From the Department of Medical Epidemiology and Biostatistics
Karolinska Institutet, Stockholm, Sweden

ADIPONECTIN: GENETIC DETERMINANTS AND RELATIONS WITH SUBCLINICAL CARDIOVASCULAR DISEASE

Stefan Gustafsson

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ABSTRACT

Cardiovascular disease (CVD) is a major cause of morbidity and premature death in Sweden and globally, which besides the substantial individual suffering, puts large restraints on the public health system. Adiponectin is a highly heritable trait, which is strongly associated with metabolic disturbances such as obesity and insulin resistance. Previous studies indicate that adiponectin may play a fundamental role in the development of CVD. However, further knowledge about pathways linking circulating adiponectin, genetic loci, and markers of early CVD is needed. Therefore, the overall aim of this thesis was to assess the genetic determinants of adiponectin, and the role of adiponectin in the development of subclinical CVD.

In Study I, we investigated the association between adiponectin and cardiac geometry and function in two cross-sectional samples of elderly and found that high adiponectin levels were associated with poorer cardiac function in men. This association was dependent on N-terminal pro-brain natriuretic peptide and this was more pronounced in individuals with prior CVD, which could indicate a counter-active effect in response to decreased cardiac function, potentially mediated by natriuretic peptides.

In Study II, we examined the role of adiponectin in vascular pathology in a cross-sectional study design where higher adiponectin levels were associated with a lower lipid-content in plaques and higher vessel wall elasticity, indicating less arterial pathology.

In Study III, we assessed oxidative stress and inflammatory markers in relation to adiponectin, where adiponectin was positively associated with the anti-oxidant glutathione and inversely associated with lipid peroxidation as well as epidermal growth factor. Our findings suggest that adiponectin is associated with a more beneficial oxidative stress profile.

In Study IV, we explored the impact of rare genetic loci on circulating adiponectin levels on a genome-wide scale. Besides several independent variants around the adiponectin gene, we found a rare coding variant in a gene upstream of adiponectin receptor 2 that was associated with higher adiponectin levels. If replicated in an independent sample, these findings can provide new insight to adiponectin biology.

In conclusion, we found that individuals with poor cardiac function had higher levels of adiponectin and the results suggested that natriuretic peptides had an important role in a potentially counter-active mechanism. In contrast, high adiponectin levels were associated with a more beneficial arterial, oxidative stress and inflammation profile. Finally, rare variation around the adiponectin gene and a potentially novel locus upstream of the adiponectin receptor 2 gene was associated with adiponectin levels.

Keywords: adiponectin, cardiovascular disease, oxidative stress, inflammation, rare genetic variation.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which are referred to by their Roman numerals:

I. Adiponectin and cardiac geometry and function in elderly: results from two community-based cohort studies. *European Journal of Endocrinology* 2010 Mar; 162(3):543-50.
II. Associations of circulating adiponectin with measures of vascular function and morphology. *Journal of Clinical Endocrinology & Metabolism* 2010 Jun; 95(6):2927-34.
III. Oxidative stress and inflammatory markers in relation to circulating levels of adiponectin. *Obesity* 2012. In press.
IV. Rare genetic variation in relation to circulating adiponectin. Manuscript.

CONTENTS

LIST OF ABBREVIATIONS

BCD LDL	Baseline conjugated dienes of low density lipoproteins
BMI	Body mass index
CCA	Common carotid artery
CI	Confidence interval
CRP	C-reactive protein
CVD	Cardiovascular disease
EDV	Endothelial-dependent vasodilation
EGF	Epidermal growth factor
eGFR	Estimated glomular filtration rate
EIDV	Endothelial-independent vasodilation
FMD	Flow-mediated dilation
GSH	Reduced glutathione
GSM	Gray scale median
GSSG	Oxidized glutathione
GWA	Genome-wide association
HDL	High-density lipoprotein
ICAM-1	Intercellular adhesion molecule-1
IL	Interleukin
IM-GSM	Intima-media gray scale median
IVS	Interventricular septal thickness
LDL	Low-density lipoprotein
LV	Left ventricular
LVEDD	LV end-diastole diameter
LVM	LV mass
LVMI	LVM index
MCP-1	Monocyte chemotactic protein-1
NT-proBNP	N-terminal pro-brain natriuretic peptide
OxLDL	Oxidized low density lipoprotein
PIVUS	Prospective Investigation of the Vasculature in Uppsala Seniors
PW	Posterior wall thickness
QC	Quality control
RWT	Relative wall thickness
SNP	Single nucleotide polymorphism
T2D	Type 2 diabetes
TAOC	Total anti-oxidant capacity
TGSH	Total glutathione
TNF	Tumor necrosis factor
ULSAM	Uppsala Longitudinal Study of Adult Men
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor

1 BACKGROUND

1.1 Cardiovascular Disease and Adipose Tissue

Cardiovascular disease (CVD) is the leading cause of death, accounting for one third of the total number of deaths in the world.[1] The prevalence of CVD is increasing, e.g. by 2030, 40.5% of all Americans are expected to have some form of CVD.[2] In addition to substantial individual suffering, CVD puts a large burden on public health care with substantial costs that are expected to increase.[2, 3] In the United States the direct medical costs of CVD are expected to triple between 2010 and 2030.[2]

More than 17 million people in the world died from CVD in 2008 and a large proportion of the CVD deaths are preventable.[1] Besides advancing age, gender, and genetic factors, most CVD risk factors are modifiable, such as tobacco use, physical inactivity, unhealthy diet, hypertension, diabetes, high blood lipid levels, and obesity. As major risk factors of CVD, obesity and overweight are major contributors to the disease burden worldwide[4] and of great public health importance as major causes of morbidity and mortality.[5] Obesity is accompanied by an activation of pro-inflammatory pathways[5-7] and also linked to increased oxidative stress.[8, 9] Inflammation is suggested to play a key role in the adverse effects of obesity, i.e. in type 2 diabetes (T2D) and CVD.[5] Similarly, harmful oxidative stress plays a critical role in obesity-associated conditions such as diabetes, hypertension and atherosclerosis.[8]

Given the increased CVD burden in the world, a better understanding of pathways leading to CVD is of great importance in order to identify new biomarkers, to better understand early CVD changes and to develop new pharmacological treatments. The view on adipose tissue has changed from a rather inactive tissue, with the main function of energy storage, to a bioactive organ expressing several secretory proteins, termed adipokines, providing a new link between adiposity and the development of CVD.[10] An adipokine that has attracted much attention in recent years is adiponectin, which was first identified in 1996 as the most abundantly expressed transcript of adipose tissue.[11] Unlike most other proteins produced by adipose tissue, plasma concentrations of adiponectin decreases with increasing body mass index (BMI).[12] Further, previous studies have linked adiponectin levels to a more beneficial cardiometabolic profile showing an inverse association with traits such as T2D,[13] hypertension,[14] triglycerides,[15] and myocardial infarction.[16]

1.2 Adiponectin and the Adiponectin Receptors

Adiponectin levels differ significantly between sexes, where women have higher levels than men.[15] An inhibitory influence of androgens on adiponectin levels might offer a potential explanation for this difference.[17, 18] The adiponectin levels are also higher in elderly, which can partly be explained by decreased renal function in aging and reduced adiponectin clearance.[19] Several pharmaceutical agents are known to increase adiponectin such as lipid-lowering drugs[20] and insulin-sensitizing thiazolidinediones (peroxisome proliferator activator receptor-γ agonists).[21] Another example of the physiological importance of adiponectin for many biological systems is that it has been suggested to play a role in cancer.[22]

Adiponectin is mainly expressed in adipose tissue, but expression has been detected in other tissues such as cardiomyocytes.[23] The protein consists of 244 amino acids and has

several domains, including a N-terminal signal sequence, a variable domain, a collagen-like domain, and a C1q-like globular domain at the C-terminus,[11, 24] where the three-dimensional structure of the globular domain of adiponectin shows structural similarities with tumor necrosis factor-α (TNF-α).[25] Adiponectin is mainly found in its full-length form but also in small amounts as a globular domain fragment.[26] Adiponectin molecules assemble into several multimeric forms, which can be categorized as low-molecular weight (LMW), medium-molecular weight (MMW), and high-molecular weight (HMW) adiponectin.[27] It has been indicated that the different forms might differ in biological effect,[28, 29] although this remains to be fully established.

Three adiponectin receptors have been identified - Adiponectin repector 1 (*ADIPOR1*), Adiponectin receptor 2 (*ADIPOR2*),[30, 31] and T-cadherin.[32] *ADIPOR1* and *ADIPOR2* are seven-transmembrane domain proteins, structurally different from G-protein-coupled receptors.[30] Both these receptors are important for the regulation of glucose and lipid metabolism. They are predicted to have varying affinity for different forms of adiponectin and *ADIPOR1* is associated with the activation of adenosine monophosphate kinase pathways, whereas *ADIPOR2* activates peroxisome proliferator-activated receptor-α pathways.[30, 31] Further, *APPL1* is suggested to be an adapter protein for *ADIPOR1/2* signaling and to play a key role in the signal transduction of these receptors.[33] T-cadherin is a suggested receptor for MMW and HMW adiponectin which was isolated after *ADIPOR1/2*.[32] Genetic variation within the T-cadherin gene (*CDH13*) has been associated with circulating adiponectin levels.[34-38] It is not clear how T-cadherin function since it does not contain an intracellular domain for signal transduction.[32]

1.3 Adiponectin and the Heart

The role of adiponectin in relation to cardiac geometry and function has attracted some attention in previous studies. Most of these studies were performed in small samples and in selected materials, e.g. patients with preexisting disease, such as T2D, hypertension or obesity, and have reported inverse associations between adiponectin and left ventricular (LV) mass,[39-43] and diastolic dysfunction.[41, 43] Similarly, one sample of healthy Japanese men found an inverse association between lower levels of adiponectin and LV hypertrophy assessed by electrocardiography (ECG).[44]

In contrast to the beneficial cardiometabolic profile that has been reported for adiponectin in most studies, some studies have indicated that adiponectin increases in cardiac dysfunction. In a moderately large study of healthy individuals, circulating adiponectin increased in parallel with brain natriuretic peptide (BNP) and was positively associated with higher LV end-diastolic diameter which could indicate depressed cardiac function.[45] The potential relationship between adiponectin and natriuretic peptides in the presence of cardiac dysfunction has also been indicated by studies showing increased levels of both adiponectin and N-terminal pro-brain natriuretic peptide (NT-proBNP) in patients with heart failure.[46-48]

1.4 Adiponectin and the Vasculature

Several epidemiological studies have reported an association between adiponectin and vascular pathology.[49-59] Previous studies have mainly focused on the intima-media thickness (IMT) and an inverse association has been reported in some[49-52] but not all studies,[53] possibly due to small study samples and concomitant diseases such as T2D[49, 51] or end-stage renal disease.[51] The association between adiponectin and carotid plaques has been investigated in a few studies, but to our knowledge, none have found a significant association.[50, 54] Also, the impact of adiponectin on morphological measures of echogenicity using the gray scale median (GSM) in the IM has not been studied before, and plaques remain to be studied in detail. Adiponectin have been negatively associated with arterial stiffness in some studies,[55-57] but not all.[52, 58, 59] Finally, studies on endothelial function and adiponectin have been inconclusive.[60, 61]

1.5 Adiponectin, Oxidative Stress, and Inflammation

Some previous epidemiological studies, most conducted in small samples and/or individuals with existing disease, have shown an association between adiponectin and pro- and anti-oxidative markers.[8, 62-66] The association between adiponectin and glutathione remains to be fully elucidated, but an association between adiponectin and the ratio of oxidized and reduced glutathione has been suggested.[62, 63] An inverse association between adiponectin and measures of lipid peroxidation has been reported in some studies,[8, 64-66] but not all.[67]

Previous studies have also indicated that the expression of adiponectin is down-regulated by inflammatory cytokines such as TNF-α and potentially IL-6.[68] Further, adiponectin exhibits anti-inflammatory effects on macrophages.[69] However, in contrast with its anti-inflammatory effects, some studies have reported increased adiponectin levels in inflammatory and auto-immune conditions such as rheumatic diseases.[70]

1.6 Genetic Determinants of Adiponectin Levels

The adiponectin-encoding gene (*ADIPOQ*) have orthologous genes in several species, such as monkey, dog, rat, mouse, chicken, and the domain structures are highly conserved among species indicating its importance for biological functions.[24, 71] The heritability estimates for adiponectin are moderate to high, with 30-70% of the phenotypic variability explained by genetic variation.[72-74]

Early candidate gene studies that mainly focused on variation within *ADIPOQ* yielded inconclusive results.[75-80] A study applying deep re-sequencing of *ADIPOQ* indicated that seven single nucleotide polymorphisms (SNPs) within the region had an independent effect on adiponectin levels.[81] The discrepancies could be explained by limited sample sizes, as well as heterogeneity of the SNPs studied, and ethnic differences.

One way to identify novel genetic loci and to overcome some of the limitations in candidate gene studies is to perform genome-wide association (GWA) studies in sufficiently large study samples. GWA studies of European individuals have identified genome-wide significant signals at *ADIPOQ*.[34, 82, 83] Further, a robust signal has been identified at the ADP-ribosylation factor-like 15 gene (*ARL15*), where the variant

associated with adiponectin also was associated with a higher risk of CHD and moderately associated with T2D.[83] Studies in Asian populations have identified a strong GWA-significant signal at *CDH13*,[36-38] and a moderate to strong signal has later been found in Europeans.[34, 35] An uncommon haplotype at *KNG1-ADIPOQ* was identified in a study of Asians[38] and a follow-up study identified a rare coding variant within *ADIPOQ*, explaining up to 17.1% of the phenotypic variability.[84] However, depending on which allele an individual carried at this non-synonymous variant, the amino acid change affected the binding-affinity of the antibodies in the adiponectin immunoassay, which resulted in artificially different levels of adiponectin between carriers of the different alleles.[84] A recent large meta-analysis of European individuals, with additional samples from other ethnicities, confirmed two known loci (*ADIPOQ*, *CDH13*) and identified eight novel loci. A multi-SNP risk score, calculated as the additive effect of the adiponectin-decreasing alleles was associated with higher T2D, triglycerides, waist-hip ratio, fasting insulin and 2-h glucose from an oral glucose tolerance test, but somewhat unexpectedly lower BMI.[34]

Although several genetic regions have been identified to be associated with adiponectin levels, a large fraction of the expected phenotypic variability due to genetic variation remains to be explained. Previous studies have mainly focused on common variants with a minor allele frequency above 5% and uncommon or rare variants with a lower frequency remain largely unexplored on a genome-wide level.

2 AIMS

The overall aim of this thesis was to assess the genetic determinants of adiponectin, and to investigate the relation between adiponectin and markers of early CVD. The specific aims for each study were:

I. To elucidate the association between adiponectin and cardiac geometry and function in two cross-sectional samples of elderly, and to further assess the role of natriuretic peptides.
II. To evaluate associations between circulating adiponectin and comprehensive measures of vascular function and morphology in a large sample of individuals from the community.
III. To investigate the importance of a broad set of well-recognized oxidative stress and inflammatory markers, representing different pathways, in relation to adiponectin in a large community-based sample of elderly.
IV. To examine the impact of the mutational burden of rare genetic variants on adiponectin levels.

3 STUDY SAMPLES

Data from the Prospective Investigation of the Vasculature in Uppsala Seniors (PIVUS) was used in all studies, whereas the Uppsala Longitudinal Study of Adult Men (ULSAM) study was used in Study I and IV. The clinical characteristics of all individuals with adiponectin measurements in PIVUS and ULSAM are presented in **Table 1**. Further, the IMPROVE study was used in the replication stage of Study IV.

Table 1. Clinical characteristics of all individuals with available adiponectin measurements in PIVUS and ULSAM.

	PIVUS (n=1,007)	**ULSAM** (n=1,205)
Age	70.1 (0.2)	71.0 (0.6)
Women (%)	50	0
BMI (kg/m^2)	27.0 (4.3)	26.3 (3.4)
Fasting plasma glucose (mmol/L)	6.0 (1.8)	5.8 (1.4)
Anti-diabetic treatment (%)	6	6
Total cholesterol (mmol/L)	5.4 (1.0)	5.8 (1.0)
HDL cholesterol (mmol/L)	1.5 (0.4)	1.3 (0.3)
Lipid lowering treatment (%)	16	9
Systolic blood pressure (mmHg)	150 (22)	147 (19)
Antihypertensive treatment (%)	31	35
Creatinine (µmol/L)	80 (19)	94 (15)
Smoking (%)	Never: 48 Previous: 41 Current: 11	Never: 38 Previous: 42 Current: 20
Adiponectin (mg/L)	7.1 (4.3)	10.4 (4.3)

3.1 The Prospective Investigation of the Vasculature in Uppsala Seniors

The PIVUS study has been described in detail previously[85] and online at http://www.medsci.uu.se/pivus/pivus.htm. Individuals at the age of 70 years, living in the community of Uppsala, Sweden, between April 2001 and June 2004 were randomly selected from the community register and invited to participate. Invitations were sent out in a randomized order by letter within 2 months of their 70th birthday in order to standardize for age. Out of 2,025 subjects invited, 1,016 subjects participated (50.1%) with an equal proportion men and women. All participants were asked to answer a questionnaire with detailed questions about their medical history, smoking habits and regular medication.

The physical examination was performed in the morning after an overnight fast with no medication or smoking allowed after midnight. During the examination, participants were in supine position in a quiet room at constant temperature for a total of four hours. At least 30 minutes passed between the different tests performed. Lipid variables and blood glucose were measured with standard laboratory techniques. Blood pressure was measured with a calibrated mercury sphygmomanometer in the non-cannulated arm and the mean of three recordings was used.

In order to characterize cardiovascular disorders and medications of non-participants, data on such factors was collected for 100 individuals that did not participate in PIVUS. Compared with the participants, these individuals had on average a similar frequency of myocardial infarction, coronary revascularization, anti-hypertensive treatment, statin

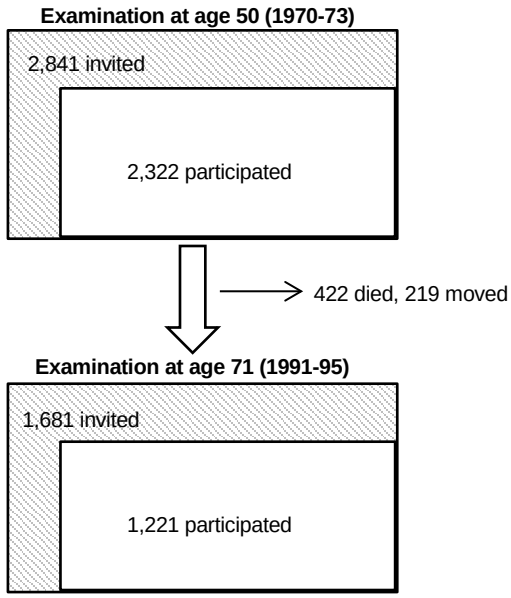
use, and insulin treatment, but higher a frequency of diabetes, congestive heart failure and stroke.

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

3.2 The Uppsala Longitudinal Study of Adult Men

The ULSAM study has been described elsewhere[86] and on the Internet at http://www.pubcare.uu.se/ULSAM/. The study is of cohort design and was initiated in 1970-73 when all 50-year-old men living in Uppsala County, Sweden were invited to participate. These individuals, born between 1920 and 1924, were selected from the register of the County Council. At the first examination at age 50, 2,841 men were invited out of which 2,322 participated (81.7%). The participants were then re-examined at the approximate age of 71 years, where all participants in the first examination at age 50 were traced by their ten-digit social security number and invited to participate. The re-examination was carried out between August 1991 and May 1995 in which 1221 out of 1681 invited subjects participated (73%), excluding 422 subjects that had died and 219 that had moved out of the Uppsala region (**Figure 1**).

Figure 1. Study design of the Uppsala Longitudinal Study of Adult Men.



The participants were asked to answer a questionnaire on general and medical background. Further, smoking habits were assessed during an interview. The examination was performed in a fasting state, where lipid variables and blood glucose were measured with standard laboratory techniques. After at least 10 minutes of rest, blood pressure was measured with a calibrated mercury sphygmomanometer and the mean of two recordings was used.

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

3.3 The IMPROVE study

The IMPROVE study has been described before.[87] It is a longitudinal study designed to investigate if the carotid artery intima-media thickness (IMT), as well as overall IMT progression can predict new vascular events in high-risk individuals of CVD. This observational study recruited participants at seven centres in Finland, France, Italy, the Netherlands, and Sweden; including 3,711 individuals (men and women) aged from 54 to 79 years and free from CVD at baseline, but with at least three vascular risk factors. The study was approved by the Ethics committee of Karolinska Institutet and the participants provided informed consent for general participation and for genotyping.

4 STUDY DESIGNS AND METHODS

4.1 General Methods

4.1.1 Transformation of Adiponectin

In Study I-III, we standardized adiponectin values through Z-score transformation, separately in men and women and then pooled the transformed values. The distribution of the transformed variable using this method is centered on a mean of 0 with a standard deviation of 1. This mean and standard deviation is the same for men and women, which will adjust for the potentially confounding effect of sex when performing a regression analysis with the transformed variable.

In Study 4, adiponectin is the outcome in the regression models and since phenotypic outliers can have a larger impact on the results when focusing on rare genetic variation, we applied an inverse normal transformation of adiponectin according to Blom. As for the Z-score transformation, this was done separately for men and women and the pooled.

4.1.2 Significance Level and Multiple Testing

In Study I-III, a P-value < 0.05 was considered to be statistically significant. Given the prior knowledge from previous studies indicating that adiponectin was associated with cardiovascular pathways, this alpha-level was in general considered strict enough when discussing the results. However, in order to assess the impact of multiple testing on the 22 tests performed in Study III, we performed a Holm-Bonferroni correction[88] to highlight the number of tests for the reader.

Regarding Study IV, previous studies had shown that much of the adiponectin phenotypic variance is explained by genetic factors and that a large fraction of the source of this heritability remains to be discovered. However, which genetic regions to focus on are not known, so we tested all transcript regions available in the UCSC transcript database. This untargeted approach calls for strict adjustment for multiple testing and a P-value < 1.7×10^{-6} was considered significant in this study (Bonferroni-correction for 30,000 tests). The Bonferroni-correction approach aims to eliminate false positives with an overall family-wise error rate of 0.05. Another approach to adjust for multiple testing is the false discovery rate which is more liberal in the sense that it controls for the proportion of accepted false positives in relation to the total number of accepted signals. However, given the risk of including false positives and that we had limited resources to assess this in an independent replication sample; the Bonferroni-approach was selected for the present study.

4.1.3 Adiponectin Measurements

Adiponectin in samples from PIVUS, frozen for 1-3 years, was analysed with a double-antibody radioimmunoassay (RIA; Millipore, Billerica, MA, USA, previously Linco research). At low levels (2–4 µg/ml), the within-assay coefficient of variation (CV) was 6% and the between-assay CV was 14%, whereas at high levels (26–54 µg/ml) the within-assay CV was 3% and the between-assay CV was 8%.

In ULSAM, adiponectin was measured in samples frozen in -70ºC for 11±2 years, without previous thaw-freeze cycles. A validated in-house time-resolved immunofluorometric assay based on commercial reagents from R&D Systems (Abingdon, UK) was used. The within-assay and between-assay CV averaged less than 5 and 10%, respectively.

The same method described for PIVUS was used to measure adiponectin in the replication sample IMPROVE (Study IV).

4.1.4 Measurement and Definition of Additional Covariates

NT-proBNP was measured in PIVUS and ULSAM with a sandwich immunoassay on an Elecsys 2010 (Roche Diagnostics, Basle, Switzerland) at an analytical range of 20 to 35,000 ng/l. The total coefficient of variation was 3.3% at a level of 209 ng/l and 3.0% at a level of 7.4 ng/l.

BMI was calculated as the ratio between weight (kg) and height squared (m^2). The homeostasis model assessment insulin resistance index (HOMA-IR) was defined as (fasting plasma glucose × serum insulin)/22.5.[89] Diabetes was defined as diabetes medication or elevated fasting glucose (in blood ≥ 6.1 mmol/L or in plasma ≥ 7 mmol/L).[90] Estimated glomular filtration rate from cystatin C (eGFR), representing kidney function, was calculated according to a previous publication[91] as eGFR ($mL/min/1.73m^2$) = $79.901 \times (\text{cystatin C in mg/L})^{-1.4389}$.

4.2 Study I

4.2.1 Exclusions

In PIVUS, 954 participants were included after the exclusion of participants without valid adiponectin measurements (n=9), echocardiography readings (n=30), or all clinical characteristics needed for multivariable analysis (n=23). An echocardiographic examination was performed in a random sub-sample of 482 individuals in ULSAM. After further exclusion of individuals without valid adiponectin measurements (n=16) or all clinical measures used in the analyses (n=39), the eligible sample was 427 individuals.

4.2.2 Echocardiographic Examination

The echocardiographic examination was performed approximately one week after the main examination using comprehensive two-dimensional and Doppler echocardiography, with an Acuson XP124 cardiac ultrasound unit (Acuson, California, USA), in both study samples.[92] The echocardiographic measures included LV end-diastole diameter (LVEDD), left atrial diameter, ejection fraction, LV isovolumic relaxation time, E/A ratio, interventricular septal thickness (IVS), LV posterior wall thickness (PW). LV relative wall thickness (RWT) was calculated as (IVS+PW)/LVEDD and LV mass (LVM) as $0.8 \times (1.04 \times ([IVS+LVEDD+PW]^3 - LVEDD^3) + 0.6$ g.[93] LV mass index (LVMI) was obtained by indexing LVM divided by height to the power of 2.7 ($LVM/m^{2.7}$).[94] Ejection fraction was calculated from M-mode recordings according to the method of Teichholz.[95] LV geometry was categorized

as: normal (LVMI ≤51 g/m$^{2.7}$; RWT ≤0.45), eccentric LV hypertrophy (LVMI >51 g/m$^{2.7}$; RWT ≤0.45), concentric remodeling (LVMI ≤51 g/m$^{2.7}$; RWT >0.45), or concentric LV hypertrophy (LVMI >51 g/m$^{2.7}$; RWT >0.45).[96]

4.2.3 Statistical Main Analysis

In order to promote normality, E/A-ratio and fasting glucose values were logarithmically transformed. The relation of adiponectin with echocardiographic measures (LVMI, RWT, LVEDD, left atrial diameter, ejection fraction, isovolumic relaxation time, E/A-ratio) as dependent variables was tested in separate linear regression models. Age- and sex-adjusted regression models were performed, as well as multivariable linear regression models adjusted for age, sex, BMI, systolic blood pressure, antihypertensive treatment, anti-diabetic treatment, lipid lowering medication, log fasting blood glucose, total cholesterol, HDL cholesterol, creatinine, and smoking (no, former, current). All analyses were performed in Stata 10.1 (Stata Corporation, College Station, TX).

4.2.4 Additional Analyses

In order to investigate the potentially mediating role of natriuretic peptides in the relation of adiponectin with cardiac function, NT-proBNP was added to the multivariable-adjusted models with ejection fraction, isovolumic relaxation time or E/A-ratio as outcomes. We further assessed if sex was an effect-modifier of the studied associations, by adding two-way interaction terms between sex and adiponectin to each model with the cardiovascular variables as outcomes. Also, to avoid a potentially confounding effect of co-morbidities with heart disease, the analyses were also performed in sub-samples consisting of 804 (PIVUS) and 356 (ULSAM) individuals without history of myocardial infarction, heart failure, angina pectoris or interventional treatment of the coronary arteries, or with significant valvular diseases detected on the echocardiogram. Adiponectin levels in relation to groups of cardiac geometry were calculated as age- and sex-adjusted least square means of adiponectin grouped by categories of cardiac geometry in the PIVUS study.

4.3 Study II

4.3.1 Exclusions

After the exclusion of individuals without valid measures of adiponectin (n=9) or missing data on any of the clinical covariates needed for multivariable analysis (n=25), the eligible study sample consisted of 981 participants.

4.3.2 Measurements of Vascular Function and Morphology

The examination of the carotid artery was done by external B-mode ultrasonography (Acuson XP128 with a 10MHz linear transducer, Acuson Mountain View, California, USA) as has been described before.[97] Frozen ultrasound images were saved by the ultrasonographer and further digitized and imported into the Artery Measurement Software (AMS)[98] for analysis of IMT and GSM. The occurrence of plaques was recorded on both sides for the common carotid artery (CCA), the bulb and the internal

carotid artery. IMT was evaluated in the far wall in the CCA, 1–2 cm proximal to the bulb, excluding overt plaques from the measurement. The borders of the IMT were identified by the AMS software and IMT was calculated from about 100 discrete points through a 10 mm long segment. The IMT value presented was the mean value from both sides. Intima-media segments to be evaluated for IM-GSM were defined by a manually placed region of interest (ROI) and IM-GSM was analyzed from individual pixels within the ROI on a scale from 0 (black) to 256 (white). The GSM was calculated as the mean from both sides, with the adventitia as a reference for white and the blood as a reference for black. The GSM of plaques was analyzed by placing the ROI within a plaque if such existed, and by the use of the same software as IM-GSM. If plaques were present in both carotids, the GSM in the most echolucent plaque was selected, since previous studies have demonstrated echolucent plaques to be the most pathological form.[99, 100]

Pulse wave analysis was performed with a micromanometer-tipped probe (Sphygmocor, Pulse Wave Medical Ltd, Sydney, Australia) using the mean value of 10 recordings. The central pulse pressure was calculated as the difference between central systolic blood pressure and central diastolic blood pressure. CCA distensibility was calculated as the percentage change in the maximum to minimum diameter divided by the central pulse pressure obtained by pulse-wave analysis.[101]

Flow-mediated dilation (FMD) was assessed with B-mode ultrasound imaging 2-3 cm over the elbow, after placing a cuff below the elbow, inflated to a pressure of at least 50 mmHg over resting systolic blood pressure for 5 min. FMD was calculated as the maximal brachial artery diameter after cuff release minus diameter at rest, divided by diameter at rest. We placed an arterial cannula in the brachial artery, and forearm blood flow (FBF) was calculated from at least five measurements by venous occlusion plethysmography (Elektromedicin, Kullavik, Sweden) with the strain-gauge technique. Endothelial-dependent vasodilation (EDV) was defined as FBF during infusion of 50 µg/min of acetylcholine minus resting FBF, divided by resting FBF. Endothelial-independent vasodilation (EIDV) was defined as FBF during infusion of 10 µg/min of sodium nitroprusside minus resting FBP, divided by resting FBF.[85] The CV of the ultrasound assessments when repeating the measurements were 3% for baseline brachial artery diameter, 29% for FMD, 8% for EDV, and 10% for EIDV.[85]

4.3.3 Statistical Main Analysis

CCA distensibility, EDV, EIDV and fasting glucose were logarithmically transformed in order to achieve normality. In separate linear regression models, the association between adiponectin and measures of vascular morphology (IMT, plaque presence, plaque GSM and IM-GSM) as well as vascular function (log CCA distensibility, FMD, log EDV and log EIDV) was assessed. Adjustments were made for potential confounders including age, sex, BMI, systolic blood pressure, antihypertensive treatment, antidiabetic treatment, lipid lowering medication, log fasting blood glucose, total cholesterol, HDL cholesterol, creatinine, and smoking (no, former, current). Stata version 10.1 (Stata Corporation, College Station, TX) was used for all analyses.

4.3.4 Additional Analyses

A two-way interaction term of adiponectin and sex was incorporated with each vascular measure as dependent variables in separate models in order to test the effect modification by sex. In order to assess the potentially confounding effect of co-morbidities with pre-existing clinical CVD, analyses were also performed in sub-samples consisting of 822 individuals without history of myocardial infarction, heart failure, angina pectoris or interventional treatment of the coronary arteries, or with significant valvular diseases detected on the echocardiogram.

4.4 Study III

4.4.1 Exclusions

To exclude individuals with acute infections, chronic inflammatory disorders or blood malignancies, participants with CRP > 10 mg/L or leukocyte count > 10×10^9 cells/L (n=65) were excluded from the present study. Further exclusions were made of participants without valid measures of adiponectin (n=1) or of those missing any covariate used in the regression analyses (n=21). Thus, 929 participants were eligible for the study.

4.4.2 Measurements of Oxidative Stress and Inflammatory Markers

Baseline conjugated dienes of LDL (BCD-LDL) were measured using a previously validated method,[102] with a CV for within-assay and between-assay of 4.4 and 4.5%, respectively. After precipitation of serum LDL with buffered heparin, BCD-LDL was measured spectrophotometrically. Levels of serum oxidized LDL were determined with enzyme-linked immunoabsorbent assay (ELISA) kits (Mercodia, AB); within-assay CV was 6.3% and between-assay CV was 4.7%. Autoantibodies to OxLDL were measured with an ELISA kit (BioMedica), with intra-assay and inter-assay CVs of 4.3 and 6.0%, respectively. All forms of glutathione, conjugated dienes (CD) and total anti-oxidative capacity (TAOC) values were measured and calculated as described previously.[103] In brief, TGSH and GSSG were measured through enzymatic reactions, and GSH was further calculated as the difference between TGSH and GSSG. Conjugated dienes, formed after double-bound rearrangements in polyunsaturated fatty acid residues in a free radical reaction, were measured spectrophotometrically. TAOC was measured by assessing the ability of serum samples to inhibit linolenic acid peroxidation. Homocysteine was measured by using Axis® Homocysteine Enzyme Immunoassay (Axis-Shield Diagnostics Ltd.) with an intra-assay coefficient of 6.8%.

High sensitive C-reactive protein (CRP) and intercellular adhesion molecule-1 (ICAM-1) was measured with commercially available ELISAs (Medix Biochemica, Kauniainen, Finland and R&D System Europe Ltd, Abingdon, Oxon, UK, respectively). The leukocyte count was measured by standard laboratory techniques at Uppsala University Hospital. Remaining inflammatory markers were analyzed with the Evidence® array biochip analyzer (Randox Laboratories Ltd, Crumlin, UK).[104] The functional sensitivity for the inflammatory markers was as follows: IL-6: 0.3 pg/mL; IL-8: 1.5 pg/mL; TNF-α 1.8 pg/mL; MCP-1 19.4 pg/mL; VCAM-1: 31 ng/mL; E-selectin: 3.1 ng/mL; P-selectin: 11.2 ng/mL; L-selectin 32.8 ng/mL; leukocyte count 0.2×10^9 cells/L. IL-1α, IL-1β, IL-2, IL-4, IL-10, and interferon-γ were included in the

Evidence® array biochip cytokine panel, but were not evaluated further due to insufficient sensitivity for measurements in the present sample.

4.4.3 Statistical Main Analysis

In order to achieve a normal distribution of the residuals of the regression models, all oxidative and inflammatory markers except GSH, conjugated dienes, TAOC, BCD-LDL, OxLDL, MCP-1, P-selectin, L-selectin, and leukocyte count were transformed, as well as the covariates HOMA-IR and triglycerides. Natural log was used for all transformations, except TNF-α for which a reciprocal root transformation was performed.

The association between adiponectin and outcomes including oxidative stress (conjugated dienes, homocysteine, TAOC, OxLDL, OxLDL antibodies, BCD-LDL, GSH, TGSH, glutathione disulfide), circulation interleukins (IL-6, IL-8), other cytokines (TNF-α, MCP-1, EGF, VEGF), cell adhesion molecules (VCAM-1, ICAM-1, E-selectin, P-selectin, L-selectin) and systemic inflammatory markers (CRP, leukocyte count) was assessed in age- and sex-adjusted, as well as multivariable-adjusted models. The multivariable-adjusted models were adjusted for age, BMI, antihypertensive treatment, log HOMA-IR, anti-diabetic treatment, total cholesterol, high-density lipoprotein (HDL), log triglycerides, lipid-lowering medication, eGFR, and smoking (no, former, current).

The statistical software package Stata 11.2 (Stata Corporation, College Station, TX, USA) was used for all analyses.

4.4.4 Additional Analyses

To address the potential effect modification by sex, previous CVD or BMI, we added two-way interaction terms to the multivariable-adjusted models, in separate models for adiponectin × sex, adiponectin × previous CVD, and adiponectin × BMI. We also re-ran the multivariable-adjusted model stratified by sex, previous CVD and overweight (BMI < 25 and BMI ≥ 25) in separate models. In an exploratory analysis, we also tested if the ratio of reduced and oxidized glutathione, calculated as GSH/GSSG, was associated with adiponectin in age- and sex-adjusted, as well as multivariable-adjusted models. The multivariable regression model was further performed after excluding all individuals with signs of chronic kidney disease (eGFR < 50 mL/min/1.73m^2) for all significant associations in the main multivariable-adjusted model.

4.5 Study IV

4.5.1 Exclusions

In PIVUS, the analyses were performed in 839 individuals after exclusions based on missing microarray genotyping data (n=34), failing sample quality control (QC) (n=33), missing adiponectin measurements (n=1), and individuals with diabetes (n=109). Using the same exclusion criteria for ULSAM, 984 individuals were included, after the exclusion of participants with missing microarray genotyping data (n=5),

failing sample QC (n=37), missing adiponectin measurements or covariates (n=74), and individuals with diabetes (n=121).

4.5.2 Preparation of Genotype Data and Imputation

Genotyping was performed using the Illumina OmniExpress and Illumina Metabochip in PIVUS, and Illumina Omni2.5M and Illumina Metabochip in ULSAM. For both PIVUS and ULSAM, sample QC was first performed for the Omni-chip. For individuals that passed this QC, the QC for Metabochip was then performed. The SNP QC was performed for each data source separately, and the quality-controlled data of OmniExpress or Omni2.5 and Metabochip was then merged. Samples were excluded from both studies based on: 1) genotype call rate <95%; 2) heterozygosity >3 standard deviations; 3) gender discordance; 4) duplicated samples; 5) identity-by-descent match; and 6) ethnic outliers. General SNP exclusion criteria of genotyped data before imputation included: 1) monomorphic SNPs; 2) Hardy-Weinberg equilibrium P-value$<1\times10^{-6}$; 3) genotype call rate<0.99 (SNPs with MAF<5%) or <0.95 (SNPs with MAF≥5%); and 4) MAF<1%. In ULSAM, for Omni2.5, further SNP exclusions were made if a SNP had large position disagreements, did not map in the genome, mapped more than once in the genome or had bad probe assays.

In PIVUS, 958 out of 982 samples passed QC for the OmniExpress, and out of those, 949 passed QC on the Metabochip. Further, 645,318 out of 733,202 OmniExpress SNPs, and 123,771 out of 185,801 Metabochip SNPs passed QC; hence, the genotyped data in PIVUS consisted of 949 samples and 738,879 SNPs.

In ULSAM, 1,179 out of 1,216 samples passed QC for the Omni2.5, which further passed QC and were available on the Metabochip. Further, 1,531,196 out of 2,379,855 Omni2.5 SNPs and 119,775 out of 185,801 Metabochip SNPs passed QC; hence, the genotyped data in ULSAM consisted of 1,179 samples and 1,621,833 SNPs.

Imputation was performed after the QC of the genotype data for each cohort in IMPUTE v.2.2.2 using haplotypes from the 1000 Genomes, March 2012 release (multi-ethnic panel on NCBI build 37 [b37]). Population substructures were captured by principal components (PCs), calculated in PLINK 1.07 using the multidimensional scaling approach.[105]

4.5.3 Statistical Main Analysis

We investigated if the proportion of rare variants within a gene, where the individual carried the minor allele, was associated with adiponectin levels, using the statistical method implemented in GRANVIL 2.0.1.[106] A burden test like this is used to increase power to detect associations of rare or uncommon variants with the outcome. The regression analysis was performed with inverse normal transformed adiponectin as the dependent variable and adjusting for PC1, PC2, as well as BMI in non-diabetic individuals. Rare variants were defined to have a MAF<1% or MAF<5% and SNPs with an information quality metric below 0.4 (indicating suboptimal imputation) were excluded from the analysis. Gene boundaries were defined based on the UCSC Human

Genome database transcript regions' start and stop position on NCBI build 37 and extracted using the UCSC Table Browser (http://genome.ucsc.edu/cgi-bin/hgTables). The association tests were performed using: 1) all available markers; 2) coding markers only; and 3) non-synonymous markers only, where the marker lists were built from the publically available 1000 Genomes annotation file. The association results from GRANVIL for PIVUS and ULSAM were combined in a sample size-weighted Z-score meta-analysis assuming fixed effects in the software METAL,[107] separately for each subset of markers and for the two MAF cutoffs.

4.5.4 Additional Analyses

In order to identify independent SNPs around the *ADIPOQ* gene that are associated with adiponectin, an association test was performed using SNPTEST 2.4.1,[108] in a forward selection conditional analysis, where independent signals where considered down to a P-value of 1×10^{-5}. We then tested if the rare variant burden test of the *ADIPOQ* gene obtained from GRANVIL was independent of these SNPs by conditioning the rare variant burden test on the genotype dosages of the independent *ADIPOQ* SNPs.

In order to assess the predicted impact on protein level of the coding marker selected for replication, we performed an analysis in two bioinformatics tools used to annotate coding non-synonymous SNPs, Polyphen-2[109] and PANTHER.[110]

4.5.5 Replication in the IMPROVE Study

The genotyping of the variant selected for replication (chr12:1702929) in IMPROVE was performed using a custom SNP genotyping array from Applied Biosystems and called using Applied Biosystems' SDS 2.3 software (call rate 97.4%). In total, 3,340 individuals had valid measures for adiponectin and available genotype data, and after further exclusions of individuals with diabetes, 2,412 were included in the analysis for which the regression model described for PIVUS and ULSAM was performed.

5 RESULTS

5.1 Adiponectin and Measures of Cardiac Geometry and Function

In multivariable-adjusted models including BMI, circulating adiponectin was inversely associated with ejection fraction in PIVUS, as well as in ULSAM (**Table 2**). For each standard deviation increase of adiponectin, ejection fraction decreased with 1.2% (PIVUS) or 1.3% (ULSAM) after taking potential confounders into account.

Table 2. Association between adiponectin and ejection fraction in PIVUS and ULSAM.

	Associations between adiponectin and ejection fraction (%)			
	Age- and sex-adjusted		Multivariable-adjusted	
	β (95% CI)	P-value	β (95% CI)	P-value
PIVUS; n=810	-0.73 (-1.25; -0.20)	0.007	-1.16 (-1.73; -0.58)	< 0.0001
ULSAM; n=385	-0.91 (-1.86; 0.04)	0.059	-1.35 (-2.41; -0.29)	0.013

In PIVUS, adiponectin was inversely associated with LVMI, RWT, and left atrial diameter in age- and sex-adjusted analyses. Consistent with this, adiponectin concentrations were significantly lower in individuals with eccentric hypertrophy, concentric remodeling, and concentric hypertrophy compared with individuals with normal cardiac geometry (**Figure 2**). The associations of adiponectin with LV geometry were non-evident in multivariable-adjusted analyses. In secondary analyses, associations between adiponectin and LV mass (without indexing for height) were almost identical to those incorporating LVMI. Exploratory analyses demonstrated that BMI was the most important confounder of the associations of adiponectin and cardiac geometry, as the addition of BMI to the age- and sex-adjusted analyses rendered all associations non-significant (P=0.52; 0.14; and 0.91). In ULSAM, adiponectin was not significantly associated with measures of cardiac geometry in any model.

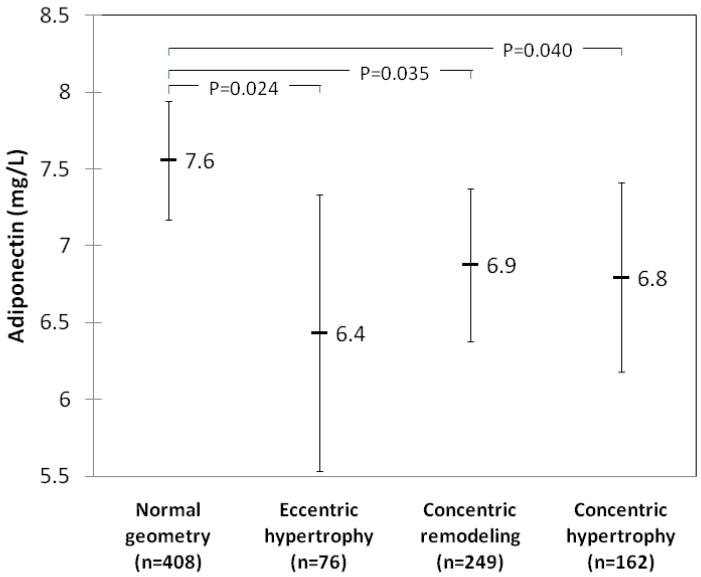


Figure 2. Age- and sex-adjusted least square means of adiponectin grouped by categories of cardiac geometry in the PIVUS study (N=954).

Sex was found to be an effect modifier only in the association of adiponectin with ejection fraction (P-value for interaction=0.063); the association was evident in men (β, -1.62; 95% confidence interval [CI], -2.50, -0.75; P-value<0.0001), but not in women (β, -0.27; 95% CI, -1.02, 0.47; P-value=0.47). The inverse relation between adiponectin and ejection fraction remained similar when excluding individuals with known prior CVD (β, -0.69; 95% CI, -1.28, -0.11; P-value=0.019; β, -1.62; 95% CI, -2.70, -0.55; P-value=0.003, in PIVUS and ULSAM, respectively). Exploratory analyses revealed that HDL cholesterol and BMI increased the magnitude of the association between adiponectin and ejection fraction when added to the age- and sex-adjusted model. LV mass had no effect on the association between adiponectin and ejection fraction when added to the multivariable model, nor did further adjustment for CRP or HOMA-IR (data not shown).

To address the role of natriuretic peptides in mediating the association of adiponectin with cardiac function, we added NT-proBNP to the multivariable-adjusted model with ejection fraction as outcome. The association between adiponectin and ejection fraction was attenuated in both PIVUS (β, -0.68; 95% CI, -1.25, -0.10; P=0.021) and ULSAM (β, -0.75; 95% CI, -1.84, 0.35; P=0.18). Consistent with the above sex-stratified analyses, the association between adiponectin and ejection fraction was attenuated, but still significant in men and non-significant in women. Further, the attenuation was less pronounced in individuals without prior CVD (β, -0.69 and -0.61; 95% CI, -1.28, -0.11 and –1.20, -0.03, without and with adjustment for NT-proBNP, respectively) compared with a sub-sample with individuals having prior CVD (β, -2.30 and -1.00; 95% CI, -3.90, -0.69 and –2.76, -0.75, without and with adjustment for NT-proBNP, respectively).

With our sample sizes, we had at least 85% (PIVUS) or 81% (ULSAM) statistical power to detect an increment to the model R^2 of 0.01 (PIVUS) or 0.02 (ULSAM) for all measures of cardiac function and geometry (at α=0.05).

5.2 Adiponectin and Measures of Vascular Function and Morphology

The main results are presented in **Table 3**. There was a positive association between adiponectin and increased vascular function, measured as CCA distensibility, independent of potential confounders. CCA distensibility increased with 0.04 %/mmHg for each standard deviation increase of adiponectin in both the age- and sex-adjusted model, as well as in the multivariable-adjusted model. Further, adiponectin was positively associated with measures of vascular morphology (IM-GSM and plaque GSM) independent of the confounders included in the regression models. For each standard deviation increase of adiponectin, there was an increase of 2.1 and 3.1 units of IM-GSM and plaque GSM, respectively, in the multivariable-adjusted model.

Table 3. Association between adiponectin and measures of vascular function and morphology that remained significant after adjustment for potential confounders.

	Associations with adiponectin			
	Age- and sex-adjusted		Multivariable-adjusted	
	β (95% CI)	P-value	β (95% CI)	P-value
log CCA distensibility (%/mmHg); n=856	0.04 (0.00; 0.07)	0.025	0.04 (0.00; 0.07)	0.034
IM-GSM; n=957	4.73 (3.30; 6.16)	< 0.0001	2.06 (0.54; 3.58)	0.008
Plaque GSM; n=575	5.91 (3.42; 8.40)	< 0.0001	3.11 (0.36; 5.86)	0.027

Adiponectin was also positively associated with log EDV and log EIDV in age- and sex-adjusted analyses, but the association did not remain significant in the multivariable-adjusted model. Exploratory analysis demonstrated BMI and HDL cholesterol to confound the association with both EDV and EIDV. No association was found between adiponectin and the other outcomes tested.

The associations of adiponectin with IM-GSM, plaque GSM and CCA distensibility were of similar effect size in men and women (data not shown). The effect size of the relation of adiponectin with IM-GSM and CCA distensibility were similar when excluding individuals with prior CVD compared with the total population (β, 2.06; 95% CI, 0.34, 3.78; P=0.019 and β, 0.05; 95% CI, 0.01, 0.09; P=0.007, respectively). The association between adiponectin and plaque GSM was highly significant in individuals with prior CVD (β, 6.43; 95% CI, 1.61, 11.26), but did not remain significant in a healthy subsample (β, 1.47; 95% CI, -1.93, 4.86). Substituting BMI with waist circumference or waist-hip ratio in the multivariable-adjusted models had no substantial effect on the associations of adiponectin with the vascular measures. Also, further adjustment for HOMA-IR or the presence of any type of medication had no effect on the results.

In the total sample we had at least 86% statistical power to detect an increment to the model R^2 of 0.010 for all vascular measures (at α=0.05), except plaque GSM for which we had 88% power to detect an increment of the model R^2 of 0.015.

5.3 Oxidative Stress and Inflammatory Markers in relation to Adiponectin

In age- and sex-adjusted, as well as multivariable-adjusted models, adiponectin was positively associated with GSH and log TGSH, whereas a negative association with conjugated dienes was observed (**Table 4**). A positive association between adiponectin and TAOC was borderline significant in age- and sex-adjusted analysis. A positive association between adiponectin and homocysteine was borderline significant in both age- and sex-adjusted and multivariable-adjusted models.

Table 4. Outcomes that remained significantly associated with adiponectin, independent of potential confounders.

	Associations with adiponectin			
	Age- and sex-adjusted		Multivariable-adjusted	
	β (95% CI)	P-value	β (95% CI)	P-value
GSH (µg/mL); n=920	22.61 (10.05; 35.16)	0.00043	20.43 (6.78; 34.09)	0.0034
log Total GSH (µg/mL); n=921	0.02 (0.01; 0.03)	0.004	0.02 (0.003; 0.03)	0.02
Conjugated dienes (µmol/L); n=928	-1.62 (-2.33; -0.92)	< 0.0001	-0.81 (-1.46; -0.16)	0.015
log EGF (pg/mL); n=928	-0.07 (-0.14; -0.01)	0.033	-0.08 (-0.15; -0.01)	0.033

Adiponectin was inversely associated with log EGF in age- and sex-adjusted and multivariable-adjusted models (**Table 4**). Further, adiponectin was negatively correlated with MCP-1, log E-selectin, and log CRP in age- and sex-adjusted analyses, but not in multivariable-adjusted models.

Adiponectin remained associated with conjugated dienes, log CRP, and GSH in the age- and sex-adjusted models when correcting for multiple testing using the Holm-Bonferroni method, but no association remained significant in the multivariable-adjusted models when considering multiple testing (corrected overall critical P-values were 0.00263 and 0.00227 respectively).

No effect modification by sex was significant for any of the tested associations. The effect of adiponectin on OxLDL was clearly different between individuals with previous CVD (β, 12.49; 95% CI, 6.43, 18.55) and individuals without previous CVD (β, -3.38; 95% CI, -6.80, 0.04), which was supported by a highly significant interaction term of adiponectin and CVD status (P-value=0.00047). A similar pattern was observed in analyses stratified by overweight status, where individuals with BMI <25 had an inverse association with OxLDL (β, -1.01; 95% CI, -5.83, 3.81), while individuals with BMI ≥25 displayed a positive association (β, 1.43; 95% CI, -2.44, 5.29) with a significant interaction term (P=0.006). All markers significant in the multivariable-adjusted model remained significant and with small changes in effect size after exclusion of individuals with kidney failure (eGFR < 50 mL/min/1.73m^2). Adiponectin was not significantly associated with the ratio of reduced and oxidized glutathione in age- and sex-adjusted, or multivariable-adjusted models (P-value=0.43 and P-value=0.36, respectively). The results were also similar when we added total energy intake from a 7-day dietary registration to the multivariable model.

5.4 Rare Genetic Variation and Adiponectin

Quantile-quantile (QQ) plots of P-values from the meta-analyses showed no systematic deviation from the null, but with a tail of low P-values indicating positive signals. The main results are shown in **Table 5**.

The proportion of rare variants where an individual carried the minor allele within the transcript region of *ADIPOQ* (chromosome 3; b37 position range 186,560,462 - 186,576,250) was significantly associated with adiponectin levels in our analysis defining rare variants based on MAF <5% (Z=-4.86; P-value=1.16×10^{-6}). Three independent SNPs (rs115527175, rs17300539, rs113365229) from the *ADIPOQ* region were identified as independent by conditional association tests in a forward selection approach. When adjusting the mutational load for independent SNPs around the *ADIPOQ* gene, the mutational load did not remain significant and the signal was only driven by the lead SNP rs115527175.

The transcript region of F-box and leucine-rich repeat protein 14 (*FBXL14*; chromosome 12; b37 position range 1,675,159 - 1,703,331) was significantly associated with adiponectin when restricting the analysis to coding (Z=4.82; P-value=1.41×10^{-6}) or non-synonymous variants (Z=5.19; P-value=2.06×10^{-7}). However, the most significant analysis, restricted to non-synonymous variants only, included a single marker at chr12:1702929. The SNP is a missense variant where a nucleotide change from major allele T to minor allele G leads to a shift from asparagine to histidine at position 102 (N102H) of the encoded protein. Polyphen-2 classified the variant as probably damaging and PANTHER reported a high probability of a functional significance. The association between adiponectin and the variant at chr12:1702929 was not replicated in IMPROVE (β, -0.04; P-value, 0.87).

Table 5. GRANVIL association results significant after adjustment for multiple testing.

			PIVUS			ULSAM			PIVUS+ULSAM	
Gene	Marker subset	MAF cutoff	Marker count	Mean MAF	P-value	Marker count	Mean MAF	P-value	Z-score	P-value
ADIPOQ	All	5%	45	0.7%	0.011	40	0.9%	1.97×10^{-5}	-4.86	1.16×10^{-6}
FBXL14	Coding	1%	5	0.5%	0.0028	3	0.5%	0.00014	4.82	1.41×10^{-6}
FBXL14	Non-synonymous	1%	1	0.7%	0.013	1	0.6%	1.74×10^{-6}	5.19	2.06×10^{-7}

6 DISCUSSION

6.1 Adiponectin and the Cardiovascular System

The main finding of Study I was the inverse association between adiponectin and ejection fraction in elderly men, independent of potential confounders including BMI. The inverse association was also was also evident in a sub-sample after exclusion of individuals with any previously known CVD. Further, our analyses indicated that NT-proBNP act as a partial mediator of the association of adiponectin with ejection fraction as the association was greatly attenuated when adding NT-proBNP to the model, although remaining significant.

In contrast to the beneficial cardiometabolic profile of adiponectin,[13-16] high adiponectin levels have been demonstrated to be associated with mortality and hospitalizations in patients with poor cardiac health; mainly heart failure.[46, 111] This unexpected association could have one or several explanations, including a counter-active response with a potentially mediating role by natriueric peptides, a confounding effect by cardiac cachexia, and adiponectin resistance.

Adiponectin levels have been shown not to predict incident heart failure in healthy individuals in ULSAM,[112] as well as in the Framingham Heart Study,[113] whereas a recent prospective study in individuals with prevalent CHD reported that higher adiponectin levels were associated with a higher risk of heart failure and death. However, after adjustment for CVD severity at baseline, this association was no longer significant, which further indicate that the higher levels of adiponectin may be a compensatory response to an initial poor cardiac health.[114]

Based on the previously reported associations of increased adiponectin and BNP concentrations with heart failure[46-48] or LV dilation,[45] we hypothesized that adiponectin would be involved in the same cardioregulatory pathway as BNP. Our analyses showed that adjustment for NT-proBNP partially attenuated the association between adiponectin and ejection fraction. Further, a recent study in asymptomatic older men reported a positive association of adiponectin with measures of poor cardiac health, which was mainly attenuated by NT-proBNP.[115] A potential explanation could be that BNP is on the causal pathway by which adiponectin is associated with LV systolic function. Previous studies have shown that naturetic peptides exhibit a lipolytic effect on human adipose tissue through a cGMP-dependent pathway[116-118] that may explain the linkage between adiponectin and BNP. Circulating adiponectin has been suggested to be a marker of total stimulated adipose tissue triacylglycerol lipolytic capacity *in vivo* in humans.[119] Another study demonstrated that infusion of synthetic atrial natriuretic peptide (ANP) increased levels of total and high-molecular weight adiponectin in patients with heart failure.[120] In addition, another study has shown that both ANP and BNP increased the adiponectin mRNA expression in a dose-dependent manner, mediated through the cGMP-dependent pathway.[121]

The observation that higher adiponectin is associated with adverse outcomes in individuals with prevalent disease, but not the progression of the disease, might also suggest that cardiac cachexia, which is seen in many heart failure patients, could lead to lower fat mass and consequently higher adiponectin. However, the present study was performed in healthy individuals without overt disease, and the inverse association between adiponectin and systolic function was also present in a healthier sub-sample (after exclusion of all individuals with any prior CVD) and after adjustments for BMI

along with other potential confounders. These observations, along with the prior study of similarly healthy elderly individuals from Japan,[45] suggest that cardiac cachexia is unlikely to be the sole reason for the associations of circulating adiponectin, NT-proBNP and LV systolic function. Nevertheless, it should be noted that the attenuation by adding NT-proBNP to the model of the association of adiponectin with ejection fraction was even more pronounced in a sub-sample of individuals with prior CVD.

Previous publications have also suggested that increased adiponectin levels in patients with poor cardiac health might be a response to adiponectin resistance, where levels are increased to counter a poorer response to adiponectin which is dysregulated in the diseased state.[122]

We observed associations of adiponectin with ejection fraction in men, but not in women. It has been shown that females have higher levels than males[15, 123] and that this sex difference in circulating levels of adiponectin might be explained by an inhibitory influence of androgens.[17, 18] However, the reason for why the association between adiponectin and systolic function was present in only men remains to be elucidated.

Previous studies have reported an inverse association between adiponectin and cardiac geometry; primarily LV mass,[39-43] but also LV hypertrophy assessed by ECG.[44] Consistent with this, adiponectin levels differed significantly between individuals with normal geometry and individuals with categories of hypertrophy and/or remodeling, adjusted for age and sex (**Figure 1**). However, further adjustment for BMI rendered this association not significant, indicating that BMI is a main confounder of the association between adiponectin and cardiac geometry in our samples.

In Study II, higher serum adiponectin was associated with less arterial stiffness (higher CCA distensibility) and increased vascular echogenicity (higher IM-GSM and plaque GSM), independently of potential confounders including BMI. The results were comparable for men and women. The inverse association with arterial stiffness is in line with some previous studies,[55-57, 124] but not all.[52, 58, 59] In 456 elderly men and women, high levels of adiponectin were associated with decreased arterial stiffness[55] and similar results were seen in 98 T2D patients and 116 controls.[57] In line with this, low adiponectin levels predicted a worsening of arterial stiffness over a one-year period in 142 postmenopausal women.[56] In a more recent case-control study of systemic lupus erythematosus, higher adiponectin was associated with less arterial stiffness[124] and an inverse association was further found between HMW adiponectin and arterial stiffness in 269 subjects without a history of cardiovascular disorders.[125]

The number of plaques in the carotid arteries was not associated with adiponectin, which is in line with previous studies.[50, 54] In Study II, adiponectin was positively associated with plaque GSM in age- and sex-adjusted analysis, as well as multivariable-adjusted models, indicating that that adiponectin is associated with plaque composition rather than plaque presence. However, HMW adiponectin was inversely associated with both the presence and extent of coronary plaques, assessed by computed tomography angiography in a study of patients with suspected CAD. HMW adiponectin was further related to vulnerability characteristics of the coronary plaques.[126] To our best knowledge, this is the first study investigating in detail the association between adiponectin and echogenicity of the vessel wall and arterial plaques in a larger population using GSM techniques. Consistent with our finding, a study evaluating an ultrasound imaging technique of plaques in 638 women diagnosed with diabetes demonstrated that lower levels of adiponectin were associated with echolucent

plaques.[127] Similarly, a smaller study performed in 66 diabetic and 119 non-diabetic patients reported that adiponectin was associated with atherogenic lipoproteins, plaque volume and lipid-rich plaques assessed with intravascular ultrasound.[128] Plaque morphology can be classified according to GSM readings, where plaques with a low GSM (echolucent) are characterized by high lipid content and haemorrhages, whereas a high GSM (ecogenic) is associated with a fibrous content.[129] Our finding that adiponectin is positively associated with plaque GSM, indicates that adiponectin protects against the ecolucent plaques which is considered to be the most pathological form. The ecolucent plaques have been associated with a higher risk of cardiovascular events,[99] since plaques with a high lipid content are more instable and prone to rupture, causing clinical events.[130] Similarly, we also found a strong positive association with IM-GSM independently of potential confounders. Although not described in previous studies, the results of IM-GSM fit into the same context as plaque GSM, since IM-GSM is closely related to the plaque GSM.[97] A low IM-GSM is associated with CVD risk factors, such high BMI and low HDL,[131] and the echogenicity of apparently plaque-free CCA IM complexes measured with the GSM is an significant predictor of CVD mortality.[132] The association between adiponectin and IM-GSM was independent of prior CVD unlike the relation with plaque GSM, which was present only in a diseased subsample. One possible explanation could be that individuals in the healthy subsample have less diseased vessels, and that the role of adiponectin would be less evident than in individuals with more extensive atherosclerosis.

Our analyses showed that levels of adiponectin were positively associated with lower fat content in the IM and plaques as well as with higher wall elasticity. Lipid accumulation in the vascular wall is a process consisting of several steps; beginning with the infiltration of an excess of low-density lipoprotein (LDL) particles, which are modified through oxidation and other enzymatic modification, and then further internalized by macrophages followed by foam cell formation.[133] Adiponectin is inversely associated with LDL particle size[134] as well as apolipoprotein B or E[135] and cellular studies have revealed that both globular and full-length adiponectin inhibit the generation of reactive oxygen species, thus preventing oxidative stress.[136, 137] Also, in Study III, adiponectin was associated with higher levels of the principal anti-oxidant glutathione and lower levels of lipid peroxidation. Adiponectin suppress the expression of macrophage scavenger receptors, reducing the uptake of oxidized LDL and foam cell formation.[138] Our results in Study III indicate an inverse association between adiponectin and oxidized LDL in individuals without previous CVD, whereas the opposite was observed for individuals with CVD. Adiponectin also suppress the growth of macrophage progenitor cells and mature macrophage function;[139] thus, possibly restraining the formation of foam cells and early atherosclerosis. An *in vivo* study in mice showed that adiponectin attenuated neointimal thickening and an *in vitro* experiment further demonstrated that adiponectin suppressed the proliferation and migration of smooth muscle cells, stimulated by HB-EGF.[140] Also, HDL is postulated to play a protecting role against atherosclerosis by the removal of excess cholesterol via the liver.[141] Adiponectin has been demonstrated to increase the HDL assembly in the liver; thus providing another complementary mechanism linking adiponectin to atherosclerosis.[142]

The distensibility of the cell wall is associated with an increase of collegenous material and degeneration of elastic fibers.[143] However, the association between adiponectin and changes in the vascular matrix remains to be fully examined. Lower insulin sensitivity is associated with increased stiffness,[144] thus providing another possible mechanism linking adiponectin to CCA distensibility, since adiponectin is inversely associated with

T2D and insulin resistance.[145] Further, the stiffness of the CCA is related to the presence of atherosclerosis;[146] thus the mechanisms described above, linking adiponectin to vascular morphology, might share common pathways with mechanisms linking adiponectin to vascular function.

6.2 Adiponectin, Oxidative Stress, and Inflammation

We found that higher levels of adiponectin were associated with higher levels of glutathione (GSH and total GSH), lower levels of lipid peroxidation products (conjugated dienes), and lower levels of the growth factor EGF.

A few previous studies have investigated the association between adiponectin and the ratio of reduced and oxidized glutathione in selected materials with small sample sizes. In 12 obese psoriasis patients, an inverse correlation between adiponectin and the ratio of GSSG/GSH was reported[62] and similarly adiponectin correlated positively with the GSG/GSSG ratio in 120 subjects with or without diabetes and with or without non-alcoholic fatty liver disease.[63] Further, adiponectin was shown to restore the GSH/GSSG ratio after it was reduced when treating HepG2 cells with palmitate.[63] We did not find a significant association with the GSH/GSSG ratio, but a strong association with absolute levels of GSH and TGSH.

In line with our results, several studies have reported that lower adiponectin associates with increased levels of lipid peroxidation byproducts,[8, 64-66] but not all.[67] A negative correlation between adiponectin and 8-epi-PGF2α was found in 105 Japanese men and women without diabetes, CVD or renal disease.[65] Further, 8-epi-PGF2α was inversely correlated with adiponectin in 259 Japanese men and women with normal glucose tolerance, impaired glucose tolerance or diabetes; however, not significantly when adjusting for the full set of confounders.[66] No association with urinary 8-epi-PGF2α was found in 76 non-diabetic, hypercholesterolemic patients.[67] Moreover, an inverse association between adiponectin and thiobarbituric acid-reacting substance (TBARS), as well as urinary 8-epi-PGF2α was found in 140 Japanese individuals[8] and an inverse association of adiponectin with TBARS was reported in 1,178 Japanese men.[64] However, no significant association between one-year change in adiponectin and one-year change in TBARS was found.[64]

Adiponectin can both affect and be affected by the oxidative environment. Previous studies have suggested that the oxidative environment could influence adiponectin secretion and assembly. One study in rats suggested that adiponectin secretion might be decreased in response to an oxidative environment[147] and an *in vitro* study indicated that adiponectin oligomerization is dependent on the redox environment.[148] Strong correlations were found between the mRNA expression levels of the adiponectin receptors (ADIPOR1 and ADIPOR2) and glutathione peroxidase-1 in 60 morbidly obese patients with non-alcoholic fatty liver disease[149] suggesting shared/linked factors regulating gene expression. Further, adiponectin has been to shown to protect against elevated oxidative stress levels. A study showed that addition of adiponectin maintained normal oxidative stress levels and GSH levels in cells exposed to OxLDL.[150] An *in vitro* experiment revealed that hypoxia-induced lipid peroxidation in cells was completely attenuated after treatment with adiponectin and that treatment with adiponectin partly attenuated hypoxia-induced loss of intracellular GSH. Further, in an *in vivo* experiment, adiponectin-overexpressing transgenic mice had significantly lower levels of lipid peroxidation products (hypoxia-induced) compared with wild-type

mice. The levels of GSH were also higher in the lungs of the transgenic mice compared with wild-type.[151]

We found an inverse association between adiponectin and EGF. A previous study has found an inverse association between mRNA expression of adiponectin and heparin-binding epidermal growth factor-like growth factor (HB-EGF), which is structurally similar to EGF. Further, the EGF receptor (EGFR) activation and transverse aortic constriction-induced cardiac hypertrophy was higher in adiponectin knockout mice compared with wild-type, and treatment with adiponectin reduced the EGFR activity.[152] In an *in vitro* experiment, adiponectin suppressed the proliferation and migration of smooth muscle cells, stimulated by HB-EGF. Adiponectin also attenuated DNA synthesis induced by EGF.[140]

Our results indicated an inverse association between adiponectin and OxLDL in individuals without prior CVD, whereas somewhat unexpectedly, a positive association was observed in individuals with prior CVD. A similar pattern was seen for normal-weight versus overweight individuals. Previous large studies have indicated an inverse association between adiponectin and OxLDL, including one study of 2,985 individuals[153] and another study of 1,309 postmenopausal women without coronary artery disease or diabetes.[154] However, in contrast, a significant positive association was found in 106 pregnant women, but not in matched non-pregnant women.[155] As previously discussed, some studies have found that adiponectin is associated with worse cardiovascular outcomes and have suggested that this might be part of a compensatory mechanism in response to poor CVD health.[114] The observed positive association between adiponectin and OxLDL among those with prior CVD, could potentially be explained by a similar phenomenon, where the increased levels represent a counter-active response to the diseased state.

6.3 Genetic Determinants of Adiponectin

Previous GWA studies investigating associations between common genetic variants and adiponectin levels have identified strong signals, mainly at *ADIPOQ*,[34, 82, 83] *ARL15*,[34, 83] and *CDH13*.[34, 36-38] Some studies have investigated rare variation in selected regions after re-sequencing of *ADIPOQ*[81] and analysis of rare haplotypes at *KNG1-ADIPOQ*,[38] but rare variation across the entire genome in relation to adiponectin levels remains an unexplored path. To our best knowledge, no previous studies have investigated the association between circulating adiponectin levels and the proportion of rare alleles within a gene carried by an individual on a genome-wide scale. In our analysis, we identified two potential regions where the proportion of minor alleles at rare variants was associated with adiponectin after taking multiple testing into account – *ADIPOQ* and *FBXL14*.

In analyses including all markers, the transcript region of *ADIPOQ* was significantly associated with lower levels of adiponectin. However, this mutational burden test signal was explained by one single variant in *ADIPOQ*, i.e. the burden test signal was correlated with an *ADIPOQ* SNP and we found no evidence of a burden test signal once we accounted for this SNP in the regression model. This SNP was identified in a standard association test, so in this case, the burden test did not add anything new in addition to a regular SNP-based test.

The transcript region of *FBXL14* was significantly associated with higher adiponectin levels when restricting the analysis to coding or non-synonymous markers, which appeared to be driven by a single non-synonymous marker at chr12:1702929. This SNP is a missense variant where the rare allele has a potentially damaging effect on the protein. However, since this variant was not replicated in the IMPROVE study, the results should be interpreted with caution. The lack of replication could potentially indicate a false positive finding, but could also be due to low statistical power or high between-study heterogeneity given that IMPROVE is a study of high-risk individuals of CVD whereas the discovery samples are population-based. If the variant is a true positive finding, it remains to be established through which pathways this variant is associated with adiponectin levels; however, the presence of *ADIPOR2* in the region is intriguing.

ADIPOQ has the most apparent connection with adiponectin levels out of the two genes identified in the present study. We have not found a connection between adiponectin and the gene product of *FBXL14*. However, interestingly, *FBXL14* lies upstream of the adiponectin receptor 2, which makes this region an interesting target for further analyses. *ADIPOR2* encodes the Adiponectin receptor 2 which is an intermediate-affinity receptor for full-length adiponectin that seems affect peroxisome proliferator-activated receptor-α signalling pathways and fatty acid oxidation.[30, 31] No prior GWA studies have reported an association of the *ADIPOR2* locus with adiponectin levels. Even if we did not replicate this finding in IMPROVE, it seems likely that the finding is a true positive given the high probability of a biological link between circulating adiponectin and the genetic region around an adiponectin receptor.

6.4 Strengths and Limitations

The main strengths of this thesis include the two large community-based samples of elderly with detailed phenotypes representing different aspects of the CVD process. These individuals are well-characterized which also allows for adjustment for relevant confounding factors. In addition, with our dense genotype data we can investigate the impact of rare genetic variation on adiponectin levels, which has not been studied in detail previously.

There are several limitations that should be mentioned. First, adiponectin aggregates into several oligomeric forms, and this thesis focuses on total adiponectin, i.e. the sum of all forms rather than separate measures for each fraction. Some studies have indicated differences in biological activity between different forms of adiponectin with regard to metabolic abnormalities.[28, 29] However, this remains to be fully established and yet other studies have found similar associations of total adiponectin and the HMW fraction (which are highly correlated) with for example T2D.[156] Second, since our samples include elderly individuals of Northern European ancestry, the generalizability to other age groups and ethnicities is unknown. However, it is expected that the lower variation in age and ethnic background of our studies provide a high internal validity and reduce the effect of confounding with regard to these factors. Third, we cannot assess causality in Study I-III due the cross-sectional and observational design of the studies, nor can it be determined if the outcomes are related to the longitudinal tracking of adiponectin concentrations. Fourth, in Study I, ejection fraction was measured according to the Teichholz formula using M-mode in both cohorts. This way to evaluate ejection fraction has its known limitations, since it does not take into consideration dyskinesia in other segments than those included in the M-mode

recording. Despite this limitation, this way of measuring ejection fraction is still used very frequently in the clinical setting, a substantial part of the literature regarding the predictive power of ejection fraction, and inclusion criteria in landmark heart failure treatment trials are based on this method. Fifth, the sample sizes in Study IV were relatively modest to investigate the impact of rare genetic variants where few individuals carry the minor allele, meaning that several findings that did not reach multiple testing-corrected significance can represent true associations with adiponectin, and may merit further exploration in future studies. Sixth, in Study IV, our replication stage was not ideally designed given the limited sample size of the replication sample and the fact that we genotyped just one SNP instead of sequencing the whole region (to find other rare variants that could contribute to the mutational load). Finally, the samples were stored in -70°C for 1-3 years (PIVUS) and 11±2 years (ULSAM) before being analyzed. However, at the time of collection, the samples were ideally treated to limit any influence on stability of samples. The effect of frozen storage on adiponectin levels has to our best knowledge been investigated under a maximum of 30 months, reporting no discernible effect of mean plasma adiponectin levels.[157] We are not aware of any other studies with longer follow-up, but most peptide hormones has been shown to be fairly stable, some peptides even during long-term storage up to 25 years.[158]

7 CONCLUSIONS

I. In our two community-based samples of elderly, adiponectin concentrations were inversely associated with ejection fraction in men, even after adjustment for potential confounders including BMI. These associations were partially attenuated by additional adjustment for NT-proBNP, indicating that adiponectin may be associated with depressed LV systolic function through pathways shared with natriuretic peptides; potentially as a counter-active response to poor cardiac health. Further studies are required to establish the biological pathways linking adiponectin, natriuretic peptides and cardiac function.

II. Higher circulating adiponectin was associated with a lower fat content in the vessel wall and plaques. Adiponectin was also positively associated with higher vessel wall distensibility; the distensibility is a measure of wall elasticity, and a higher value is equivalent to lower arterial stiffness. Together, these findings imply that adiponectin is associated with less arterial pathology. The pathways linking adiponectin with higher vessel echogenicity and elasticity need to be further investigated using other study designs.

III. Adiponectin levels were positively associated with the primary anti-oxidant glutathione (GSH and TGSH) and negatively associated with products of lipid peroxidation (conjugated dienes). Together these associations suggest that adiponectin is associated with a more beneficial oxidative stress profile, potentially through shared pathways. Further studies are needed to investigate whether anti-oxidative effects may be a mechanism linking adiponectin with type 2 diabetes and/or cardiovascular disease.

IV. Our results indicated that individuals who carry a larger proportion of minor alleles at rare variants within *ADIPOQ* have on average lower levels of adiponectin, but this signal was driven by one SNP in the gene. Further, a significant positive association was observed in analyses including a single non-synonymous marker at chr12:1702929 in a gene upstream of *ADIPOR2*. If replicated in independent samples, our results could be of importance for the understanding of adiponectin biology.

8 FUTURE PERSPECTIVES

8.1 Study Design

Many of the epidemiological studies in the adiponectin research field as well as in this thesis are of cross-sectional design and causality cannot be assessed. Even though these studies provide important information regarding in which pathways adiponectin might be involved, large longitudinal studies are needed since they offer the possibility to investigate changes in adiponectin as well as the outcome over time, which is needed to elucidate the causal role of adiponectin in different cardiometabolic disorders. In addition, Mendelian randomization studies should also be mentioned as an interesting cross-sectional alternative to investigate the causal role of adiponectin in the development of subclinical and overt disease.

Some previous studies have indicated that different oligomeric forms of adiponectin elicit different biological effects. This remains to be fully established, but future studies should aim at measuring each fraction to investigate this possibility. Standardization of assay procedures to measure the different forms of adiponectin would be warranted.

To further study the influence of genetic variation on adiponectin levels, larger sample sizes are merited. A recent large meta-analysis including a total of 45,891 individuals in the ADIPOGen consortium confirmed known adiponectin loci and identified several novel loci,[34] and this type of effort could be expanded to study rare genetic variants in relation to adiponectin.

8.2 Adiponectin as a Biomarker and Utility in Risk Prediction

Adiponectin has several attractive features as a biomarker such as a high levels in plasma[23] and high stability.[159] However, there are also several problems associated with the use of adiponectin as a biomarker. First, given that the mean levels differs with regard to gender[15] and ethnicity,[160, 161] more data is needed to find reference values for what constitutes a normal adiponectin level in the different subgroups. This is further complicated by the fact that adiponectin, through dysregulation or as part of a compensatory mechanism, is increased in conditions such as heart failure; hence, high adiponectin levels are not always associated with a healthy profile. Adiponectin is associated with a mostly beneficial health profile, e.g. an inverse relation to BMI,[12] T2D,[13] hypertension,[14] and triglycerides.[15] However, given the high correlation between adiponectin and several CVD risk factors, it remains to be established how much adiponectin can add to established risk factors in the prediction of CVD risk in a clinical setting.

8.3 New Biological Pathways and Pharmaceutical Targets

Several pathways linked to the regulation of adiponectin, as well as adiponectin signaling have been established or suggested, but more research is needed. In order to fully elucidate the signal transduction and downstream effect after adiponectin binds to its receptors, a targeted approach can be used to study selected pathways *in vitro* and *in vivo*. Further, an untargeted approach, using techniques such transcriptomics,

proteomics, and metabolomics can give further hints about the intra- and extra-cellular pathways that are related to adiponectin.

Administering adiponectin as a drug has proven difficult,[162] but agents that increase adiponectin levels as well as agonists targeting the adiponectin receptors might offer a solution. Research about adiponectin as a pharmaceutical agent is in early development and it can be mentioned that an adiponectin-based peptide compound acting as an adiponectin receptor agonist in cancer cells was recently developed.[162]

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