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# GENETIC PREDISPOSITION AND DIETARY FACTORS IN RELATION TO ADIPONECTIN AND INSULIN RESISTANCE

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*To my parents*

# ABSTRACT

Diabetes mellitus is a global health problem, owing to the high prevalence and enormous associated economic burden. Insulin resistance is a critical condition to the development of type 2 diabetes (T2D). Adiponectin, a hormone secreted by the adipose cells, has attracted much attention for its insulin-sensitizing and anti-diabetic effects.

The overall aim of this thesis was to have a better understanding of the roles of ethnicity, genetic variants and dietary factors in relation to adiponectin and insulin resistance by means of different analytical approaches.

In Study I, using path analysis, we examined potential mediators including body fatness, adiponectin levels, and inflammation for the extent they mediate the ethnic differences in insulin resistance among Singaporean Chinese, Malays and Indians. General adiposity explained the difference in insulin resistance between Chinese and Malays, whereas abdominal fat distribution, inflammation, and unexplained factors contributed to excess insulin resistance in Asian Indians as compared with Chinese and Malays.

In Study II, we carried out a genome-wide association study to identify genetic variants that influence adiponectin levels in East Asian populations. The top signal from *CDH13* explains a substantial part of variation in high-molecular-weight (HMW) adiponectin levels, but its effect on circulating HMW adiponectin levels did not appear to translate into effects on insulin-resistance related metabolic traits, suggesting that compensatory mechanisms exist that lead to greater 'adiponectin sensitivity'.

In Study III, the question whether changes in adiponectin levels causally influence insulin sensitivity was addressed by a Mendelian randomization design in a cohort of Swedish men. Genetically determined adiponectin levels influence euglycemic clamp-measured insulin sensitivity to the same degree as the observed epidemiological associations. Thus, the observed association between higher adiponectin levels and increased insulin sensitivity is likely to represent a causal relationship.

In Study IV, we examined relations between serum selenium levels and measures of glucose and insulin metabolism, as well as risk of T2D longitudinally in Swedish men. There was no clear evidence of an effect of selenium status on various measures of insulin sensitivity or β-cell function. Selenium levels were also not associated with risk of T2D. These results do not support a role for selenium supplementation as a broad approach for the prevention of T2D.

In conclusion, mediators of ethnic differences in insulin resistance differed markedly in the Singaporean populations. In East Asians, *CDH13* strongly influences adiponectin levels and associates with a beneficial metabolic profile when controlling for circulating adiponectin. Inferred from genetics, the positive relationship between adiponectin and insulin sensitivity appears to be causal. There is no evidence of an effect of selenium intake on glucose and insulin metabolism or risk of T2D in the Swedish population.

# LIST OF PUBLICATIONS

* Denotes equal contribution or joint direction of the project work

I. Gao H, Salim A, Lee J, Tai ES, van Dam RM: Can body fat distribution, adiponectin levels and inflammation explain differences in insulin resistance between ethnic Chinese, Malays and Asian Indians? *International journal of obesity* 2012;36:1086-1093

II. Gao H, Kim YM, Chen P, Igase M, Kawamoto R, Kim MK, Kohara K, Lee J, Miki T, Ong TH, Onuma H, Osawa H, Sim X, Teo YY, Tabara Y*, Tai ES*, van Dam RM*: Genetic variation in *CDH13* is associated with lower plasma adiponectin levels, but greater adiponectin sensitivity in East Asian populations. (Accepted, *Diabetes*)

III. Gao H, Fall T, van Dam RM, Flyvbjerg A, Zethelius B, Ingelsson E*, Hägg S*: Evidence of a causal relationship between adiponectin levels and insulin sensitivity: a mendelian randomization study. *Diabetes* 2013;62:1338-1344

IV. Gao H, Hägg S, Sjögren P, Lambert P, Ingelsson E*, van Dam RM*: Serum selenium in relation to measures of glucose metabolism and incidence of type 2 diabetes in an older Swedish population. (Submitted)

Other relevant publication not included in thesis:

Wu Y*, Gao H*, [45 other authors], Mohlke KL*, Tai ES*: A meta-analysis of genome-wide association studies for adiponectin identifies a novel locus near *WDR11-FGFR2* in East Asians. (Provisionally accepted, *Human Molecular Genetics*)

# CONTENTS

# LIST OF ABBREVIATIONS

| | |
|---|---|
| AAC | acetyl coenzyme A carboxylase |
| AMPK | 5'-adenosine monophosphate-activated protein kinase |
| BMI | body mass index |
| CRP | c-reactive protein |
| GPx | glutathione peroxidase |
| GWAS | genome-wide association study |
| HDL-C | high-density lipoprotein cholesterol |
| HMW | high-molecular-weight |
| HOMA-β | homeostasis model assessment of β-cell function |
| HOMA-IR | homeostasis model assessment of insulin resistance |
| HWE | Hardy-Weinberg equilibrium |
| IV | instrumental variable |
| IVGTT | intravenous glucose tolerance test |
| LD | linkage disequilibrium |
| MAF | minor allele frequency |
| MR | mendelian randomization |
| NEFAs | non-esterified fatty acids |
| OGTT | oral glucose tolerance test |
| PPAR | peroxisome proliferator activated receptor |
| QC | quality control |
| RBP4 | retinol-binding protein 4 |
| RCT | randomized clinical trial |
| ROS | reactive oxygen species |
| SNP | single nucleotide polymorphism |
| SP2 | the Singapore Prospective Study Programme |
| T2D | type 2 diabetes |
| TNF-α | tumor necrosis factor-α |
| TZDs | thiazolidinediones |
| ULSAM | the Uppsala Longitudinal Study of Adult Men |
| WHR | waist-hip ratio |

# 1 BACKGROUND

## 1.1 Diabetes

### 1.1.1 Prevalence of diabetes

Diabetes mellitus is one of the most common non-communicable diseases in the world nowadays. It has three major types, namely type 1 diabetes, type 2 diabetes (T2D) and gestational diabetes. T2D used to be called non-insulin dependent diabetes and accounts for at least 90 percent of all diabetic cases. The global prevalence of diabetes by International Diabetes Federation (IDF) was estimated to be 8.3% in 2011 and this number is projected to reach 9.9% with a burden of 438 million individuals in 2030 [1]. The economic cost incurred is heavy and 471 billion USD were spent due to diabetes in 2012 [1], not to mention the indirect and intangible costs.

A remarkable feature is that developing countries now experience more serious situation than developed countries, as 4 out of 5 people with diabetes live in low- and middle-income countries. In addition, almost half of the global burden of diabetes now falls in Asia, mainly due to large population size in China and India. The estimated number for 2011 was reported to be 92.4 million in China [2] and 62.4 million in India [3].

Moreover, T2D is often undiagnosed and according to the IDF statistics, 50% of the people were unaware of their condition. Patients are usually only diagnosed due to serious macrovascular and microvascular complications which include cardiovascular diseases, kidney failure (diabetic nephropathy), neurological complications (diabetic neuropathy) and eye diseases (diabetic retinopathy).

### 1.1.2 Pathophysiology of the disease

T2D is a disorder in glucose metabolism characterized by hyperglycemia. It results from a combination of defects in insulin secretion and insulin action and is hallmarked by resistance to effects of insulin in the liver, skeletal muscle and adipose tissues. As a result, the pancreatic β-cells increase the release of insulin to compensate for the reduced insulin action, until β-cell dysfunction occurs when the high demand cannot be met.

The relationship between β-cell function and insulin sensitivity is nonlinear following a hyperbolic shape and β-cells adapt to changes in insulin sensitivity by improved functional responsiveness and an increase in volume/mass [4, 5]. The degree of abnormality of insulin release by the β-cells proved to be the primary determinant of differences in glucose tolerance between individuals. Moreover, β-cell function declines progressively and the risk could be pre-existing, or genetically determined [4, 6].

The heritability of T2D is high according to twin studies and family studies [5]. However, known genetic variants explained only <10% of the estimated genetic contribution. Chronic over-nutrition and a failure to contain this fuel surfeit is the primary cause for the development of T2D [7]. Overweight and obesity are highly associated with risk of diabetes. However, many obese individuals manage to maintain normal glucose levels and do not develop diabetes in their entire life which implies the existence of interplay between genetics and environment. Moreover, early-life environment could regulate fatal and neonatal programming through epigenetic effects and hyperglycemic intrauterine environment is believed to contribute to the pathogenesis of T2D [8, 9].

## 1.2 Insulin resistance

### 1.2.1 Definition

Insulin is secreted in the β-cells of the pancreatic islets of Langerhans in response to elevated blood glucose levels after a meal. The major tissues insulin targets to lower blood glucose levels include the liver, skeletal muscles and adipose tissue. Insulin suppresses gluconeogenesis in hepatic cells and promotes glucose uptake in the muscles and adipocytes. Insulin sensitivity measures the responsiveness of tissues to insulin action and conversely, insulin resistance refers to a condition where the body is less sensitive to the functions of insulin. As a result, high level of insulin is usually observed under insulin-resistant condition.

### 1.2.2 Diagnostic tests

The gold-standard method to measure insulin sensitivity is the glucose clamp, or more specifically, the hyperinsulinemic euglycemic clamp [10]. As the name suggests, in this test insulin is infused at a constant high rate to reach a hyperinsulinemic status. This stimulates glucose uptake in skeletal muscles and adipose tissue and suppressed glucose production in the liver. Glucose is infused during the entire process in order to keep glucose concentration at constant levels. At steady-state the glucose infusion rate is assumed to equal glucose disposal rate which measures tissue sensitivity to exogenous hyper insulin levels. However, this test requires sophisticated equipment and is time-consuming and labor-intensive, and is thus rarely used in large epidemiological studies.

There are indirect measures of insulin sensitivity based on dynamic tests. One of such is the intravenous glucose tolerance test (IVGTT) and the most widely used method is the minimal model analysis of frequently sampled intravenous glucose tolerance test (FSIGT) [11]. FSIGT involves frequent blood samples for the measurement of glucose and insulin concentrations, and is slightly less laborious than the clamp method. There are also measures that are based on the oral glucose tolerance test (OGTT), a test widely used in clinical settings. Some OGTT-derived indices for the assessment of insulin sensitivity include the Matsuda index [12], the Stumvoll index [13], the Gutt index [14] and the Belfiore index [15].

In large epidemiological studies, it is common to use simple fasting measures of blood glucose and insulin concentrations to estimate insulin sensitivity and β-cell function. The Homeostasis model assessment index of insulin resistance (HOMA-IR) [16] uses simple equation to compute a surrogate index of insulin resistance based on steady-state basal glucose and insulin concentrations. However, it correlated more weakly with euglycemic clamp-measured insulin sensitivity, as compared with the OGTT-derived indices [17].

### 1.2.3 The role of obesity in insulin resistance and T2D

Mammalian adipose tissues consist of white and brown adipose. The brown fat is involved in thermogenesis and in human it is mainly found in newborn infants. Instead, the white adipose tissue has an important role in maintaining whole-body glucose homeostasis as well as lipid metabolism and adipocyte dysfunction is closely linked to insulin resistance and T2D.

Since the discovery of leptin in 1994 [18], the adipose tissue is increasingly recognized for its function as an endocrine organ. The white adipose tissue secretes numerous cytokines and hormones collectively known as adipocytokines, or adipokines [19]. Most of them, such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), leptin, retinol-binding protein 4 (RBP4) are pro-inflammatory, while adiponectin (introduced in the next chapter) has a unique anti-inflammatory and insulin sensitizing property [19-21]. This is important because chronic low-grade inflammation has been implicated in obesity and insulin resistance. C-reactive protein (CRP) is an acute-phase protein that serves as an important marker of systemic inflammation and is also related to insulin resistance [22].

Hypertrophic adipocytes increase secretion of many adipokines including monocyte chemoattractant protein-1 (MCP-1), also known as chemokine (C-C motif) ligand 2 (CCL2). MCP-1/CCL2 recruits additional macrophages which secretes large amount of TNF-α, resulting in an inflammatory state [23]. Increased lipolysis and decreased triglyceride storage lead to higher circulating levels of non-esterified fatty acids (NEFAs) and triglycerides. This causes ectopic lipid accumulation and impairs insulin-stimulated glucose uptake in skeletal muscle, which is believed to be a primary cause of insulin resistance [24]. Similarly, excess fatty acids in the liver decrease the responsiveness of the hepatic cells to insulin.

High levels of NEFAs may be the most critical determinant of insulin sensitivity. One proposed mechanism [25] is that increased intracellular concentrations of fatty acid metabolites, such as fatty acyl-coenzyme A (fatty acyl-CoA), diacylglycerol (DAG), and ceramides lead to phosphorylation of insulin receptor substrates (IRS) at serine/threonine site, and this disrupts the insulin signaling pathway by deactivating the phosphatidylinositol 3–kinase (PI 3-kinase). As a result, downstream insulin signaling and glucose transport is compromised.

Fat distribution is another important contributor of insulin resistance and abdominal obesity is closely associated with adverse metabolic consequences [26, 27]. Visceral adipose tissue is more lipolytic and less insulin-sensitive than the subcutaneous adipose tissue [28]. Together with the fact that the visceral fat depot is in closer proximity to the liver, portal NEFA levels are elevated in abdominal obesity. The expected results are increased hepatic glucose production and peripheral hyperglycemia [29].

### 1.2.4 Ethnic differences

Marked differences in insulin resistance exist between ethnic groups [30]. At the same degree of body fatness, whites have been consistently observed to have the lowest level of insulin resistance among major ethnic groups in the world [31-33].

Focusing on Asia where the burden of T2D is the heaviest, Asians, and particularly Asian Indians, are more insulin resistant than whites for a given degree of adiposity [34]. For the same body mass index (BMI), Asian Indians tend to have a higher level of body fat than whites [35] and they were more insulin resistant than whites for the same level of total body fat [36, 37], likely contributed by their abdominal fat distribution [38-40], differences in adipocyte cell size [37] and a higher ratio of total body fat to lean mass [36]. In addition, lower adiponectin levels [30, 31, 33, 41-43], higher CRP levels [43-45] as compared with whites independent of BMI, and dietary factors may contribute to the higher susceptibility of Asian Indians in developing T2D [46, 47].

Several studies in the U.S. have also demonstrated greater insulin resistance in East Asians as compared with whites for a given BMI [32, 48, 49]. For example, Chinese-American and Japanese-American women were more insulin resistant than white women after adjusting for waist circumference [48]. Asian Americans have also been shown to be more likely to develop T2D than whites after adjustment for BMI [50]. Lower adiponectin levels in East Asians than BMI-matched whites, as reported for Japanese men [51] and for Koreans men and women [52], may contribute to these ethnic differences in insulin resistance and T2D. In a Canadian study, Chinese had lower adiponectin levels than whites in both men and women after considering differences in waist circumference [30].

There is also evidence for substantial differences in insulin resistance between ethnic groups in Asia. Asian Indians are more insulin resistant and glucose intolerant than Chinese and Malays [53], but reasons for these ethnic differences are not well understood. One of the major contributing factors could be adiposity and fat distribution, as Asian ethnic groups differ from each other in body composition [54]. However, this does not fully explain the ethnic difference in insulin resistance. In Singapore, for example, Malays have the highest levels of adiposity, but not the highest levels of insulin resistance [53]. Circulating adipokines and inflammatory markers may contribute to some of the differences in insulin resistance between Asian ethnic groups,

but to what extend these factors mediate the relation between ethnicity and insulin resistance independently and inter-connectively remains largely inconclusive.

### 1.2.5 Dietary antioxidants

Oxidative stress manifests an imbalance between the production of reactive oxygen species (ROS) and antioxidant defenses [55]. Increased ROS levels and excessive oxidative stress trigger insulin resistance, impair glucose tolerance and β-cell function, and accelerate the development of T2D [56-58]. Some of the most recognized dietary antioxidants include vitamin C, vitamin E, selenium and carotenoids.

A meta-analysis showed that the intake of antioxidants was associated with 13% reduction in the risk of T2D and this was mainly attributed to vitamin E and carotenoids [59]. Several longitudinal studies have found that dietary β-carotene (a subtype of carotenoids) and α-tocopherol (a subtype of tocopherol, or vitamin E) independently predicted risk of T2D [60-62], possibly mediated by an improvement in insulin sensitivity [62]. However, supplementation of β-carotene and α-tocopherol in randomized clinical trials (RCTs) revealed no benefit in the prevention of T2D and indicated that the relationship may not be causal [59].

Flavonoids are compounds with anti-oxidant capacities. A recent large prospective study found an association between consumption of foods rich in anthocyanin (a sub-class of flavonoids), especially berries and apples/pears, and a lower risk of T2D[63].

In contrast to anti-oxidants, excess iron is believed to elicit toxic effects mainly related to oxidative stress, due to the generation of ROS during a redox cycle [64]. High body iron store, as reflected by serum ferritin levels, has been associated with increased risk of T2D and it was suggested that dietary iron caused insulin resistance through down-regulation of adiponectin [65, 66].

*Selenium*

Selenium is an essential trace mineral. It exists predominantly as selenocysteine and selenomethionine in foods, but the content varies greatly depending on the soil conditions. Selenocysteine (Se-Cys) is a cysteine analogue with a selenium-containing selenol group replacing the sulfur-containing thiol group. Proteins that include a selenocysteine amino acid are called selenoproteins. The most abundant selenoprotein in the plasma is selenoprotein P [67, 68]. Glutathione peroxidases (GPx) is a family of enzymes with peroxidase activity that detoxify peroxides and hydroperoxides. They are involved in antioxidant defense and protect against oxidative stress. GPx are also selenoproteins accounting for 10-30% of plasma selenium [67] and it contains selenium as selenocysteine in the catalytic site.

Selenium has been suggested to have insulin-mimetic and anti-diabetic properties [69, 70], but results from existing studies on its association with risk of T2D are inconclusive [70]. In cross-sectional studies, higher selenium status has been associated with both a lower and a higher prevalence of diabetes [71-73]. Most RCTs concluded

no overall efficacy of selenium supplementation on risk of diabetes and glucose control [74-76]. Importantly, selenium supplementation in clinical trials is usually given in high doses and effects may thus not reflect effects of smaller variation in dietary selenium intakes. To date, little evidence is available from prospective cohort studies of selenium status in relation to glucose tolerance and risk of diabetes. In addition, there is a lack of studies studying selenium intake in relation to detailed measures of insulin processing, secretion and sensitivity.

Selenium content in foods varies greatly depending on soil conditions and as a result, selenium intakes calculated using food composition databases are inaccurate. In contrast, serum/plasma selenium is a reliable biomarker for selenium status [77, 78]. The correlation between serum selenium and selenium intake has been shown to be reasonably high [79]. Besides, erythrocyte selenium and toenail selenium are good measures of long-term selenium intake.

## 1.3 Adiponectin

### 1.3.1 Structure and circulating forms

Adiponectin is a 30-kDa protein secreted by the adipocytes. It is the most abundant adipokine in the circulation and its levels remain relatively constant [80, 81]. The protein is composed of four domains: an N-terminal signal peptide, a variable region, a collagenous domain, and a C-terminal globular domain. Adiponectin exists in full-length as multimer complexes, and also as a globular fragment. The three major oligomeric forms include the low-molecular-weight (LMW) trimer, the middle-molecular-weight (MMW) hexamer and the high-molecular-weight (HMW) 12-18mer [82]. The different isoforms could have distinct properties and exert diverse biological functions, although this is not completely understood yet. Therefore, multimer distribution, in addition to total adiponectin concentration, should also be considered in the interpretation of adiponectin levels and health outcomes [83].

### 1.3.2 Receptors and signaling pathways

Three adiponectin receptors are known to date. Adiponectin receptor 1 (AdipoR1) and adiponectin receptor 2 (AdipoR2) are transmembrane receptors having an inverse topology as compared with the G protein-coupled receptors [84]. A third receptor, T-cadherin, belongs to the cadherin family of proteins and has not been well-characterized for its function with adiponectin. It lacks an intracellular domain and is attached to the membrane by a glycosylphosphatidylinositol (GPI) anchor [85].

AdipoR1 binds to globular adiponectin with high affinity and AdipoR2 has intermediate affinity for both globular and full-length adiponectin [84]. Both receptors are abundantly expressed, although the relative abundance varies in tissues. T-cadherin is a receptor exclusively for hexameric and HMW adiponectin [85] and is expressed in the endothelial and smooth muscle cells [86].

Adiponectin mainly signals through the adenosine monophosphate (AMP)-activated protein kinase (AMPK) and the peroxisome proliferator activated receptor-α (PPAR-α)

signaling pathways [87, 88]. However, individual isoforms seem to have different biological activities and activate different signal transduction pathways. In skeletal muscle, trimeric adiponectin activates AMPK, whereas the hexamer and HMW form activate NF-kB pathway [89]. In the liver, AMPK are stimulated by full-length adiponectin only [87]. Therefore, it is possible that adiponectin controls its ligand signaling by different oligomerization states. Without an intracellular domain, T-cadherin is thought to have no role in signal transduction, although it is capable of binding adiponectin.

### 1.3.3 Functions in glucose and fatty acid metabolism

Although adiponectin is primarily produced by the adipose tissue, as a paradox, blood levels of adiponectin are inversely associated with obesity [90]. In addition, a sexual dimorphism is observed where adiponectin levels are higher in women than in men, but this is not explained by fat mass [91] and could be partially accounted for by the inhibitory effect from testosterone [92, 93].

It is also well-established that adiponectin levels are inversely associated with degree of insulin resistance [94, 95] and lower adiponectin is also associated with higher risk of T2D [96]. In concordance, hypoadiponectinemia is observed in insulin resistance-related conditions such as metabolic syndrome, hypertension, dyslipidemia, and oxidative stress [97]. However, it remains a question whether the inverse relationship between adiponectin levels and degree of insulin resistance represents a causal relationship.

In the liver, adiponectin lowers glucose levels by suppressing gluconeogensis and sensitizes hepatic cells to the effects of insulin by a reduction in lipid content [21, 98]. This is achieved by the inhibition of the expression of gluconeogenic enzymes and the phosphorylation of acetyl coenzyme A carboxylase (ACC). In skeletal muscle, it activates AMPK, thereby stimulating phosphorylation of ACC, fatty acid oxidation and glucose uptake [87, 99]. In particular, HMW adiponectin has been suggested to be the bioactive form having stronger associations with insulin sensitivity and suppression of hepatic glucose production than other forms of adiponectin [83, 100-102].

In obese or obese T2D individuals, activation of AMPK signaling and fatty acid oxidation by globular adiponectin is reduced, not due to the expression of adiponectin receptors, but as a result of downstream signaling after receptor binding [103]. APPL1, an adaptor protein, has been suggested to be a key molecule in signal transduction linking adiponectin receptors and AMPK activation [104].

### 1.3.4 Heritability and genetic predisposition

Genetic determinants account for a substantial proportion of the variation in plasma adiponectin, as estimated to be 30 -70% [105-107].

Genome-wide scans based on LOD scores have identified signals on chromosome 5 and 14 in Europeans [107], on chromosome 9 in Pima Indians [106], and on

chromosome 15 in East Asians [105]. The region flanking the adiponectin gene locus on chromosome 3 was first reported to be responsible for the linkage to adiponectin levels in an Amish population through an initial linkage scan plus fine-mapping using single nucleotide polymorphisms (SNPs) [108]. However, linkage studies had limited accuracy in the ascertainment of the exact genomic location harboring the signal and more importantly, very few linkage studies (for any traits) have been replicated - probably due to the power being too low in all such studies.

With the advancement of the Genome-wide association studies (GWAS) era, a substantial larger number of adiponectin-associated loci have been identified using the SNPs as markers. These include the adiponectin gene *ADIPOQ* [109-112], *CDH13* which exhibited as a prominent signal in Asians [113-116], *ARL15* [109] and *FER* [112]. A recent multi-ethnic large GWAS meta-analysis identified 10 novel loci and this increased the known loci to 14 in total. Many of the newly identified loci, such as *IRS1*, *PEPD*, *GPR109A,* and *ZNF664*, have functional relevance with insulin resistance and T2D [117].

The *ADIPOQ* gene is located on chromosome 3q27 and consists of 3 exons spanning 17kb. Many common polymorphisms in the promoter, exon and introns, as well as rare non-synonymous mutations have been associated with obesity, glucose and insulin metabolism and T2D in diverse populations [118]. Variants in the adiponectin receptor genes *ADIPOR1* and *ADIPOR2* have also been found to associate with insulin resistance and T2D, but not always replicated across populations. Potential reasons that limit the reproducibility include undetected population structure, false-positive results, small sample sizes, between-study heterogeneity, imprecise measurement of phenotypes and gene-environment interactions [119].

Another gene highly associated with adiponectin levels is *CDH13* on chromosome 16 and it contains 14 exons and spans 1.2 Mb. This gene encodes for T-cadherin which binds hexameric and HMW adiponectin in the vasculature and endothelial cells [85]. Despite the strong association of *CDH13* with adiponectin, follow-up studies for its roles in metabolic disorders are lacking. Polymorphisms in the *CDH13* gene are not broadly studied and the mechanisms of T-cadherin in adiponectin signaling and insulin resistance are unclear at the moment.

# 2 AIMS OF THE THESIS

The overall aim of this thesis was to have a better understanding of the role of ethnicity, genetic variants and dietary factors as determinants of adiponectin levels and insulin resistance by means of different analytical approaches. The specific aims were:

- To evaluate to what extent body fatness, adiponectin levels, C-reactive protein and their interconnections mediate the relation between ethnicity and insulin resistance by path analysis in an Asian context. (Study I)

- To identify common genetic variants associated with adiponectin levels on a genome-wide scale and examine their associations with insulin resistance and related metabolic risk factors in East Asians. (Study II)

- To elucidate the potential causal effect of adiponectin on insulin sensitivity measured by euglycemic insulin clamp in a cohort of Swedish men using a Mendelian randomization approach. (Study III)

- To investigate prospectively the relationship between baseline selenium concentration as a surrogate of selenium dietary intake and measures of glucose metabolism and incidence of type 2 diabetes in Swedish men. (Study IV)

# 3 METHODS

## 3.1 Participants

### 3.1.1 The Singapore Prospective Study Programme (SP2)

Participants of SP2 previously participated in cross-sectional studies carried out from 1982 to 1998, namely the Thyroid and Heart Study (1982-1984) [53], the National Health Survey (1992) [120], the National University of Singapore Heart Study (1993-1995) [53], and the National Health Survey (1998) [121]. Each of these studies was based on a random sample of Singapore residents with the minority groups (Malays and Asian Indians) being over-sampled. From 2003 to 2007, all 10,747 participants from these studies were invited to participate in the SP2 study. Of these, 559 had deceased at the time of study, 6 emigrated, and 102 were excluded because of errors in their identity card numbers. Qualified participants were contacted and 7,774 completed the questionnaire. The remainder was invited to a health screening which 5,164 subjects attended. **Figure 1** is a flow-chart of the design of this study.

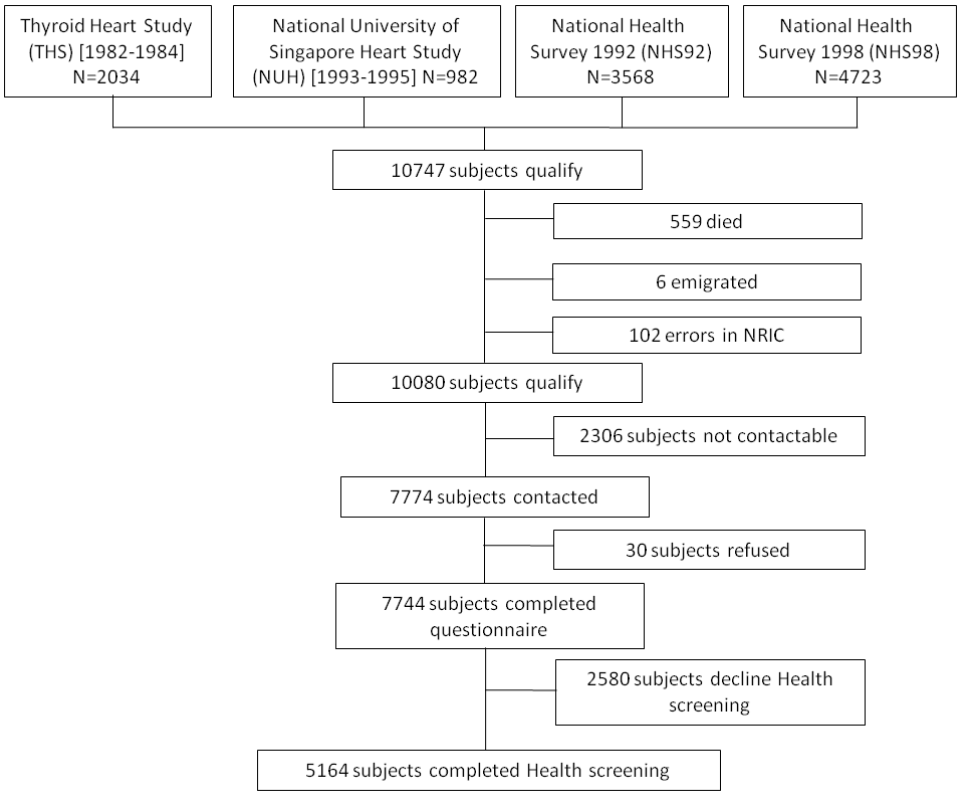


**Figure 1.** Design of the Singapore Prospective Study Programme

Written informed consent was obtained from all participants and ethics approval was obtained from the Singapore General Hospital and the National University Hospital Institutional Review Boards.

### 3.1.2 The Uppsala Longitudinal Study of Adult Men (ULSAM)

The Uppsala Longitudinal Study of Adult Men (ULSAM) was initiated between September 1970 and September 1973 with an invitation of all 50-year-old men living in Uppsala County, Sweden. Of the invited, 82% participated in the investigation. There are five follow-ups to date, at 60, 70, 77, 82 and 88 years of age. **Figure 2** is an overview of the design of this cohort study and detailed information can be found at the cohort website (http://www.pubcare.uu.se/ULSAM/). The main sample used for this thesis work comes from the third investigation during 1991-1995 when the subjects were approximately 71 years old. A total of 1,221 men (73% of those invited, i.e. men still alive and residing in Uppsala County) participated and the examination included a medical questionnaire, blood pressure and anthropometric measurements, collection of blood samples, a 75-gram oral glucose tolerance test, and insulin sensitivity measurements.

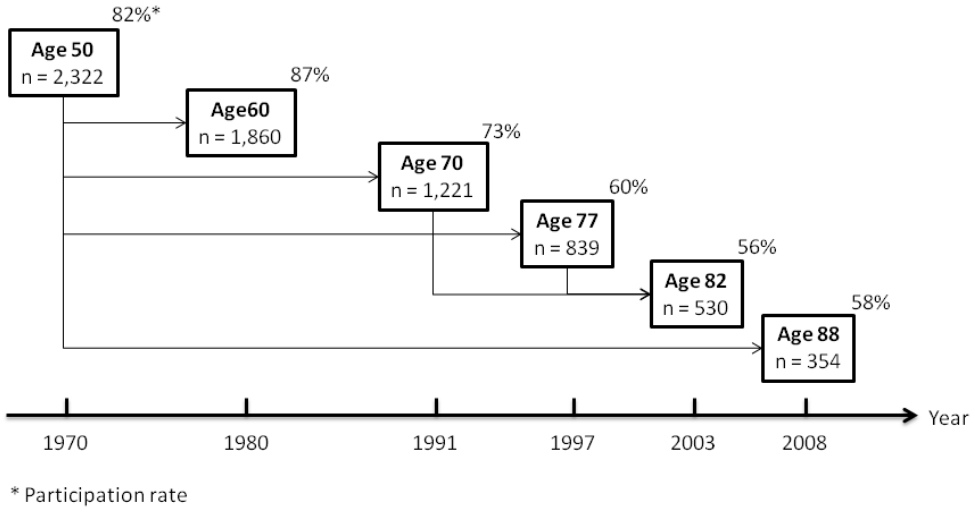


**Figure 2**. Overall design of the Uppsala Longitudinal Study of Adult Men.

The ULSAM study was approved by the Ethics Committee of Uppsala University, and all participants provided written informed consent.

## 3.2 Measurements

### 3.2.1 SP2 (Study I and II)

Height was measured using a wall mounted measuring tape and weight was measured using a digital scale. Waist circumference was measured midway between the lower rib margin and the iliac crest and hip circumference was measured at the widest point over the greater trochanters. BMI was computed as weight (kg) divided by height square ($m^2$). Demographic data including ethnicity and lifestyle information such as smoking status and alcohol intake were assessed using standardized questionnaires. Total physical activity was measured using a locally validated questionnaire covering activity in four domains (household, occupational, leisure-time and transport).

Fasting blood samples were analyzed for glucose using enzymatic methods (ADVIA 2400, Siemens, Germany), for insulin using micro-particle enzyme immunoassay (Abbot AXSYM, Abbott Laboratories, Chicago, IL), for high-sensitivity CRP using an immuno-turbidimetric assay (Roche Diagnostics, Rotkreuz, Switzerland), and for total and HMW adiponectin using an enzyme linked immune-sorbent assay (Sekisui Medical Co Ltd, Japan). Insulin resistance was assessed by HOMA-IR calculated as: (fasting insulin in mIU/L x fasting glucose in mmol/L) / 22.5 [16]. The intra and inter batch coefficient of variations percent were as follows: glucose (2.5, 6.6), insulin (4.0, 4.5), total adiponectin (18.1, 15.9), HMW adiponectin (6.8, 18.3) and CRP (0.6–1.3, 2.3–3.1).

### 3.2.2 ULSAM (Study III and IV)

*At baseline (50 years old)*

An IVGTT was performed where blood glucose and serum insulin levels were measured between 0 and 60 min after the intravenous glucose load. Proinsulin concentrations were analyzed using the two-site immunometric assay technique [122]. Glucose tolerance was evaluated by the K-value calculated as $K=\ln 2 \cdot 100/T_{1/2}$ where $T_{1/2}$ is the time in minutes required for the concentration to be reduced by half its value. Early serum insulin response was represented by the insulin peak and expressed as the mean value of the serum insulin concentrations determined at 4, 6 and 8 minutes. The insulin index was defined as the ratio between peak serum insulin response and fasting serum insulin concentration. Based on fasting glucose and insulin concentrations, the homeostasis model assessment index for insulin resistance and β-cell function (HOMA-IR and HOMA-B) were calculated [16].

T2D was ascertained by elevated fasting blood glucose (≥ 6.1 mmol/L, which equals plasma glucose ≥ 7.0 mmol/L) or use of anti-diabetic medicine.

Selenium was determined in serum using the graphite-furnace atomic absorption spectrometric method [123]. Samples were diluted (1+9) with a solution containing nickel (to reduce the volatility of selenium) and nitric oxide (to keep samples free of precipitates) and measured by a standard additions method.

Information about cigarette smoking status was based on data from a standardized interview. Current smokers were further classified according to the quantity of cigarettes smoked (<10 or >=10 cigarettes per day) based on information from the questionnaire. Leisure time physical activity was recorded in the questionnaire in four levels: sedentary, moderate, regular and athletic. Education level was also assessed on the questionnaire and was re-grouped into three categories: 7 years or 8 years was defined as low education; 12 years as medium education; and ≥3 years of college or completion of university graduate exam as high education.

*At 20-year follow-up (70 years old)*

Participants at the age 70 investigation underwent a 75-gram OGTT with measurement of plasma glucose and immunoreactive insulin. The intra-individual coefficient of variation (CV) for fasting plasma glucose was 3.2%. The insulinogenic index, calculated as [(insulin at 30 min) – (insulin at 0 min)]/ [(glucose at 30 min) – (glucose at 0 min)], was used as a measure of glucose-stimulated insulin secretion indicative of β-cell function [124]. Proinsulin levels were measured by the two-site immunometric assay technique [122].

*In vivo* sensitivity to insulin was determined by the euglycemic insulin clamp, according to the procedure described by DeFronzo *et al*. (1979) [10], but with a higher insulin infusion rate per body surface area to better suppress liver glucose output (56 mU $min^{-1}$ $(m^2)^{-1}$ instead of 40 mU $min^{-1}$ $(m^2)^{-1}$). After a primary dose in the initial 10 min, continuous infusion of insulin lasted for 110 min and hepatic glucose production was assumed to be entirely suppressed. Glucose disposal (M) was calculated as the total amount of glucose infused during the last 60 min (the steady state) of the clamp divided by kg body weight and minutes. The insulin sensitivity index (M/I ratio) was derived by dividing M by the steady-state mean insulin concentration (I). M/I thus represents the amount of glucose metabolized per unit of plasma insulin and was given in 100 × mg $kg^{-1}$ $min^{-1}$ $mU^{-1}$ L.

T2D was defined by elevated fasting glucose levels and/or use of anti-diabetic medicine. Elevated glucose levels were assessed as fasting plasma glucose ≥7.0 mmol/L at age 70 (the same criterion was used at age 60 and age 77).

Serum adiponectin was measured in plasma samples frozen at -70 °C for 11 ± 2 years, without previous thaw-freeze cycles and using a validated in-house time-resolved immunofluorometric assay (TR-IFMA) with reagents from R&D Systems (Abingdon, UK). The intra- and inter-assay coefficient of variation averaged less than 5% and 10%, respectively, as described in detail previously [125].

## 3.3 Genotyping

### 3.3.1 SP2 (Study II)

2,865 blood-derived DNA samples from the SP2 Chinese participants were genotyped using Illumina HumanHap 550, 610 Quad, and 1Mduov3 BeadChips (http://www.illumina.com). The data went through quality control (QC) procedures which were described in details elsewhere [126]. SNPs with call rate < 0.95, minor allele frequency (MAF) < 0.01 or Hardy-Weinberg equilibrium (HWE) *P*-value < $1\times10^{-6}$ were filtered out. 431 individuals did not pass the QC due to high rates of missingness, excessive heterozygosity, cryptic relatedness, discordant ethnicity and gender discrepancy. Imputation was done with IMPUTE (http://mathgen.stats.ox.ac.uk/impute/impute.html) on 22 autosomes using NCBI build 36 HapMapII CHB and JPT data (release 22) as the reference panel. Imputation results

of SNPs that were actually genotyped were replaced with experimentally determined genotypes before the association tests were conducted.

### 3.3.2 ULSAM (Study III)

The ULSAM participants at age 70 have undergone prior genotyping on the Human CardioMetabo beadchip (Metabochip; http://www.illumina.com/support/array/array_kits/ humancardio-metabo_beadchip_kit.ilmn), which is designed to interrogate 200,000 markers of interest for cardiovascular and metabolic diseases. Of the 1,221 individuals with genotype data, we removed those with genotyping call rate < 0.99 (n = 5), failing sex check (n = 1), close relatedness (n = 36), or large heterozygosity (n = 7). Quality control also ensured that all SNPs had good call rate (> 0.99) and did not deviate from HWE ($P > 1 \times 10^{-6}$). In total, genotypes of 183,357 SNPs were available for 1,175 individuals.

## 3.4 Statistical analysis

### 3.4.1 Path analysis in Study I

Path analysis is an extension of regression analysis that simultaneously performs a series of regression analyses in complex networks. It is suitable to elucidate complicated inter-relationships between exposure variables of interest, multiple potential mediators, and health outcomes [127, 128]. Compared with ordinary regression analysis, path analysis has the advantage that it allows examination of the potential causal processes underlying an observed relationship and to estimate the relative importance of alternative paths of influence in a complicated system [127]. Particularly, path analysis also allows a disentanglement of the direct and indirect effects which can provide more insights into the complicated relationships.

In our study path analysis was carried out to evaluate mediators of pair-wise ethnic differences in insulin resistance. A hypothetical model was proposed based on previous biological knowledge of the relationships between ethnicity, insulin resistance, adipokines and inflammation (**Figure 3**). BMI and the BMI-adjusted waist circumference (waistR) were chosen based on previous analysis results to represent body fatness in the model, total adiponectin level to represent adipokines, and inflammation was represented by CRP levels. Covariates considered included age, alcohol, smoking and physical activity. One-way arrows represent causal effects and two-way arrows represent correlations between the variables. Endogenous variables which are variables having at least one incoming arrow were depicted with associated error terms.

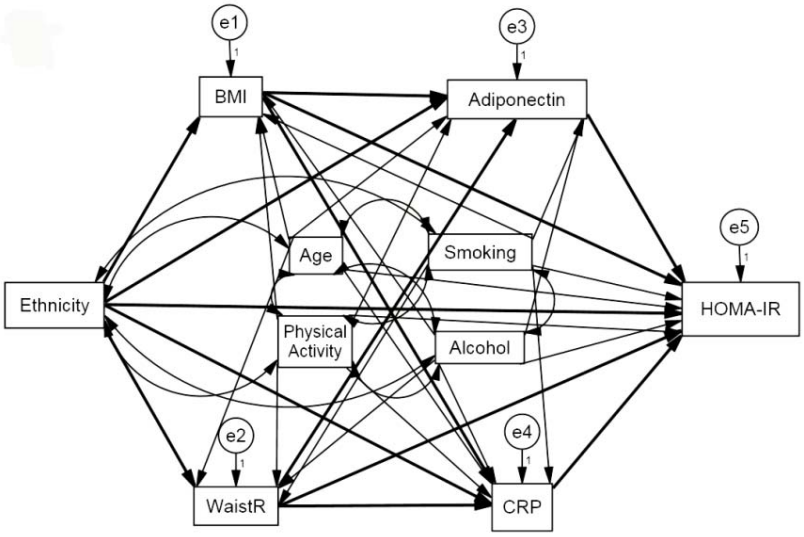


**Figure 3**. Full path diagram for the hypothetical model.

We performed path analysis based on the full hypothesized model and paths with non-significant path coefficients were removed to form a reduced model. Path analysis was performed again based on the reduced model. Model fit in comparison with the saturated and independent models was assessed based on the Bayesian Information Criterion (BIC).

Amos 18.0 [129] was used to run the path analysis and Stata/SE 10.0 (Stata Corporation, College Station, Texas) for other analyses. All statistic tests were two-sided and the level of significance was set at $\alpha$=0.05.

### 3.4.2 Genome-wide association analysis in Study II

A genome-wide association study for total and HMW adiponectin was carried out on 2,434 post-QC samples of the SP2 study. Adiponectin levels were natural log-transformed and standardized to the z-scores. Additive genetic model adjusting for age and sex was used to test the associations and BMI was further adjusted for in a second model. The software used for the association study was SNPTEST v2.2.0 (https://mathgen.stats.ox.ac.uk/genetics_software/snptest/snptest.html). Samples genotyped on different chips were treated as separate studies and the results were meta-analyzed under fixed-effect model weighted by inverse variance using METAL (http://www.sph.umich.edu/csg/abecasis/metal/). Sample size weighted meta-analysis was also performed and results were similar. Genomic control was applied to each study as well as the first-round meta-analysis results to correct for inflation.

Genotypes for the top SNP from the meta-analysis, rs4783244 in the *CDH13* gene, were successfully called for 2,429 individuals in the SP2 sample. Among them 39 had missing adiponectin levels and 4 had missing values for HOMA-IR. After further excluding those without age (n = 1), BMI (n = 2) and those taking diabetic medicine (n = 101), 2,282 individuals remained for the analysis.

For the replication analysis, a total of 3,290 Japanese from the Nomura [130] and AAC studies [131] (1,226 with both total and HMW adiponectin levels) and 1,610 Koreans in the Yangpyeong Study [132] (total adiponectin levels only) with clinical data and genotypes for rs4783244 were included.

Levels of adiponectin were measured using different kits in the studies involved, so standardization to mean of zero and variance of one was uniformly performed on adiponectin and other metabolic variables to facilitate cross-study comparisons and meta-analyses. Multiple linear regressions based on additive and general genetic models were used with different adiponectin forms and metabolic risk factors (log-transformed if necessary) as dependent variable and genotype, age and sex as independent variables. In addition, for metabolic traits, multivariable models that also included HMW adiponectin were evaluated. Bonferroni-corrected threshold $\alpha \leq 5\times10^{-8}$ was considered genome-wide significant and $\alpha \leq 0.005$ was used as a cutoff for the tests on rs4783244 and 10 metabolic variables (calculated as 0.05/10). All tests were 2-sided.

### 3.4.3 Mendelian randomization in Study III

Mendelian randomization (MR) is a method that uses genetic variants (instrumental variables, IVs) as robust proxies for an environmentally modifiable exposure to assess and quantify potential causal relationships with health outcomes [133, 134]. Because genotypes that influence the exposure are assigned at conception, they are unlikely to be related to confounders such as lifestyle, socioeconomic and environmental risk factors or reverse causation.

*Hypothetical model*

**Figure 4** illustrates the key relationships in the study of the influence of adiponectin on insulin sensitivity using a MR design. Solid arrows represent causal influences that are expected and dotted arrows are causal influences that are assumed not to exist. Genetic variants from *ADIPOQ* gene are used as IVs based on three main assumptions: (1) The IVs are robustly associated with the exposure of interest, i.e. adiponectin levels; (2) the IVs are independent of confounders for the association between adiponectin and insulin sensitivity in observational studies; (3) the IVs are independent of the outcome insulin sensitivity given adiponectin levels and the confounders. Moreover, we hypothesize that part of the effect of adiponectin on insulin sensitivity is mediated by adiposity which is represented by BMI and waist circumference.

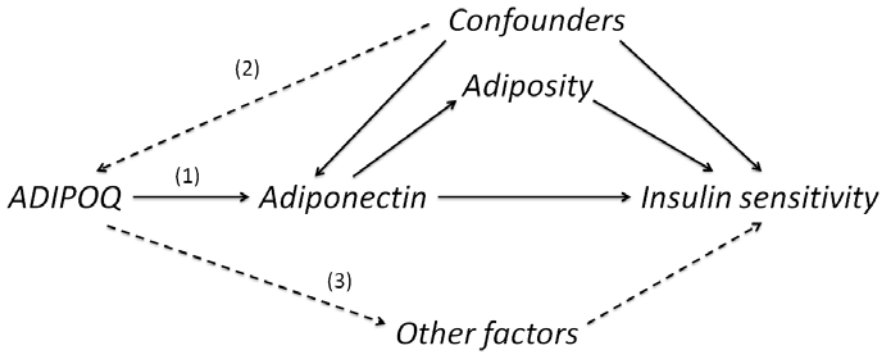


**Figure 4.** Hypothetical model for the relationship between *ADIPOQ*, adiponectin and insulin sensitivity

*Selection of instrumental variables*

We searched the genomic region ± 50kb of the *ADIPOQ* gene and identified 16 SNPs that were present on the Metabochip. Pair-wise linkage disequilibriums (LDs) were then assessed and of the SNPs that were significantly associated with adiponectin, we selected rs17300539 and rs3774261 that were the top associated SNPs in their respective LD block as IVs. We also selected rs6444175 ($r^2 = 0.506$ with rs3774261) and further generated an un-weighted allele score from rs17300539 and rs3774261 ($r^2 = 0.137$). Thus, we had three single SNPs and one allele score as IVs of serum adiponectin for subsequent MR analyses.

Among the 1,221 participants, 199 were excluded for the following reasons: unavailable clamp data (n = 61), unavailable measurement of adiponectin (n = 15), or presence of T2D (n = 123). Of the remaining 1,022 participants, 942 participants with genotypes for *ADIPOQ* were eligible for the analysis.

Adiponectin and insulin sensitivity index (M/I ratio) were natural log transformed and outliers (≥ 4SD from the mean) were truncated. The observed effects of adiponectin on M/I ratio, as well as effects of IVs on M/I ratio were obtained by linear regression under additive genetic models with adjustment for age. In further analyses, BMI and waistR were included as covariates into the model to evaluate effects independent of adiposity. Residual plots and cubic spline curves were plotted to visualize the degree of deviation from linearity.

IV analysis was then performed using a two-stage least square (2SLS) approach with the Stata package 'ivreg2'. In brief, the first stage was a conventional linear regression assessing the association between the SNPs and serum adiponectin. The predicted value of adiponectin from the model was saved and used as an independent variable in the second stage where the dependent variable was insulin sensitivity index (M/I ratio). This beta coefficient, or the IV estimate, reflects an unconfounded effect of genetically determined adiponectin level on insulin sensitivity. Endogeneity, or the difference between the IV estimate and the observed effect size, was examined by a Durbin-Wu-Hausman Chi-square test implemented in the package. The percentage of change in

insulin sensitivity with x% increase in adiponectin estimated by each instrument was calculated by $((1 + x\%)^{\beta}-1) \times 100\%$ where β is the corresponding IV estimate. To test the generalizability of our results, we performed secondary analyses including the 123 individuals with T2D. In order to examine potential effect modification by adiposity, we conducted a BMI-stratified analysis by defining overweight using the median BMI level in our sample (BMI ≥ 25.7kg/m$^2$).

Data were analyzed using Stata/IC (version 12.1, StataCorp, College Station, TX). Two-sided *P*-values < 0.05 were considered significant.

### 3.4.4 Longitudinal analysis in Study IV

Of the 2,322 participants at the age 50 examination, 393 individuals were excluded for the following reasons: serum selenium data not available (n = 274), presence of diabetes at baseline (n = 115) or missing glucose measurements (n = 8). In total, 1,925 non-diabetic individuals formed the baseline study sample of the analysis in this study. After 20 years of follow-up, among the 1,211 participants at age 70, 1,026 had baseline serum selenium and 936 non-diabetic individuals were included for the analyses on glucose metabolic measures.

Selenium levels were analyzed both as categorical variables in tertiles and as continuous variable (presenting betas per 1-SD increment). Continuous outcome variables were natural log transformed to improve normality of the distribution and also presented as change in SD-unit. Observations beyond 4SD from the mean were defined as outliers and truncated. Logistic regression analysis was used to examine the association between baseline selenium levels and risk of T2D at follow-up. The relationship between selenium and glucose metabolism measures at baseline and after 20 years of follow up was examined by linear regression analysis. Two sets of models were used: model 1 adjusted for age at baseline; model 2 further adjusted for potential confounders including BMI, cigarette smoking, leisure time physical activity, and education levels. *P*-values for trend were calculated by assigning the median value to each selenium tertile category and modeling it as continuous variable.

In a sensitivity analysis, we evaluated the association of baseline selenium levels and incident diabetes risk using the follow-up data at age 60 and 77 and compared the estimates with those from the age 70 follow-up.

Analyses were performed using Stata/IC (version 12.1, StataCorp, College Station, TX). Two-tailed *P*-values < 0.05 were considered significant.

# 4 RESULTS & DISCUSSIONS

## 4.1 Study I

In this study we examined the mediating effects of body fatness, adiponectin and CRP on the differences of insulin resistance between three major ethnic groups in Singapore using path analysis.

The degree of insulin resistance as reflected by HOMA-IR was highest in Asian Indians, intermediate in Malays, and lowest in Chinese. CRP was higher in Asian Indians and Malays than in Chinese, and Indian women had particularly high levels of CRP. Total and HMW adiponectin were highest in Chinese and lowest in Asian Indians when women were compared, but these ethnic differences were less marked among men. BMI was lowest in the Chinese and was similar for Malays and Asian Indians. However, Asian Indians had a larger WHR and a larger waist circumference than Malays and Chinese. WHR was not significantly different between Chinese and Malay men.

By linear regression, ethnic difference in insulin resistance between Malays and Chinese disappeared after adjusting for BMI and waist or only BMI. The difference in HOMA-IR between Asian Indians and Chinese was also reduced after adjusting for BMI and waist, but remained statistically significant. Further adjustment for adiponectin and CRP levels explained slightly more of the ethnic difference in HOMA-IR (**Table 1**).

**Table1.** Associations between ethnicity and the HOMA-index of insulin resistance based on linear regression analysis with and without adjustment for potential intermediates.

| | **Male** | | | **Female** | | |
|---|---|---|---|---|---|---|
| | **Model 1** | **Model 2** | **Model 3** | **Model 1** | **Model 2** | **Model 3** |
| Chinese | Ref. | Ref. | Ref. | Ref. | Ref. | Ref. |
| Malay | 0.17 | *-0.01* | *-0.01* | 0.28 | *-0.05* | *-0.02* |
| Asian Indian | 0.48 | 0.25 | 0.23 | 0.50 | 0.17 | 0.15 |
| BMI, kg/m2 | | 0.58 | 0.48 | | 0.56 | 0.42 |
| WaistR, cm | | 0.13 | 0.10 | | 0.13 | 0.10 |
| Adiponectin, ug/ml | | | -0.19 | | | -0.21 |
| CRP, mg/l | | | 0.11 | | | 0.13 |
| $R^2$ | 0.06 | 0.38 | 0.42 | 0.12 | 0.38 | 0.44 |

Model 1, unadjusted; Model 2, adjusted for BMI and waistR; Model 3, further adjusted for adiponectin and CRP. Italic values indicate statistically non-significant at $\alpha$=0.05 level.

*Malay-Chinese comparison*

The effect of ethnicity on insulin resistance when Malays were compared with Chinese could be fully explained by the considered mediators as indicated by the non-significant direct path between ethnicity and HOMA-IR (**Figure 5A and 5B**).

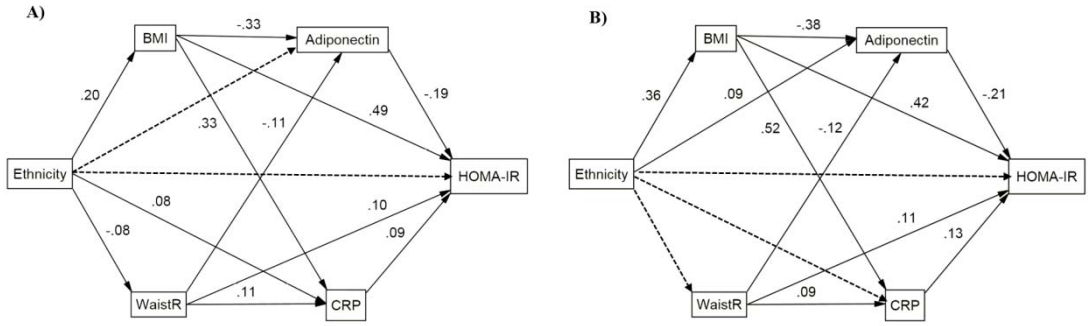


**Figure 5A, 5B.** Path coefficients in the reduced models for Malay-Chinese comparison of differences in insulin resistance in men (A) and women (B).

Ethnic Malays represent millions of people from different Southeast Asian Island populations including Malaysians, Filipinos and Indonesians who are experiencing a rapid increase in chronic disease burden [135]. Studies have demonstrated that overall ethnic Chinese from East Asia and Malays from Southeast Asia were genetically closer than either of them with Asian Indians [136]. More specifically, although culturally distinct, Malays in Singapore had a greater genetic similarity with Chinese than Asian Indians [136, 137]. Greater general adiposity of Malays as compared with Chinese explained their greater insulin resistance in our study and targeting excess weight gain in Malays should have high priority in public health efforts for chronic disease prevention [135].

*Indian-Chinese comparison*

BMI alone accounted for a substantial proportion of the observed difference in insulin resistance between Asian Indians and Chinese. In addition, CRP, but not adiponectin, also contributed independently to the greater insulin resistance in Asian Indians as compared with Chinese. A smaller proportion was attributable to effects of adiposity through adiponectin and CRP and effects of CRP independent of BMI (**Figure 5C and 5D**).

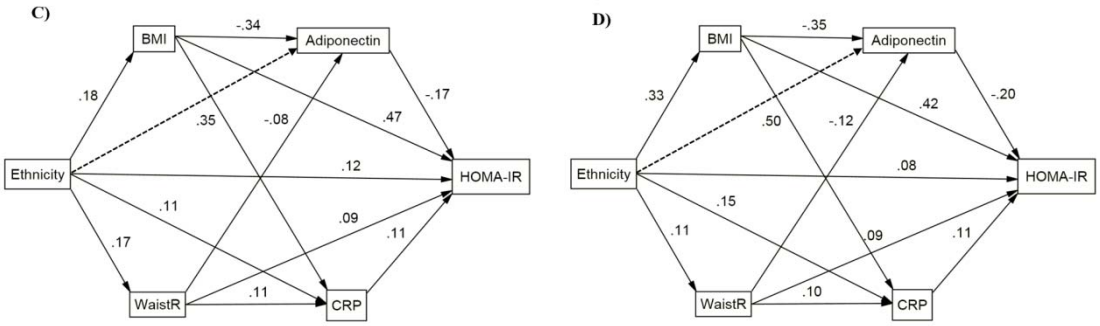


**Figure 5C, 5D.** Path coefficients in the reduced models for Indian-Chinese comparison of differences in insulin resistance in men (C) and women (D).

Two previous studies [30, 36] found higher insulin resistance in Asian Indians than in Chinese, which is consistent with our results. One of them [36] suggested that the

differences in total body fat mass could explain the higher insulin resistance in Asian Indians as compared with Chinese. In our study the difference in insulin resistance between Asian Indians and Chinese partly remained after adjusting for BMI and waist circumference. This discrepancy in results might be due to the use of anthropometry instead of direct body fat measurements in our study or the relative small sample size in the previous study.

*Indian-Malay comparison*

The difference in insulin resistance between Asian Indians and Malays was not mediated by BMI (**Figure 5E** and **5F**) and this was in agreement with the similar BMI of Asian Indians and Malays. Instead, abdominal adiposity contributed to the greater insulin resistance in Asian Indian men and women as compared with their Malay counterparts. A substantial independent contribution of adiponectin and CRP to Indian-Malay differences in insulin resistance was observed in women, but not in men.

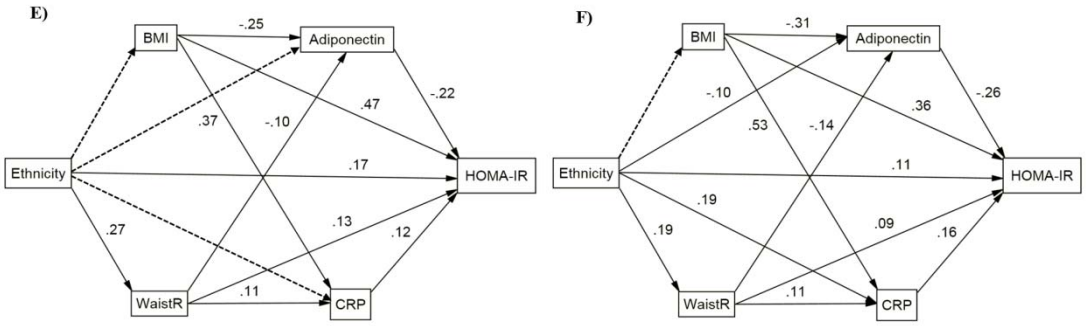


**Figure 5E, 5F**. Path coefficients in the reduced models for Indian-Malay comparison of differences in insulin resistance in men (E) and women (F).

The comparison between Asian Indians and Malays highlighted the role of abdominal fat distribution in the contribution to insulin resistance and the effects of abdominal adiposity also appeared to mediate through adiponectin levels. In line with this finding, results from previous studies in Asian populations suggest that intra-abdominal fat may reduce adiponectin levels to a greater extent than subcutaneous fat [138-140] although other studies did not support this [141, 142].

## 4.2 Study II

In this study, we conducted a GWAS of total and HMW adiponectin in a Chinese population living in Singapore. With an extension to other East Asian populations, we also examined a genetic variant in *CDH13* in relation to insulin resistance and associated metabolic traits.

Among the populations involved in this study, Singaporeans were generally younger than Japanese and Korean participants, whereas Koreans had a higher BMI, higher triglyceride levels, higher HOMA-IR, and lower HDL-cholesterol (HDL-C) levels than the other populations. The substantial differences in adiponectin levels between study populations may be partly due to differences in laboratory methods and have been addressed by standardization of adiponectin levels in the data analysis. As expected, blood adiponectin levels were inversely correlated with insulin resistance (measured by HOMA-IR or fasting insulin), fasting glucose, triglycerides and CRP and directly correlated with HDL-C in our study populations.

*GWAS results*

In the GWAS in Singapore Chinese, signals reaching genome-wide significance ($5 \times 10^{-8}$) mapped exclusively to the *CDH13* and *ADIPOQ* gene (**Figure 6**). The strongest signal in *CDH13* was rs4783244 located in the intron region. With regard to other previously reported loci, associations with total and HMW adiponectin levels reached genome-wide significance for *ADIPOQ* (rs10937273) and we observed nominally significant associations for *GPR109A* (rs601339), *CMIP* (rs2925979) and *PEPD* (rs731839). We focused on the top hit *CDH13* SNP rs4783244 in this study.

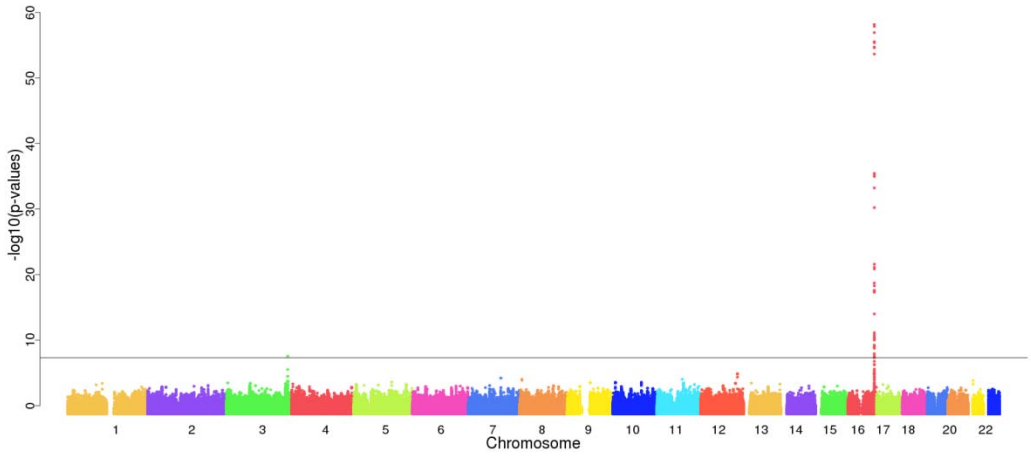


**Figure 6**. Manhattan plot for the whole-genome association with HMW adiponectin in Singapore Chinese.

*Association of the CDH13 variant with different adiponectin forms*

In the combined data from Singapore Chinese, Japanese and Korean cohorts, total adiponectin levels significantly decreased by 0.34 SD on the log scale for each

additional T allele rs4783244 in *CDH13* (95% CI: -0.38 to -0.30, **Table 2**). This *CDH13* variant was even more strongly associated with HMW adiponectin levels and the HMW-to-total adiponectin ratio based on the Singaporean Chinese and Japanese data. Adjustment for BMI did not substantially affect these effect estimates and similar results were obtained for the general genetic model. The *CDH13* rs4783244 variant explained more than 4% of variation in total adiponectin (Singapore: 4.5%, Korea: 5.5%, Japan: 4.1%) and more than 6% of variation in HMW adiponectin levels (Singapore: 8.3%, Japan: 6.5%).

**Table 2.** Association between rs4783244 in *CDH13* and different forms of adiponectin

| | N | β (95% CI) | *P*-value |
|---|---|---|---|
| **Total adiponectin** | | | |
| Singaporean Chinese | 2,282 | -0.32 (-0.37, -0.26) | $2.7 \times 10^{-27}$ |
| Japanese | 1,266 | -0.34 (-0.42, -0.27) | $4.2 \times 10^{-19}$ |
| Koreans | 1,610 | -0.37 (-0.44, -0.30) | $2.4 \times 10^{-25}$ |
| Meta-analysis | 5,158 | -0.34 (-0.38, -0.30) | $2.0 \times 10^{-70}$ |
| **HMW adiponectin** | | | |
| Singaporean Chinese | 2,282 | -0.43 (-0.48, -0.37) | $1.7 \times 10^{-51}$ |
| Japanese | 3,290 | -0.38 (-0.42, -0.34) | $3.3 \times 10^{-64}$ |
| Meta-analysis | 5,572 | -0.40 (-0.43, -0.36) | $1.1 \times 10^{-117}$ |
| **HMW-to-total adiponectin ratio** | | | |
| Singaporean Chinese | 2,282 | -0.48 (-0.54, -0.43) | $9.7 \times 10^{-62}$ |
| Japanese | 3,290 | -0.36 (-0.44, -0.29) | $1.7 \times 10^{-19}$ |
| Meta-analysis | 5,572 | -0.44 (-0.49, -0.40) | $3.2 \times 10^{-83}$ |

Results are age and sex adjusted. Total and HMW adiponectin were natural log transformed and all the adiponectin forms were standardized to the z-scores. An additive genetic model is assumed and each β represents the effect of one copy of the T allele on the respective form of adiponectin.

*Association of the CDH13 variant with metabolic traits*

At a Bonferroni-corrected threshold of $P \leq 0.005$, no significant association between rs4783244 in *CDH13* and metabolic risk factors was observed in Singaporean Chinese or in Japanese (Model 1). Because *CDH13* is known to code for a receptor for HMW adiponectin, we reassessed these associations after adjustment for HMW adiponectin levels (Model 2). In a meta-analysis of the Singaporean Chinese and Japanese samples, the minor allele T in rs4783244 was significantly associated with lower BMI (β = -0.15, 95% CI: -0.19 to -0.11, $P = 3.5\times10^{-14}$), lower HOMA-IR (β = -0.16, 95% CI: -0.20 to -0.12, $P = 9.2\times10^{-16}$), higher HDL-C (β = 0.16, 95% CI: 0.12 to 0.19, $P = 2.1\times10^{-17}$) and lower triglycerides (β = -0.16, 95% CI: -0.19 to -0.12, $P = 1.3\times10^{-16}$) when HMW adiponectin levels were adjusted for. After further adjusting for BMI, associations were weaker, but remained significant (**Figure 7**). These associations were significant but also weaker when we adjusted for total adiponectin instead of HMW adiponectin. In a sensitivity analysis, there was minimal difference in effect estimates between fixed- and random-effect meta-analysis and the estimates retained genome-wide significant with random-effect analysis.

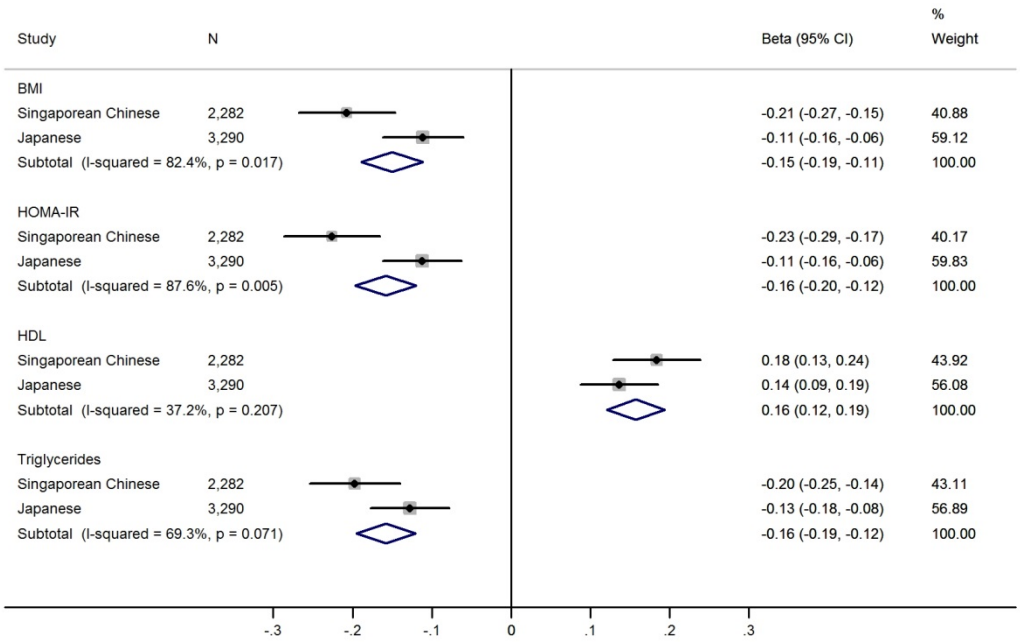


**Figure 7**. Effect estimates of rs4783244 in *CDH13* on selected metabolic traits across studies; age, sex and HMW adiponectin adjusted.

Results from previous studies also provided little support for an association between variants at the *CDH13* locus and metabolic traits. In Filipino women, no significant associations with metabolic risk factors were detected for rs3865188 in *CDH13* (LD with rs4783244, $r^2 = 0.85$) except for a nominal association ($P = 0.042$) with waist circumference [115]. Similarly, a Swedish study reported that rs11646213, a SNP upstream of *CDH13*, in minimal LD with rs4783244 ($r^2 = 0.08$), was not associated with metabolic risk factors [143]. In a Taiwanese study, the significant associations between rs4783244 and waist circumference, glucose and triglyceride levels did not remain after adjustment for BMI, although the adiponectin-lowering T allele was paradoxically still associated with a reduced risk for diabetes, the metabolic syndrome and stroke [116].

Recently, Japanese researchers reported an association between another *CDH13* SNP (rs12051272) and BMI, fasting insulin, fasting glucose, HOMA-IR, and fasting triglycerides only after controlling for adiponectin levels [114]. This SNP is in close proximity and moderate LD ($r^2 = 0.66$) with rs4783244, which we studied. Together with our findings, current evidence suggests a complex relationship between variants at the *CDH13* locus and metabolic traits that is only evident after controlling for their effects on blood adiponectin levels.

*Hypothetical mechanism*

The association between the variants at *CDH13* and plasma HMW adiponectin may be explained by the function of the T-cadherin receptor that it encodes. T-cadherin is a receptor for hexameric and HMW adiponectin that is expressed in the vasculature

[144], cardiac myocytes [145] and epithelial cells in the lung [146]. We believe that the T allele at rs4783244 is associated with increased binding of HMW adiponectin to the T-cadherin receptor resulting in the sequestration of HMW adiponectin in these tissues, thus removing it from the blood. Consistent with this explanation, ablation of the T-cadherin receptor increased plasma adiponectin levels in mice [144-146].

To explain the paradoxical observation that the T allele at rs4783244 that is associated with lower blood levels of HMW adiponectin is associated with a more favorable metabolic profile than would be expected based on HMW adiponectin levels, we hypothesize that the rs4783224 variant at the *CDH13* locus may have an indirect effect on an individual's sensitivity to circulating adiponectin. In this hypothesis, the chronically low levels of plasma adiponectin associated with the T allele may result in up-regulation of adiponectin receptors AdipoR1/R2. Consistent with this proposed mechanism, chronic elevation of plasma adiponectin led to down regulation of AdipoR2 in adipose tissue in mice [147]. Furthermore, the expression of AdipoR1/R2 was up-regulated in insulin-resistant women with the polycystic ovary syndrome [148], who would be expected to have low blood adiponectin levels. The greater expression of adiponectin receptors could counter-balance the low adiponectin levels resulting in the lack of association between rs4783244 and the metabolic profile in unadjusted analyses. However, when the blood adiponectin levels are controlled for, then the greater 'adiponectin sensitivity' results in an association between the T allele and a more favorable metabolic profile.

## 4.3 Study III

In this study, we examined the causal effect of increased adiponectin levels on improved insulin sensitivity in humans using a Mendelian randomization approach with *ADIPOQ* SNPs as instruments in a population-based cohort study of 71-year-old men in Sweden.

*Association between the instruments and serum adiponectin*

**Table 3** shows the 16 *ADIPOQ* SNPs available on the Metabochip and their associations with adiponectin levels in the ULSAM cohort. Based on LD structure, they were assigned to 11 different blocks. Seven SNPs belonging to two different LD blocks showed highly significant associations with serum adiponectin (all $P \leq 4.8 \times 10^{-7}$). The three SNPs chosen as instrumental variables, namely rs17300539, rs3774261 and rs6444175 were all strongly associated with adiponectin levels. As expected, the allele score generated from rs17300539 and rs3774261 was even more strongly associated with adiponectin level as compared with each of the single *ADIPOQ* SNPs. The instruments explained 3.5 to 6.0% (rs17300539: 4.3%, rs3774261: 3.8%, rs6444175: 3.5%, all three SNPs combined: 6.0%) of the total variance in adiponectin levels.

**Table 3**. *ADIPOQ* SNPs available on the Metabochip and their associations with serum adiponectin.

| **SNP or allele score** | | **Position on chr3** | **Alleles*** | **EAF** | **HWE *P*-value** | **Association with adiponectin, age adjusted** | |
|---|---|---|---|---|---|---|---|
| | | | | | | Beta (95% CI) | *P*-value |
| rs3917086 | (1) | 188007420 | A/C | 0.03 | 0.621 | 0.02 (-0.08, 0.12) | 0.668 |
| rs864265 | (2) | 188036986 | T/G | 0.15 | 0.015 | -0.06 (-0.10, -0.01) | 0.020 |
| rs822387 | (3) | 188038731 | C/T | 0.07 | 0.660 | 0.16 (0.10, 0.22) | $4.8 \times 10^{-7}$ |
| rs17300539‡ | (3) | 188042154 | A/G | 0.07 | 0.189 | 0.21 (0.14, 0.27) | $1.1 \times 10^{-10}$ |
| rs16861209 | (3) | 188045808 | A/C | 0.07 | 0.088 | 0.21 (0.15, 0.28) | $1.5 \times 10^{-10}$ |
| rs16861210 | (3) | 188049192 | A/G | 0.08 | 0.550 | 0.19 (0.13, 0.25) | $1.1 \times 10^{-9}$ |
| rs16861194 | (4) | 188042119 | G/A | 0.09 | 0.855 | -0.05 (-0.11, 0.01) | 0.106 |
| rs822396 | (5) | 188049571 | G/A | 0.17 | 0.475 | 0.01 (-0.03, 0.06) | 0.562 |
| rs3774261‡ | (6) | 188054253 | A/G | 0.38 | 0.950 | 0.11 (0.07, 0.14) | $1.3 \times 10^{-9}$ |
| rs6773957 | (6) | 188056399 | A/G | 0.38 | 0.950 | 0.11 (0.07, 0.14) | $1.4 \times 10^{-9}$ |
| rs6444175‡ | (6) | 188062438 | A/G | 0.29 | 0.438 | 0.11 (0.08, 0.15) | $5.3 \times 10^{-9}$ |
| rs3774262 | (7) | 188054508 | A/G | 0.09 | 0.185 | 0.03 (-0.03, 0.09) | 0.327 |
| rs9853541 | (8) | 188095161 | A/G | 0.38 | 0.121 | 0.00 (-0.04, 0.04) | 0.927 |
| rs11708293 | (9) | 188096021 | G/A | 0.04 | 1.000 | -0.04 (-0.13, 0.04) | 0.322 |
| rs11716002 | (10) | 188096058 | G/A | 0.24 | 0.105 | 0.01 (-0.03, 0.05) | 0.616 |
| rs17301514 | (11) | 188096103 | A/G | 0.13 | 1.000 | 0.01 (-0.04, 0.06) | 0.757 |
| Allele score§ | | | | | | 0.40 (0.30, 0.51) | $1.2 \times 10^{-13}$ |

EAF, Effect allele frequency. SNPs were first sorted based on physical position in the *ADIPOQ* gene and then aggregated by LD group indicated by the number in the bracket. All the SNPs had call rate >0.96. *The first allele is the effect allele. ‡SNPs selected for instrumental variable analysis. §The allele score was created using genotypes for rs17300539 and rs3774261 ($R^2 = 0.137$).

The three SNPs selected as IVs have been repeatedly reported for their association with adiponectin in individuals of European descent [109, 110, 149-152]. This confirms that the SNPs we selected are robust instruments for adiponectin levels in European populations.

*Association between the instruments and insulin sensitivity*

All the instrumental variables including the three *ADIPOQ* SNPs and the allele score were significantly associated with insulin sensitivity represented by the M/I ratio ($P \leq 0.022$ for all SNPs and $P = 8.0 \times 10^{-4}$ for the allele score). Adjustment for adiposity represented by BMI and waistR weakened these associations, but some of the instruments remained significantly associated with the M/I ratio (rs6444175, $P = 0.011$; allele score, $P = 0.042$).

Studies of the relationship between *ADIPOQ* and insulin sensitivity in candidate gene studies have been less consistent [153]. The 3q27 region in which the *ADIPOQ* gene is located was reported for its linkage signal with seven metabolic traits, and was suggested for an association with obesity and insulin sensitivity [154]. However, individual *ADIPOQ* SNPs, including some of the IVs in our study, did not show an association with parameters for glucose and insulin metabolism [109, 111, 152]. However, in a recent large study, Dastani *et al*. demonstrated that several individual SNPs which were significant in their GWAS of adiponectin levels, including a few *ADIPOQ* SNPs, were associated with T2D and related traits. Furthermore, they found a multi-SNP genotypic risk score based on the identified hits to be strongly associated with metabolic traits related to insulin resistance [117]. Therefore, even though *ADIPOQ* has been established as major determinant of adiponectin levels, its associations with measures of insulin sensitivity have not been consistent which may have been due to lack of statistical power to detect this indirect effect. Of note, prior studies have not assessed associations of *ADIPOQ* variants and insulin sensitivity measured with intravenous methods in a larger study sample.

*Instrumental variable analysis of the effect of adiponectin on insulin sensitivity*

To examine the potential causal effect of adiponectin on insulin sensitivity in an IV analysis, we assessed the first-stage F-statistic in which an empirical value >10 indicates sufficient strength of the genetic variant as a proxy of the exposure. The large F-statistics ensured that the SNPs chosen were strong instruments for adiponectin levels (**Table 4**).

**Table 4**. Instrumental variable estimated and observed association between adiponectin and insulin sensitivity.

| | | IV estimate | | Observational estimate | | |
|---|---|---|---|---|---|---|
| Instrumental variable | First stage F statistic | Beta (95% CI) | *P*-value | Beta (95% CI) | *P*-value | Endogeneity† *P*-value |
| Age adjusted | | | | | | |
| rs17300539 | 42.6 | 0.47 (0.10, 0.84) | 0.014 | | | 0.878 |
| rs3774261 | 37.6 | 0.68 (0.28, 1.08) | $9.1 \times 10^{-4}$ | 0.50 | $1.2 \times 10^{-33}$ | 0.364 |
| rs6444175 | 34.7 | 0.81 (0.38, 1.23) | $2.1 \times 10^{-4}$ | (0.42, 0.58) | | 0.136 |
| Allele score* | 56.7 | 0.60 (0.27, 0.93) | $3.3 \times 10^{-4}$ | | | 0.528 |
| Age, BMI and waistR adjusted | | | | | | |
| rs17300539 | 38.5 | 0.27 (-0.08, 0.61) | 0.131 | | | 0.955 |
| rs3774261 | 29.3 | 0.39 (-0.01, 0.78) | 0.055 | 0.28 | $1.3 \times 10^{-14}$ | 0.572 |
| rs6444175 | 28.1 | 0.55 (0.13, 0.96) | 0.010 | (0.21, 0.34) | | 0.178 |
| Allele score | 46.7 | 0.34 (0.03, 0.65) | 0.034 | | | 0.677 |

*The allele score was created using genotypes for rs17300539 and rs3774261 ($R^2 = 0.137$). †Endogeneity was assessed by Durbin-Wu-Hausman test and reflects whether the difference between the IV estimate and the observational estimate was statistically significant.

The IV-estimated effect size of adiponectin levels on insulin sensitivity was highly significant and consistent for all instruments. In addition, endogeneity tests suggested there was no statistical difference between the IV estimate and the observational estimate (**Table 4**). This was consistent for each of the individual SNP instruments and also the allele score, demonstrating that genetically determined adiponectin levels affect insulin sensitivity to the same degree as expected based on the observed association. In our secondary analyses including individuals with T2D, the results were similar to those from our main analyses.

For some of the IVs, we still observed an association between the IV-estimated adiponectin and insulin sensitivity after adjustment for BMI and waistR (**Table 4**), although the effect sizes were substantially attenuated. This suggests that at least part of the causal beneficial effect of adiponectin levels on insulin sensitivity was mediated by lower adiposity as measured by BMI and waist circumference. BMI-stratified analysis suggested that the association between increased adiponectin levels and higher insulin sensitivity was stronger in the group with higher BMI, for which the observed causal relationship between adiponectin and insulin sensitivity also persisted. However, in the group with lower BMI, we did not have enough power to robustly confirm or refute an association due to limited sample size.

The role of adiposity is important to dissect the causal relationship between adiponectin and insulin sensitivity, as will be discussed in Future perspectives.

*Estimated impact of adiponectin on insulin sensitivity*

Based on the IV estimates in the age-adjusted model (ranged between 0.47 and 0.81), we estimated the relationship between percent change in adiponectin levels and corresponding percent change in insulin sensitivity. For example, a 10% increase in serum adiponectin level would lead to an improvement of insulin sensitivity ranging between 4.6% and 8.0%. Similarly, excluding the mediation through obesity, the

percent increase in insulin sensitivity associated with a 10% elevation in adiponectin level was estimated to be between 2.6% to 5.3%. However, due to the large confidence intervals for the IV estimates, these numbers should be interpreted with caution.

## 4.4 Study IV

In this study, we examined relations between serum selenium levels and measures of glucose and insulin metabolism, as well as risk of diabetes after 20 years of follow-up in 1,925 participants from the ULSAM cohort.

The mean concentration of serum selenium in our study population was 75.6 μg/L. At baseline, serum selenium was not significantly associated with fasting glucose levels or glucose tolerance represented by the K-value from the IVGTT. Higher serum selenium levels were associated with a lower insulin peak during the IVGTT indicating lower early insulin response. This association persisted after adjustment for potential confounders (selenium as continuous variable, standardized β = -0.08; 95% CI: -0.14 to -0.03, *P* for trend = 0.026). However, this is likely a chance finding due to the multiple tests we performed and does not have an obvious biological meaning. Selenium was not substantially associated with HOMA measures of insulin resistance or insulin secretion.

At follow-up after 20 years, we observed no clear associations with any glucometabolic traits as well as adiponectin levels. Among 1,024 individuals with baseline selenium levels and follow-up data, 88 developed diabetes. Selenium levels were not substantially associated with a higher risk of diabetes during follow-up in either age-adjusted or multivariable models (**Table 5**). Analysis with selenium as a continuous variable also revealed no association of baseline selenium levels with incident risk of diabetes.

**Table 5**. Association of baseline serum selenium (at age 50 years) with diabetes risk during follow-up at age 60 years, age 70 years and age 77 years.

| Study | | N | Low Se tertile | Middle Se tertile | High Se tertile | Continuous Se (per SD of Se) | *P* for trend* |
|---|---|---|---|---|---|---|---|
| Age 60 exam | Diabetes (yes/no) | 53/1,486 | 15/501 | 15/489 | 23/496 | | |
| | Model 1 | 1,539 | 1 | 0.99 (0.48, 2.05) | 1.51 (0.78, 2.92) | 1.15 (0.87, 1.52) | 0.201 |
| | Model 2 | 1,539 | 1 | 0.91 (0.43, 1.95) | 1.28 (0.64, 2.61) | 1.11 (0.81, 1.51) | 0.437 |
| Age 70 exam | Diabetes (yes/no) | 88/936 | 24/302 | 33/314 | 31/320 | | |
| | Model 1 | 1,024 | 1 | 1.31 (0.76, 2.27) | 1.21 (0.69, 2.10) | 1.04 (0.82, 1.31) | 0.544 |
| | Model 2 | 1,024 | 1 | 1.31 (0.73, 2.36) | 1.25 (0.68, 2.27) | 1.06 (0.83, 1.38) | 0.497 |
| Age 77 exam | Diabetes (yes/no) | 91/565 | 27/179 | 34/182 | 30/204 | | |
| | Model 1 | 656 | 1 | 1.23 (0.71, 2.14) | 0.97 (0.55, 1.70) | 0.97 (0.76, 1.25) | 0.876 |
| | Model 2 | 656 | 1 | 1.16 (0.65, 2.08) | 0.97 (0.54, 1.75) | 0.96 (0.74, 1.25) | 0.880 |

Logistic regression was used and results as OR (95% CI). The low Se tertile is the reference category. Model 1: adjusted for age; Model 2: adjusted for age at baseline, BMI, cigarette smoking, leisure time physical activity, and education at the baseline examination.

In sensitivity analyses, 53 individuals free of diabetes at baseline developed diabetes by the age 60 follow-up. A total of 91 incident diabetes cases were identified in the age 77 examination after 27 years of follow-up since baseline at age 50. The odds ratio estimates based on continuous selenium levels were similar to those observed at age 70 with no evidence for an association of selenium levels with incident diabetes (**Table 5**).

Two large RCTs to address the effect of selenium on future risk for disease have been conducted to date, including the Selenium and Vitamin E Cancer Prevention Trial (SELECT) [75] and the Nutritional Prevention of Cancer (NPC) trial [76]. Results from these two trials suggested a lack of beneficial effect of selenium supplementation on T2D and the NPC trial reported that the risk significantly increased with greater baseline selenium levels defined by tertiles [76]. To be noted, both trials were conducted in the United States where selenium supplementation is common.

Comparison and interpretation of the findings for the effects of selenium in different studies is not straightforward. Firstly, baseline selenium levels are greatly different across populations, due to factors such as soil selenium content, dietary patterns, selenium supplements used and genetics [155, 156]. For example, selenium intakes are considerably lower in Europe than in USA and Canada [155]. In Sweden, the estimated intake is 31 to 38 μg per day [156] while in USA the daily intake ranged from 60 to 220 μg [155]. Serum or plasma selenium concentrations also vary substantially by country [155, 157]. In our cohort, serum selenium averaged 76 μg /L, which is even lower than most participants of low baseline selenium levels in SELECT and NPC trial [75, 76]. It is possible that selenoenzyme activities have already reached a maximum in the trials [158], whereas in most of the ULSAM participants it did not.

Secondly, selenium supplementation in the clinical trials used high doses and the resulting circulating selenium levels were substantially higher than would result from dietary intake only. This is even more pronounced if the baseline levels were already different. Selenium is a trace element with a relatively narrow range between deficiency and toxicity [159, 160]. Protective effects of selenium on diabetes and glucose metabolism, if these exist, may only be found at optimal levels that enhance selenoprotein activities. A loss of benefit or even adverse effects could occur outside the optimal range. In a prospective study of similar design and baseline selenium levels to our study, no significant association was detected between selenium and hyperglycemia [161]. Therefore, the role of selenium in the development of diabetes and hyperglycemia remains to be elucidated.

Insulin resistance can be induced by ROS [162] and several selenoproteins have an antioxidant property [163]. Thus, selenium could exert a protective effect on insulin sensitivity by alleviating oxidative stress. However, experimental evidence has been conflicting. On the one hand, selenoproteins such as GPx1 and selenoprotein P have been shown to interfere with insulin signaling and contribute to the development of insulin resistance [70, 164-166]. Mice overexpressing GPx1developed diabetic phenotypes, including hyperglycemia, increased insulin production and insulin levels, and elevated β-cell mass [167, 168]. On the other hand, selenium-containing micronutrients such as sodium selenate can serve as an insulin-mimetic [69]. Sodium selenate has been demonstrated to improve peripheral insulin sensitivity measured by the euglycemic clamp in rats [169] and increased the expression of PPAR-γ, a transcription factor that sensitizes the body to insulin, in diabetic *db/db* mice [170]. One human trial found that adding selenium to a hypocaloric diet led to a borderline

significant reduction of fasting insulin and HOMA-IR in 84 centrally-obese women [171].

Adiponectin is an adipocyte-secreted hormone and plays an important role in glucose metabolism, especially in the regulation of insulin sensitivity [95, 96, 172, 173]. Selenium treatment decreased adiponectin levels possibly by suppressing adiponectin production in Otsuka Long-Evans Tokushima Fatty (OLETF) rats [174]. A few studies have examined the effects on adiponectin levels when selenoprotein P, a major class of selenoproteins [67], was knocked out/down in mice, but results from different studies have been inconsistent [175, 176]. A human study involving 36 patients with diabetes reported an inverse association of selenoprotein P with circulating adiponectin, but not with the quantitative insulin sensitivity index [175]. In a recent randomized trial from UK where selenium status is comparable to Sweden, baseline plasma selenium was inversely associated with adiponectin concentrations, but selenium supplementation of six months at doses 100, 200 and 300 μg/day had no effect on adiponectin levels [177]. Results of our study did not support an association between baseline selenium levels and adiponectin levels after 20 years of follow-up, but further research is needed to clarify the inconsistency of results across studies.

## 4.5 Strengths and limitations

### 4.5.1 Strengths

A major strength of the SP2 study is the inclusion of a multi-ethnic sample consisting of Chinese, Malays and Asian Indians, which well represents the populations in East and South East Asia. Their susceptibility to (central) obesity, insulin resistance, as discussed in Study I, makes it an important research focus to study Asians for the etiology and prevention of T2D. In addition, HMW adiponectin was measured together with total adiponectin levels in all the participants with blood samples. This is valuable because HMW adiponectin has been shown to be the form of highest bioactivity and a stronger association with various metabolic traits [83, 100-102].

The strengths of the ULSAM study include the longitudinal design and the long follow-up period. Moreover, the study was undertaken in a homogeneous, age-standardized sample from Uppsala in Sweden, and therefore population stratification which could, for example, violate assumptions of Mendelian randomization in Study III is unlikely. Moreover, the ULSAM cohort has detailed measurements of many metabolic variables related to insulin and glucose metabolism. Importantly, it is the largest single-center, population-based cohort in the world with insulin sensitivity measured by the euglycemic insulin clamp technique. Compared with indirect measurements and surrogate indices, this method directly measures whole body glucose disposal at a given level of insulinemia under steady-state conditions and is therefore regarded as the gold standard for measuring insulin resistance [178]. This partially compensated for the relatively modest sample size of the ULSAM study when analyzing insulin sensitivity as the main outcome in Study III and Study IV.

### 4.5.2 Limitations

To date, the SP2 study only contains cross-sectional data and this does not allow us to study the temporal relationship between exposure and outcome. This study also suffered from a moderate response rate (~60%). In 1998, non-participants were contacted and they were similar to the participants for the collected information including demographic, socio-economic status, diabetes and hypertension status. However, it is not known whether they differ from the participants in other aspects.

The ULSAM study was based on elderly men of Northern European descent and hence, the generalizability to women, other age groups and other ethnicities is unknown. In addition, there was substantial loss to follow-up in the two most recent investigations, although this thesis only used studies with participation rate above 60%.

In both SP2 and ULSAM studies, adiponectin was only measured once due to cost, research interest and other reasons. It would be desirable to have repeated measurements of adiponectin in future cohort studies, so as to discern changes over time and evaluate its temporal relationship with metabolic outcome of interest. Besides, in both studies, adiposity was assessed by anthropometry instead of direct techniques which are more accurate but were not feasible in our relatively large study. It would be ideal to measure adiposity in different depots and differentiate visceral abdominal fat from subcutaneous fat. As another limitation, in ULSAM dietary intake was not

assessed at baseline. Due to this, in study IV we cannot rule out the possibility of confounding by unmeasured dietary factors or other unmeasured or imperfectly measured confounders.

*Underlying assumptions*

We proposed the path model in Study I based on previous research findings, but the studied risk factors might not be in the causal pathway towards insulin resistance or T2D. In addition, other adipokines and inflammatory markers may also contribute to ethnic differences in insulin resistance [179], but were not considered in our study.

In Study II, we started with a GWAS to search for genetic variants associated with adiponectin levels. As is for all GWAS, this is based on the 'common disease common variants' assumption. We did identify *CDH13* and a few other loci strongly associated with total and HMW adiponectin, but rarer variants (MAF < 1%) are unlikely to be captured by the GWAS approach due to a lack of statistical power.

The choice of the instrumental variables and the fulfillment of the assumptions of the Mendelian randomization are critical in Study III. *ADIPOQ* is the most established gene influencing adiponectin levels and genetic variation in *ADIPOQ* affects adiponectin production without affecting insulin sensitivity in other known ways. Therefore, by focusing on *ADIPOQ*, concerns over the strength and potential pleiotropic effects of the instrument could largely be eliminated.

For study IV, our longitudinal assessment of selenium as an exposure in relation to glucometabolic profiles and diabetes risk was based on serum selenium measured at baseline, which assumes that this single measurement reflects dietary selenium intake over the long follow-up years. Even though serum selenium is a good indicator of long-term dietary selenium intake, it is still desirable to have repeated sampling to minimize the possibility of residual confounding due to substantial changes in individual intake of this element.

# 5 CONCLUSIONS

I. Mediators of ethnic differences in insulin resistance differed markedly depending on the ethnic groups compared. General adiposity explained the difference in insulin resistance between Chinese and Malays, whereas abdominal fat distribution, inflammation, and unexplained factors contributed to excess insulin resistance in Asian Indians as compared with Chinese and Malays. Interventions targeting excess weight gain can reduce ethnic disparities in insulin resistance among Asian Indians, Chinese and Malays.

II. A genetic variant in *CDH13* explains a substantial part of variation in HMW adiponectin levels in East Asian populations. However, this effect of *CDH13* on circulating HMW adiponectin levels did not appear to translate into effects on insulin-resistance related metabolic traits, suggesting that compensatory mechanisms exist that lead to greater 'adiponectin sensitivity'.

III. Genetically determined adiponectin levels influence insulin sensitivity to the same degree as the observed epidemiological associations. The observed association between higher adiponectin levels and increased insulin sensitivity is likely to represent a causal relationship.

IV. No effect of selenium intake on insulin sensitivity, insulin secretion, or risk of type 2 diabetes was identifiable in older Swedish men. Taken together with prior studies including randomized clinical trials, the body of evidence argues against a role for selenium supplementation as a broad approach to prevent diabetes in the older population.

# 6 FUTURE PERSPECTIVES

## 6.1 Clinical utility of adiponectin as a biomarker

Clinical trials usually involve substantial investment of resources. Validated biomarkers, when used in clinical studies, can help detect the efficacy of the drug early (for example, in stage I or stage II) that would otherwise only be known at late stages and thus reduce unnecessary cost. In addition, a good predictive biomarker could aid in the selection of patients to treat [180].

Thiazolidinediones (TZDs) are synthetic ligands of PPAR-γ that have been widely used to treat diabetes [181] and many studies have shown that adiponectin levels increased in response to TZD treatment [20, 182]. Thus adiponectin could be viewed as a promising biomarker of PPAR–γ activation.

Despite the robust inverse association of adiponectin with insulin resistance and T2D, current research largely focuses on pathogenesis of this protein and studies on the clinical utility of adiponectin in predicting the risk of T2D are still lacking. Because adiponectin correlates well with established metabolic risk factors of T2D [90, 95], it remains to be assessed in a formal statistical approach whether adiponectin improves risk prediction in addition to existing predictors in a cost-effective manner.

Adiponectin has also been studied as a biomarker for gestational diabetes (GDM). Since the placenta does not produce adiponectin, plasma adiponectin which solely comes from the adipose tissue is an independent marker of maternal adipose homeostasis [183]. In a study involving 445 pregnant women, decreased maternal adiponectin levels positively correlated with GDM diagnosed in the second trimester and was proposed to be a potential early marker of susceptibility to GDM [184].

In view of adiponectin's anti-diabetic property, interestingly and paradoxically, elevated adiponectin levels have been associated with increased risk of all-cause and cardiovascular mortality for individuals at-risk, for example, elderly people [185], those with renal diseases [186], and heart failure [187, 188]. It is possible that adiponectin levels increase in response to vascular stress as a compensatory effect or due to adiponectin resistance, but further studies are needed to better understand this. Therefore, the effect of adiponectin on cardiovascular diseases should be interpreted with caution at the moment.

## 6.2 Further characterization of the causality between adiponectin and insulin sensitivity

Our results in Study III support the hypothesis that adiponectin levels are causally related to the degree of insulin sensitivity in humans. However, the role of adiposity in the relationship between adiponectin and insulin sensitivity is debatable. The degree of adiposity is associated with both adiponectin levels and insulin sensitivity [90, 172, 189] and thus could confound the adiponectin-insulin sensitivity relationship. What we observed in Study III was attenuated associations of the SNPs and insulin sensitivity

with adjustment for adiposity, which does not support a role of adiposity as a confounder, but instead implies it as a partial mediator for the relationship between adiponectin and insulin sensitivity. Previous studies also suggested that adiponectin influenced insulin sensitivity independent of adiposity [21, 95]. As proposed by Kadowaki and *et al.*, obesity caused by environmental risk factors could interact with genetic factors leading to reduced adiponectin levels, which in turn plays a role in the development of insulin resistance, T2D and metabolic disease [97]. Due to limited statistical power, we were not able to fully explore the role of adiposity in the causal effect of adiponectin on insulin sensitivity, but suggested it as a partial mediator which might work by antagonizing the secretion of pro-inflammatory cytokines through reduced adiposity.

Therefore, in order to establish causality, further studies on the inter-relationship between adiposity, adiponectin and insulin sensitivity are warranted.

## 6.3 Adiponectin as a therapeutic target in diabetes treatment

Experimental studies have provided convincing evidence for an effect of adiponectin on insulin sensitivity when adiponectin levels are altered in transgenic and adiponectin-deficient models [190, 191]. In a recent study using adiponectin knockout mice, high-fat diet and adiponectin supplementation were associated with changes in lipid, carbohydrate and amino acid metabolomic profiles in skeletal muscle. Conversely, the replenishment of adiponectin reversed euglycemic clamp-measured insulin sensitivity by a certain degree [192].

One of the logical strategies for the treatment of insulin resistance and T2D is to increase adiponectin levels. Direct administration of adiponectin is difficult, but therapeutic agent that target adiponectin signaling could achieve this. For example, the anti-diabetic drug TZDs, are known to ameliorate insulin resistance partly by increasing adiponectin levels through transcriptional activation [97]. In addition, lifestyle and surgical interventions with weight loss have also led to a significant increase of adiponectin concentration [193, 194]. Motivated by our results in Study III, if a causal relationship exists between adiponectin and insulin sensitivity, dietary factors that affect adiponectin levels would be of interest, as already shown for coffee [195] and alcohol [196] in RCTs. Another viable strategy to enhance insulin sensitivity is to up-regulate adiponectin receptors and this includes an approach to develop receptor agonists that can mimic adiponectin actions. Some studies have reported proteins or short-peptides that could function as adiponectin receptor agonist [197, 198] and further research in this area is promising.

Compared with currently used anti-diabetic drugs, novel drugs that may enhance the effect of adiponectin possess several potential advantages [21]. For example, in addition to its anti-diabetic and lipid-lowering effects, adiponectin also has an anti-inflammatory property which potentially prevents atherogenesis, although this still need to be supported by further evidence given the association between adiponectin and cardiovascular disease death. Moreover, anti-diabetic drugs commonly incur

weight gain and adiponectin seems to avoid this. However, our findings on *CDH13* in Study II may be a cautionary tale for drug development that not all ways to increase adiponectin are beneficial, especially when it concerns the vascular systems.

Future research on the therapeutic potentials of adiponectin could also focus on the changes of metabolic profiles in relation to the presence/alteration of adiponectin, using a combination of genomic, transcriptomic, metabolomic and other 'omic' approaches.

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The cover illustration is a painting of lotus drawn by my grandpa who passed away 14 years ago. I miss you grandpa and I hope you can see this thesis and feel proud of me!

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