different modifiers for the THBS4387P allele among the different ethnic populations.

We examined the THBS4 A387P variant as part of our study to explore potential gene variants as risk factors for MI and the effect of interaction between different variants on MI. So far 11 different variants have been analyzed, and some have been published (Zheng et al. 2002; Butt et al. 2003; Xie et al. 2003). To test the hypothesis that the THBS4 variant constitutes increased risk for MI we carried out a case control study by genotyping the THBS4 A387P in a large number of patients with MI from the genetically isolated Newfoundland population. This approach reduces the effects of genetic admixture in the studied subjects, a common challenge facing case control studies. The population of the island of Newfoundland consists mainly of descendants of English and Irish settlers who arrived in the $17^{th}$ and $18^{th}$ Centuries (Bear et al. 1987; Martin et al. 2000). The geographical and social isolation of this island has ensured very little inward migration for several hundred years and thus has led 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.

Blood samples were collected from 500 consecutive MI patients (306 males and 194 females) and 500 normal controls (214 males and 286 females) of the genetically isolated Newfoundland population. Patients categorized in the MI group represented those with symptoms and biochemical evidence suggestive of MI (Troponin I values >2.0 μg/L or >0.5 μg/L). 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 noncardiac and nonthrombotic related events. Ethics approval

for this study was granted by the Human Investigations Committee of Memorial University and by the Health Care Corporation of St. John's.

Genotyping of the THBS4 A387P polymorphism was conducted by using Taq Man SNP genotyping technology on real-time PCR. The primers used were: forward, GCACTAGGTCTGCACTGACATTG; and reverse, CCGGTTCTGCTTTGATAACAACA. The probes used were: 387A allele, AAATGGACCGTGCGTT, which was labeled at the 5' end with VIC; 387P allele, AAATGGAGCGTGCGTT, which was labeled at the 5'end with FAM. Both probes also had a quencer dye TAMRA at 3'.

Pearson $\chi^2$ 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.

Our study showed that both patients and controls were in Hardy-Weinberg equilibrium for the A387P polymorphism. The gene frequency of THBS4 A387P in the Newfoundland population was 23.1%, similar to other white populations. Slightly increased carrier frequency, allele frequency, and homozygous frequency of the 387P allele were observed in patients with MI compared with controls (43.6% versus 41.8%, 24.9% versus 23.1%, and 6.2% versus 4.4%, respectively), but the differences did not reach statistical significance (Table 2).

The distribution of the THBS4 A387P variant was further analyzed by subgrouping patients and controls according to age and sex (Table 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 2 corresponding age groups.

The 387P allele showed a tendency toward higher prevalence in female patients with MI compared with the controls of same sex and gave an OR of 1.34 (0.930, 1.944), but the difference was not statistically significant (P=0.132; Table2). However, the prevalence of homozygotes for the 387P allele was significant higher in female patients compared with the female controls, giving an odds ratio of 2.96 (1.29, 6.78; P=0.008). In contrast, there was a tendency toward reduced prevalence of the 387P allele and homozygosity in male patients with MI compared with the male controls (41.8% versus 45.3% and 4.6% versus 6.1%). These differences were not statistically significant.

Genetic predisposition in multifactorial disease, such as MI, results from the coeffect of multiple genes, gene–gene, and/or gene–environment interactions. These collectively produce an additive or synergistic effect which affects risk for disease. Individual genetic changes may produce small or insufficient effects to impart significant pathogenic risk. Therefore, it is not surprising to see that most of the genetic variants associated with MI are characterized as polymorphisms rather than mutations. Gene–gene interaction as a significant factor in genetic predisposition for MI has been clearly demonstrated in our previous studies (Butt et al. 2003; Xie et al. 2003).

Table 2 Distribution of Heterozygote and Homozygote for THBS4 387P Genotypes Among MI Patients With Different Onset Ages, Genders, and Compared With Age and Gender Matched NC

| | MI | NC | OR (95% CI) | P |
|---|---|---|---|---|
| THBS4 A387P | | | | |
| CC+GC | 218/500 (43.6%) | 209/500 (41.8%) | 1.08 (0.84,1.38) | 0.565 |
| CC | 31/500 (6.2%) | 22/500 (4.4%) | 1.44 (0.82,2.52) | 0.204 |
| Males | | | | |
| CC + GC | 128/306 (41.8%) | 97 /214 (45.3%) | 0.87(0.61,1.23) | 0.428 |
| CC | 14/306 (4.6%) | 13 /214 (6.1%) | 0.74(0.34,1.61) | 0.448 |
| Females | | | | |
| CC + GC | 90/194 (46.4%) | 112/286 (39.2%) | 1.34 (0.93,1.94) | 0.132 |
| CC | 17/194 (8.8%) | 9/286 (3.1%) | 2.96 (1.29,6.78) | 0.008 |
| Age $\leq$ 50Y | | | | |
| CC + GC | 25/54 (46.3%) | 154/378 (40.7%) | 1.25 (0.71,2.22) | 0.463 |
| CC | 3/54 (5.5%) | 18/378 (4.8%) | 1.18 (0.34,4.14) | 0.736 |
| Age > 50Y | | | | |
| CC + GC | 193/446 (43.3%) | 55/122 (45.1%) | 0.93 (0.62,1.39) | 0.758 |
| CC | 28/446 (6.2%) | 4/122 (3.3%) | 2.04 (0.70,5.94) | 0.181 |

CC is 387PP and GC is 387AP.

In our present study, the 387P allele showed a tendency toward increased prevalence in the MI patients group compared with the controls. The carrier frequency of the 387P allele (GC+CC) was only slightly higher in patients with MI compared with the controls (43.6% versus 41.8%; OR 1.08). However, such increases become remarkable when comparing the prevalence of homozygosity for the 387P allele between patients and controls (6.2% versus 4.4%; OR 1.436). Further analysis of the 387P allele in subjects subgrouped according to sex showed that the higher prevalence of 387P alleles in MI patients only occurs in females. Female MI patients compared with control females gave odds ratios of 1.34 (P=0.132) and 2.96(P=0.008), respectively, when compared for prevalence of carriers (genotype of CC+GC) and homozygotes (genotype of CC). This indicates that the 387P allele may be a weak and dosage sensitive risk factor for MI. Other female-specific factors, genetic or otherwise, may have modifying effects on the relationship of 387P with MI risk.

Based on our investigation it is not clear whether the 387P allele has any effect on MI risk for male patients. We noticed a trend of reduced prevalence for 387P in carriers and homozygous males in the MI group compared with male controls (41.8% versus 45.3% and 4.6% versus 6.1%). However, the difference did not reach statistical significance. Further study on increased sample size will be useful.

Our results showed an increased prevalence of the 387P allele in patients with MI. This is in contrast with the results from the Netherlands study, in which the 387P alleles were associated with a reduced risk for MI (Boekholdt et al. 2002). Several differences are

worth noting, however. The Netherlands study examined patients with early onset MI, a situation where one would expect strong predisposing factors to be most relevant. Our study examines a much older MI population where the etiology for MI may be significantly different. Secondly, the Netherlands study population had a high proportion of males (≈80%) compared with our study (≈50%).

Finally, we conclude that THBS4 A387P polymorphism is associated with MI in females, especially in female homozygosity condition.

Acknowledgments

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Chapter 5

# THBS4 1186G>C(A387P) is a Sex Dependent Risk Factor for MI

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Abstract

Background: Case-control studies have successfully identified many genetic associations for complex diseases, but suffer from lack of reproducibility in the same population. Demonstrating weak genetic effects requires large sample sizes to minimize statistical bias. Based on a study examining 500 myocardial infarction (MI) patients and 500 controls from the genetically isolated Newfoundland population, we previously reported that thrombospondin-4 1186G>C variant associates with MI in females. To validate this sex-dependent association with the THBS–4 variant, we analyzed an additional 532 patients and 514 controls from the same population, and the combined cohort consisting of 1,032 patients and 1,014 controls.

Methods: Genotyping of THBS–4 1186G>C was conducted using Taq Man SNP genotyping technology on real-time PCR.

Results: The genotype distributions of THBS–4 1186G>C in the validation and combined cohorts were similar with those in our initial study which supports genetic homogeneity in the studied population. The association of the CC genotype with MI in females (OR = 2.96; P = 0.008), reported in our initial cohort, failed to achieve statistical significance in our validation cohort (OR = 1.53; P= 0.307), but was confirmed in the combined cohort (OR = 2.14; P=0.009). In contrast to the results from the initial cohort was a significant association of the CC genotype with later onset MI in the validation (OR = 2.37; P = 0.029) and combined cohorts (OR: 2.22, P = 0.011). Moreover, the larger studied

population gave statistical power to associate the CC genotype with risk of MI in the total patient population (OR=1.58, P=0.023).

Conclusion: Homozygosity for the THBS-4 1186C variant is a weak risk factor for MI especially in older females.

Key words:

Myocardial infarction, thrombospondin-4 1186 G>C, association study

## Introduction

Case-control association analysis is a commonly used study design in the field of complex trait genetics. The genetic associations, however, are not consistently reproducible due to inadequate sample size, population stratification, complexity of mechanisms, multiplicity of causative genes, confounding gene-environment interactions, and complex dependency of the associations (Ioannidis et al. 2001, Hirschhorn and Altshuler 2002, Colhoun et al. 2003, Hegele 2002). Therefore, cautious replication of studies is usually necessary (Ioannidis et al. 2001). Thrombospondins-4 (THBS-4) is one of five members of the THBS family of multidomain extracellular matrix proteins (named THBS-1 to 5) which all have been detected in vascular tissue (Wight et al. 1985, Lawler et al. 1993, Stenina et al. 2003). Members of the THBS are involved in a wide range of processes involving the vessel wall, including smooth muscle cell proliferation (Majack et al. 1986) and migration (Taraboletti et al. 1990) and associated with various extracellular matrix proteins (Narouz-Ott et al. 2000). THBS-4 appears to be more specific to cardiac and skeletal muscle (Lawler et al. 1993). A variant in the coding region of THBS-4 gene, 1186G>C (A387P) (rs1866389) is predicted to affect folding and secretion of the protein and disruption of the calcium binding site (Bornstein 1992, Geourjon and Deleage 1994), and may affect the cellular dynamics of the vessel wall (Stenina et al. 2003). The inhibitory effects of the THBS-4 1186G>C on endothelial cell (EC) repair functions, coupled with its stimulatory effects on smooth muscle cell (SMC), may predispose the vasculature to the initiation and development of atherosclerotic

lesions (Stenina et al. 2003). We had previously reported a sex dependent association of the THBS-4 1186G>C variant with myocardial infarction (MI) in an association study using 500 MI patients and 500 controls from the genetically isolated Newfoundland population (Cui et al. 2004). To confirm the results of our initial study, we carried out a validation study for THBS-4 1186G>C by examining an independent cohort from the same population and combining it with our previous results to give a final study population consisting of 1,034 patients and 1,014 controls.

## Material and Methods

### Subjects:

Blood samples were collected from 1,032 consecutive MI patients (640 males and 392 females) and 1,014 normal controls (477 males and 537 females) of the genetically isolated Newfoundland population. These samples included the 500 MI patients and 500 healthy controls used in our initial case control study. 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) or greater than 0.5 μg/L (Access II, Beckman-Coulter Corp.) were used in this group. These levels of cardiac Troponin I are consistent with a degree of myocardial damage that constitutes MI according to World Health Organization criteria. 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 Investigations Committee of Memorial University and by the Health Care Corporation of St. John's.

Newfoundland population:

The island portion of the Canadian province of Newfoundland and Labrador is located in the Atlantic Ocean. The population of the island of Newfoundland mainly originated from the descendants of small number of Irish and southwest English settlers who arrived in the $17^{th}$ and $18^{th}$ centuries (Bear et al. 1987). The geographical and social isolation of this island has ensured very little inward migration for several hundred years (Martin et al. 2000) 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.

Genotyping of the THBS-4 A387P polymorphism:

Genomic DNA was isolated from peripheral blood using standard salt precipitation methods. Genotyping of the THBS-4 1186G>C variant was conducted using Taq Man SNP genotyping technology on real-time PCR (ABI Prism® 7000 Sequence Detection System) which gives call rate of 99.996%. Primers and probes were designed using

Primer Express 2.0 software according to Applied Biosystems' guidelines (Foster city, CA, USA). The primers were GCACTAGGTCTGCACTGACATTG and CCGGTTCTGCTTTGATAACAACA. The probe for the 1186G allele is VIC-AAATGGACCGTGCGTT-TAMRA, and the probe for the 1186C allele is FAM-AAATGGAGCGTGCGTT-TAMRA. PCR reactions were carried out in 96-well optical reaction plates and each reaction consisted of 0.3μl genomic DNA (10 ng/μL) as template, 2.5μl of TaqMan Universal PCR Master Mix, 700 nM (each) primer and 200 nM (each) probe in a total volume of 5μl. After activation of uracil-N-glycolase (UNG) (2 min; 50°C) and AmpliTaq Gold (10 min; 95°C), 42 cycles of denaturation (15 s; 95°C) and elongation (1 min; 58°C) were used for two-step PCR. The fluorescent signals of the two reporter dyes were directly determined after PCR. The four distinct clusters were manually categorized as 1186GG (VIC), 1186CC (FAM), 1186GC (VIC and FAM) and no amplification control (NTC) based on the VIC to FAM ratio.

Prevalence determination and statistical analysis:

The prevalence of each gene variant was calculated by counting the total carrier frequency including heterozygotes (GC) and homozygotes (CC). The allele frequencies were determined by gene counting. To determine whether the polymorphisms were in Hardy–Weinberg equilibrium, the actual and predicted genotype counts for controls were compared using Chi square analysis with two degrees of freedom. Pearson Chi Square statistical analyses was performed 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. A two-tailed P<0.05 was considered statistically significant. All analyses were performed using SPSS for Windows (Version 11.0, SPSS, Chicago, Illinois, USA). Genetic power was calculated using the method for case-control studies of discrete traits posted on the web page of the Psychiatric and Neurodevelopmental Genetics Unit (PNGU) at Harvard Medical School (http://pngu.mgh.harvard.edu/~purcell/cgi-bin/cc2.cgi).

Results

The genotype distributions of THBS-4 1186G>C (A387P) in both the MI patient and the control populations are given in table 3. For comparison, the previously published data form table2 our initial study were also included in the table 3. In all cohorts the genotype distributions of THBS-4 1186G>C in both the MI patients and the healthy control population were all in Hardy–Weinberg equilibrium. The calculated genetic power to detect an overall association was 45% in the initial cohort and 74% in the combined cohort. The results for the validation cohort are similar to that of the initial cohort. When data from both were combined the CC, but not GC, genotype showed a significantly increased frequency in MI patients (6.40%) compared with the controls (4.14%) (OR=1.581, 95% CI 1.063-2.352, P=0.023). However, the total carrier frequency (GC+CC) did not show distribution disequilibrium between the MI patients (41.38%) and the controls (40.93%) (OR=1.019, 95% CI 0.854-1.215, P=0.837).

Table 3. Distributions of genotypes of THBS4 1186G>C (A387P) in MI patient and NC populations.

| | Genotype | MI (%) | NC (%) | OR ( 95%CI ) | P value |
|---|---|---|---|---|---|
| Initial cohort* | GC | 187/500 (37.4) | 187/500 (37.4) | 1.00(0.77,1.29) | 1.000 |
| | CC | 31/500(6.2) | 22/500 (4.4) | 1.44(0.82,2.52) | 0.204 |
| | GC+CC | 218/500(43.6) | 209/500(41.8) | 1.08(0.84,1.38) | 0.565 |
| Validation cohort | GC | 174/532 (32.7) | 186/514 (36.2) | 0.86(0.66,1.11) | 0.236 |
| | CC | 35/532 (6.6) | 20/514 (4.0) | 1.74(0.99,3.06) | 0.052 |
| | GC+CC | 209/532(39.3) | 206/514(40.1) | 0.97(0.76,1.24) | 0.793 |
| Combined cohort | GC | 361/1032 (35.0) | 373/1014 (36.8) | 0.93(0.77,1.11) | 0.395 |
| | CC | 66/1032 (6.4) | 42/1014 (4.1) | 1.58(1.06,2.35) | 0.023 |
| | GC+CC | 427/1032(41.4) | 415/1014(40.9) | 1.02(0.85,1.22) | 0.837 |

* Cui et al. Arterioscler Thromb Vasc Biol. 2004; 24(11):e183-4

Table 4. Genotype Distribution of THBS4 1186G>C (A387P) in the onset age and gender based MI patients and NC subpopulations.

| | Genotype | MI (%) | NC (%) | OR ( 95%CI ) | P value |
|---|---|---|---|---|---|
| Initial cohort* | | | | | |
| Onset age ≤ 50 | CC + GC | 25/54 (46.3) | 154/378 (40.7) | 1.25 (0.70,2.22) | 0.463 |
| | CC | 3/54 (5.6) | 18/378 (4.8) | 1.18 (0.34,4.14) | 0.736 |
| Onset age >50 | GC+CC | 193/446(43.3) | 55/122(45.1) | 0.93 (0.62,1.40) | 0.758 |
| | CC | 28/446 (6.3) | 4/122 (3.3) | 1.93 (0.62,5.75) | 0.181 |
| Male | GC+CC | 128/306 (41.8) | 97 /214 (45.3) | 0.87 (0.61,1.23) | 0.428 |
| | CC | 14/306 (4.6) | 13 /214 (6.1) | 0.74 (0.34,1.61) | 0.448 |
| Female | GC+CC | 90/194 (46.4) | 112/286 (39.2) | 1.34 (0.93,1.94) | 0.132 |
| | CC | 17/194 (8.8) | 9/286 (3.2) | 2.96 (1.29,6.78) | 0.008 |
| Validation cohort | | | | | |
| Onset age ≤ 50 | CC + GC | 30/85 (35.3) | 94/233 (40.3) | 0.81 (0.48,1.35) | 0.414 |
| | CC | 6/85 (7.1) | 12/233 (5.2) | 1.40 (0.51, 3.85) | 0.515 |
| Onset age >50 | GC+CC | 179/447 (40.1) | 112/281 (39.9) | 1.01 (0.73,0.87) | 0.960 |
| | CC | 29/447 (6.5) | 8/281 (2.9) | 2.37 (1.07,5.26) | 0.029 |
| Male | GC+CC | 129/334 (38.6) | 106/263 (40.3) | 0.93 (0.67,1.30) | 0.676 |
| | CC | 22/334 (6.6) | 9/263 (3.4) | 1.99 (0.90,4.40) | 0.084 |
| Female | GC+CC | 80/198 (40.4) | 100/251 (39.8) | 1.02 (0.70,1.50) | 0.904 |
| | CC | 13/198 (6.6) | 11/251 (4.4) | 1.53 (0.67,3.50) | 0.307 |
| Combined cohort | | | | | |
| Onset age ≤ 50 | GC+CC | 55/139(39.6) | 248/611(40.6) | 0.96 (0.66, 1.40) | 0.825 |
| | CC | 9/139 (6.5) | 30/611 (4.9) | 1.34 (0.62,2.89) | 0.453 |
| Onset age >50 | GC+CC | 372/893(41.7) | 167/403(41.4) | 1.01 (0.80,1.28) | 0.941 |
| | CC | 57/ 893 (6.4) | 12/ 403 (3.0) | 2.22 (1.18,4.19) | 0.011 |
| male | GC+CC | 257/640(40.2) | 203/477(42.6) | 0.91 (0.81,1.15) | 0.420 |
| | CC | 36 / 640 (5.6) | 22 / 477 (4.6) | 1.23 (0.72,2.12) | 0.450 |
| Female | GC+CC | 170/392(43.4) | 212/537(39.5) | 1.17 (0.90,1.53) | 0.234 |
| | CC | 30 / 392 (7.7) | 20 / 537 (3.7) | 2.14 (1.20,3.83) | 0.009 |

* Cui et al. Arterioscler Thromb Vasc Biol. 2004; 24(11):e183-4

The THBS-4 1186G>C was further analyzed by sub-grouping patients and controls according to age and sex (Table 4). The corresponding data from our initial study were also included in table 4 for comparison. To determine the relationship with age, the THBS-4 1186G>C was analyzed in the MI patients who were divided into those with an early age of onset ($\leq$ 50 years) and those with a later age of onset (>50 years). The control population was also divided into the two corresponding age groups. There was no significant distribution disequilibrium for the genotypes CC + CG and CC between the early onset MI patients and the age matched controls in either the validation or the combined cohort. In the later onset MI patients, the prevalence of CC genotype was significantly increased compared with age matched controls in both of the validation cohort (OR= 2.37, P=0.029) and combined cohort (OR= 2.22, P=0.011). This effect was not seen in the initial cohort, although the trend was present. Examination of the effect of sex showed that the prevalence of the CC genotype remained significantly higher in female patients compared with the sex matched controls (OR=2.14, P = 0.009) in the combined cohort. This confirms the observation made in our initial study. Only the trend was evident in the validation cohort which did not achieve statistical significance.

## Discussion

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. Case control studies using genetically isolated populations are of advantage due to a relatively homogenous genetic

background. However, the effect of a weak genetic risk factor can easily be influenced by any of a number of different genetic and environmental factors which may vary among individuals in the same population. Cases-control studies for investigating weak risk factors, therefore, require the study of large populations in order to show an effect. The THBS-4 1186G>C was suggested to impart a weak sex dependent predisposition for MI in our initial study based on the fact that only the CC genotype showed significant risk for MI in females (Cui et al. 2004). In order to eliminate a possible statistical error, due a small study population, we analyzed an addition 532 patients and 514 controls from the same population and combined this with results from the original 500 patients and 500 controls in the initial study.

The genotype distribution of THBS-4 1186G>C in MI patients and controls are similar in both our initial and present study (table 3 and 4). This supports a homogenous genetic background of the Newfoundland population. The sex dependent association of the CC genotype with MI is further confirmed in the combined cohort of this study (OR: 2.14, P=0.009) although only the trend was present in the validation cohort. This is at least partially due to the lower odds ratio indicated in the validation cohort and insufficient power using a small sample size. The equilibrium distribution of the CC genotype was detected in the later onset MI patients compared with the age matched controls in the validation cohort (OR: 2.37, P = 0.029) and combined cohort (OR: 2.22, P = 0.011) but not in the initial cohort, although a trend of increasing prevalence was present in the initial cohort (OR: 1.93, P = 0.18). Furthermore, similar CC genotype prevalences were

found in the initial, the validation, and combined cohorts, it is only in the combined cohort that the difference in prevalence in MI patients compared with controls becomes statistically significant (OR: 1.58, P= 0.023) for the unsubgrouped populations. The greater statistical power gained by using the larger combined populations is the main explanation for this effect. In the present study, the genetic power was increased from 45% to 74% when the samples size increased from 500 to 1014. The findings were inconsistent in achieving statistical significance when the smaller cohorts were examined. This illustrates the importance of a large scale case control study for investigating weak causative genetic predisposition.

In summary, we validated our previous case control study for the THBS-4 1186G>C variant using a combined cohort consisting of subjects from the initial study and an additional 532 MI patients and 514 healthy controls from the same genetically isolated Newfoundland population. The combined cohort consisted of 1,032 patients with MI and 1,014 healthy controls. The genotype distributions of THBS–4 1186 G>C in the expanded population was similar with those obtained in our initial study and supports genetic homogeneity within the Newfoundland population used. The sex-dependent association of the CC genotype with MI reported in our initial study was confirmed in this study. This suggests that some female-specific factors, genetic or otherwise, may have modifying effects on the relationship of CC genotype with MI risk. Moreover, the larger population studied here gave the necessary statistical power to establish an association of the CC genotype with the entire MI population and more specifically later

onset MI patients. Based on these results, we conclude that the THBS-4 1186C variant is a weak risk factor for MI when individuals are of homozygous status, and such predisposition may be more important for older females.

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Chapter 6

# MTHFR Gene Variants, 677C>T and 1298A>C Play Independent but Contradictory Roles in MI

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Abstract

Studies examining the role of methylenetetrahydrofolate reductase (MTHFR) gene variants, 677C>T and 1298A>C, in myocardial infarction (MI) have yielded discordant results. This is possibly due to interaction of complex genetic and environmental modifiers which vary among populations. To reduce such influences, a large case-control study using a genetic homogenous population would be of great advantage. We, therefore, carried out a large cohort for studying these two variants in 1,032 MI patients and 1,014 controls from the genetically isolated Newfoundland population. Genotyping results showed a significantly increased prevalence of 1298C (OR=1.369, P<0.001), but only a trend toward decreased prevalence of 677T (OR=0.847, P=0.062) in patients compared with the controls. Combined genotype analysis showed distribution disequilibrium of 677CT+TT/1298AA (OR=0.700, P<0.001) and 677CC/1298AC+CC (OR=1.205, P=0.048) in patients. The combined genotype 677CT+TT/1298AC+CC presented only a trend toward increased prevalence in patients (OR=1.221, P=0.056). Analysis of estimated haplotype-frequencies demonstrated linkage disequilibrium for the 667T-1298C (OR=3.206, P<0.001), but not for 677C-1298C (OR=1.106, P = 0.133) in patients. A further age based sub-population study showed the distribution disequilibrium of 1298C (AC+CC) with late onset MI patients (OR=1.513, P=0.001). These results suggest: 1) the 677T is a weak protective factor against MI but its effect is silenced when it is in cis with 1298C; 2) the 1298C is a risk factor for later onset MI and

its effect is reduced by an in trans 677T. These effects may be responsible for the lack of agreement among previous association studies examining MTHFR gene variants in MI.

Keywords

Newfoundland population, MTHFR gene variants, Myocardial infarction, Association, Haplotype

## Introduction

Methylenetetrahydrofolate reductase (MTHFR) is essential for homocysteine metabolism (Frosst et al. 1995). The two MTHFR common variants, 677C>T and 1298A>C especially the 677C>T have been extensively studied as risk factors for CAD including MI mainly due to its association with increased levels of homocysteine which are commonly observed in CAD patients (Danesh and Lewington 1998). Outcomes from a large number of previous association studies between 677T and CAD and MI were controversial though several meta analysis were included (Brattstrom et al. 1998; Wald et al. 2002; Klerk et al. 2002; Lewis et al. 2005). In contrast, the correlation between CAD and the 1298C allele has been less studied and only a few studies with inconsistent results were reported (Kolling et al. 2004; Strauss et al. 2005). Given that increases in homocysteine caused by MTHFR 677C>T and 1298A>C are relatively small, the effects of 677T and 1298C on CAD are expected to be weak. High prevalence of the two variants in different ethnic populations and discordant results in previous association studies for CAD suggest that the potential effects of both alleles in CAD may be universal but influenced by multiple yet unknown genetic and/or environmental factors which vary among different ethnic populations. To assess the suspected weak genetic effects from the 677T and 1298C, large study populations are required to provide sufficient statistical power to detect possible allele-allele interactions. Use of genetically isolated populations help to reduce the effects of other genetic modifiers and/or environmental conditions. For this purpose, we performed a case-control study in 1032

MI patients and 1014 healthy controls from a genetically isolated Newfoundland population. Our study not only focuses on determining the different distributions of each individual gene variant in the studied population, but also on investigating a possible intragenic allelic interaction between 677T and 1298C alleles through combined genotype analysis and haplotype calculation.

## Material and Methods

### Subjects:

Blood samples were collected from 1032 consecuitive MI patients (640 males and 392 females) and 1014 normal controls (477 males and 537 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) or greater than 0.5 μg/L (Access II, Beckman-Coulter Corp.) 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 Investigations Committee of Memorial University and by the Health Care Corporation of St. John's.

Newfoundland population

The island portion of the Canadian province of Newfoundland and Labrador is located in the Atlantic Ocean. The population of the island of Newfoundland mainly originated from the descendants of small number of Irish and southwest English settlers who arrived in the $17^{th}$ and $18^{th}$ centuries (Bear et al. 1987). The geographical and social isolation of this island has ensured very little inward migration for several hundred years (Martin et al. 2000) and thus has lead to a small population (530 000 individuals; Statistics Canada 2001) with a relatively homogenous genetic background. The living habit and diet are similar in the total population. This makes this population ideal for study of complex multifactorial disease such as MI.

Genotyping of the MTHFR 677C>T and 1298A>C:

Genomic DNA was isolated from peripheral blood using standard salt precipitation methods (Miller et al. 1988). Genotyping of the 677C>T and 1298A>C were conducted by using TaqMan SNP genotyping technology on real-time PCR (ABI Prism® 7000 sequence Detection System). The primers and probes for 677C>T and were obtained from the Validated TaqMan SNP genotyping kit supplied by Applied Biosystems (ABI; Foster City, CA). The primers for 1298A>C were: forward: GGAGGAGCTGCTGAAGATGTG; and reverse: CCCGAGAGGTAAAGAACAAAGACTT. The probes used were: 1298A allele: ACCAGTGAAGAAAGTGT, which was labelled at the 5' end with VIC; 1298C allele:

CAGTGAAGCAAGTGT, which was labelled at the 5'end with FAM. Both probes also had a quencer dye TAMRA at the 3' end. PCR reactions were carried out in 96-well optical reaction plates and each reaction consisted of 0.3µl genomic DNA (100 ng/µl) as template, 2.5µl of TaqMan Universal PCR Master Mix, 700 nM (each) primer and 200 nM (each) probe in a total volume of 5µl. After activation of UNG (2 min; 50°C) and AmpliTaq Gold (10 min; 95°C), 40 cycles of denaturation (15 s; 95°C) and elongation (1 min; 60°C) were used for two-step PCR. The fluorescent signals of the two reporter dyes were directly determined after PCR. The four distinct clusters were manually categorized as 1298A or 677C (VIC), 1298C or 677T (FAM), 1298A/C or 677C/T (VIC and FAM) and no amplification control (NTC) based on the VIC to FAM ratio.

Prevalence determination and 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. Tests of Hardy–Weinberg equilibrium (HWE) were carried out for all loci among MI patients and controls separately by the Chi-square test. 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 estimated haplotype frequencies from the unphased diploid genotype was calculated via the method of maximum likelihood from genotype data through the use of the expectation-maximization (EM) algorithm under the assumption of HWE (Fallin and Schork 2000).

Haplotype frequencies for various marker combinations were estimated for MI patients and controls separately. Linkage disequilibrium between the 677C>T and 1298A>C variants was calculated as D', which ranges from 0 (no linkage disequilibrium) to 1 or –1 (complete linkage disequilibrium) (Lewontin 1988).

## Results

### Genotyping MTHFR 677C>T and MTHFR 1298A>C

The genotype distributions of both 677C>T (CC, CT and TT) and 1298A>C (AA, AC and TT) in the controls were all in the Hardy–Weinberg equilibrium. A high degree of linkage disequilibrium was observed between the C677T and A1298C polymorphisms (D' = –0.714 for case, D'= -0.903 for control). The negative sign of the D' indicates that the 677C-1298C (or 677T-1298A) alleles were linked. The distributions of individual and combined genotypes of the 677C>T and 1298A>C in the MI patients and the controls are given in Table 5. The 677T allele (CT and TT) showed a trend toward a lower prevalence in the MI patients compared with the controls (53.2% vs. 57.30%), (OR=0.874, 95% CI 0.711-1.009, P=0.062). This trend was also reflected by a lower odds ratio among patients with TT genotype compared with those with only CT genotype (OR: 0.818 vs. 0.917, P value: 0.157 vs. 0.328). In contrast, the 1298C allele (AC and CC) presented a significantly increased prevalence in the MI patients (60.37%) compared with the controls (52.66%) (OR=1.369, 95% CI 1.149-1.632, P <0.001).

Combined genotype analysis and calculation of estimated haplotype frequency

The distributions of combined genotypes between 677C/T and 1298A/C in MI patient and control groups are depicted in Table 5. As expected, the frequency of double homozygosity for wild type alleles (677AA/1298CC) were found equally in both patient and control groups (11.82% vs. 11.83%). Significant distribution disequilibrium of the combined genotypes, 677CT+TT/1298AA (OR=0.700, P<0.001) and 677CC/1298AC+CC (OR=1.205, P=0.048) were detected in MI patients. In contrast, the combined genotype for both variant alleles, 677CT+TT/1298AC+CC, showed a trend toward increased frequency in MI patients (OR: 1.221, 95% CI 0.995-1.498, P=0.056).

To further characterize the interaction between the 677T and 1298C, all possible combined genotypes between the 667A/T and 1298A/C were calculated at the haplotype level. The estimated haplotype frequencies for all four possible genotypes in both MI patient and control populations are presented in the table 5. The 677T-1298A haplotype shows significantly reduced frequency in the MI patients compared with the controls (OR: 0.782; 95% CI 0.685-0.894; P<0.001). In contrast, the 677T-1298C haplotype had a significantly increased frequency in the MI patients compared with the controls (OR: 3.206; 95% CI 1.956-5.255; P<0.001). However, the frequencies of the cis 677C-1298C were similar in both patients and controls.

Table 5: MTHFR genotype distributions and haplotypes frequency estimation in MI patient and NC populations

| Genotype | MI (n = 1032) | NC (n = 1014) | OR ( 95%CI ) | P value |
|---|---|---|---|---|
| 677 CC | 46.80% | 42.70% | | |
| CT | 43.22% | 45.36% | 0.917(0.770,1.091) | 0.328 |
| TT | 9.98% | 11.93% | 0.818(0.619,1.081) | 0.157 |
| CT+TT | 53.20% | 57.30% | 0.847(0.711,1.009) | 0.062 |
| 1289 AA | 39.63% | 47.34% | | |
| AC | 48.93% | 42.31% | 1.307(1.098,1.556) | 0.003 |
| CC | 11.43% | 10.36% | 1.118(0.846,1.477) | 0.434 |
| AC+CC | 60.37% | 52.66% | 1.369(1.149,1.632) | <0.001 |
| Combined genotypes | | | | |
| 677CC/1298AA | 11.82% | 11.83% | 0.999 (0.764,1.306) | |
| 677CC /1298AC+CC | 34.98% | 30.6 % | 1.205(1.002,1.449) | 0.048 |
| 677CT+TT/1298AA | 27.81% | 35.51% | 0.700(0.580,0.844) | <0.001 |
| 677CT+TT/1298AC+CC | 25.39% | 21.80% | 1.221(0.995,1.498) | 0.056 |
| Haplotype frequencies | | | | |
| 677C-1298A | 35.74% | 34.93% | 1.038 (0.913, 1.180) | 0.572 |
| 677T-1298A | 28.36% | 33.58% | 0.782 (0.685, 0.894) | <0.001 |
| 677C-1298C | 32.67% | 30.46% | 1.106 (0.970, 1.262) | 0.133 |
| 677T-1298C | 3.23% | 1.05% | 3.206 (1.956, 5.255) | <0.001 |

Table 6. Distribution of MTHFR genotypes among MI patients with different onset age compared with age and gender matched NC.

| | MI | NC | OR ( 95%CI ) | P value |
|---|---|---|---|---|
| Age ≤ 50 | | | | |
| 677 CC | 70/139 (50.36%) | 260/608 (42.76%) | | |
| CT+TT | 69/139 (49.64%) | 348/608 (57.24%) | 0.736 (0.509, 1.065) | 0.104 |
| 1298 AA | 62/139 (44.60%) | 281/608 (46.22%) | | |
| AC+CC | 77/139 (55.40%) | 327/608 (53.78%) | 1.067 (0.737, 1.546) | 0.731 |
| Age >50 | | | | |
| 677 CC | 413/893 (46.25%) | 173/406 (42.61%) | | |
| CT+TT | 480/893 (53.75%) | 233/406 (57.39%) | 0.863 (0.681, 1.093) | 0.222 |
| 1298 AA | 347/893 (38.86%) | 199/406 (49.01%) | | |
| AC+CC | 546/893 (61.14%) | 207/406 (50.99%) | 1.513 (1.194, 1.916) | 0.001 |
| Males | | | | |
| 677 CC | 304/640 (47.50%) | 201/477 (42.14%) | | |
| CT+TT | 336/640 (52.50%) | 276/477 (57.86%) | 0.805 (0.634, 1.022) | 0.075 |
| 1298 AA | 260/640 (40.63%) | 228/477 (47.80%) | | |
| AC+CC | 380/640 (59.37%) | 249/477 (52.20%) | 1.338 (1.054, 1.700) | 0.017 |
| Females | | | | |
| 677 CC | 179/392 (45.66%) | 232/537 (43.20%) | | |
| CT+TT | 213/392 (54.34%) | 305/537 (56.80%) | 0.905 (0.697, 1.176) | 0.456 |
| 1298 AA | 149/392 (38.01%) | 252/537 (46.93%) | | |
| AC+CC | 243/392 (61.99%) | 285/537 (53.07%) | 1.442 (1.106, 1.880) | 0.007 |

Influence of gender and onset age

The distributions of the two MTHFR variants were further analyzed by sub-grouping patients and controls according to age and sex (Table 6). In the age based population study, the MI patients were divided into those with an early age of onset ($\leq$ 50 years) and those with a later age of onset (>50 years). The control population was also divided into the two corresponding age groups. There was no significant distribution disequilibrium of the 677T in either early onset or later onset patient populations. The 1298C allele (AC+CC) showed a significant MI risk only in the late onset MI patients compared with the age matched controls (OR: 1.513, 95% CI 1.194-1.916; P = 0.001). In the sex based population study, the 1298C allele (AC+CC) showed distribution disequilibrium in both male (OR: 1.338, 95% CI 1.054-1.700; P = 0.017) and female MI patients (OR: 1.442, 95% CI 1.106-1.880; P = 0.007).

## Discussion

Investigation of the suspected weak genetic effects, such as, MTHFR 677T and 1298C, require large study populations to obtain sufficient statistical power and identify possible allele-allele interactions. Using a genetically isolated population for study subjects can reduce the diversity of genetic and/or environmental modifiers. The present case-control study was carried out in large population including1032 MI patients and 1014 healthy controls from a genetically isolated Newfoundland population. As a fact of geographic isolation, Newfoundland has had very little inward migration and living habits are

relatively similar within the population. All these make the Newfoundland population ideal for examination of association and co-effects of allele-allele interactions. Both MTHFR variants 677T and 1298C are prevalent in the Newfoundland population making this population of great advantage in the present study.

In the present study, the 677T allele (CT and TT) showed a trend toward reduced prevalence in the MI patients compared with controls (OR: 0.847, P = 0.062) and evidence of a gene dosage effect for 677T. This trend is more clearly seen in the combined genotype analysis, in which 677CT+TT/1298AA had a significantly reduced prevalence in patients compared with the controls (OR: 0.700, P<0.001). This suggests that an independent but weak protective role of the 677T allele in MI. In contrast with 677T, 1298C (AC and CC) presents a significantly increased prevalence in patients compared with controls (OR: 1.369, P<0.001). This result was supported by distribution disequilibrium of the combined genotype, 677CC/1298AC+CC (OR: 1.205, P = 0.048), in the MI patient population. Therefore, the 1298C allele is thought to be an independent risk factor for MI. Further analysis of these two gene variants by sub-grouping patients and controls according to age and sex associated 1298C with later onset MI in both males and females. This suggests that the 1298C allele may be more important for later onset MI without sex specific influence.

Genetic predisposition in a multifactorial disease, such as MI, results from the co-effect of multiple genes, gene-gene and/or gene-environment interactions. These collectively

produce an additive or synergistic effect which affects risk for disease. Individual genetic changes may produce small or insufficient effects to impart significant pathogenic risk. Gene-gene interaction as a significant factor in genetic predisposition for MI has been clearly demonstrated in our previous studies (Butt et al. 2003; Xie et al. 2003), and genetic predisposition for CAD from co-effect of intragenic allelic interaction has also been previously reported for other genes (Rodriguez-Esparragon et al. 2005). In the present study, the 677T and 1298C variants showed independent but opposing effects on MI. Although the combined genotype, 677CT+TT/1298AC+CC, showed a trend to increased prevalence in patients compared with the controls, the difference failed to achieve statistical significance (OR; 1.221, P= 0.056). This reveals a potential co-effect of cancelling out by opposing effects from allele-allele interaction between 677T and 1298C.

The suggested allele-allele interaction was further characterized by calculating the estimated haplotype frequencies for all possible haplotypes. Interestingly, there was a significantly increased frequency of the 677T-1298C haplotype (OR=3.206, P<0.001), but not 677C-1298C (OR=1.106, P=0.133), in the MI patients compared with the controls. This suggested that the co-effect of cancelling out due to the interactions between 677T and 1298C occurs when the two alleles are in trans. The protective effect of 677T seem to be silenced when it is in cis with 1298C. The identified intragenic allelic interactions between 677T and 1298C may have also influenced results of previous association studies showing discordant results.

The cis 677T-1298T haplotype is extremely rare (0.0032 to 0.0034) based on a meta-analysis which calculated the halotype frequencies in pooled European, Australian and North America populations (Ogino and Wilson 2003). In the present study, the Newfoundland population showed much higher frequencies of the combined genotype of 677CT+TT/1298AC+CC than other population (Ogino and Wilson 2003). Therefore, it is not surprising to find a higher frequency for cis 677T-1298T in patients (0.0323) and controls (0.0105). This is possible due to a founder effect. A similar a high frequencie of cis 677T-1298T has also been reported from other studies (Isotalo and Donnelly 2000; Isotalo et al. 2000; Dekou et al. 2001).

A similar case control study was reported from a German group (Kolling et al. 2004). In this study, the 677T and the 1298C were genotyped in 2,121 angiographic CAD patients and 617 controls which were collected from consecutive Caucasian patients with normal coronary angiograms and without ventricular wall motion abnormalities. The two variants were not associated with the presence of angiographic CAD in this study. Comparing this study with ours, there are several differences which may affect outcome. 1) The control population in the German study was small compared with the case population (617 vs. 2,121); 2) The case and control populations in the German study were collected mainly from the southern part of Germany, Munchen area, close to Austria, Switzerland and Liechtenstein. The effect of ethnic diversity and existence of different ethnic specific genetic modifiers would potentially complicate results. 3)

Environmental effects, such as diets, may be different in the two studied populations; 4) The targeted clinical conditions in the two studies are different. The angiographic CAD used in the German study is an earlier event in atherosclerosis that may or may not progress to atherothrombosis (MI). It is presently not clear at which stage MTHFR 667T and 1298C are involved. If the two variants are involved in late events in the pathogenesis of MI, different outcomes between the two studies are not surprising.

The 667T and 1298C have been previously shown to affect folate metabolism and plasma homocysteine levels (Brattstrom et al. 1998; Friedman et al. 1999). Moreover, the association between MTHFR genotype and bone phenotypes depending on plasma folate status have also been reported (McLean et al. 2003; Abrahamsen et al. 2006). The design of the present study did not permit a comparison of the genotypes with biochemical parameters, or other factors, like smoking, diabetes and hypertension. Data on folic acid taken in the studied population are also not available. It is possible that other unidentified genetic or environmental factor may also be involved in modification of the effect of 677T and/or 1298C, and the co-effect of the allele-allele interaction. Further studies are planed in this area to elucidate the relationship with biochemical risk factors and other environmental factors mentioned above.

In summary, we performed a large case control association study using the genetically isolated Newfoundland population to investigate the correlation between two MTHFR common variants, 677C>T and 1298A>C, and the risk for MI. Our results suggest

independent but opposite roles of MTHFR 677T and 1298C in MI. The MTHFR 677T allele presents an independent protective role against MI. In contrast, the MTHFR 1298C is an independent risk factor for MI which may mainly predisposes for later onset MI. The results from the combined genotype analysis and estimated haplotype frequency calculation suggest the existence of complicated allele-allele interaction between 677T and 1298C. A co-effect of cancelling out effects through interactions between 677T and 1298C may occur mainly when the two alleles are in trans, and the protective effect of 677T may be silenced when the it is in cis with 1298C. The identified intragenic allelic interactions between 677T and 1298C may contribute to discordant results from previous association studies.

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# Chapter 7

# Conclusion

We undertook a large case control association study with 1,032 MI patients and 1,014 healthy controls from a genetically isolated population, the Newfoundland population, to investigate the genetic effects of the THBS4 A387P, MTHFR 677C>T and 1298A>C variants in the etiology of MI. We conclude that 1) homozygosity for the THBS4 29926C variant is associated with MI, especially in older females. 2) the 677T is a weak protective factor against MI but its effect is silenced when it is in cis with 1298C; 3) the 1298C is a risk factor for later onset MI and its effect is reduced by an in trans 677T. These effects may be responsible for the lack of agreement among previous association studies examining MTHFR gene variants in MI.

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