

From DEPARTMENT OF CLINICAL SCIENCES,
DANDERYD HOSPITAL
Karolinska Institutet, Stockholm, Sweden

ADIPONECTIN IN ATHEROSCLEROSIS

Jonas Persson



**Karolinska
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Cover legend: Adiponectin inhibits the development of atherosclerosis in experimental animal models. In the present thesis, data is suggestive of a causal protective role of adiponectin in early atherosclerosis

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ABSTRACT

Adiponectin is a protein secreted from adipocytes, circulating at high levels in plasma (3–30 µg/mL), and in overweight subjects it is down-regulated. Adiponectin inhibits the development of atherosclerosis in experimental animal models but whether adiponectin is anti-atherogenic in humans is not known. Furthermore, epidemiological studies on plasma adiponectin with regard to cardiovascular outcome are contradictory.

We therefore explored the relationship between plasma adiponectin, cardiovascular disease (CVD) and mortality in three separate studies in which the investigated subjects differed with respect to age, cardiovascular risk factors, and prevalence of CVD. Furthermore, we examined the relationship between plasma adiponectin and intima-media thickness (IMT) in the common carotid artery (CCA), in the bifurcation of the carotid artery (Bif) at baseline and after 30 months progression. In addition, we analysed the associations of 1,214 single nucleotide polymorphisms (SNPs) in five loci *ADIPOQ*, *ADIPOR1*, *ADIPOR2*, *CDH13*, and *ARL15* in relation to plasma adiponectin. The relationship between plasma adiponectin raising alleles and IMT measures was also investigated. We also explored the use of gene expression profiles, measured with Affymetrix® microarray technology, from carotid atherosclerotic plaques and from peripheral blood mononuclear cells for improving the prediction of ischaemic events, in addition to established risk factors.

In paper I, we demonstrated that low plasma adiponectin was associated with myocardial infarction in young individuals (<60 years) in an age- and gender-matched case-control study of 244 survivors of first-time myocardial infarction and corresponding controls. The relationship was independent of fasting glucose, high-density lipoprotein cholesterol, hypertension and creatinine. In paper II, we showed that high plasma adiponectin was associated with cardiovascular and all-cause mortality in 292 subjects who all had prevalent CVD and underwent carotid endarterectomy (CEA). No relation to ischaemic events (i.e. ischaemic stroke or myocardial infarction) was evident. In paper III, low plasma adiponectin was associated with cardiovascular and coronary events in a large cohort of 3,430 high-risk subjects with three or more cardiovascular risk factors, but without prevalent CVD. Plasma adiponectin was inversely associated with Bif-IMT at baseline and progression of CCA-IMT in men. We also showed that four SNPs in *ADIPOQ* were associated with plasma adiponectin and the finding was replicated in independent cohorts (n=6,576). The sum of plasma adiponectin raising alleles was inversely associated with Bif-IMT in men but not in women. The association could, however, not be replicated in a smaller cohort of low risk subjects. In paper IV, we showed that adding gene expression profiles, from atherosclerotic plaques extirpated by CEA in 127 patients, to classical risk factors could improve prediction of ischaemic events.

In summary, prevalent CVD is an important divider for the relationships between plasma adiponectin and outcome measures; Low plasma adiponectin is associated with adverse outcome in subjects without prevalent CVD, whereas in subjects with prevalent CVD high plasma adiponectin is associated with mortality. Plasma adiponectin is inversely associated with early atherosclerosis and progression of atherosclerosis in men. Adiponectin raising alleles are inversely associated with early atherosclerosis in men, which supports a causal protective role of adiponectin in early atherosclerosis.

Additionally, gene expression profiling from atherosclerotic plaques improves prediction of ischaemic events in subjects with prevalent CVD.

Key words: Adiponectin, atherosclerosis, intima-media thickness, genetics, myocardial infarction, mortality, gene expression profiling, risk assessment, ischaemic stroke

LIST OF PUBLICATIONS

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LIST OF ABBREVIATIONS

APPL1	Adaptor protein containing pleckstrin homology domain, phosphotyrosine binding (PTP) domain and leucine zipper motif
Bif	bifurcation of the carotid artery
BiKE	Biobank of Karolinska carotid Endarterectomies
BMI	body mass index
C/EBP	CCAAT/enhancer binding protein
CAD	coronary artery disease
CCA	common carotid artery
CEA	carotid endarterectomy
CHD	coronary heart disease
CI	confidence interval
CVD	cardiovascular disease
FFA	free fatty acids
GoDARTs	Genetics of Diabetes Audit and Research Tayside study
GWA	genome-wide association
HDLc	high-density lipoprotein cholesterol
HMW	high molecular weight
ICA	internal carotid artery
IL	interleukin
IMPROVE	Carotid Intima Media Thickness (IMT) and IMT Progression as Predictors of Vascular Events in a High Risk European Population
IMT	intima-media thickness
LDLc	low-density lipoprotein cholesterol
LMW	low molecular weight
LPL	lipoprotein lipase
MMP	matrix-degrading metalloproteinases
MMW	middle molecular weight
NASCET	North American Symptomatic Carotid Endarterectomy Trial
NFAT	nuclear factor of activated T-cells
OR	odds ratio
PAR	population attributable risk
PBMC	peripheral blood mononuclear cell
PIVUS	Prospective Investigation of the Vasculature in Uppsala Seniors
PPAR	peroxisome proliferator-activated receptor
SBP	systolic blood pressure
SCARF	Stockholm Coronary Atherosclerosis Risk Factor Project
SNP	single nucleotide polymorphism
SREBP	sterol-responsive element-binding protein
TIMP1	tissue inhibitor of metalloproteinases 1
TNF- α	tumor necrosis factor α
TZD	thiazolidinediones

1 INTRODUCTION

Atherosclerosis is a progressive condition in which the walls of arteries become thickened and thereby obstruct the vessel lumina, which leads to serious consequences such as myocardial infarction, ischaemic stroke, and critical limb ischaemia. The onset of atherosclerosis occurs at an early age and entails dysfunction of the innermost layer of arteries, called the endothelium. Over decades, lipids are deposited in the vessel walls, which results in local inflammation and development of arterial plaques. These plaques remain clinically silent until they are large enough to limit the flow of arterial blood to different tissues and thus give rise to clinical manifestations of atherosclerosis, such as angina pectoris and intermittent claudication. Atherosclerotic plaques can also burst and expose thrombogenic debris from the plaque core to clotting factors and platelets in the blood. This brings about formation of a thrombus that occludes the artery and induces cell death distal to the site of the occlusion, which culminates in the life-threatening conditions mentioned above (i.e., myocardial infarction, ischaemic stroke, and critical limb ischaemia). The process of atherosclerosis is driven by multiple factors, including heredity, male gender, smoking, exposure to pollution, diet, high blood pressure, high blood cholesterol, unfavourable intrauterine environment before birth, and overweight.

Adipose tissue is an energy storage depot, and it also functions as an endocrine organ secreting numerous metabolically active molecules called adipokines. These bioactive molecules are involved in energy homeostasis and inflammation in a wide array of tissues, among them adipose tissue, liver, skeletal muscle, brain, and arteries. Obesity is characterized by low-grade systemic inflammation and dysfunctional adipose tissue. In that context, adipokines that promote inflammation and insulin resistance are up-regulated, whereas the anti-inflammatory and insulin-sensitizing protein *adiponectin* is down-regulated.

The research reported in this thesis focused on the role of the anti-inflammatory adipokine adiponectin in development of early atherosclerosis, onset of myocardial infarction, and ischaemic stroke. Furthermore, work was done to evaluate the usefulness of gene-expression patterns in atherosclerotic plaques and blood cells to predict incident myocardial infarction and ischaemic stroke.

1.1 RISK FACTORS FOR CARDIOVASCULAR DISEASE

Today, ischaemic heart disease and cerebrovascular disease are the leading causes of death globally, and it has been projected that that will continue to be the case until 2030 (Mathers and Loncar, 2006). It has also been predicted that by 2030 those two conditions will become the third and sixth leading causes of disease burden (measured as loss of healthy life) after unipolar depressive disorders and HIV/AIDS (Mathers and Loncar, 2006). Risk factors for cardiovascular disease (CVD) include non-modifiable characteristics such as age, male gender (Anderson et al., 1991), and family history of CVD (Murabito et al., 2005; Marenberg et al., 1994; Lloyd-Jones et al., 2004), and also modifiable risk factors like smoking, dyslipidaemia, hypertension, diabetes mellitus, obesity, psychosocial factors, lack of regular physical activity (O'Donnell et al., 2010; Yusuf et al., 2004), and low birth-weight (Barker et al., 1989; Rich-Edwards et al., 1997). Risk factor patterns differ somewhat for myocardial infarction and ischaemic stroke. Dyslipidaemia accounts for the largest population attributable risk (PAR) for myocardial infarction (49%; Yusuf et al., 2004) whereas hypertension is responsible for the highest PAR for stroke (52%; O'Donnell et al., 2010). It seems that regular physical activity is more important to avoid ischaemic stroke, than myocardial infarction (PAR values 29% and 12%, respectively). It should also be mentioned that cardiac disorders give rise to ischaemic stroke (PAR value 8.5%; O'Donnell et al., 2010).

1.2 PATHOPHYSIOLOGY OF ATHEROSCLEROSIS

Early stages of atherosclerosis are present in adolescents and young adults. Autopsies of young soldiers (mean age 22 years) who suffered a violent death in the Korean war showed that 77% had evidence of coronary artery disease (CAD) varying from fibrous thickening to complete occlusion of one or more of the main branches (Enos et al., 1953). However, symptoms of atherosclerosis usually manifest in middle-aged and older people. Diffuse intima thickness is believed to be related to atherosclerosis, because it can be observed in arteries that are prone to this disease (i.e. coronary, carotid, and femoral arteries) but not in those that are apparently resistant to atherosclerosis (i.e., splenic and intracranial arteries; Nakashima et al., 2002). The diffuse intima thickness is accompanied by fatty streaks (Stary et al., 1994), and the key initiation process in atherosclerosis is subendothelial retention of low-density lipoprotein cholesterol (LDLc; **Figure 1**; Skalen et al., 2002). The LDLc is oxidized (Sparrow and Olszewski, 1993) and subsequent endothelial dysfunction and

inflammation contribute to the development of atherosclerosis (Healy, 1990; Hansson et al., 2006). The activated endothelium expresses adhesion molecules (Nakashima et al., 1998; Cybulsky and Gimbrone, 1991) which make monocytes and T-cells adhere (Nakashima et al., 1998). Cytokines secreted by inflammatory cells in the underlying intima stimulate the leukocytes to migrate into the subendothelial space (Boring et al., 1998; Gu et al., 1998). The monocytes transform into macrophages (Hansson et al., 1991; Nakashima et al., 2007) after being exposed to oxLDL (Shashkin et al., 2005). The macrophages engulf oxidized LDLc and change into foam cells (Ley et al., 2011) and arterial plaques develop. T-cells contribute to disease progression by secreting pro-inflammatory mediators which amplifies the inflammatory response (Zhou et al., 2000; Stemme et al., 1995). As atherosclerosis evolves, macrophage foam cells and smooth muscle cells undergo apoptosis and necrosis, and a necrotic core develops in the plaque (Tabas, 2010).

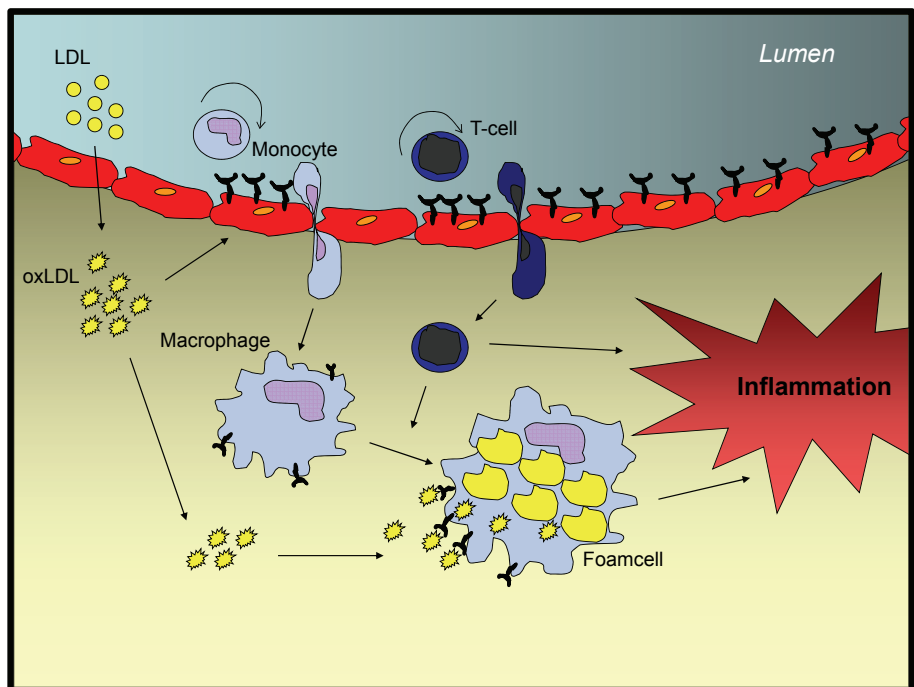


Figure 1. Initiation of atherosclerosis in the arterial wall. LDLc = low-density lipoprotein cholesterol; oxLDL = oxidized LDLc; Adapted from Andersson et al., 2010.

1.3 MYOCARDIAL INFARCTION

The rupture of an atherosclerotic plaque in a coronary artery with subsequent luminal thrombus formation and myocardial infarction were first described in Denmark more than 160 years ago. The Danish artist Bertel Thorvaldsen died suddenly at the Royal theatre in Copenhagen on 24 March 1844. In the autopsy performed two days later, Dr Carl Emil Fenger described *“several atheromatous plaques, one of which quite clearly had ulcerated, pouring the atheromatous mass into the arterial lumen”*, referring to the left coronary artery (Fenger et al., 1844). In a landmark paper published in 1912 in the *Journal of the American Medical Association*, the physician Dr James B Herrick, practising in Chicago, Illinois, in the United States, was the first to report the clinical features of myocardial infarction, which were divided into three groups: (i) death is instantaneous and perhaps painless; (ii) the attack is angina, the pain severe, the shock profound, and death follows within minutes; (iii) mild symptoms and *“...slight angina attacks without the ordinary causes (such as walking)...”*. Notably, Herrick reviewed descriptions of ligation of coronary arteries in experimental animal studies and investigations/case reports of autopsies in humans, and we still rely on such methods today when exploring the pathophysiology of myocardial infarction (Gonon et al., 2011; Arbustini et al., 1999).

There are three main aetiologies of luminal thrombosis: plaque rupture, erosion, and calcified nodules (Virmani et al., 2000). The main features of plaque rupture are shown in **Figure 2**. The collagen-rich fibrous cap covering an atherosclerotic plaque is broken down by matrix-degrading metalloproteinases (MMPs) secreted by macrophages (Galis et al. 1995; Shah et al. 1995). The cap becomes thinner and then bursts and exposes the necrotic core to clotting factors and platelets in the blood. Thereafter, a thrombus is formed in the lumen of the artery, which leads to total or partial occlusion and cell death of myocardium distal to that location (Gronholdt et al., 1998). Plaque erosion is characterized by superficial erosion of thick, proteoglycan-rich fibrous cap accompanied by less infiltration of inflammatory cells and the presence of more smooth muscle cells (Farb et al., 1996). Less commonly (in 2–7% of cases) are thrombi attributed to calcified nodules that are characterized by eruptive nodular calcification with an underlying fibrotic calcified plaque (Virmani et al., 2000). In 298 consecutive cases of fatal myocardial infarction, it was found that 75% had plaque

rupture, and 25% had plaque erosion, which was more common in females (37%) than in males (18%; Arbustini et al., 1999).

Among individuals with first time myocardial infarction, 28.9% die outside hospital, and another 9.5% die within 28 days (Dudas et al., 2011). Among male survivors of a first-time myocardial infarction the estimated long-term risk of fatal CVD was 13.8/1000 years for those aged 55–64 and 34.6/1000 years for those aged 65–74; the corresponding figures for women were 11.9/1000 years and 30.1/1000 years (Dudas et al., 2012). Furthermore, it has been reported that the 3-year risk of heart failure after a myocardial infarction is 33% for individuals aged 65–84 (Shafazand et al., 2011). In summary, myocardial infarctions are dangerous and survivors have high risk of developing heart failure.

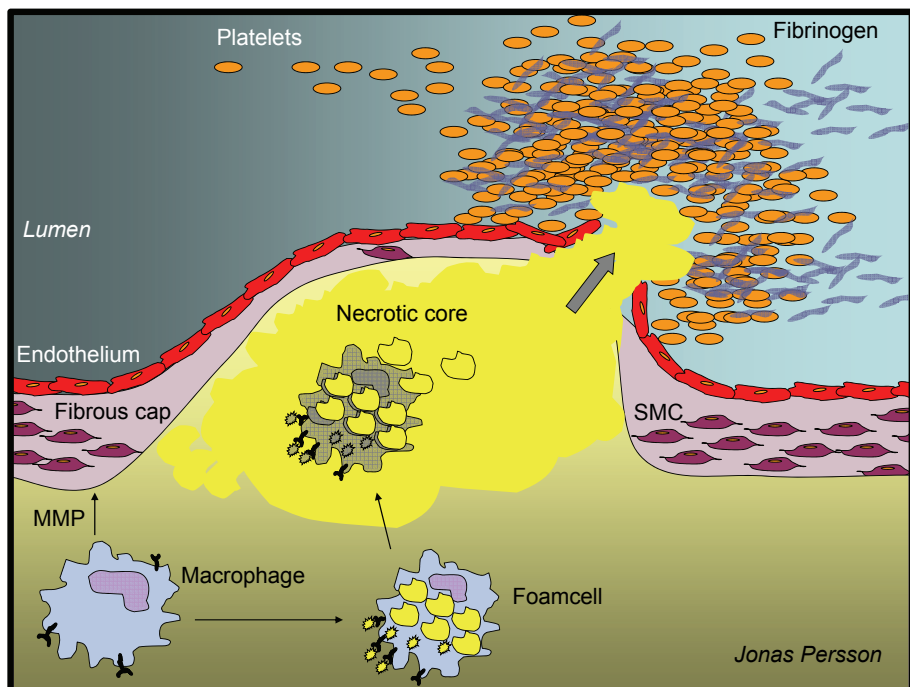


Figure 2. Plaque rupture causing luminal thrombosis. MMP matrix-degrading metalloproteinases; SMC = smooth muscle cells.

1.4 ISCHAEMIC STROKE

Ischaemic stroke occurs due to disturbance in the blood vessels supplying blood to the brain and are classified into different subtypes based on etiology; atherothrombotic stroke, cardio embolic stroke, small vessel disease, other causes and undetermined cause (Adams et al., 1993). The frequencies of the subtypes have been estimated to 13%, 27%, 23%, 2%, and 35%, respectively in subjects with a first time ischaemic stroke (Kolominsky-Rabas et al., 2001). In an autopsy study of 142 cases of ischaemic stroke who died within 30 days of the index event the corresponding numbers were 12%, 75%, 1%, 8%, 3% (Ogata et al., 2008). Atherothrombotic stroke occurs due to occlusion of large-arteries or artery to artery embolism. The process is associated with atherosclerosis in cervical arteries and major intracranial arteries and a thrombotic occlusion is believed to share the pathophysiological features of plaque rupture in coronary atherosclerosis. Thirty-six percent of subjects with a first time stroke are dead within a year and 58% dies within five years (Hankey et al., 2000).

1.5 ESTIMATING RISK IN CARDIOVASCULAR DISEASE

It is important that an individual's total burden of cardiovascular risk is assessed in a clinical setting when recommending lifestyle and therapeutic interventions. The concept of evaluating multiple risk factors to determine a person's risk in this context was introduced in 1991 in a paper from the Framingham Heart study (Anderson et al., 1991). The investigation, comprising 5,573 individuals who were initially free of CVD, evaluated age, gender, systolic blood pressure (SBP), total cholesterol, high-density lipoprotein cholesterol (HDLc), left ventricular hypertrophy, smoking, and diabetes mellitus in relation to cardiovascular morbidity and mortality. A European risk scoring scheme was launched in 2003 (Conroy et al., 2003) and national scoring systems are now available for European countries.

Although methods for estimation of cardiovascular risk have been established and are being used, the majority of events occur in individuals who are classified as being at low or intermediate risk. One in five patients who suffer from coronary heart disease (CHD) lack one or more of the four conventional risk factors: hyperlipidaemia, diabetes mellitus, smoking, and hypertension (Khot et al., 2003). Thus improvement of risk estimation is a priority in research in this area. Identification of new biomarkers that can provide useful information to complement the established cardiovascular risk factors has long been regarded as a plausible approach to achieving better risk

estimation. However, as of 2012, such biological indicators have proven to be of only limited value as supplementary strategies in CVD risk assessment (Perk et al., 2012).

Genetic variants for estimation of cardiovascular risk

Several genetic variants have been found to be associated with myocardial infarction and coronary artery disease in genome wide association (GWA) studies (Kathiresan and Srivastava, 2012). Thus, it is an appealing idea to use genetic variants to improve cardiovascular risk assessment. In addition to traditional risk factors, a genetic risk score, based on 12 single nucleotide polymorphisms (SNPs) associated with CHD in GWA studies slightly improved risk assessment in two different cohorts, area under the curve (AUC) 0.801 versus 0.809; $p = 0.0073$ (Davies et al., 2010). In another investigation it was found that a genetic risk score composed of 13 SNPs associated with coronary disease was independently correlated with cardiovascular events, although this resulted in only modest improvement of risk reclassification (Thanassoulis et al., 2012). Furthermore, other studies evaluating risk scores based on genetic variants have failed to demonstrate enhanced prediction of incident CVD (Lluis-Ganella et al., 2012 ; Paynter et al., 2010). In summary, the approach of analysing genetic variants, in addition to traditional risk factors have yielded very limited value in cardiovascular risk assessment.

Transcripts for estimation of risk

In CVD, gene expression profiles (mRNA levels for multiple gene loci) in peripheral-blood mononuclear cells (PBMC) have been analysed with regards to various phenotypes, such as hypertension (Chon et al., 2004), chronic heart failure (Cappuzzello et al., 2009), thoracic aortic aneurysms (Wang et al., 2007), and extent of coronary artery stenosis (Wingrove et al., 2008). Gene expression profiles from carotid endarterectomy specimens have been found to be related to plaque instability (Papaspnyridonou et al., 2006) but it is not known whether the gene expression profiles from plaques or PBMCs can predict ischaemic events. In paper IV, we explored the ability of gene expression profiles from carotid atherosclerotic plaques and from PBMCs to improve the prediction of ischaemic events in addition to established risk factors.

1.6 OVERWEIGHT, OBESITY, AND ADIPOSE TISSUE FUNCTIONS

1.6.1 Epidemiology

Overweight and obesity (body mass index [BMI] ≥ 25 and ≥ 30 kg/m², respectively) have emerged as a major threat to future health in high-income countries, and, along with growing wealth in middle- and low-income countries, the magnitude of this problem is expected to increase throughout the world. Being overweight or obese constitutes the fifth most common cause of death and account for 7% of global mortality (WHO Burden of Disease 2004–2008 update, 2009). In 2008, more than 1.4 billion adults were overweight (**Figure 3**), and that figure includes over 200 million men and nearly 300 million women who were obese. In Sweden, the prevalence of overweight and obesity are 53% and 18%, respectively (WHO Global Health Observatory <http://apps.who.int/gho/data/>), and there was a clear trend towards increasing mean BMI in the Swedish population from 1980 to 2008 (**Figure 4**).

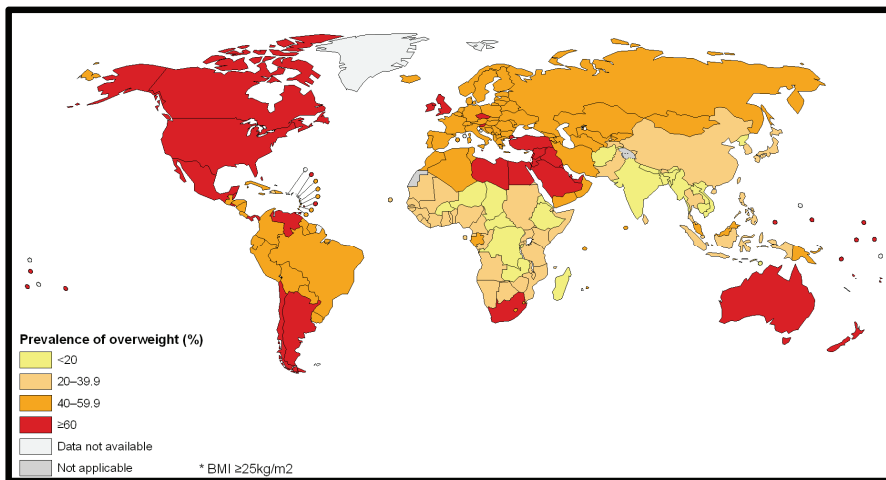


Figure 3. Prevalence of overweight in the world 2008. (WHO Global Health Observatory <http://apps.who.int/gho/data/>, November 2012); BMI = body mass index.

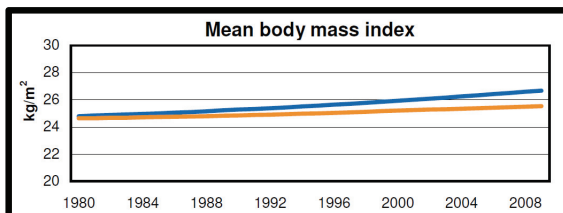


Figure 4. Mean body mass index in Sweden from 1980 to 2008 in men (blue) and women (yellow). (WHO Global Health Observatory <http://apps.who.int/gho/data/>, November 2012); BMI = body mass index.

1.6.2 Adipose tissue stores energy and secretes hormones

Adipose tissue is an energy storage organ that is mainly found subcutaneously and in the visceral cavity, although there are also other depots, around the heart, kidneys, blood vessels, and lungs, and in the bone marrow. Dietary fat enters the circulation from the small intestine in the form of chylomicrons, and there is a continuous flow of fatty acids between adipose tissue, liver, and muscle (**Figure 5**; Frayn et al., 2006). Lipoprotein lipase (LPL) present in adipose tissue and skeletal muscle cleaves triglycerides from the chylomicrons. In the liver, hepatic lipase is involved in the uptake of triglycerides from the remnants of the chylomicron particles. The adipose tissue, muscle, and liver store triglycerides in a regulated manner, and the triglycerides are mobilized for β -oxidation when needed. The liver releases triglyceride fatty acids as very-low-density lipoprotein (VLDL) particles, which are taken up by other organs, including muscle and adipose tissue.

Adipose tissue serves not only as an energy storage organ, but is also responsible for secreting numerous factors called *adipokines*, which are involved in lipid metabolism, insulin sensitivity, inflammation, vascular haemostasis, and regulation of energy balance (**Table 1**). A formal definition of adipokines is yet to be presented, but here this term refers to cytokines that are produced by adipocytes or stromal cells in the adipose tissue. Adipose tissue and adipocytes become dysfunctional during the expansion that occurs in overweight and obese individuals (**Figure 6**). The adipose tissue becomes less sensitive to insulin (Xu et al., 2003b), and the production of numerous pro-inflammatory adipokines is increased, whereas the secretion of anti-inflammatory adipokines (e.g. adiponectin) is reduced. Macrophages are recruited to the adipose tissue (Weisberg et al., 2003) through the actions of cytokines, including chemokine(C-C motif) ligands (CCL; Keophiphath et al., 2010), IL-6 (Fried et al., 1998), IL-18 (Fischer et al., 2005) and TNF- α (Hotamisligil et al., 1993). T-cells promote the recruitment of macrophages (Nishimura et al., 2009), thus resulting in secretion of more pro-inflammatory adipokines, and the dysfunctional pro-inflammatory adipose tissue maintains a chronic inflammation in a feed-back loop.

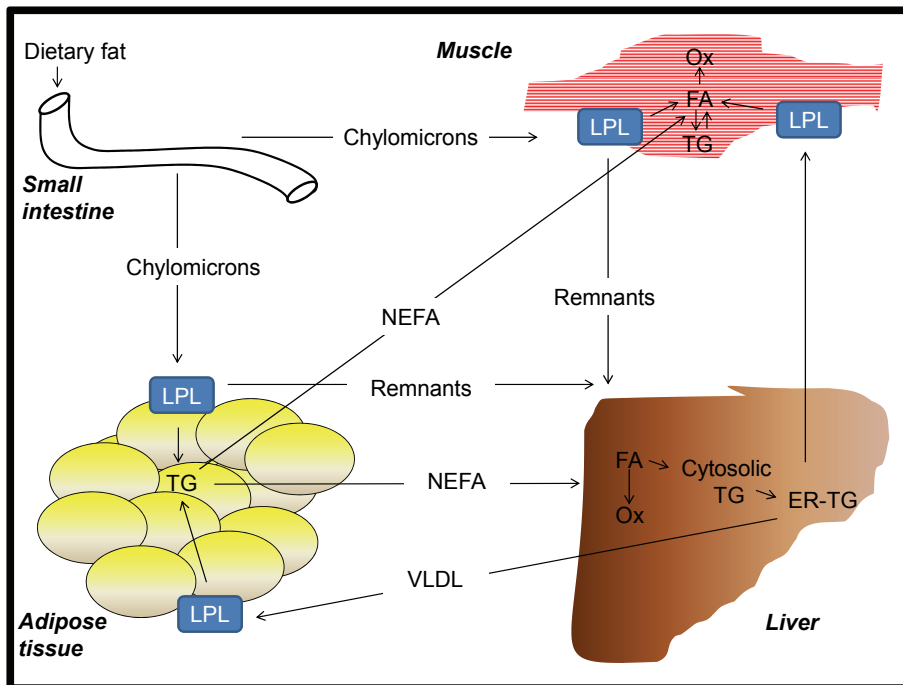


Figure 5. Exchange of lipids between gut, adipose tissue, muscle, and liver. LPL = lipoprotein lipase; TG = triglycerides; NEFA = non-esterified fatty acids; Ox = β -oxidation; FA = fatty acids; ER = endoplasmic reticulum; VLDL = very-low-density lipoprotein. Adapted from Frayn et al., 2006.

The association between obesity and mortality that is related to vascular causes is well established (Whitlock et al., 2009). However, body fat distribution measured as waist-hip-ratio has a greater impact than BMI on the risk of incident CVD (Canoy et al., 2007, Pischon et al., 2008, Yusuf et al., 2005), indicating that abdominal adipose tissue is of particular interest. Visceral and subcutaneous adipose tissue differs regarding adipokine secretion profiles in that visceral fat expresses higher levels of adipokines involved in inflammation (Fried et al., 1998; Samaras et al., 2010). In summary, the adipose tissue is a metabolic active tissue and an energy storage organ, which becomes dysfunctional in overweight and obesity. Visceral adipose tissue is more important with regard to cardiovascular risk than subcutaneous adipose tissue.

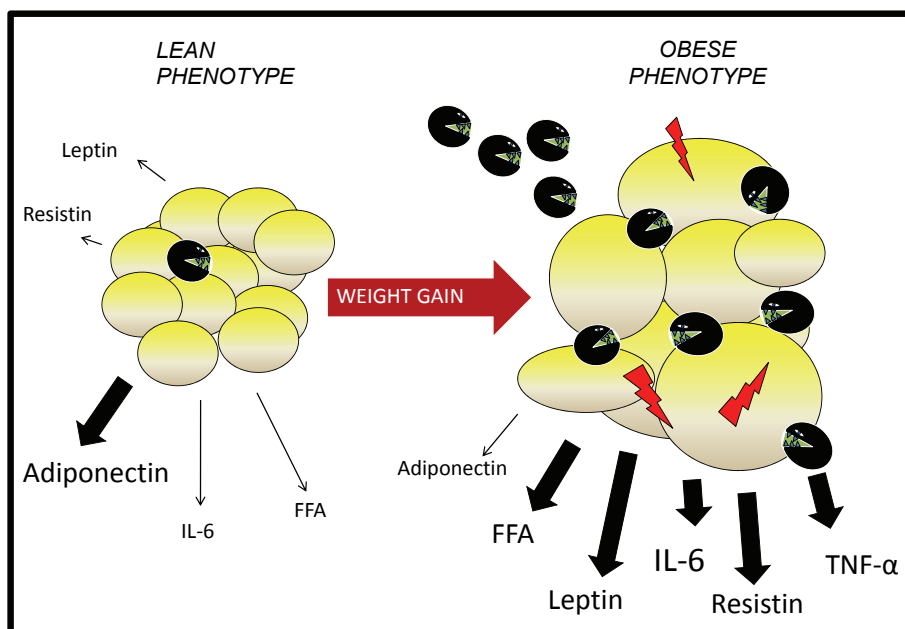


Figure 6. Adipose tissue becoming dysfunctional as the result of a positive energy balance. Inflammatory cells are recruited from the circulation. IL-6 = interleukin-6; FFA = free fatty acids; TNF- α = tumour necrosis factor α .

1.7 ADIPONECTIN - AN ADIPOKINE

Adiponectin is a 30-kD protein that is produced by adipocytes and circulates at high levels ($\approx 10\text{--}30\text{ }\mu\text{g/mL}$) in human plasma. It was discovered in 1995–1996 by four independent research groups (Hu et al., 1996; Maeda et al., 1996; Nakano et al., 1996; Scherer et al., 1995). In experimental models, adiponectin has been shown to regulate appetite and energy expenditure (Xu et al., 2003a), and to have insulin sensitizing and anti-inflammatory effects in the liver and skeletal muscle (**Figure 7**; Yamauchi et al., 2001; Xu et al., 2003a). In addition adiponectin have anti-atherogenic effects in the arterial wall. Clinical studies show that plasma adiponectin concentration is strongly associated with gender (higher in females), age (positively correlated), body mass index (inversely correlated) and T2DM (low; Hotta et al., 2000; Arita et al., 1999).

Table 1. Overview of key adipokines

Adipokine	Primary source	Levels in plasma	Binding partner/Receptor	Function	Plasma level correlation with obesity
Leptin	Adipocytes	5–50 ng/mL	Leptin receptor	Appetite control	↑
Resistin	Peripheral blood mononuclear cells	2–100 ng/mL	Unknown	Promotes insulin resistance	↑
Retinol-binding protein 4	Liver, adipocytes, macrophages	4–20 µg/mL	Retinol (vitamin A)	Implicated in systemic insulin resistance	↑
Lipocalin 2	Adipocytes, macrophages	40–100 ng/mL	Unknown	Promotes insulin resistance	↑
ANGPTL2	Adipocytes, other cells	1.0–4.0 ng/mL	Unknown	Local and vascular inflammation	↑
TNF-α	Stromal vascular cells, adipocytes	0.5–15 pg/mL	TNF receptor	Inflammation, antagonism of insulin signalling	↑
IL-6	Adipocytes, stromal vascular fraction cells, liver, muscle	1.0–3.0 pg/mL	IL-6 receptor	Inflammation, Changes with source and target tissue	↑
IL-18	Stromal vascular fraction cells	100–500 pg/mL	IL-18 receptor, IL-18-binding protein	Broad-spectrum inflammation	↑
CCL	Adipocytes, stromal vascular cells	5–70* ng/mL	CCR2	Monocyte recruitment	↑
Visfatin	Adipocytes, macrophages	10–30 ng/mL	Unknown	Monocyte chemotactic activity	↑
SFRP5	Adipocytes	3–20 ng/mL	WNT5a	Suppression of pro-inflammatory WNT signalling	→
Adiponectin	Adipocytes	3–30 µg/mL	Adiponectin receptors 1 and 2, T-cadherin	Anti-inflammatory, insulin sensitizer	↓

**CCL-5 in serum; ANGPTL2 = Angiopoietin-like protein 2; TNF = tumor necrosis factor alpha; IL = interleukin; CCL = Chemokine (C-C motif) ligand; SFRP5 = secreted frizzled-related protein 5; Adapted from Ouchi et al., 2011.*

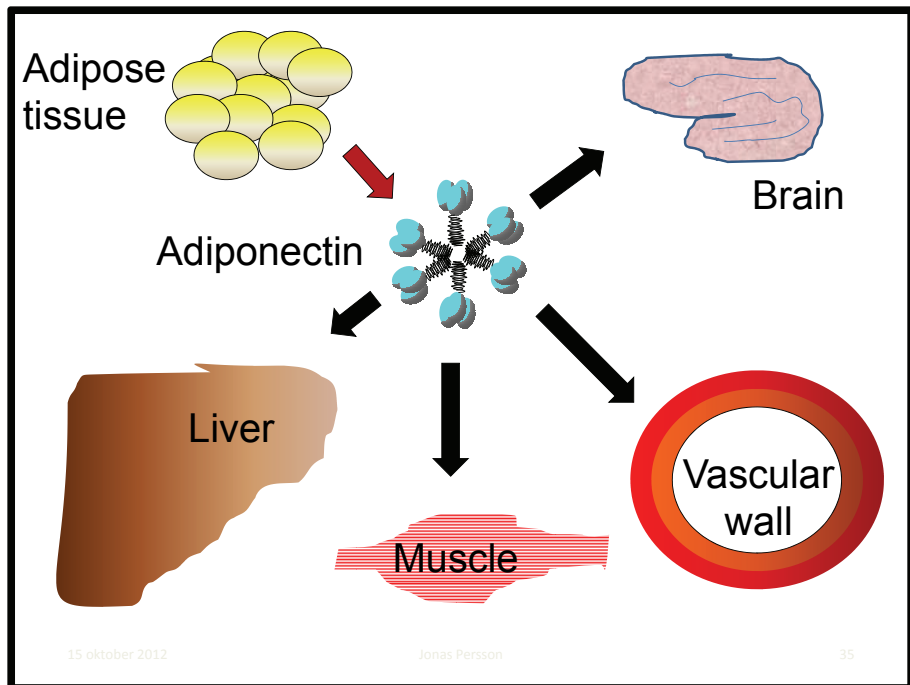


Figure 7. Adiponectin and effects on different types of tissue. Experimental studies have shown that the adipose-tissue-derived protein adiponectin has effects on the liver (decreases glucose output and fat accumulation, anti-inflammatory), skeletal muscle (increases glucose uptake, decreases fat accumulation), brain (appetite and energy expenditure), and the vascular wall (decreases inflammation, endothelial adhesion of leukocytes, and foam cell formation).

1.8 EFFECTS OF ADIPONECTIN ON ATHEROSCLEROSIS

Studies conducted at the beginning of the 21st century showed that adiponectin protected against atherosclerosis in apolipoprotein E knock-out mice (Okamoto et al., 2002; Yamauchi et al., 2003b) and in rabbits (Li et al., 2007). However, it was later observed that atherosclerosis was not accelerated in adiponectin knock-out mice crossed with apolipoprotein E knock-out or low-density lipoprotein receptor knock-out mice (Nawrocki et al., 2010).

Monocyte migration through the endothelium, conversion into macrophages, and subsequent foam cell formation are essential for atherosclerotic plaque formation (**Figure 1**), and adiponectin inhibits these processes in experimental settings. Adiponectin lowers expression of endothelial adhesion molecules (Ouedraogo et al.,

2007; Ouchi et al., 1999) and primes human monocytes to differentiate into anti-inflammatory M2 macrophages rather than pro-inflammatory M1 macrophages (Lovren et al., 2010). Adiponectin also blocks foam cell formation by suppressing scavenger A receptors (Ouchi et al., 2001) and by promoting efflux of cholesterol (Tsubakio-Yamamoto et al., 2008). The lipid accumulation in foam cells (Tian et al., 2009) and T-cell recruitment to atherosclerotic plaques (Okamoto et al., 2008) is reduced by adiponectin. Furthermore, adiponectin suppresses proliferation of smooth muscle cells (Matsuda et al., 2002; Motobayashi et al., 2009). In summary, the vast majority of the studies in the literature show that adiponectin has anti-inflammatory effects and protective effects but whether the effects are valid in humans is not known.

1.9 PLASMA ADIPONECTIN AND CARDIOVASCULAR OUTCOME

Given that the vast majority of experimental data indicate that adiponectin has anti-inflammatory and anti-atherogenic effects it seems that high plasma levels of this protein should be associated with better outcome compared to lower levels. In a case-control study conducted in 2003 in men undergoing coronary angiography, it was found that low adiponectin concentration was associated with coronary atherosclerosis (Kumada et al., 2003). Furthermore, in 2004 Pischon and co-workers showed that a low plasma adiponectin level was predictive of myocardial infarction in male subjects in a nested case-control study performed as part of the Health Professionals Follow-up Study (Pischon et al., 2004). However, in 2006 it was reported that high adiponectin was predictive of all-cause mortality and cardiovascular mortality in male patients with coronary atherosclerosis who underwent coronary angiography (Cavusoglu et al., 2006). The cited investigations imply that low levels of adiponectin are predictive of myocardial infarction in cohorts of healthy subjects, but high levels of this protein are associated with future mortality in high-risk subjects and in 2006 this was denoted as the adiponectin paradox (Teoh et al., 2006). This paradox was also apparent in a population-based study of 2 484 which showed that low plasma adiponectin was predictive of non-fatal cardiovascular events in females (Dekker et al., 2008). However, in that investigation, higher adiponectin was a significant predictor of all-cause and CVD mortality in both sexes, and this was more apparent in subjects with prevalent CVD.

Some studies have been neutral with regard to a relationship between adiponectin and cardiovascular outcome. In a nested case-control study of men it was observed that plasma adiponectin was not associated with incident coronary heart disease (Sattar et al., 2006). Also, in the British Women's Heart and Health Study plasma adiponectin was correlated with measures of insulin resistance, but it was not associated with incident coronary heart disease either before or after adjustments for risk factors (Lawlor et al., 2005).

Studies of the association of plasma adiponectin with recurrent events after an acute coronary syndrome agree with the paradox. High adiponectin was found to predict myocardial infarction or death after an acute coronary syndrome in the trial entitled *Pravastatin Or atorvastatin Evaluation and Infection Trial-Thrombolysis in Myocardial Infarction 22* (PROVE IT-TIMI 22; Wilson et al., 2011).

Isoforms of adiponectin in relation to outcome

High molecular weight (HMW) adiponectin (see page 22) has been suggested to be more biologically active than other species of adiponectin (Pajvani et al., 2004). However, the data on that subject in epidemiology is conflicting. In a nested case-control study of women included in the Nurses' Health Study, low total plasma adiponectin and low levels of HMW adiponectin were equally associated with incident CHD (Pischon et al., 2011). In another nested case-control study of older women, no correlation was found between HMW adiponectin and incident CHD (Sattar et al., 2008). In non-diabetic obese men, it was noted that the MMW/HMW adiponectin ratio, but not total adiponectin, was associated with myocardial infarction (Baessler et al., 2011).

Plasma adiponectin in relation to cerebrovascular disease

It appears that adiponectin is not or associated with stroke. This conclusion is supported by an investigation in which HMW adiponectin concentration was not correlated with incident stroke in postmenopausal women (Ogorodnikova et al., 2010), and a Swedish nested case-referent study of men showing no relationship between plasma adiponectin and stroke (Soderberg et al., 2004). Moreover, a nested-case control study of older subjects (70–82 years) indicated that low adiponectin was associated with ischaemic stroke, but this correlation was not seen after adjustment for other risk factors for stroke (Stott et al., 2009).

Genetic variants and cardiovascular disease

There are a number of candidate gene studies that have examined the association of genetic variants in *ADIPOQ* in relation to cardiovascular disease. In brief, the genetic variant rs1501299 have been associated with CHD both women and men with T2DM (Qi et al., 2006; Qi et al., 2005). Furthermore two genetic variants in the promoter, rs17300539 and rs266729, have been associated with CHD (Gable et al., 2007) and the rs266729 have also been associated with ischaemic stroke (Hegener et al., 2006). In a GWA study in a genetic variant in the loci *ARL15* associated with plasma adiponectin concentration was also found to be associated with CHD (Richards et al., 2009).

Association of genetic variants in ADIPOQ with intima-media thickness

Plasma adiponectin is inversely associated with carotid intima-media thickness (cIMT) in both adults (Saarikoski et al., 2010; Iglseder et al., 2005; Nilsson et al., 2006) and juveniles (Pilz et al., 2005) independently of established risk factors. An overview of the genetic variants that have been studied in relation to cIMT is shown in **table 2**. In the Relationship between Insulin Sensitivity and Cardiovascular disease (RISC) study, the genetic variant rs266729 in the promoter region of the *ADIPOQ* was associated with cIMT regardless of the plasma concentration of adiponectin or CVD risk factors (Patel et al., 2008). The correlation between rs266729 and cIMT was also evident in obese subjects after adjustments for age and gender in the Carotid Atherosclerosis Progression Study (CAPS; Bevan et al., 2011). Furthermore, an association between the genetic variant rs2241767 in *ADIPOQ* cIMT has been identified in a subgroup of African Americans in the Multi-Ethnic Study of Atherosclerosis (MESA;Wassel et al., 2011). In the Salzburg Atherosclerosis Prevention program in subjects at High Individual Risk study (SAPHIR), the two genetic variants rs2241766 and rs1501299 in the *ADIPOQ* locus were analysed and found to be correlated with plasma adiponectin levels but not with cIMT (Mackevics et al., 2006).

Table 2. Studies of genetic variants of ADIPOQ and carotid intima-media thickness

Study	SNP	Association with plasma adiponectin	Association with CCA-IMT	Association with ICA-IMT
CAPS (Bevan et al., 2011) n=990	rs266729	yes	yes	n/a
	rs17300539	yes	no	n/a
	rs2241766	no	no	n/a
	rs1501299	yes	no	n/a
	rs822395	no	no	n/a
RISC (Patel et al., 2008) n=1,306	rs266729	yes	yes	n/a
	rs17300539	yes	no	n/a
	rs16861194	yes	no	n/a
	rs2241766	no	no	n/a
SAPHIR (Mackevics et al., 2006) n=1,745	rs2241766	yes	no	no
	rs1501299	yes	no	no
MESA (Wassel et al., 2011) n= 2,847	rs11711353	n/a	no	n/a
	rs822396	n/a	no	n/a
	rs12495941	n/a	no	n/a
	rs7649121	n/a	no	n/a
	rs9877202	n/a	no	n/a
	rs9882205	n/a	no	n/a
	rs2241767	n/a	no*	n/a
	rs1063537	n/a	no	n/a
	rs1063538	n/a	no	n/a
	rs1063539	n/a	no	n/a
	rs1403697	n/a	no	n/a

*Associated with IMT in a subgroup of African Americans; CCA = common carotid artery; ICA = internal carotid artery; IMT = intima-media thickness; CAPS = the Carotid Atherosclerosis Progression Study; RISC = the Relationship between Insulin Sensitivity and Cardiovascular disease study ; SAPHIR = Salzburg Atherosclerosis Prevention program in subjects at High Individual Risk study; MESA = the Multi-Ethnic Study of Atherosclerosis; n/a = not available.

1.10 ADIPONECTIN RECEPTORS AND DOWNSTREAM SIGNALLING

Two transmembrane receptors, AdipoR1 and AdipoR2 have been identified (Yamauchi et al., 2003a), which are crucial for adiponectin signalling (Yamauchi et al., 2007). Also, a cell-surface glycoprotein, T-cadherin, which binds adiponectin have been identified and is important for the cardioprotective effects of adiponectin (Denzel et al., 2010).

There are several ways in which the two adiponectin receptors AdipoR1 and AdipoR2 achieve downstream signalling (**Figure 8**). The adaptor protein containing pleckstrin homology domain, phosphotyrosine binding (PTP) domain and leucine zipper motif (APPL1), interacts directly with both AdipoR1 and AdipoR2 (Mao et al., 2006), and this also involves LKB1 leading to the activation of AMP-activated protein kinase

(AMPK; Zhou et al., 2009), PPAR- α and p38 MAP kinase (Kadowaki and Yamauchi, 2005). AMPK activates eNOS in endothelial cells which leads to nitric-oxide production (Chen et al., 2003). Furthermore, AdipoR1 and AdipoR2 inhibit inflammation through suppressing NF- κ B (Xu et al., 2011). Recently, it was shown that adiponectin inhibits apoptosis through attenuation of the ceramide content in cells (Holland et al., 2011).

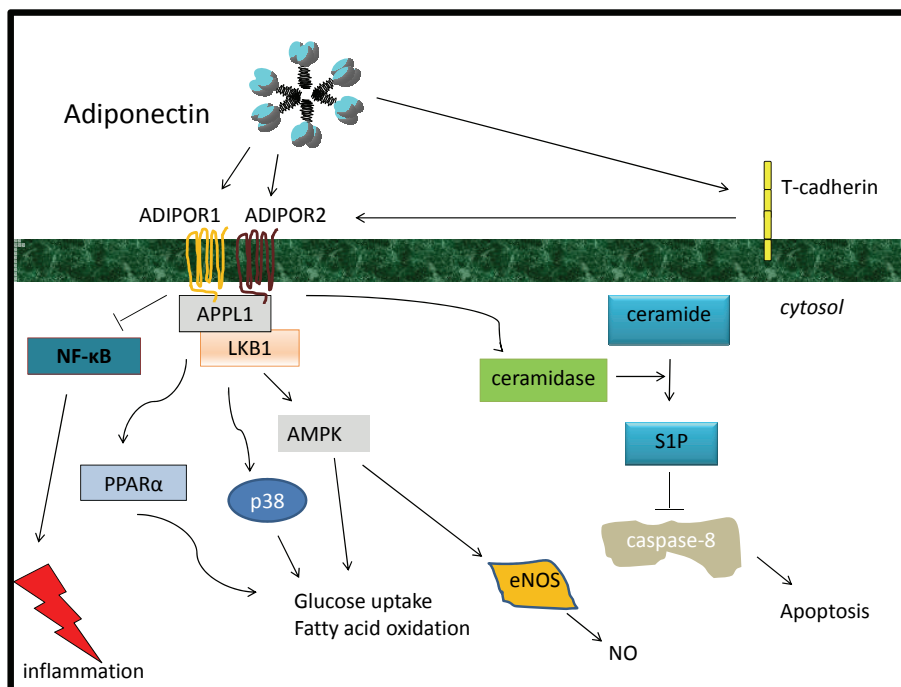


Figure 8. Schematic diagram of downstream pathways of adiponectin receptors. AdipoR1 = adiponectin receptor 1; AdipoR2 = adiponectin receptor 2; APPL1 = Adaptor protein containing pleckstrin homology domain, phosphotyrosine-binding (PTP) domain and leucine zipper motif; NF- κ B = nuclear factor-kappa-light-chain-enhancer of activated B cells; PPAR- α = peroxisome proliferator-activated receptor- α ; AMPK = AMP-activated protein kinase; eNOS = endothelial nitric oxide synthases; NO = nitric oxide; S1P=sphingosine 1-phosphate. Adapted from (Hui et al., 2012).

1.11 ADIPONECTIN REGULATION

There is a large heritability in plasma adiponectin concentrations 35-60% (Comuzzie et al., 2001; Miljkovic-Gacic et al., 2007; Heid et al., 2010) and the parameters age, sex and BMI accounts for approximately 25% of the variability (Miljkovic-Gacic et al., 2007). The gene coding for adiponectin, *ADIPOQ*, is located on chromosome 3q27 and *ADIPOQ* gene span is 16 kb and includes three exons. The promoter of the adiponectin gene *ADIPOQ* (locus 3q27) contains multiple transcriptional binding sites (**Figure 9**),

which suggests that adiponectin is regulated by many upstream signals. This assumption is supported by the findings of clinical studies showing that plasma adiponectin concentration is strongly associated with gender (higher in females), age (positively correlated), body mass index (inversely correlated), T2DM (low), type 1 diabetes mellitus (T1DM; high), triglycerides (inversely correlated), and HDLc (positively correlated). Moreover, there are various interlinked determinants of the production of adiponectin that are related to obesity, T2DM, and sex hormones.

1.11.1 Obesity, T2DM and inflammation

Increased fat mass results in a hypoxic environment (Ozcan et al., 2004), which in turn suppresses adiponectin expression via hypoxia-induced factor-1 α (Zappala and Rechler, 2009). Obesity is also associated with a low-grade chronic inflammation that increases the production of IL-6, TNF- α , and IL-18 (Fischer et al., 2005), all of which restrict adiponectin gene expression. TNF- α inhibits transcription of adiponectin through various pathways, including suppression of peroxisome proliferator-activated receptor (PPAR)- γ (Zhang et al., 1996), CCAAT/enhancer binding protein (C/EBP; Kita et al., 2005; Ron et al., 1992), and sterol-responsive element-binding protein (SREBP; Sewter et al., 2002). IL-6 has also been reported to inhibit adiponectin (Fasshauer et al., 2003). IL-18 represses adiponectin expression via a protein called nuclear factor of activated T-cells (NFAT; **Figure 9**; Chandrasekar et al., 2008).

Experimental studies have shown that insulin stimulates expression of the adiponectin gene in adipocytes (Blumer et al., 2008; Pereira and Draznin, 2005), whereas deletion of insulin receptors in those cells leads to increased plasma levels of adiponectin (Bluher et al., 2002). Clinical investigations have revealed a negative correlation between levels of insulin and adiponectin (Mohlig et al., 2002). Furthermore, the plasma concentration of adiponectin is elevated in individuals with defective insulin receptors (Semple et al., 2006). Thus, the regulating effects of insulin on adiponectin production are unclear.

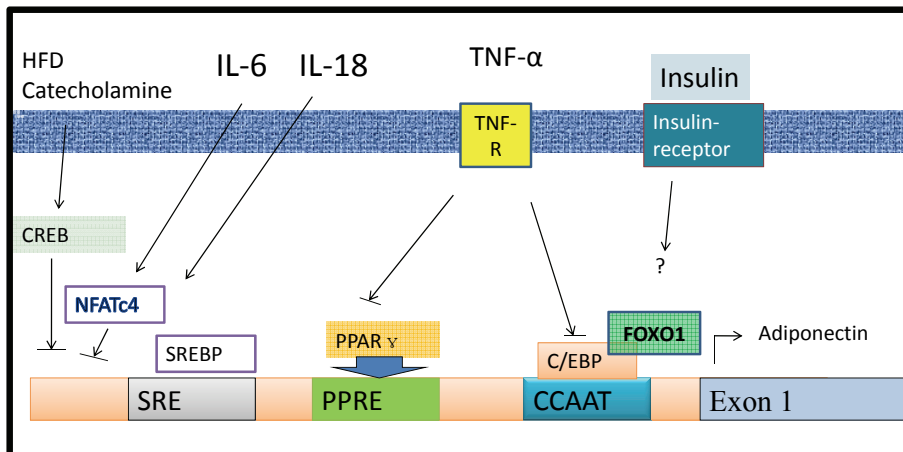


Figure 9. Schematic diagram of the ADIPOQ promoter and some transcription factors and upstream regulators involved in transcription of adiponectin. The promoter contains E-boxes (Rahmouni and Sigmund, 2008), PPAR- γ -responsive element (PPRE; Iwaki et al., 2003), a CCAAT-box (Schaffler et al., 1998), C/EBP α enhancers (Park et al., 2004), and a sterol regulatory element (SRE; Kita et al., 2005; Doran et al., 2008). Mediators of inflammation, tumour necrosis factor (TNF)- α , interleukin(IL)-6, and IL-18 β -adrenergic stimulation (Fasshauer et al., 2001) inhibit adiponectin expression via different pathways as depicted. The effect of insulin on adiponectin transcription is not clear. cAMP-responsive element-binding protein (CREB) contributes to obesity-induced down-regulation of adiponectin expression (Qi et al., 2009). Nuclear factor of activated T-cells (NFAT) suppresses adiponectin expression (Kim et al., 2006). Deletion of the PPAR- γ gene decreases plasma levels of adiponectin in mice (He et al., 2003). There are FoxO1-responsive elements in the adiponectin promoter (Qiao and Shao, 2006). Expression of mRNA in adipocytes is increased by over expression and decreased by blocking of C/EBP α (Qiao et al., 2005). Sterol-regulatory element-binding protein (SREBP) stimulates transcription of adiponectin (Seo et al., 2004). HFD = high fat diet; TNF-R = TNF receptor; Adapted from Liu et al., 2009.

1.11.2 Testosterone lowers plasma adiponectin

Plasma levels of adiponectin are 1.6 times higher in females than in males. Testosterone has been reported to reduce the plasma concentration of this protein in transsexual female patients (Berra et al., 2006), healthy men (Page et al., 2005), hypogonadal men (Lanfranco et al., 2004), and hypogonadal men with diabetes (Kapoor et al., 2007). Also, in laboratory mice, it was observed that castration led to a lower concentration of HMW adiponectin, whereas the level of total adiponectin remained unchanged (Xu et al., 2005).

1.11.3 Genetic variants

The gene is organized in two linkage disequilibrium (LD) blocks (Heid et al., 2006). In earlier candidate gene studies, the genetic variant rs17300539 in the promoter and the first LD-block have been associated with plasma adiponectin levels (Menzaghi et al., 2007). Another genetic variant, rs266729, in the promoter have been associated with plasma adiponectin levels in some (Hoefle et al., 2007; Warodomwicht et al., 2009), but not all studies (Hivert et al., 2008). In the second LD block, the variant rs1501299 have been found to be associated with plasma adiponectin concentration (Menzaghi et al., 2007). Genetic variants in other loci, *CDH13* and *ARL15*, have been associated with plasma adiponectin concentration in genome wide association (GWA) studies (Chung et al., 2011; Ling et al., 2009; Richards et al., 2009). In one population based GWA study from 2010, one genetic variant rs17366568 in the *ADIPOQ* locus explained 3.8% of the plasma adiponectin variance (Heid et al., 2010). Thirty-three SNPs in the same locus explained 6.7% of the variance and the authors state that *ADIPOQ* is the only major gene for plasma adiponectin. However, a large GWA study (n=45,891) that was published in 2012 revealed 8 new novel independent loci associated with plasma adiponectin levels and also confirmed *ADIPOQ* and *CDH13* to be associated with plasma adiponectin (Dastani et al., 2012).

1.11.4 Interventions in humans

As expected from experimental studies, thiazolidinediones (TZDs) have been found to raise plasma adiponectin concentrations in clinical trials (Riera-Guardia and Rothenbacher, 2008; Ogasawara et al., 2009). Glimepiride increases levels of both adiponectin and HDLc in subjects with T2DM (Araki et al., 2009). Weight loss also increases plasma adiponectin (Kopp et al., 2005; Behre et al., 2007; Simonyte et al., 2010) but, notably, does not enhance the production of this protein in adipose tissue (Behre et al., 2007; Simonyte et al., 2010), indicating that post-translational modifications have an important impact on plasma adiponectin. Angiotensin-converting enzyme inhibitors (Hermann et al., 2006; Krysiak et al., 2010) and angiotensin II receptor antagonists (Makita et al., 2008; Mori et al., 2007) increase the plasma concentration of adiponectin. Fibrates have been reported to increase plasma adiponectin levels in human subjects with hypertriglyceridaemia (Koh et al., 2005). Furthermore, polyunsaturated fats augment adiponectin secretion in obese mice and

obese humans (Itoh et al., 2007), and it seems that alcohol amplifies expression of this protein in adipose tissue in women (Joosten et al., 2008).

1.11.5 The molecule and post-translational modifications

Adiponectin is synthesized as a 244 amino-acid polypeptide which assembles and circulates in plasma in three different isoforms: a low molecular weight (LMW) trimer, a middle molecular weight (MMW) hexamer, and a HMW multimer (composed of 12–36 molecules; **Figure 10**; Holland and Summers, 2008). The formation of HMW adiponectin in cells is regulated by post-translational modifications (Liu et al., 2008; Wang et al., 2008), and intermolecular disulfide bonds are crucial for this multimerization (Waki et al., 2003). HMW adiponectin is suggested to be the most bioactive of the three isoforms with respect to insulin sensitivity (Pajvani et al., 2004). Once synthesized and secreted, the various complexes are quite stable in the circulation and do not inter-convert *in vivo* (Schraw et al., 2008).

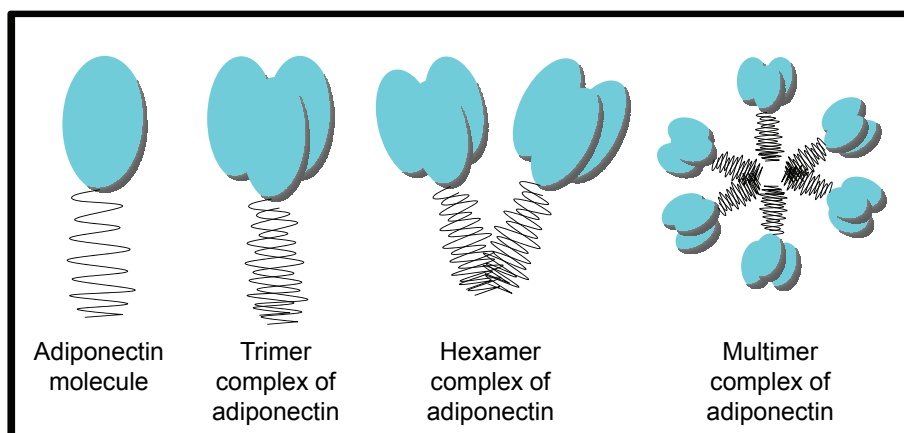


Figure 10. Isoforms of plasma adiponectin.

2 HYPOTHESIS AND AIMS

The main hypothesis of the present research was that adiponectin exerts protective effects against atherosclerosis in the arterial walls in humans. The overall objective was to study adiponectin in relation to atherosclerosis, myocardial infarction, ischaemic stroke, and mortality. The specific aims were as follows:

1. To investigate the association of plasma levels of adiponectin with cardiovascular events and mortality in cohorts that differed with regard to cardiovascular risk.
2. To analyse the relationship between plasma adiponectin concentration and early atherosclerosis and progression of atherosclerosis.
3. To study genetic variants in the adiponectin pathway and association with plasma adiponectin and early atherosclerosis.
4. To evaluate whether gene expression profiling of carotid artery plaques and PBMCs can predict incident ischaemic events in a high-risk population.

3 METHODS

3.1 STUDY SUBJECTS

The studies in paper I, II, III, and IV differed with respect to design, prevalence of CVD and cardiovascular risk factors in the subjects, as schematically presented in **Figure 11**.

The Stockholm Coronary Atherosclerosis Risk Factor Study (SCARF), Papers I and III

From 1996 to 2001, 387 survivors of first-time myocardial infarction below the age of 60 years were recruited in Sweden at Danderyd University Hospital, Karolinska University Hospital, and Norrtälje Hospital. Age- and gender-matched controls (n = 387) were also recruited. Plasma samples were obtained and risk factor assessment was performed three months after enrolment. Statin therapy was not mandatory at the beginning of the recruitment period, whereas the patients enrolled later during the

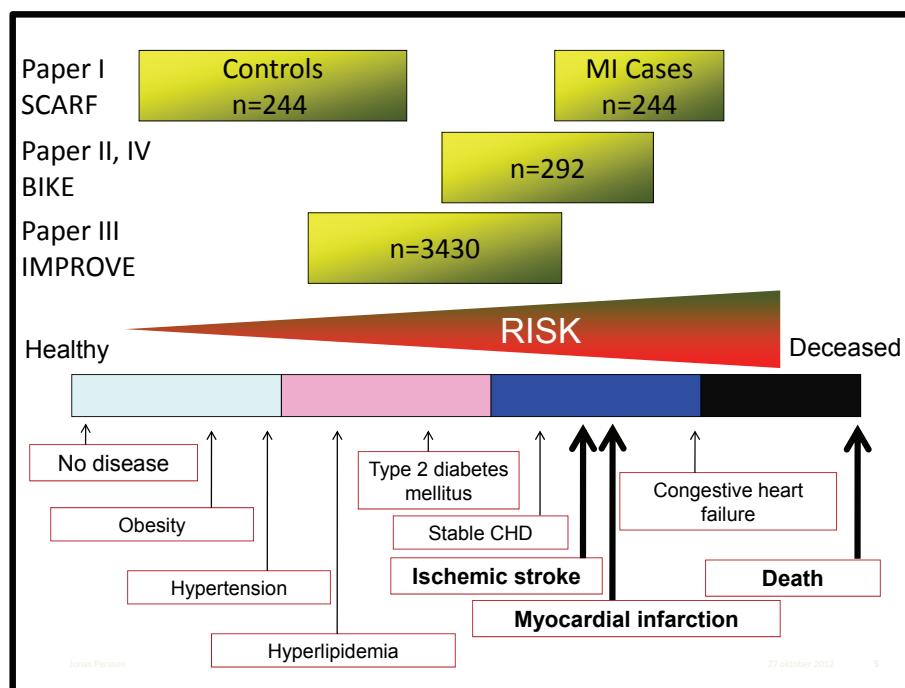


Figure 11. Schematic diagram of the risk of cardiovascular morbidity and mortality in Paper I-IV. The studies differed with respect to design, rate of risk factors, and prevalence of cardiovascular disease; SCARF = Stockholm Coronary Atherosclerosis Risk Factor Study; IMPROVE = Carotid Intima Media Thickness (IMT) and IMT Progression as Predictors of Vascular Events in a High Risk European Population; BiKE = Biobank of Karolinska Carotid Endarterectomies; MI = myocardial infarction.

period were given statins at the index event, and thus 32% of the study subjects received such treatment.

In the investigation reported in paper I, patients were excluded if they lacked a valid control, missed data on biomarkers (e.g. CRP) or if CRP was above the 10th percentile (**Figure 12**). Thus 244 cases and 244 age- and gender-matched controls remained for the analysis. All subjects gave informed consent, and the study was approved by the local ethics committee.

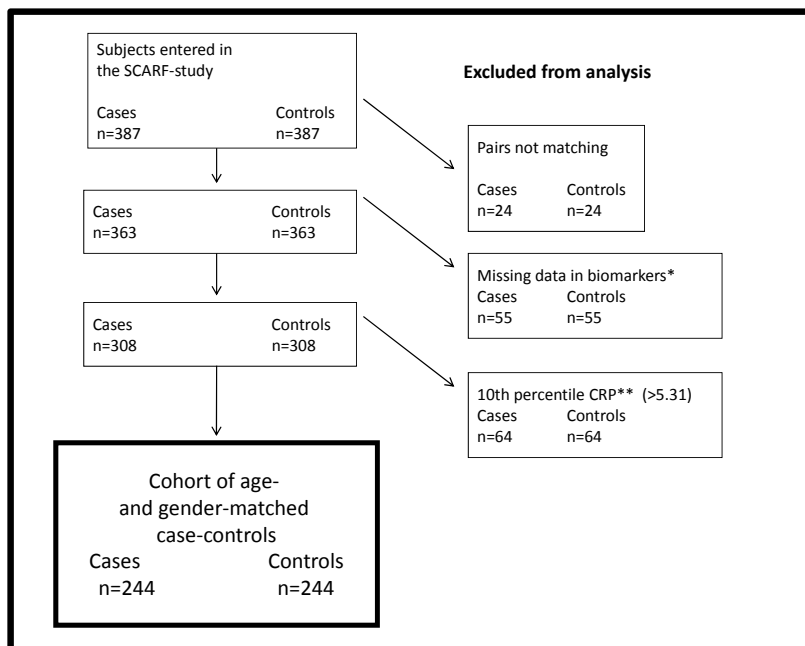


Figure 12. Selection of participants analysed in SCARF. *Missing in case or corresponding control; **10th percentile amongst 363 cases and 363 controls, in case and / or corresponding control; SCARF = Stockholm Coronary Atherosclerosis Risk Factor Study.

The Biobank of Karolinska carotid Endarterectomies (BiKE), Papers II and IV

Since 2001, patients undergoing carotid endarterectomy (CEA; **Figure 13**; Eastcott et al., 1954) at Karolinska University Hospital have been consecutively included in BiKE, and enrolment is still in progress. As of today (November 2012), the BiKE database covers 620 patients with samples of atherosclerotic plaque tissue, plasma, and PBMCs, and information on clinical characteristics. Either of the following inclusion criteria is to be fulfilled: (i) symptomatic stenosis according to the criteria in the North American Symptomatic Carotid Endarterectomy Trial (NASCET; Barnett et al., 1998);

(ii) asymptomatic stenosis with 70% diameter of the common carotid artery or the bifurcation of the carotid artery, as assessed by ultrasound measurement. The study reported in paper I included 292 subjects who had plasma samples deposited in the BiKE database from 2002 to 2007 and complete follow-up until 31 December 2010.

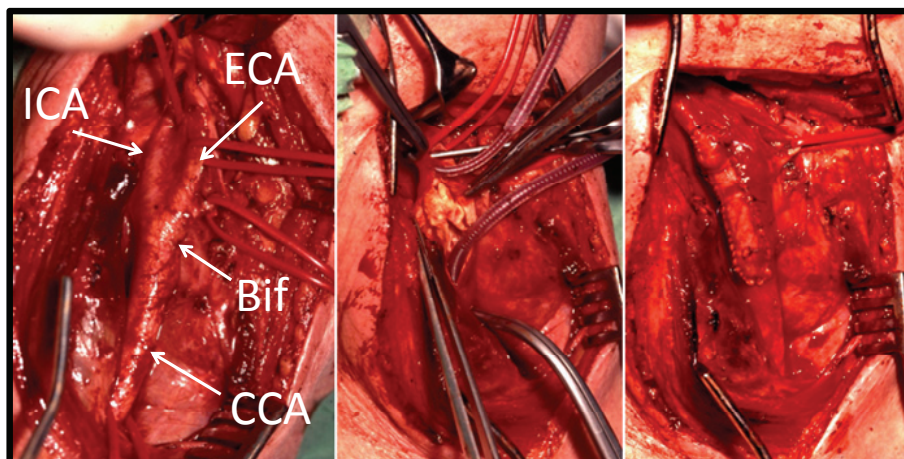


Figure 13. Carotid endarterectomy. The carotid artery is exposed (left), after which the plaque obstructing the bifurcation (Bif) is removed (middle), and the arteriotomy is closed with a patch (right). ICA = internal carotid artery; ECA = external carotid artery; CCA = common carotid artery; Photo: Ulf Hedin.

The study described in paper IV included 127 subjects with available microarray data from carotid plaque tissues and 98 subjects with microarray data from PBMCs (97 overlapping with the former group).

The subjects included in the BiKE have a high cardiovascular risk: they all have documented atherosclerosis; 75% have had a cerebrovascular event before inclusion; 60% are current or former smokers; the mean age is 70 years. However, there is an important selection bias that should be taken into account, namely, that in the opinion of a surgeon the patients in this cohort would benefit from a CEA, a procedure that is associated with risks of peri- and postoperative complications.

The Carotid Intima-Media Thickness (IMT) and IMT Progression as Predictors of Vascular Events in a High-Risk European Population (IMPROVE) study, Paper III.

The IMPROVE study is a multicenter, longitudinal observational investigation involving seven recruitment centres in five European countries: Finland, France, Italy, the

Netherlands, and Sweden. The primary objective is to evaluate progression of cIMT 15 months after inclusion in relation to future cardiovascular events. Among other things, the secondary objectives are to study the relationships between genetic variants in candidate genes that play a role in atherosclerosis, and to assess the effect of the interactions between gene polymorphisms and vascular risk factors (VRFs).

From March 2004 to April 2005, 21,000 patients were screened, and 3,711 of those individuals were enrolled in the IMPROVE study (1,050 in Finland, 501 in France, 1,095 in Italy, 532 in the Netherlands, and 533 in Sweden). Adults between the ages of 55 and 79 years were eligible for inclusion if they had three or more of following risk factors: male sex or female at least 5 years since menopause; LDL > 4.1 mmol/L or treatment with lipid-lowering drugs; triglyceride level > 2.2 mmol/L after diet or treatment with triglyceride-lowering drugs; HDLc < 1.0 mmol/L; diastolic blood pressure (DBP) > 90 mmHg and/or systolic blood pressure (SBP) >140 mmHg or treatment with anti-hypertensive drugs; blood glucose level > 6.1 mmol/L or treatment with insulin or oral hypoglycaemic drugs; smoking 10 cigarettes per day for at least 30 months; family history of CVD. The exclusion criteria were stipulated as any of the following: abnormal anatomical configuration of neck and muscles; marked tortuosity and/or depth of the carotid vessels, and/or uncommon location of arterial branches; history of myocardial infarction, angina pectoris, stroke, TIA, aortic aneurysm, or intermittent claudication; revascularization of carotid, coronary, or peripheral arteries; congestive heart failure (NYHA Class III–IV); history of serious medical conditions that might limit life span.

In the present study (Paper III) persons without detectable plasma adiponectin or with ambiguous sex, cryptic relatedness (Voight and Pritchard, 2005) or non-European descent, or a genotyping call rate < 95% were excluded, which left 3 430 subjects for analysis.

3.2 REPLICATION COHORTS

We utilized five different cohort for replications of associations between genetic variants and phenotype in paper III.

The Malmö Diet and Cancer study - Cardiovascular cohort (MDC-CV)

The Malmö Diet and Cancer study is a large population-based prospective investigation including 30,446 subjects enrolled in 1991–1996. From 1991 to 1994, members of the MDC cohort were also asked to participate in a sub-study of epidemiology of carotid artery disease, which was designated the MDC Cardiovascular cohort (MDC-CV). In all, 6,103 subjects were included in the cardiovascular arm, and IMT measurement data on the right carotid artery were obtained. Of those individuals, 1 690 with valid IMT measurements and genetic variants (below) determined were chosen to replicate associations between genetic variants and Bif-IMT.

SCARF

A total of 738 subjects from the SCARF project were included for replication of associations between genetic variants and plasma adiponectin (above).

Whitehall II

The Whitehall II study (Marmot and Brunner, 2005) included 10,308 civil servants in 20 major government departments in London 1985 until 1988. Subjects with valid plasma adiponectin measurements and analysed genetic variants (below; n= 1,929 subjects) were selected for replication of association between genetic variants and plasma adiponectin.

The Genetics of Diabetes Audit and Research Tayside study (GoDARTs)

The GoDARTs is an ongoing investigation of holding data on patients with T2DM (n=9,439) and controls (n = 8,187) in November 2012 (<http://diabetesgenetics.dundee.ac.uk>). A total of 2,941 GoDARTs subjects with valid plasma adiponectin measurements and analysed genetic variants (below) were included for replication of associations of genetic variants with plasma adiponectin.

The Prospective Investigation of the Vasculature in Uppsala Seniors (PIVUS) study

The PIVUS study was initiated in 2001 to investigate the usefulness of different measures of endothelial function for evaluating vascular function. In all, 1,016 70-year old subjects took part in the study and 968 subjects with valid plasma adiponectin

measurements and genetic variants analysed (below) were included for replication of associations of genetic variants with plasma adiponectin.

3.3 MEASUREMENTS OF ADIPONECTIN IN PLASMA

Paper I

Plasma adiponectin concentration was determined using a commercially available radioimmunoassay kit (Millipore Corporation, Linco Research, Inc, St. Charles, MO, USA.; #HADP-61HK), which was semi-automated and standardized in the laboratory. A lyophilized adiponectin standard (200 µg/L) stock solution was used to prepare dilutions. The analytical sensitivity of the method was estimated to typically be about 0.78 µg/L (equal to the lowest standard). The intra-and inter-assay variations were determined to be approximately 1.8–6.2% and 6.9–9.3%, respectively, at plasma sample concentrations of 3, 6, and 15 µg/L (1:500 dilution).

Paper II

In this study, plasma adiponectin was measured using an ELISA kit (Catalogue no. E09, Human Adiponectin ELISA, Mediagnost, Reutlingen, Germany) with intra-assay variation of <10.7%. Inter-assay variation was 8.3 % at 5 mg/L and 9.7 % at 10 mg/L.

Paper III

A double antibody radioimmunoassay (Millipore, Billerica, MA, USA) was used to analyse the adiponectin concentration in plasma samples that had been stored at –80 °C. The total coefficient of variation was 15.2% at low levels (2–4 µg/mL) and 8.8% at high levels (26–54 µg/mL).

3.4 GENOTYPING

Paper I

In the work reported in paper I, genotypes were determined using a fluorescence-based allelic discrimination method (Applied Biosystems). DNA was prepared from peripheral blood cells by use of a genomic DNA isolation kit (Qiagen Inc., Valencia, CA, USA), and the probes and primers for rs1501299 and rs266729 were obtained using Assay-by-Design (Applied Biosystems). The primers were tested by PCR on DNA prepared from PBMCs. PCR products were analysed on an agarose gel to verify unclonal primer sequences. Genotyping was carried out on an ABI 7000 instrument (Applied Biosystems, Foster City, CA, USA).

rs266729 probe Allele 1: VIC-CTC AGA TCC TGC CCT TC-MGBNFQ

rs266729 Probe Allele 2: 6-FAM TCA GAT CCT GCG CTT C-MGBNFQ

rs266729 forward: CAT CAG AAT GTG TGG CTT GCA
rs266729 reverse: GGC ACG CTC ATG TTT TGT TTT
rs1501299 Probe Allele 1: VIC-TG AAT GCC TTC ATA TAG T-MGBNFQ
rs1501299 Probe Allele 2: 6FAM-ATG AAT GAC TTC ATA TAG TT-MGBNFQ
rs1501299 Primer forward: TTT CAT CAC AGA CCT CCT ACA CTG A
rs1501299 Primer reverse: TCT CCC TGT GTC TAG GCC TTA GTT A

Paper III

SNPs associated with adiponectin levels in *ADIPOQ*, *CDH13*, and *ARL15* (Richards et al., 2009; Heid et al., 2010; Chung et al., 2011), or in candidate genes, *ADIPOR1* and *ADIPOR2* (Yamauchi et al., 2007; Yamauchi et al., 2003a), were determined in the analysis ($n = 1,214$). The Illumina iSelect CardioMetabo200K array (containing loci associated with cardiovascular and metabolic traits; Morris et al., 2012) included 1,172 SNPs that were supplemented with 42 SNPs genotyped by Illumina GoldenGate technology. All genotyping was performed at the SNP Technology Platform, Uppsala University, Sweden. SNPs were excluded for failing Hardy-Wienberg equilibrium ($p < 1 \times 10^{-6}$) or having a low call rate ($< 95\%$). In all, 884 SNPs (*ADIPOQ* [$n = 360$], *ADIPOR1* [$n = 59$], *ADIPOR2* [$n = 67$], *ARL15* [$n = 238$], and *CDH13* [$n = 160$]) were analysed. The number of independent signals was determined in PLINK software (Purcell et al., 2007) to be 488.

Paper IV

DNA samples from patients were genotyped using Illumina Human 610WQuad BeadArray at the SNP Technology Platform at Uppsala University. The GenomeStudio™ software from Illumina was used for genotype calling and quality control, and the MACH 1.0 algorithm (Center for Statistical Genetics, University of Michigan, Ann Arbor, MI, USA) was employed for imputation on the basis of references from the 1000 Genomes Project (1000 Genomes Project Consortium, 2010). The average call rate per SNP was 99.84%. Replicate genotyping of 12 samples demonstrated an overall concordance of 99.99%. Twenty-nine SNPs associated with early onset myocardial infarction (Coronary Artery Disease Genetics Consortium, 2011; Schunkert et al., 2011; Kathiresan et al., 2009) were used to create a genotype score. Four SNPs were omitted for failing Hardy-Weinberg disequilibrium ($p < 0.01$), a frequency mismatch (> 0.2 compared to reported frequencies), or unsatisfactory imputation quality. Genotype score was calculated as the sum of all risk alleles for each subject.

3.5 TRANSCRIPTS

In paper IV, total RNA was isolated using the RNeasy Mini Kit (Qiagen) and treatment with the RNase-free DNase set (Qiagen) according to the manufacturer's instructions. RNA quality was determined on an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), and RNA concentration was measured on a NanoDrop spectrophotometer (Thermo Scientific). Low-concentration and low-quality samples were omitted. RNA samples were hybridized to Affymetrix HG-U133 plus 2.0 oligonucleotide arrays and scanned at the Karolinska Institute Bioinformatics and Expression Analysis core facility. The resulting CEL files were pre-processed by robust multi-array average (RMA) normalization (Irizarry et al., 2003), as implemented in the Affymetrix Power Tools 1.10.2 package apt-probeset-summarize. As part of the RMA normalization, all expression measurements were \log_2 transformed. Low-expression probe sets with average expression levels less than the genome-wide median value were omitted from the analysis.

3.6 INTIMA-MEDIA THICKNESS MEASURES

In an autopsy study conducted in 1986, the B-mode ultrasound imaging of the distance between lumen–intima and media–adventitia interfaces reflected the intima-media complex, and hence this distance is referred to as IMT (Pignoli et al., 1986). It is believed that IMT is related to atherosclerosis, because it is increased in arteries prone to atherosclerosis but not in arteries that seem to be resistant to such disease (Nakashima et al., 2002).

At each of the participating centres in paper III, ultrasound measurements of the carotid artery were obtained by certified sonographers using a Techos system (Esaote, Genoa, Italy) with a 5–10 Mhz linear array probe, and these data were stored on VHS tapes at enrolment, at 15 months, and at 30 months. The ultrasound systems were calibrated with a phantom before the start of the study and after one year. IMT measurements were obtained by readers at the co-ordinating centre (the Department of Pharmacological Sciences, University of Milan, Italy) using a dedicated software (M'Ath, Metris SRL France; Beux et al., 2001) that allows semi-automatic edge detection of the echogenic lines of the intima-media complex. The far walls of the left and right common carotid artery (CCA), the first centimetre of the CCA, the bifurcation of the carotid artery (Bif), and the internal carotid artery (ICA) were visualized in anterior, lateral, and posterior projections (**Figure 14 A**). The same sonographer

followed 62.2% of the participants throughout the study. All scans for each patient were analysed by a single reader without knowledge of the subjects' identity. Mean and maximum values for the CCA and the Bif were analysed in the study reported in paper III (**Figure 14 B**), but the IMPROVE database also contains mean and maximum values for the ICA, and the first centimetre of the CCA.

Intra-observer variability was measured in 125 subjects, and, at baseline, the absolute intra-observer differences (mean \pm SD) between duplicate scans were 0.031 ± 0.030 and 0.089 ± 0.161 , for CCA-IMT_{mean}, Bif-IMT_{mean}, respectively. Inter-observer variability was measured in 32 subjects, and the inter-observer differences between duplicate scans of the same carotid segments were 0.045 ± 0.041 and 0.101 ± 0.081 , respectively.

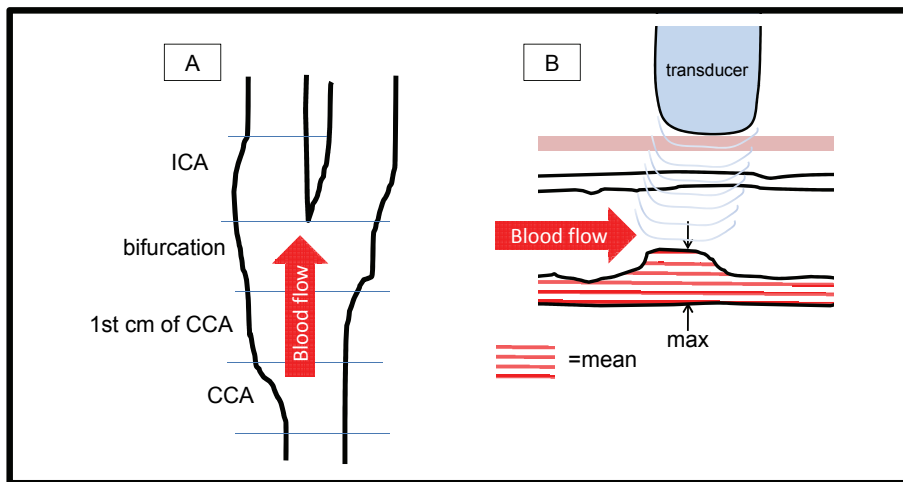


Figure 14. Schematic diagram of measurements of carotid intima-media-thickness. (A) Measurements are obtained from four segments of the carotid artery: the common carotid artery (CCA), the first centimetre of CCA, the bifurcation, and the internal carotid artery (ICA). The proximal and distal ends of the carotid artery are shown at the bottom and top, respectively. (B) Measurement of far-wall IMT in one segment. The proximal and distal ends of the carotid artery are shown to the left and right, respectively.

Also considering the same carotid segments, the intra-sonographer intra-class correlation coefficients for duplicate scans were 0.95 and 0.92, and the inter-sonographer intra-class correlation coefficients for duplicate scans were 0.89 and 0.95.

3.7 MERGING REGISTERS AND BIKE

The BiKE database was merged with the Swedish Cause of Death Register and the Swedish Hospital Discharge Register to acquire follow-up data for papers II and IV. Myocardial infarction was defined according to the International Classification of Diseases (ICD)-10 codes I21–I23, I24.8, and I24.9, as reported to the two registers mentioned above until 31 December 2010. The sensitivity and the positive predictive value for myocardial infarction have previously been estimated to 94% and 83%, respectively (Hammar et al., 2001). Ischaemic stroke was defined as stipulated in ICD-10 code I63. It has been estimated that 95% of stroke cases are covered in the Swedish Hospital Discharge Register (The National Board of Health and Welfare, 2009) and that the sensitivity and the positive predictive value for stroke in the same register is between 84-98% and 68.5-98.6%, respectively (Ludvigsson et al., 2011). Medical records of deceased subjects were obtained from hospitals and general practitioners for review to determine cause of death.

3.8 STATISTICS

Computer software

The software SPSS Statistics versions 15.0–19.0 (IBM, Armonk, NY, USA) were used in three of the present studies (Papers I, II, and III), and Statistical analysis system 9.2 (SAS Institute Inc., Cary, NC, USA) was also employed in paper I. Furthermore, PLINK (Purcell et al., 2007) was used in analyses comparing genetic variants and phenotypes, and METAL (Willer et al.) was used for meta-analyses (Paper III). In paper IV, the *coxph* function from the survival R/Bioconductor (Gentleman et al., 2004; 2012) package was employed using default settings.

Conditional logistic regression (Paper I)

Logistic regression provides a flexible means of analysing associations between a binary outcome and a number of continuous and categorical exposure variables. In paper I (a cross-sectional case-control study of age- and gender-matched sets [n = 244]) conditional logistic regression analyses were performed conditioned on the matching factors age and gender to evaluate the associations of traditional risk factors, biomarkers, genetic variants of *ADIPOQ*, and plasma adiponectin with myocardial infarction. It was assumed that there were no interaction effects.

Linear regression

The relationship between plasma adiponectin concentration and intima-media thickness variables (Paper III)

Linear regression analyses were stratified for gender due to the significant differences in adiponectin levels between men and women. The basic model included adjustment for age, and the full model included adjustments for age, BMI, T2DM, SBP, current smoking, triglycerides, high-density lipoprotein cholesterol (HDLc), and C-reactive protein. Tests for gender by plasma adiponectin interaction were performed on the entire cohort. The assumption of normality was assessed by normal-probability plots of the residuals.

The relationship between genetic variants and phenotypes (paper III)

Linear regression (assuming an additive genetic model) was performed in PLINK [24] to assess gender-stratified associations between SNPs and log-transformed plasma adiponectin levels. Adjustments were made for population structure, age, BMI, and T2DM. METAL software [26] was used in a meta-analysis of the gender-specific results and the replication results. To adjust for multiple testing, the p-value required for statistical significance was corrected for the number of independent loci tested: in discovery, $p < 0.05/488 = 1.02 \times 10^{-4}$; in replication, $p < 0.05/4 = 0.0125$.

The relationship between genetic variants and IMT variables (paper III)

A gene score from adiponectin raising alleles was generated (see page 47). The relationships between gene score and IMT measures was analysed using a basic model (adjusting for population structure and age) and a full model (adjusting for population structure, age, body mass index, SBP, HDLc, triglycerides, C-reactive protein, current smoking, and T2DM), and an additional model including adiponectin was also investigated. An interaction term (allelic score by gender) was included when women and men were evaluated together. Multiple testing corrections were not applied in analyses of IMT variables, because the phenotypes are closely interrelated. Baseline IMT variables were log-transformed before analyses, whereas progression of IMT was not.

Cox proportional hazard regression (papers II, III, and IV)

In two of the studies (papers II and III), Cox proportional hazards models were used to evaluate the association of plasma adiponectin with incidence of cardiovascular events and mortality over time. In paper IV, we evaluated the ability to predict

ischaemic events with the aid of transcripts from carotid artery specimens and PBMCs with the same statistical method . This method is an extended version of logistic regression, which takes time-to-event into account. The central assumption when using Cox proportional hazards models is that the risk of exposure is proportional over time.

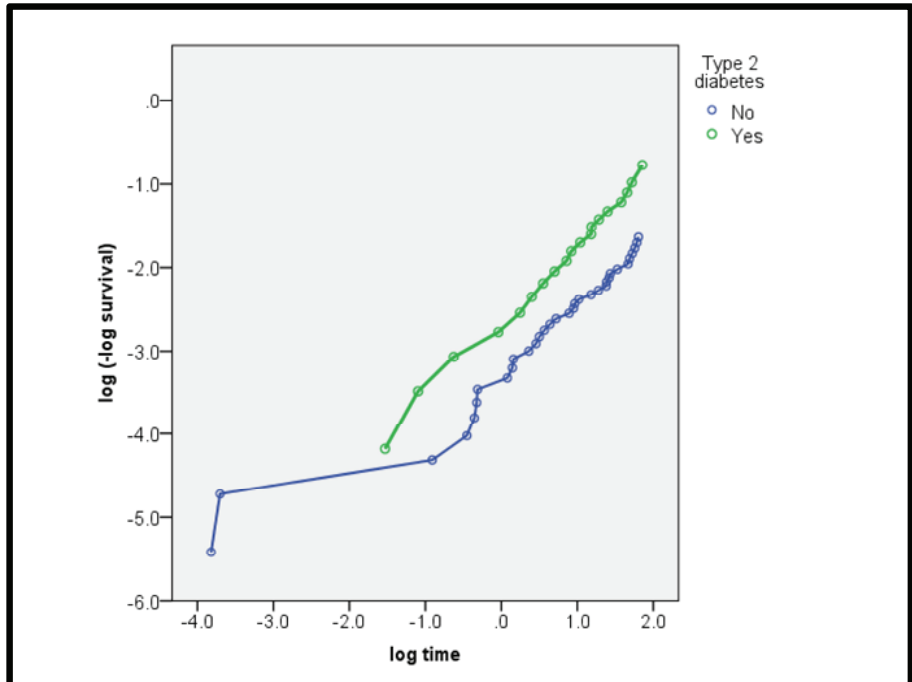


Figure 15. Log (-log survival function) curves for type 2 diabetes mellitus and mortality in the BiKE cohort. Log (-log survival) on the y-axis is plotted against log time on the x-axis for subjects having an event with (green) and without (blue) type 2 diabetes mellitus.

The proportional hazards assumption can be assessed in three ways, using a graphical, goodness-of-fit, or time-dependent variable approach. The assumptions were tested by using “log-log curves” for categorical variables and the time-dependent variable approach for continuous variables. An example of the graphical approach from paper II regarding the proportional hazards assumption for T2DM is given in **Figure 15**. Survival function of the variable T2DM is used as exposure and mortality as outcome in that calculation. Log (-log survival) on the y-axis is plotted against log time to event on the x-axis for subjects with and without T2DM. An interpolation line is drawn between the points representing subjects with events with and without exposure to

T2DM. The interpolation lines should be parallel, if the proportional hazards assumption is fulfilled. Martingales residuals were plotted to assess non-linearity of effects.

Leave-one-out cross-validation

In the investigation reported in paper IV, the Cox proportional hazards model was used to assess association of genetic profiling with prediction of ischaemic events. To our knowledge, at the time we performed that study, there was no replication cohort to supply microarray data on atherosclerotic plaques. Furthermore, there were no follow-up data to validate our results of the use of transcripts from carotid artery plaques to predict ischaemic events. The cohort we investigated was not large enough to split into a discovery and a replication cohort, and thus we carried out leave-one-out cross-validation (**Figure 16**). Briefly, we split the data set into two parts: one covering 125 subjects and the other covering one subject for the plaque data set. The part of the cohort comprising 125 subjects was used to create a score from genes that was associated with ischaemic events. The ability of the score to predict whether the other part of the cohort (one subject) had or had not had an event was evaluated. The procedure was repeated an additional 125 times, and the number of times the prediction was correct constituted an estimate of the accuracy of the method. This type of cross-validation is an established approach used to verify the prediction of outcome in cancer studies (Borup et al., 2010; Tibshirani et al., 2002). The R-code for the scheme is available in the supplementary material for paper IV. Receiver operating characteristics-curves were plotted for prediction of ischaemic events at 300 days.

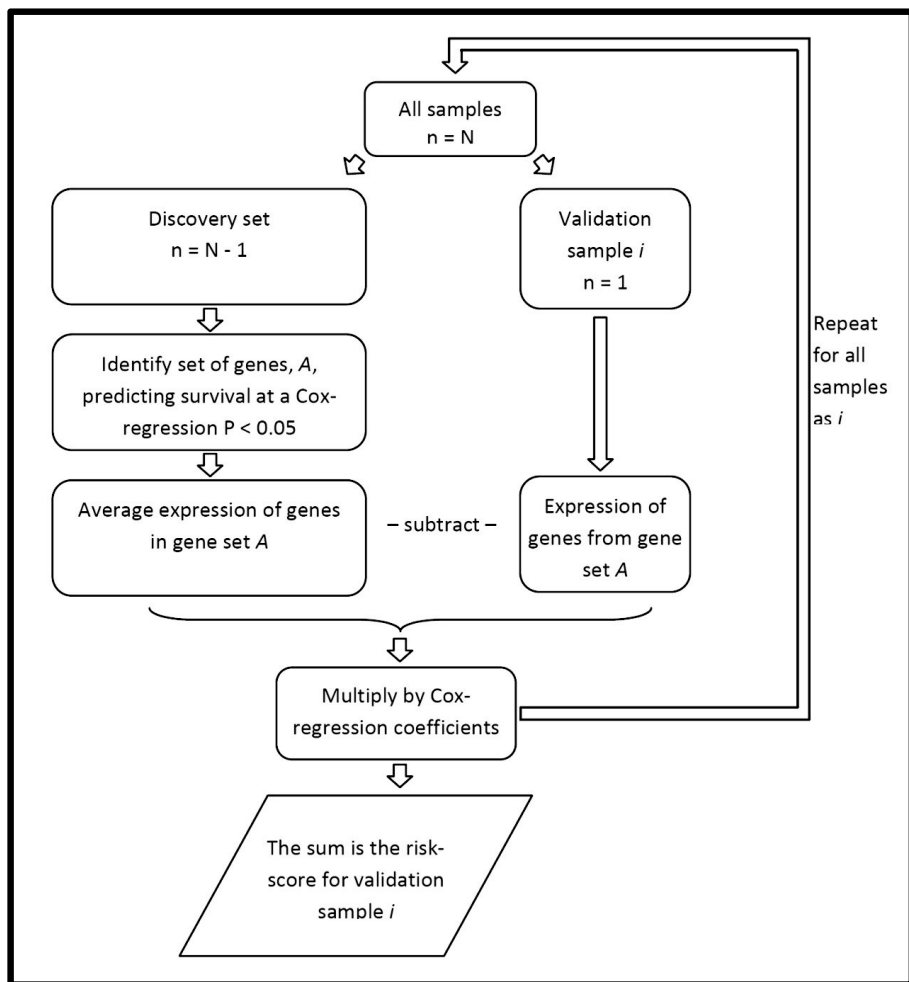


Figure 16. Flow diagram of the leave-one-out cross-validation scheme.

4 RESULTS AND COMMENTS

4.1 LOW PLASMA ADIPONECTIN IS ASSOCIATED WITH MYOCARDIAL INFARCTION IN YOUNG INDIVIDUALS (PAPER I)

Plasma adiponectin in relation to myocardial infarction

Low plasma adiponectin have been associated with myocardial infarction in some studies but data are conflicting. We therefore investigated if plasma adiponectin was associated with myocardial infarction in this case-control study of young subjects (age < 60 years).

We found that cases had lower plasma adiponectin than controls: median 4.5 (interquartile range [IQR] 3.1–6.3) vs. 6.6 (IQR 5.1–8.8) mg/L ($p < 0.001$). Furthermore, plasma adiponectin was inversely associated with myocardial infarction in univariate analysis: odds ratio (OR) per mg/L adiponectin 0.70 (95% confidence interval [CI] 0.64–0.78). The variables that were associated with myocardial infarction in univariate analysis (i.e., history of hypertension, smoking, CRP, creatinine, HDLc, glucose, proinsulin, and BMI) were explored by multivariate analysis with backward stepwise removal of variables that did not change model-fitting criteria ($p > 0.05$). Adiponectin together with glucose, HDLc, creatinine, and history of hypertension was independently associated with myocardial infarction (**Table 3**). Considering all the variables included in the model, glucose had the largest (standardized estimate) and adiponectin the smallest impact on the association with myocardial infarction.

Adiponectin is inversely associated with inflammation, insulin resistance, obesity, and dyslipidaemia. Therefore, further investigation included analyses of adiponectin in relation to myocardial infarction, adjusting for CRP, proinsulin, BMI, and HDLc. Quartiles of adiponectin were inversely associated with myocardial infarction across quartiles of CRP, proinsulin, HDLc, and BMI groups (**Figure 17**). In agreement with other studies (Pischon et al., 2004), the data reported in paper I revealed a strong association between low plasma adiponectin and myocardial infarction.

All of the subjects in this investigation were survivors of first-time myocardial infarction (i.e., cases of fatal infarctions were excluded), and hence there is a potential survival bias in the results. LDL cholesterol concentrations were lower in the patients than in the controls, because 32% ($n = 79$) of the patients but none of the controls

were on lipid-lowering therapy at the time of blood sampling. Nevertheless, the relationship found between adiponectin and myocardial infarction was consistent through the statistical models presented in paper I. In conclusion, this study showed that low plasma adiponectin is associated with myocardial infarction in individuals under the age of 60 years.

Table 3. Variables independently associated with myocardial infarction

Variable	Odds ratio (95% CI)	<i>p</i>	Standardized estimates β $\pi/\sqrt{s.d.}$
Glucose (mmol/L)	3.54 (1.97–6.37)	< 0.001	1.07
HDL cholesterol (mmol/L)	0.101 (0.028–0.365)	< 0.001	–0.54
Creatinine (μmol/L)	1.063 (1.030–1.097)	< 0.001	0.51
History of hypertension (yes vs. no)	3.60 (1.40–9.29)	0.008	0.36
Adiponectin (mg/L)	0.872 (0.762–0.998)	0.047	–0.29

*Multivariate conditional logistic regression model in the age- and gender-matched SCARF cohort (cases, *n* = 244; controls, *n* = 244); Variables are sorted descending based on standardized estimates of impact in the model; CI = confidence interval; β = regression coefficient; s.d. = standard deviation of the variable.*

Genetic variants in ADIPOQ, plasma adiponectin and myocardial infarction

From the literature (Hoefle et al., 2007; Qi et al., 2005; Bacci et al., 2004), we chose the two CVD-associated genetic variants rs266729 and rs1501299 to be genotyped. We found that rs266729, which is located in the promoter region and the first haploblock of *ADIPOQ*, was associated with plasma adiponectin concentrations both before (*p* = 0.035) and after adjustment for case-control status (**Figure 18**). Plasma adiponectin concentrations were lower in individuals with the rare G/G genotype than in heterozygotes. However, the rare genotype G/G for rs266729 was not correlated with myocardial infarction, OR 1.37 (95% CI 0.62 – 3.01) and 1.36 (95% CI 0.63 – 2.97), compared to heterozygotes and the common C/C genotype, respectively. The rs1501299 variant, which is located in the second haploblock, was not associated with either plasma adiponectin or myocardial infarction (data not shown). In conclusion,

our results show that the rare allele of a genetic variant in the promoter of *ADIPOQ* is associated with lower plasma adiponectin levels.

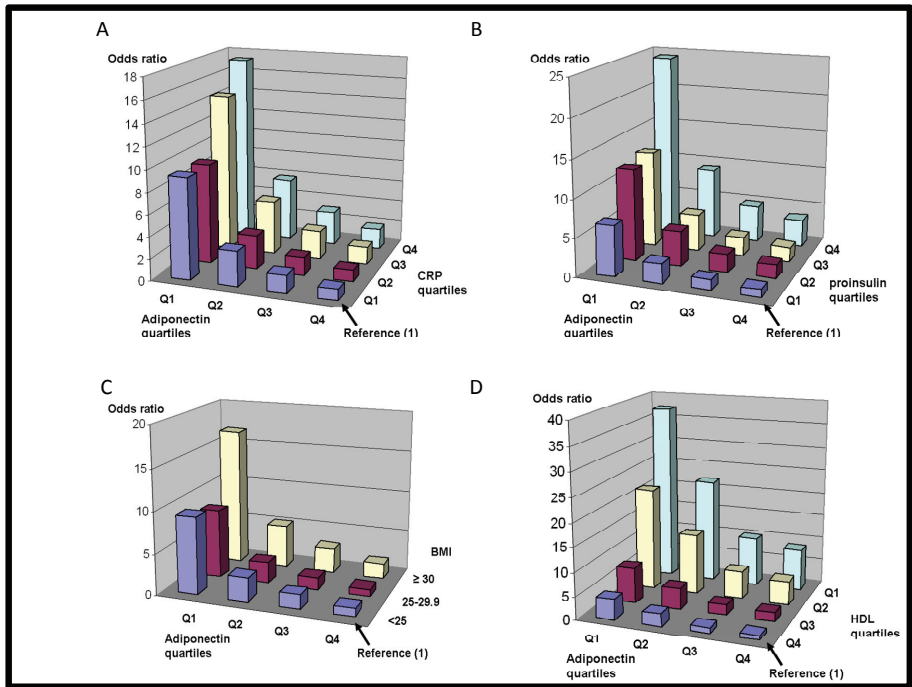


Figure 17. Association between adiponectin quartiles and myocardial infarction. Odds ratios for age- and gender-matched models adjusted for CRP quartiles (A), proinsulin quartiles (B), body mass index (BMI) group (C), and quartiles of high-density lipoprotein (HDL) cholesterol; Q = quartile.

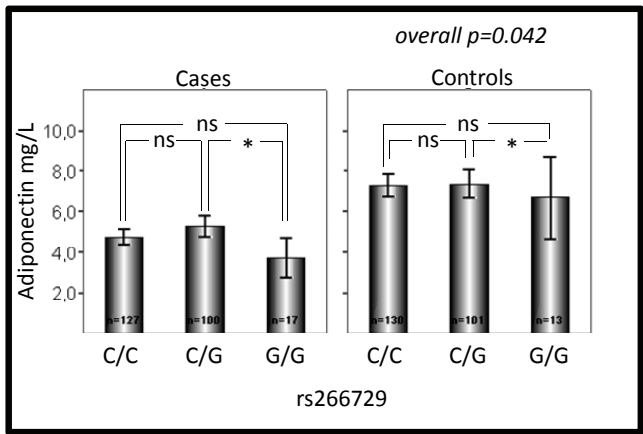


Figure 18. Plasma adiponectin in relation to rs266729 after adjusting for subject status (case or control). The bars represent mean plasma adiponectin concentrations; The error bars indicate 95% confidence intervals, and the p values are given for overall effects using one way ANOVA; *p = 0.024; ns = p not significant.

4.2 HIGH PLASMA ADIPONECTIN CONCENTRATION IS ASSOCIATED WITH ALL-CAUSE MORTALITY IN PATIENTS WITH CAROTID ATHEROSCLEROSIS (PAPER II)

The relationship between plasma adiponectin and outcome in high risk subjects with prevalent CVD undergoing CEA has not been studied before. We hypothesized that high adiponectin might be associated with all-cause mortality but yet protective against ischaemic events. Thus we analyzed the relationships between plasma adiponectin and all-cause mortality, cardiovascular mortality and ischaemic events in the BiKE.

Subjects in the BiKE cohort differ markedly from those in the SCARF and IMPROVE studies. The BiKE patients are older (mean age 70 years) and have a heavy burden of cardiovascular risk and prevalent CVD, as indicated by the following: 64% are current or former smokers, 79% have a history of hypertension, 23% have T2DM, and 31% have CHD. All the subjects had carotid artery stenosis susceptible to CEA at inclusion, and 24% have a contralateral carotid artery stenosis. Thus, all members of this cohort have clinically evident atherosclerosis but, notably, they are also eligible for CEA.

In the current analysis, 52 patients died (**Table 4**), and 73 had an ischaemic event (ischaemic stroke [$n = 52$] and/or myocardial infarction [$n = 28$]) at a median follow-up time of 5.2 years. The main finding reported in paper II is that a high plasma concentration of adiponectin was associated with all-cause mortality and cardiovascular mortality independently of major determinants of plasma adiponectin, such as age, T2DM, CRP, and inflammation (**Table 5; Figure 19**). Plasma adiponectin was not associated with ischaemic events (**Table 5**).

Table 4. Cause of death in BiKE

Cardiovascular disease,	n = 27	<i>n</i>	Other causes,	n = 25	<i>n</i>
Myocardial infarction		8	Cancer		12
Atherosclerosis		8	Suicide		2
Congestive heart failure		5	Infection		4
Ischaemic stroke		3	Lung disease		2
Intracranial bleeding		3	Trauma/Complications		2
			Neurological disorder		2
			Liver failure		1

Table 5. Plasma adiponectin association with mortality and ischaemic events

	Total Mortality		Cardiovascular mortality		Ischaemic events	
	OR (95% CI)	p	OR (95% CI)	p	OR (95% CI)	p
Model 1						
Adiponectin, per s.d. increase	1.81 (1.24–2.66)	0.002	2.21 (1.30–3.77)	0.003	0.89 (0.68–1.18)	0.427
Model 2						
Adiponectin, per s.d. increase	2.06 (1.32–3.22)	0.002	2.17 (1.22–3.89)	0.009	1.00 (0.73–1.36)	0.981

Model 1: stratified for gender and adjusted for age; Model 2: as in Model 1 and adjusted for type 2 diabetes mellitus, body mass index, C-reactive protein, and interleukin-6; OR = odds ratio; C = confidence interval; s.d. = standard deviation.

This paper is the first study of plasma adiponectin in relation to mortality, cardiovascular mortality and incidence of ischaemic events in patients undergoing CEA. To increase the power of the analysis of the relationship between biomarkers and possible plaque-rupture, we chose to focus our study on association of plasma adiponectin with ischaemic events rather than with myocardial infarction and ischaemic stroke separately. This was based on assumption that these two conditions share pathophysiological features (Ogata et al., 2011), and carotid plaque instability is associated with unstable angina pectoris (Lombardo et al., 2004). Our hypothesis was that although adiponectin is associated with mortality in high-risk subjects (Cavusoglu et al., 2006), this protein might protect against ischaemic events. No such relationship was found. Moreover, there was no evident association between adiponectin and myocardial infarction or ischaemic stroke analysed separately (ORs per s.d. increase were 0.90 [95% CI 0.58–1.40] and 0.83 [95% CI 0.58–1.17], respectively, after adjusting for age and stratifying for gender).

In summary, this study showed that plasma adiponectin is associated with all-cause and cardiovascular mortality in patients with atherosclerosis undergoing CEA. No relationship was found between plasma adiponectin and ischaemic events only.

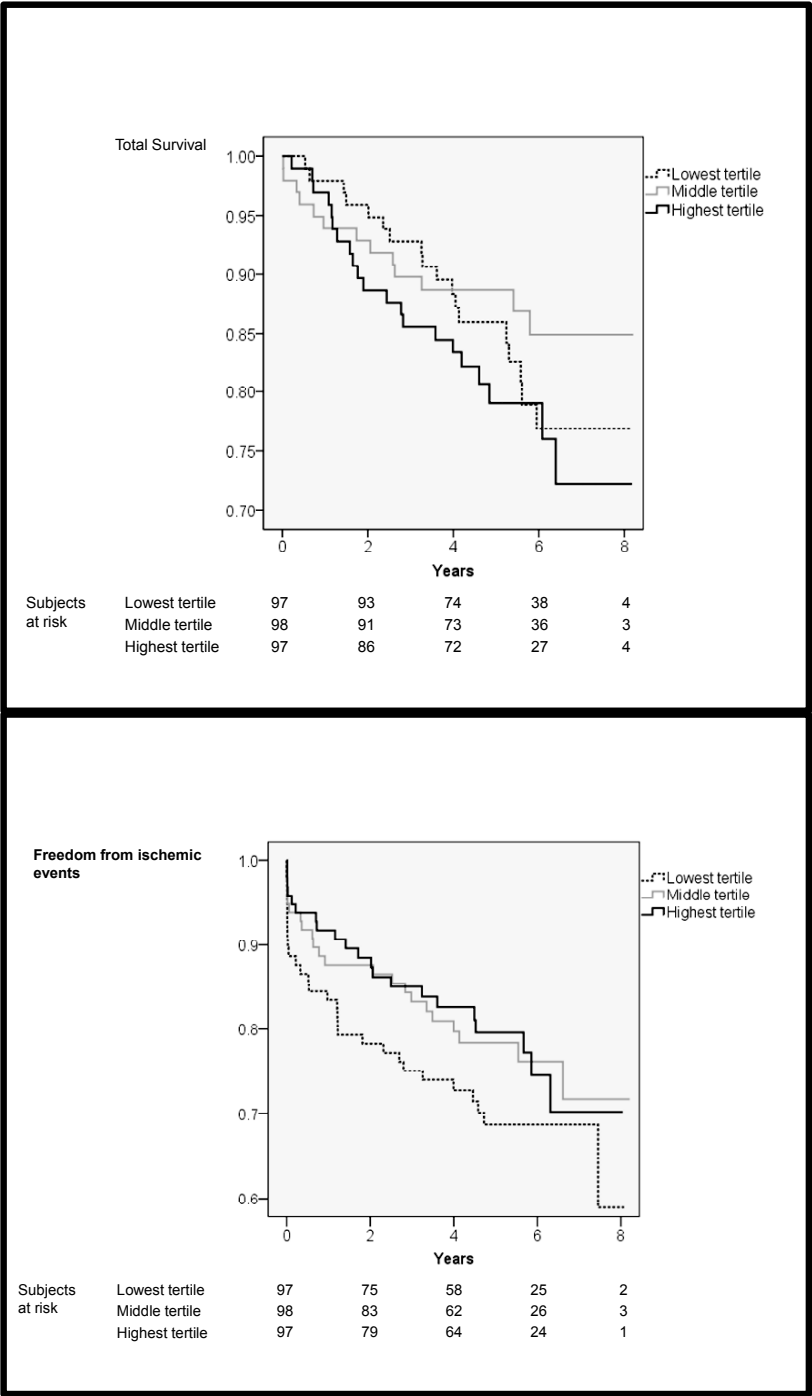


Figure 19. *Kaplan-Meier curves of total survival (top panel) and freedom from ischaemic events (bottom panel) in gender-specific tertiles of plasma adiponectin.*

4.3 ROLE OF ADIPONECTIN IN EARLY ATHEROSCLEROSIS AND INCIDENT CARDIOVASCULAR DISEASE IN HIGH-RISK EUROPEAN SUBJECTS (PAPER III)

Adiponectin is protective against atherosclerosis in experimental models (Yamauchi et al., 2003b; Okamoto et al., 2002; Li et al., 2007) but whether these effects are valid in humans is unknown. Therefore, we investigated the relationships between plasma adiponectin, genetic variants in the adiponectin pathway and carotid IMT in a large cohort (n=3,430) of high-risk subjects. Carotid IMT is a validated ultrasound measure of early atherosclerosis.

In young individuals low plasma adiponectin is associated with myocardial infarction (paper I) and in high risk subjects with prevalent CVD high adiponectin is paradoxically associated with cardiovascular and all-cause mortality (paper II). In paper III, we also examine the incidence of cardiovascular events in relation to plasma adiponectin in this cohort of high-risk subjects without prevalent CVD.

Plasma adiponectin in relation to intima-media thickness

Plasma adiponectin was inversely associated with Bif-IMT_{mean} and Bif-IMT_{max} in females, males, and the whole cohort in the basic model. These relationships were independent of established risk factors and CRP in males and the whole cohort, but not in females (**Table 6**). Plasma adiponectin was also inversely correlated with CCA-IMT_{mean} and CCA-IMT_{max} in females and males separately, and in the whole cohort (data shown in paper III, Table 2). This relationship was abolished after further adjustments for established risk factors and CRP (full model). No plasma adiponectin by gender interactions with regards to baseline IMT measures were observed (data not shown).

Plasma adiponectin was also associated with 30 months progression of CCA-IMT_{mean} and CCA-IMT_{max} in males but not in females, and there were significant plasma adiponectin–gender interactions (**Table 7**).

Table 6. Association of plasma adiponectin with baseline IMT in the bifurcation of the carotid artery.

	Variable	Model	β	95% CI		p
Females	Bif-IMT _{mean}	Basic	-0.013	-0.021	-0.005	0.002
		Full	-0.006	-0.015	0.003	0.185
	Bif-IMT _{max}	Basic	-0.011	-0.021	-0.001	0.029
		Full	-0.006	-0.017	0.004	0.247
Males	Bif-IMT _{mean}	Basic	-0.020	-0.029	-0.011	< 0.001
		Full	-0.018	-0.027	-0.009	< 0.001
	Bif-IMT _{max}	Basic	-0.023	-0.033	-0.012	< 0.001
		Full	-0.019	-0.03	-0.008	0.001
Whole cohort*	Bif-IMT _{mean}	Basic	-0.017	-0.023	-0.010	< 0.001
		Full	-0.012	-0.018	-0.006	< 0.001
	Bif-IMT _{max}	Basic	-0.017	-0.024	-0.009	< 0.001
		Full	-0.013	-0.020	-0.005	0.001

*Basic model: adjusted for age; Full model: adjusted for age, body mass index, type 2 diabetes mellitus, systolic blood pressure, SBP, current smoking, triglycerides, HDLc, and C-reactive protein; *additional adjustment for gender; Bif = bifurcation of the carotid artery; IMT = intima-media thickness; mean = average of mean IMT values obtained from left and right measurements; max = highest of all the maximal IMT values obtained from left and right measurements; CI = confidence interval.*

Table 7. Association between plasma adiponectin and progression of the IMT in the common carotid artery.

		Females				Males				p_{int}^*
		β	95% CI		p	β	95% CI		p	
CCA- IMT_{mean}	Basic	0.0002	-0.0016	0.0019	0.867	-0.0027	-0.0048	-0.0007	0.008	0.034
	Full	0.0007	-0.0012	0.0025	0.475	-0.0022	-0.0043	3.0×10^{-5}	0.047	0.018
CCA- IMT_{max}	Basic	0.0029	-0.0031	0.0089	0.347	-0.0074	-0.0141	-0.0007	0.031	0.020
	Full	0.0031	-0.0035	0.0097	0.354	-0.0071	-0.0141	-0.0001	0.045	0.024

*Basic model and Full model: see table 6; *adiponectin – gender interaction; CCA = common carotid artery; other abbreviations as in table 6.*

Genetic variants in five candidate genes in relation to plasma adiponectin

We determined genetic variants in the adiponectin gene locus, *ADIPOQ* (Heid et al., 2010), and we also genotyped genetic variants in loci *CDH13* (chr.16q23.3) and *ARL15* (chr.5p15.2), which had previously been found to be associated with plasma adiponectin in GWA studies. In addition, genetic variants in adiponectin receptor loci, *ADIPOR1* (chr.1q32.1) and *ADIPOR2* (chr.12p13.31), were determined. The receptors may be involved in regulation of plasma levels of adiponectin. In all, we analysed 1,214 genetic variants, and, after pruning, 488 independent signals in the five different loci were evaluated regarding associations with plasma adiponectin.

Seven SNPs, rs6773957, rs3774261, rs17300539, rs1501299, rs16861210, rs6444175, and rs16861209 were associated with plasma adiponectin ($p < 1.02 \times 10^{-4}$) and all were located at the *ADIPOQ* locus (**Figure 20, top**). Of the seven SNPs, three were highly correlated ($R^2 \geq 0.95$) with another SNP. Four SNPs were less correlated with each other ($R^2 \leq 0.84$; **Figure 20, bottom**) and were selected for replication of association with plasma adiponectin in replication cohorts (SCARF, PIVUS, GoDARTs, and Whitehall II). The associations of the four SNPs with plasma adiponectin were replicated in a meta analysis (**Table 8**). The top SNP differed with regard to gender in the discovery cohort. However, the lead SNPs in the replication cohorts did not differ according to gender (**Table 8**).

Table 8. Association of genetic variants in ADIPOQ with plasma adiponectin in the discovery cohort, replication cohort and test for heterogeneity between sexes.

SNP	Minor Allele	Major allele	Direction of effect	Females Disc. P	Males Disc. p	Whole Disc. p	Repl. p	Heterogeneity between sexes in repl. p
rs16861210	A	G	+	0.0177	0.0003	3.6×10^{-5}	4.8×10^{-12}	0.11
rs6444175	A	G	+	1.7×10^{-5}	0.1782	4.6×10^{-5}	1.2×10^{-7}	0.19
rs6773957	A	G	+	2.3×10^{-5}	0.0438	6.5×10^{-5}	3.7×10^{-11}	0.52
rs17300539	A	G	+	0.0132	6.8×10^{-5}	8.5×10^{-6}	3.8×10^{-14}	0.33

Summary of online table 4 and 5 in paper III; Disc. = discovery cohort; repl. = replication cohorts

Association of adiponectin raising alleles with IMT

A gene score was generated from the four independent genetic variants that were associated with plasma adiponectin (above). One point was given to each subject in the study for each adiponectin-raising allele, thus each subject received a score between 0 and 8. The gene score was inversely associated with Bif- IMT_{mean} and Bif- IMT_{max} in men after adjustment for established risk factors and CRP (beta values – 0.0052 [95% CI –0.0094 to –0.0011, $p = 0.014$] and –0.0057 [95% CI –0.0106 to –0.0007, $p = 0.026$], respectively; **Figure 21**). Surprisingly, there was a positive association between the gene score and Bif- IMT_{max} in females, but this was noted only after (i.e., not before) adjustments for established risk factors and CRP (beta values 0.0061 [95% CI 0.0016–0.0107, $p = 0.008$] and 0.0038 [95% CI –0.0008–0.0085, $p = 0.107$], respectively). Further adjustments for adiponectin did not have any an impact on the beta value in the analyses in men or women. The association between allele score and Bif- IMT_{mean} and Bif- IMT_{max} differed according to gender for both measures ($p < 0.001$ for the gender–allele score interaction). However, the associations could not be replicated with regards to Bif- IMT_{max} in the MDC-CV cohort for males (beta -0.003 [95 % CI -0.010-0.003], $p = 0.320$), females (-0.0005 [-0.0065-0.0055] $p = 0.870$) or in the whole cohort (p for interaction = 0.306).

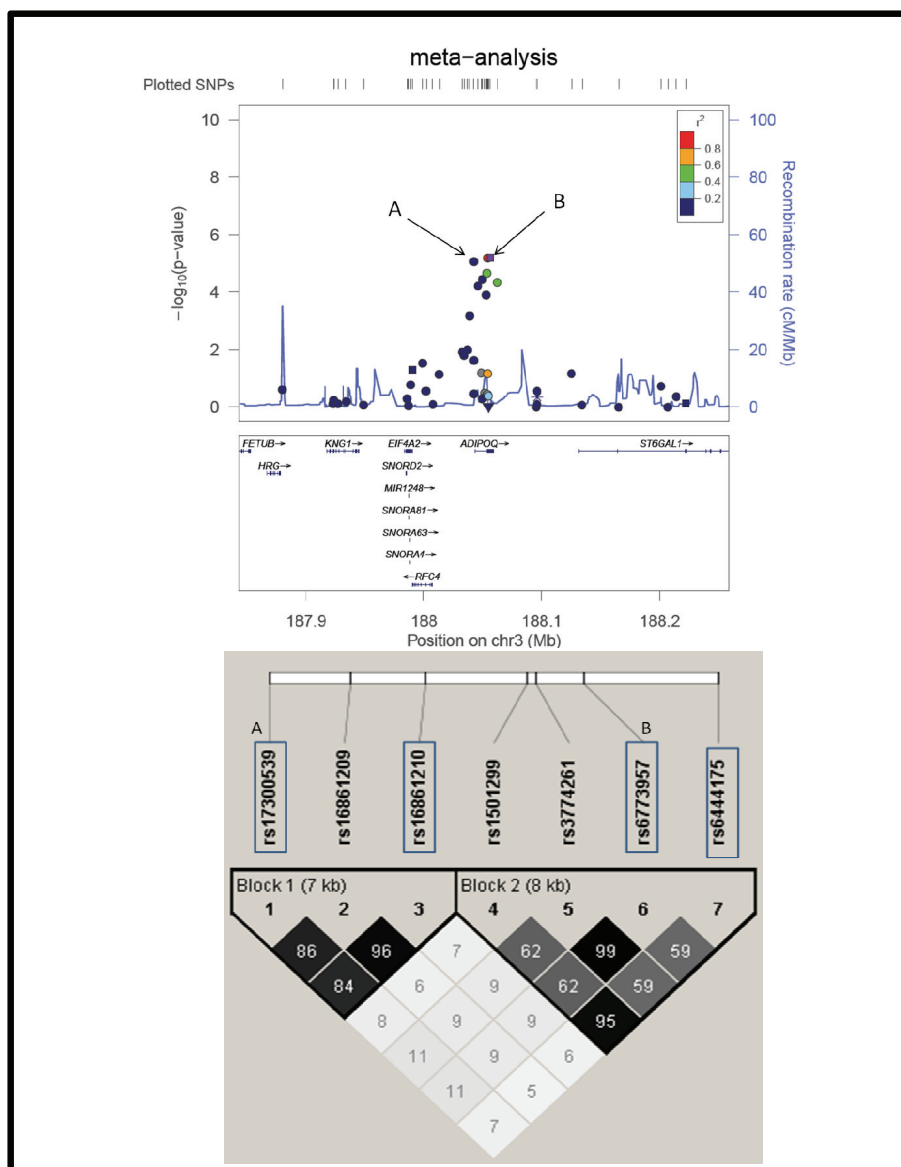


Figure 20. Association between SNPs in the ADIPOQ locus and adiponectin levels in the IMPROVE cohort. (Top) The purple square represents the lead SNP in the meta-analysis; A = lead SNP in the male-specific analysis; B = lead SNP in the female-specific analysis; (Bottom) Linkage-disequilibrium structure of the SNPs that reached significance in the meta-analysis; The SNPs highlighted with a box are those replicated and included in the gene score; Figure by Rona J Strawbridge.

The gene score was not correlated with progression of Bif-IMT or CCA-IMT (data not shown).

We were unable to replicate the association between gene score and IMT of the bifurcation in the MDC-CVA cohort, possibly because of a lack of power due to that cohort being half the size of the IMPROVE cohort. Furthermore, MDC-CV cohort includes subjects at lower risk compared to the IMPROVE subjects, who are older and have three or more vascular risk factors.

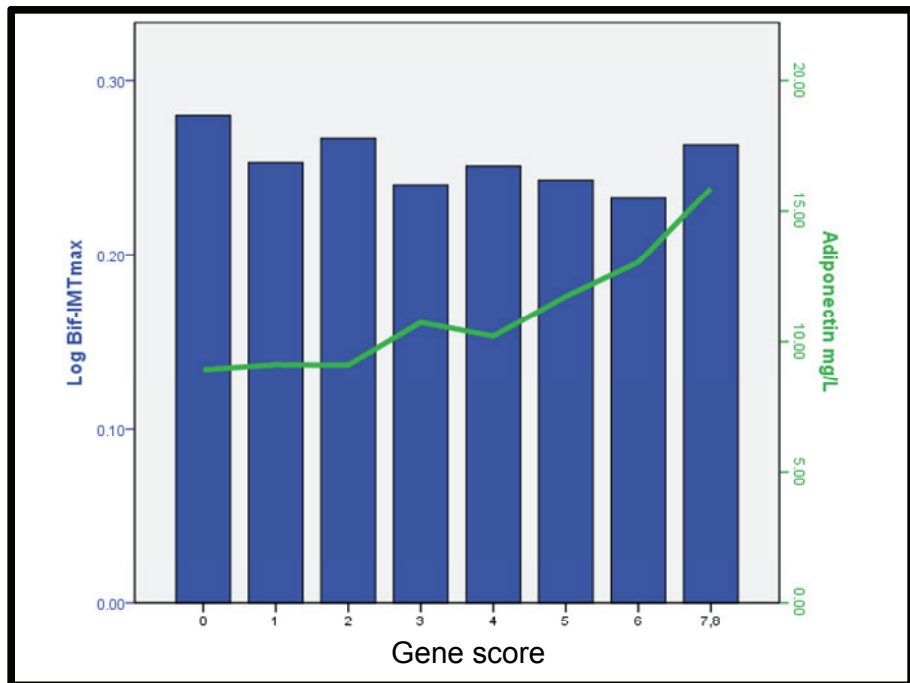


Figure 21. Relationship between gene score, plasma adiponectin and carotid bifurcation IMT in males. The gene score (number of adiponectin raising alleles) is positively associated with plasma adiponectin concentration (green line) inversely associated with Bif-IMT_{max} (blue bars) in males ($p=0.014$ in basic model); Bif = bifurcation; IMT = intima-media thickness.

Plasma adiponectin in relation to cardiovascular events in high-risk subjects without prevalent cardiovascular disease

Plasma adiponectin was inversely associated with risk of incident cardiovascular events, OR per s.d increase of adiponectin were 0.69 (95% CI 0.56-0.84) after adjustment for age and gender. The association was evident also after further adjustments for BMI, T2DM, SBP, HDLc, triglycerides, and current smoking, OR 0.76 (95% CI 0.61-0.95). Corresponding ORs for coronary events were 0.58 (95% CI 0.44-0.77) and 0.64 (95% CI 0.47-0.87). The Kaplan-Meier plot in **Figure 22** clearly shows that the subjects in the lowest plasma adiponectin tertile had higher risk of cardiovascular events than those in the middle and highest tertiles.

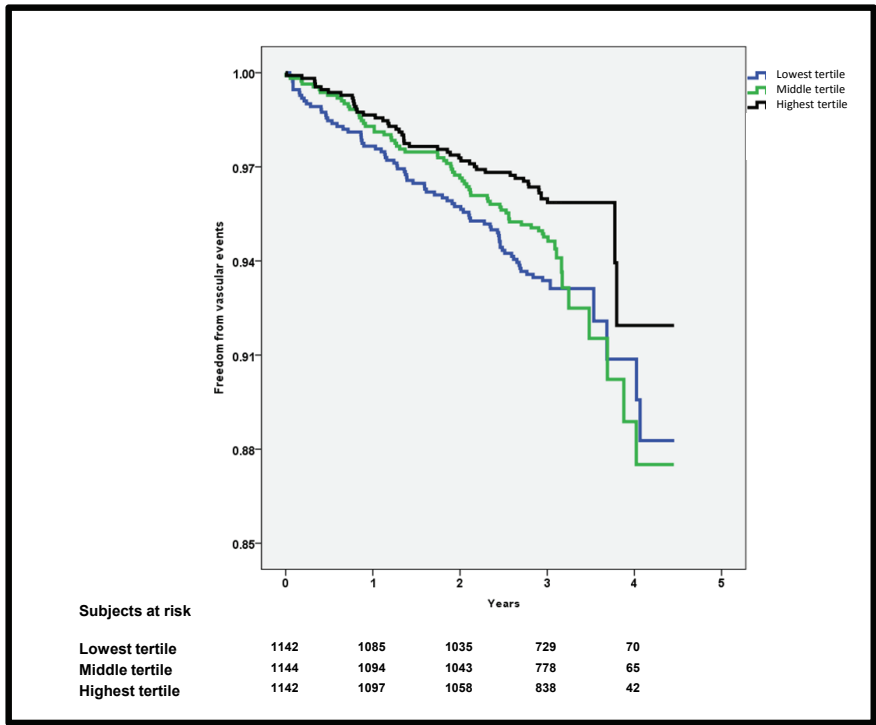


Figure 22. Kaplan-Meier plot of freedom from cardiovascular events. Subjects are divided into gender-specific tertiles of plasma adiponectin concentrations (ranges for females and males, respectively: lowest, 0.54–10.4 and 0.34–6.04 mg/L; middle, 10.4–18.9 and 6.04–10.6 mg/L; highest, 18.9–87.8 and 10.6–86.6 mg/L).

In summary there was a strong inverse relationship between plasma adiponectin levels, Bif-IMT and progression of CCA-IMT in men. Low plasma adiponectin was

associated with cardiovascular events in the whole cohort of high-risk subjects without prevalent CVD. There was an inverse relationship between of genetic variants, which determines plasma adiponectin, and Bif-IMT in men. The data presented speaks in favour of a causal protective effect of adiponectin in early atherosclerosis among men as stipulated in experimental animal models.

4.4 PREDICTION OF ISCHAEMIC EVENTS ON THE BASIS OF TRANSCRIPTOMIC AND GENOMIC PROFILING IN PATIENTS UNDERGOING CAROTID ENDARTERECTOMY (PAPER IV)

One out of five subjects that have a myocardial infarction lack one or more of the common risk factors; hyperlipidaemia, diabetes mellitus, smoking, and hypertension (Khot et al., 2003) which makes novel markers for cardiovascular risk a priority for research. Thus, in paper IV we examined (i) the global transcriptome of carotid artery plaques, (ii) the global transcriptome of peripheral blood mononuclear cells, and (iii) selected genetic variants associated with myocardial infarction regarding their ability to predict ischaemic events in patients with established atherosclerosis and undergoing CEA.

The area under the curve value (AUC) for the prediction of ischaemic events when using only age and risk factors (i.e. gender, LDLc, and smoking status) was 0.66 in the plaque data set (**Figure 23: Table 9**). Adding the gene expression score obtained from the carotid plaques improved the prediction of ischaemic events to AUC 0.77. Because of the limited number of events, $n = 25$, the number of variables used in the main analysis was restricted. However, a sensitivity analysis including three more variables (creatinine, eGFR, and T2DM) was also conducted (**Table 9**). The contribution of genotype score in prediction of ischaemic events was sparse (**Table 9**).

The gene expression score obtained from PBMCs provided very little extra information compared to classical risk factors only in the PBMC data set (AUC 0.68 vs. 0.67; **Figure 23**).

Table 9. Prediction of ischaemic events in the plaque data set

	AUC
Genotype score only	0.55
Expression score only	0.72
Classical risk markers	0.66
Classical risk markers and expression score	0.77
Classical risk markers and genotype score	0.67
Classical risk markers, eGFR, creatinine and T2DM	0.69
Classical risk markers, expression score and genotype score	0.79
Classical risk markers, eGFR, creatinine, T2DM, expression score and genotype score	0.83

eGFR = estimated glomerular filtration rate; T2DM = type 2 diabetes mellitus; AUC = area under the curve.

Inasmuch as it is assumed that PBMCs roll along the endothelium and are involved in the pathogenesis of atherosclerosis, plaque rupture, and arterial thrombosis, we both expected and hoped that gene expression profiles for such cells would have a greater impact on prediction of ischaemic events. Of course, considering feasibility, it is much easier to obtain peripheral blood mononuclear cells than plaques from endarterectomies, and hence the method of genomic profiling for prediction of ischaemic events is limited to subjects undergoing surgery on peripheral arteries.

It would have been desirable to validate the findings in an independent cohort, but, at the time we conducted this study, no replication cohort was available that had undergone microarray analysis of carotid artery plaques. Therefore, we applied the leave-one-out cross-validation method confirm the findings.

The aim of this study was not to identify new genes and targets involved in atherosclerosis and plaque rupture, but rather to predict ischaemic events regardless of which transcripts are important for pathophysiology of atherosclerosis and plaque rupture. No transcripts of single genes were predictive of ischaemic events after correction for multiple testing. Nevertheless, several transcripts from the carotid artery plaques (paper IV, supplement, Table 2) could predict ischaemic events before adjustments for multiple testing. It is plausible that these transcripts can play a role in the development of atherosclerosis. However, before proceeding with mechanistic investigations, it would be necessary to confirm the value of those transcripts in another cohort. It should also be kept in mind that a carotid artery

plaque is a heterogeneous tissue and thus conclusions regarding mechanistic pathways are not possible.

In the same manner as previously (paper II), we chose to examine the associations with ischaemic events in this study to enhance the power of the analyses. It is not surprising that gene profiles from diseased atherosclerotic tissue can predict ischaemic events, because carotid plaque instability is associated with unstable angina pectoris (Lombardo et al., 2004). Also, atherothrombotic ischaemic stroke and myocardial infarction have pathophysiological features in common (Ogata et al., 2011).

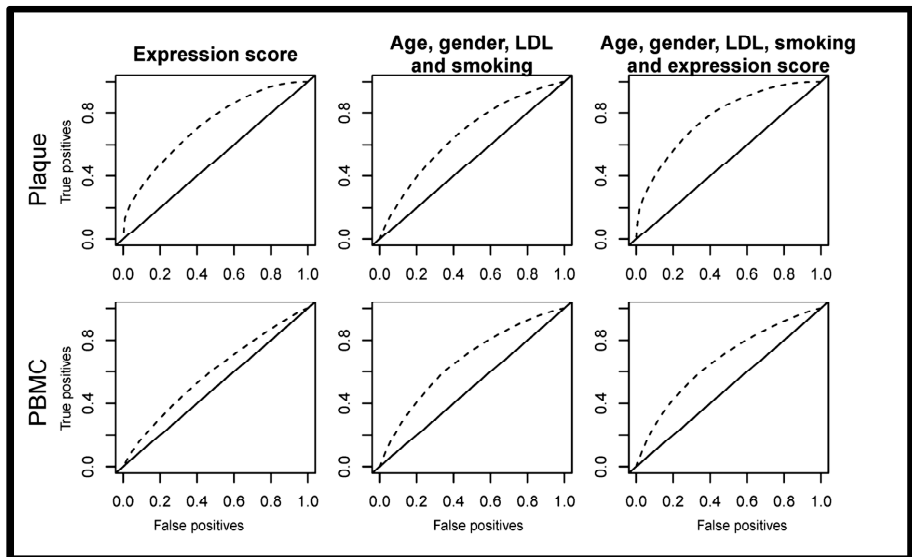


Figure 23. Receiver operating characteristic curves for different risk factors at 300 days. The plots represent the plaque data set (upper row) and the PBMC data set (lower row), and the calculations were based on the following: gene expression profiles only (left); the established risk factors age, gender, low-density lipoprotein (LDL) cholesterol, and smoking (middle); both gene expression profiles and the four established risk factors (right). The straight diagonal lines illustrate prediction by pure chance (area under the curve [AUC] 0.5), and the dashed curves describe the prediction achieved using the given predictive variables. A theoretical curve along the top left corner would indicate perfect prediction (AUC 1.0).

The main finding of the study presented in paper IV is that gene expression profiles from atherosclerotic plaques extirpated by carotid endarterectomy can improve prediction of ischaemic events when added to classical risk factors in subjects with

established atherosclerosis. Genotype score and gene expression profiles from PBMCs provide limited additional information about future events as compared to classical risk factors only.

5 GENERAL DISCUSSION

The research underlying this thesis was roughly outlined in 2006, and, at the time, it was known that plasma adiponectin is inversely correlated with CHD (Kumada et al., 2003) and myocardial infarction (Pischon et al., 2004) in men, and experimental data suggested that this protein exerts protective anti-inflammatory and anti-atherosclerotic effects (Okamoto et al., 2002; Yamauchi et al., 2003b). However, conflicting epidemiological data later emerged showing that high levels of adiponectin were associated with mortality in high risk subjects (Cavusoglu et al., 2006) and community dwelling subjects (Dekker et al., 2008). Therefore, to further elucidate the role of adiponectin in cardiovascular epidemiology, we explored the relationship between plasma adiponectin and outcome in three separate studies in which the subjects investigated differed with respect to age, prevalence of cardiovascular disease, and cardiovascular risk factors. We found plasma adiponectin to be inversely associated with myocardial infarction in younger individuals (aged <60 years) regardless of inflammation, anthropometric measurement, dyslipidaemia, and insulin resistance (paper I). Our results also showed that plasma adiponectin, predicted all-cause and cardiovascular mortality in subjects that all had prevalent CVD (paper II). Furthermore, in a large cohort of high-risk individuals with three or more cardiovascular risk factors but, notably, without prevalent CVD, low plasma adiponectin was associated with future cardiovascular and coronary events (paper III).

5.1 ADIPONECTIN IN RELATION TO EARLY ATHEROSCLEROSIS

In the largest investigation to this date (paper III; November 2012) on plasma adiponectin in relation to IMT measures, plasma adiponectin was strongly associated with baseline Bif-IMT and progression of CCA-IMT over 30 months in males. Also, the sum of adiponectin raising alleles in four genetic variants of *ADIPOQ* were inversely associated with Bif-IMT in males which speaks in favour of a causal protective effect of adiponectin in the arterial wall in humans which is stipulated in animal models.

The significant relationships between plasma adiponectin and Bif-IMT and between the gene score and Bif-IMT in our study were confined to males. Females had 1.7

times higher plasma median adiponectin levels, and hence it can be hypothesised that females in general might have “enough” plasma adiponectin in the circulation to inhibit inflammation and protect against early atherosclerosis. Hypothetically, males might be more vulnerable to the variation in levels of circulating adiponectin because they have a lower baseline concentration of this protein. Thus, lifelong exposure of low or high plasma adiponectin determined by genetic variants could be more important in men than in women. The gene score of in the IMPROVE cohort determined 1.0 % of the plasma adiponectin concentrations, and, in addition to gender, it appears that population structure plays an important role (paper III, Online Table 2). This indicates that some of the variation in plasma adiponectin is determined by genetic variation that is not accounted for in our analyses of gene score in relation to IMT.

The association of plasma adiponectin raising alleles in males were confined to Bif-IMT, whereas there was no relation to CCA-IMT. This indicates that adiponectin is more involved in regulating inflammatory activity in regions of the arterial tree that are more prone to atherosclerosis where the blood flow is disturbed. Also, it is known that Bif-IMT is associated to a greater extent with risk factors for coronary heart disease and prevalent coronary heart disease, whereas CCA-IMT has a more pronounced relationship with risk factors for stroke and prevalent cerebrovascular disease (Ebrahim et al., 1999).

5.2 GENETIC VARIANTS IN *ADIPOQ* AND IMT

Associations between genetic variants of *ADIPOQ* and CCA-IMT have been analysed in the RISC (Patel et al., 2008) and CAPS (Bevan et al., 2011) studies, which both showed that the rs266729 is related to CCA-IMT. In the RISC study, rs266729 was not correlated with plasma adiponectin concentrations, whereas in the CAPS study the rare allele in rs266729 was related to lower plasma levels of this protein. In the IMPROVE cohort, rs266729 was associated with plasma adiponectin (discovery meta-analysis, $p = 0.0235$), but it did not reach the Bonferroni-corrected significance level when all the genetic variants tested ($n = 488$) were taken into account.

It should be noted that there are several methodological differences between our investigation and other studies on genetic variants of candidate genes in the

adiponectin pathway in relation to IMT. We measured only far-wall IMT, whereas both far- and near-wall measurements were used to calculate mean common cIMT in the RISC study. Furthermore, we determined 488 independent SNPs potentially involved in the regulation of adiponectin, but the RISC study and the CAPS study determined four and five *ADIPOQ* SNPs, respectively. Also, we used a gene score created from the top four adiponectin-raising alleles to analyse associations between genetic variants and IMT variables, whereas relationships between single genetic variants and IMT were evaluated in the CAPS and RISC investigations. In addition to the differences in methodology, there were dissimilarities with regard to cohort composition: RISC and CAPS are smaller cohorts ($n = 1306$ and $n = 990$, respectively); the mean age of cohort members was 43 years in the RISC study, 53 years in the CAPS study, but 64 years in the IMPROVE study. Furthermore, subjects with T2DM were excluded from the RISC investigation, and only 5.1% of the CAPS subjects had T2DM, whereas in the IMPROVE cohort subjects had three or more vascular risk factors of which one was blood glucose level > 6.1 mmol/L or pharmacological treatment for T2DM.

5.3 ADIPONECTIN AND MYOCARDIAL INFARCTION

We showed that low plasma adiponectin is associated with first-time myocardial infarction (paper I) in younger individuals. There are no experimental models to evaluate effects of adiponectin on plaque vulnerability but studies in humans have shown that adiponectin correlates with vulnerable features of coronary plaques (Kunita et al., 2012; Otake et al., 2008; Iwata et al., 2008). Furthermore, adiponectin has also been found to increase the secretion of the tissue inhibitor of metalloproteinases 1 (TIMP1; Kumada et al., 2004) which inhibits the fibrous cap degrading enzyme MMP-9 (**Figure 2**). As mentioned before, adiponectin inhibits the conversion of monocytes into to pro-inflammatory M1 macrophages (Lovren et al., 2010), suppresses foam cell formation (Tian et al., 2009; Ouchi et al., 2001) and inhibits accumulation and T-cell recruitment to atherosclerotic plaques (Okamoto et al., 2008). In addition, experimental studies have shown that the recruitment of endothelial progenitor cells, involved in endothelium repair, is dependent on adiponectin (Eren et al., 2009; Nakamura et al., 2009; Sambuceti et al., 2009). In summary, there is a substantial amount of epidemiological and experimental data

that suggest that adiponectin inhibits plaque instability, although hard evidence is yet to be presented.

5.4 PARADOXAL ASSOCIATION BETWEEN PLASMA ADIPONECTIN AND OUTCOME IN HIGH-RISK SUBJECTS

The association between elevated plasma adiponectin concentration and mortality in high-risk subjects that was noted in paper II is difficult to explain given the protective effects of adiponectin observed in experimental models (Okamoto et al., 2002; Yamauchi et al., 2003b). It can be speculated that adiponectin receptors in target tissues are down-regulated in individuals with advanced atherosclerotic disease (paper II), congestive heart failure (Kistorp et al., 2005), or renal insufficiency (Ohashi et al., 2008), and that adiponectin concentrations in plasma reach higher levels as the result of compensatory up-regulation.

After acute coronary syndromes, high levels of adiponectin on admission is associated with future major adverse events including congestive heart failure (Ang et al., 2009; Wilson et al., 2011; Lindberg et al., 2012) opposite to what would be expected from experimental data by us and others which shows that adiponectin inhibits ischemia-reperfusion injury (Gonon et al., 2008; Shibata et al., 2005). In the majority of patients who have suffered a myocardial infarction, the plasma concentration of adiponectin decreases after the event (Ang et al., 2009) and we can only speculate about the mechanisms. One plausible mechanism is that myocardial infarction triggers acceleration of chronic atherosclerosis accompanied by monocyte/macrophage recruitment (Dutta et al., 2012). Adiponectin modulates the actions of monocytes/macrophages (Ouchi et al., 2001; Tian et al., 2009; Tsubakio-Yamamoto et al., 2008) and the protein is consumed. Subjects who cannot utilize/consume the protein and benefit from its protective effects are at higher risk of future events. This theory is complex and speculative but supported by that increased plasma adiponectin concentration at 7 weeks after ACS is a better predictor of prognosis than the value recorded at baseline (OR per 1 log unit increase in delta adiponectin 5.42 [2.78–10.55] vs. 1 log unit increase in adiponectin 2.06 [0.92–4.63]; Ang et al., 2009).

5.5 PREVALENT CVD IS AN IMPORTANT DIVIDER FOR THE RELATIONSHIPS BETWEEN PLASMA ADIPONECTIN AND OUTCOME MEASURES

Again, high plasma adiponectin was positively associated with mortality and cardiovascular mortality in high-risk subjects with prevalent CVD (paper II), whereas in subjects without prevalent CVD (paper I and paper III) we showed that low plasma adiponectin was associated with cardiovascular and coronary events. The inverse association between plasma adiponectin levels and myocardial infarction has been found in other cohorts of subjects without prevalent CVD (Pischon et al., 2004; Pischon et al., 2011). No relationship was found between plasma adiponectin and incident CHD in a large nested-case control study in which the prevalence of CVD was 17% in controls and 32% in cases (Sattar et al., 2006). The latter investigation also included a meta-analysis, which showed that plasma adiponectin was inversely associated with incident CHD when cohorts with prevalent cardiovascular disease were excluded (Sattar et al., 2006). In summary, prevalent CVD is an important divider for the relationships between plasma adiponectin and outcome measures.

5.6 ISOFORMS OF ADIPONECTIN IN PLASMA

Experimental studies have suggested that HMW adiponectin is biologically more active than LMW and MMW adiponectin (Kobayashi et al., 2004), and the epidemiological data is conflicting (Pischon et al., 2011; Inoue et al., 2007; Sattar et al., 2008). Adiponectin isoforms were not accounted for in our studies. This might have attenuated associations between plasma adiponectin and IMT, cardiovascular outcome, and total mortality.

5.7 MERGING BIOBANKS WITH THE SWEDISH CAUSE OF DEATH REGISTER AND THE SWEDISH HOSPITAL DISCHARGE REGISTER

In two of our studies (papers II and IV), the BiKE database was merged with the Swedish Cause of Death Register and the Swedish Hospital Discharge Register at the Swedish National Board of Health and Welfare. All Swedish citizens have a personal identification number which gives a unique opportunity to obtain records from the registers above after ethical vetting. The risk of violation of integrity by unauthorized or authorized persons is low but should nonetheless be acknowledged. However, the benefits of the utility provided by merging registries in Sweden to improve

healthcare should be taken advantage of in a responsible and cautious manner so as not to undermine important epidemiological work that is yet to be performed.

There are limitations when merging biobanks with the registries above. Outcomes like myocardial infarction or stroke should be validated against medical records to make it possible to judge the quality of the data that are acquired. Validation studies from the beginning of 1990's and forward have been conducted regarding the different outcomes reviewed in a recent paper (Ludvigsson et al., 2011). The results of some validation studies are dated. For example the definition of myocardial infarction has changed since then (Thygesen et al., 2012). Notwithstanding, this approach has advantages in that no subjects are lost to follow-up with regard to mortality, and follow-up data can be obtained for large databases at a low cost.

5.8 FUTURE PERSPECTIVES

There is a fair amount of data indicating that adiponectin has a casual protective effect on atherosclerosis, plaque vulnerability, and arterial thrombogenesis and the research presented in paper III speaks in favour of an anti-atherogenic protective causal role of adiponectin in early atherosclerosis in men. However, there are lot of work to be conducted before therapeutic interventions in the adiponectin pathway can be implemented.

The adiponectin receptors are expressed in a wide array of tissues and cells, including monocytes, macrophages, adipocytes, skeletal muscle, brain, and liver, and they are a prerequisite for the effects of adiponectin in experimental models (Yamauchi et al., 2007; Yamauchi et al., 2003a). The presence and the function or functions of these receptors in the vascular wall have not been elucidated in humans and thus represent an important target for future studies.

An adiponectin-mimicking peptide has also been tested and found to inhibit neovascularization in an experimental model of macula degeneration (Lyzogubov et al., 2012). It would be highly interesting to conduct small-scale studies to evaluate the effects of peptides, that mimic adiponectin on surrogate markers for cardiovascular disease. If such research is successful, and the peptides are tolerated

by humans, it will be possible to perform large clinical trials aimed at preventing acute coronary syndromes.

Little is known about the effects of plasma adiponectin on platelet aggregation and thrombus formation, and the data that are available are divergent. Two studies have demonstrated that adiponectin augments activation of human platelets (Riba et al., 2008a; Riba et al., 2008b), whereas another investigation showed that the platelet aggregation response to ADP is decreased after pre-incubation with adiponectin (Restituto et al., 2010). In adiponectin knock-out mice, a deficiency of adiponectin led to enhanced thrombus formation and platelet aggregation (Kato et al., 2006). In humans, adiponectin has been inversely associated with platelet activation (Shoji et al., 2006) and with markers for platelet aggregation (Bigalke et al., 2010). Considering that plasma adiponectin is inversely associated with myocardial infarction but positively correlated with adverse outcome after ACS (above), there are large gaps to be explored in the understanding of both the regulation of adiponectin secretion in prevalent CVD and the effects of adiponectin on platelet aggregation, thrombus formation and plaque rupture.

6 CONCLUSIONS

Adiponectin and atherosclerosis

- I. Low adiponectin is associated with myocardial infarction in young individuals (<60 years).
- II. Low adiponectin is associated with incident cardiovascular events in high-risk subjects without prevalent CVD.
- III. High adiponectin is associated with all-cause mortality and cardiovascular mortality in subjects with prevalent CVD.
- IV. Plasma adiponectin is inversely associated with IMT in the bifurcation of the carotid artery in men but not in women.
- V. Plasma adiponectin is inversely associated with progression of IMT in the common carotid artery in men but not in women.
- VI. Four common genetic variants in the locus of the adiponectin gene *ADIPOQ* are associated with plasma adiponectin levels.
- VII. The sum of adiponectin-raising alleles of the four genetic variants is inversely associated with IMT in the bifurcation of the carotid artery in men but not in women.

In summary, prevalent CVD is an important divider for the relationships between plasma adiponectin and outcome. Low plasma adiponectin is associated with adverse outcome in subjects without prevalent CVD whereas in subjects with prevalent CVD high plasma adiponectin is associated with mortality. Plasma adiponectin is inversely associated with early atherosclerosis and progression of atherosclerosis in men. Plasma adiponectin raising alleles are inversely associated with early atherosclerosis in men, which supports a causal protective role of adiponectin against early atherosclerosis.

Prediction of ischaemic events

- I. Gene expression profiling of carotid artery plaques improves prediction of ischaemic events in addition to established risk factors in subjects undergoing carotid endarterectomy.

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9 POPULÄRVETENSKAPLIG SAMMANFATTNING

Adiponectin är ett protein som utsöndras från fettceller och cirkulerar i blodet. Överviktiga individer har lägre nivåer av proteinet i blodet än normalviktiga. Dessutom har män ca 40% lägre nivåer än kvinnor. Proteinets skyddande effekter mot åderförkalkning i djurstudier. Det är okänt om proteinet har liknande effekter hos människor. Studier som har beskrivit förhållandet mellan nivån av proteinet i blodet och hjärt-kärlsjukdom har varit motsägelsefulla.

I tre studier utforskade vi relationen mellan koncentrationen av adiponectin i blodet med insjuknande i hjärt-kärlsjukdom och död. Deltagarna skilde sig åt i ålder, förekomst av riskfaktorer och tidigare diagnostiserad hjärt-kärlsjukdom.

I den första studien studerades 244 hjärtinfarktpatienter som jämfördes med friska personer. Vi kunde visa att låga nivåer av proteinet var associerat med hjärtinfarkt hos individer som var under 60 år. Relationen var oberoende av andra faktorer som blodsocker, högt blodtryck, njurfunktion och det "goda" kolesterolet, HDL.

I den andra studien ingick 292 patienter som alla hade en känd hjärt-kärlsjukdom och genomgick halskärls-kirurgi. Den visade, paradoxalt i jämförelse med den första studien, att höga nivåer av proteinet i blodet var associerat med framtida död i hjärt-kärlsjukdom.

I den tredje studien ingick 3430 individer med flera riskfaktorer för hjärt-kärlsjukdom men som inte hade insjuknat då studien startade. Resultatet visade att låga nivåer av proteinet var associerat med framtida hjärt-kärlhändelser. Exempel på händelser är hjärtinfarkt, stroke, hjärtkirurgi, ballongvidgning av hjärtats kranskärl, kärlkirurgi och död i hjärt-kärlsjukdom. Vidare undersöktes tjockleken på halspulsåderns två innersta lager (IMT). IMT är ett mått på åderförkalkning. Studien visade ett samband mellan låg protein-nivå och hög IMT hos män, men inte hos kvinnor. Vi visade också att fyra vanliga genetiska varianter i adiponectin-genen korrelerade till protein-nivån i blodet. De män i studien som hade genetiska varianter som höjde protein-nivåer hade lägre IMT, vilket talar för att adiponectin kan ha skyddande effekter mot åderförkalkning hos män.

I en fjärde separat studie, som ej behandlar adiponectin, undersökte vi om uttryck av ca 20 000 gener i kärlvägg med åderförkalkning kunde förutsäga insjuknande i hjärtinfarkt och stroke. Bitar av kärlvägg hade opererats ut i samband med halskärls-

kirurgi på 126 patienter. Som tillägg till klassiska riskfaktorer som ålder, kön, blodfetter och rökning, förbättrade metoden riskbedömningen avseende insjuknande i framtida hjärtinfarkt och stroke.

Sammanfattningsvis visar de tre studierna om adiponectin att nivå av protein i blodet är relaterat till framtida hjärt-kärlhändelser. Förekomst av känd hjärt-kärlsjukdom är betydelsefullt för relationen mellan adiponectin i blodet och framtida hjärt-kärlhändelser. Hos individer utan känd hjärt-kärlsjukdom är låga nivåer relaterat till hjärt-kärlhändelser medan hos individer med tidigare känd hjärt-kärlsjukdom är höga nivåer relaterat till död i hjärt-kärlsjukdom. Vi visar också att proteinet kan ha skyddande effekter mot åderförfattning hos män men detta visas ej hos kvinnor. Ytterligare studier av adiponectinets effekter i samband med hjärt-kärlsjukdom är starkt motiverat.

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