"Epidemiology of plasma lipids and the metabolic syndrome in a multi-ethnic population"

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SUMMARY

Cardiovascular disease (CVD) imposes a significant burden in terms of morbidity and mortality in developed countries. Asia is likely to see an increase in the burden of these diseases in the next several decades. In developed countries, most CVD relate to atherosclerosis. Atheroscleorsis is a complex, multifactorial disorder. As such, multiple risk factors contribute to the pathogenesis of CVD. It has been observed that some cardiovascular risk factors, particularly obesity, glucose intolerance, hypertension and dyslipidemia, occur in the same individual more often than can be expected by chance. This cluster of abnormalities has become known as the metabolic syndrome. While the pathogenesis of the metabolic syndrome, and its link to obesity, insulin resistance is an important part of it.

Through the studies described in this thesis, we have shown that the metabolic syndrome is common, and is associated with a 2-3 fold increase in the risk of CVD in the Singapore population. Over half of the CVD events occurring in our population are attributable to the metabolic syndrome. We further show that the metabolic syndrome is not always associated with the presence of central obesity, even when defined using lower cut-points designed for use in Asian populations. Nevertheless, even in the absence of central obesity, individuals with multiple metabolic risk factors are insulin resistant, and experience a greater risk of CVD. To better understand the pathogenesis of the metabolic syndrome, we carried out a study to determine whether a biochemical signature of disordered protein metabolism, first identified in obese individuals, was independently associated with insulin resistance. We found that this signature of increased branch chain amino acid catabolism was indeed associated with insulin resistance independent of obesity. Through a genetic association study, we also found that polymorphisms at the APOA1/C3/A4/A5 locus were important risk factors for dyslipidemia (of the sort associated with the metabolic syndrome). We describe a novel interaction between a polymorphism at the APOA5 locus and plasma triglycerides, which may contribute to the development of hypertriglyceridemia at relatively low levels of obesity.

In addition to defining the burden of disease associated with the metabolic syndrome, these studies cast important light on some of the pathways involved in the pathophysiology of the metabolic syndrome.

LIST OF TABLES

LIST OF FIGURES

PUBLICATIONS

This thesis is based on the following 5 publications

1. Heng D, Ma S, Lee JJ, Tai BC, Mak KH, Hughes K, Chew SK, Chia KS, Tan CE, Tai ES. Modification of the NCEP ATP III definitions of the metabolic syndrome for use in Asians identifies individuals at risk of ischemic heart disease. Atherosclerosis. 2006 Jun;186(2):367-73.
2. Khoo CM, Liew CF, Chew SK, Tai ES. The impact of central obesity as a prerequisite for the diagnosis of metabolic syndrome. Obesity (Silver Spring). 2007 Jan;15(1):262-9. PubMed PMID: 17228055.
3. Lee J, Ma S, Heng D, Tan CE, Chew SK, Hughes K, Tai ES. Should central obesity be an optional or essential component of the metabolic syndrome? Ischemic heart disease risk in the Singapore Cardiovascular Cohort Study. Diabetes Care. 2007 Feb;30(2):343-7.
4. Tai ES, Tan MLS, Stevens RD, Low YL, Muehlbauer MJ, Goh Ilkayeva OR, Wenner BR, Bain JR, Lee JJM, Lim SC, Newgard CB. Insulin resistance is associated with a metabolic profile suggesting enhanced protein catabolism in Chinese and Asian Indian men. Manuscript submitted to Diabetologia
5. Tai ES, Teo YY, Tan JT, Chew SK, Chia KS. Genetic variation at the APOA1/C3/A4/A5 loci and their associations with dyslipidemia. Manuscript in preparation

The following papers also provided important background and motivation for the work presented in this thesis

1. Lai CQ, Tai ES, Tan CE, Cutter J, Chew SK, Zhu YP, Adiconis X, Ordovas JM. The APOA5 locus is a strong determinant of plasma triglyceride concentrations across ethnic groups in Singapore. J Lipid Res. 2003 Dec;44(12):2365-73.
2. Ang LW, Ma S, Cutter J, Chew SK, Tan CE, Tai ES. The metabolic syndrome in Chinese, Malays and Asian Indians. Factor analysis of data from the 1998 Singapore National Health Survey. Diabetes Res Clin Pract. 2005 Jan;67(1):53-62.

3. Tan CE, Ma S, Wai D, Chew SK, Tai ES. Can we apply the National Cholesterol Education Program Adult Treatment Panel definition of the metabolic syndrome to Asians? Diabetes Care. 2004 May;27(5):1182-6.

4. Tai ES, Ordovas JM. Clinical significance of apolipoprotein A5. Curr Opin Lipidol. 2008 Aug;19(4):349-54.

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Chapter 1 INTRODUTION & LITERATURE REVIEW

1.1 Health burden associated with cardiovascular disease

Socio economic development, accompanied by rapid urbanization, has resulted in an epidemiologic transition in the burden of diseases from those associated with infection and malnutrition, to those associated with non-communicable chronic diseases. Cardiovascular diseases, ischemic heart disease in particular, represent some of the major causes of morbidity and mortality in developed countries today. These diseases also have a significant economic impact. Coronary heart disease (CHD) is the leading cause of death in most industrialized countries[1]. The cost of heart disease and stroke in the United States, including health care expenditures and lost productivity from deaths and disability, is projected to be more than $475 billion in 2009 (http://www.cdc.gov/NCCDPHP/publications/AAG/dhdsp.htm accessed on 7 may 2009).

In developing countries, this transition is still in progress and many populations in Asia can be expected to experience in a doubling of the burden of cardiovascular disease (CVD) over the next several decades. The increase in CHD mortality and morbidity in Asia has now been documented in Malaysia, Singapore, Korea, China, the Philippines, Thailand, and India[2]. Several reasons have been provided for the increase in CHD in Asia[3]. Firstly, a decrease in mortality from infection and nutritional deficiencies will result in more individuals will reach middle and old age. It is anticipated that the greatest increases in life expectancy will occur in Asia [4]. Secondly, lifestyle and economic changes associated with urbanization are likely to increase to higher levels of risk factors for CHD. In most countries in Asia, serum levels of total cholesterol have shown a secular rise that has occurred in parallel with the increase in CHD mortality[2]. Serum total cholesterol tended to be lower in rural compared to urban parts of Asia[2]. Further evidence that urbanization is important comes from the experience of migrant populations. Japanese living in California and Hawaii experience higher rates of CHD than those living in Japan[5]. CHD mortality amongst Chinese living in Singapore is several-fold higher than that seen amongst Chinese living in China or Hong Kong[6]. Comparison of Chinese living in a rural village in the South of China to Chinese

living in an urban environment (Hong Kong or Australia) revealed greater sub-clinical atherosclerosis, as measured by carotid intima-media thickness[7].

In Singapore, rapid socio-economic development in the past four to five decades has resulted in a doubling of the age-standardized mortality from ischemic heart disease between the 1960s and the 1980s [8]. Today, coronary artery disease/Ischemic heart disease (CAD/IHD) and its antecedent syndromes remain the second most common cause of death in Singapore and are increasing with the aging population (http://www.moh.gov.sg/mohcorp/statistics.aspx?id=5526). In the Singapore Burden of Disease Study 2004 conducted by the Ministry of Health, diabetes mellitus, ischemic heart disease and stroke were the top 3 leading cause of premature death and ill-health in Singapore, and together accounted for more than one-quarter (28%) of the total disease burden. Ischemic heart disease, stroke and lung cancer were also the major contributors to the premature mortality burden (unpublished Data. Personal communication, Dr Derrick Heng).

In summary, cardiovascular disease is imposes significant morbidity, mortality and cost upon developed countries, including Singapore. More importantly, cardiovascular disease can be prevented through intervention to reduce the levels of risk factors (see next chapter). For these reasons, the ability to identify individuals at increased risk of CVD, understand the pathways involved in its pathogenesis, will facilitate these preventive activities. Rapid socio-economic development, which is occurring in most rapidly in many countries in Asia will make the lessons learned in Singapore relevant to the rest of Asia, which houses two-thirds of the world's population.

1.2 The multi-factorial nature of cardiovascular disease

Although cardiovascular disease comes in many forms, the epidemiologic transition is producing a change in the patterns of cardiovascular disease, in addition to increased rates of cardiovascular disease[1]. CVD related to atherosclerosis (coronary heart disease and ischemic stroke) are the major forms of CVD that affect developed countries.

Atherosclerosis is a disease affecting the intima of large and medium sized arteries. It is an inflammatory process that appears to begin with the accumulation of lipid in the sub-endothelium of the intima[9]. This is followed by the influx of inflammatory cells including macro-phages and lymphocytes. This leads to the migration and proliferation of smooth muscle cells and the formation of fibrous tissue, which gradually occludes the artery. In addition to occlusion of the artery, ongoing inflammation can lead to erosion and thinning of the fibrous cap of the atheromatous plaque, resulting in rupture and sudden, total occlusion of an artery. When this occurs in a coronary artery, this results in a myocardial infarction. The processes involved in the initiation and promotion of plaque formation as well as their eventual rupture are complex and multifactorial. It should therefore come as no surprise that cardiovascular disease is a complex, multifactorial disease and that the identification of individuals at increased risk of CVD requires us to consider multiple risk factors.

The Framingham heart Study was initiated in 1948 with the aim of securing epidemiologic data on CVD, which encompassed the establishment of the relation of "risk factors" to CVD[10]. Through the Framingham heart study, it was found that considering multiple risk factors significantly improved our ability to predict CVD [11-13]. Over the years, many different risk factors for CVD have been identified. Some of these risk factors are non-modifiable and examples include age, a family history of premature CAD or gender, whereas others are potentially modifiable, such as obesity, dyslipidemia, hypertension, cigarette smoking and diabetes mellitus. In a large case-control study for myocardial infarction, it was estimated that up to 90% of population attributable risk for myocardial infarction was related to a few, potentially modifiable risk factors[14].

1.3 Clustering of obesity, dyslipidemia, hypertension and glucose intolerance-The metabolic syndrome

As early as the 1920's, investigators observed and reported that several metabolic traits (which are now known to be CVD risk factors) tended to cluster, in the sense that that they occurred in the same individuals more often than could be expected by chance alone[15]. In 1967, Crepaldi described a series of 6 patients who exhibited diabetes mellitus, dyslipdemia and obesity, in whom a hypocaloric, low calorie diet resulted in improvements in all three of these parameters[16]. In 1977, Haller used the term "metabolic syndrome" for associations of obesity, diabetes mellitus, hyperlipoproteinemia, hyperuricemia and Hepatic steatosis when describing the additive effects of risk factors on atherosclerosis[17]. In 1977 and 1978, Gerald B. Phillips developed the concept that risk factors for myocardial infarction concur to form a "constellation of abnormalities" (i.e., glucose intolerance, hyperinsulinemia, hyperlipidemia [hypercholesterolemia and hypertriglyceridemia] and hypertension) that is associated not only with heart disease, but also with aging, obesity and other clinical states. He suggested there must be an underlying linking factor, the identification of which could lead to the prevention of cardiovascular disease; he hypothesized that this factor was sex hormones[18, 19]. Since that time, this clustering has been observed in multiple populations. In 1988, in his Banting lecture, Gerald M. Reaven proposed insulin resistance as the underlying factor and named the constellation of abnormalities Syndrome X. Reaven did not include abdominal obesity, which has also been hypothesized as the underlying factor, as part of the condition[20].

1.4 Defining the metabolic syndrome in the population

Since 1999, a number of agencies have proposed definitions of the metabolic syndrome. These included the World Health Organization (WHO), The European Group for the study of Insulin Resistance (EGIR), the National Cholesterol Education Program Adult Treatment Panel III (NCEP ATP III) and the International Diabetes Federation. These definitions are summarized in table 1.

	WHO	EGIR	NCEP ATPIII	IDF
Glucose	*FPG >6.0 mmol/l (110 mg/dl)/IGT/DM	*FPG >6.0 but not DM	FPG >6.0 mmol/l (110 mg/dl)	FPG >5.6 mmol/l (100 mg/dl)
Insulin	*Highest quartile for population			-
Blood pressure	160/90	140/90	130/85	130/85
Triglyceride	>=1.7 mmol/l (150 mg/dl)	>2 mmol/l (180 mg/dl)	>=1.7 mmol/l (150 mg/dl)	>=1.7 mmol/l (150 mg/dl)
High Density lipoprotein Cholesterol	Men <0.9 mmol/l (35 mg/dl) Women <1.0 mmol/l(40 mg/dl)	<1.0 mmol/l (40 mg/dl)	Men <1.0 mmol/l (40 mg/dl) women <1.3 mmol/l (50 mg/dl)	Men<1.0 mmol/l (40 mg/dl) Women <1.3 mmol/l(50 mg/dl)
obesity	WHR > 0.9 in men or > 0.85 in women and/or BMI > 30 kg/m2	WC > 94 cm in men or > 80 cm in women	WC > 102 cm in men or > 88 cm in women	*Ethnic specific WC
Albuminuria	UAE ≥ 20 µg/min or albumin:creatinine ratio ≥ 20 mg/g.			

Table 1. Definitions of the metabolic syndrome. WHO=World Health Organizatoinl EGIR=European Group for the Study of Insulin Resistance; NCEP ATPIII=National Cholesterol Education Panel Adult Treatment Panel III; IDF=International Diabetes Federation; WC=waist circumference; WHR=Waist Hip Ratio; UAE=Urine albumin excretion

The two earlier definitions from the WHO and the EGIR included an assessment of insulin resistance. While this recognizes the central role of insulin resistance in the pathogenesis of the metabolic syndrome, it posed problems in clinical use because the measurement of plasma insulin was not routinely available. Furthermore, the population distribution of plasma insulin is not known for most populations to which the metabolic syndrome is relevant. Finally, there has been little effort to standardize measurement of insulin which means that a definition that includes a plasma insulin in the highest quartile for the population is subject to differences related to the assay used for the measurement.

In 2001, the National Cholesterol Education Program, in formulating the recommendations of Adult Treatment Panel III, elected to include a definition of the metabolic syndrome that included only commonly measured risk factors for CVD, and identified individuals with the metabolic syndrome as candidates for therapeutic lifestyle modification in order to reduce the risk of CVD[21]. The diagnostic criteria included an assessment of each of the 4 traits most commonly associated with insulin resistance, namely central obesity, dyslipidemia, hypertension and glucose intolerance. This was not meant to be a comprehensive list of all traits that cluster together, but rather a set of commonly available measurements that could be used clinically (personal communication, Prof Scott Grundy). This simple, readily applied, recommendation has led to a large increase in the number of publications related to the metabolic syndrome. In 2006, the international diabetes federation adopted the same criteria (with some minor variations (some of which are relevant to the projects in this thesis)[22].

1.5 The assessment of obesity in Asian populations.

The assessment of obesity in humans (particularly in clinical practice) is largely dependent on the use of simple anthropometric measurements. The most commonly used is the body mass index,

which is the weight in kg of an individual divided by the square of the height. This was developed in caucasians as an estimate of fat mass. The World Health Organization categorizes individuals based on the BMI into those who are overweight (BMI>=25 kg/m^2), and obese BMI>=30 kg/m^2. While the BMI is highly correlated with the degree of adiposity, it is not a precise measure of adiposity, which requires more detailed, intensive methods such as underwater weighing or isotope dilution.

As mentioned in a previous section, the rates of CVD is increasing rapidly in Asia, and in more developed countries like Singapore, rates are as high, if not higher than in Western countries. The same is true of type 2 diabetes mellitus. Singapore has a high prevalence of T2DM. All of this is occurring despite an average BMI in these countries that is well below the cut-off point of 25 kg/m^2 that defines overweight in the current WHO classification. There is increasing evidence that the associations between BMI and percentage of body fat, and body fat distribution differ across populations. Specifically, BMI may underestimate the degree of adiposity in Asian populations. For example, persons in Singapore have a greater degree of adiposity than Caucasians, for the same body mass index[23]. This means that, if we want to identify overweight or obese individuals with the same degree of adiposity in Singapore, as, for example, in the United States, we would have to define overweight or obesity using lower cut-points for the BMI.

The Marseilles physician Dr. Jean Vague, in 1947, observed that upper body obesity appeared to predispose to diabetes, atherosclerosis, gout, and calculi[24]. Over time, this has evolved into the belief that intra-abdominal fat, contributes more to insulin resistance than subcutaneous fat. A number of hypotheses have been advance to explain this observation. It has been suggested that visceral fat is metabolically distinct from subcutaneous fat[25]. In addition, the anatomical position of the intra-abdominal fat depot is such that visceral fat drains via the portal system to the

liver, which is an important organ involved in glucose homeostasis, and that this results in a greater impact on hepatic glucose metabolism. Obviously,intra-abdominal fat mass cannot be readily measured in humans. However, it has been shown that anthropometric measures of central obesity, offer an estimate of intra-abdominal fat mass[26]. Several anthropometric measures have been utilized for this purpose but the one most common used in recent years is the waist circumference, which has been shown to correlate with intra-abdominal fat mass using imaging techniques[27]. Recently, it has been shown that the ethnic difference in the relationship between BMI and adiposity also applies to relationship between waist circumference and intra-abdominal fat mass[28-30]. It has been shown that for the same waist circumference, individuals of different ethnic background carry different amounts of intra-abdominal fat. As such, the use of the waist circumference in different ethnic groups may result in a systematic over -or under-estimation of intra-abdominal fat mass. In the Asian context, the intra-abdominal fat mass is under-estimated by the waist circumference[29, 30].

In this regard, we have previously shown that the presence of multiple metabolic risk factors (particularly diabetes mellitus and dyslipidemia) occurs at lower levels of waist circumference than the 102cm in men, and 88 cm in women proposed in the NCEP ATPIII recommendations[31]. On this basis , we have suggested that a definition of central obesity in Asians use a cut-off of 90cm in men and 80 cm in women, a recommendation that has been adopted by both the American Heart Association/National Heart Lung and Blood Institute (AHA/NHLBI)[21], as well as the International Diabetes Federation (IDF)[22].

The use of these modified criteria for the assessment of central obesity led to a large increase in the prevalence of central obesity in the Singapore population (table 2).

Table 2. Impact of changing the criteria for defining central obesity on its prevalence by gender and ethnic group in Singapore (from Tan CE et al[31]).

	NCEP ATPIII criteria		Asian criteria	
	Men (waist circumference >102cm)	Women (waist circumference >88 cm)	Men (waist circumference >90cm)	Women (waist circumference >80 cm)
Chinese	3.7(3.0–4.7)	6.9(5.9–8.2)	26.2(24.2–18.2)	21.0(19.3–23.0)
Malay	4.8(2.9–7.8)	22.0(17.6–27.0)	29.8(24.9–35.2)	43.2(37.7–48.9)
Indian	8.6(5.3–13.6)	25.5(19.5–32.6)	41.4(34.5–48.7)	53.8(46.2–61.2)

Data are % (95% CI). *Prevalence standardized to 1998 Singapore population, weighted for age, sex, and
ethnic distribution;

The inclusion of the Asian criteria for central obesity in the definition of the metabolic syndrome increased the prevalence of the metabolic syndrome from 12.2% (95% CI 11.3–13.2) to 17.9% (16.8–19.0)[31], which is similar to that observed in the United States and other developed countries. However, equating the prevalence to that in other countries is a poor reason to modify the diagnostic criteria. The definition of the metabolic syndrome by the NCEP ATP III was for the purpose of identifying individuals at increase risk of cardiovascular disease. As such, it is important to ascertain the impact of these new criteria on the risk of cardiovascular disease in our population. This constitutes the aim of the first study in this thesis.

1.6 The role of obesity in the pathogenesis of insulin resistance and the metabolic syndrome

Obesity is a key feature of the metabolic syndrome, and an important determinant of insulin resistance. Many studies, including our own, have shown that higher levels of obesity are associated with greater levels of insulin resistance [32]. The pathways linking obesity and insulin resistance are complex and heterogeneous. Two important pathways involve a) fatty acids and b) inflammation.

1.6.1 Fatty acids as the link between obesity and insulin resistance.

Adipose tissue was the site where excess energy was stored, in the form of triglycerides (TGs), and where that energy, when needed elsewhere in the body, was released in the form of fatty acid (FA). During hyperinsulinemia, as occurs in the post-prandial state, triacylglycerol clearance from lipoproteins and fatty acid trapping are specifically up-regulated in adipose tissue[33, 34]. Furthermore, adipose tissue is the only site of release of fatty acids when energy is needed (as occurs in the fasting state). Obesity is associated with increased levels of free fatty acids, which may have an important role in the pathogenesis of insulin resistance associated with obesity. Over 40 years ago, Randle et al[35] proposed a mechanism for fat-induced insulin resistance that implicated fatty acid oxidation as causing the inactivation of mitochondrial pyruvate dehydrogenase and ultimately leading to decreased glucose uptake. They speculated that increased fat oxidation was responsible for the insulin resistance associated with obesity and hypothesized that intracellular fatty acid accumulation would lead to an increase in the intramitochondrial acetyl coenzyme A (CoA)/CoA and NADH/NAD^+ ratios, leading to inhibition of pyruvate dehydrogenase and increasing concentrations of intracellular citrate. The citrate accumulation would inhibit phosphofructokinase, a key rate-controlling enzyme in glycolysis, increasing intracellular glucose-6-phosphate concentrations and inhibiting hexokinase II activity. The inhibition of hexokinase II activity would result in an increase in intracellular glucose concentrations and decreased muscle glucose uptake. However, their work was done in isolated rat heart muscle, which may be metabolically very different from resting human skeletal muscle. Recent work using NMR spectroscopy to measure intracellular levels of metabolites under hyperinsulinemia clamp conditions have suggested that the insulin resistance resulting from

excess free fatty acids results from reduced glucose transport and it's subsequent phosphorylation [36], which is much earlier in the glycolytic process than proposed by Randle. These defects in glucose transport and hexokinase were similar to previous findings in patients with type 2 diabetes [37]. and in the lean, normoglycemic offspring of diabetic patients[38]. Subsequent studies have suggested that exposure to fatty acids could lead to a serine/threonine phosphorylation cascade and increased serine phosphorylation of IRS-1 (and possibly IRS-2) at critical sites which block tyrosine phosphorylation of IRS-1[39]. The latter is an important event in the insulin signaling cascade.

1.6.2 Inflammation as the link between obesity and insulin resistance

Obesity is a pro-inflammatory state. Multiple studies have shown an association between obesity and the levels of inflammatory markers including fibrinogen, TNF-alpha, IL-6, and C-reactive protein (CRP)[40-44]. Adipose tissue from obese mice and humans is infiltrated with macrophages[45, 46] In these studies, adipose tissue was shown to contain bone marrow–derived macrophages, and the content of these macrophages tracked with the degree of obesity [45, 46]. In fact, more than 40% of the total adipose tissue cell content from obese rodents and humans was comprised of macrophages compared with approximately 10% in lean counterparts [45]. These macrophages produce inflammatory cytokines, which play an important role in the pathogenesis of insulin resistance. In fact, the pathway involving serine/threonine phosphorulation of IRS-1 leading to attenuated insulin signaling was first described in relation to the ability of the proinflammatory cytokine TNF-□impairs insulin action[47-49]. Specifically, phosphorylation of these serine residues impedes the normal association of IRS-1 with the insulin receptor, thereby impairing downstream propagation of insulin signaling [39, 50].

In summary, a number of metabolic pathways have been implicated that may link obesity and insulin resistance. Several, including those involving fatty acids and inflammation, converge on

the insulin signaling cascadee. Specifically, elevated levels of free fatty acids, amino acids and TNF-alpha, are associated with obesity and through a variety of mechanisms, result in the phosphorylation of serine and threonine residues on IRS-1. In turn, this results in reduced phosphorulation of IRS-1 at tyrosine residues, a key step in the insulin signaling cascade.

1.7 Obesity as a pre-requisite risk factor for defining the metabolic syndrome

The literature linking obesity (particularly central obesity) and insulin resistance are so compelling that, in making their recommendations for the diagnosis of the metabolic syndrome, the international diabetes federation lists central obesity as a prerequisite risk factor for the diagnosis of the syndrome. In this regard, the IDF definition differs from that of the AHA/NHLBI, where the presence of central obesity is "optional". It has been suggested that the proportion of individuals without central obesity who have three or more components of the metabolic syndrome is small [51, 52]. It is also felt that in the U.S., for the most part, the same individuals will be identified by either definition so that differences in the definitions are probably insignificant[21] . However, this has not been assessed in various populations, particularly in populations comprising ethnic groups from Asia. Furthermore, the impact of central obesity as an pre-requisite risk factor as opposed to an optional component of the metabolic syndrome has not been extensively assessed in relation to either insulin resistance or the risk of ischemic heart disease (IHD). This is particularly pertinent given the finding that, obesity (as assessed by BMI) only explains 22% of the variance in insulin resistance in humans (Abassi), raising the possibility that insulin resistance may occur in the absence of significant obesity, or that some individuals may be prone to develop the features of insulin resistance a relatively low levels of obesity. The second and third studies in this thesis will specifically address these issues.

1.8 Disordered protein metabolism in the pathogenesis of insulin resistance.

While insulin resistance is often thought of as a disorder of carbohydrate metabolism, it is increasingly obvious that the metabolism of other nutrients is also disordered in the insulin resistant state. While an extensive body of literature exists in relation to fatty acids and their role in the pathogenesis of insulin resistance, disordered protein metabolism has also been implicated in the pathogenesis of insulin resistance and has been much less extensively studied. Felig et al reported high amino acid levels were observed in obese individuals which declined after weight loss as early as 1969 [53]. Linn et al found that 6 months of high protein intake induced several features of insulin resistance including increased fasting glucose level, impaired suppression of hepatic glucose output by insulin, and enhanced gluconeogenesis[54]. Using NMR techniques (similar to those described in relation to the study of free fatty acids and insulin resistance), investigators have reported that amino acids – like free fatty acids – directly inhibit skeletal muscle glucose transport/phosphorylation [55]. Furthermore, activation of mTOR/S6K by AAs leads to serine phosphorylation of insulin receptor substrate-1, and thereby interferes with early steps of insulin signaling [56, 57]. It was recently demonstrated that obese humans exhibit a signature of elevated branch chain amino acids (BCAAs) suggesting enhanced branch chain amino acid catabolism [58]. It was further shown that a diet supplemented with branch chain amino acids synergistically increased the effect of a high fat diet on insulin resistance in rats, and result in phosphoryation of serine/threonine residues on IRS-1. While the link between obesity and fatty acids and inflammation seem obvious, the link between obesity and elevated amino acid levels are less obvious. In their paper, Newgard et al suggested that the rise in BCAA in blood of insulin resistant subjects may be due in part to increased protein intake in obese individuals, an idea supported by rat feeding studies in which supplementation of a high fat diet with BCAA caused a rise in circulating BCAA and their metabolites, and contributed to development of insulin resistance. However, in their experiments, the animals which consumed a high fat diet supplemented with branch chain amino acids gained less weight, and yet were more insulin resistant, than those that just consumed a high fat diet. This suggested that the elevations in amino acids may have an impact on insulin resistance that is independent of obesity. The extent

to which abnormal protein metabolism this contributes to insulin resistance independent of obesity is incompletely understood. The fourth study in this thesis explores this by examining the relationship between free fatty acids, inflammatory markers and plasma amino acids and insulin resistance, after controlling for obesity.

1.9 Dyslipidemia in the metabolic syndrome-The atherogenic lipoprotein phenotype

1.9.1 Lipid metabolism in health individuals

Lipids are carried in the plasma as part of lipoproteins. There are particles that contain a lipid core, and are surrounded by a layer of phospholipids. Embedded in the phospholipid layer are apolipoproteins, which are proteins that facilitate the interaction between lipoproteins and other proteins (eg enzymes) or receptors. In this fashion, these apoliproteins regulate the metabolism of the lipids in the lipoproteins and also the transfer of lipid to and from lipoproteins.

Lipoproteins can be divided into several major classes based on their size or density. They also contain different apolipoproteins on their surface. The largest of the lipoproteins are chylomicrons, which are produced in the intestine following the ingestion of a meal. They contain large amounts of triglycerides (TG). In the circulation, chylomicrons are acted upon by lipoprotein lipase (an enzyme that is bound to the inner surface of the capillaries), and hydrolyse lipids. The free fatty acids and glycerol released from the chylomicrons can then be stored or used for energy production. This portion of the pathway, involving the transfer of lipids from the diet into tissues where they can be stored or used, is called the exogenous pathway of lipoprotein metabolism (figure 1).

There is also an endogenous pathway through which lipids synthesized in the liver are transferred to other tissues. The lipid packages lipids into very low density lipoproteins (VLDL). These are the predominant triglyceride containing lipoproteins present in the plasma in the fasting state and elevated levels give rise to elevated fasting plasma triglycerides. VLDL particles are secreted into the circulation where they are acted upon by lipoprotein lipase and are remodeled to smaller, denser particles called low density lipoproteins. Low density lipoproteins (LDL) interact with a number of receptors, most importantly the LDL-receptor, and cholesterol is taken up into tissues for utilization or back to the liver for excretion as bile acids. Excess levels of LDL lead to deposition of LDL in the sub-endothelium of the arteries, where they initiate the process that is atherosclerosis. For many years, it was thought that LDL was the predominant lipoprotein involved in the pathogenesis of atherosclerosis. More recently, it has been appreciated that some of the TG rich lipoproteins can also cross the endothelium and may participate in the atherosclerotic process[59]. In a similar manner, while plasma cholesterol (particularly LDL-cholesterol) is a well established risk factor for CVD, the independent contribution of TG has been recognized in the last 20 years[60]. This seems to be particularly important in Asian populations[61].

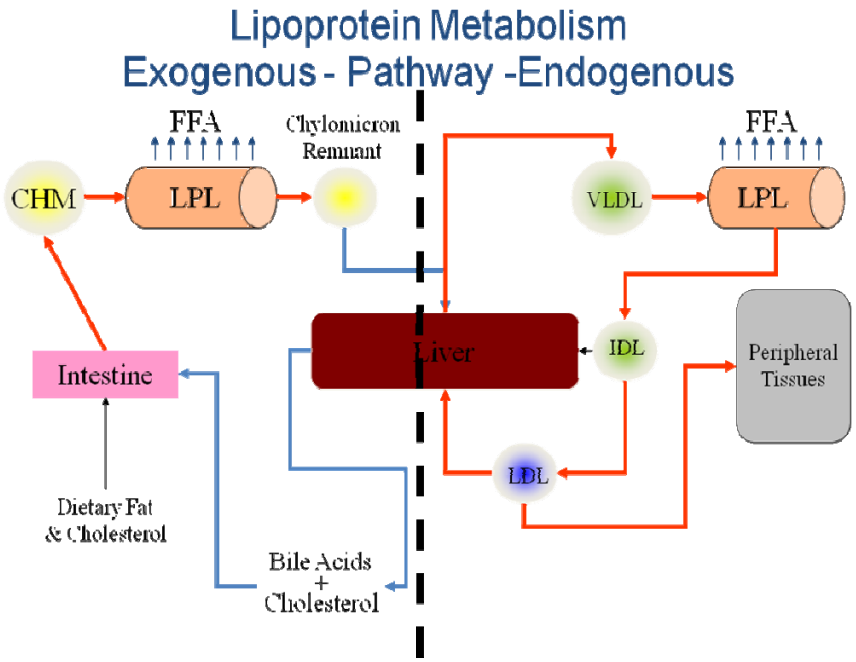


Figure 1. Exogenous and Endogenous pathways for lipoprotein metabolism

There exists a third pathway for lipid transport, which is reverse cholesterol transport. This is the pathway through which excess cholesterol in the tissues are returned to the liver for excretion in the bile and involves high density lipoproteins (HDL). Small lipid poor particles containing mostly apolipoprotein A-I (APOA-I) are secreted by the intestine. They interact with transport proteins in the peripheral tissues and acquire cholesterol and phospholipid, increasing in size (figure 2). The cholesterol in HDL then has one of several fates. It can be taken up by the liver through a process of selective cholesterol uptake. The HDL particle docks with a receptor call scavenger receptor class B type I. Cholesterol is taken up into the liver and the apolipoproteins and phospholipids are re-cycled to take up more cholesterol. Another important pathway for reverse cholesterol involves the transfer of cholesterol from HDL to VLDL and LDL, in exchange for triglyceride. This occurs via a protein called cholesterol ester transfer protein (CETP). This reverse cholesterol transport is thought to be responsible for a large part of the observation that low HDL-cholesterol is one of the most important risk factors for CVD.

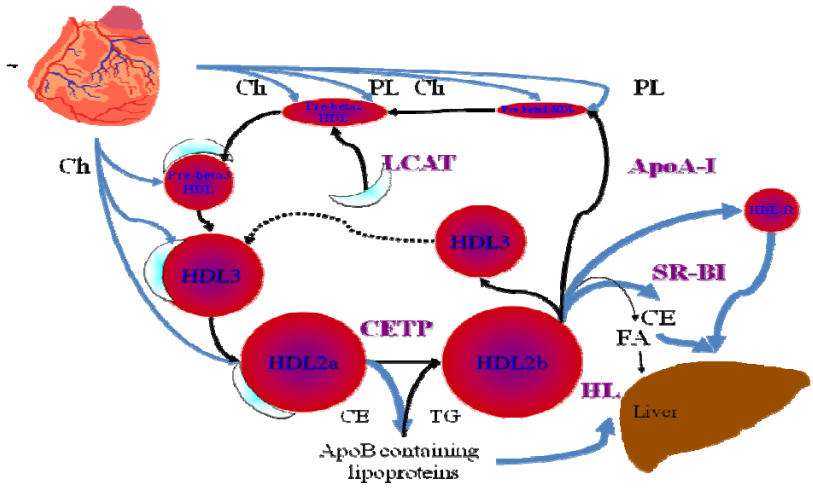


Figure 2. Pathway for reverse cholesterol transport. CETP=cholesterol ester transfer protein; LCAT=lecithin cholesterol acyl transferase; Ch: cholesterol; PL =phospholipids; CE=Cholesterol ester; TG: triglyceride; HL: Hepatic Lipse; SR-BI: Scavenger Receptor Class B Type I; FA=fatty acids.

The rate of this CETP mediated lipid transfer is determined by 3 factors, the importance of which, in relation to the dyslipidemia associated with insulin resistance, will become obvious in the next section. The 3 factors are:

1) The availability of donor particles (VLDL or LDL)

2) The availability of acceptor particles (HDL)

3) The concentration and activity of CETP.

1.9.2 Lipoprotein metabolism in the insulin resistant state

Dyslipidemia is a key feature of the metabolic syndrome. Specifically, insulin resistance is associated with a particular pattern of dyslidemia, which includes elevated levels of plasma triglycerides and low HDL-C. Normally, insulin inhibits hepatic VLDL secretion, so that in the fed state, VLDL secretion is low. Insulin resistance causes loss of this inhibition, leading to increased VLDL secretion. As such, in insulin resistant individuals, there is increased VLDL production leading to a moderate degree of hypertriglyceridemia[62]. While the precise molecular mechanisms of insulin resistance– driven hepatic VLDL overproduction remain undefined, a major factor is believed to be the increased flux of fatty acids from adipose tissue to the liver in this state. The liver is quite efficient in taking up fatty acids from the circulation. Although β-oxidation of fatty acids is one important metabolic fate in the liver, many fatty acids delivered to the liver by the circulation are re-esterified into TG[63]. Newly synthesized hepatic TG can be loaded onto apoB-100 and incorporated into VLDL for secretion resulting in elevated plasma triglycerides. In addition, LPL activity is reduced in obese or insulin resistant subjects. As described, LPL is important for the hydrolysis of TG in VLDL, and this also contributes to the elevated TG levels observed in the insulin resistant state.

In addition to causing elevated plasma triglyceride levels, the greater availability VLDL particles (as donors for CETP mediated lipid transfer) drives an increased rate of CETP-mediated exchange, thus resulting in a greater siphoning of cholesterol out of HDL and reduction in HDL-C levels[64]. Furthermore, the CETP-mediated transfer process enriches HDL with triglycerides, resulting in a TG-rich HDL that is a better substrate for hepatic lipase[65] Obese insulin-resistant patients have TG-enriched HDL and also have higher levels of hepatic lipase activity[66]. Hydrolysis of HDL TG by hepatic lipase leads to the formation of smaller HDL particles that are more rapidly catabolized[67] (Figure 4). results in greater exchange of triglycerides on VLDL for cholesterol on HDL. For these reasons, there is often an inverse relationship between TG and HDL-C in the plasma, and both eleveated TG and low HDL-C are features of the metabolic syndrome.

It is also important to note that HDL-C can also be low for reasons that are unrelated to elevated VLDL. For example, reduce APOA-I production, the major structural apolipoprotein on HDL, can also impact on HDL-cholesterol levels.

I have only described those aspects of lipoprotein metabolism that are pertinent to the next portion of this thesis. There are many other mechanisms (reviewed in [62]) that may contribute to the dyslipidemia associated with obesity and insulin resistance which are beyond the scope of this thesis.

1.10 The *APOA1/C3/A4/A5* locus and dyslipidemia

There exists, on chromosome 11 in humans, a cluster of genes encoding several apoliproteins that play a key role in the metabolism of triglyceride rich lipoproteins and HDL (the lipoproteins that are most disordered in the metabolic syndrome[68]. This is the *APOA1/C3/A4/A5* locus.

As already described, APOA-I is the major structural apolipoprotein in HDL and reduced expression of this protein can result in reduced HDL-C. Overexpression of the human *APOA1* gene (hAPOA1) in mice increased plasma HDL concentrations and protected the animals from the development of diet-induced atherosclerosis [69]. Conversely, *APOA1* knockout mice exhibited decreased HDL levels and developed atherosclerotic lesions [70].

Apolipoprotein C-III (APOC-III) is a component of triglyceride-rich lipoproteins and HDL. It is synthesized mainly in the liver and to some extent in the intestine[68]. The major physiological role of APOC-III appears to be as an inhibitor of lipoprotein lipase[71]. Therefore, plasma APOC-III concentrations are positively associated with triglyceride concentrations. Consistent with this role, overexpression of the human *APOC3* gene in mice resulted in dramatically increased plasma triglyceride concentrations, and increased atherosclerosis[72]. Conversely, mice lacking the *APOC3*

gene had lower plasma triglyceride levels resulting from the faster clearance of postprandial triglycerides [73].

Apolipoprotein A-IV (APOA-IV) was first identified as a component of chylomicrons and HDL [74, 75][15,16]. Although the role of APOA-IV remains unclear, it has been shown that the overexpression of APOA-IV at supraphysiological levels in mice had a protective effect against the formation of diet-induced aortic lesions. However, these mice also showed elevated triglyceride, HDL-cholesterol, and total cholesterol levels. An apolipoprotein homolog of rat APOA-IV in human plasma.

Apolipoprotein A-V (APOA-V) on TG rich lipoproteins may enhance LPL activity, either directly or by facilitating the binding of VLDL to LPL attached to heperan sulphate proteoglycans.

Multiple polymorphisms at the APOA1/C3/AIV/AV locus are associated with altered levels of plasma lipids[76]. The most consistent associations have been demonstrated for variants at the *APOC3* and *APOA5* loci.

The SstI polymorphism at the APOC3 locus has been most extensively studied and in most, though not all [77], populations studied, the presence of the minor allele is associated with elevated TG. In 1995, Li et al found a number of polymorphisms in the APOC3 promoter, which were in strong linkage disequilibrium with the SstI polymorphism (which is in an intergenic region) [78]. It was shown that the minor alleles at these promoter polymorphisms (particularly those at positions -482 and -455 were associated with impaired suppression of APOC3 promoter activity by insulin. Li hypothesized that these “functional” polymorphisms in the APOC3 promoter were responsible for the observed association between the SstI polymorphism and blood triglycerides.

Strong associations have also been observed in relation to polymorphisms at the APOA5 locus and TG. In a German population, 2 haplotypes at the *APOA5* locus were associated with hypertriglyceridemia. One contains 4 SNPs in Strong LD and another containing a coding SNP S19W. It was found that the haplotype containing the 4 SNPs was strongly associated with all the minor alleles for all 3 *APOC3* SNPs (SStI, -482 and -455) whereas the other haplotype was not. Interestingly, in this population, no association was observed between any of the *APOC3* SNPs and plasma TG [79]. In a similar study carried out in the United Kingdom, a similar pattern of associations were observed. The S19W polymorphism was independently associated with plasma triglyceride. However, in this study, polymorphisms at the APOC3 locus were associated with elevated TG, but it was suggested that the TG raising effect of the APOC3 SNPs may reflect LD between the -1131T>C polymorphism and the *APOC3* polymorphisms. Two small studies in Taiwan[80] and one in India[81] also identified polymorphisms at this locus as being important determinants of plasma TG. However, only a limited number of SNPs were studied and the independent role of *APOC3* SNPs was not clearly delineated. Nor did these studies examine SNPs in the *APOA4* locus. In a previous study, we studied polymorphisms at the APOA5 locus. The S19W was rare in our population as it was in other populations of Chinese ethnicity. However, no data is available in relation to other SNPs at this locus and how they may contribute to TG in our population. Furthermore, another coding SNP has been identified in Chinese in Taiwan and in the United States[82]. This SNP has not been examined in our population. It remains unclear whether the associations between polymorphisms at the different loci in this cluster represent independent associations. Thus, to fully understand the contribution of the APOA1/C3/A4/A5 locus to dyslipidemia (particularly in relation to elevated TG and low HDL, the typical dyslipidemia associated with the metabolic syndrome) in study 5, we genotyped 62 SNPs spanning this gene cluster.

Subsequently, another study reported that the polymorphism at position -455 modified the association between plasma insulin and TG rich lipoproteins in humans[83]. This raised the possibility that polymorphisms at this locus may modulate the association between obesity (which is associated with elevated plasma insulin) and blood lipids. This finding has not been replicated and in this study, we will also explore any interactions between obesity and genetic variants within the *APOA1/C3/A4/A5* cluster.

Chapter 2 AIMS

The following are the aims of the 5 studies that comprise this thesis

Study 1 Modification of the NCEP ATP III definitions of the metabolic syndrome for use in Asians identifies individuals at risk of ischemic heart disease

1) To examine the risk of ischemic heart disease (IHD) associated with the MS in an Asian population

2) to examine the effect of modifying the NCEP ATP III criteria for the MS (lowering of the WC to 80 cm in women and 90 cm in men) on the risk of IHD associated with the MS.

Study 2 The Impact of Central Obesity as a Prerequisite for the Diagnosis of Metabolic Syndrome

Following the publication of data from study 1, the International Diabetes Federation recommended criteria for the diagnosis of the metabolic syndrome, in which the presence of central obesity was required to be present as one of the criteria for the metabolic syndrome. This was in distinct contrast to the NCEP ATP III and subsequently, the American Heart Association (AHA)/National Heart Lung and Blood Institute, which required the presence of multiple risk factors for the diagnosis of the metabolic syndrome, but did not specify a requirement for the presence of central obesity. Thus study 2 was carried out:

1) To compare the prevalence of metabolic syndrome (MS) defined according to the American Heart Association (AHA)/National Heart Lung and Blood Institute (NHLBI) and the International Diabetes Federation (IDF)

2) to compare the phenotype (insulin resistance and other cardiovascular risk factors) associated with MS when defined according to these definitions. on

Study 3 Should Central Obesity Be an Optional or Essential Component of the Metabolic Syndrome? Ischemic heart disease risk in the Singapore Cardiovascular Cohort Study

In study 2, we carried out a cross-sectional analysis to examine the the clinical and biochemical profile associated with different definitions of the metabolic syndrome. This study was an extension of study 2, in which we examined, in a prospective manner, the impact of these different definitions on the risk of IHD. The primary aim of this study was:

1) To determine the impact of central obesity as an "essential" rather than "optional" component of the metabolic syndrome on the risk of IHD in a healthy Asian population

Study 4 Insulin resistance is associated with a metabolic profile of altered protein metabolism in Chinese and Asian-Indian men

1) to determine which of the elements of the metabolic profile associated with obesity is independently associated with IR?;

2) When controlled for BMI, which metabolic features differ in the two ethnic groups living in Singapore?

Study 5 Polymorphsism at the APOA1/C3/A4/A5 locus are associated with plasma lipids and modulate the association between obesity and dyslipdiemia in the Singapore Population.

1) To examine associations between polymorphisms at the APOA1/C3/A4/A5 locus and plasma lipids
2) To explore the possibility that polymorphisms at this locus modulate the relationship between obesity and dyslipidemia

Chapter 3 STUDY POPULATIONS AND METHODS

3.1 Study populations

The studies in this thesis utilized data from 4 previous cross-sectional studies in the Singapore population. These were the thyroid heart study (THS), the National University Hospital Heart Study (NUHHS), the 1992 National Health Survey (NHS92) and the 1998 National Health Survey (NHS98). These studies were combined based on the availability of the appropriate variables for the hypotheses that were tested in each population. Subsequently, I was the principal investigator of a study that recalled all subjects from these 4 studies between 2003-2007. This became known as the Singapore Prospective Study Program and provided additional data and materials for some of the studies performed as part of this thesis.

3.1.1 Thyroid heart study (THS)

We do not actually use data from this study in the studies that form part of this thesis. This is because waist circumference, a key variable for the definition of the metabolic syndrome, was not measured as part of this study. As such, while I will describe the sampling methodology, no details as to measurements made or other procedures will be described. If required, these have been published[84]. However, the study participants do form part of the sampling frame for the National University Hospital Heart Study and therefore, I felt it was important to describe the sampling methodology here.

The THS used a four stage sampling design. In the first three stages, stratified systematic sampling was used to select census districts and then within these, reticulated units and households. The proportions of Malay and Indian households were increased to 25% and 15% respectively to increase statistical efficiency in comparing findings between ethnic groups. For the fourth stage, field officers visited the selected houses and listed persons aged 18 years and over, after which a random selection of these persons was made using a sampling fraction of between

0.5 and 0.67. Two attempts were made to persuade the persons to attend the clinic at Singapore General Hospital between 1982 and 1985. A final response of 2143 (60 3%) in persons aged 18 to 69 years was obtained, with the response rates by ethnic group and sex being: Chinese males 64.6%o, females 60.2%; Malay males 52.4%, females 52.1%; and Indian males 66.0%, females 64.3%. Comparisons were made between the respondents, the sample, and the sampling frame, and no major discrepancies were found by age, sex, and ethnic group; the youngest age group had slightly lower response rates than the older ones.

3.1.2 National University Hospital Heart Study (NUHHS)

This cross sectional survey was .of a random sample of persons aged 30 to 69 years from the general population of Singapore. The sample was obtained from two sources-the Thyroid and heart study (described above) and electoral registers of five divisions, each in a different part of the Singapore (north, south, east, west, and centre). There was disproportionate sampling in relation to ethnic groups to obtain equal numbers of subjects in each of the six gender-ethnic groups. The required sample was 180 subjects in each gender-ethnic group giving a total of 1080 subjects. Assuming that 20% of the subjects would not be recruitable because of death, migration, infirmity, or relocation (which was high in Singapore at that time due to massive urban redevelopment), and assuming a response rate of 75%, a total of 1800 persons was selected. Of these, 419 (23.3%) were not recruitable and 983 responded, giving a response rate of71.2%. Of the 983 subjects, 22 were 70 years or over and were excluded, leaving 961 persons aged between 30 and 69 years.

Morning clinics were held from June 1993 to December 1995, with both genders and three ethnic groups seen concurrently. Subjects were asked to fast from 21.00 hours the previous evening. Questionnaires were administered by a nurse trained in interview techniques with questions on age, gender, ethnic group, occupation, exercise, cigarette smoking, and alcohol consumption.

Anthropometric measurements were carried out by a nurse trained in the techniques. Height (to the nearest 0.1 cm) and weight (to the nearest 0.1 kg) were measured on a SECA machine without shoes and in light clothing after emptying all pockets. Waist circumference (smallest measurement between the costal margins and the iliac crests) and hip circumference (at the level of the greater trochanters) were measured with a tape to the nearest 0.5 cm with the subject standing. Blood pressures were taken using the standard mercury sphygmomanometer. The same doctor did the measuring to remove inter-observer variation, and it was carried out according to the MONICA project protocol[85] to reduce intra-observer variation. Measurement took place between 09.00 and 11.00 hours to remove diurnal variation. Phases 1 and 5 were recorded and the mean of two readings used for the analyses.

Venous blood samples were taken with the subject in a sitting position. Venepuncture took place between 10.00 and 12.00 hours to remove diurnal variation, and after at least 10 minutes rest. All measurements were made in the Department of Laboratory Medicine, National University Hospital, within 1 hour of collection. Lipids were measured enzymatically on an autoanalyser (Ektachem, Kodak)-total cholesterol and triglyceride directly and HDL cholesterol after precipitation, with LDL cholesterol calculated using the Friedewald formula. Plasma glucose was measured in blood collected in fluoride oxalate by a specific enzyme assay on the Kodak Analyser. Subjects with a glucose concentration > 5.5 mmol/l who were not currently on medication for diabetes subsequently had an oral glucose tolerance test (after at least 10 hours' fasting) of 75 g of dextrose in 296 ml of carbonated orange (Trutol 75, Custom Laboratories Inc, USA). They then had measurements of plasma glucose at fasting and 2 hours after the oral glucose. Those with a fasting plasma glucose concentration <5.5 mmol/l are very unlikely to have diabetes. Subjects currently taking medication for diabetes were also classified as diabetic.

3.1.3 1992 National Health Survey (NHS92)

The NHS92 was conducted between September and November 1992 at six community centers distributed around Singapore Island. The survey team moved systematically each fortnight, through Silat, To a Payoh, Fengshan, Ang Mo Kio, Ulu Pandan, and Chong Pang community centers over the 3-month period, to provide island-wide coverage of survey sites and proximity to those selected. A total of 4,915 individuals were randomly selected from a sample of all household units in Singapore, obtained from the Department of Statistics' National database on dwellings in Singapore. The characteristics of the selected sample conformed to that of the resident population. Systematic sampling, followed by disproportionate stratified sampling by ethnic groups, was used to select the sample for the survey. The two minority groups, Malays and Asian Indians, were oversampled to give an ethnic distribution of 60% Chinese, 20% Malays, and 20% Asian Indians. This was to ensure sufficient numbers for statistical analysis, and representative results were weighted back during the analysis of findings. From the 4,915 eligible individuals randomly selected from a sample of all household units in Singapore, 3,568 Singapore residents aged between 18 and 69 years finally participated in the survey. The response rate was 72.6%. Although response rates did differ between ethnic groups, the non responders were contacted and information was sought regarding their demographic and socioeconomic profile and diabetes and hypertension status. This was to ensure that the prevalence of these diseases would not be underestimated during the survey. Characteristics of the non-responders were similar to those of the survey respondents.

The protocols and procedures used in the NHS '92 were based on the 1990 Mauritius Non-communicable Diseases Survey [86] and the World Health Organization's Monitor Trends in Cardiovascular Diseases protocol [87]. An interviewer-administered questionnaire was used to capture data on socio-demographic factors, dietary intake, physical activity, smoking and alcohol consumption.

All biochemistry analyses were done at the biochemistry Laboratory of the Singapore General Hospital, which is accredited by World Health Organization International Quality Assurance

programs and the College of American Pathologists program . Fasting blood specimens for lipids (10 ml plain tubes), and glucose (2 ml fluoride oxalate tubes) were taken from all respondents after an overnight fast of 10 h. All subjects (1,315 men and 1,426 women) except those with diabetes on oral hypoglycemic medications or insulin were then given an oral glucose tolerance test (OGTT) (75 g of anhydrous glucose made up to 250 ml of solution with water). All blood specimens were centrifuged at room temperature (27–30ºC) on-site to separate the sera, which were then placed in separate tubes on ice for daily dispatch to the central biochemistry laboratory. Plasma glucose and lipid measurements were performed on the same day as collection. Glucose measurements were done by the glucose oxidase method using the Vitros 700 Chemistry Analyzer (Rochester, NY), with an intra-assay coefficient of variation (CV) of 1.2% and an interassay CV of 1.5%. Total cholesterol (intra-assay CV 3.0% and interassay CV 4.1%) and triglyceride (TG) (intra-assay CV 1.4% and interassay CV 1.9%) were measured directly by enzymatic methods using Kodak Ektachem clinical chemistry slides. HDL cholesterol (intra-assay CV 2.6% and interassay CV 4.4%) was separated from LDL and VLDL by precipitation with dextran sulfate and magnesium chloride and then measured by enzymatic methods as in cholesterol measurements. The slides were then read on the Vitros 700 Chemistry Analyzer. LDL cholesterol was calculated by Friedewald's equation.

Height (to the nearest millimeter) was recorded in all subjects without shoes, and weight (in kilograms) was measured in subjects in light clothing using electronic weighing scales (SECA model 220). Waist (defined as the narrowest part of the body below the costal margin) was measured using a non-elastic tape measure. At least two readings of blood pressure were taken from respondents who had rested adequately before measurement, using a standard mercury sphygmomanometer. If the two readings differed by more than diastolic 15 mmHg or systolic 25 mmHg, a third reading was performed. The mean values of the closest two readings were calculated.

3.1.4 1998 National Health Survey (NHS98)

NHS98 was a cross-sectional study conducted between September amd November 1998 through six survey centers across the Singapore Island. To ensure comparability of the data with that obtained from NHS92, almost identical methodology was employed. A target sample size of 5000 subjects was determined in order to have 80% power to detect a 10-15% difference in the prevalence of common diseases and risk factors. The sampling was divided into 2 phases. In the first phase, to account for non-respondents, 11,200 households were selected from the National Database on Dwellings. These households were selected based on their proximity to the six survey centers as well as based on house-type (as a proxy for socio-economic status (SES) to ensure a representative distribution of households in Singapore. In the second phase, a random sample of 7500 individuals (between ages 18-69 years) were selected from the 11,200 households, with an oversampling of Malays and Asian-Indians to ensure that prevalence estimates for these ethnic groups were reliable. Participants were contacted first by mail, then by telephone. Interviewers subsequently also conducted a home visit. A response rate of 64.5% (4723/7500) was achieved comprising 3228 Chinese (64%), 849 Malays (21%) and 646 Asian-Indians (15%).

Fasting blood samples were drawn for measurement of glucose and lipids in all subjects in after a 10 hour overnight fast. As in the NHS92, the tubes were centrifuged on site and then sent to the Biochemistry Laboratory (Department of Pathology, Singapore General Hospital) for analysis on the same day. Serum lipid and glucose concentrations were measured using kits from Boehringer Mannheim Systems (Mannheim, Germany) and read on a BM/Hitachi 747 analyzer (Roche Diagnostics, Corp. Indianapolis, IN). Total cholesterol [intra-assay coefficient of variation (CV) 0.8%, interassay CV 1.7%], triglyceride (intra-assay CV 1.5%, interassay CV 1.8%) and glucose (intra-assay CV 0.9%, interassay CV 1.8%) were measured using enzymatic colorimetric assays. HDL-C (intra-assay CV 2.9%, interassay CV 3.6%) was measured using a homogenous colorimetric assay, whereas low-density lipoprotein cholesterol (LDL-C) (intra-assay CV 0.9%,

interassay CV 2.0%) was measured using a homogenous turbidimetric assay. Insulin levels were measured using immunoassay (Abbott ASYM Abbott Laboratories, Chicago, IL).

Height (to the nearest millimeter) was recorded in all subjects without shoes, and weight (in kilograms) was measured in subjects in light clothing using electronic weighing scales (SECA model 220). Waist (defined as the narrowest part of the body below the costal margin) was measured using a non-elastic tape measure. At least two readings of blood pressure were taken from respondents who had rested adequately before measurement, using a standard mercury sphygmomanometer. If the two readings differed by more than diastolic 15 mmHg or systolic 25 mmHg, a third reading was performed. The mean values of the closest two readings were calculated.

DNA was isolated from blood samples using DNA blood Midi kits (Qiagen, Hilden, Germany) following the manufacturer's recommended protocol. DNA samples for 2936 Chinese, 788 Malays and 598 Asian-Indians were available for analysis.

3.1.5 The Singapore Prospective Study Program (SP2)

Between 2004 and 2007, I was the principal investigator of this study which invited 10,445 subjects from all four studies described above (the THS, the NUHHS, NHS92 and NHS98) to participate in a repeat examination. Subjects deceased at the time of follow-up (as shown by data linkage to the Registry of Births and Deaths) were excluded (n- 517). Also excluded were 6 subjects who had emigrated and 85 who had errors in the records regarding their identity card number (which were used to re-contact the subjects).

Subjects were contacted to obtain an appointment for investigators to administer the questionnaire at a subject's home. Three home visits were made on 3 different occasions, including one weekend and weekday, before a subject was deemed non-contactable. After this procedure was completed, 2,673 subjects were non-contactable. Of those subjects who could be contacted, 30 (0.3%) refused to participate. All subjects were invited to attend a health examination for additional tests and collection of biologic specimens shortly after the home visit. A total of 7,742 (74.1% response rate) subjects completed the questionnaire; 5,157 of them (66.6% of those who completed the questionnaire or 49.4% of all eligible subjects) also attended the health examination (Figure 3). Ethics approval was obtained from 2 institutional review boards (the National University of Singapore and the Singapore General Hospital). Informed consent was obtained before the study was conducted.

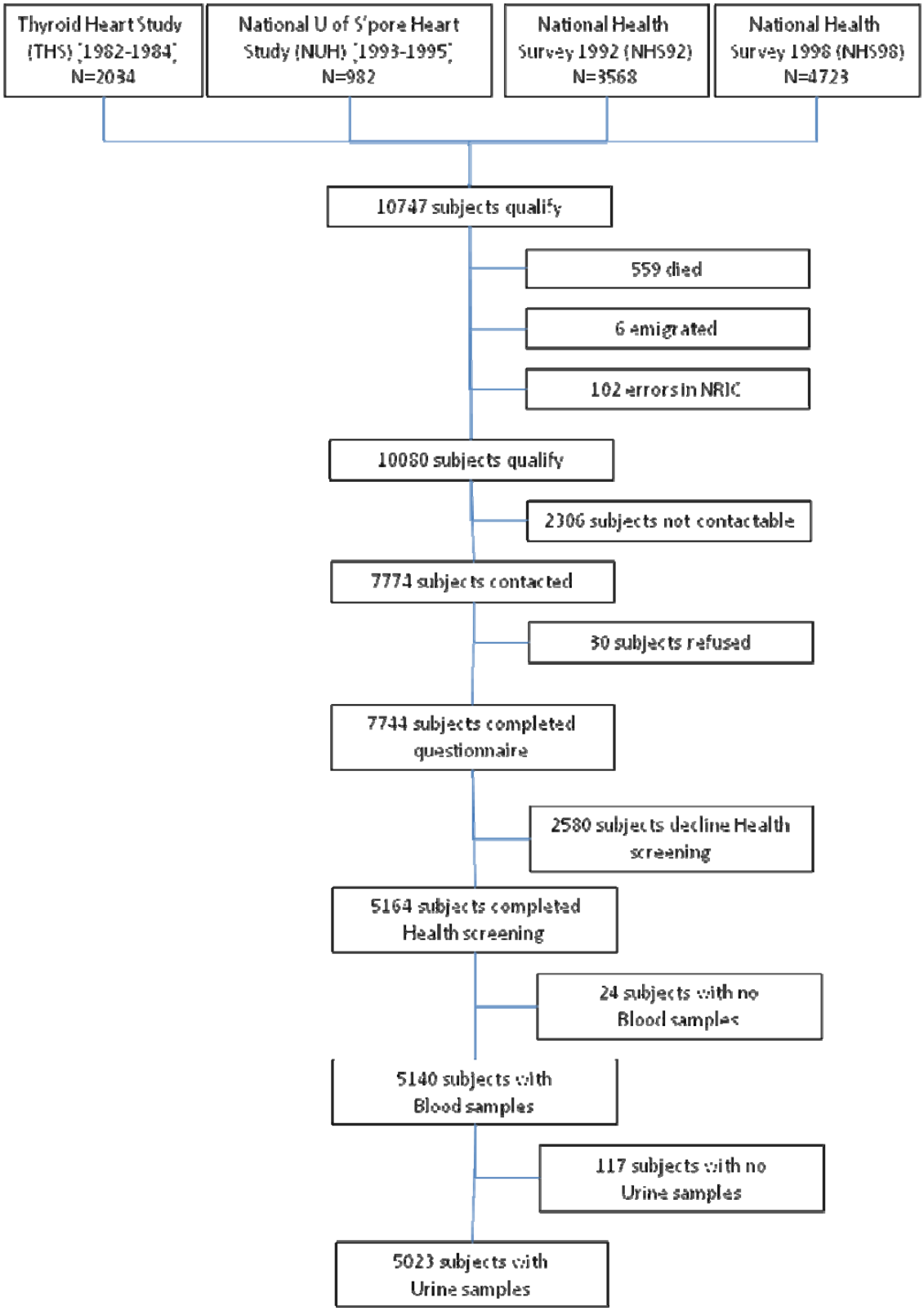


Figure 3. Subject recruitment for the Singapore Prospective Study Program

Data on demographic and lifestyle factors (alcohol consumption, smoking), as well as medical history (including physician-diagnosed hypertension, diabetes mellitus, and hyperlipidemia), were collected by using interviewer administered questionnaires. For the health examination, participants were examined the morning following a 10-hour overnight fast. Venous blood was drawn and collected in plain and fluoride oxalate tubes and was stored at 4^{o}C for a maximum of 4 hours prior to processing. A random urine specimen was also collected. All biochemical analyses of blood were carried out at the National University Hospital Referral Laboratory, which is accredited by the College of American Pathologists. Serum total cholesterol, triglyceride, and high density lipoprotein cholesterol were measured by using an automated autoanalyzer (ADVIA 2400; Bayer Diagnostics, Tarrytown, New York). Low density lipoprotein cholesterol levels were calculated by using the Friedewald formula. Plasma glucose was also assayed with enzymatic methods (ADVIA 2400) by using blood collected in fluoride oxalate tubes. The intraday and interday coefficients of variation for total cholesterol, triglyceride, high density lipoprotein cholesterol, plasma glucose were 0.80%–1.57% and 0.93%– 1.15%, 0%–3.85% and 1.27%–3.40%, 0.56%–0.65% and 1.18%–2.00%, and 0%–0.93% and 1.68%–1.83%, respectively. Random urinary creatinine was measured by using a commercial assay (Immulite; Diagnostic Products Corporation, Gwynedd, United Kingdom for urinary albumin and Roche Diagnostics GmbH, Mannheim, Germany for creatinine). The lower detection limits for urinary creatinine was 0.027 mmol/l.

Height was measured without shoes by using a wallmounted stadiometer. Weight was measured in light clothing by using the same digital scale (SECA, model 782-2321009; Vogel & Halke, Hamberg, Germany) for all participants. Participants were instructed to remove any objects such as keys and mobile phones before measurement. An automated blood pressure monitor (Dinamap Pro100V2; Criticon, Norderstedt, Germany) was used to take 2 blood pressure readings from participants after 5 minutes of rest. A third reading was performed if the difference between the 2 readings of systolic blood pressure was greater than 10 mm Hg or of diastolic

blood pressure was greater than 5 mm Hg. Mean values of the closest 2 readings were calculated. The respective inter- and intra- observer coefficients of variation for systolic blood pressure were 0.51%–10.20% and 0%–2.5%, whereas they were 0.41%– 7.50% and 0%–2.5% for diastolic blood pressure.

3.2 SPECIFIC DESIGN AND METHODS FOR STUDIES

3.2.1 Study 1

Study 1 was a cohort study. We combined the individuals who participated in the NUHHS and the NHS92 as illustrated in figure 4. Outcomes were obtained by linking individual records (using unique national registry identity card numbers) to three national registries as previously described [88, 89]. These were: (i) the Registry of Births and Deaths; (ii) the Central Claims Processing System and its predecessor the Hospital Inpatient Discharge System. These databases capture inpatient discharge information from all hospitals in Singapore, both public and private; (iii) the Singapore Myocardial Infarct Registry, a population based registry with comprehensive coverage of acute myocardial infarction occurring in Singapore. All obtained outcome measures were in coded form using the Ninth Revision of the International Classification of Diseases (ICD-9). An IHD event was defined as the occurrence of acute myocardial infarction or IHD (ICD-9 410-414) recorded in the aforementioned registries. The first IHD event was used for analysis. Subjects were censored as of 31 December 2002 or their date of event occurrence or death, whichever occurred first.

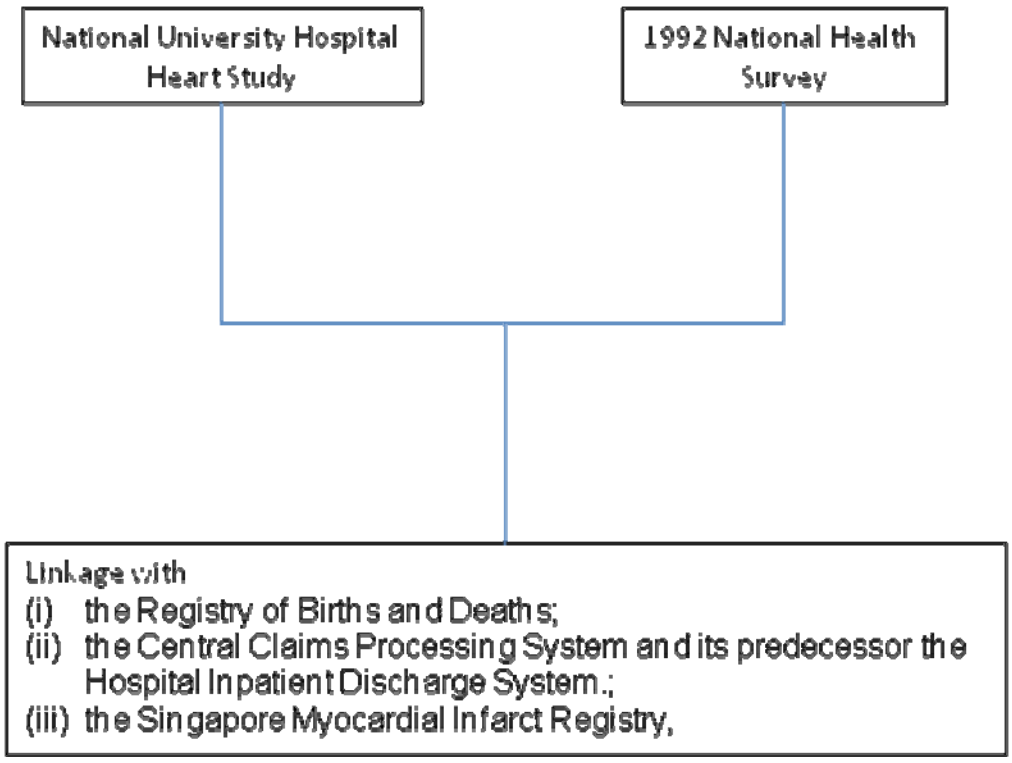


Figure 4. Establishing a cohort for studies 1 and 3.

This analysis included 3954 subjects (3156 from the 1992 National Health Survey and 798 from the National University of Singapore Heart Study). We excluded 367 subjects with diabetes mellitus, 61 subjects with pre-existing IHD and 31 subjects with both. A further 27 subjects were excluded in this analysis due to missing data in the fields used.

The presence of three out of five of the following criteria constituted a diagnosis of the MS in accordance with the NCEP ATP III recommendations[90]:

1) WC> 102 cm in men or 88 cm in women;

2) fasting TG≥1.7 mmol/l;

3) HDL-C < 1.0 mmol/l in men or <1.3 mmol/l in women;

4) blood pressure≥130/85mmHg or hypertensive on medication;

5) fasting glucose≥6.0 mmol/l.

For the modified Asian criteria, we used a waist circumference of 90 cm in men and 80 cm inwomen to define central obesity [31]. The other criteria were unchanged. Diabetes mellitus was diagnosed in those with fasting plasma glucose >7.0 mmol/l or if the study subject gave a history of diabetes mellitus for which they were receiving treatment in the form of oral hypoglycemic agents or insulin.

Statistical analysis

Statistical analyses were performed using SPSS version 11.2 for windows (SPSS Inc. Chicago, IL). Continuous variables were expressed as means with standard deviation in parenthesis. Categorical variables are expressed in percentages. Time-to-event analyses were conducted using Cox proportional hazards regression (stratified by the study from which the data was derived). This allowed the baseline hazard function to differ for subjects from each of the two studies in order to take into account any period effects on IHD incidence. Hazard ratios (HR) and 95% confidence intervals (95% CI) for IHD amongst those who satsfied only the Asian criteria for MS and those who satisfied both the Asian and the NCEP ATP III criteria compared to those who satisfied neither set of criteria were estimated. We first conducted the analyses separately for men and women and in each ethnic group (Chinese, Malay and Asian Indian). The patterns of associations were similar (although some failed to reach statistical significance due to the small number of events in some groups). We also tested for homogeneity of effect by including the interaction term MS×gender and MS×ethnic group in the model. Neither of these terms were

statistically significant (p = 0.555 for gender and p = 0.863 for ethnic group). As such, men and women from all three ethnic groups were combined and analyses were carried out after adjustment for age, sex, ethnicity and current cigarette smoking.

3.2.2 Study 2

This study was a cross-sectional study and utilized data from the NHS98. By the time this study was carried out, the IDF[22] and AHA/NHLBI[21] had adopted the definition of central obesity used in study 1 as part of their recommendations for defining the metabolic syndrome in persons of Asian ethnicity. Furthermore, both groups included individuals with diabetes mellitus in the definition.

Each individual was categorized according to the five criteria for the metablic syndrome [21, 22]

1) elevated triglycerides: ≥150 mg/dl (1.7 mmol/l);
2) reduced HDL cholesterol: < 40 mg/dl (1.03 mmol/l) in male subjects and < 50 mg/dl (1.29 mmol/l) in female subjects;
3) elevated blood pressure: systolic ≥130 mmHg or diastolic ≥85 mmHg or on treatment for hypertension;
4) elevated fasting plasma glucose: ≥100 mg/dl (5.6 mmol/l) or on treatment for type 2 diabetes; and
5) central obesity, using the waist circumference for South Asians/Asians: >90 cm in male subjects and >80 cm in female subjects.

The population was then divided into four mutually exclusive groups according to the presence of central obesity and the presence of multiple other features of MS. Group 1 comprised those individuals without central obesity and who exhibited two or fewer of the other risk factors. Group 2 comprised those individuals with central obesity but who exhibited none or one of the other risk factors. Group 3 comprised those individuals who exhibited three or more risk factors, of which central obesity was not one. These individuals would be classified as having MS by the AHA/ NHLBI criteria but not by the IDF criteria. Group 4 comprised those individuals who exhibited central obesity and at least two other risk factors. These individuals would be classified as having MS using either the AHA/NHLBI or the IDF criteria.

Insulin resistance was calculated using homeostasis model assessment. Insulin resistance were estimated using the Homeostatic Model of Assessment (HOMA) as described by Matthews et al[91]. These estimates have been demonstrated to correlate with the 'gold-standard' measures for insulin resistance (euglycaemic clamp) and β-cell function (hyperglycaemic clamp). The estimates were calculated using the formulae: HOMA-IR = Fasting plasma glucose (mmol/L) x Fasting insulin (μU/ml) ÷ 22.5.

Using the Framingham risk score based on LDL-C level [13], we calculated the estimated total coronary heart disease risk over a 10-year period for every individual included in the analysis.

Statistical analysis

Statistical analyses were performed with SPSS/PC statistical program (version 11.5 for Windows; SPSS, Inc., Chicago, IL). Values are expressed as means ± standard deviation. The differences in clinical characteristics and body composition among the groups were compared using ANOVA. TG and insulin resistance were log-transformed to improve the skewness of the distribution. Subsequently, their means were back-transformed for presentation in the tables. Post hoc analyses were carried out using a Bonferroni correction. All proportions were compared using the Chi-square2 test. $P< 0.05$ was considered statistically significant. To compare proportions after adjustment for age, we utilized multinomial logistic regression.

3.2.3 Study 3

This study was a cohort study and utilized the same study population and dataset as study 1.

The central obesity/metabolic syndrome status of individuals was obtained using the criteria set out by IDF [22] and AHA/ NHLBI [21]:

1) elevated triglycerides: ≥150 mg/dl (1.7 mmol/l);
2) reduced HDL cholesterol: < 40 mg/dl (1.03 mmol/l) in male subjects and < 50 mg/dl (1.29 mmol/l) in female subjects;
3) elevated blood pressure: systolic ≥130 mmHg or diastolic ≥85 mmHg or on treatment for hypertension;
4) elevated fasting plasma glucose: ≥100 mg/dl (5.6 mmol/l) or on treatment for type 2 diabetes; and
5) central obesity, using the waist circumference for South Asians/Asians: >90 cm in male subjects and >80 cm in female subjects.

Using these five metabolic score components, individuals were categorized into three central obesity/metabolic syndrome groups: *1*) no metabolic syndrome, which includes individuals with less than three metabolic syndrome components; *2*) central obesity and metabolic syndrome, which includes individuals with elevated waist circumference and two or more other components; and *3*) no central obesity and metabolic syndrome, which includes individuals with low waist circumference but three or more other components.

Statistical analyses

Statistical analyses were performed using SPSS (version release 13.0.1, 2004; Chicago, IL). Categorical variables were expressed in percentages and continuous variables in means ± SD

unless otherwise specified. Incidence rates for first IHD events were calculated for each of the central obesity/ metabolic syndrome groups, and Cox proportional hazards regression was used to obtain adjusted hazard ratios (HRs) for first IHD events. The time-to-IHD event was the difference between the date of the first IHD event and the date of entry into the study. Subjects without IHD were censored at 31 December 2002 or the date of non-IHD death, whichever occurred first. HRs were adjusted for age, sex, ethnic group, study, LDL cholesterol, smoking, and alcohol intake. Interaction terms, created using the three central obesity/ metabolic syndrome groups, with ethnic group (P = 0.387), sex (P = 0.911), and study (P = 0.081) showed no significant interaction; thus, the analysis was done with ethnic group, sex, and study combined. An interaction term consisting of follow- up time and the three central obesity/ metabolic syndrome groups was used to test the proportional hazards assumption (15) for occurrence of IHD events and was found not to be significant (P = 0.351), indicating proportional hazard over time. The attributable percent among those with metabolic syndrome (AP_E: the percent of risk IHD among those with metabolic syndrome that is due to metabolic syndrome) and attributable percent among the total population (AP_T: that is the percent of risk of IHD among the whole population that is due to the metabolic syndrome) was also calculated for both AHA/NHLBI and IDF criteria with and without diabetes.

3.2.4 Study 4

This study was a case-control study. We selected a subset of individuals who participated in SP2. In this study, we chose to study men only, in order to reduce the potential confounding effects of gender as well as metabolic alterations occurring at different phases of the menstrual cycle. Furthermore, we chose to study only Chinese and Asian Indian men, as these were the ethnic groups that show the greatest differences in IR in our population [92, 93]. In order to examine the metabolic and hormonal correlates of IR as opposed to obesity, we first separated Asian Indian and Chinese men into the upper and lower tertiles of IR, as measured by homeostasis model assessment (HOMA)[91]. These opposing tertiles corresponded to HOMA indices of ≤1.06 and HOMA ≥ 1.93. We next excluded all those individuals who were taking medications for diabetes or hyperlipidemia, those who did not provide a urine specimen, and those with missing height and weight information. This left 1042 men (56 Asian Indians with low HOMA, 132 Asian Indians with high HOMA, 547 Chinese with low HOMA and 307 Chinese with high HOMA). Of the 56 Asian Indians with low HOMA index, 30 had BMI between 21 and 29 kg/m2 (non-obese). For each of these 30 subjects, up to a maximum of 3 subjects with BMI within 0.3 kg/m2 were selected from each of the other 3 categories. The final study subjects comprise of 30 Indians with low HOMA, 53 Indians with high HOMA, 97 Chinese with low HOMA and 83 Chinese with high HOMA, giving a total sample size of 263 men. Results from analysis of metabolites in plasma were available on all 263 men. As for urinary analysis, 5 men had urine samples which were too dilute (<1.5μmol creatinine/mL) and therefore urinary analysis results were only carried out in 258 men.

Assessment of physical activity

Physical activity was assessed by interviewer-administered questionnaire with a recall period of the previous 3 months. The questionnaire was adapted from several sources[94-97] and covered transportation, occupation, leisure time and household activities. A metabolic equivalent of task

(MET) value was assigned to each reported activity according to the compendium by Ainsworth et al [98, 99].

Weekly energy expenditure in physical activity (Kcal/ week) was computed as: Hours spent on activity per day x numbers of day per week x METS x Body weight in kg. The energy expenditure for transportation, household and leisure time activity was calculated from light, moderate and vigorous activity whereas energy expenditure for occupation activity was from moderate and vigorous activity.

Transportation activity: The questions on transportation activity were adapted from National Health Survey 2004 questionnaire[100]. To assess transportation activity, participants were asked about their involvement in active transportation (i.e. walking or cycling for transport at least 10 minutes). In addition, the duration, frequency and the intensity of the activity were also recorded. The intensity was assessed by asking the participants whether the intensity of walking or cycling was light, moderate or vigorous.

Occupational activity: Questions on occupational activity were based on the previously validated Modifiable activity questionnaire [96] which was designed for the use in different populations [101]. For occupational activity, individuals were asked to list all jobs held during the past 3 months. For each job entry, data was collected for the average job schedule (number of weeks in the last 3 months, days per week, and hours per day worked). Activity on the job was determined by the number of hours spent sitting at work and the most common physical activities performed when not sitting, which were categorized into light, moderate and vigorous activity. Light activities include job activities involving standing still without heavy lifting, occasional short distance walking, general office work and similar activities. Moderate activities include continuous walking, carrying light loads and heavy cleaning, etc while vigorous activities include all those

activities with energy demands approaching those of heavy lifting, construction, etc. 4 METs and 7 METs were assigned to moderate and vigorous activity respectively and no credit was given to light activity.

Leisure time activity: The leisure time activity component was adapted from the Minnesota leisure time activity questionnaire which has been validated and evaluated against other physical activity questionnaires, accelerometers, fitness tests, and criterion methods on adults [102-109]. The leisure time activities were grouped into four general categories: walking and miscellaneous (i.e bicycling, hiking, etc), conditioning exercise (ie, home exercise, health club exercise, jogging, running, etc), water activities (i.e water skiing, swimming, sailing, etc) and sports (i.e. bowling, tennis, soccer, etc) and covered 48 popular activities. The participants were asked if they performed any of the activities under these four categories or any other leisure time activities during the past 3 months. For each activity, participants identified the frequency per week/ month and the average minutes of participation in each activity for each time.

Household activity: The household activity component was adapted from the validated Yale physical activity questionnaire, which has been shown to be acceptable and reliable [94, 110-112]. Three categories of common household activities were covered in this section: housework, yard work and caretaking (elderly persons or children). Participants were asked the type of activity performed, the frequency (the number of times per week), and the duration (length of time spent in the activity per day).

Biochemical measurements.

For this study, in addition to the biochemical measurements that have already been described, a

panel of metabolic, hormonal, and inflammatory marker assays were performed at the Sarah W. Stedman Nutrition and Metabolism Center, Duke University. Commercial radioimmunoassays were used to measure leptin (Millipore, St. Charles, MO) and total insulin-like growth factor-I (IGF-I, Beckman-Coulter, Webster, TX). Searchlight chemiluminescent technology (Thermo Pierce-Endogen, Woburn, MA) was used for measuring three IGF-1 binding proteins (IGFBP1, IGFBP2, IGFBP3) using a custom multiplex assay. Adiponectin was measured via technology from Meso Scale Discovery (MSD, Gaithersburg, MD), as was a 9-plex cytokine panel for GM-CSF, IFN-γ, IL-10, IL-12p70, IL-1β, IL-2, IL-6, IL-8, and TNFβ. Total free fatty acids, ketones and 3-hydroxybutyrate (reagents from Wako, Richmond, VA) and lactate (Roche, Indianapolis, IN) were measured on a Hitachi 911 autoanalyzer.

Acylcarnitines and amino acids were analyzed in plasma by tandem mass spectrometry (MS/MS), and urine organic acids by gas chromatography/mass spectrometry (GC/MS) as described previously [58, 113-117]. All MS analyses employed stable-isotope-dilution with internal standards from Isotec (St. Louis, MO), Cambridge Isotope Laboratories (Andover, MA), and CDN Isotopes (Pointe-Claire, Quebec, CN). A list of all internal standards utilized in these studies has been published [114].

Characteristics and performance of the assays have been previously published [118]. In general, the assays that were used in this study gave coefficients of variantion <10% for hormones and cytokines, <5% for the conventional metabolites measured on the Hitachi 911 autoanalyser, and <15% for acylcarnitines and amino acids measured by GC/MS.

Ethics approval was obtained from two Institutional Review Boards (National University of Singapore and Singapore General Hospital). Informed consent was obtained before conduct of study.

Statistical analyses.

Statistical analysis was carried out using STATA (StataCorp LP, College Station, Texas, USA). Categorical variables were compared using the chi-square test. Continuous variables were compared using analysis of variance. We first used an F-test to identify those variables for which at least 1 of the 4 groups showed a statistically significant difference from the others. Subsequently, the levels of metabolites in high and low HOMA groups were compared using analysis of covariance, with and without adjustment for age and BMI. A p-value < 0.05 was taken as statistically significant.

3.2.5 Study 5

This study was a cross sectional study and utilized data and DNA from the NHS98. For the purpose of this study, we only used data from Chinese Subjects, as this was the largest ethnic group.

Genotyping

Genotyping was carried out at different times for the 4 loci examined in this study. As such, the methods differed somewhat.

APOA5

Genotyping at the APOA5 locus was carried out as previously described[119]. Briefly, we identified 12 SNPs on the National Center for Biotechnology Information Human SNP Database and tested their informativeness in these ethnic groups on a pilot experiment including 278 subjects (i.e., three 96-well plates of DNA samples including 147 Chinese, 93 Malays, and 38 Asian- Indians). Based on this study, five of the SNPs were either monomorphic or had average frequencies of the minor allele of < 0.05 in our population, and Three other SNPs (ss4383598, ss2990302, and ss1943494) were not given further consideration, because of their low reliability for genotyping across all three populations. At the end, five previously reported SNPs [79, 120, 121]were included for the analyses presented in this research. Subsequently, we genotyped an additional SNP [82] which had been identified in Chinese populations from Taiwan and the United States using a Taqman assay.

APOC3

18 SNPs were selected from the dbSNP, which comprised all SNPs validated by frequency at the time of the selection on 16 April 2004. Genotyping for all 18 SNPs was performed in an initial sample of 517 individuals comprising 367 Chinese, 80 Malay and 70 Indians. Of the 18 SNPs, four did not display any variability, and variation in SNP rs5135 was seen in one heterozygous individual. These SNPs were not evaluated further. We then examined the patterns of linkage disequilibrium between these SNPs using Haploview[122]. The pattern of R^2 across the locus is shown in Figure 1. Based on these data, to increase the efficiency of genotyping, tagSNPs were selected using pairwise tagging R^2 between the remaining 13 SNPs. Using a tagging R^2 cut-off of 0.80, two SNPs (rs5142 & rs5141) were found to be in strong LD with rs5128. Although rs33989105 and rs35523410 (also known as -482 and -455 respectively) showed a high degree of LD, both SNPs were included due to previous data suggesting that these were functional. On this basis, we selected 11 SNPs to be genotyped in our population. Five DNA fragments encompassing all 18 polymorphisms were amplified in a single multiplex polymerase chain reaction and genotyping was carried out using the ABI Prism SNaPshot multiplex system (Applied Biosystems, Foster City, CA).

APOA1 and APOA4

For these 2 loci, we selected SNPS from dbSNP as we had done for APOC3. However, genotyping of SNPs at these loci were carried out using the Sequenom MassARRAY platform (Sequenom, San Diego, CA, USA) without selection.

Statistical Analysis

To study the association between serum lipid concentrations and the individual SNPs, we used linear regression models under various inheritance models (additive, dominant and recessive). The serum lipid concentrations investigated were total cholesterol (TC), TG, HDL-C and LDL-C. To test for interactions between genetic variants and obesity, the interaction term SNPXBMI was

introduced into the model. To account for multiple testing, we used Bonferroni corrections and declared statistical significance when $p<0.001$.

Chapter 4 RESULTS

4.1 Study 1: The impact of modifying the definition of central obesity in Asian populations on the association between the metabolic syndrome and ischemic heart disease

Table 3 shows the baseline characteristics of the subjects included in this analysis.

Table 3. Baseline characteristics (mean±SD) of non-diabetic participants from the 1992 National Health Survey and the National University of Singapore Heart Study.

		Male (n = 1905)	Female (n = 2049)
Age (years)		38.6 (13.0)	38.9 (12.3)
Waist (cm)		80.5 (10.4)	72.3 (10.7)
BMI (kg/m^2)		23.2 (3.8)	23.3 (4.6)
Total cholesterol (mmol/l)		5.35 (1.04)	5.31 (1.03)
Triglyceride (mmol/l)		1.65 (1.62)	1.20 (0.77)
HDL cholesterol (mmol/l)		1.08(0.29)	1.31 (0.35)
LDL cholesterol (mmol/l)		3.57 (0.94)	3.46 (0.96)
Ethnic group (%)	Chinese	59.7%	60.9%
	Malay	21.3%	20.1%
	Asian Indian	19.1%	19.1%

*Hypertension (%)		10.6%	8.2%
Current smokers (%)		35.2%	2.8%

*Hypertension was diagnosed if blood pressure >140/90 mmHg or the subject was gave a history of treatment with anti-hypertensive medication. A subject was considered a current smoker if he/she smoked daily.

Table 4 shows the prevalence of the individual features of the MS using both the NCEP ATPIII criteria and the modified Asian criteria. The lowering of the cut-off for waist circumference led to an increase in the prevalence of central obesity from 2.8% to 17.1% in men and 8.7% to 21.8% in women. Correspondingly, the prevalence of the MS defined by the modified Asian criteria rose by 5.1% in men and 4.1% in women.

Table 4. Prevalence [percentages (95% CI)] of features of the metabolic syndrome amongst non-diabetic participants of the 1992 National Health Survey and the National University of Singapore Heart Study according to the NCEP ATP III criteria and the modified Asian criteria.

	Male (n = 1905)	Female (n = 2049)
TG≥1.7 mmol/l	32.9 (30.8 - 35.0)	16.8 (15.3 - 18.5)
Low HDL-C (<1.0 mmol/l in men and <1.3 mmol/l in women)	46.9 (44.7 – 49.2)	51.3 (49.1 – 53.5)
Fasting plasma glucose >=6.0 mmol/l	8.7 (7.5 - 10.1)	7.2 (6.2 - 8.4)
BP≥130/85 mmHg or hypertensive on treatment	29.6 (27.6 - 31.7)	23.0 (21.2 – 24.9)
Central obesity (NCEP Criteria)	2.8 (2.1 - 3.6)	8.7 (7.6 - 10.0)
Central obesity (Asian Criteria)	17.1 (15.4 - 18.8)	21.8 (20.1 – 23.7)
Metabolic syndrome (NCEP ATP III criteria)	14.1 (12.6 - 15.8)	12.3 (11.0 – 13.8)
Metabolic syndrome (modified Asian Criteria)	19.2 (17.5 – 21.0)	16.4 (14.9 - 18.1)

We subsequently divided the study population into those without the MS, those who satisfied only the Asian criteria for MS and those who satisfied both the Asian and the NCEP criteria. After 38,157.4 person years of follow up amongst subjects with neither diabetes mellitus nor IHD at baseline, an incident IHD event occurred in 93 subjects. The MS was associated with step-wise increase in the risk of IHD beginning with those who satisfied the modified Asian criteria only to

those who satisfied both sets of criteria (figure 1).

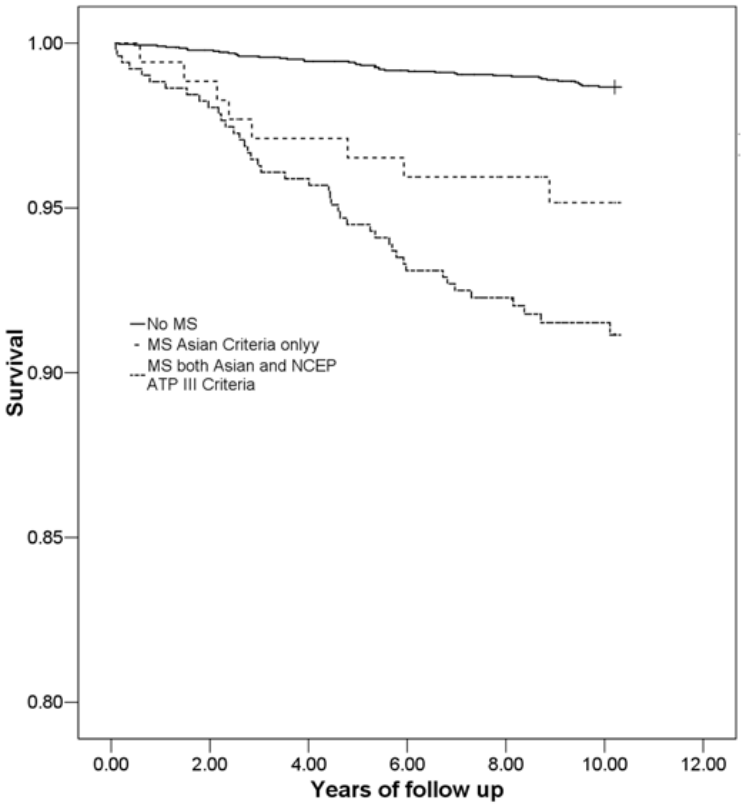


Figure 5. Survival curves for subject who satisfied the NCEP ATP III criteria for the metabolic syndrome (MS NCEP), the modified Asian Criteria (MS Asian) and neither (No MS) in relation to ischemic heart disease. Analyses were carried out using Kaplan Meier analysis.

These associations were observed even after adjustment for age, sex, ethnic group, current cigarette smoking (table 5) with adjusted hazard ratios of 2.13 (95% CI 0.99-4.58) and 3.09 (95% CI: 1.96-4.88) respectively for the modified Asian Criteria alone and the NCEP ATP III criteria. Additional adjustment for serum low density lipoprotein cholesterol concentration did not alter the findings (data not shown).

Table 5. Risk of Ischemic Heart Disease Events associated with the metabolic syndrome amongst non-diabetic participants of the 1992 National Health Survey and the National University of Singapore Heart Study according to the NCEP ATP III criteria and the modified Asian criteria. Hazard ratios were estimated using Cox proportional hazard's regression (stratified by the study from which the data was derived) and adjusted for age, gender, ethnic group and current cigarette smoking.

	Person-years	Number of events	Hazard ratio	95% CI	p-value
No metabolic syndrome	32064.8	42	1.00	-	-
Metabolic syndrome-Asian criteria only	1565.9	8	2.13	0.99,4.58	0.052
Metabolic syndrome (NCEP ATP III criteria)	4526.7	43	3.09	1.96,4.88	<0.005
Total	38157.4	93			

When each set of criteria were applied separately, the hazard ratio for IHD associated with the MS diagnosed according to the Asian criteria was 2.87 (95% CI 1.85-4.44) and that according to the NCEP ATP III criteria was 2.78 (95% CI 1.80-4.31). Based on these hazard ratios and the prevalence of the MS according to each set of criteria found in Table 3, the population attributable risk (PAR) associated with the MS when the Asian criteria are applied is 24.4%. The PAR associated with the MS when the NCEP ATP III criteria are applied is 18.8%. Use of the Asian criteria, as opposed to the NCEP ATP III criteria, to define the MS resulted in an increase in sensitivity from 52.7% to 61.8% with a corresponding decrease in the specificity from 72.8% to 70.3%.

4.2 The role of central obesity in the definition the metabolic syndrome

4.2.1 Study 2: Impact on associations with insulin resistance

Table 6 shows the prevalence of each of the individual features of the metabolic syndrome by gender and ethnic group.

Table 6. Prevalence of individual features of the metabolic syndrome by gender and ethnic group. The 1998 Singapore National Health Survey.

Metabolic features	Men			Women		
	Chinese	Malay	Asian Indian	Chinese	Malay	Asian Indian
	n=1467	n=403	n=307	n=1756	n=445	n=337
	%	%	%	%	%	%
Waist circumference >90 cm in men or >80 cm in women	25.4	32.0	44.6	18.9	43.4	56.4
Fasting plasma glucose >=5.6 mmol/l or treatment for diabetes mellitus.	48.5	56.6	56.0	29.9	46.7	44.5
Systolic blood pressure >=130 mmHg or diastolic blood pressure >= 85 mmHg or treatment for hypertension	36.5	39.7	37.1	20.4	35.5	21.7
Fasting triglyceride >1.7 mmol/l	34.6	45.2	48.5	14.5	24.7	21.1
High density lipoprotein cholesterol < 1.0 mmol/l in men or 1.3 mmol/l in women	20.7	33.0	45.9	23.9	36.2	65.6

The prevalence of the metabolic syndrome defined according to the recommendations of the IDF was 20.2% and that defined by the AHA/NHLBI recommendations was 26.9%. 6.7 percent of the population who did not meet the criteria for central obesity exhibited three or more features of the metabolic syndrome.

Table 7 shows these prevalences by gender and ethnic group. The p-values for the differences in proportions of each group by ethnicity was highly statistically significant ($p<0.005$) both before and after adjustment for age. A greater proportion of Asian Indians exhibited three or more features of the MS which included central obesity. This was observed in both men and women. In contrast, more Malays exhibited three or more risk factors without central obesity. This phenotype was more common in men than in women. The degree of discordance between the AHA/NHLBI and the IDF criteria was greater in Chinese and Malay men than Asian Indian men ($p=0.023$ after age adjustment) and in Chinese than Malay and Asian Indian women ($p<0.005$ after age adjustment).

Table 8 shows the insulin resistance, and other phenotypic characteristics associated with the four mutually exclusive groups defined in the methods section of this manuscript. Individuals in group 3 ($\geq$3 risk factors without central obesity) were indistinguishable from those in group 4 ($\geq$3 risk factors including central obesity) in relation to age, blood pressure, total cholesterol and LDL-cholesterol. However, the latter had higher fasting glucose and insulin resistance and were more likely to have diabetes mellitus. They also had lower TG and higher HDL-C compared to those in group 3. The Framingham risk score was higher in group 3 than group 4.

Table 7. Prevalence of various metabolic groups by gender and ethnic group. The 1998 Singapore National Health survey.

		Group 1 No central obesity and ≤2 risk factors (%)	Group 2 Central Obesity with 0 or 1 risk factor (%)	Group 3 No central obesity but with ≥3 risk factors (%)	Group 4 Central obesity with ≥ other 2 risk factors (%)	Percentage of individuals meeting AHA/NHLBI criteria but not IDF criteria (%)[1]	p-value[2]	p-value[3]
Male	Chinese	64.6	6.6	10.0	18.8	34.6	<0.005	0.021
	Malay	54.8	6.7	13.2	25.3	34.2		
	Asian Indian	45.0	8.5	10.4	36.2	22.4		
Female	Chinese	77.7	6.7	3.4	12.1	22.1	<0.005	<0.005
	Malay	52.6	13.0	4.0	30.3	11.8		
	Asian Indian	41.5	22.0	2.1	34.4	5.7		

[1] group 3/group 3 + group 4

[2] p-value for comparing the distribution of individuals in all four groups by ethnic group estimated using chi-square analysis Analyses were carried out separately for males and females.

[3] p-value for comparing the distribution of individuals in group 3 and group 4 by ethnic group estimated using chi-square analysis Analyses were carried out separately for males and females.

Table 8. Phenotypic characteristic (mean±SD) of various metabolic groups. The 1998 Singapore National Health Survey.

	Group 1 No central obesity and ≤2 risk factors n=3045	Group 2 Central Obesity with 0 or 1 risk factor n=402	Group 3 No central obesity but with ≥3 risk factors n=316	Group 4 Central obesity with ≥ other 2 risk factors n=952	p-value[1]	p-value[2]
Age	35.1 ± 11.4	40.2 ± 10.8	44.7 ± 12.0	46.0 ± 11.6	<0.005	0.43
Body mass index (kg/m^2)	21.5 ± 2.7	27.7 ± 3.8	23.5 ± 2.2	28.5 ± 3.9	<0.005	<0.005
Waist circumference (cm)	73.9 ± 7.6	90.2 ± 9.0	82.4 ± 5.8	93.6 ± 8.0	<0.005	<0.005
Fasting glucose (mmol/L)	5.41 ± 0.99	5.54 ± 1.19	6.50 ± 2.20	6.92 ± 2.57	<0.005	<0.005
SBP (mmHg)	116 ± 14	121 ± 13	134 ± 18	135 ± 19	<0.005	>0.99
DBP (mmHg)	70 ± 10	75 ± 9	82 ± 11	83 ± 11	<0.005	>0.99
Total cholesterol (mmol/L)	5.27 ± 1.00	5.59 ± 1.03	6.00 ± 1.14	6.03 ± 1.13	<0.005	>0.99
Fasting triglyceride (mmol//L)	1.00 ± 1.55	1.17 ± 1.40	2.49 ± 1.54	2.32 ± 1.75	<0.005	<0.005
HDL cholesterol (mmol/L)	1.48 ± 0.36	1.36 ± 0.30	1.05 ± 0.25	1.11 ± 0.28	<0.005	0.01
LDL cholesterol (mmol/L)	3.24 ± 0.89	3.67 ± 0.92	4.03 ± 1.02	4.13 ± 1.04	<0.005	0.66
Insulin resistance	1.26 ± 1.72	2.10 ± 1.72	2.10 ± 1.70	3.32 ± 1.93	<0.005	<0.005
Framingham risk score	3.8 ± 4.9	4.3 ± 4.1	14.3 ± 10.7	12.8 ± 10.0	<0.005	0.002
Diabetes mellitus, %	2.2	3.0	15.2	25.5	<0.005	
Exercise regularly, %	15.9	13.5	20.6	17.3	0.06	
Current smoking, %	13.4	10.7	27.8	16.6	<0.005	
Alcohol consumption, %	38.9	29.9	41.8	32.1	<0.005	

[1] p-value for univariate ANOVA comparing all four groups

[2]p-value for post-hoc test comparing group 3 and group 4 following Bonferroni correction for multiple testing

Table 9 shows the same phenotypic characteristics of individuals who met the criteria for the MS as recommended by the AHA/NHLBI and the IDF. In this table, unlike table 8, the two groups are not mutually exclusive. In fact, those who meet the criteria according to the IDF definition are a subset of those who meet the criteria according to the AHA/NHLBI definition. The two sets of criteria identify individuals with very similar levels of cardiovascular risk factors.

Table 9. Comparison of cardiovascular disease risk factor levels between those with the metabolic syndrome identified by the American Health Association/National Heart Lung and Blood Institute (AHA/NHLBI) criteria and the International Diabetes Federation (IDF) criteria.

Definition of the metabolic syndrome	AHA/NHLBI N=1268	IDF N=952
Age	45.7 (CI 22.3-69.1)	46.0 (CI 22.8-69.2)
Body mass index (kg/m^2)	27.3 (CI 18.9-35.7)	28.5 (CI 20.7-36.3)
Waist circumference (cm)	90.8 (CI 72.8-108.8)	93.6 (CI 77.6-109.6)
Fasting glucose (mmol/L)	6.81 (CI 1.01-12.61)	6.92 (CI 1.78-12.06)
SBP (mmHg)	134 (CI 96-172)	135 (CI 97-173)
DBP (mmHg)	83 (CI 61-105)	83 (CI 61-105)
Total cholesterol (mmol/L)	6.02 (CI 3.76-8.29)	6.03 (CI 5.96-6.10)
Fasting triglyceride (mmol//L)	2.43 (CI -1.25-6.11)	2.32 (CI 1.18-5.84)
HDL cholesterol (mmol/L)	1.10 (CI 0.54-1.66)	1.11 (CI 0.55-1.67)
LDL cholesterol (mmol/L)	4.11 (CI 2.04-6.17)	4.13 (CI 2.05-6.21)
Insulin resistance	2.99 (CI -.087-6.85)	3.32 (CI -0.54-7.18)
Diabetes mellitus, %	22.9 (CI 20.6-25.3)	25.5 (CI 22.9-28.4)
Exercise regularly, %	18.1 (CI 16.1-20.3)	17.3 (CI 15.0-19.8)
Current smoking, %	19.4 (CI 17.5-21.3)	16.6 (CI 14.4-19.1)
Alcohol consumption, %	34.5 (CI 32.0-37.2)	32.1 (CI 29.3-35.2)
Framingham risk score	13.1 (CI -7.3-33.5)	12.8 (CI -7.2-32.8)

CI = 95% Confidence interval of the parameter for the population

4.2.2 study 3: Impact on associations with cardiovascular disease

In this cohort study, there were 4,334 participants after excluding 92 participants with preexisting IHD and 27 with missing data. These comprised 2,546 Chinese, 909 Malays, and 879 Asian Indians. There were 2,087 male subjects and 2,247 female subjects. The mean duration of follow up was 9.6 ± 1.5 years and totaled 41,400 person-years. A total of 135 first IHD events were reported. Table 10 shows that the prevalence of the three central obesity/metabolic syndrome groups were: no metabolic syndrome, 73.8%; central obesity/metabolic syndrome, 17.7%; and no central obesity/ metabolic syndrome, 8.5%. Using the Asian criteria for waist circumference, the prevalence of metabolic syndrome according to the IDF was 17.7% and 26.2% according to the AHA/NHLBI. The prevalence of the three groups was also different among the ethnic groups, with Asian Indians having the highest prevalence of metabolic syndrome in both the presence (28.6%) and absence (9.3%) of central obesity (Table 10).

Table 10 further describes the characteristics of the three central obesity/ metabolic syndrome groups. Compared with those with three or more metabolic syndrome components without central obesity, those with three or more components with central obesity were older, more obese, and had higher blood pressure. In addition, they were more likely to be female and of Malay or Asian-Indian ethnicity. However, in contrast, individuals with three or more metabolic syndrome components without central obesity had higher plasma triglyceride and higher fasting glucose values than those with three or more components with central obesity. Also, in this group, there was a higher proportion of current smokers (25.8%) compared with the group with three or more components with central obesity (15.6%) or no metabolic syndrome (18.1%).

Table 10: Characteristics of study population by central obesity/metabolic syndrome groups. CO=central obesity. Met S=metabolic syndrome

Characteristics	No CO, No Met S	CO, No Met S	CO, Met S	No CO, Met S
N (row %)	2942 (67.9)	258 (6.0)	766 (17.7)	368 (8.5)
Gender[1]				
Males	1407 (47.8)	98 (38.0)	339 (44.3)	243 (66.0)
Females	1535 (52.2)	160 (62.0)	427 (55.7)	125 (34.0)
Ethnic group [1]				
Chinese	1911 (65.0)	106 (41.4)	309 (40.3)	220 (59.8)
Malays	573 (19.5)	64 (24.8)	206 (26.9)	66 (17.9)
Asian Indians	458 (15.6)	88 (34.1)	251 (32.8)	82 (22.3)
Age (yrs) [2]	36.4 (11.9)	41.9 (10.8)	48.8 (12.0)	47.9 (12.2)
Total-Cholesterol (mmol/l) [2]	5.2 (0.9)	5.5 (0.9)	5.9 (1.1)	5.8 (1.1)
LDL-C (mmol/l) [2]	3.4 (0.9)	3.8 (0.9)	3.9 (1.0)	3.8 (1.0)
HDL-C(mmol/l) [2]	1.3 (0.3)	1.1 (0.3)	1.0 (0.3)	0.9 (0.2)
TG	0.9	1.2	1.9	2.2
Fasting glucose (mmol/l) [2]	5.3 (0.9)	5.4 (1.5)	6.5 (2.4)	6.8 (2.8)
Systolic blood pressure (mmHg) [2]	114.2 (15.2)	119.2 (14.2)	137.4 (21.9)	136.3 (20.3)
Diastolic blood pressure (mmHg) [2]	67.1 (10.8)	72.9 (9.7)	81.9 (11.9)	80.9 (11.5)
Waist Circumference (cm) [2]	71.1 (8.1)	89.6 (7.4)	92.6 (8.4)	79.8 (6.6)
BMI (kg/m^2) [2]	21.6 (2.9)	28.3 (3.7)	29.0 (3.9)	23.8 (2.5)
Diabetes Mellitus [1]	41 (1.4)	5 (1.9)	120 (16.7)	59 (14.9)
Current Smoker[1]	549 (18.7)	31 (12.0)	119 (15.6)	95 (25.8)
Alcohol intake[1]	294 (10.0)	22 (8.5)	69 (9.0)	55 (14.9)
Study[1]				
NHS 92	2514 (85.5)	170 (65.9)	434 (56.7)	291 (79.1)
NUHHS	428 (14.5)	88 (34.1)	332 (43.3)	77 (20.9)

[1] categorical variables: numbers and column percentages

[2] continuous variables: means and standard deviation, except for triglyceride (TG) where median values are shown

Table 11 shows the risk of IHD for the three central obesity/metabolic syndrome groups including and excluding individuals with type 2 diabetes. The highest incidence rates were for the central obesity/ metabolic syndrome and no central obesity/metabolic syndrome groups, which included diabetic patients at 8.8 and 9.5 per 1,000 person-years, respectively. Compared with the no metabolic syndrome group, individuals with central obesity/metabolic syndrome and no central obesity/metabolic syndrome had significantly increased risks for IHD with adjusted HRs of 2.8 (95% CI 1.8–4.2) and 2.5 (1.5– 4.0), respectively. A comparison of the central obesity/metabolic syndrome and no central obesity/ metabolic syndrome groups showed no significant difference in risk of IHD between them, with HR 1.0 (95% CI 0.6–1.5) and an absolute rate difference of 0.7 (95% CI: -4.7 to 3.2) per 1,000 person-years).

Table 11: Association of central obesity/metabolic syndrome groups with risk of ischemic heart disease (HR; 95%CI)

	No. of events (%)	Person years	Incidence rate per 1000 py	HR (95%CI)*	HR (95%CI) **
No CO, No Met S	39 (1.3)	28875.9	1.4 (0.9-1.8)	1.0	1.0
CO, No Met S	5 (1.9)	2442.8	2.0 (0.7-4.8)	1.0 (0.4-2.6)	1.0 (0.4-2.6)
CO, Met S	59 (7.7)	6715.9	8.8 (6.6-11.0)	2.6 (1.7-4.1)	2.7 (1.8-4.2)
No CO, Met S	32 (8.7)	3365.9	9.5 (6.5-13.4)	2.7 (1.7-4.5)	2.8 (1.7-4.5)
CO, Met S -vs- No CO, Met S				1.0 (0.6-1.5)	1.0 (0.6-1.5)

* adjusted for age, gender, ethnic group and study

** adjusted age, study, ethnic group, gender, smoking (none/occasional/ex-smoker vs current), alcohol intake (none/occasional vs >=once/mth)

Individuals with the metabolic syndrome using either the IDF or AHA/ NHLBI criteria were found to have an increased risk of IHD (Table 12). The exclusion of diabetic patients did not remarkably change the risk estimates for either the IDF or AHA/NHLBI criteria and adjusted HRs were similar (HR 2.3 [95% CI 1.5–3.6] using both criteria). The AP_E among those with metabolic syndrome according AHA/NHLBI criteria was higher (84%) than the IDF criteria (76%). Similarly, the AP_T was also found to be higher when the AHA/NHLBI criteria were used (57.6%) compared with the IDF criteria (36.4%). Although the AP_E did not change when diabetic patients were excluded from the analyses, the APT was lowered to 48.0 and 28.0% for the AHA/NHLBI and IDF criteria, respectively.

Table 12—Risk of IHD (HR (95% CI) for individuals with the metabolic syndrome according to IDF and AHA criteria

	N of events (%)	Person-year	Incidence rate (per 1,000 person-years)	HR (95% CI) *	HR (95% CI)†
Including diabetic patients					
IDF criteria					
No MetS	76 (2.1)	34,685	2.1 (1.7–2.7)	1.0	1.0
MetS	59 (7.7)	6,716	8.8 (6.6–11.0)	3.9 (2.8–5.5)	2.1 (1.4–3.1)
AHA criteria					
No MetS	44 (1.4)	31,319	1.4 (0.9–1.8)	1.0	1.0
MetS	91 (8.0)	10,082	9.0 (7.2–10.9)	6.3 (44–9.0)	2.7 (1.8–4.0)
Excluding diabetic patients					
IDF criteria					
No MetS	58 (1.7)	32,994	1.8 (1.3–2.2)	1.0	1.0
Met S	38 (6.7)	5, 013	7.6 (5.2-10.0)	4.2 (2.8-6.3)	2.3 (1.5-3.6
AHA criteria					
No Met S	40 (1.3)	30,541	1.3 (0.9-1.7)	1.0	1.0
MetS	56 (5.7)	7466	7.5 (5.5-9.5)	5.6 (3.7-8.4)	2.3 (1.5-3.6)

*Unadjusted HRs. †Adjusted HRs for age, study, ethnic group, sex, LDL cholesterol, smoking (nonsmoker vs. current smoker), and alcohol intake (none/occasional vs. ≥1/month). MetS metabolic syndrome.

4.3 Study 4: Disordered amino acid metabolism and it's associations with insulin resistance.

Table 13 presents demographic and clinical characteristics of the Chinese and Asian-Indian study participants, stratified by insulin resistance. Individuals from the top tertile of insulin resistance in both ethnic groups had HOMA indices 3-4 times higher than those from the lowest tertile. These differences were driven almost exclusively by elevations in fasting insulin levels in the high HOMA groups. Despite our attempts to control for BMI, BMI was slightly higher in individuals with higher levels of IR. Hip circumference was identical between the groups, whereas waist circumference, and consequently, waist-to-hip ratio, differred significantly between high and low HOMA subjects in both ethnic groups. The HDL-C were significantly lower in both groups of Asian Indian subjects than in Chinese subjects, and HDL-C levels were lower in the high versus low HOMA subjects within both ethnic groups, whereas LDL-C levels were unaffected by HOMA tertile or ethnic origin. TG levels were clearly elevated in the high HOMA subjects in both the Chinese and Asian Indian groups. Finally, blood pressure (both systolic and diastolic) increased modestly in the high HOMA versus low HOMA subjects within both ethnic groups.

Table 13. demographic and clinical characteristics (mean±SD) of study subjects by insulin resistance and ethnic group.

	Chinese, Low HOMA (n=97)	Chinese, High HOMA (n=83)	Indian, Low HOMA (n=30)	Indian, High HOMA (n=53)	p-value
Cardiovascular risk factors					
Age	46.82 ± 11.49	51.25 ± 11.35	50.84 ± 11.54	49.54 ± 7.05	0.037
Height	169.42 ± 5.70	169.39 ± 6.85	172.80 ± 6.64	170.54 ± 7.54	0.067
Weight	68.66 ± 7.34	70.48 ± 8.59	72.15 ± 7.57	72.73 ± 8.89	0.018
BMI (body mass index)	23.89 ± 1.94	24.52 ± 2.13	24.16 ± 2.05	24.95 ± 2.10	0.017
Waist	86.69 ± 6.32	90.60 ± 6.99	88.42 ± 12.41	93.18 ± 6.97	0.000006
Hip	99.52 ± 5.27	99.98 ± 5.50	99.11 ± 12.67	101.50 ± 5.95	0.299
WHR (waist-hip-ratio)	0.87 ± 0.05	0.91 ± 0.05	0.89 ± 0.08	0.92 ± 0.05	0.000002
SBP (systolic blood pressure)	129.13 ± 13.06	138.83 ± 18.09	126.42 ± 16.07	133.91 ± 16.04	0.0002

	Chinese, Low HOMA (n=97)	Chinese, High HOMA (n=83)	Indian, Low HOMA (n=30)	Indian, High HOMA (n=53)	p-value
DBP (diastolic blood pressure)	80.41 ± 9.80	84.63 ± 11.62	79.66 ± 10.14	82.56 ± 10.86	0.034
Insulin (mU/L)	3.60 ± 1.07	12.98 ± 6.09	3.53 ± 1.22	14.76 ± 11.57	1.455e-28
Fasting glucose (mmol/L)	4.73 ± 0.50	5.29 ± 0.97	4.76 ± 0.57	5.83 ± 2.33	0.000002
Cholesterol	5.17 ± 0.86	5.35 ± 0.82	5.09 ± 0.90	5.35 ± 0.83	0.292
Triglyceride	1.30 ± 0.61	1.85 ± 0.96	1.38 ± 0.99	1.95 ± 1.22	0.000015
Cholesterol: HDL ratio	4.24 ± 1.02	4.68 ± 1.22	4.98 ± 1.53	5.21 ± 1.13	0.000016
HDL	1.33 ± 0.27	1.26 ± 0.24	1.15 ± 0.30	1.11 ± 0.20	0.000006
LDL	3.25 ± 0.74	3.25 ± 0.74	3.29 ± 0.76	3.33 ± 0.87	0.924
Fasting insulin (HOMA_IR)	0.75 ± 0.22	3.02 ± 1.58	0.74 ± 0.24	3.70 ± 3.02	1.222e-27
C-Reactive Protein (CRP)	3.63 ± 16.07	2.32 ± 3.34	1.85 ± 2.76	5.50 ± 17.39	0.4743
Smoking					
Yes	35.1	42.0	36.7	38.0	
No	64.9	58.0	63.3	62.0	0.826
Hypertension					
Yes	32.0	55.4	33.3	28.3	
No	68.0	44.6	66.7	71.7	0.002
Taking anti-hypertensive medications					
Yes	13.2	25.8	12.0	7.0	
No	86.8	74.2	88.0	93.0	0.047

Table 14 shows the dietary intake and physical activity in these individuals. None of the subjects in this study were vegans. As a proportion of total energy intake, Asian Indians consumed less protein and more saturated fat than Chinese. However, no dietary differences were noted between high and low HOMA individuals in either ethnic group. There was a trend towards less physical activity in individuals with high HOMA that was not statistically significant.

Table 14. Dietary intake and physical activity (mean±SD) in subjects by insulin resistance and ethnic group.

	Chinese, Low HOMA (n=97)	Chinese, High HOMA (n=83)	Indian, Low HOMA (n=30)	Indian, High HOMA (n=53)	p-value
Dietary intake					
Total calories (kcal) consumed per day	2343.17 ± 964.75	2246.71 ± 722.34	2106.34 ± 936.68	2270.09 ± 675.18	0.607
Total fat (g) consumed per day	73.65 ± 39.63	69.14 ± 29.47	67.81 ± 41.04	73.97 ± 23.15	0.716
Total CHO (g) consumed per day	333.75 ± 127.10	322.23 ± 105.88	297.71 ± 122.08	323.61 ± 106.50	0.552
Total protein (g) consumed per day	84.55 ± 37.22	82.39 ± 28.96	70.20 ± 30.71	73.23 ± 22.98	0.061
Total saturated fat (g) consumed per day	29.17 ± 17.30	27.11 ± 11.73	28.41 ± 20.79	31.45 ± 11.41	0.451
Percent energy from fat	27.70 ± 4.95	27.43 ± 5.71	28.25 ± 6.41	29.56 ± 5.39	0.15
Percent energy from carbohydrate	57.50 ± 5.56	57.64 ± 6.89	57.27 ± 5.75	56.76 ± 5.72	0.868
Percent energy from protein	14.50 ± 1.65	14.65 ± 1.69	13.47 ± 2.07	12.92 ± 1.18	4.478e-09
Percent energy from SFA	10.82 ± 2.64	10.67 ± 2.24	11.49 ± 3.39	12.53 ± 3.05	0.001
Total weight of rice & alternatives (g) consumed per day	554.19 ± 231.31	514.51 ± 169.48	404.05 ± 220.46	465.06 ± 221.85	0.004
Starch (g/day)	178.15 ± 76.87	174.72 ± 62.24	172.59 ± 53.71	163.17 ± 68.63	0.656
Sugar (g/day)	122.28 ± 61.48	125.02 ± 83.04	116.92 ± 61.47	130.24 ± 138.89	0.925
Physical activity (kcal per week)	796.74 ± 1116.91	672.071 ± 802.78	932.14 ± 865.12	873.29 ± 956.13	0.537

Table 15 shows the metabolite concentrations in the various groups. Free fatty acid levels did not differ between individuals with and without insulin resistance. The most marked differences were observed amongst the amino acids. Chinese subjects with high HOMA had higher levels of several amino acids than Chinese subjects with low HOMA. These included alanine, proline, valine, leucine/isoleucine, phenylalanine, tyrosine, glutamate/glutamic acid and ornithine. With the exception of phenylalanine, all these differences remained statistically significant when adjusted for age and BMI. Associations in the same direction were

observed in Asian Indians but reached statistical significance only for alanine, tyrosine and glutamate/glutamic acid. We also measured a group of 20 urinary organic acids in all of the subject groups. Lactate and pyruvate levels were increased in urine in the high HOMA subjects of both ethnic groups; these changes were accompanied by highly significant increases in blood lactate levels as a function of HOMA, again in both ethnic groups. Interestingly, isobutyrylglycine was significantly lower in the high HOMA vs low HOMA group in Asian Indians but not in Chinese, and isovalerylglycine showed a similar trend. Methylmalonate, methylsuccinate and hydroxymethylglutarate levels were all higher in urine of Asian Indian compared to Chinese subjects, but did not change significantly with HOMA in either ethnic group. Finally, among 45 acylcarnitine species measured by MS/MS, C10:1 and C8:1-DC were low in insulin resistant individuals, but only in Asian Indians and not in Chinese. C3 and C5 acylcarnitines, previously shown to be elevated in association with the increase in branched-chain amino acids in obese versus lean subjects from the Southeastern United States [58] trended higher in the high HOMA group in both the Asian Indian and Chinese men, but this change did not achieve statistical significance.

Table 15. Metabolite concentrations by insulin resistance and ethnic group. Units of measure—acylcarnitines and amino acids, μM; urinary organic acids, mmol/mol creatinine. Abbreviations--AC, acylcarnitine; DC, dicarboxyl. Analytes that showed statistically significant differences between any 1 group and the others are highlighted in grey.

Metabolites	Chinese, Low HOMA	Chinese, High HOMA	p-value unadj	p-value adj age	p-value adj age bmi	Indian, Low HOMA	Indian, High HOMA	p-value unadj	p-value adj age	p-value adj age bmi	p-value interaction
Amino Acids											
Gly (plasma)	323.530	315.751	0.2302	0.2078	0.283	335.762	320.775	0.1222	0.111	0.100	0.5383
Ala (plasma)	388.547	424.497	0.0024	0.0023	0.0048	414.825	482.272	0.0006	0.0003	0.001	0.1487
Ser (plasma)	132.873	135.764	0.4531	0.5270	0.4536	129.731	120.747	0.1903	0.2266	0.3553	0.1090
Pro (plasma)	170.005	197.519	0.0002	0.0002	0.0007	196.922	204.526	0.4573	0.5057	0.6961	0.1234
Val (plasma)	285.194	309.05	0.005	0.0023	0.0044	279.868	293.943	0.2985	0.3565	0.4232	0.5302
Leu/Ile (plasma)	202.756	216.067	0.0071	0.0110	0.0321	193.412	202.959	0.1545	0.1903	0.2131	0.6633
Met (plasma)	31.186	31.830	0.4376	0.4145	0.4917	31.119	32.346	0.3257	0.3518	0.1724	0.698
His (plasma)	77.644	75.100	0.0946	0.1298	0.0843	80.126	75.586	0.0535	0.0606	0.0732	0.4708
Phe (plasma)	69.67	72.994	0.0202	0.0482	0.0879	67.701	71.542	0.0818	0.068	0.0555	0.8423
Tyr (plasma)	69.006	77.855	1.3e-06	2.7e-06	2.3e-05	69.014	80.587	0.0002	0.0002	0.0002	0.4136
Asx (plasma)	69.484	67.357	0.2545	0.3847	0.4303	66.071	68.197	0.3559	0.3194	0.4772	0.1857
Glx	95.52	113.093	2.2e-05	2.9e-5	0.0001	94.105	111.714	0.0142	0.0153	0.0196	0.9963

Metabolites	Chinese, Low HOMA	Chinese, High HOMA	p-value unadj	p-value adj age	p-value adj age bmi	Indian, Low HOMA	Indian, High HOMA	p-value unadj	p-value adj age	p-value adj age bmi	p-value interaction
(plasma)											
Orn (plasma)	103.063	118.721	0.0005	0.0009	0.0017	100.578	112.372	0.0747	0.0779	0.1157	0.6289
Cit (plasma)	34.723	35.590	0.4822	0.6875	0.7016	37.376	36.412	0.6315	0.7592	0.6435	0.4255
Arg (plasma)	79.464	80.861	0.6569	0.8501	0.6616	84.942	78.014	0.2034	0.2056	0.2774	0.1643
Plasma Acylcarnitines											
C2 AC (plasma)	8.507	8.262	0.5331	0.2267	0.1923	8.486	8.635	0.7995	0.7468	0.6258	0.5800
C3 AC (plasma)	0.549	0.555	0.8553	0.9586	0.9467	0.527	0.585	0.1975	0.1829	0.2350	0.3181
C4/Ci4 AC (plasma)	0.261	0.286	0.1668	0.3529	0.2467	0.274	0.253	0.5488	0.6016	0.7340	0.2019
C5:1 AC (plasma)	0.051	0.054	0.5427	0.8347	0.7399	0.050	0.051	0.8759	0.852	0.6257	0.7965
C5's AC (plasma)	0.138	0.146	0.3412	0.3471	0.6472	0.144	0.154	0.4555	0.5609	0.6962	0.8458
C4-OH AC (plasma)	0.042	0.042	0.8826	0.7846	0.6750	0.040	0.038	0.6273	0.7254	0.7278	0.6453
C6 AC (plasma)	0.026	0.021	0.5336	0.5105	0.5011	0.024	0.024	0.9901	0.9569	0.9081	0.7427
C5-OH/C3-DC AC (plasma)	0.054	0.053	0.8263	0.6604	0.4418	0.051	0.055	0.6625	0.6075	0.7061	0.6147
Ci4-DC/C4-DC AC (plasma)	0.052	0.054	0.7213	0.8363	0.8853	0.052	0.059	0.4444	0.437	0.3475	0.6838

Metabolites	Chinese, Low HOMA	Chinese, High HOMA	p-value unadj	p-value adj age	p-value adj age bmi	Indian, Low HOMA	Indian, High HOMA	p-value unadj	p-value adj age	p-value adj age bmi	p-value interaction
C8:1 AC (plasma)	0.154	0.164	0.5799	0.6664	0.9199	0.181	0.187	0.8169	0.6289	0.6370	0.9172
C8 AC (plasma)	0.27	0.249	0.2218	0.2271	0.2195	0.253	0.216	0.3189	0.3311	0.5026	0.6639
C5-DC AC (plasma)	0.056	0.051	0.1705	0.2182	0.2372	0.069	0.061	0.2007	0.2125	0.3213	0.6723
C6:1-DC/C8:1-OH AC (plasma)	0.03	0.029	0.5302	0.4178	0.398	0.041	0.037	0.2806	0.285	0.2154	0.5651
C6-DC AC (plasma)	0.092	0.083	0.0698	0.0570	0.0558	0.085	0.09	0.5354	0.4697	0.3817	0.1292
C10:3 AC (plasma)	0.065	0.064	0.8946	0.8134	0.6536	0.097	0.102	0.7453	0.6273	0.5653	0.7046
C10:2 AC (plasma)	0.023	0.020	0.4707	0.4054	0.5181	0.019	0.035	0.0499	0.0521	0.0580	0.0328
C10:1 AC (plasma)	0.219	0.199	0.0722	0.0704	0.1002	0.241	0.189	0.006	0.0077	0.0192	0.1363
C10 AC (plasma)	0.430	0.390	0.1744	0.1982	0.1890	0.409	0.350	0.3006	0.3106	0.4489	0.7468
C7-DC AC (plasma)	0.008	0.008	0.9802	0.6381	0.6420	0.006	0.014	0.0799	0.0970	0.1017	0.0786
C8:1-DC AC (plasma)	0.035	0.033	0.4494	0.3643	0.2630	0.046	0.039	0.0435	0.0548	0.0836	0.1427
C10-OH/C8-DC AC (plasma)	0.077	0.068	0.0433	0.0370	0.0255	0.069	0.076	0.2978	0.2445	0.1474	0.0475
C12:1 AC	0.167	0.149	0.0613	0.0200	0.0191	0.164	0.155	0.5913	0.6423	0.8312	0.6116

Metabolites	Chinese, Low HOMA	Chinese, High HOMA	p-value unadj	p-value adj age	p-value adj age bmi	Indian, Low HOMA	Indian, High HOMA	p-value unadj	p-value adj age	p-value adj age bmi	p-value interaction
(plasma)											
C12 AC (plasma)	0.165	0.148	0.0811	0.0755	0.0807	0.182	0.166	0.3990	0.4187	0.6416	0.9505
C12-OH/C10-DC AC (plasma)	0.014	0.015	0.3903	0.7655	0.7375	0.014	0.016	0.1951	0.1625	0.1206	0.4378
C14:2 AC (plasma)	0.060	0.055	0.2082	0.1906	0.3180	0.063	0.057	0.2484	0.2798	0.5638	0.7328
C14:1 AC (plasma)	0.115	0.101	0.0529	0.0319	0.0373	0.112	0.108	0.7825	0.8094	0.9245	0.4432
C14 AC (plasma)	0.045	0.044	0.8541	0.798	0.8634	0.052	0.057	0.2683	0.2386	0.1518	0.3009
C14:1-OH/C12:1-DC AC (plasma)	0.025	0.024	0.5352	0.2589	0.3388	0.024	0.027	0.2556	0.2388	0.1823	0.2051
C14-OH/C12-DC AC (plasma)	0.014	0.016	0.0833	0.1532	0.1584	0.015	0.015	0.9540	0.9253	0.8149	0.3716
C16:2 AC (plasma)	0.010	0.009	0.2994	0.1677	0.1580	0.011	0.010	0.1998	0.2541	0.3163	0.6676
C16:1 AC (plasma)	0.027	0.025	0.1187	0.0257	0.0227	0.029	0.027	0.5191	0.5959	0.7885	0.8535
C16 AC (plasma)	0.143	0.142	0.6874	0.4737	0.3244	0.138	0.148	0.2255	0.2023	0.1486	0.1899
C16:1-OH/C14-DC AC (plasma)	0.0102	0.0096	0.3496	0.1895	0.0976	0.0103	0.0108	0.6296	0.5898	0.6627	0.3520

Metabolites	Chinese, Low HOMA	Chinese, High HOMA	p-value unadj	p-value adj age	p-value adj age bmi	Indian, Low HOMA	Indian, High HOMA	p-value unadj	p-value adj age	p-value adj age bmi	p-value interaction
C16-OH/C14-DC AC (plasma)	0.00759	0.00736	0.6429	0.4812	0.4459	0.00928	0.00855	0.4824	0.5487	0.5074	0.6852
C18:2 AC (plasma)	0.078	0.076	0.5949	0.401	0.4695	0.080	0.079	0.8162	0.9384	0.7658	0.9542
C18:1 AC (plasma)	0.157	0.159	0.8048	0.8739	0.7325	0.144	0.158	0.1441	0.1036	0.0537	0.2411
C18 AC (plasma)	0.062	0.064	0.3985	0.6536	0.6888	0.0612	0.0613	0.9815	0.9857	0.7807	0.6571
C18:2-OH AC (plasma)	0.0149	0.0131	0.1071	0.0129	0.0154	0.0114	0.0118	0.807	0.7348	0.7805	0.2753
C18:1-OH/C16:1-DC AC (plasma)	0.00899	0.00853	0.4023	0.3576	0.2685	0.009	0.008	0.1631	0.1897	0.2515	0.5086
C18-OH/C16-DC AC (plasma)	0.0101	0.0106	0.3725	0.6717	0.6111	0.0077	0.011	0.0013	0.0011	0.0002	0.0162
C20:4 AC (plasma)	0.0130	0.0124	0.4483	0.5368	0.4253	0.0138	0.0134	0.7186	0.7608	0.8755	0.9005
C20 AC (plasma)	0.007	0.008	0.2645	0.4466	0.4493	0.006	0.008	0.1629	0.1225	0.1596	0.2786
C20:1-OH/C18:1-DC AC (plasma)	0.009	0.010	0.1892	0.2868	0.2966	0.0105	0.0101	0.6800	0.7745	0.7896	0.2878
C20-OH/C18-	0.0112	0.0109	0.6093	0.4491	0.5465	0.0110	0.0114	0.7230	0.7217	0.4238	0.5504

Metabolites	Chinese, Low HOMA	Chinese, High HOMA	p-value unadj	p-value adj age	p-value adj age bmi	Indian, Low HOMA	Indian, High HOMA	p-value unadj	p-value adj age	p-value adj age bmi	p-value interaction
DC AC (plasma)											
C22 (plasma)	0.0069	0.0078	0.1179	0.1421	0.1743	0.0071	0.0079	0.2589	0.2662	0.3579	0.8842
Conventional metabolites											
Lactate (plasma)	2.493	2.895	7.5e-06	3.5e-06	1.1e-05	2.244	3.022	2.4e-08	1.6e-08	5.7e-08	0.017
Free fatty acids (plasma)	0.396	0.425	0.2354	0.3687	0.4965	0.381	0.423	0.2898	0.2566	0.2073	0.7687
total ketones (plasma)	75.258	67.145	0.4832	0.2755	0.2726	59.133	51.660	0.5188	0.5306	0.7681	0.9733
3-OH butyrate (plasma)	52.804	46.277	0.4505	0.2525	0.2683	40.867	35.585	0.5272	0.5466	0.8147	0.9302
Urinary Organic Acids											
Lactate (urine)	7.294	9.07	0.0078	0.0233	0.02	6.904	8.795	0.0143	0.0099	0.013	0.9188
Pyruvate (urine)	4.392	5.287	0.0013	0.0019	0.0037	4.362	5.988	0.0543	0.0572	0.0446	0.2927
Methylmalonate (urine)	1.037	0.945	0.361	0.1494	0.1437	1.562	1.528	0.9428	0.9828	0.9769	0.8690
Ethylmalonate (urine)	4.040	4.178	0.5062	0.4475	0.4734	4.585	4.391	0.6375	0.6748	0.6582	0.4277
Succinate (urine)	2.17	1.893	0.2673	0.1384	0.1941	1.802	1.746	0.8316	0.9249	0.7522	0.5984
Methylsuc	0.679	0.716	0.2686	0.6453	0.6099	0.9244	0.785	0.1387	0.1475	0.1448	0.0288

Metabolites	Chinese, Low HOMA	Chinese, High HOMA	p-value unadj	p-value adj age	p-value adj age bmi	Indian, Low HOMA	Indian, High HOMA	p-value unadj	p-value adj age	p-value adj age bmi	p-value interaction
cinate (urine)											
Fumarate (urine)	0.735	0.791	0.2147	0.2527	0.2445	0.766	0.814	0.6025	0.5077	0.4839	0.9285
Isobutyrylglycine (urine)	0.527	0.511	0.5862	0.4722	0.7091	0.634	0.456	0.0141	0.0153	0.0084	0.0165
Glutarate (urine)	0.506	0.690	0.1310	0.2468	0.1947	0.506	0.385	0.1150	0.1119	0.0785	0.1140
Butyrylglycine (urine)	0.608	0.643	0.4926	0.539	0.3933	0.631	0.617	0.8558	0.8876	0.8561	0.5991
Isovalerylglycine (urine)	0.415	0.438	0.347	0.3494	0.1975	0.438	0.355	0.0715	0.0655	0.0627	0.0291
Hydroxy-3-methylglutarate (urine)	2.643	2.745	0.3128	0.3477	0.282	3.067	2.965	0.6893	0.7307	0.6532	0.3728
Malate (urine)	1.443	1.574	0.5011	0.918	0.4734	1.368	1.812	0.1789	0.1613	0.6582	0.3948
Adipate (urine)	0.824	1.024	0.0098	0.0159	0.0287	1.096	1.106	0.9424	0.9319	0.7929	0.2045
□-Ketoglutarate (urine)	6.995	8.057	0.058	0.1302	0.1912	8.496	8.86	0.6866	0.6605	0.6122	0.5025
Hexanoylglycine (urine)	0.264	0.261	0.8765	0.6605	0.6963	0.238	0.253	0.6288	0.6219	0.5635	0.6589
Suberate (urine)	0.824	1.091	0.0006	0.0009	0.0017	1.01	1.103	0.3353	0.3325	0.4182	0.1933

Metabolites	Chinese, Low HOMA	Chinese, High HOMA	p-value unadj	p-value adj age	p-value adj age bmi	Indian, Low HOMA	Indian, High HOMA	p-value unadj	p-value adj age	p-value adj age bmi	p-value interaction
Orotate (urine)	0.654	0.591	0.1722	0.2615	0.2160	0.520	0.560	0.5201	0.4934	0.5718	0.2071
Homovanillate (urine)	2.045	1.982	0.4623	0.4728	0.4871	2.340	2.148	0.3376	0.3950	0.4545	0.4945
Citrate (urine)	176.553	177.057	0.9652	0.8174	0.8238	185.415	175.078	0.6089	0.6954	0.6845	0.6249

Table 16 shows the hormonal and inflammatory profile of the various groups. In both ethnic groups, high HOMA was associated with elevated plasma leptin and reduced adiponectin, but in addition, leptin levels were clearly elevated in the Asian Indians compared to the Chinese regardless of HOMA group. IGF-1 levels were negatively associated with HOMA in Chinese, but this was no longer statistically significant after adjustment for age and BMI. More strikingly, IGF-1 levels were higher in Chinese than in Indian subjects irrespective of the degree of IR. IGFBP1 levels were sharply decreased as a function of HOMA in both ethnic groups, but overall levels were not different between Chinese and Asian Indian subjects. Among cytokines, TNFα levels were elevated in high HOMA versus low HOMA tertiles, but these changes only reached statistical significance for Chinese subjects and were attenuated after adjustment for age and BMI.

Table 16. Hormone and cytokine profiles of subjects by insulin resistance and ethnic group.

Hormones/Cytokines	Chinese, Low HOMA	Chinese, High HOMA	p-value unadj	p-value adj age	p-value adj age bmi	Indian, Low HOMA	Indian, High HOMA	p-value unadj	p-value adj age	p-value adj age bmi	p-value interaction
GM-CSF	9.109	2.648	0.2773	0.2767	0.3565	0.527	1.017	0.6109	0.5973	0.7298	0.4406
IFN-□	2.174	1.736	0.2697	0.262	0.3151	1.888	2.028	0.7549	0.7071	0.8696	0.3884
IL-10	21.980	7.479	0.3180	0.3079	0.3969	3.903	6.625	0.6482	0.6308	0.7932	0.4402
IL-12p70	29.336	11.284	0.2572	0.2609	0.3366	7.762	23.939	0.2271	0.2395	0.2448	0.1829
IL-1□	0.483	0.186	0.1328	0.1582	0.1437	0.459	0.244	0.2869	0.3368	0.2777	0.8019
IL-2	4.351	3.864	0.7831	0.8086	0.9049	6.275	7.286	0.7985	0.8043	0.6251	0.6902
IL-6	3.225	1.774	0.5495	0.3109	0.4088	1.285	1.460	0.4632	0.3469	0.3608	0.6578
IL-8	3.594	3.021	0.4510	0.3163	0.2199	4.629	7.360	0.5316	0.5398	0.5574	0.2912
TNF-□	4.373	4.835	0.0336	0.1736	0.1515	4.881	5.342	0.4999	0.3988	0.3694	0.9964
Leptin	6.767	10.531	7.9e-11	1.6e-10	1.6e-09	10.291	14.228	0.0143	0.0147	0.0437	0.8964

Hormones/Cytokines	Chinese, Low HOMA	Chinese, High HOMA	p-value unadj	p-value adj age	p-value adj age bmi	Indian, Low HOMA	Indian, High HOMA	p-value unadj	p-value adj age	p-value adj age bmi	p-value interaction
IGF1	204.228	185.466	0.0410	0.2625	0.5032	173.232	161.482	0.2467	0.1632	0.1339	0.6478
IGFBP1	23581.284	17016.292	0.0066	0.0002	0.0013	24548.879	15555.552	0.0230	0.0319	0.0593	0.5867
IGFBP2	1074002.9	909345.3	0.2711	0.0666	0.1282	1106115.4	695282.46	0.0127	0.0172	0.0270	0.3276
IGFBP3	990534.53	894708.08	0.0506	0.0843	0.1371	857774.02	877290.3	0.7730	0.8308	0.9015	0.1842
Adiponectin	7670.0674	6338.04	0.0074	0.0007	0.0056	8808.630	6334.192	0.0024	0.0043	0.0048	0.2123
IGF1:IGFBP3 ratio	0.000247	0.000231	0.5284	0.5749	0.5567	0.000233	0.000202	0.2108	0.2208	0.3120	0.7040

4.4 Study 5: Genetic variants at the APOA1/C3/A4/A5 locus and their role in the pathogenesis of dyslipidemia.

Table 17 shows the the characteristics of the study population.

Table 17. Clinical characteristics of study population.

	Males (n=1305)	Females (n=1585)
Age (Years)	37.6 ± 12.0	37.4 ± 11.9
Triglyceride (mmol/L)[a]	1.39 (0.31-11.78)	1.01 (0.29-10.79)
HDL cholesterol (mmol/L)	1.26 ± 0.31	1.56 ± 0.36
LDL cholesterol (mmol/L)	3.53 ± 0.94	3.23 ± 0.92
Total cholesterol (mmol/L)	5.50 ± 1.03	5.31 ± 1.03
Waist Circumference (cm)	83.8 ± 9.77	72.9 ± 8.40
BMI (kg/m^2)	23.4 ± 3.68	22.0 ± 3.56
Waist-to-hip ratio	0.87 ± 0.06	0.78 ± 0.05
Fasting glucose (mmol/L)	5.64 ± 0.86	5.42 ± 1.04
Systolic blood pressure (mmHg)	124.8 ± 14.6	116.6 ± 16.0
Diastolic blood pressure (mmHg)	77.3 ± 10.6	70.4 ± 10.7

Data are means ± SD, unless otherwise stated.

[a] Geometic mean and range shown as distribution was skewed.

Table 18 shows the allele frequencies of the polymorphisms studied along with an assessment of deviation from Hardy-Weinberg Equilibrium. A total of 62 SNPs were genotyped. 28 SNPs with mean allele frequency < 0.01 (23 in total) or extreme deviations from Hardy Weinberg Equilibrium (5 in total)

were not included in any further analyses. This left a total of 34 SNPs that were included for association testing.

Table 18. Single Nucleotide polymorphisms genotyped in this study. SNPs highlighted in grey were not considered in subsequent analyses.

	Rs#	HWE p-value	MAF
Apolipoprotein A5			
1	rs2266788 (1259T>C)	0.001	0.184
2	rs2075291	0.737	0.059
3	rs2072560 (+476G>A)	0.001	0.184
4	rs3135506 (56C>G)	0.0319	0.193
5	rs651821 (-3A>G)	1	0.001
6	rs662799 (-1131T>C)	0.102	27.6
Apolipoprotein A4			
8	rs675 (Thr347Ser)	1	0.0003
9	rs5109	0.1315	0.005
10	rs5108	1	0.001
11	rs5107	1	0.001
12	rs5106	1	0.0005
13	rs2238008	0.0099	0.001
14	rs5105	1	0.0002
15	rs12721043	1	0.001
16	rs2234668	0.0661	0.004
17	rs5104 (Hinc II)	0.8107	0.308
18	rs6413456	0.6866	0.034
19	rs6413455	1	0
20	rs5101	1	0.001
21	rs5100	0.8899	0.365
22	rs5099	9x10-4	0.004
23	rs5098	1x10-5	0.007
24	rs5096	0.6941	0.374
25	rs5095	3x10-4	0.003
26	rs5094	0.5183	0.058
27	rs2239013	0.367	0.039
28	rs5093	0.0791	0.213
29	rs5092	0.2343	0.372
30	rs12721041	0.081	0.004
31	rs5091	0.0011	0.001

32	rs5090	1	0.002
33	rs5089	1x10-20	0.012
Apolipoprotein C3			
34	rs33989105 (-482)	0.4852	0.421
35	rs35523410 (-455)	0.2572	0.434
36	rs618354	1	0.0002
37	rs2070669	3x10-04	0.443
38	rs2070668	0.6497	0.45
39	rs4520 (1100 C>T)	0.5693	0.409
40	rs5134	7x10-33	0.145
	Rs#	HWE p-value	MAF
41	rs5132	0.6985	0.081
42	rs5130	0.1635	0.425
43	rs5128 (Sst I)	1	0.286
44	rs4225	1.2x10-31	0.256
Apolipoprotein A1			
45	rs12721025	0.6986	0.037
46	rs5081	0.5369	0.079
47	rs5080	1	0
48	rs5079	1	0
49	rs5077	1	0
50	rs5076	1x10-5	0.05
51	rs5075	1	0
52	rs17243102	0.2913	0.332
53	rs17243109	0.8244	0.039
54	rs5073	1x10-20	0.012
55	rs5072	0.2745	0.29
56	rs2070665	0.141	0.292
57	rs17249477	0.6337	0.036
58	rs5070 (317 C>T)	0.4283	0.285
59	rs670 (-75 G>A)	0.0043	0.271
60	rs17243123	0.0234	0.042
61	rs12718467	0	0.423
62	rs12691374	1	0.001

None of the SNPs showed a statstically significant association with either total cholesterol or LDL-cholesterol. However, multiple SNPs across all 4 loci showed statistically significant associations with both triglyceride and HDL-cholesterol (tables 19 and 20). The strongest associations for both TG an HDL-C were at the APOA5 locus. Since 4 of the 5 SNPs that showed a statistically significant association with TG are know to be in strong linkage disequilibrium in both our population and in populations of European ancestry. These are rs2266788, rs2072560, rs651821 and rs662799 and the rare alleles for these polymorphisms are present on haplotype*2 described in the previous studies[79, 119-121]. In addition, rs2075291 which had been identified in association with TG in Chinese subjects in the US [82] and in Taiwan also showed a strong association with both TG and HDL.

Figure 6 shows the pattern of linkage disequilibrium for the SNPs that showed the strongest associations with TG. It can be seen that that the polymorphisms that show a statistically significant association with triglyceride represent 3 blocks of relatively high LD, corresponding to the APOA5, APOA4 and APOA1/C3 loci. In the apoA5 block, it was also noted that rs2075291 showed a strong association with TG, but the degree of LD between other SNPs within that block was relatively low. In particular, the R2 between this SNP and rs662799, the SNP that shows the strongest association with TG and HDL-C in this study, was only 0.14. To test the hypothesis that multiple variants at these loci contribute to the variation of TG and HDL in this population, we carried out additional analyses adjusting for rs662799 (the top hit from the haplotype previously associated with TG) as well as rs2075291. We found that adjustment for rs662799 attenuated the association between all other SNPs and TG such that none of the SNPs showed a statistically significant association with TG, except for rs662799. Adjustment for rs2075291 also attenuated the associations between other SNPs and TG, although may remained statistically significant. Interestingly, adjustment for rs2075192 significantly attenuated the association between rs662799 and TG (the p-value increased from 9.6 x 10-19 in the unadjusted analysis to 7.1 x 10-11 in the adjusted analysis).

Table 19. Associations between SNPs at the APOA1/C3/A4/A5 locus and triglycride amongst Chinese in NHS98. The numbering of the SNPs is kept in line with table 18 even though SNPs with allele frequencies <0.01 have been removed from the table.

Apolipoprotein A5		BETA*	Unadjusted	Adjusted for rs2075291	Adjusted for rs662799
1	rs2266788	0.11	**$1.3x10^{-7}$**	$4.9x10^{-9}$	0.998
2	rs2075291	0.20	**$3.2x10^{-11}$**	NA	$7.1x10^{-11}$
3	rs2072560	0.10	**$5.7x10^{-9}$**	$6.7x10^{-11}$	0.007
4	rs3135506 (56C>G)	-0.26	0.248	0.183	0.211
6	rs662799 (-1131T>C)	0.14	**$9.6x10^{-19}$**	7.1x10-^{11}	NA
Apolipoprotein A4					
17	rs5104 (Hinc II)	0.09	**$5.6x10^{-8}$**	$2.2x10^{-6}$	0.005
18	rs6413456	-0.08	0.493	0.676	0.869
21	rs5100	0.06	**$3.2x10^{-5}$**	$1.9x10^{-4}$	0.171
24	rs5096	0.06	**$3.3x10^{-5}$**	$2.8x10^{-4}$	0.145
26	rs5094	-0.01	0.701	0.902	0.701
27	rs2239013	-0.02	0.589	0.619	0.932
28	rs5093	-0.03	0.143	0.037	0.696
29	rs5092	0.07	**$1.7x10^{-6}$**	$2.2x10^{-5}$	0.044
Apolipoprotein C3					
34	rs33989105 (-482)	-0.01	0.388	0.529	0.607
35	rs35523410 (-455)	0.01	0.534	0.617	0.551
37	rs2070669	-0.04	0.017	0.002	0.32
38	rs2070668	-0.03	0.045	0.001	0.977
39	rs4520 (1100 C>T)	-0.05	0.003	0.003	0.454
41	rs5132	-0.05	0.08	0.21	0.433
42	rs5130	0.04	0.018	0.002	0.394
43	rs5128 (Sst I)	0.07	**$8x10^{-6}$**	$1.3x10^{-6}$	0.033
Apolipoprotein A1					
45	rs12721025	-0.02	0.693	0.956	0.879
46	rs5081	-0.05	0.067	0.187	0.457
50	rs5076	-0.09	0.01	0.037	0.021
52	rs17243102	0.05	0.001	0.001	0.417
53	rs17243109	0.00	0.959	0.756	0.66
55	rs5072	0.07	**$2.4x10^{-5}$**	$9.5x10^{-6}$	0.077
56	rs2070665	0.06	**$2.1x10^{-4}$**	$6.7x10^{-5}$	0.117
57	rs17249477	-0.06	0.15	0.303	0.532
58	rs5070 (317 C>T)	-0.04	0.009	0.009	0.433
59	rs670 (-75 G>A)	-0.01	0.609	0.326	0.581
60	rs17243123	-0.11	0.003	0.01	0.006
61	rs12718467	0.09	0.003	0.004	0.017

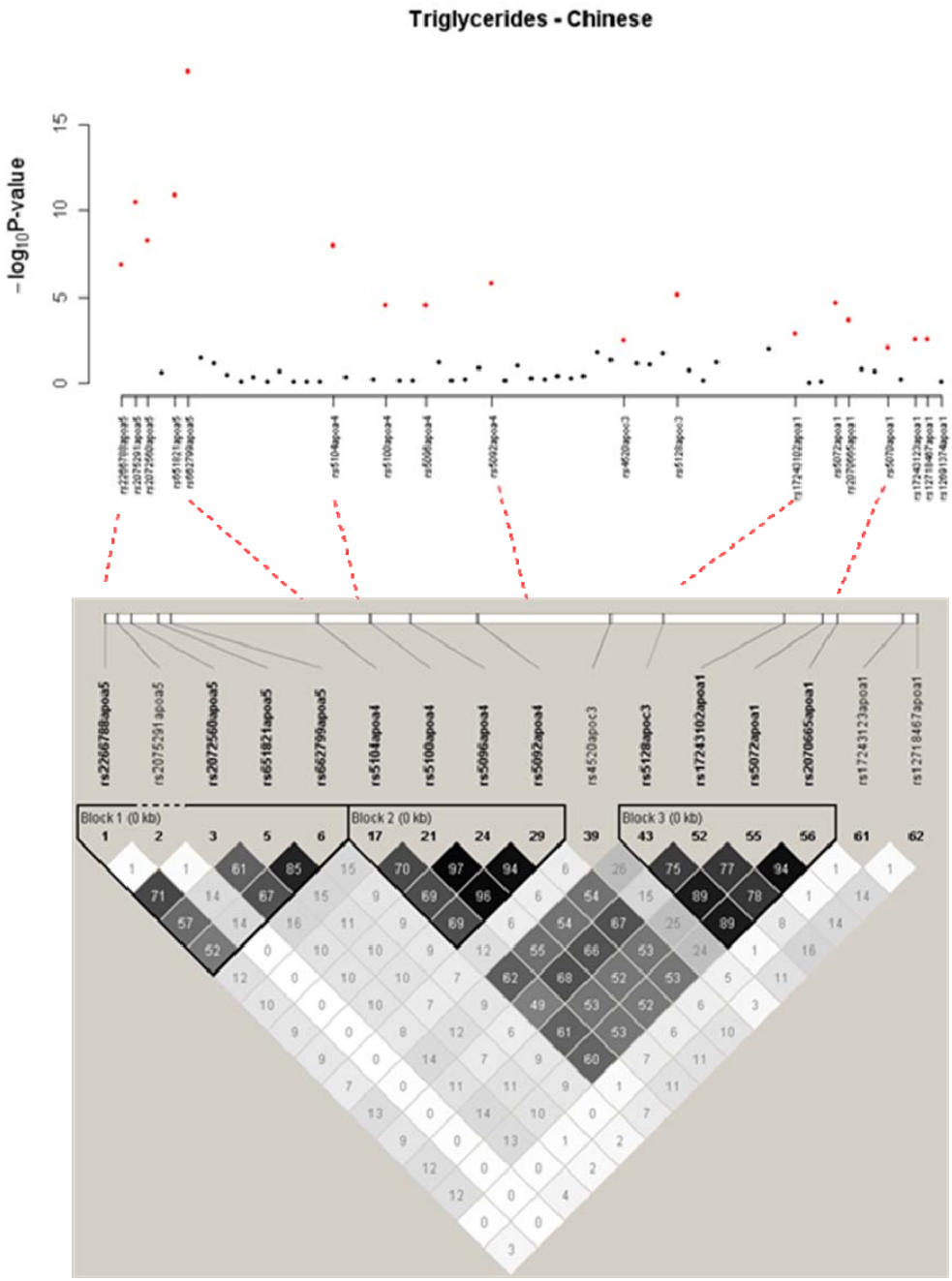


Figure 6. Pattern of linkage disequilibrium for the SNPs at the APOA1/C3/A4/A5 locus that show the strongest associations with TG in the Chinese population in NHS98.

The associations with HDL-C were a little different (table 20 and figure 7). As with TG, the strongest associations for HDL-C were with SNPs at the APOA5 locus. However, SNPs that showed an association with HDL-C were generally found to involve only 2 haplotype blocks (in the APOA5 and APOA1 loci) with effectively no evidence of linkage disequilibrium between them. Adjustment for 2 SNPs at the APOA5 locus has little impact on the association between SNPs in APOA1 and HDL-C.

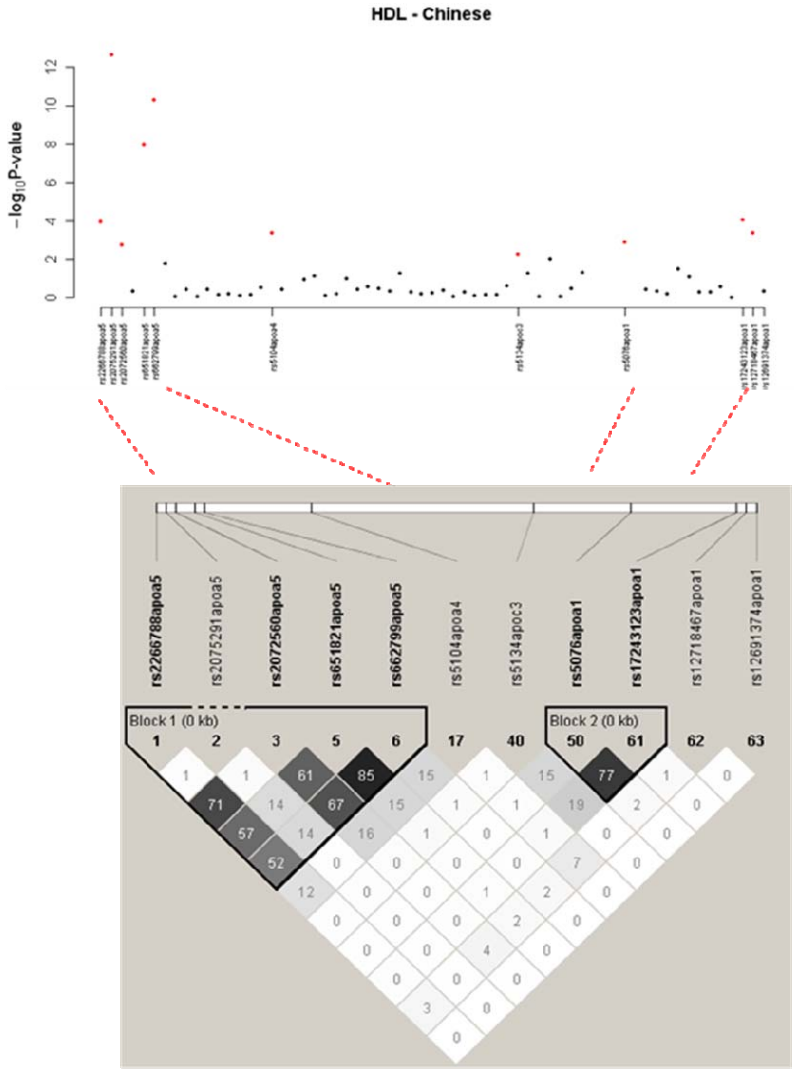


Figure 7. Pattern of linkage disequilibrium for the SNPs at the APOA1/C3/A4/A5 locus that show the strongest associations with HDL-C in the Chinese population in NHS98.

Table 20. Associations between SNPs at the APOA1/C3/A4/A5 locus and triglycride amongst Chinese in NHS98. The numbering of the SNPs is kept in line with table 18 even though invariant SNPs have been removed from the table.

Apolipoprotein A5		BETA	Unadjusted	Adjusted for rs2075291	Adjusted for rs662799
1	rs2266788	-0.05	**$3.3x10^{-4}$**	5.2x10-5	0.831
2	rs2075291	-0.14	**$8.2x10^{-11}$**	NA	1.9x10-6
3	rs2072560	-0.03	0.009	0.001	0.002
4	rs3135506 (56C>G)	0.12	0.447	0.34	0.406
6	rs662799 (-1131T>C)	-0.06	**$2.4x10^{-8}$**	0.005	NA
Apolipoprotein A4					
7	rs9282601	-0.04	0.019	0.024	0.009
17	rs5104 (Hinc II)	-0.04	0.001	0.003	0.189
18	rs6413456	0.05	0.536	0.776	0.549
21	rs5100	-0.02	0.124	0.178	0.608
24	rs5096	-0.01	0.16	0.271	0.589
25	rs5095	-0.10	0.258	0.259	0.441
26	rs5094	0.02	0.286	0.47	0.682
27	rs2239013	-0.02	0.351	0.434	0.234
28	rs5093	0.01	0.502	0.098	0.942
29	rs5092	-0.02	0.103	0.191	0.767
Apolipoprotein C3					
34	rs33989105 (-482)	0.00	0.863	0.853	0.191
35	rs35523410 (-455)	0.01	0.314	0.279	0.044
37	rs2070669	0.01	0.605	0.231	0.593
38	rs2070668	0.00	0.769	0.153	0.264
39	rs4520 (1100 C>T)	0.01	0.294	0.363	0.747
41	rs5132	0.03	0.066	0.152	0.204
42	rs5130	0.00	0.749	0.72	0.164
43	rs5128 (Sst I)	-0.03	0.021	0.01	0.427
Apolipoprotein A1					
45	rs12721025	-0.03	0.325	0.245	0.287
46	rs5081	0.04	0.046	0.143	0.204
50	rs5076	0.08	0.001	0.002	0.001
52	rs17243102	-0.01	0.396	0.27	0.424
53	rs17243109	-0.02	0.404	0.296	0.312
55	rs5072	-0.02	0.043	0.018	0.749
56	rs2070665	-0.02	0.096	0.055	0.993
57	rs17249477	0.02	0.442	0.683	0.752
58	rs5070 (317 C>T)	0.01	0.36	0.488	0.737
59	rs670 (-75 G>A)	0.00	0.987	0.609	0.965
60	rs17243123	0.10	**$1.6x10^{-4}$**	0.001	1.8x10-4
61	rs12718467	-0.08	**$3.7x10^{-4}$**	4.9x10-4	0.003

We also tested the hypothesis that these SNPs identified might modulate the association between obesity and TG or HDL-C. We only examined interactions for SNPs that showed the greatest independent effects on the traits of interest. For TG, we examined interactions between waist circumference and rs662799 and rs2075291 at the APOA5 locus. For HDL-C, we also examined rs17243123 at the APOA1 locus. The interaction term for rs662799 and waist circumference was statistically significant (p=0.002). The nature of the interaction is depicted in figure 8. The presence of the rare allele was associated with a greater effect of central obesity on TG, in a dose dependent manner.

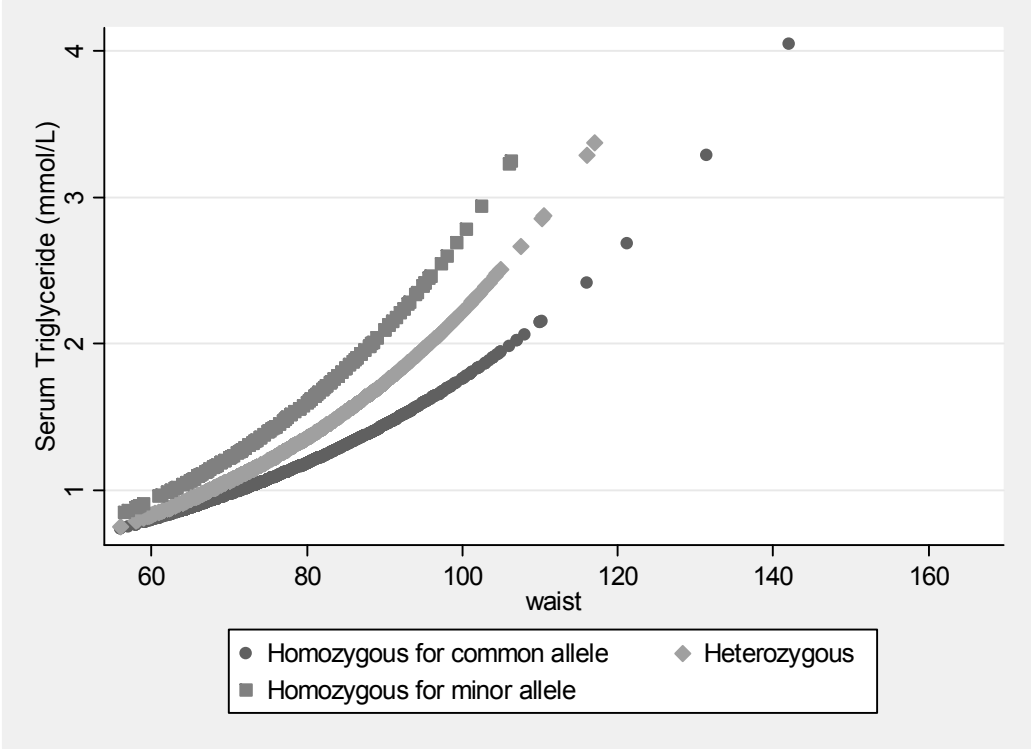


Figure 8. Interaction between rs662799 and waist circumference in relation to serum triglycerides. The p-value for the interaction term rs662799Xwaist circumference was 0.0054.

Chapter 5 DISCUSSION

5.1 Major findings and implications

5.1.1 Study 1

Several things are evident from our first study. First, the metabolic syndrome is common. In studies that included those with diabetes mellitus, the prevalence was 20-30% of Caucasian populations [123-126]. When individuals with diabetes mellitus were excluded, the prevalence was 10-15% [127, 128]. Second, in several populations, the presence of the MS identifies persons at increased risk of cardiovascular disease (CVD), CVD mortality or total mortality. This has been demonstrated in both cross sectional studies [125, 129-131] and prospective studies [124, 127, 128, 132-135]. Finally, measures taken to reduce CVD risk are as effective in this sub-population of individuals as in those without the MS [135, 136]. For these reasons, it has been suggested that the diagnosis of MS is an effective means to target individuals for more intensive measures to reduce the morbidity and mortality attributable to CVD.

As in Caucasian populations, the metabolic syndrome is also common in Asian populations. Amongst non-diabetic individuals, the prevalence was 12.5% in Korea[137] according to NCEP ATP III criteria. In this study, prior to the exclusion of those with diabetes mellitus and pre-existing IHD, the prevalence was 12.9%. Dyslipidemia is particularly common in our population with 46.9% of men and 51.3% of women exhibiting low HDL-C concentration. This is in line with our previous findings showing that insulin resistance and low HDL-C concentration are common findings in our population, particularly amongst those of Malay and Asian Indian ethnic groups [93]. However, to our knowledge, there is no data linking the MS to CVD in these populations. Our study demonstrates, for the first time, that the metabolic syndrome is associated with a three-fold increased risk of IHD events in Singapore. This increased risk remained statistically significant even after adjustment for age, sex and current cigarette smoking. These findings are in line with the findings of previous studies in Caucasian populations [124, 127, 128, 132-135] but in contrast to those in American Indians who participated in the Strong Heart Study [138]. This highlights the importance

of the metabolic syndrome in Asian populations, which are likely to be most affected by the rising burden of CVD in the next several decades [4]. Furthermore, the effect of the metabolic syndrome on the risk of IHD did not appear to be modified by gender or ethnic group. Although the metabolic syndrome has been associated with increased risk of CVD in women [123, 125, 126], at least 1 study has suggested that the effect may be modified by gender [131].

One other aspect of our study is important. Data from Singapore had previously demonstrated that for any given percentage of body fat, the body mass index (BMI) of Singaporeans were 3 kg/m^2 lower than that in Caucasians [139]. A review of other Asian data also concluded that Asians had a higher percentage body fat at a lower BMI compared with Caucasians[23]. Subjects of Chinese descent have been shown to have similar risk of developing glucose intolerance at lower BMI compared with indigenous Europeans [140, 141]. A study conducted in Asian Indians suggested that these findings involving BMI also apply to other anthropometric indices such as the WC [142]. We have previously shown, in a cross sectional analysis of the Singapore population, that a WC of 80 cm in women and 90 cm in men best predicted the presence of at least two other features of the MS[31] and suggested that these may be more appropriate as cut-offs to identify those with the MS in an Asian Population.

This modification of the diagnostic criteria resulted in an increase in the prevalence of the MS to levels similar to those in the United States population. However, the ultimate test of clinical utility for these modified Asian criteria for the metabolic syndrome lies in their ability to identify persons at risk of CVD. Our study shows that the suggestion to modify the NCEP ATP III criteria by reducing the level of WC used to define central obesity is appropriate, in relation to predicting the risk of IHD. The lower WC, in association with other features of the metabolic syndrome, was associated with increased risk of IHD.

The magnitude of the risk associated with this definition of the metabolic syndrome in our population (OR2.13; 95% CI 0.99-4.58) was similar or greater than that when the NCEP ATPIII criteria were used in Western populations. In a review by Earl S. Ford in 2005[143], a meta-analysis of 8 studies that used the NCEP ATP III definition of the metabolic syndrome showed an odds ratio of 1.65; 95% CI1.38-1.99) for cardiovascular disease.

Furthermore, modification of the diagnostic criteria increased the sensitivity of the metabolic syndrome for identifying those at risk of IHD with little loss in specificity and increased the population attributable risk associated with the metabolic syndrome.

Our study confirms our belief that the metabolic syndrome is a predictor of IHD in Asians without diabetes mellitus. However, if the NCEP ATP III criteria are to be used in this population, there is a need to lower the cut-off for waist circumference to improve the identification of those at risk of CVD. There remain many unanswered questions. It has been suggested the metabolic syndrome identifies a subgroup of subjects amongst those with diabetes mellitus which are at particularly high risk of cardiovascular disease [131]. Our study was not adequately powered to make this assessment (data not shown). Furthermore, we were unable to assess the potential role of adding other features of the metabolic syndrome to the diagnostic criteria. Potentially, these could include albuminuria, as proposed by the WHO [144] or C-reactive protein, which has been shown to add to the prognostic significance of the MS [134, 135].

5.1.2 Study 2.

Our data suggests that central obesity is important for the identification of individuals with insulin resistance and glucose intolerance. Those individuals who had central obesity and at least 2 other features of the metabolic syndrome (in line with the IDF recommendations) were the most insulin resistant and the most likely to have diabetes mellitus. Even amongst those with central obesity but with less than 1 other risk factor, significantly elevated insulin resistance was observed in comparison to the normal population.

However, central obesity does not appear to be a pre-requisite for the presence of the other cardiovascular risk factors associated with the metabolic syndrome. In fact, a significant proportion of individuals in our population (ranging from 10-13% in men and 2-4% in women) exhibited multiple features of the metabolic syndrome even in the absence of central obesity. These individuals would be diagnosed as having the metabolic syndrome according to the AHA/NHLBI criteria and not by the IDF criteria. These individuals have elevated blood pressure and LDL-C which are comparable to levels observed in those who meet the IDF definition of the MS. In addition, they have higher fasting TG and lower HDL-C, both of which are independent risk factors for cardiovascular disease [60, 145]. The findings of higher TG and lower HDL-C in this group of individuals are a consequence of the definition applied. Since these individuals are not centrally obese, qualification for the diagnosis of metabolic syndrome with three or more risk factors means that the other risk factors, including hypertriglyceridemia or low HDL-C, would need to be more common.

The originally stated rationale for the criteria is that the syndrome components are associated with insulin resistance. This seems like a valid reason for attempting to diagnose the metabolic syndrome since insulin resistance has been shown to be a predictor of both diabetes mellitus and cardiovascular disease [146-149]. Precise assessment of insulin resistance is technically difficult. Less precise estimates of insulin

resistance, using fasting insulin as a surrogate measure, are also difficult to use clinically in the absence of well-standardized assays for plasma insulin. Thus a robust, biologically valid construct for the identification of insulin resistant individuals is desirable for use in clinical practice. In this regard, our study suggests that the IDF recommendation might be more appropriate since it identifies a segment of the metabolic syndrome population that is more insulin resistant. This may be particular relevant for the identification of those at increased risk of diabetes mellitus, given previous findings that, amongst features of the metabolic syndrome, it is the factors associated with insulin resistance that best predict diabetes mellitus [150]. The greater degree of glucose intolerance identified using the IDF criteria would seem to support this conclusion.

In contrast, if the purpose of defining the metabolic syndrome is to identify individuals at increased risk of cardiovascular disease, it is possible that the IDF definition misses a significant proportion of the population at increased risk. The IDF definition of the metabolic syndrome identifies only a subset of those identified by the AHA/NHLBI definition. The latter includes additional individuals who exhibit high levels of several other cardiovascular disease risk factors including greater age, higher LDL-C, higher serum triglyceride, lower HDL-C and higher blood pressure. Each of these is an independent risk factor for cardiovascular disease. The higher Framingham risk score of individuals in this group would seem to support this hypothesis. Given these findings, the AHA/NHLBI definition may be more effective in identifying those at increased risk of cardiovascular disease.

Our data also suggests that other factors may influence the phenotype associated with central obesity or insulin resistance. Examples include age, cigarette smoking and alcohol intake, all of which are significantly associated with the presence or absence of other features of the MS in the face of central obesity. Although our study highlights the importance of the role that lifestyle factors may play in the pathogenesis of the metabolic syndrome, this association between llifestyle factors and alcohol are not novel. It has been shown in several previous studies that smoking and alcohol consumption have an

adverse effect on the risk of the metabolic syndrome and it's associated features. [151-158]. Specifically, in relation to the lipid related features of the MS, cigarette smoking is known to cause low HDL-C (one of the features of the MS) through its effects on lipid and lipoprotein metabolism (reviewed in Chellend Campbell et al[159]) while alcohol ingestion, particularly in excess, causes hypertriglyceridemia[160-163].

In conclusion, our study suggests that harmonization of the diagnostic criteria alone does not solve the problems associated with defining the metabolic syndrome. The AHA/NHLBI and the IDF definitions for MS identify different segments of the population. Importantly, there is a subset of individuals who meet the AHA/NHLBI recommendation for MS but will be missed if IDF recommendation is used. Although these individuals exhibit no central obesity, they have similar risk factors for cardiovascular disease and may benefit from similar risk reduction therapy.

5.1.3 Study 3

Our study showed that the prevalence of the metabolic syndrome is 17.7% based on IDF criteria but 26.2% based on AHA/NHLBI criteria. This meant that 8.5% of the population had three or more metabolic syndrome components in the absence of central obesity while using the Asian definition. Thus, making central obesity an "essential" rather than "optional" component in diagnosing metabolic syndrome fails to identify a fairly large proportion of individuals who otherwise would be classed as having the metabolic syndrome.

Our study also showed that individuals with central obesity/metabolic syndrome and no central obesity/metabolic syndrome groups are at similar risk of IHD. This suggests that including central obesity as an "essential" component for the diagnosis of metabolic syndrome, as proposed by IDF, does not add more to the identification of individuals at increased risk of IHD. These findings from our study suggest, at least in this Asian population that having central obesity as an "optional" component of the metabolic syndrome instead of an "essential" component identifies a significantly greater number of individuals at increased risk of IHD. From the absolute measures, the AP_E and AP_T using the AHA/NHLBI criteria was higher than the IDF criteria. Thus, individuals with the metabolic syndrome, using the AHA/NHLBI criteria, can attribute a higher proportion of their risk of IHD to the metabolic syndrome. Similarly, a higher proportion of the risk of IHD for the total study population can be attributed to metabolic syndrome using the AHA/NHLBI rather than the IDF criteria.

The IDF based their recommendation on the strength of the evidence linking waist circumference with CVD and the other components of the metabolic syndrome [164, 165] and states that central obesity is an early step in the etiological cascade leading to the full metabolic syndrome. Our findings refute neither of these premises. Indeed, one study [166] in Japan has shown that visceral adiposity was a crucial determinant on the degree of insulin resistance associated with the presence of other metabolic syndrome components. In that study, the presence of three or more metabolic syndrome components was associated with a lesser degree of insulin resistance if visceral adiposity was not one of the three

components (versus if it was). However, in relation to identifying individuals at increased risk of IHD, it does appear that central obesity adds to the risk of IHD in much the same way as the other four risk factors. This is in line with the findings of Katzmarzyk et al[167] who showed that increasing waist circumference was associated with increased risk of CVD mortality when added to the other components of the metabolic syndrome. However, the presence of central obesity as one of the components of metabolic syndrome does not appear to alter the association between the presence of other multiple components and the risk of IHD. Thus, while making central obesity an essential requirement may make etiological sense and may be relevant to the identification of the insulin-resistant individual, the evidence that this approach is important for the identification of individuals at risk of IHD is limited at this time.

Several factors could also explain our findings. First, central obesity may not cause IHD directly but rather through the associated risk factors and thus may not have a strong influence on the risk of IHD in this study until after the other CVD (metabolic syndrome) risk factors associated with central obesity have developed. Second, waist circumference is an imperfect surrogate of abdominal adiposity [168], and using it might lead to misclassification of individuals. Finally, central obesity is important, but the threshold for Asians may need to be further lowered. The underlying purpose for diagnosis of the metabolic syndrome is to identify individuals who are at increased risk of developing diabetes and CVD and to apply preventive measures [21, 22]. We found in Asians that both individuals with and without central obesity and other metabolic syndrome components are at similar risk of IHD. The current AHA/NHLBI [21] proposal includes all of these individuals, while a sizable number who do not have central obesity but have the metabolic syndrome are omitted by the IDF [22] criteria and thus identifies a greater proportion of those at increased risk of IHD. However, we cannot comment at this time on the relevance of central obesity as an essential component of the metabolic syndrome in relation to the risk of diabetes due to lack of follow-up data. It may well be that the impact differs from that for IHD. Finally, of note is the high proportion of current smokers in the group of individuals without central obesity but with the metabolic syndrome compared with the other two groups. Although this has been adjusted for in the analysis to determine the risk of IHD for each group, it is still important to remember that the focus on the metabolic syndrome

should not lead to negligence of the other CVD risk factors that need to be addressed at the individual level.

In conclusion, this study has shown that the risk of IHD is increased in individuals with the metabolic syndrome with or without central obesity. However, the prevalence of metabolic syndrome is increased by 8.5% if central obesity is “optional” rather than “essential” and thus identifies more individuals at risk of IHD. Apart from metabolic syndrome, other CVD risk factors in individuals should also be considered and appropriately managed.

5.1.4 Study 4

In this study, by using a comprehensive metabolic profiling approach, we have found a striking association between a subset of amino acids and insulin resistance in Chinese subjects living in Singapore. In a previous study that applied the same tools to a group of obese (BMI 37 kg/m^2) versus lean (BMI 23 kg/m^2) African-American and Caucasian subjects residing in the Southeastern United States, striking increases in amino acid levels were also noted in the obese and insulin resistant subjects, including branched chain amino acids (BCAA) and several of their metabolites [58]. The current findings strongly support the idea that these changes are related to IR rather than obesity per se, since we observed the changes in high versus low tertiles of IR in a group of Chinese subjects with a mean BMI of approximately 24 kg/m^2.

We recognize that this conclusions is based on the fact that insulin resistant and insulin sensitive subjects in this study were matched for BMI. BMI is an imperfect measure of adiposity and therefore, there remains the possibility that the associations between plasma amino acids and insulin resistance are a consequence of residual confounding by adiposity, despite our attempts to correct for this by matching, and subsequently adjusting for BMI. Nevertheless, our conclusions are supported by the finding that rats fed a high fat diet supplemented with BCAA had reduced food intake and lower body weight relative to rats fed unsupplemented high fat diet, but had the same degree of insulin resistance [58].

Unfortunately, the number of Asian Indian subjects available limited the power of this study to detect associations with insulin resistance in this ethnic group. For this reason, several of the associations observed in Chinese were not statistically significant in Asian Indians. Nevertheless, the effects of insulin resistance on BCAA and related amino acids trended in the same direction as observed in Chinese. Furthermore, the effects on downstream catabolic products (alanine, glutamine/glutamate, lactate and pyruvate) were

sufficiently large to reach statistical significance. As such, we believe that the observations in Chinese are also applicable to Asian Indians (although this needs to be confirmed in larger study).

BCAA are major donors of amino groups for the synthesis of the gluconeogenic amino acids alanine and glutamate, and their levels are also closely correlated with tyrosine and phenylalanine in this and several other recent studies [58, 116, 169]. In situations of impaired BCAA oxidation (such as maple syrup urine disease), serum levels of alanine are reduced [170]. In contrast, the current study documents increases in the levels of pyruvate, lactate, alanine, and glutamate/glutamine in insulin resistant individuals, consistent with a model of increased supply and catabolism of BCAA in insulin resistant subjects.

What is the source of the increased supply of BCAAs in insulin resistant subjects? As essential amino acids, BCAA levels in blood are controlled by dietary intake, their rate of utilization for the anabolic processes of protein biosynthesis and cell growth, their rate of catabolism through transamination and the branched-chain keto acid dehydrogenase complex, and the rate of protein turnover/hydrolysis. In a prior study, it was reported that obese individuals consumed more protein than lean individuals (a difference that was of borderline statistical significance) [58]. However, in the present study, whereas dietary protein intake differed between ethnic groups, it did not differ between individuals with high or low HOMA. We suggest that the increased supply of BCAA in individuals with high HOMA instead results from increased protein catabolism. In support of this idea, Gougeon et al compared a number of measures related to protein metabolism in three groups of individuals who were lean, obese without diabetes and obese with diabetes [171]. As in our prior study [58] obesity was associated with increased protein intake. However, this difference disappeared after correcting for fat-free mass. When they compared obese individuals with and without type 2 diabetes mellitus, no difference in protein intake was observed, but net negative nitrogen balance was observed in subjects with type 2 diabetes mellitus and not in those with normal glucose tolerance.

Despite similar BMI, insulin resistant and insulin sensitive individuals in the current study had markedly different waist circumferences. The differences observed in waist circumference suggest that despite similar BMI, individuals who are insulin resistant may have reduced lean body mass and increased adiposity. This has been termed 'sarcopenic obesity [172], and its presence is consistent with the strongly elevated plasma leptin levels observed in the individuals with high HOMA from both ethnic groups. Conversely, levels of the insulin sensitizing hormone adiponectin fall in both high HOMA groups. One possibility raised by our findings is that sarcopenic obesity could represent a condition of increased protein catabolism associated with insulin resistance, a finding that is supported by the aforementioned study showing that net protein balance is diminished in individuals with type 2 diabetes mellitus [171]. Also consistent with this idea, insulin resistance is independently correlated with poor muscle strength and with accelerated loss of muscle strength and quality in older subjects with type 2 diabetes [173-176].

Down-regulation of the growth hormone axis has also been implicated in the link between insulin resistance and sarcopenia. Levels of IGF-1 were reduced in insulin resistant individuals in the present study, similar to prior findings in obese, insulin resistant North American subjects [58]. This could potentially result in decreased anabolic utilization of amino acids, including BCAA, for protein synthesis and cell growth, contributing to their rise in the circulation. However, these differences disappeared after adjustment for age and BMI. Interestingly, Chinese subjects had higher IGF-1 levels than Indian subjects, possibly contributing to the particular susceptibility of the latter group to develop metabolic diseases such as type 2 diabetes mellitus. We also considered other possible explanations for the increased protein catabolism observed in these individuals. It has been suggested that increased inflammation associated with obesity and insulin resistance may result in accelerated muscle catabolism [177, 178]. However, the levels of pro-inflammatory cytokines were only minimally elevated in our insulin resistant individuals.

When BMI is controlled as in the current study, no correlation beween free fatty acid levels and HOMA isevident, at least for the two ethnic groups studied here. Increases in free fatty acids have been reported in obese and insulin resistant subjects in many prior studies, leading to extensive studies on the potential "lipotoxic" effects of these agents in development of insulin resistance [179-184]. Another striking finding of our

study is the dramatic lowering of IGFBP1 in insulin resistant compared to the more insulin sensitive groups, an observation made for both ethnic groups. Insulin is known to suppress production of IGFBP1 in the liver [185]. These findings suggest that the elevated plasma insulin in insulin resistant individuals is largely effective for suppression of lipolysis and free fatty acid levels, as well as hepatic IGFPB1 production. However, these same elevated insulin levels fail to control the levels of BCAA and related amino acids. Whether this is indicative of early and selective insulin resistance at the level of suppression of protein turnover and amino acid catabolism or whether the reduced IGF-1 levels play a more prominent role is a matter that will require further investigation.

Several limitations of the study should be noted. Firstly, we have only studied men, and the relevance of tese findings to women is not known at this time. Secondly, we have only studied these individuals in the fasting or post-absorptive state. We have previously shown that high fat feeding results in abnormalities in lipid metabolism that is primarily observed in the fed state [180]. Additional studies in the fed state are likely to yield even greater insight into the metabolic perturbations involved in insulin resistance. Thirdly, HOMA is an imprecise method for assessing insulin resistance. However, we feel that the selection of individuals from opposing tertiles of HOMA index allowed us to clearly differentiate insulin sensitive and insulin resistant individuals. The observation that these groups truly differed in terms of insulin resistance are further supported by the clear differences in the levels of blood lipids (triglyceride and HDL-C) and adiponectin between groups.

In conclusion, extensive evidence supports the role of free fatty acids and inflammation in the pathogenesis of obesity associated insulin resistance. Our study complements this knowledge by showing that insulin resistance is not always associated with obesity, and when insulin resistance develops at relatively low body weight, IR is no longer associated with excess free fatty acids and inflammation but instead with a metabolic signature that suggests enhanced protein catabolism. This enhanced protein catabolism, and the corresponding hyperaminoacidemia, may have several pathophysiologic consequences [55, 56, 58, 186]. Studies in animals suggest that elevated BCAAs may increase IR resistance through increased mTOR and JNK

activity. In particular, elevated BCAAs could potentiate the effects of a high fat diet on insulin resistance [58], which may be particularly relevant in Asian Indians (who eat a diet higher in saturated fatty acids in our study) and may contribute to the development of greater degrees of insulin resistance at relatively low body weight. Furthermore, the increased flux of gluconeogenic precursors to the liver may increase the contribution of gluconeogenesis to hepatic glucose output. Finally, this enhanced protein catabolism may contribute to the link between insulin resistance and sarcopenia. While previous studies have focused mainly on dietary protein intake in relation to IR [56], our study suggests that strategies that reduce muscle protein catabolism and increase muscle protein synthesis might be useful in combating development of insulin resistance even in the absence of obesity. Consistent with this idea, the inclusion of resistance training in addition to aerobic training optimizes the benefits of exercise on insulin resistance [187]. Further work will be required to fully understand the utility of such therapies for influencing metabolic variables associated with insulin resistance described in this study.

5.1.5 Study 5

We had previously established that SNPs at the APOA1/A4/C3/A4 locus were important determinants of plasma triglycerides in our population[76]. This study extends our previous findings in several ways. Firstly, we have found that SNPs at the APOA5 locus are, by far and away, the most important common variants associated with plasma triglycerides within this gene cluster. In fact, we believe that our data supports the hypothesis that SNPs at the APOA5 locus explain most of the association signals previously observed with SNPs at other loci within this gene cluster. This should not come as a total surprise. Talmud et al[121] previously reported that SNPs at the APOA5 locus probably explained the associations between SNPs at the APOA4 locus and plasma TG. What was rather surprising was that this was the case even for polymorphisms at the APOC3 locus. Our study included the SstI polymorphism at the APOC3 locus as well as the promoter polymorphisms that have been implicated in the regulation of APOC-III expression by insulin and were thought to be functional [78]. Furthermore, Olivier had previously genotyped 49 SNPs across the APOA1/C3/A4/A5 cluster and reported relatively high LD across the cluster. Their analyses also supported the presence of recombination events between APOC3 and APOA5 disrupting the cluster, which is in line with the relatively low degree of linkage disequilibrium between the SNPs at the APOA5 locus and SNPs at the APOC3 locus. For example, the R^2 between rs662799 and rs5128 (SstI) is only 0.13. Our study thus shows that genetic variants with relatively large effects, (rs662799 at the APOA5 locus alone explains close to 2% of the variance of TG), can result in association signals at distant loci despite low LD, and that the demonstration of relevant biological functionality, as for the APOC3 promoter polymorphisms, does not protect this.

It is not clear how these polymorphisms result in altered TG. While it has been reported that APOA-V present on TG rich lipoproteins may enhance LPL activity, either directly or by facilitating the binding of VLDL to LPL attached to heperan sulphate proteoglycans[188]. This could result in enhanced lipolysis of the TG in VLDL. However, in a study conducted in a British population, rs662799 was associated with

plasma TG concentrations, but not APOA-V concentrations[189]. As such, it would appear that these polymorphisms did not exert their effects on plasma triglyceride concentrations through their effects on plasma levels of APOA-V. In a nested case control study which included 997 cases and 2031 controls derived from the prospective Epic-Norfolk Population Study[190], no association was observed between plasma APOA-V concentrations and the risk of incident fatal, or non-fatal coronary artery disease. However, rs662799 did show an association with increased risk of coronary artery disease providing further evidence that the effects of this polymorphism on the risk of CAD is not related to its effects on the plasma levels of APOA-V. One possibility that has been raised, given the very low concentrations of APOA-V in the plasma [191], is that APOA-V may have an intra cellular function, which is supported by data slowing that APOA-V is associated with intra-cellular lipids in cell-lines [192].

The 2 polymorphisms at the APOA5 locus (rs2075291 and rs662799) merit some additional comment. They both show a strong association with TG and in both instances, these associations remain statistically significant even when adjusted for the presence of the other SNP. This has been taken as evidence that these SNPs contribute independently to TG and perhaps represent 2 functional SNPs at this locus. However, closer examination of the data shows that, even though the association between rs662799 and TG remains statistically significant after adjustment for rs2075291, there is a significant reduction in the association signal with the p-value increasing from 9.6×10^{-19} to 7.1×10^{-11}, suggesting that at least some of the association signal for rs662799 is explained by rs2075291. The R^2 between these 2 SNPs is 0.14 (in the region of that between rs662799 and rs5128 at the APOC3 locus) and we believe that his could represent a situation where a single functional SNP with a large effect could explain the association signal from both these SNPs.

Our data does not rule out the possibility that more than 1 variant within this cluster shows an association with blood lipids. In fact, for HDL-C, there is a polymorphism in the APOA1 promoter (rs17243123) which shows a statistically significant association with HDL-C (borderline for TG) which hardly changes where adjusted for the APOA5 SNPs, which is hardly surprisingly given that the R^2 between this SNP and the 2 APOA5 SNPs is only 0.0046. Thus, it seems that there are at least 2 variants within this cluster that affect levels of HDL-C. One is at the APOA5 and probably has it major effect through elevations of TG rich lipoproteins, which in turn provides additional substrate for neutral lipid transfer between HDL and VLDL. The other is at the APOA1 locus which appears to have an effect that is largely independent of TG.

Our data also shows that rs662799 modulates the association between central obesity and elevated TG. The presence of the rare allele for this SNP is associated with a higher TG in the presence of greater waist circumference. Such a gene-environment interaction may explain why some features of the metabolic syndrome are present, even in individuals with relatively low levels of central obesity [193, 194].

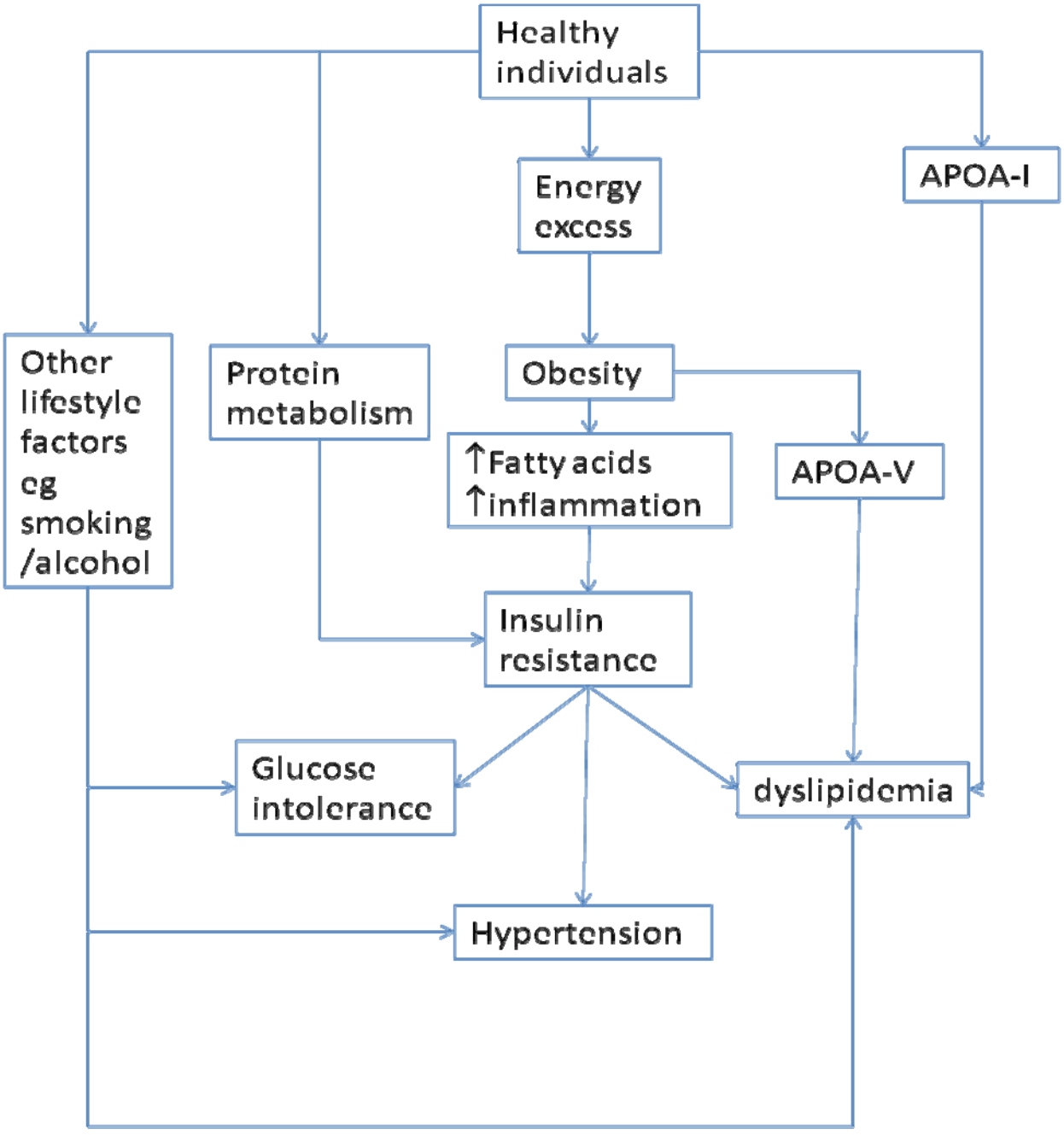
In conclusion, our study clearly implicates the APOA5 locus in the pathogenesis of elevated TG and low HDL-C. One or more SNPs at this locus explain most of the associations previously observed between SNPs at the APOA1, APOA3 and APOA4 loci and TG, despite the fact that some of these other SNPs have demonstrated functional significant, that these gene clusters span a large distance (over 60kb) and that the LD is relatively low (R^2 in the region of 0.15 or less). These findings may have significant implications for efforts to identify functional polymorphisms that are responsible for the association signals found in completed and ongoing genome-wide association studies. Furthermore, the APOA5 SNPs also connote of developing elevated TG and low HDL-C in association with central obesity. This may explain the observation that some individuals develop dyslipidemia at relatively low levels of obesity, and also suggest that targeting APOA-V expression might be a useful strategy for the treatment of dyslipidemia associated with the metabolic syndrome.

5.2 Bringing it all together

Through a series of studies, we have demonstrated that the metabolic syndrome is both common, and important for defining the risk of CVD in the Singapore population, as it is in other populations.

57.6% of ischemic heart disease events are attributable to the presence of the metabolic syndrome. We have also shown that central obesity is an important component of the metabolic syndrome but it's presence is not essential. In the absence of central obesity, individuals can still develop multiple features of the metabolic syndrome and they are at just as much risk for CVD as those without central obesity. Our studies have identified two pathways that could result in this situation. Firstly, we have shown that insulin resistance, and with it many of the features of the metabolic syndrome, is associated with a metabolic signature suggesting increased protein catabolism and a sarcopenic form of obesity. Secondly, we have shown that polymorphisms at the APOA1/C3/A4/A5 loci are associated with the dyslipidemia associated with the metabolic syndrome that that, a polymorphism at the APOA5 locus may increase an individuals susceptibility to develop dyslipidemia in the face of central obesity. Finally, we have also shown that smoking and alcohol intake are associated with the presence of multiple features of the metabolic syndrome in the absence of obesity. These pathways are illustrated in figure 9. The schema begins with a healthy individual at the top of the figure. A central portion of the pathway involves energy excess which leads to the development of obesity. In turn, this leads to insulin resistance (involving excess free fatty acids and inflammation). In turn, insulin resistance causes the other features of the metabolic syndrome. This portion of the pathway is very much in line with the IDF definition of the metabolic syndrome. However, the AHA/NHLBI recommendation, recognizes that these metabolic abnormalities can occur in the absence (or at least at lesser levels) of obesity. Lifestyle factors (smoking and alcohol in study 2), abnormalities of protein metabolism and polymorphisms at the APOA1 and APOA5 loci may facilitate this pathophysiologic process either directly or by modulating the impact of obesity on some of the features of the metabolic syndrome.

Figure 9. Unifying hypothesis for studies in this thesis.



Chapter 6 LIMITATIONS AND METHODOLOGICAL CONSIDERATIONS

6.1 Bias and confouding

All observational studies (such as those used in the studies descrided in this thesis) are prone to bias and confounding. In the following sections, I will discuss some of these issues in relation to the studies conducted and described.

6.1.1 Selection bias

Despite efforts to select a random sample of the Singapore population in all the studies used in this thesis, response rates were in the region of 60%. Some efforts were made to determine whether there was a non-response bias. In NHS98, non-participants were contacted to obtain information regarding their demographic and socio-economic profile, diabetes and hypertension status. Characteristics of the non-participants were similar to the survey participants. Nevertheless, we cannot be certain that the participants did not differ from thenon-participants in other ways. The healthy volunteer effect, where participants are healthier than the general population, is a well known phenomenon. Indeed, we know that in SP2, participants who provided blood samples and participated in the health examination, were less likely to report a history of diabetes mellitus and hypertension. This may result in a misrepresentation of the population's health/risk-profile and we may have underestimated the prevalence of disease in our population.

This type of bias may be less important studies 4 and 5, which are focused more on the etiology of the disease. In general, we feel that it was because the subjects for this study were not ascertained on the basis of the disease, that selection bias is unlikely to be a major issue in relation to the validity of the findings from these studies. It is possible that the rather low response rate (particularly in study 4), may limit the generalizability of the findings. After all, SP2 was a study in which less than half the eligible participants provided blood samples. Nevertheless, even if we believe that our findings do not apply to those who did not attend the health examination (which we believe is very unlikely given the similarity of

some of our findings to those from studies conducted in other populations), 50% is still a sizeable portion of the population and our findings would still be an important health issue.

6.1.2 Misclassification or measurement error

The possibility that measurement error or misclassification could result in some degree of bias must also be considered. In relation to the exposures (metabolic syndrome and other CVD risk factors), some laboratory error could have occurred. We believe that such errors are random (since the measurements were made without any knowledge of the subjects health status). Furthermore, the use of standardized methodology in all 4 population studies would tend to reduce the measurement error. This was possible because many of the investigators (and sometimes the research staff) were common across the studies. Finally, the use of accredited labs with good quality control measures (as evidenced by the small coefficients of variations for the various assays carried out) would also tend to reduce such errors. Nevertheless, for some variables which show considerable intra-individual variation, such as plasma triglyceride, the fact that measurements were made at only a single time point would add to the measurement error. We do believe that these measurement errors are non-differential (unless the disease or genetic variant studied also affects the variability of the measurement-there is no real evidence for this) and as such, these measurement errors are likely to bias the association towards the null hypothesis. Taken together with the potential for a health volunteer effect described in the preceding section, it is possible that we underestimated the population attributable risk associated with the metabolic syndrome in studies 1 and 3. However, this does not change our conclusion that the metabolic syndrome is both common, and important for the health of the Singapore population.

In carrying out these studies, we also had to deal with the fact that the ascertainment of IHD events in this study was carried out using data from several registries, which could be unreliable. We are currently unable to provide direct evidence as to the reliability of the registry data. However, we can say that data

from the myocardial infarction registry was derived using the WHO MONICA protocol. The methodology is therefore well established. Furthermore, submissions to the central claims processing system and its predecessor, the hospital in-patient discharge system are a regulatory requirement for all hospitals in Singapore and most, if not all, hospital admissions are represented. The discharge diagnoses are completed by doctors directly involved in the management of the patients admitted to the hospital and we believe that they are reasonably accurate. We concede that patients who are overseas at the time of their IHD event would be missed. However, our own experience as clinicians suggests that these would be small in number. Nevertheless, some degree of misclassification is likely to have occurred. This could bias the results in one of two ways. If the misclassification is non-differential (as we believe it is) then it would tend to bias the associations towards the null hypothesis which, as described in the preceding paragraph, would lead to us underestimating the impact of the metabolic syndrome on CVD. On the other hand, it is possible that the diagnosis of CVD is made more readily, or more like to be suspected, in patients with features of the metabolic syndrome such as diabetes, hypertension or dyslipidemia. This would tend to inflate the associations observed. While we cannot exclude this possibility, we have found that the effect estimates associated with the metabolic syndrome on CVD are similar to those observed in other studies (as discussed in study 1). In addition, other studies that we have carried out using this dataset looking at different risk factors [88, 89] also show that the effect sizes associated with these risk factors were similar to those observed in studies with much more detailed assessment of outcomes such as the Framingham Heart Study. Furthermore, we have also shown that the metabolic syndrome is associated with mortality (which is much less prone to misclassification than CVD)[195]. As such, we believe that any bias that has occurred to misclassification of the outcomes is likely to be similar to those observed in other studies and unlikely to significantly affect our conclusions.

One other issue that we face, due to the fact that studies 1 and 3 are based on registry linkage, is that some of our study subjects may have emigrated. In this instance, we would not have captured the occurrence of IHD events, had they occurred. We do not feel that this has a large impact on the conclusions drawn for several reasons. Firstly, the emigration rate form Singapore is relatively low (<5%).

Secondly, it is our belief that emigration would be non-differential in terms of the presence or absence of the metabolic syndrome. As such, any impact would be to bias the association towards the null hypothesis, resulting in an underestimate of the effect of the metabolic syndrome. Thus, our conclusions from these studies remain valid.

6.1.3 Confounding

Confounding may also be responsible for some of the findings observed. In fact, in genetic association studies, we exploit this to delineate the importance of a particular locus to a disease. For example, if we accept the hypothesis suggested in study 5, that a single functional variant is responsible, through linkage disequilibrium, for the associations observed between plasma TG and rs2075291 and rs662799 at the APOA5 locus, then the observed associations between these 2 SNPs and TG are both a consequence of confounding.

Confounding takes on a special meaning in genetic association studies. Population stratification is a particular form of confounding that affects genetic association studies. This is particularly relevant given that Singapore is a multi-ethnic population, and the three ethnic groups exhibit differences in the prevalence and levels of various metabolic syndrome related traits. Thus, any SNPs that have different allele frequencies in different ethnic groups would exhibit associations with these traits, which are a consequence of confounding by ethnic group. To a large extent, study 5 is protected against this kind of confounding. Firstly, we restricted the study to Chinese. This does not exclude the possibility of population stratification occurring as a consequence of sub-sets of the Chinese population having different population histories. However, we have recently carried out genome-wide association studies in our Singapore population, which shows that the Chinese population in Singapore is quite homogenous genetically. Thirdly, many of the associations observed in study 5 replicate those carried out in other populations. As such, we do not believe that the associations observed are a consequence of population stratification.

It is unlikely that the associations that we have described, between the metabolic syndrome and CVD, are a consequence of confounding. This is because multiple randomized controlled trials targeting the features of the metabolic syndrome in patients with type 2 diabetes mellitus (the ultimate patient with the metabolic syndrome), such as blood glucose [196], hypertension[197], dyslipidemia [198, 199] or the combination[200], results in reduced cardiovascular morbidity and mortality.

6.2 Establishing the temporality of the associations observed

In addition to dealing with bias and confounding, one of the challenges of observational studies is to establish that a risk factor is antecedent to the development of the disease being studied. Several of these studies are cross-sectional studies (study 2, study 4). Even though the associations are robust, the temporal relationship between risk factor and disease cannot be established. As such, it remains possible that the associations between the features of the metabolic syndrome and lifestyle factors (study 1) and metabolic abnormalities (study 4) are a consequence of reverse causation. Fortunately, these observations are not made in isolation. For example, the impact of hyper-aminoacidemia might have on insulin resistance (described in study 5) is supported by experimental models in animals, which not only replicate our findings in humans, but provide a mechanistic basis for this association (thus giving biological plausibility). Nevertheless, it will be important to follow up these studies with experimental study designs that will formally test the causality of the associations observed. Although study 5 is also a cross-sectional study, in this instance, we are dealing with somatic mutations which have been present since birth and as such, establishing temporality is not an issue.

6.3 Issues related to multiple testing

Multiple testing can give rise to false positive findings. This issue has been highlighted by recent genetic association studies which have examined the associations of millions of genetic variants in association with multiple phenotypic traits. Two approaches have been taken to reduce the likelihood of false positive findings related to multiple testing. One is to take a more stringent cut off for defining statistical significance. This was carried out in relation to the genetic association studies in study 5, in which we used a p-value <0.001 (Bonferoni correction) to declare an association statistically significant, which we believe is actually rather conservative (table 19 and 20). In addition, the associations that we report are in well validated loci/variants. As such, we do not believe that the chance of a false positive finding in this

regard is minimal. However, we do appreciate that the interaction between variants at the APOA5 locus and waist circumference (figure 8) is novel and despite a p-value of 0.002 for the test of interaction and replication of this observation in an independently collected population with similar measurements is an important next step. The issue of multiple testing has been much less prominent in non-genetic studies, in part because the technologies that allow the testing of millions of hypotheses in parallel are less readily available. Nevertheless, multiple testing is an issue in studies such as study 4, in which we have measured multiple metabolites and hormones (tables 15 and 16), we have used the traditional p-value of <0.05 to define statistical significance. I recognize that this fails to take into account multiple testing. However, several things enhance my belief that these findings are true associations, and not a consequence of chance resulting from multiple testing. Firstly, the major observation that plasma amino acids may impact insulin resistance is supported by animal studies, which showed that the supplementation of a high fat diet with branch chain amino acids results in greater insulin resistance in a rat model of obesity and insulin resistance. Secondly, in addition to the initial observation that formed the basis for the hypothesis tested in study 4[58] since the publication of this data, 2 additional studies [201, 202] have also found similar association of amino acids and insulin resistance. Although these latter studies are not indentical, in terms of the analytical methods, to ours, I believe that they lend some support to the findings reported in study 4.

For all these reasons, despite the limitations described, we believe that the findings of these studies are valid, and relevant to a broad segment of the population.

Conclusion

Through this series of studies, we have demonstrated that the metabolic syndrome, is an important risk factors for IHD in the Singapore population (and by extrapolation, to much of Asia), Studies 2 and 3 add further to this by providing some insight as to the appropriateness of the various definitions of the metabolic syndrome. I realize that since the time that these studies were published, there has been significant tought/controversy with regards the utility of the metabolic syndrome in the clinical setting. Increasingly, I believe that the real utility of the metabolic syndrome lies is an a construct within which we can attempt to understand the pathophysiology that gives rise to diabetes mellitus and cardiovascular disease. In addition to providing insight as to appropriate definitions, studies 2 and 3 also suggest the possibility that some individuals may be more susceptible to develop risk factors for diabetes and cardiovascular disease at lower levels of obesity. Studies 4 and 5 provide some potential pathways through which these may occur (protein metabolism and genetic factors) and I hope that these will form the basis of future investigation in this field.

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