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THE GENETIC AND MOLECULAR MARKERS OF ISCHEMIC STROKE: RISK, PROGNOSIS, AND TREATMENT

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To my family

ABSTRACT

Stroke is a major cause of mortality and morbidity worldwide, with ischemic stroke (IS) being the predominant type. With the current ageing population, IS burden will inevitably escalate leading to increasing demand for more effective prevention, diagnosis, and treatment strategies. The overall aim of this thesis was to elucidate various factors affecting IS in different stages, from risk to prognosis and subsequently treatment, by exploring potential genetic and/or molecular markers of IS in the hope to better understand this complex multi-factorial vascular disease.

In Study I, we estimated the familial risk of IS in a very large, nation-wide population-based study by exploring the effects of sibling kinship, sex, and age in the heritability of IS. We found a 60% increased risk for IS in individuals having a sibling with prior stroke, and the risk was stronger for full siblings compared to half siblings. Having a sibling with early IS doubled the risk of early IS. No sex differences were found in the familial inheritance of IS.

In Study II, we assessed the common familial risk between IS and MI in a large, population-wide matched cohort study where we observed a 44% increased risk for MI in individuals having a sibling with prior IS and a 41% increased risk for IS in individuals having a sibling with prior MI, indicating shared familial aggregation between these two conditions.

In Study III, we explored the utility of sub-acute C-reactive protein (CRP) measurement in the prediction of outcomes after IS in a large prospective cohort of Singaporean acute IS patients, and whether CRP addition improved a conventional prognostic model. Only CRP at high levels was significantly associated with outcomes independent of other risk factors. In addition, comparison of conventional prognostic models with and without CRP showed significantly better fit in predictor model improvement upon CRP addition.

In Study IV, we examined whether the efficacy of B-vitamin in reducing total homocysteine (tHcy) was modified by ethnicity in a Singaporean IS population. The magnitude of reduction in tHcy with B-vitamin therapy did not differ between ethnic groups despite differences in dietary intake and genetic makeup.

In conclusion, data from the Swedish registers showed that full siblings exposed to IS have a higher risk of IS compared to those unexposed, and that early exposure to IS doubled the risk of IS compared to those unexposed. Similar increased risks for MI and IS when exposed to IS and MI respectively suggested shared familial aggregation between these two conditions. Data from the Singapore IS patients cohorts suggested the potential added value of CRP measurement towards prediction of future outcomes, and that the effect of B-vitamins in lowering tHcy may be generalizable across Asian IS populations.

Keywords: ischemic stroke, family history, heritability, siblings, sex, onset age, myocardial infarction, c-reactive protein, prognosis, homocysteine, ethnicity, risk factors

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numeral (I-IV):

I. Kasiman K, Lundholm C, Sandin, S, Malki N, Sparén P, Ingelsson E. Familial effects on ischemic stroke: The role of sibling kinship, sex, and age of onset. *Circ Cardiovasc Genet*. 2012;5:226-233.

II. Kasiman K, Lundholm C, Sandin, S, Malki N, Sparén P, Ingelsson E. Common familial effects on ischemic stroke and myocardial infarction: A Swedish national cohort study. *Manuscript*.

III. Kasiman K, Da Silva DA, Lee JH, Chang HM, Wong MC, Chen CP. CRP paper. C-reactive protein and its prognostic utility for poor functional outcomes and recurrent vascular events after ischemic stroke. *Manuscript*.

IV. Kasiman K, Eikelboom JW, Hankey GJ, Lee SP, Lim JP, Lee JH, Chang HM, Wong MC, Chen CP. Ethnicity does not affect the homocysteine-lowering effect of B-vitamin therapy in Singaporean stroke patients. *Stroke*. 2009;40(6):2209-2211.

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LIST OF ABBREVIATIONS

AF	Atrial fibrillation
AUC	Area under curve
CDR	Cause of death register
CHD	Coronary heart disease
CI	Confidence interval
CRP	C-reactive protein
CVD	Cardiovascular disease
DNA	Deoxyribonucleic acid
FA	Folic acid (folate)
GWAS	Genome-wide association studies
HDR	Hospital discharge register
ICD	International Classification of Diseases
IQR	Inter-quartile range
IS	Ischemic stroke
LACI	Lacunar infarct
MGR	Multi-generation register
MI	Myocardial infarction
mRS	Modified Rankin Scale
OCSP	Oxfordshire Community Stroke Project
OR	Odds ratio
PACI	Partial anterior circulation infarct
POCI	Posterior circulation infarct
ROC	Receiver operating characteristic
ROS	Reactive oxygen species
RR	Relative risk
SD	Standard deviation
SNP	Single nucleotide polymorphism
TACI	Total anterior circulation infarct
tHcy	Total homocysteine
TIA	Transient ischemic attack
TOAST	Trial of Org 10172 in Acute Stroke Treatment
VITATOPS	VITAmins TO Prevent Stroke
WHO	World Health Organization

1 INTRODUCTION

Stroke is a major cause of mortality and morbidity worldwide, with ischemic stroke (IS) being the predominant type (approximately 80%) found in stroke patients [1, 2]. With the current ageing population, the number of people suffering from stroke will inevitably escalate leading to increasing demand for more effective prevention, diagnosis and treatment strategies. Therefore, it is important to identify early risk factors as a strategy of primary prevention. Equally imperative is to identify factors which may predict recurrence of the disease for those already burdened for planning secondary prevention and treatment.

The evidence for IS being a heritable disease has increased over the years. Although strong familial inheritance of stroke risk has been previously demonstrated in prior family and twin studies, where a stronger genetic effect over environmental ones has been suggested [3-7], few studies have addressed potential effects of sex, age of onset, or sibling kinship on familial risk of IS [5, 6, 8-16], and the possible interactions among these factors have not been studied.

Recent genome-wide association study (GWAS) analyses suggested that genetic determinants of stroke share some pathogenic mechanisms with coronary heart disease (CHD) [17], but the extent of this overlap is unknown. Several studies in first-degree relatives have explored the associations between family history of IS and the risk of IS, or family history of myocardial infarction (MI) and the risk of MI, or family history of either these diseases and the risk of cardiovascular disease in general [4, 8, 18-28]. However, few studies have explored the co-inheritance of familial factors of one condition affecting the risk of the other [20, 28, 29].

C-reactive protein (CRP), a peripheral marker of inflammation and generalized atherosclerosis, has been associated with stroke risk in the general population [30, 31] and long-term mortality after stroke [32]. Previous studies of prognostic utility of CRP in stroke patients were mainly performed with samples collected within 24 hours of stroke onset in predominantly Caucasian populations [33-37]. Most studies suffered from various methodological issues, such as low sample size, unadjusted confounders, inadequate assessments of clinical utility and additional benefit of this marker beyond current well-established predictors [38, 39], with less than half using validated functional outcome measures [40]. It is therefore unknown if results from these studies can be generalized to non-white populations where stroke subtypes differ [41] and CRP measurement time window differs.

Elevation of total homocysteine (tHcy) has been linked to atherosclerotic vascular morbidity and mortality in a dose-dependent fashion [42]. B-vitamins consumption has been shown to lower tHcy [43]. A previous study demonstrated the reduction of plasma tHcy by 3.8 μmol/L after one year treatment of B-vitamin therapy, and that the *MTHFR* gene did not modify this effect [44]. However, it is still uncertain if this reduces incidence of major vascular events. If the efficacy and safety of this therapy in stroke patients can be confirmed in ongoing trials [45], it is also important to determine the consistency of treatment effect among patients of different ethnicities particularly in Asia where the global burden of stroke is the highest, hence the potential impact of therapy may be greatest in this group of people.

2 BACKGROUND

Stroke is a major cause of mortality and neurological disability in adults worldwide [1, 2]. According to the World Health Organization (WHO), approximately 15 million people are affected by this debilitating condition on an annual basis, where two-thirds of these either die or left permanently disabled [46]. **Figure 1** displayed stroke with the second highest proportion of deaths within cardiovascular mortality worldwide. Complex and multifactorial in nature, stroke is best considered as a syndrome. The WHO defined it as a "rapidly developed signs of focal (or global) disturbance of cerebral function lasting longer than 24 hours (unless interrupted by death), with no apparent non-vascular causes".

In the USA, around 795,000 strokes occur annually, and approximately 75% are incident cases [47]. This is similar to the number of annual stroke incidence found in Sweden [48]. In Singapore, stroke is the fourth highest cause of death comprising of 8.4% of total number of deaths in 2010, behind cancer, ischemic heart disease and pneumonia [49]. A previous community-based prevalence survey conducted in Singapore showed a crude stroke prevalence of approximately 4% for adults aged 50 years and above with neurological diseases. Higher rates were observed among men and with increasing age. Chinese men displayed the highest prevalence of approximately 5% while Malay women had the lowest with around 3% [50]. Another prospective cohort study performed in the early/mid-1980s and 1990s found a stroke incidence of 1.8/1000 person-years with rates highest among Malay women [51].

Worldwide mortality from various forms of cardiovascular diseases in millions

- CHD 7.2 (43%)
- Stroke 5.5 (33%)
- Other forms of HD 2.4 (14%)
- Hypertensive HD 0.9 (5%)
- Inflammatory HD 0.4 (2%)
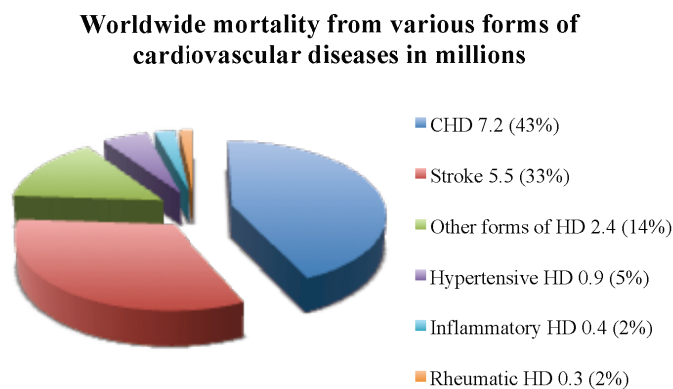- Rheumatic HD 0.3 (2%)

Figure 1. Proportion of deaths attributable from stroke in cardiovascular diseases mortality worldwide [Adapted from the WHO].

Conventional stroke risk factors are generally similar to those of coronary heart disease (CHD), which can be divided into modifiable and non-modifiable. Modifiable risk factors include high blood pressure, diabetes mellitus, cardiac diseases (such as

atrial fibrillation (AF), heart failure, and myocardial infarction (MI)), high cholesterols, cigarette smoking, alcohol consumption, obesity, sedentary lifestyle, and unhealthy dietary habits [52]. Proper monitoring and control of these modifiable risk factors greatly reduced stroke incidence, especially with the control of high blood pressure and cigarette smoking having the greatest contribution. Age, gender, ethnicity, and genetics are non-modifiable risk factors. Despite these vast arrays of risk factors, a substantial portion of stroke risk remains unexplained [53]. Risk factors as informative as they may be in helping to manage this condition, the consequences vary from individuals to individuals. Among stroke survivors, having a stroke before greatly increases the risk for subsequent strokes. Unfortunately, recurrences of the episodes happen to approximately one-quarter of this group within five years and the severity of disability accompanying each recurrent stroke, when survived, is higher with each increasing number of strokes.

With the current ageing population, the number of people suffering from stroke will inevitably escalate leading to increasing demand for more effective prevention, diagnosis and treatment strategies. The grim projection of clinical, social, and economical burden, and high cost of treatment accompanying this condition, preventive strategies should take precedence. Therefore, it is important to identify early risk factors as a strategy of primary prevention. Equally imperative is to identify factors which may predict recurrence of the disease for those already burdened as means of secondary prevention and treatment.

Approximately 80% of strokes are ischemic while the remaining 20% are hemorrhagic. Ischemic stroke, in general, involves cerebral artery blockage by atherosclerotic plaque or embolus. Atherosclerosis and extracranial arteries or myocardium due to co-existing conditions such as MI, mitral stenosis, endocarditis, AF, dilated cardiomyopathy, or congestive heart failure are usually involved. Hemorrhagic stroke, on the other hand, involves bleeding into the brain, and usually consist of two sub-types: (1) intracerebral hemorrhage where weakened cerebral vessels rupture forming a localised hematoma within the parenchymal cerebral spaces, and (2) subarachnoid hemorrhage which occurs outside of the brain releasing into the cerebrospinal fluid. Pathophysiology of these two types of strokes is different, and mortality risks between the different types of strokes vary. Another condition the transient ischemic attack (TIA), often termed 'mini-stroke' due to its similarity to stroke, happens when stroke symptoms are resolved in less than 24 hours leaving no further noticeable symptoms or deficits. Often, TIA acts as a warning sign of an impending stroke for the individual, and having one increased the risk of stroke. The work in this thesis will focus only on ischemic stroke.

2.1 ISCHEMIC STROKE

Ischemic stroke, the majority (approximately 80%) type of stroke, is heterogeneous and can be caused by manifestations of several different pathologies resulting in the disruption of cerebral blood supply due to cerebral artery blockage by atherosclerotic plaque (thrombus) or embolus.

2.2 DEFINITION OF ISCHEMIC STROKE SUBTYPES

The most commonly and widely used ischemic stroke subtypes definition today are the Oxfordshire Community Stroke Project (OCSP) [54] and the Trial of Org 10172 in Acute Stroke Treatment (TOAST) [55] classification systems.

2.2.1 Oxfordshire Community Stroke Project classification

The OCSP [54] classification categorized IS according to clinical presentation into four subtypes based largely on initial symptoms, providing a basic estimation of the magnitude and location of cerebral infarct: (1) total anterior circulation infarct (TACI) – cerebral infarction affecting the *entire anterior circulation* (both cortical and subcortical) supplying one side of the brain, (2) partial anterior circulation infarct (PACI) – cerebral infarction affecting *part of the anterior circulation* (more restricted and predominantly cortical) supplying one side of the brain, (3) posterior circulation infarct (POCI) – cerebral infarction affecting the *posterior circulation* (vertebrobasilar arterial territory) supplying one side of the brain, and (4) lacunar infarct (LACI) – *occlusion of one of the penetrating arteries* (deep perforating arterial territory) that provides blood to the brain's deep structures.

2.2.2 Trial of Org 10172 in Acute Stroke Treatment classification

The TOAST [55] classification categorized IS according to pathology into five subtypes based on clinical symptoms, neuroimaging data, and other diagnostic tests when available: (1) large-vessel disease – large extra-cranial or intra-cranial artery stenosis usually of atherosclerotic nature, (2) small-vessel disease – equivalent to a lacunar infarct within the white matter and deep grey matter in the brain, (3) cardioembolic stroke – arterial thrombus or embolus of cardiac origin, (4) stroke of other determined etiology, and (5) stroke of undetermined etiology – two possible causes, no identified causes, or incomplete investigation.

2.3 RISK FACTORS

IS risk factors for both incidence and recurrence are largely the same. They are commonly categorized into modifiable and non-modifiable risk factors. However, modifiable or not, many of these factors each have their own heritable components.

2.3.1 Modifiable risk factors

Modifiable risk factors such as high blood pressure, diabetes mellitus, cardiac diseases (such as AF, heart failure, and MI), high cholesterols, cigarette smoking, alcohol consumption, obesity, sedentary lifestyle, and unhealthy dietary habits have been well accepted as stroke risk factors [52]. Increased blood pressure contributes greatly to stroke, and that proper management lead to a significant reduction of stroke incidence and recurrence [56-58]. This very much resonates with cigarette smoking [59]. High total cholesterol and low-density lipoprotein and low high-density lipoprotein had been associated with increased risk of ischemic heart disease but this increased risk is more modest among IS [60]. Beneficial effect accompanying the moderate consumption of

alcohol has been suggested. However, high alcohol consumption has the opposite association and is a risk factor, possibly acting through elevation of blood pressure and subsequently cardiac diseases [61]. High salt intake and lack of physical activity also contribute towards stroke risk [62]. Moderate- to heavy-intensity physical activity had been shown to have protective effect against IS [63].

2.3.2 Non-modifiable risk factors

Age, gender, ethnicity, and genetics are non-modifiable risk factors. Age is the strongest risk factor for stroke; stroke risk has an approximately two-fold increase with every increasing decade after the age of 55 years old [64]. Men has a higher stroke incidence and mortality compared to women, however, with the considerable elevation of stroke rates in older age group, more women are suffering from this condition as women tend to live longer [65, 66]. Different ethnic groups have shown different stroke incidence, and different stroke subtypes. Asians, African Americans and Hispanic Americans are shown to have higher incidence compared to the Caucasians [47, 67]. Intracranial atherosclerosis is more prevalent among the Asians [68-73], Africans Americans, and Hispanics Americans [74], while extracranial carotid stenosis is most common among Caucasians [41]. Genetic predisposition to stroke had been demonstrated in family history and twin studies, and this is especially prominent among younger individuals [3-7, 26].

2.3.3 Other risk factors

Inflammation, infection, hypercoaguable markers, usage of hormone replacement therapy and oral contraceptives, and socioeconomic factors are some of the potential risk factors that have been been associated with IS [75-78].

2.4 ISCHEMIC STROKE AND MYOCARDIAL INFARCTION

As previously mentioned, IS and another cardiovascular diseases, CHD, shared many similar risk factors, and as such, its corresponding outcome MI [79]. With the overwhelming overlap of risk factors, it is not the least surprising to consider that the pathogenic mechanism between the two conditions may be common [17]. However, the extent of this overlap is still largely unknown. Familial aggregation studies in first-degree relatives have explored the associations between family history of IS and the risk of IS, or family history of MI and the risk of MI, or family history of either these diseases and the risk of cardiovascular disease in general [4, 8, 18-28]. Moreover, a recent meta-analysis of 11 MI-associated single nucleotide polymorphisms (SNPs) looking at the associations with IS found that the major common loci associated with MI risk did not have effects of similar magnitude on overall IS [80]. Nevertheless, suggestions of moderate associations to specific IS subtypes were not excluded.

2.5 GENETICS AND ISCHEMIC STROKE

Although conventional risk factors account for a substantial contribution towards stroke risk, there are still unaccounted residual risk which may be explained by genetics.

Genetic susceptibility of stroke has previously been supported whereby family and twin studies showed the existence of familial aggregation of stroke risk. Inheritance of rare monogenic causes of stroke had been extensively studied and these studies had been largely successful in the identification of monogenic condition in specific ischemic stroke subtypes (**Table 1**). However, for the more common multifactorial form of IS, the identification of underlying genetic variants have been much slower and proved to be more challenging.

Table 1. Examples of monogenic causes of ischemic stroke (Adapted from Dichgans, 2007) [81]

Monogenic disorder	Inheritance mode	Associated gene	Stroke mechanism
Cerebral autosomal dominant arteriopathy with subcortical infarcts and leucoencephalopathy (CADASIL)	Autosomal dominant	*NOTCH3*	Small vessel disease
Ehlers-Danlos syndrome type IV	Autosomal dominant	*COL3A1*	Arterial dissection
Fabry's disease	X-linked	*GAL*	Large-artery disease, small vessel disease
Homocysteinuria	Autosomal recessive	*CBS* and others	Large-artery disease, cardioembolism, small vessel disease, arterial dissection
Marfan syndrome	Autosomal dominant	*FBN1*	Cardioembolism, arterial dissection
Mitochondrial myopathy, encephalopathy, lactacidosis, and stroke (MELAS)	Maternal	Mitochondrial DNA	Complex (microvascular and neuronal factors)
Sickle-cell disease	Autosomal recessive	*HBB*	Large-artery disease, small vessel disease, hemodynamic insufficiency
Pseudoxanthoma elasticum	Autosomal recessive	*ABCC6*	Large-artery disease, small vessel disease

The identification of the *PDE4D* [82] and *ALOX5AP* [83] genes associated with IS by the Icelandic deCODE genetics group in early studies were promising. However, subsequent studies carried out in other populations have shown replication to be inconsistent [84, 85]. Genes involved with atherosclerosis, the renin-angiotensin system, lipid metabolism, inflammation, hemostasis, and so on, have generated an extensive list

of genetic variants associated with IS, however, few have been successfully replicated [86, 87].

Typically, genetic studies in stroke have investigated genetic risk factors alone, not accounting for environmental factors which are as important in stroke development. However, interactions between genes, and gene and environment have been suggested. To elucidate these, studies would require large sample sizes in order to detect these associations especially in IS where effect sizes are typically very modest.

2.5.1 Familial aggregation

The evidence for IS being a heritable disease has increased over the years, with increasing number of studies showing familial aggregation of stroke. Family and twin studies have suggested genetic influence in the susceptibility to IS [3-7], but the number of genetic variants associated with stroke is modest [88-90]. Twin studies provide data suggesting strong familial inheritance of stroke risk [3-7]. Concordance rates of stroke are significantly higher in monozygotic twins compared with dizygotic twins of approximately five-fold increase in stroke prevalence between the two [3, 4].

Parental history of stroke has been associated with increased stroke risk [5], and a recent analysis of this Framingham cohort showed a tripled increased risk of stroke in children whose parents had a stroke before 65 years old [27]. Self-reported family history may be highly inaccurate owing to significant reporting or misclassification bias when compared with a validated family history. A systematic review found few studies carried out with detailed stroke phenotype, consideration of the number of affected or unaffected relatives, and influence of family history on stroke development [91]. A population-based study carried out in Sweden a few years ago teased out the difference of genetic influence between the two major types of stroke, ischemic and hemorrhagic stroke [13]. Sibling concordance of stroke was found, however no increased risk for spouses of individuals with stroke was observed, suggesting a greater effect of genetic factors rather than environmental ones.

2.5.2 Candidate gene association studies

The candidate gene association study is probably the most commonly used method to study genetics in IS, as with other complex diseases, before the advent of the whole genome association study. Candidate gene study is hypothesis-driven, i.e. it utilises the knowledge of biological pathways to select genes of interest, in order to study associations between these genetic variations and phenotypes. Genes in pathways such as those involved in the renin-angiotensin-aldosterone system, endothelial dysfunction and nitric oxide release, homocysteine metabolism, coagulation and hemostasis, and inflammation, to name a few, have been extensively studied for associations with IS [84, 92-101]. Despite a large number of studies carried out, replicable results are limited.

2.5.3 Genome-wide association studies

Genome-wide association studies (GWAS) have revolutionised the field of genetics in general. It has allowed novel genetic loci to be identified in a hypothesis-free setting, i.e. no *a priori* knowledge of the gene's biological pathway within the disease of interest is taken into consideration. However, GWAS performed in stroke have largely been

disappointing. Only a few genetic variants associated with IS had been compellingly identified such as the 4q25, 9p21, and 7p21.1 loci [102-106], replicated on some studies but not all and usually involved a more specific stroke subtyping as a phenotype.

GWAS in stroke was first performed in 2007 by the International Stroke Genetic Consortium (ISGC) and the Wellcome Trust Case Control Consortium 2 (WTCCC2) in an effort to characterize whether there is a common genetic risk factor underlying stroke risk, and to create a form of genotypes database made available to the public for re-analyses [107]. More than 400,000 unique SNPs were genotyped in 249 Caucasian white IS patients and 268 Caucasian white neurologically normal controls. Most significant SNPs were found within or near interesting candidate loci including genes involved in potassium transport, *KCNIP4* and *KCNK17*, and marker for differentiated vascular smooth muscle cells, the aortic preferentially expressed gene-1 (*APEG-1*). This study suggested that stroke is not caused by a single common variant, in agreement with a study of unrelated families with affected and unaffected siblings, the Siblings With Ischemic Stroke (SWISS) study [89].

Recent study attempting to validate associations between stroke-related GWAS SNPs failed to replicate previous findings despite reasonable power [108]. They found different results by ancestry, highlighting the importance of conducting studies in different populations. They also found modest IS associations with modifiable risk factors such as body mass index and lipids SNPs, however validation studies need to be carried out to confirm these findings. Genetic association with chromosome 6p21.2 was also found in another IS GWAS, and this association became more prominent with a more specific subtype of IS, the large artery atherosclerosis (LAA) [109]. This reiterated the importance of specific definition of phenotypes required to elucidate this association. Gene cluster related to lipid metabolism, *APOL1-APOL4*, at chromosome 22q12.3 were also found to have potential associations with IS and LAA risks amongst Europeans. Replications in non-Europeans have yet to be carried out.

2.6 BIOMARKERS AND ISCHEMIC STROKE

As with genetics, a large number of studies have been carried out in an effort to identify clinically useful stroke biomarkers. In order to achieve that, associations of serum markers with IS alone is no longer sufficient. These markers have to be sensitive enough to be detected in early ischemia, fast enough to be measured, and specific enough to IS. The continuous search for biomarkers to integrate into clinical panels may one day better stratify individuals into different risk groups for more appropriate treatment, and establish one or more as surrogate prognostic markers for future clinical trials, gearing towards a more individualized clinical care [110].

Several candidate biomarkers which have been evaluated include markers involved in inflammation, oxidative stress, glial activation, hemostasis, and endothelial dysfunction [31, 77, 110-132]. To date, only lipoprotein-associated phospholipase A2, an enzyme which hydrolyzes oxidized phospholipids, is included in blood test panel for both IS and coronary artery disease risk assessment [119, 133].

2.6.1 Inflammation and ischemic stroke

Higher risks of stroke and CHD have been associated with inflammation [134]. Several inflammatory markers have been investigated in the past to evaluate stroke risk and other vascular outcomes [127]. However, the usefulness or validity of these markers independent of conventional risk factors with regards to stroke diagnosis or prognosis is still inconclusive.

Briefly, within the ischemic cascade, neurons, astrocytes, microglia, and endothelial cells are activated upon cerebral reperfusion immediately following ischemia. This, in turn, triggers a series of inflammatory responses from the release of transient cytokines such as interleukin-1ß (IL-1ß) and tumor necrosis factor-α (TNF-α), followed by secondary longer-lasting inflammation such as interleukin-6 (IL-6) and interleukin-8 (IL-8). Subsequently, this progresses to the development of fever, C-reactive protein (CRP), fibrinogen, and cell adhesion molecules release. There are three major groups of cell adhesion molecules being released at this stage: (1) selectins – participate in the interaction between leukocyte and endothelial cells surrounding infarcts; (2) immunoglobulin super family – molecules such as intercellular cell adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) participate in acute phase ischemia process, and (3) integrins – participate in the intercellular adhesion, and interaction between molecules and the extracellular matrix. Over time, these cause leukocyte build-ups which then result in adhesion of vascular wall. This proceeds to stimulate productions of matrix metallopeptidases (MMPs) (e.g. MMP-9) resulting in the disruption of the blood-brain barrier, whereby vascular edema then occurs, finally giving way to hemorrhagic conversion of infarct [127].

Various studies have explored the roles of each of these inflammatory markers [34, 35, 112, 114, 117, 119, 120, 122, 135-142] and others such as leukocyte counts [143-146], fibrinogen [113, 144], amongst others, with regards to cardiovascular risk, diagnosis, and prognosis in different patient groups [115, 147], and various outcomes such as early neurological deterioration, functional outcome, recurrence, and infarct volume [115, 124] just to name a few. Many has features which would potentially be risk markers, unfortunately specificity of assays has not been elucidated, in part due to the vast variability in measurements, or just simply non-specific.

At present, no guidelines have yet been made to include inflammatory markers as part of a clinical diagnostic tool, or even recommendation of appropriate levels despite the extensive amount of studies carried out in this area.

2.6.2 Homocysteine and ischemic stroke

Elevated plasma levels of homocysteine, a sulphur-containing amino acid produced during methionine metabolism, had been associated with an increased risk of atherosclerotic and ischemic events in a dose-dependent manner [42, 148]. Vascular function impairment such as endothelial dysfunction, reactive oxygen species production, and low-density lipid oxidation are a few potential mechanisms associated with hyperhomocysteinemia.

Hyperhomocysteinemia can be determined through dietary intake, and as such, is a modifiable risk factor of stroke. Folic acid (FA) and B-vitamins, essential for the

remethylation of homocysteine to methionine (**Figure 2**), are the most important dietary determinants, and plasma tHcy levels had been shown to reduce through daily supplementation [43, 45]. Several large-scale intervention trials have been conducted to determine whether this supplementation can reduce cardiovascular risk in healthy subjects or improve survival in patients with CHD including secondary vascular ischemic events prevention [43, 45]. However, some of these trials found no significant beneficial effects of B-vitamins supplementation in spite of adequate homocysteine lowering [43, 149, 150].
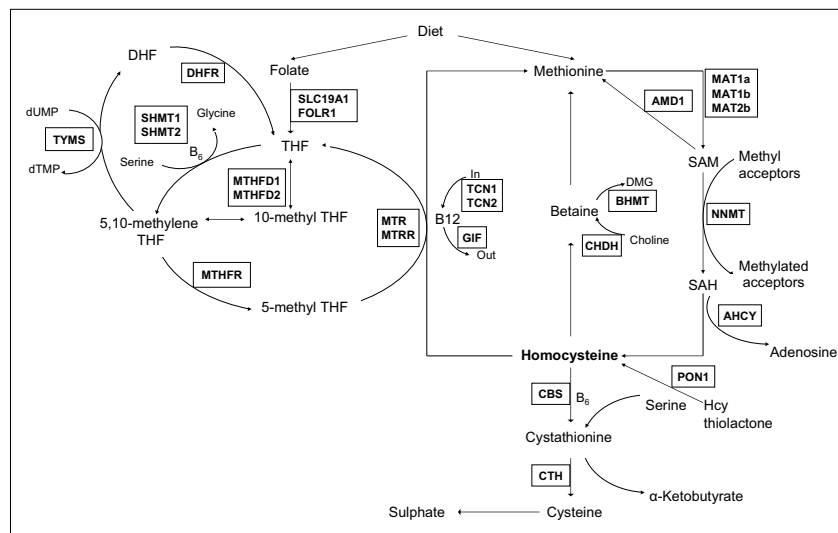
Figure 2. The homocysteine metabolism pathway (*Source*: Low *et al*., 2011) [101]

A genetic component has also been identified in the contribution towards this condition with the deficiency of the *MTHFR* enzyme, a critical enzyme involved in the remethylation pathway of homocysteine metabolism, being considered the common cause. The functional polymorphisms, *C677T* and *A1298C*, of the *MTHFR* gene affecting plasma tHcy had been associated with IS [151-154]. Thermolabile form of the enzyme is usually found in individuals affected by one or both of these polymorphisms, causing activity reduction, and hence displaying mild to moderate hyperhomocysteinemia.

Therefore, it is still debatable whether reduction of plasma tHcy levels through dietary supplementation may convey to a reduction in cardiovascular risk, and how the associated genetic variants may contribute towards vitamin effect modification on tHcy lowering.

2.7 PREVENTION AND TREATMENTS OF ISCHEMIC STROKE

Current primary and secondary prevention strategies for IS are almost identical. These include lowering of blood pressure and body mass index, anti-platelet, anti-thrombotic, anti-diabetic and lipid-lowering therapies, cessation of cigarette smoking and alcohol consumption [155-157]. Aspirin, clopidogrel, aspirin plus extended release dipyridamole, aspirin plus clopidogrel, and warfarin are examples of well-established anti-thrombotic and anti-coagulant used [157-161].

The only approved treatment of acute IS up-to-date is thrombolysis by means of intravenous (IV) recombinant tissue plasminogen activator (rtPA) within a time window of no more than 4.5 hours upon admission (stroke onset) [162-165]. rtPA greatly improves clinical outcome and reduces the after effects of stroke. An alternative administration method, intra-arterially (IA), is available; this gives an advantage of direct clot side administration in high concentration thus limiting exposure of tPA elsewhere in the system. Combination of the two administration methods has been shown to have modest improvement. The downside of this treatment is that the short time window for administration significantly reduces the proportion of acute IS patients eligible for it. In addition, due to its high risk of causing cerebral bleeding, administration of rtPA is highly dependent on the type of stroke, time of arrival to the hospital, age and, general well-being of the patient. Invasive procedures, such as endovascular or surgical, in extracranial or intracranial carotid or vertebral artery disease are available options depending on stroke subtypes [157].

3 AIMS

The overall aim of this thesis was to elucidate various factors which affect IS in different stages, from risk to prognosis and subsequently treatment, by exploring potential genetic and/or molecular markers of IS, in the hope to better understand this complex multi-factorial vascular disease.

The specific aims were:

I. To estimate the familial risk of IS using various Swedish registers by investigating the effects of sibling kinship, sex, and age in the heritability of IS whereby full or half sibling relations, sex of siblings, and incident IS onset age were taken into account.

II. To assess and characterize the shared familial risk between IS and MI by exploring the shared effects of sibling kinship, sex, and onset age of one disease on the risk of the other.

III. To investigate the utility of sub-acute CRP in the prediction of functional dependency, recurrent vascular events, and all-cause mortality at one-year follow-up among acute IS patients, and to assess whether the addition of CRP to the conventional clinical prediction models would improve the prediction of outcomes in acute IS patients.

IV. To determine whether treatment efficacy of B-vitamin therapy in reducing mean concentration of tHcy level is modified by ethnicity, and if so, whether this may be influenced by genetic or dietary factors indirectly measured by serum FA and vitamin B_{12} amongst IS patients.

4 STUDY MATERIALS

4.1 SWEDISH DATA SOURCES

4.1.1 Hospital Discharge Register

The Swedish Hospital Discharge Register (HDR) is a register collecting data on in-patients treated at public hospitals with a nation-wide coverage since 1987 onwards. This register contains information on dates of admission and discharges, with up to eight discharge diagnosis codes, the first representing the principal cause of hospitalization. The International Classification of Diseases, Ninth Edition (ICD-9; 1987-1996, codes 433, 434, 436, and 437.1) and Tenth Edition (ICD-10; 1997-2007, code I63), were used for IS diagnosis in this register. For diagnosis of MI, the International Classification of Diseases, Ninth Edition (ICD-9; 1987-1996, code 410) and Tenth Edition (ICD-10; 1997-2007, codes I21 and I22), were used in this Register.

4.1.2 Causes of Death Register

The Swedish Cause of Death Register (CDR) is a nationwide reporting system which documents death and causes of death since 1749, achieving nationwide coverage since 1911. The same ICD codes as in the HDR were used to determine IS and MI as primary causes of death for this register.

4.1.3 Multi-Generation Register

The Swedish Multi-Generation Register (MGR) is a national register consisting of all individuals born in 1932 onward who had been registered in Sweden since 1961. This register has the ability to connect individuals to their biological and adoptive parents, and henceforth, siblings through the unique Swedish personal identity numbers (PINs) assigned to all individuals born in Sweden or who relocate to Sweden for a period of one year or longer. These PINs can be used to link data in various national registers, such as the HDR, the CDR, and other national registers.

4.1.4 Other national registers

The Swedish National Census Data (1990) and the Swedish Education Register (after 1990) collect information such as highest formal education levels for all individuals living in Sweden. Education levels categorized into four main groups – primary school, secondary-technical school, secondary-theoretical school, and college/university – were used as proxy for socioeconomic status.

4.2 SINGAPORE DATA SOURCES

4.2.1 SingHealth Cohort Study

The SingHealth Cohort Study is a prospective, single-centre, hospital-based cohort study whereby all consenting patients with Singapore residency admitted to the Inpatient Stroke Programme or referred to the Outpatients Stroke Clinics at the Singapore General

Hospital, the largest tertiary public hospital, with a recent (less than one month) IS or TIA between May 2002 and 2004, were eligible for recruitment.

In attempting to assemble an inception cohort of patients who have been identified at an early and uniform point in the course of their disease, patients admitted or referred to a large integrated stroke programme within the largest tertiary hospital in Singapore were targeted. The vast majority of stroke patients in Singapore are admitted to or referred to hospital for management. The Department of Neurology's Stroke Programme manages the overwhelming majority of stroke patients admitted or referred to the Singapore General Hospital, which accounts for a large and representative proportion of strokes in Singapore. Neither patients who died before hospital admission, nor patients who refused consent for blood sampling and follow-up could be recruited. The long-term objectives of the study are to develop effective new therapies for stroke and improve our understanding of the pathophysiology of stroke.

The specific aims of the study were to determine: (1) the clinical utility of measures of homocysteine and inflammatory biomarkers (CRP, IL-6, IL-10, IL-18, TNF-α and albumin) to prognosticate outcome following stroke; (2) whether the inflammatory biomarkers can be used efficiently in combination or alone as indicators of inflammation; (3) if fasting plasma homocysteine and inflammatory biomarkers measured after admission for IS in consecutive acute stroke patients predict functional outcome (as measured by the modified Rankin Scale [mRS]), recurrent vascular events (stroke, M, peripheral vessel occlusion), and mortality from vascular causes at six months and one year follow-up; (4) factors (including genetic) affecting fasting plasma homocysteine; (5) if other genetic factors (such as genetic markers for hypertension, *APOE* and *ACE* gene polymorphisms) affect outcome; and (6) the frequencies of the candidate hypertension and stroke gene polymorphisms in Singaporean stroke patients.

By the end of recruitment period of May 2004, a total of 997 acute IS or TIA patients were included in the study.

4.2.2   The VITATOPS Trial Sub-Study

This is a sub-study of the VITAmins TO Prevent Stroke (VITATOPS) trial – an international, multi-centre, randomized, double-blinded, placebo-controlled clinical trial – primarily designed to examine the efficacy and safety of B-vitamin therapy in secondary stroke prevention. Patients were randomized into two treatment arms, one receiving B-vitamins (a combination of 2.0 mg FA, 0.5 mg vitamin B_{12} and 25 mg B_6) and the other receiving placebo, both given as a single tablet, once daily.

Patients included were recruited within seven months of IS or transient ischemic attack (TIA) who had agreed to take the study medications, follow-up, consented, and agreed to have their fasting blood collected. Patients taking any of the B-vitamins, methotrexate, pregnant, or had limited life expectancy were not eligible for the study. Five-hundred-and-five (254 placebo and 251 vitamin-treated) patients were eligible for analyses in the Singapore General Hospital (SGH) site between July 2000 and March 2006.

5 STUDY DESIGNS AND METHODS

5.1 FAMILIAL EFFECTS ON IS (STUDY I)

5.1.1 Ischemic stroke individuals

Individuals with IS as primary diagnosis or primary cause of death between January 1, 1987, and December 31, 2007, were identified in the Swedish HDR and the Swedish CDR, respectively. IS was defined according to the International Classification of Diseases, Ninth Edition (ICD-9) Diagnostic Codes 433, 434, 436, and 437.1, and International Classification of Diseases, 10th Edition (ICD-10) Diagnostic Code I63 as registered in the HDR or CDR after January 1, 1987.

5.1.2 Exposed sibling-pair

To be included in our study, siblings of stroke individuals from the HDR had to be free from stroke at the time of stroke diagnosis in the sibling, thus making up the subjects of interest in our study (called "exposed study participant" from now on). This study participant and their sibling with IS were hence the exposed sib-pair; or in other words, an exposed study participant was defined as a stroke-free individual having a sibling with IS at baseline.

5.1.3 Unexposed sibling-pair

An unexposed study participant was defined as a stroke-free individual having a sibling without prior IS at the time of inclusion (called "unexposed study participant" from now on).

5.1.4 Matched cohort analysis

Our study design (**Figure 3**) was a nationwide matched cohort study based on pairs of siblings born and still living in Sweden, identified through the MGR. Each exposed sib-pair was then matched with up to five unexposed pairs (from the MGR). The matching was done by birth year of both siblings and the age of IS onset of the sibling (i.e. calendar time). We only allowed for selection of one sib-pair per family, so there are no first-degree relatives in the dataset, beyond the chosen sib-pairs; however, since the MGR does not contain information on individuals in the grandparent generation, there could be first and second cousins.

Following this design, we included a total of 30,735 exposed and 152,391 unexposed study participants. Information on sibling status (full or half), sex, birth date, hospitalizations with IS, and deaths (including causes of death) were collected. Study participants were followed up until an IS event, death, or end of follow-up period at December 31, 2007, whichever came first.
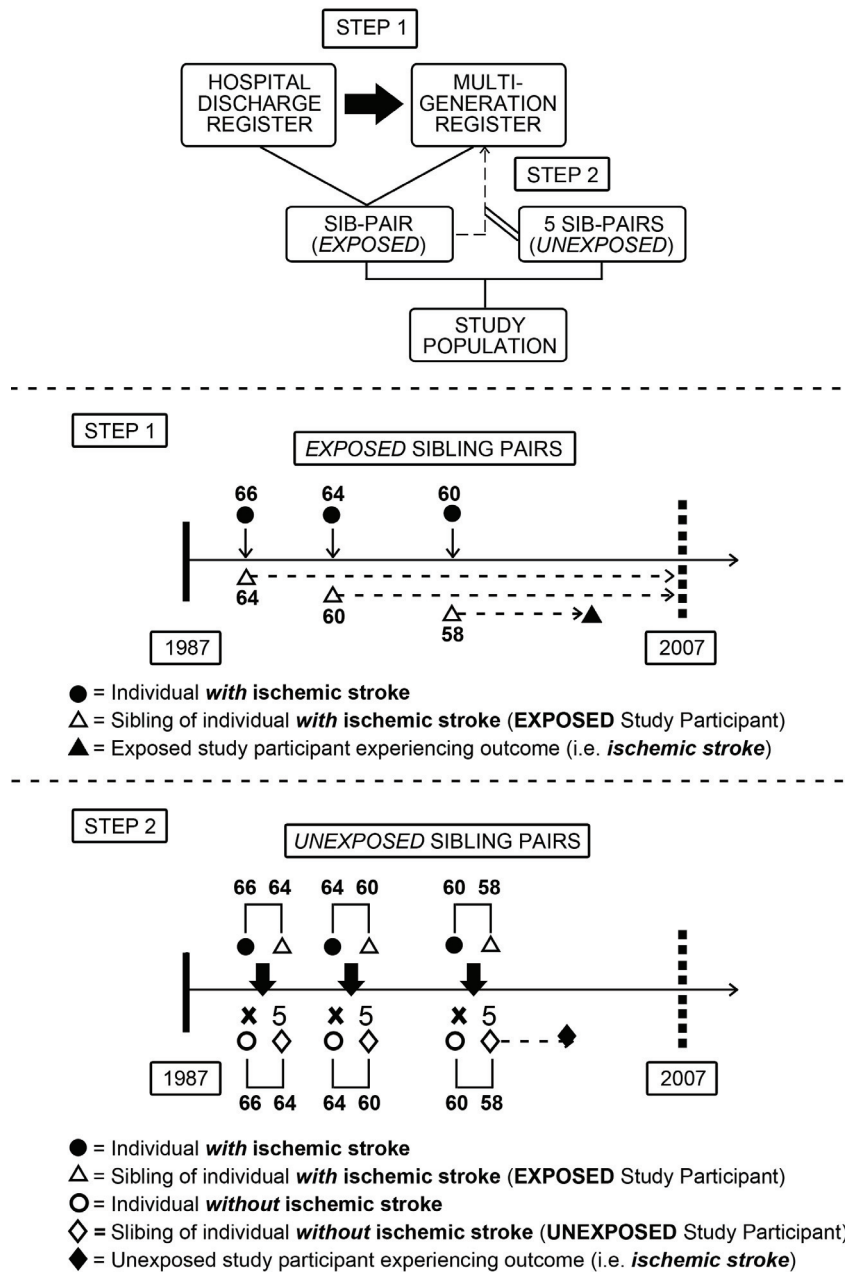
STEP 1

HOSPITAL DISCHARGE REGISTER → MULTI-GENERATION REGISTER

STEP 2

SIB-PAIR (*EXPOSED*)

5 SIB-PAIRS (*UNEXPOSED*)

STUDY POPULATION

STEP 1

EXPOSED SIBLING PAIRS

66 64 60

64
60
58

1987   2007

● = Individual *with* ischemic stroke
△ = Sibling of individual *with* ischemic stroke (**EXPOSED** Study Participant)
▲ = Exposed study participant experiencing outcome (i.e. *ischemic stroke*)

STEP 2

UNEXPOSED SIBLING PAIRS

66 64   64 60   60 58

● △   ● △   ● △

✗ 5   ✗ 5   ✗ 5
○ ◇   ○ ◇   ○ ◇

1987   2007

66 64   64 60   60 58

● = Individual *with* ischemic stroke
△ = Sibling of individual *with* ischemic stroke (**EXPOSED** Study Participant)
○ = Individual *without* ischemic stroke
◇ = Slibing of individual *without* ischemic stroke (**UNEXPOSED** Study Participant)
◆ = Unexposed study participant experiencing outcome (i.e. *ischemic stroke*)

Figure 3. Study population matched-cohort flow chart (*Source*: Kasiman *et al*., 2012) [26]

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5.1.5 Statistical analyses

We calculated the time from the incident IS event in the sibling (or the corresponding time of entry of unexposed study participants) until an IS event (hospitalization or death), censoring due to death from other causes or end of follow-up at December 31, 2007, in the study participant. The relative risks (RRs) of IS comparing exposed study participants (individuals having a sibling with IS) with unexposed study participants (individuals having a sibling without IS) were estimated from stratified Cox regression models, using the matching factors to define strata to account for the matched study design. The crude age-, sex-, and education-adjusted RRs and 95% Wald-type confidence intervals (CIs) of incident IS events in exposed versus unexposed were estimated.

To assess the role of familial risk of early stroke onset, we also analyzed the risk of stroke before the age of 55. The Cox models were also fitted in subgroups stratified by sibship (full or half siblings), sex, sex of the sibling, and by age of IS in the sibling (early: <=55; late:>55). Proportional hazard assumptions were tested using Schoenfeld residuals [166]. Two-tailed significance values were given with $p<0.05$ regarded as significant. Data were analyzed using SAS version 9.2 (SAS Institute Inc., Cary, North Carolina, USA) and STATA for Windows version 12 (Stata Corporation LP, College Station, Texas, USA) packages.

5.2 COMMON FAMILIAL EFFECTS ON IS AND MI (STUDY II)

5.2.1 Ischemic stroke-exposed matched cohort analysis

First, we identified individuals with IS as primary diagnosis or primary cause of death. To be included in this dataset, siblings (identified via linkage made through their biological parents in the MGR) of IS individuals from the Hospital Discharge Register had to be free from MI at the time of IS diagnosis in the sibling, thus making up the subjects of interest (called "IS-exposed study participant" from now on). Outcome of interest in the study participants was incident MI. In other words, an IS-exposed study participant was defined as an MI-free individual having a sibling with IS at baseline. Collectively, these IS-exposed study participants and their respective siblings formed the IS-exposed sib-pair. Up to five IS-unexposed sib-pairs were randomly selected via matching by birth year of both siblings and IS onset age of the sibling, i.e. calendar year, to each IS-exposed sib-pair through the MGR. IS-unexposed study participants were defined as MI-free individuals having a sibling without prior IS at the time of inclusion. Using this design, a total of 31,659 IS-exposed and 143,728 IS-unexposed study participants were included in the IS dataset.

5.2.2 Myocardial infarction-exposed matched cohort analysis

Here, we identified individuals with MI as a primary diagnosis or primary cause of death. To be included in this dataset, siblings (identified via linkage made through their biological parents in the MGR) of MI individuals from the Hospital Discharge Register had to be free from IS at the time of MI diagnosis in the sibling, thus making up the subjects of interest (called "MI-exposed study participant" from now on). Outcome of

interest in the study participants was incident IS. In other words, an MI-exposed study participant was defined as an IS-free individual having a sibling with MI at baseline. Collectively, these MI-exposed study participants and their respective siblings formed the MI-exposed sib-pair. Up to five MI-unexposed sib-pairs were randomly selected via matching by birth year of both siblings and MI onset age of the sibling, i.e. calendar year, to each MI-exposed sib-pair through the MGR. MI-unexposed study participants were defined as IS-free individuals having a sibling without prior MI at the time of inclusion. A total of 62,766 MI-exposed and 265,974 MI-unexposed study participants were included in the MI dataset.

5.2.3 Statistical analyses

We calculated the time from study entry of participants (incident IS or MI event in the sibling for exposed or time of matching for unexposed) until study exit (MI or IS events, censoring due to death from other causes, or end of follow-up at 31 Dec 2007). Primarily, two types of RRs were estimated from stratified Cox regression models using matching factors to define strata to account for the matched study design: (1) the RRs of IS comparing MI-exposed study participants (individuals having a sibling with MI) with unexposed study participants (individuals having a sibling without MI); and (2) the RRs of MI comparing IS-exposed study participants (individuals having a sibling with IS) with unexposed study participants (individuals having a sibling without IS). The relative risks and 95% Wald-type CIs of incident IS and MI events in exposed versus unexposed were estimated.

Sensitivity analyses were also carried out via two alternate models by fitting a time-varying covariate to the aforementioned main models. In the analyses assessing relative risks of MI, the time-varying covariates in the two alternate models, as delineated in **Figure 4**, were: (1) incident IS prior to incidence of MI (in other words, the occurrence of incident IS before the outcome of interest which was incidence of MI in these analyses) [Time-varying covariate model 1]; and (2) MI in sibling *after* having a sibling with IS (in other words, an additional exposure to sibling risk of MI after being exposed to sibling risk of IS) [Time-varying covariate model 2] (**Figure 4a**). Similarly, in analyses assessing relative risks of IS, time-varying covariates were: (1) incident MI prior to incidence of IS in study participants (in other words, the occurrence of incident MI before the outcome of interest which was incidence of IS) [Time-varying covariate model 1]; and (2) IS post-MI in sibling (in other words, an additional exposure to sibling risk of IS after being exposed to sibling risk of MI) [Time-varying covariate model 2] (**Figure 4b**).

To assess the role of familial risk of early IS onset and early MI onset, we also analysed the risks of IS and MI before the age of 55. In addition, the Cox models were fitted in subgroups stratified by sibship (full/half siblings), sex, sex of the sibling and by onset age of IS or MI in the sibling (early: up to age 55; late: after age 55). Sensitivity analyses following the aforementioned alternate models were also implemented in these sets of analyses.

Proportional hazard assumptions were confirmed using Schoenfeld residuals [166]. Two-tailed significance values were given with $p<0.05$ regarded as significant. Data were analysed using SAS version 9.2 (SAS Institute Inc., Cary, North Carolina, USA)

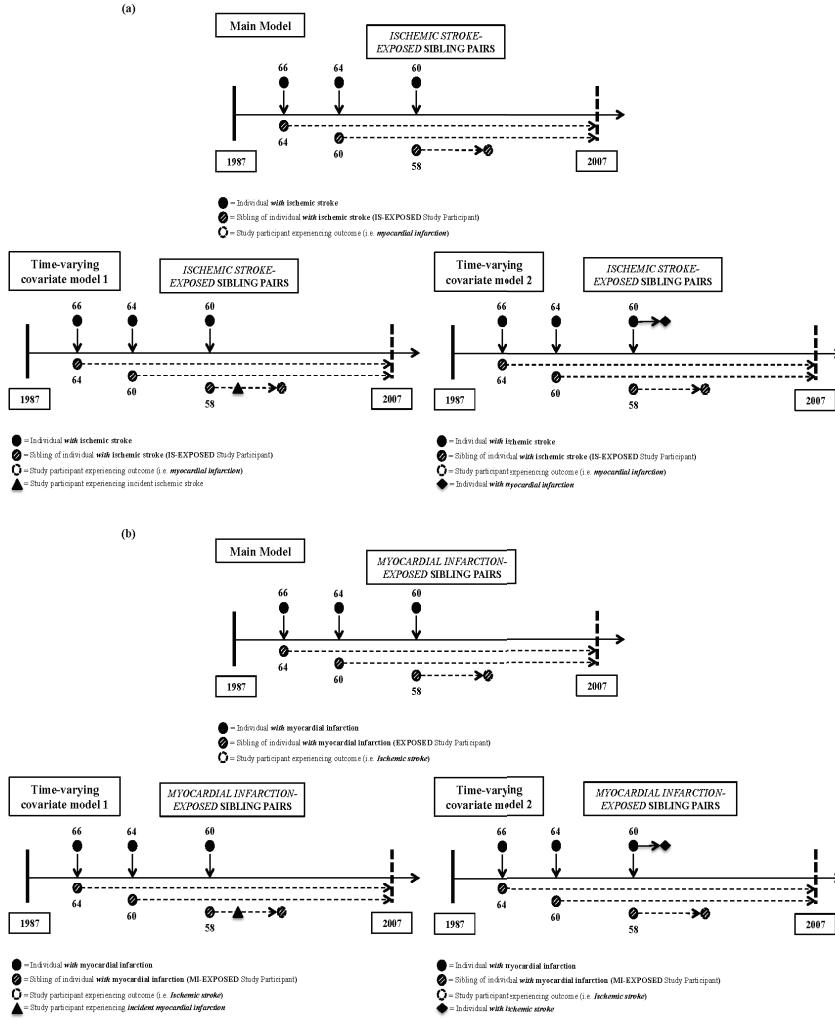and STATA for Windows version 11.2 (Stata Corporation LP, College Station, Texas, USA) packages.

Figure 4. (a) Main model of matched cohort design to estimate the relative risks of IS comparing MI-exposed study participants (individuals having a sibling with MI) with unexposed study participants (individuals having a sibling without MI) and its two alternative models; and (b) main model of matched cohort design to estimate the relative risks of MI comparing IS-exposed study participants (individuals having a sibling with IS) with unexposed study participants (individuals having a sibling without IS) and its two alternative models

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5.3 CRP AND PROGNOSIS FOLLOWING IS (STUDY III)

5.3.1 Patients with acute ischemic stroke and TIAs

Patients diagnosed with recent (within 30 days of onset) TIA or IS were prospectively recruited when admitted to the acute stroke inpatient program or referred to the outpatient stroke clinics at the Department of Neurology, Singapore General Hospital between May 2002 and May 2004 (**Figure 5**).
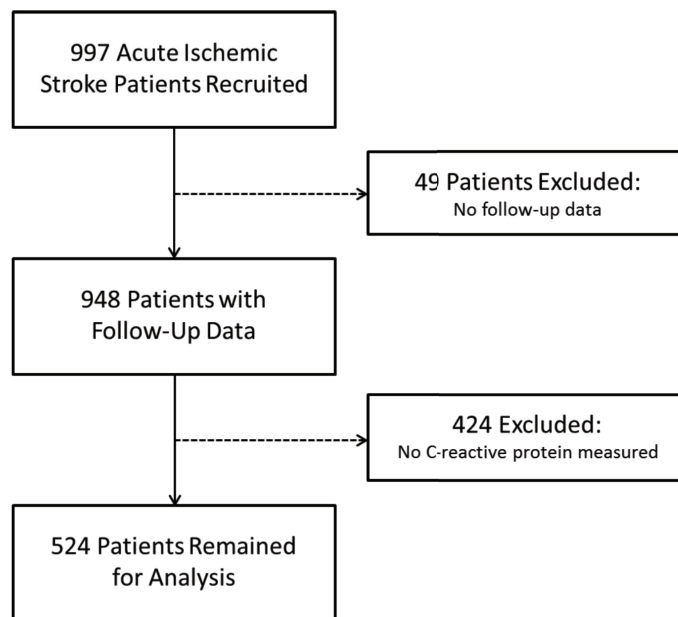

Figure 5. Study flowchart of patients included for this analysis

5.3.2 Methods

5.3.2.1 Clinical assessment

Brain imaging using either computed tomography (CT) or magnetic resonance imaging (MRI) was performed routinely within 72 hours upon admission. Patients with hemorrhagic stroke were excluded from the study. Information on vascular risk factors (such as hypertension, hyperlipidemia, ischemic heart disease, diabetes mellitus, atrial fibrillation, previous stroke, smoking and peripheral artery disease), stroke subtypes and neurovascular imaging were obtained through thorough investigation according to standardized criteria upon admission. IS was classified according to the OCSP Classification; TACI, PACI, POCI and LACI [54].

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5.3.2.2 Laboratory measurements

After informed consent was obtained, fasting venous blood samples were collected into EDTA anticoagulant and placed immediately on ice. Upon transport to the laboratory, samples were centrifuged at 4000 rpm for 10 minutes where plasma and cells were separated. Extracted plasma was stored in plain plastic tubes at –80°C until analysis. CRP was assayed retrospectively in plasma by high-sensitivity nephelometry.

5.3.2.3 Outcome measurements at one-year follow-up

Study participants were assessed for functional and vascular outcomes at one year following the stroke onset by investigators blinded to baseline data. Functional outcome was evaluated using the mRS [167]. The mRS (**Table 2**) was dichotomized to indicate functional independence (0-2) or dependence (3-6). Vascular outcomes were defined as a composite of recurrent IS, non-fatal MI, amputation or death due to any vascular cause. All-cause mortality included vascular and non-vascular deaths. A home visit or telephone interview was performed if the patient was unable to attend the clinic for assessment.

Table 2. Categories in the modified Rankin Scale (mRS) (Adapted from Risselada *et al*. 2010) [168]

Scale	Definition
0	No symptoms at all
1	No significant disability despite symptoms; able to carry out all usual duties and activities
2	Slight disability; requiring some help, but able to look after own affairs without assistance
3	Moderate disability; requiring some help, but able to walk without assistance
4	Moderately sever disability; unable to walk without assistance and unable to attend to own bodily needs without assistance
5	Severe disability; bedridden, incontinent and requiring constant nursing care and attention
6	Dead

5.3.3 Statistical analyses

To assess the associations between CRP, baseline features, and outcomes, the Fisher's exact or the Pearson's-χ^2 tests were used. For normally-distributed continuous variables, the student's *t*-test was used. The Mann-Whitney U test was used for skewdly-distributed continuous variables. Normally-distributed continuous variables were expressed in means ± standard deviation (SD), while skewdly-distributed continuous variables were expressed in medians and interquartile ranges (IQR). The Spearman's rank correlation coefficients were used to assess associations between two continuous variables.

Binary logistic regression models were used to assess the association of CRP in quartiles with the outcomes of interest (functional dependency, recurrent vascular event, and all-cause mortality) where unadjusted odds ratios (ORs) and 95% CIs from these

models were obtained. We also carried out adjusted models whereby significantly associated factors in the univariate analyses were put into the models. Likelihood-ratio tests were used to assess the significant difference between models.

To assess discrimination of prognostic models, the area under curve (AUC) or *c*-statistics of the receiver operating characteristics (ROC) in different models were estimated. Likelihood-ratio tests were used to assess the significant difference between models with and without CRP.

All analyses were performed using STATA for Windows version 12.1 (Stata Corporation LP, College Station, Texas, USA) and the statistical significance was set at $p<0.05$.

5.4   ETHNICITY AND B-VITAMIN THERAPY (STUDY IV)

5.4.1   VITATOPS Sub-study Samples

This is a Singapore-site sub-study of the main VITAmins TO Prevent Stroke (VITATOPS) trial [45] – an international, multi-centre, randomized, double-blinded, placebo-controlled clinical trial – primarily designed to examine the efficacy and safety of B-vitamin therapy in secondary stroke prevention.

5.4.2   Methods

5.4.2.1   Study population

Patients were recruited within seven months of their IS or TIA onset. They agreed to take the study medications, to be followed-up, consented, and to have their fasting blood collected. Patients taking any of the B-vitamins, methotrexate, pregnant, or has limited life expectancy were not eligible for the study. Ethnicity was determined using the Singapore National Registration Identity Card. Eurasians were excluded. Five-hundred-and-five (254 placebo and 251 vitamin-treated) patients were eligible for analyses in the Singapore General Hospital site between July 2000 and March 2006 (**Figure 6**).

5.4.2.2   Intervention

Patients were randomized into two treatment arms, one receiving B-vitamins (a combination of 2.0 mg FA, 0.5 mg vitamin B_{12} and 25 mg B_6) and the other receiving placebo, both given as a single tablet, once daily. Patients and study personnel were blinded to treatment allocation.

5.4.2.3   Laboratory methods and genotyping

Eligible patients had 20 ml of fasting venous blood specimens collected into EDTA anticoagulant tubes at randomization to measure plasma tHcy, serum FA, and vitamin B_{12}, and for acquisition of genomic DNA. Repeat blood specimens were collected one year after randomization. Samples were centrifuged at 4000 rpm for 10 minutes upon arrival at the laboratory, where plasma and cells were separated and stored at −80°C until processed for tHcy using the fluorescent polarization immunoassay method (Abbott

Laboratories), and genomic DNA analysis. Additionally, serum FA and vitamin B_{12} were measured using the chemiluminescent microparticle immunoassay technique (Abbott Diagnostics).

DNA was isolated from blood cells using commercially available QIAamp DNA Blood Mini kit (Qiagen GmbH) according to the manufacturer's protocol. Amplification of the regions containing the two *MTHFR* polymorphisms were performed separately according to established methods using standard polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) with *Hin*fI and *Mbo*II restriction enzymes (New England BioLabs) to determine the *C677T* [153] and *A1298C* [152] genotypes, respectively. The use of both positive and negative controls in each set of analyzed samples, assayed in duplicate, and independent confirmation of results were carried out by two laboratory personnel blinded to the phenotypes to maintain quality control for the DNA analyses.

5.4.3 Statistical analyses

The primary analysis was a comparison of mean tHcy at 1 year between the placebo and B-vitamin treatment arms in the three ethnic groups based on the intention-to-treat principle. Our study provided 80% power to detect a difference in the change of tHcy levels between baseline and one year of 2.0 μmol/L between Chinese and Malays, 1.7 μmol/L between Chinese and Indians, and 2.4 μmol/L between Malays and Indians. Statistical analyses were carried out using SAS version 8.2 (SAS Institute Inc., Cary, North Carolina, USA) by an independent third-party statistician.
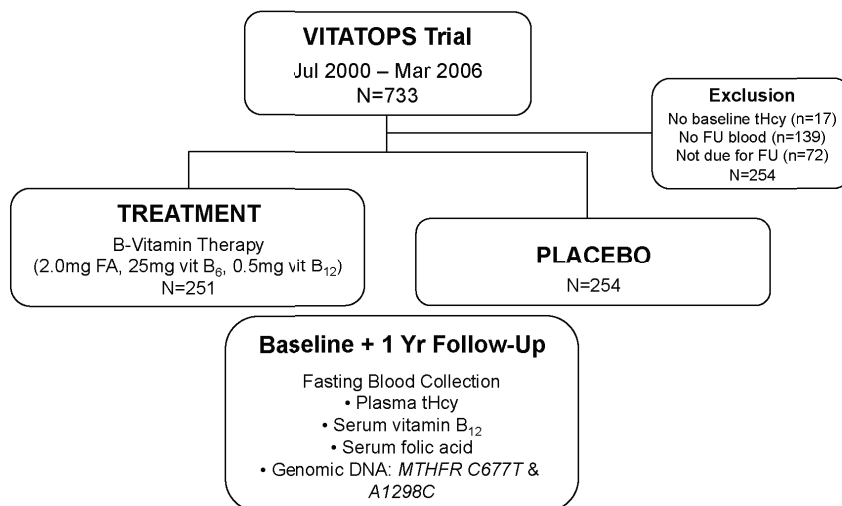

Figure 6. The VITATOPS trial sub-study population

6 RESULTS

6.1 FAMILIAL EFFECTS ON IS (STUDY I)

A total of 30,735 exposed (individuals having a sibling with IS) and 152,391 unexposed (individuals having a sibling without IS) study participants were included, generating a total of 208,080 and 1,036,809 person-years of follow-up, respectively. The numbers of incident IS events during follow-up were 717 among the exposed and 2,242 among the unexposed. Cumulative incidence of IS in our study participants was shown to be much higher in those whose siblings previously had IS (**Figure 7**).
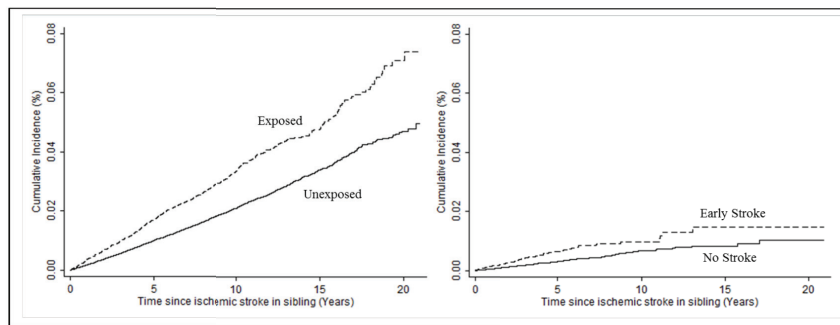

Figure 7. Left: Cumulative incidence for study participants between those whose siblings had ischemic stroke (IS) (exposed) compared to those whose siblings had not (unexposed). Right: Cumulative incidence for study participants between those whose siblings had early IS (early stroke) compared to those whose siblings had not (no stroke) (*Source*: Kasiman *et al.*, 2012) [26]

Overall, the study participants who had been exposed to IS were observed to have an approximately 60% higher risk of incident IS compared with those who had not been exposed. Having either full or half siblings with stroke was statistically significantly associated with the risk of incident IS; however, this difference in RRs between types of sibling relation was not statistically significant. There were no statistically significant differences in RRs across gender of the study participants or their siblings (**Figure 8**).
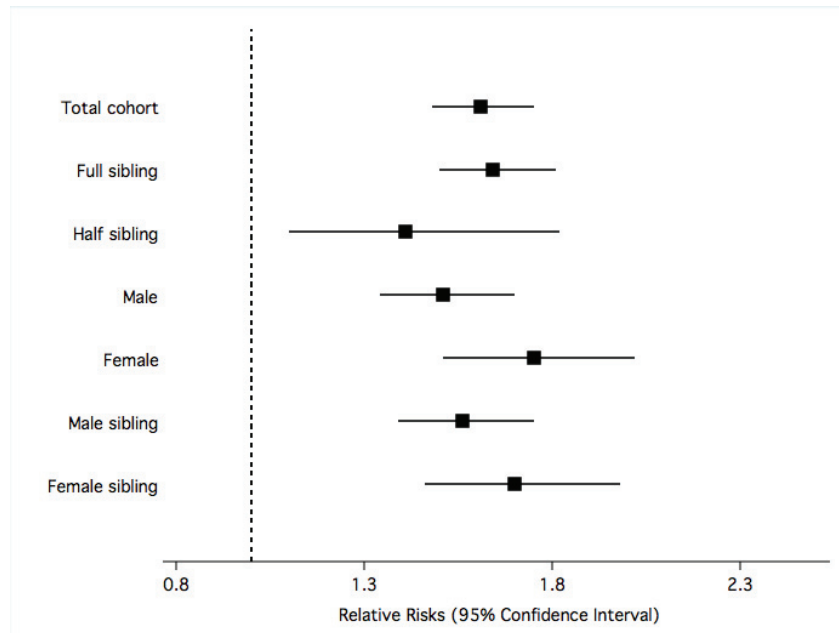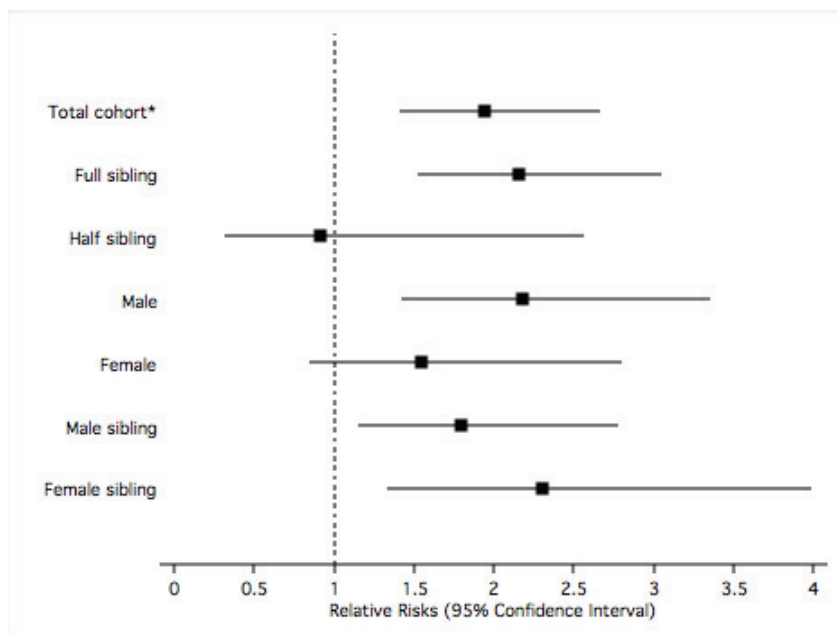
Figure 8. The relative risks (RRs) of incidence ischemic stroke among study participants when exposed to siblings with ischemic stroke compared to unexposed overall, and when stratified by sibling relation, sex, and sex of sibling

The risk of incident IS in the study participants was elevated by 1.67-fold in those exposed to siblings with early stroke compared with those unexposed and only 1.58-fold in those exposed to late stroke compared with those unexposed. The pattern of higher risk in those exposed to siblings with early stroke compared with those exposed to siblings with late stroke was consistently observed among different sibling relations, sex of study participants, and sex of their siblings.

Overall, having a sibling with an early IS significantly increased the study participants' risk of having an early IS themselves by approximately 2-fold. Having a full sibling with early stroke increased the risk of an early stroke by 2.16-fold. Such an association was not observed in half-sibling relation; however this may be owing to the relatively small number of affected half siblings in our study group. Again, higher RR could be observed among those exposed to full siblings with early stroke compared with those exposed to half siblings with early stroke, but this association was not statistically significant (p for interaction = 0.1218). Neither sex of the sibling nor of the study participant seemed to affect the increased familial risk of having an early ischemic stroke (**Figure 9**).

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*Study population used for analyses in this figure consists of a subset of individuals <=55 years old at study enrolment drawn from the original study population

Figure 9. Relative risks (RRs) of early incident ischemic stroke among study participants when exposed to siblings with early ischemic stroke compared to unexposed overall, and when stratified by sibling kinship, sex, and sex of sibling

6.2 COMMON FAMILIAL EFFECTS ON IS AND MI (STUDY II)

In this study, a total of 31,659 IS-exposed (individuals having a sibling with IS) and 143,728 IS-unexposed (individuals having a sibling without IS) study participants were included, generating a total of 199,582 and 939,337 person-years at risk of follow-up, respectively. The numbers of incident MI events during follow-up were 1,011 among the IS-exposed, and 3,294 among the IS-unexposed. In addition, a total of 62,766 MI-exposed (individuals having a sibling with MI) and 265,974 MI-unexposed (individuals having a sibling without MI) study participants were included, generating a total of 461,694 and 2,042,377 person-years at risk of follow-up, respectively. The numbers of incident IS events during follow-up were 1,314 among the MI-exposed, and 4,130 among the MI-unexposed.
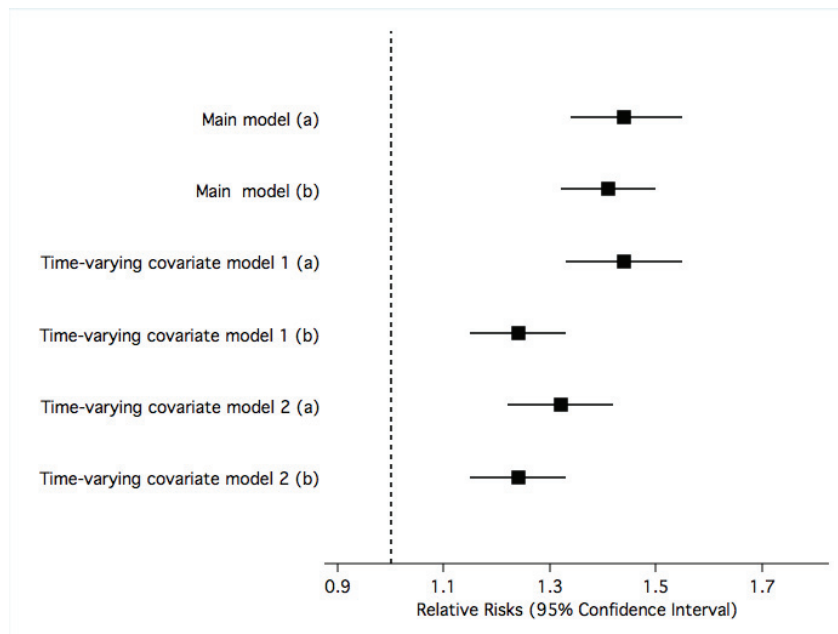

Figure 10. Relative risks (RRs) of (a) incident MI among study participants when exposed to siblings with IS compared to unexposed; and (b) incident IS among study participants when exposed to siblings with MI compared to unexposed

Overall, the study participants who had been exposed to having a sibling with IS were observed to have an approximately 40% higher risk of incident MI compared to those who had not been exposed, and a similar magnitude of increased risk of IS was observed in study participants who had been exposed to having a sibling with MI compared to those who had not been exposed. The relative risk of MI remained the same

when incident IS events in study participants post-study entrance and prior to MI or end of study were taken into account (time-varying covariate model 1). However, the relative risk of MI was reduced to 32% (from 44%) when the model was adjusted for MI in siblings (time-varying covariate model 2). The relative risk of IS was reduced to 24% (from 41%) when incident MI event post-study entrance were taken into account (time-varying covariate model 1). Similarly, the relative risk of IS was reduced to 24% when the model was adjusted for IS in siblings (time-varying covariate model 2) (**Figure 10**).

IS-exposed full siblings had higher RR compared to IS-exposed half siblings. However, this difference in RR among sibling relation was not statistically significant. On the other hand, MI-exposed full siblings had similar RR compared to MI-exposed half siblings. The pattern of higher risk in women compared to men was consistently observed when the RRs between exposed and unexposed were stratified by sex of study participants and sex of their siblings; however, the differences were not statistically significant.

Overall, the risk of MI in the study participants was elevated by 1.69-fold when their siblings had early IS compared to when their siblings did not have IS. This increased risk was halved when their siblings had late IS compared to when their siblings did not have IS. These higher estimates of MI risk in those exposed to siblings with early IS were repeatedly observed when stratified by sex of sibling and sibling relation. However, the difference of the estimates was not statistically significant.

The risk of early MI among study participants exposed to siblings with early IS was observed to be 94% higher than those whose siblings did not have IS while the risk for early IS among study participants exposed to siblings with early MI when compared to those with siblings without MI was slightly lower at 63%. Stratification by sibling relation, sex, and sex of sibling showed that the relative risks for incident MI among study participants exposed to early IS compared to unexposed approximately doubled in full sibling relations, males, and female siblings. The relative risks of incident IS among study participants exposed to early MI compared to unexposed were not as high, and stratification by sibling relation, sex, and sex of sibling showed higher risk among full siblings relations, females, and female siblings.

6.3 CRP AND PROGNOSIS FOLLOWING IS (STUDY III)

To assess the influence of different ranges of CRP on functional outcome post-stroke, CRP was categorized into quartiles due to its highly skewed distribution. The risk of functional dependence at one year doubled with each increasing quartile of CRP, with a tripled risk between the top two highest quartiles of CRP. After adjustment for age at stroke onset and stroke subtypes which predicted functional dependence in our study, the ORs remained significantly associated with functional dependency by approximately two-fold in the top two quartiles. The OR of the second bottom quartile was not significantly associated with functional dependency at one year; however, the magnitude of the OR for the association hardly changed. After additional adjustment for other markers which were significantly correlated to CRP, the association between CRP and functional dependency remained independently significant with risk increasing by approximately two-fold with each quartile of CRP (**Table 3**). A likelihood-ratio test comparing the aforementioned adjusted model with a fully adjusted model, whereby other significantly associated factors (such as hyperlipidemia and atrial fibrillation) were included, did not show significant differences of the estimates (p=0.2886).

Table 3. Odds ratios per quartile increase of CRP of functional dependency at one-year

	Unadjusted OR (95% CI)	Adjusted OR (95% CI)
Quartiles of CRP		
1st quartile (<1.33 mg/L)	1.00	1.00
2nd quartile (1.33-3.71 mg/L)	2.30 (1.00-5.29)[*]	2.33 (0.99-5.50)
3rd quartile (3.72-8.78 mg/L	4.75 (2.17-10.4)[†]	4.61 (2.05-10.4)[‡]
4th quartile (>8.78 mg/L)	11.8 (5.53-25.2)[†]	7.87 (3.56-17.4)[‡]

*p<0.05; †p<0.010;‡p<0.001

The risk of having recurrent vascular events was shown to increase in each increasing quartile of CRP. However, only the top two quartiles were shown to be significantly associated. After adjustment for age at stroke onset and stroke subtypes which predicted recurrent vascular events in our study, the ORs remained significantly associated with recurrent vascular events although the magnitude of risk seemed to decrease slightly. Upon further adjustment for other markers which were significantly correlated with CRP, the association between CRP and recurrent vascular events was attenuated at the top two quartiles. The risk also seemed to plateau at these top two quartiles (**Table 4**). A likelihood-ratio test comparing the aforementioned adjusted model with a fully adjusted model, whereby other significantly associated factors (such as hyperlipidemia and atrial fibrillation) were included, did not show significant difference of the estimates (p=0.4726).

Table 4. Odds ratios per quartile increase of CRP of recurrent vascular event at one-year

	Unadjusted OR (95% CI)	Adjusted OR (95% CI)
Quartiles of CRP		
1st quartile (<1.33 mg/L)	1.00	1.00
2nd quartile (1.33-3.71 mg/L)	1.64 (0.52-5.15)	1.62 (0.51-5.17)
3rd quartile (3.72-8.78 mg/L	4.01 (1.44-11.2)[†]	3.49 (1.24-9.86)[†]
4th quartile (>8.78 mg/L)	5.65 (2.00-15.3)[‡]	3.90 (1.39-11.0)[†]

*$p<0.05$; †$p<0.010$;‡$p<0.001$

The risk of having all-cause mortality was shown to increase in each increasing quartile of CRP. However, only the top two quartiles were shown to be significantly associated. After adjustment for age at stroke onset and stroke subtypes, the ORs remained significantly associated with all-cause mortality, although the magnitude of the risk decreased. Upon further adjustment of other markers which were significantly correlated with CRP, the association between CRP and all-cause mortality was attenuated at the top two quartiles. The risk only remained statistically significant at the highest quartile with an increase of approximately 18-fold (**Table 5**). A likelihood-ratio test comparing the aforementioned adjusted model with a fully adjusted model, whereby other significantly associated factors (such as hyperlipidemia and atrial fibrillation) were included, did not show significant difference of the estimates ($p=0.3501$).

Table 5. Odds ratios per quartile increase of CRP of all-cause mortality at one-year

	Unadjusted OR (95% CI)	Adjusted OR (95% CI)
Quartiles of CRP		
1st quartile (<1.33 mg/L)	1.00	1.00
2nd quartile (1.33-3.71 mg/L)	4.09 (0.45-37.1)	4.20 (0.45-39.0)
3rd quartile (3.72-8.78 mg/L	9.59 (1.20-76.8)[*]	7.58 (0.92-62.5)
4th quartile (>8.78 mg/L)	30.7 (4.09-230.0)[‡]	17.7 (2.29-137.4)[‡]

*$p<0.05$; †$p<0.010$;‡$p<0.001$

To assess whether the addition of CRP improves the conventional clinical predictive model, we compared three models (Model 1: age only; Model 2: age + stroke subtypes; Model 3: age + stroke subtypes + CRP quartiles) for the prediction of outcomes of interest (functional dependency, recurrent vascular events, and all-cause mortality) (**Figure 11**). CRP improved the discriminative ability of the model for all three outcomes of interest. The c-statistics increased significantly in the prediction of functional dependency, in recurrent vascular event, and all-cause mortality (**Table 6**).
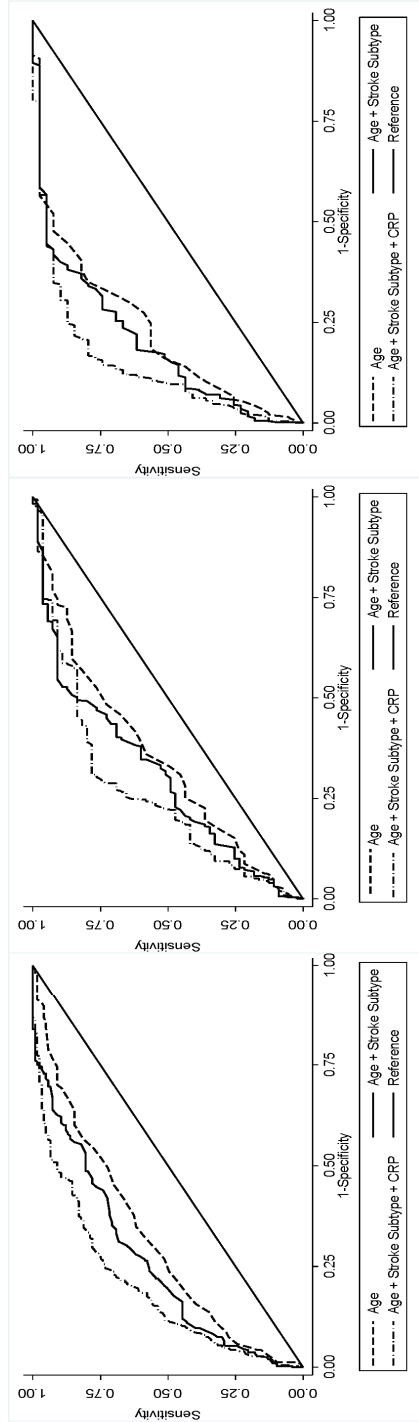
Figure 11. Receiver operating characteristics illustrating the models with and without the addition of CRP in the prediction of functional dependency (left), recurrent vascular event (middle), and all-cause mortality (right) at one-year post-stroke

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Table 6. Performance of models in predicting functional dependency, recurrent vascular event, and all-cause mortality one-year post-stroke

Models	Likelihood Ratio χ^2 Statistic*	p-value	AIC†	BIC†	AUC§ (95% CI)	p-value	Hosmer-Lemeshow χ^2‖	p-value
Functional Dependency								
Age	Ref		532.1	540.6	0.688 (0.634-0.742)		1.47	0.9931
Age+Stroke subtypes	39.2	<0.001	500.9	526.5	0.745 (0.697-0.793)	<0.001	13.6	0.0926
Age+Stroke subtypes+CRP	76.5	<0.001	469.5	507.9	0.807 (0.765-0.848)		5.08	0.7490
Recurrent Vascular Event								
Age	Ref		341.3	349.9	0.653 (0.580-0.725)		4.73	0.7862
Age+Stroke subtypes	10.1	0.0385	339.2	364.8	0.697 (0.630-0.764)	0.0127	11.32	0.1845
Age+Stroke subtypes+CRP	21.0	0.0037	334.3	372.7	0.741 (0.673-0.809)		12.34	0.1365
All Death								
Age	Ref		245.8	254.4	0.777 (0.711-0.843)		14.15	0.0779
Age+Stroke subtypes	14.1	0.0071	239.8	265.3	0.808 (0.744-0.871)	0.0095	10.82	0.2119
Age+Stroke subtypes+CRP	33.1	<0.001	226.8	265.1	0.863 (0.809-0.917)		8.40	0.3952

*Likelihood-ratio statistic
†AIC denotes Akaike Information Criterion and BIC denotes Bayes Information Criterion.
§AUC denotes Area Under Receiver Operating Curve (ROC) or C-statistic.
‖Hosmer-Lemeshow test

6.4   ETHNICITY AND B-VITAMIN THERAPY (STUDY IV)

The Singapore General Hospital site randomized 733 patients into VITATOPS between July 2000 and March 2006. Baseline tHcy was not measured in 17 patients, 139 did not undergo repeat blood collection due to death, withdrawal from the study, or refusal to have blood taken, and 72 had yet to undergo repeat blood collection by study end. Thus, 505 (254 placebo and 251 vitamin treated) patients were eligible for analyses (**Figure 6**). The ethnic distribution of study participants was representative of Singapore. Baseline demographics and risk factor profiles were similar among ethnic groups apart from a higher prevalence of ischemic heart disease in Indians (**Table 7**).

Table 7. Baseline patient characteristics and *MTHFR* genotypes across the three ethnic groups (*Source*: Kasiman *et al*., 2009) [169]

Characteristics	Chinese n=419 (83%)	Malay n=41 (8%)	Indian n=45 (9%)
Age, mean (SD)	61.9 (11.6)	59.0 (11.0)	59.0 (11.5)
Male, n (%)	274 (65.4)	26 (63.4)	29 (64.4)
Qualifying Event, n (%)			
TIA	79 (18.9)	5 (12.2)	9 (20.0)
Ischemic stroke	340 (81.2)	36 (87.9)	36 (79.9)
Stroke risk factors, n (%)			
Hypertension	300 (71.6)	33 (80.5)	35 (77.8)
Current smoking	131 (31.3)	15 (36.6)	13 (28.9)
Hypercholesterolemia	197 (47.0)	24 (58.5)	23 (51.1)
Diabetes mellitus	150 (35.8)	18 (43.9)	24 (53.3)
History of vascular disease, n (%)			
Ischemic heart disease	48 (11.5)	5 (12.2)	13 (28.9)[*]
Peripheral arterial disease	2 (0.5)	1 (2.4)	2 (4.4)
Stroke	74 (17.7)	7 (17.1)	8 (17.8)
Biochemistry, mean (SD)			
tHcy, μmol/L	13.9 (4.9)	13.4 (4.0)	13.8 (5.9)
Vitamin B_{12}, pmol/L	334 (210)	453 (254)[*]	325 (183)
Folic acid, nmol/L	18.7 (7.7)	14.0 (5.0)[†]	17.2 (7.1)
Genotypes, n (%)			
MTHFR C677T	(n=402)	(n=41)	(n=41)
CC	221 (55.0)	26 (63.4)	33 (80.5)
CT	151 (37.6)	13 (31.7)	8 (19.5)
TT	30 (7.5)[‡]	2 (4.9)	0 (0)
MTHFR A1298C	(n=410)	(n=40)	(n=44)
AA	240 (58.5)	18 (45.0)	17 (38.6)
AC	146 (35.6)	16 (40.0)	22 (50.0)
CC	24 (5.9)[‡]	6 (15.0)	5 (11.4)

There were no significant differences in tHcy between the ethnic groups. However, Malays had significantly lower FA and higher vitamin B_{12} in comparison to non-Malays. *MTHFR C677T* allele distribution differed among ethnic groups, with non-Chinese having significantly lower *T* allele prevalence. *MTHFR A1298C* allele distribution also differed, with non-Chinese having significantly higher *C* allele prevalence. There were no significant deviations of genotype distributions from expected Hardy-Weinberg equilibrium.

At one year, vitamin B_{12} remained significantly higher in Malays. There was a similar magnitude of reduction of tHcy associated with B-vitamin treatment across all ethnic groups (**Table 8**).

Table 8. Effect of one-year vitamin treatment in the three different ethnic groups (Adapted from Kasiman *et al*., 2009) [169]

Mean difference between treatment groups, (95% CI)	Chinese (n=419)	Malay (n=41)	Indian (n=45)
tHcy, μmol/L	-3.8 (-4.2 – -3.4)	-4.9 (-6.2 – -3.6)	-3.3 (-4.4 – -2.2)
Vitamin B_{12}, pmol/L	330 (304 – 357)	386 (307 – 465)	213 (128 – 298)
Folic acid, nmol/L	16.9 (16.0 – 17.8)	21.9 (19.4 – 24.4)	19.2 (16.6 – 21.8)

The *677TT* genotype was significantly associated with a higher mean tHcy compared with *677CT* and *677CC* genotypes in Chinese (**Table 9**). This trend was also observed in Indians, but not in Malays. The *MTHFR A1298C* polymorphism was significantly associated with mean baseline tHcy only in Chinese. Importantly, the interaction between vitamin treatment efficacy and *MTHFR* genotypes on tHcy difference (one year from baseline) was not significant across ethnic groups for both *C677T* (p for interaction = 0.8 Chinese; 0.9 Malays; 0.3 Indians) and *A1298C* (p for interaction = 0.4 Chinese; 0.7 Malays; 0.3 Indians).

Table 9. Effect of the *MTHFR C677T* and *A1298C* polymorphisms on plasma total homocysteine (tHcy) levels at baseline and one year (*Source:* Kasiman *et al.*, 2009) [169].

	Baseline tHcy			1-year tHcy					
	All Participants			Placebo			Vitamins		
	Mean, μmol/L (SD)			Mean, μmol/L (SD)			Mean, μmol/L (SD)		
	Chinese	Malay	Indian	Chinese	Malay	Indian	Chinese	Malay	Indian
MTHFR C677T	n=402	n=41	n=41	n=206	n=16	n=20	n=196	n=25	n=21
CC	13.5 (4.5)	13.7 (4.5)	13.1 (4.3)	14.2 (4.7)	14.8 (5.8)	13.0 (4.0)	10.2 (3.3)	10.5 (3.1)	10.0 (2.9)
CT	14.0 (4.8)	13.1 (2.9)	17.4 (10.5)	15.4 (6.8)	12.8 (0)	13.3 (4.9)	10.4 (3.4)	9.9 (3.9)	21.3 (16.3)
TT	17.1 (7.2)	10.9 (1.1)	0	15.2 (5.6)	0	0	12.5 (4.7)	8.6 (0.7)	0
p-value (ANOVA)	**0.001**	0.606	0.071	0.328	0.750	0.882	0.063	0.767	0.353
MTHFR A1298C	n=410	n=40	n=44	n=211	n=16	n=21	n=199	n=24	n=23
AA	14.6 (5.3)	13.6 (3.9)	13.7 (4.4)	15.4 (6.2)	14.8 (8.2)	13.3 (4.3)	10.9 (3.7)	9.3 (1.8)	9.8 (2.5)
AC	12.9 (4.2)	13.3 (4.7)	14.3 (7.3)	13.6 (4.8)	12.9 (3.7)	13.4 (3.9)	9.7 (3.0)	11.1 (5.5)	12.9 (8.7)
CC	13.2 (4.1)	13.6 (2.5)	11.4 (4.2)	16.1 (4.5)	19.2 (3.6)	13.0 (5.8)	9.9 (2.5)	11.0 (2.0)	10.2 (1.3)
p-value (ANOVA)	**0.003**	0.985	0.630	0.063	0.265	0.993	0.069	0.491	0.585

7 DISCUSSION

In this thesis, we have studied various genetic and molecular markers of risk, prognosis, and treatment of IS. We first estimated the familial risk of IS incidence in relation to sibling kinship, sex, and age of stroke onset in a nationwide, population-based matched cohort study. In addition, we assessed the common familial risk between IS and MI, another vascular disease with many overlapping risk factors, by exploring the shared effects of sibling kinship, sex, and onset age of one disease on the other's risk. To assess if CRP measurement at sub-acute phase has additional prognostic value in simple conventional predictor models, we first examined if CRP is a predictor of future outcomes in our IS patient. Finally, to investigate the efficacy of B-vitamins therapy in lowering tHcy level in three different Asian ethnic groups, we randomized our IS patients into two treatment arms (placebo vs vitamin-treated) and measured their plasma tHcy levels, serum vitamin B_{12}, and serum FA levels in two occasions (at baseline and at one-year follow-up).

The role of sibling kinship, sex, and age of onset in the familial effects on IS in Sweden

In the first study, we found that having a sibling with IS increased the risk of having an IS yourself by 60%. This is consistent with various studies showing familial aggregation of stroke, although the magnitude of this risk varies owing to the difference in study population and heterogeneity of stroke types [5, 6, 8, 12, 13]. This risk was further increased when restricting the analysis to having a full sibling with stroke, although having a half sibling was not associated with an increased risk. Previous studies had demonstrated the existence of familial risk between siblings and the aggregation of multiple stroke risk factors among them [6, 8]; however, to our knowledge, there are no studies to date quantifying the detailed relationship of sibling kinship in this association. Our finding of higher risk in full siblings emphasized the role of genetic factors in the transmission of familial risk for ischemic stroke.

Sex differences in the transmission of stroke within families has garnered substantial interest over the years, with a review of several population-based studies [14] and a meta-analysis [15] showing differences in the heritability of stroke and TIA between men and women. In the meta-analysis, women were shown to be more likely than men to have a history of stroke among female first-degree relatives, suggesting sex-specific transmission in stroke [15]. In our study, we did not look at this parent-offspring relationship; however, we found that, among siblings, sex of neither study participants nor siblings seemed to affect the familial risk of IS. This is consistent with studies of incident stroke that have suggested no sex differences in lifetime risk of stroke [170, 171].

Having a sibling with early onset IS doubled the risk of the study participant to have an early onset IS. This is consistent with other studies where independent association between family history of stroke and IS with early onset age was found; albeit the slight difference in the cut-off age of early stroke onset [6, 11, 12]. Both studies by MacClellan *et al.* and Schulz *et al.* have found associations between familial aggregation of stroke and younger age of onset in white and black women, and British men and women, respectively [6, 12].

Common familial effects of IS and MI in the Swedish population

IS and MI, both being diseases of vascular nature, have overlapping risk factors [79]. Several studies in first-degree relatives have explored the associations between family history of IS and the risk of IS, or family history of MI and the risk of MI, or family history of either these diseases and the risk of cardiovascular disease in general [4, 8, 18-28]. However, few studies have looked at the co-inheritance of familial factors of one condition affecting risk of the other [20, 28, 29]. Our study of common familial effects of IS and MI taking the role of sibling kinship, sex, and age of onset into account, we found that having a sibling with IS increased the risk of having a MI by 44%, and having a sibling with MI increased the risk of having an IS by 41%. This is not entirely consistent with results from Cardiovascular Health Study, whereby the incident MI risk was not associated with a positive sibling history for stroke (composite of IS/TIA) nor was the risk of incident stroke higher with a positive history for MI [28]. Our findings may be due to a much bigger sample size leading to higher statistical power, a more stringent disease definition, and/or physician-validated sibling history. Also, the Cardiovascular Health Study included an older study population, all stroke subtypes and self-reported sibling history.

Time-varying covariate/exposure analyses showed that the risk of MI associated with having a sibling with IS was not affected by having an IS yourself prior to the MI. In contrast, the risk of stroke associated with having a sibling with MI was attenuated (albeit still significant) when taking interim MI in the study participant into account. Further, taking interim events in siblings into account attenuated the sibling risks of both MI and stroke. Taking all these observations into consideration, it seems to suggest that heritability of MI is contributing towards risk of both conditions in a stronger fashion than the heritability of IS. This suggestion is consistent with another study where heritability of coronary events was found to be greater than heritability of cerebral events [20]. A recent study by Siegerink *et al*. also found that a positive family history of MI was more frequent in MI cases compared with IS cases. They showed that a family history of neither MI nor stroke were strong predictors of IS, whereas both were associated with MI, suggesting that familial clustering of MI is more prominent than IS [29].

One previous smaller study explored the association of positive sibling history of MI and the risk of MI, the association of positive sibling history of IS and the risk of IS, the association of positive sibling history of MI and the risk of IS and vice versa [28]. However, this study was unable to quantify the relationship between full and half siblings in these associations. Our study showed that sibling kinship did not substantially modify the risk of MI from exposure of familial risk of IS and vice versa. The increased risk of MI when having a half sibling with IS was shown to be more modest than the increased risk of IS when having a half sibling with MI.

The most widely studied risk factor in familial aggregation studies is probably the role of sex in the heritability of either stroke or coronary heart disease. One prior study reported the importance of sex-of-parent/sex-of-proband offspring interactions in the family history of MI in acute coronary syndrome patients [19]. Banerjee *et al.* found that maternal history of MI was twice as common in women with early acute coronary syndrome, as in men with early acute coronary syndrome. Previous population-based

studies in stroke also emphasised that women are more likely than men to have a history of stroke among female first-degree relatives suggesting sex-specific transmission of stroke. In a separate study, Banerjee and colleagues also found maternal stroke to be twice as common as paternal stroke in women with acute coronary syndrome, but not in men with the same condition, and that women with acute coronary syndrome were more likely to have stroke-affected female first-degree relatives compared to men with acute coronary syndrome having stroke-affected male first-degree relatives [18]. In our study, the risk of MI and IS in both sexes and sexes of siblings were increased in similar magnitudes as the overall risk in each disease. Interestingly, all relative risks seemed to be slightly higher amongst women compared to men. However, the differences were modest and not statistically significant. This suggested the absence of sex differences in the lifetime-shared risk of both conditions.

Having a sibling with early onset of either IS or MI increased the risk of the study participant to have an early onset of MI or IS, respectively, although the magnitude of increase differed slightly. This was expected as our previous study and other studies on familial aggregation of stroke found associations with younger age of onset within the same condition [6, 12, 26]. Moreover, many common risk factors have been strongly associated with familial risk of both conditions, which further strengthened this assumption [79].

CRP and prognosis following IS in Singaporean stroke patients

In this study, we investigated the utility of sub-acute CRP measurement for the prediction of poor outcome (functional dependency, recurrent vascular events, and all-cause mortality) one year after ischemic stroke in a large hospital-based prospective cohort of Singaporean patients. Only CRP at high levels was significantly associated with these outcomes independently of other risk factors. In addition, comparison of conventional prognostic models with and without CRP showed significantly better fit of predictor model improvement upon CRP addition in our study population.

The increased risk, with varying magnitudes, of poor outcome including death with elevated levels of CRP have been extensively reported by many studies, primarily in Caucasian populations [35, 37, 77, 172-176]. Arenillas et al. measured CRP levels in ischemic stroke and TIA patients, and found that those in the highest quintile of CRP level had a significantly increased risk of subsequent stroke or MI [33]. Another study by Di Napoli et al. similarly found that CRP levels >1.5 mg/L at discharge were significantly associated with occurrence of a new vascular event or death at one year [137]. There has been a paucity of studies investigating the functional outcomes and recurrences post-stroke in non-Caucasian populations [175-177]. A recent study by Song et al. conducted in a Korean study population demonstrated that elevated CRP was found to be a predictor of functional disability post-stroke [177]. Huang et al. also showed that a high level of CRP (>3 mg/L) predicted all-cause mortality in their patient population three months post-stroke, adjusting for potential confounders [176]. In general, our findings are consistent with these studies where only high levels of CRP were found to be significantly associated to functional dependency, recurrent vascular events, and mortality at one year after adjustment to other factors.

Some studies have observed that the magnitude of increased recurrence and dependency in patients with high CRP was not as large as that of mortality [176]. We

also observed similar findings where the adjusted risk of mortality with the highest quartile of CRP level was 18-fold compared to the 4.6-fold and 3.9-fold found in functional dependency and recurrent vascular events, respectively. Our findings seem to suggest that high level of CRP is indeed an independent prognostic marker of poor outcome in acute ischemic stroke patients.

Age, stroke subtypes, and vascular risk factors such as hypertension, diabetes mellitus have long been considered as the traditional risk factors for predicting future vascular events or in general poor prognosis. Among these factors, older age and severe stroke subtypes have been shown to be the strongest predictors of poor prognosis after stroke. This method of prognostication amongst acute ischemic stroke patients can be easily and rapidly implemented, especially in clinical settings. However, this simple conventional prediction model is limited in such a way that a more detailed risk stratification of patients to be given a more immediate attention when it comes to secondary prevention clinical management is not possible. The search for biomarkers such as CRP to improve on this conventional prediction model is ongoing. In this study, we investigated whether the addition of CRP measurement after stroke in the conventional predictive model better discriminated prognosis compared to the model without CRP. Previous studies by Whiteley and colleagues investigated the additional benefit of including inflammatory markers such as CRP in the prediction of poor outcomes after stroke, and they found no further improvement in the prediction model beyond their validated stroke prognosis model [38, 39]. Contrary to these findings, our results seem to show that addition of CRP improved the discriminative ability of the model in all three outcomes in our study population as shown in **Table 3 (to add table in results section)** and illustrated in **Figure 13**, suggesting the potential usefulness of adding CRP into the conventional prediction model.

Ethnicity and B-vitamin therapy in the lowering of tHcy among Singaporean IS patients

We did not show any significant differences in baseline tHcy between different Asian ethnic groups despite inter-ethnic differences in FA and vitamin B_{12}. Our results are consistent with an earlier community-based study in Singapore [178]. It may be hypothesized that in Malays, the effect of lower FA on tHcy may be offset by higher levels of vitamin B_{12}. By contrast, a study from the United Kingdom reported that Indian Asians had higher tHcy compared to Europeans [179], which was attributed to lower vitamin B_{12} and FA among Indian Asians.

Our study demonstrated a significantly lower prevalence of *T* alleles in non-Chinese compared to Chinese, with no *TT* genotype observed among Indians. A Canadian study showed significant differences in *MTHFR C677T* allele frequencies between Asians and Caucasians; however, the prevalence of *TT* genotype among Chinese was reported to be similar to South Asians [180]. The association of the *A1298C* polymorphism and mean baseline tHcy only in Chinese may be attributable to the low number of non-Chinese subjects. The lack of significant differences in baseline tHcy between ethnic groups despite variation in functional *MTHFR* polymorphisms suggests that the effect on tHcy of a higher frequency of *A1298 C* alleles in Indians is possibly offset by the low frequency of *C677 T* alleles; alternatively, it can indicate low statistical power to detect gene-environment interactions.

The effect of vitamin therapy on tHcy-lowering has not been extensively explored in Asian populations but is critically important because vitamin intake and response to vitamin therapy may vary across different ethnic groups due to differences in food preparation since FA is heat labile and destroyed by prolonged cooking, a feature of Malay cuisine. Moreover, vitamin B_{12} may be lower in Indians because of a vegetarian diet [179].

Strengths and Limitations

In the first two Swedish register-based studies (Study I & II), the strengths of these studies include the publicly available and financed Swedish health system, which gives nationwide coverage, resulting in a very large, representative sample, allowing us to study the familial risks of IS and MI in detail, taking sibling relations, sex of siblings, and age of onset into account. The possibility to account for socioeconomic factors, the prospectively collected data, the non-differential follow-up of both IS and MI incidence and mortality, and the inclusion of only IS instead of all strokes (which include hemorrhagic strokes which are likely to be less relevant for co-heritability with MI) are other important strengths of these studies. Moreover, sibling history of IS and MI were physician-validated. Many studies relied on self-reported questionnaires to obtain medical history of relatives making them prone to ascertainment bias, and also they are usually unable to identify sibling relations in their study populations.

Several limitations should be acknowledged as well. Due to truncation of the registers' data, inclusion into the cohort was conditional of (1) sib-pair being alive after 1987; and (2) sib-pairs older than 55 years of age in 1987 could not be included (since they would have been born before 1932). As a consequence, age at diagnosis is likely to be confounded by calendar time, since the proportion of older individuals in this study increase from 1987 to 2007; however, this potential limitation was dealt with by taking both age and calendar time into account in the study design. Furthermore, subjects with IS or MI events occurring before 1987 could be falsely classified as healthy (since the coverage of the register was not complete until 1987); thereby diluting a real recurrence ratio of one. This bias would drive the associations toward the null. In both studies, we did not have information on stroke subtypes within IS or a more specific type of MI (ST-elevation or non-ST-elevation), which may be important in the heritability of IS and MI. Finally, we did not have information on vascular risk factor information (such as hypertension, diabetes, hypercholesterolemia, diet, and physical activity), which have been suggested to be associated to familial aggregation of stroke and MI or, by themselves, have heritability component. We acknowledge that it would have improved our studies if we had this information; however, these factors would rather act as mediators than confounders, as they are along the causal pathway between familial factors and our outcome of interest, and lacking this information should not invalidate our findings.

The strengths of the third study among Singaporean acute IS patients (Study III) include a relatively large sample size of non-Caucasian stroke patients. This may be important as Asians have a different case mix and underlying pathology for their strokes. In addition, we measured CRP in blood collected in the sub-acute phase as opposed to hyper-acute phase (less than 24 hours) as done in most previous studies. Measures performed in the hyper-acute phase are problematic to generalize to the majority of

stroke patients who arrive after 24 hours. Our choice concurred with a study that measured CRP at multiple time points post-stroke whereby elevated CRP measured on the seventh day of hospital admission was found to be a stronger predictor for functional disability post-stroke compared to CRP measured within 24 hours of stroke onset, and another study that found that measurement of CRP at admission did not predict outcome and that CRP levels remained stable 28 days or more [36, 174, 177, 181]. These studies implied that measurements made soon after stroke may be spurious perhaps due to immediate inflammatory response to infection and may be less reliable for long-term risk stratification [174].

Some limitations of the third study are that CRP measurement at one-time point may not reflect long-term changes in prognostication and CRP is also associated with other inflammatory factors, many of which themselves are associated with ischemic events. It thus remains plausible that increased levels of CRP are caused by the presence of those other cytokines such as interleukin-6. More studies with even bigger sample sizes and other study designs are needed to establish a causal relationship between CRP and recurrent stroke outcomes. Guidelines for risk classification in secondary stroke prevention needs to be established, and only then we can establish whether the addition of biomarker measurements would be beneficial in conventional clinical prognostic models by either estimating net reclassification improvement or integrated discrimination improvement models in the hope of establishing a more efficient target of patients for secondary prevention.

The strength of our final study (Study IV) is the ability to examine the effect of vitamin therapy within the context of a randomized, double-blinded, placebo-controlled trial thus minimizing systematic bias. Limitations include lack of data on dietary information and socioeconomic status together with the relatively small number of non-Chinese in this study, which has limited power. Further, we acknowledge that the genetic part of the study would have benefitted from a larger study population.

8 CONCLUSIONS

I. There was a 60% increased risk for IS in individuals having a sibling with prior stroke. The risk was stronger for full siblings compared with half siblings. Moreover, having a sibling with early IS doubled the risk of early IS. No sex differences were observed in the familial inheritance of IS.

II. A shared familial aggregation between IS and MI was found with a 44% increased risk for MI in individuals having a sibling with prior IS and a 41% increased risk for IS in individuals having a sibling with prior MI.

III. CRP at high levels, measured at sub-acute phase, was found to be significantly associated with one-year outcomes (functional dependency, recurrent vascular events, and all-cause mortality) in acute IS patients independent of vascular risk factors. In addition, comparison of conventional prognostic models of age and stroke subtypes with and without CRP showed significantly better fit in predictor model improvement upon CRP addition in our study population. This suggested the potential additional value of CRP measurement in acute IS prognosis.

IV. Ethnicity did not appear to affect the tHcy-lowering effect of B-vitamin despite differences in dietary intake and genetic makeup via the prevalence of *MTHFR* polymorphisms. This indicates that the efficacy of B-vitamin therapy in lowering tHcy is likely to be generalizable across Asian populations.

9 FUTURE PERSPECTIVES

This thesis has examined various factors which affect IS from risk to prognosis and subsequently treatment, by exploring potential genetic and/or molecular markers of IS, in the hope to better understand this complex multi-factorial vascular disease. We found the substantial contribution familial information has on predicting IS incidence, and that this familial risk overlapped considerably between IS and MI. We tried to quantify these relationships further by stratifying exposures into sibling kinship, sex, and age of onset in order to estimate IS and MI risks when among full or half siblings, men or women, male sibling or female sibling, and early or late onset of diseases. Unfortunately, in our studies, we were unable to take pre-existing vascular risk factors or their management information into account. We were also not able to stratify IS into its subtypes using the ICD coding system. It would be interesting to estimate risks of specific IS subtypes as each IS subtypes have been known to be

Although further studies are needed to confirm these findings, we can conclude that public health education on IS is important. With the current trend of population towards ageing and the great economic burden of cardiovascular disease and treatments, the best management strategy is through prevention. These studies helped to emphasise the importance of understanding the predisposing risk within families so that early risk factors management could be implemented. One can only hope that reduction of vascular risk factors via the promotion of physical activity and healthy dietary habits, which include reduction of salt intake, other high vascular risk associated food and alcohol consumption, and smoking cessation, can be more efficiently carried out with an educated population.

The understanding of risk factor differences across different population and gender within each stroke types is important when planning large-scale international studies as this will have implications on future prevention programs for the general population. Large studies are increasingly in demand in the attempt to tease out effect modifications between genes and environment. Current effort is being made through the INTERSTROKE study, a large standardized international case–control study in various income levels countries, which aims to determine the importance of established and emerging risk factors for the stroke subtypes in different regions and ethnic groups [182]. As ethnicities, geographic location, and socioeconomic status seem to contribute towards increased stroke risk, perhaps through this study, a better target and more personalized aggressive management regime could be tailored.

Stroke genetics and biomarker studies often suffer from the inability to replicate findings. This is due mostly to sample size issue and phenotyping of IS subtypes. Efforts have been made in the last few years to combine large cohort studies in the attempt to increase power, however, the issue of heterogeneity is never really quite resolved. Other issues include the lack of uniformity in the measurements of biomarkers. To complicate matters further, a constellation of risk factors are now considered as complex traits possess heritable components within themselves, thus making it difficult to establish if associated markers are directly associated with IS. Only study design such as randomized controlled trial with clinically well-defined patient samples with specific subtypes can then perhaps elucidate causality.

In our final study, a sub-study of the main VITATOPS trial, we determined that treatment efficacy of B-vitamin therapy in lowering tHcy level was modified by ethnicity despite differences in dietary intake and genetic makeup indicating the likely generalizability this effect across the Asian populations. At this stage the trial was still ongoing, and hence, we did not elucidate whether this effect would actually reduce the combined incidence of recurrent vascular outcome (non-fatal stroke, non-fatal myocardial infarction, and death attributable to vascular causes) among our patients, which was the eventual goal of the main trial. Subsequent publication of the main trial's results showed that treatment of B-vitamins to recent IS or TIA patients for a median of 3.4 years had no significant effect compared with placebo, on the overall incidence of major vascular events. However, sub-group analyses demonstrated B-vitamins therapy might reduce the risk of recurrent events (stroke, myocardial infarction, or vascular death) in patients with symptomatic small vessel disease of the brain causing lacunar infarction or ICH [183]. This is in agreement with earlier investigators' report suggesting homocysteine to be a risk factor for cerebral small vessel disease [184, 185]. Post-hoc sub-analysis also supported the possibility of interaction with anti-platelet therapy as suggested by other trials, thus modifying any observable beneficial effect [186].

In conclusion, further validation studies are needed to confirm all the work in this thesis. It is important to refine the methods and study designs for future studies from what we have previously learnt. A recurring theme of better phenotyping of IS subtypes seems to echo throughout all IS research. It has been repeatedly suggested that there exists differential etiologies, mechanisms, and treatment effects between each IS subtypes. Uniformed data collection on risk factors information is vital as gene-environment interactions seem to play a big part in this complex multi-factorial disease. The emerging area of pharmacogenomics looking at the interactions between treatment drugs and genetic variants could perhaps stratify at-risk patients more efficiently.

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