Risk factors for cardiovascular events and incident hospital-treated diabetes in the population
To my family

"The knowledge of anything, since all things have causes, is not acquired or complete unless it is known by its causes."

Avicenna
Persian polymath
(c. 980-1037)
Risk factors for cardiovascular events and incident hospital-treated diabetes in the population
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Abstract


Background. Cardiovascular disease (CVD) is the leading cause of death worldwide. Well-established risk factors for CVD include increasing age, male sex, sedentary lifestyle, obesity, smoking, diabetes, hypertension, dyslipidaemia and low socio-economic status. Traditional risk factors do, however, not fully explain cardiovascular risk in general. In this thesis we focused on two conventional risk factors (smoking, blood pressure), and two unconventional risk markers (adiponectin, an adipocyte derived protein; and sialic acid (SA), a marker of systemic inflammation) for prediction of CVD events.

Aims. In Paper I we examined to what degree smoking habits modify the risk of CVD in relation to systolic blood pressure levels in middle-aged men. In Paper II we investigated the predictive role of adiponectin for risk of CVD as well as the cross-sectional associations between adiponectin and markers of glucose metabolism, also in men. In Paper III we examined if increasing pulse pressure (PP) and increasing levels of SA both increase the risk of CVD and whether their effects act in synergism. In Paper IV the association of SA with risk of incident diabetes mellitus and related complications, resulting in hospitalization, was studied.

Subjects and Methods. Two large-scale, population-based, screening studies with long follow-up periods have been used. The Malmö Preventive Project (MPP) was used with 22,444 individuals in Paper I and a sub cohort of 3,885 individuals in Paper II. The Värmland Health Survey (VHS) was used in Papers III and IV with 37,843 and 87,035 individuals, respectively.

Results. CVD risk increases with increasing systolic blood pressure levels and this risk is almost doubled in smokers. Total adiponectin level is not associated with increased risk of future CVD but it is inversely associated with markers of glucose metabolism. PP and SA both contribute to risk of future CVD. Adjustment for mean arterial pressure reduces the risk induced by PP. Elevated SA contributes to increased risk of incident diabetes and related complications leading to hospitalization.

Keywords: Adiponectin, blood pressure, cardiovascular risk, diabetes, inflammation, pulse pressure, sialic acid, smoking.

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Abbreviations

ALT Alanine aminotransferase
AST Aspartate aminotransferase
AUC Area under the curve
BMI Body mass index
BP Blood pressure
CAD Coronary artery disease
CHD Coronary heart disease
CI Confidence interval
CRP C-reactive protein
CV Coefficient of variation
CVD Cardiovascular disease
DBP Diastolic blood pressure
HDL-C High-density lipoprotein
HMW High-molecular weight
HR Hazard ratio
ICD International classification of diseases
IPR Swedish national inpatient register
IL-6 Interleukin-6
LDL-C Low-density lipoprotein
MAP Mean arterial pressure
MGR Multi-generation register
MPP Malmö preventive project
OGGT Oral glucose tolerance test
PP Pulse pressure
RERI Relative excess risk due to interaction
RR Risk ratio
SA Sialic-acid
SBP Systolic blood pressure
SD Standard deviation
tHT Treated hypertension
TNF-α Tumour necrosis factor
VHS Värmland health survey
WBC White blood count
List of papers

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

I  Payam Khalili, Peter M Nilsson, Jan-Åke Berglund, Göran Berglund
*J Hypertens*. 2002 Sep;20(9):1759-64

II  Payam Khalili, Allan Flyvbjerg, Jan Frystyk, Fredrik Lundin, Johan Jendle, Gunnar Engström, Peter M Nilsson
Total adiponectin does not predict cardiovascular events in middle-aged men in a prospective, long-term follow-up study.
*Diabetes Metab*. 2010 Apr;36(2):137-43

III  Payam Khalili, Johan Sundström, Stanley Franklin, Johan Jendle, Fredrik Lundin, Ingmar Jungner, Peter Nilsson
Combined effects of brachial pulse pressure and sialic acid as risk for cardiovascular events during 40-years of follow-up in 37,843 subjects.
*J Hypertens*. 2012 Sep;30(9):1718-24

IV  Payam Khalili, Johan Sundström, Johan Jendle, Fredrik Lundin, Ingmar Jungner, Peter M Nilsson
Sialic acid and incidence of hospitalization for diabetes and its complications during 40-years of follow-up in a large cohort: The Värmland Survey.
*Submitted*

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Background

Chest pain has long been recognized as a serious medical phenomenon and a symptom of coronary disease. Hippocrates (460-375 BC) described it: “sharp pains, irradiating soon towards the clavicle and towards the back are fatal” or “frequent recurrence of cardialgia, in an elderly person announces sudden death” [1]. Another ancient description is “cardiac disease is an inflammation in the region of the heart due to a heaping-up or stoppage of the corpuscles” [2]. This inflammatory theory predated the current debate on the role of inflammation in atherosclerosis by 2500 years. Hippocrates also accurately described stroke, prodromal symptoms, and transient ischemic attacks as “apoplexia” [3].

Cardiovascular disease (CVD), including coronary heart disease (CHD) and stroke, is the leading causes of death worldwide. Of an estimated 58 million deaths worldwide in 2005, CVD accounted for 30% [4]. A significant proportion of these deaths (46%) were of people under 70 years of age, the more productive period of life, and 79% of the disease burden attributed to CVD is found in this age group [5]. CVD is responsible for 42% of all deaths in women below 75 years of age in Europe; and the corresponding figure for men is 38% [6]. Although a decline in age-standardized CVD mortality has been observed in many affluent European countries during the recent decades, the burden of CVD remains high, especially in several eastern European countries [7].

Risk factors for CVD

Conventional risk factors

Well-established risk factors for CVD are increasing age, male sex, a sedentary lifestyle, obesity, smoking, diabetes, hypertension and dyslipidaemia, especially hypercholesterolemia. Another important risk marker is low socio-economic status [8, 9]. Various algorithms have been designed for predicting coronary risk [10-13].

The SCORE chart, recommended by the European Society of Cardiology [12, 14] estimates the 10-year risk of a first fatal atherosclerotic event. All ICD (International Classification of Diseases) codes that could be assumed to be associated with atherosclerosis are included. Individuals with a 10-year risk of cardiovascular death ≥ 5% should be considered for interventions. While SCORE estimates the risk of CVD mortality, the summarized risk of total fatal and non-fatal CVD events is higher. Data from Finland
suggests that at the level (5%) at which risk management advice is likely to be intensified, total event risk is approximately 15% [15].

‘Fixed’ factors that increase risk of CVD are increasing age and male sex. Both are used to stratify risk assessments [12]. Exposure to common risk factors such as hypertension and diabetes also increases with age. Another important risk factor is smoking which however decreases in frequency in the elderly because of selective survival and advice to quit smoking from health care providers. Other risk factors such as physical inactivity and low-socio-economic status also contribute to the increased risk [16, 17].

A sedentary lifestyle is a major risk factor for CVD [18] and responsible for about one-third of deaths due to CHD and type 2 diabetes [19]. Regular physical activity reduces the risk of cardiovascular events in healthy individuals by 30-50% [20, 21] and in subjects with coronary risk factors by approximately 40% [22].

Physical activity improves endothelial function [23], helps to control body weight and lowers the risk of developing the risk of diabetes type 2 [24]. Physical activity also prevents or delays the development of hypertension in normotensive subjects, reduces blood pressure (BP) in hypertensive subjects [25], and in addition also improves the lipid profile [26].

Overweight (BMI 25-29.9 kg/m²) and obesity (BMI ≥ 30 kg/m²) are highly related to cardiovascular morbidity, mortality and total mortality [27, 28], and obesity is becoming a worldwide epidemic in both children and adults. It has been estimated that in the USA if obesity trends continue unchecked, obesity will increasingly offset the positive effects of declining smoking rates [29]. A U-form association exists between BMI and total- and CVD- mortality. Below 22.5–25 kg/m², there is an inverse association with BMI, which is believed to be predominantly due to strong inverse associations for smoking-related respiratory disease (including cancer) [27].

Several prospective studies have found evidence of stronger associations of abdominal adiposity measures with CHD than with BMI and CHD in women [30, 31] but not in men. One case-control study showed that the waist-to-hip ratio was to a greater extent associated with myocardial infarction than was BMI in both men and women [32]. In the multi-centre European Prospective Investigation into Cancer and Nutrition (EPIC) cohort study, BMI, waist circumference, and waist-to-hip ratio were independently associated with total mortality. No direct comparisons of associations between the different measures were made [33].

Some ambiguity exists as to whether BMI, waist circumference, or waist-to-hip ratio best associates with diabetes and whether there are any sex-differences. In a meta-analysis of 32 studies no overall difference was ob-
served [34] and the European Society of Cardiology has recently suggested that in routine clinical practice there does not appear to exist strong evidence that measurements of waist or direct measurement of fat mass should replace BMI [14].

Smoking is responsible for 50% of all deaths in smokers, half of these due to CVD. Smoking enhances the development of atherosclerosis through adverse effects on endothelial function [35, 36], oxidative processes, platelet function [37], fibrinolysis and inflammation [38, 39]. There is a clear relation between the associated risk and the amount of daily smoked tobacco and strong evidence that passive smoking increases the risk of CHD [40]. In Paper I of this thesis we looked at how smoking modifies the blood-pressure induced risk of cardiovascular events and mortality.

In the past two decades it has become clear that adipose tissue is a metabolically active endocrine organ excreting several proteins including leptin, adiponectin, resistin and tumour necrosis factor-α(TNF-α). These metabolically active proteins may play a role in glucose metabolism and may affect cardiovascular risk [41]. The inflammatory cytokine TNF-α was the first adipose-derived factor suggested to represent a link between obesity, inflammation and diabetes. Increased levels of TNF-α mRNA expression in adipose tissue have been reported [42] and a number of studies have demonstrated that TNF-α can impair insulin signalling [42, 43]. Adiponectin, one of the adipokines, and sialic acid, a marker of systemic inflammation, will be further discussed in Papers II-IV of this thesis.

The risk of cardiovascular events is 2-3 times higher in patients with type 1 or type 2 diabetes [44, 45] and CVD accounts for 60% of total mortality in these subjects. Epidemiological evidence shows that the positive association between increasing blood glucose levels and the corresponding elevated CVD risk begins before diabetes manifests itself [46, 47]. In a meta-analysis of individuals without diabetes, those with the highest blood glucose levels had a relative risk of 1.3 for cardiovascular events compared with those with the lowest blood glucose levels [48]. Several important intervention studies have shown that glycaemic control is of crucial importance for avoiding both macro- and microvascular complications of diabetes [49-51].

It is estimated that about seven million premature deaths throughout the world are due to high BP [4]. Observational data involving > one million individuals have indicated that death from both CHD and stroke increases progressively and linearly starting with BP levels as low as 115 mmHg systolic and 75 mmHg diastolic upwards [52]. Treating raised blood pressure has been associated with a 35-40% reduction in the risk of stroke and at least a 16% reduction in the risk of myocardial infarction [53]. In sub-
jects aged 50 years or older, increasing pulse pressure (SBP minus DBP) has been shown to be a better predictor of cardiovascular outcomes than either SBP or DBP [54]. However, one large meta-analysis of observational data showed that pulse pressure (PP) was less predictive than both SBP and DBP. This study also confirmed the increasing contribution of PP to CVD risk above age 55 years [52]. In Paper III in this thesis we studied whether this contribution was affected by levels of sialic acid as a marker of inflammation.

Hyperlipidaemia and dyslipidaemia, especially hypercholesterolemia, plays a crucial role in the development of CVD and a strong and graded positive association exists between total cholesterol as well as LDL cholesterol and risk of CVD [55]. Reducing plasma LDL cholesterol also reduces CVD risk and must be of prime concern in the prevention of CVD. Every 1.0 mmol/L reduction in LDL-C is associated with a corresponding reduction in CVD mortality and non-fatal myocardial infarction [56]. Low concentrations of HDL-C, on the other hand, are associated with higher CVD risk and are common in high-risk patients with type 2 diabetes and, abdominal obesity and in physically inactive individuals [57]. However, recent genetic findings based on so called Mendelian randomization question the causality between HDL-C levels and the risk of CHD [58].

Moderate, rather than severe hypertriglyceridemia, is an independent risk factor for CVD but not as strong as hypercholesterolemia [59]. Recent large prospective studies reported that non-fasting TG predicts CHD risk more strongly than fasting TG levels [60, 61].

Apolipoprotein B (the main apoprotein of atherogenic lipoproteins) levels have been measured parallel with LDL cholesterol and can substitute for LDL-C [62]. However LDL-C appears to be a better index of the adequacy of LDL-lowering therapy [63]. The major apoprotein of HDL-C is Apolipoprotein A1 and one of the strongest risk markers for CHD is the apoB:apoA1 ratio [62, 64].

Novel biomarkers of cardiovascular risk
Risk-scoring systems such as SCORE and the Framingham Risk Score evaluate traditional risk factors for improvement of risk prediction. Data from the global case-control study INTERHEART suggest that nine biological or lifestyle factors (six for increased and three for decreased risk) may jointly explain up to 80-90% of all cases of myocardial infarction [64]. However, it has also been reported that traditional risk factors do not fully explain inter-individual variation in cardiovascular risk. For instance, a large proportion of individuals who develop CVD have few or no risk factors at all [65]. Therefore, during the past decade there has been in-
increased interest in “novel” biomarkers for identification of individuals at risk for developing CVD.

Two major groups of systemic biomarkers relevant to CVD have been identified: inflammatory biomarkers such as high sensitive C-reactive protein (CRP) [66, 67] and fibrinogen [68] and thrombotic biomarkers such as homocysteine [69] and lipoprotein-associated phospholipase (LpPLA₂) [70]. Several other biomarkers including N-terminal brain natriuretic pro-peptide (NT-pro-BNP) [71], D-dimer, [71] and interleukin-6 [72] have been related to incident cardiovascular events. However, due to lack of specificity, lack of dose-effect or causality relationship and lack of specific therapeutic strategies, these biomarkers have shown only limited additional value in risk assessment and preventing actions.

Inflammation

Inflammation and atherosclerosis

Inflammation plays an essential role in the initiation and progression of atherosclerotic lesions and plaque disruption [73, 74]. The infiltration and retention of LDL-C in the arterial intima initiates an inflammatory response in the artery wall [75]. Oxidation of LDL or enzymatic attack in the intima leads to the release of phospholipids that can activate endothelial cells [76] which cause increased expression of adhesion molecules and inflammatory genes by endothelial cells, preferentially at sites of hemodynamic strain [77, 78]. Activated endothelial cells express several types of leukocyte adhesion molecules [79] and induce monocytes entering the plaque to differentiate into macrophages. This step is critical for the development of atherosclerosis [80]. The activated macrophage produces inflammatory cytokines, proteases and growth factors [81]. These inflammatory processes in the arterial wall play an essential role in the progression of atherosclerotic lesions and plaque disruption [73]. Cytokines, enzymes and growth factors can induce further damage and eventually lead to focal necrosis, figure 1. Cycles of accumulation of mononuclear cells, migration and proliferation of smooth muscle cells, and formation of fibrous tissue lead to further enlargement and restructuring of the lesion and it becomes covered by a fibrous cap that overlies a core of lipid and necrotic tissue.
**Inflammation and Smoking**

Since atherosclerosis constitutes an inflammatory process and cigarette smoking has been associated with systemic inflammation, it has been hypothesized that inflammation may serve as one of the mechanisms by which cigarette smoking affects CVD [82, 83]. CRP levels and white blood cell (WBC) count are higher among current smokers compared to never-smokers [84-86]. Positive, independent relationships between the number of cigarettes/day and elevated levels of CRP and WBC counts have also been described [87].

**Inflammation and Diabetes**

Early observations of elevated inflammatory markers in diabetes were rapidly followed by prospective studies demonstrating that CRP, IL-6 and WBC were all independently associated with incident type 2 diabetes [88, 89]. The association with CRP may be stronger in women than men but this needs further investigation [90]. Several other acute phase response markers such as fibrinogen and orosomucoid as well as low serum albumin...
have also been linked to risk of type 2 diabetes. However, whether these inflammatory markers are causally linked to diabetes deserves further investigation.

**Adiponectin**

Abdominal adipose tissue, which is regarded as an important endocrine organ, secretes a wide range of biologically active adipokines such as adipin [91], leptin [92], plasminogen activator inhibitor-1[92], resistin [93] and TNF-α[94]. Increasing evidence indicates that dysregulated adipokine production caused by excess fat accumulation contributes to obesity-associated complications. Most adipokines are up-regulated in obese states, and usually act as pro-inflammatory mediators that promote the disease process. Conversely, a few adipokines are down-regulated by obesity and these factors typically exert beneficial actions on obesity-linked disorders. Adiponectin is such an example of such adipokines that has attracted much attention; it was first identified in 1995 [95] and is exclusively secreted from adipose tissue. The adiponectin monomer (30 kDa) has a structure consisting of a globular head and a collagenous tail, and this monomer is able to multimerize to form several stable complexes of low-, medium-, and high-molecular weight (HMW). Adiponectin shares sequence homology with collagens VIII and X as well as complement factor C1q [96, 97]. The three multimeric forms are all found in the circulation. Initially it was not clear which adiponectin forms were biologically active. However the current consensus is that the HMW form may be the more clinically relevant.

Adiponectin activates adenosine mono-phosphate activated protein kinase in the liver and skeletal muscle, thereby stimulating phosphorylation of acetyl-CoA carboxylase, fatty acid oxidation. This reduces tissue triglyceride (TG) content in muscle and liver. These changes increase insulin sensitivity *in vivo* [98] and there is also a strong negative correlation between plasma adiponectin concentration in humans and fat mass [99], with obesity reducing adiponectin levels while weight reduction increases adiponectin [100]. Low adiponectin levels are inversely related to high levels of CRP in patients with obesity [101, 102]. Other adipokines, such as leptin, TNF-α IL-1β IL-6, and IL-8 are also pro-inflammatory and increased in obesity [103, 104]. It has also been suggested that there is a decreased adiponectin level in current smokers and this reduction can be reversed by quitting smoking [105].
**Adiponectin and diabetes**
Adiponectin is strongly associated with insulin resistance [106-108]. Physical activity increases insulin sensitivity and increases circulating adiponectin concentrations and the expression of adiponectin receptors in skeletal muscle. Adiponectin levels begin to decrease early in the pathogenesis of diabetes, while adipose tissue increases in tandem with reduction in insulin sensitivity. Hypoadiponectinemia has also been associated with beta cell dysfunction [109]. Furthermore, it has also been linked to future development of insulin resistance and type 2 diabetes mellitus [110, 111].

Additionally, animal experiments have demonstrated that adiponectin can reduce insulin resistance and enhance the action of insulin in liver, resulting in lowering of glucose blood levels [112].

**Adiponectin and cardiovascular disease**
In 1999, a potential anti-atherogenic action of adiponectin was indicated in vitro studies, which showed that adiponectin inhibits monocyte adhesion to aortic endothelial cells and suppresses macrophages-to-foam cell transformation [113-115]. In adiponectin-deficient mice, neo-intimal thickening and increased proliferation of vascular smooth muscle cells were found in response to external arterial injury [116]. It has also been shown that adiponectin acts as an endogenous antithrombic factor [117].

Low total adiponectin is associated with increased carotid intima-media thickness [118, 119]. However, the relationship between adiponectin levels and coronary artery disease and acute coronary syndrome is not straightforward. Several cross-sectional studies of diverse populations have documented an inverse association between lower plasma levels of total adiponectin and higher prevalence of CAD [120-122]. Other studies found no association between total adiponectin levels and the incidence of CAD [123-125]. In addition, although the HMW-adiponectin has been suggested to be more closely associated with incident CHD [126], results from other studies are still contradictory [127, 128].

**Sialic acid**
Sialic acids (SAs) are acetylated derivatives of neuraminic acid and are attached to non-reducing residues of the carbohydrate chains of glycoproteins and glycolipids [129]. In human plasma, a large quantity of SA is found in several acute-phase proteins such as orosomucoid, α1-antitrypsin, haptoglobin, ceruloplasmin, fibrinogen, complement proteins and transferrin [130]. SA has also been positively correlated to TNF-α and IL-6 levels [131].

The negative charge of SA in physiological pH directly relates to the functions of SA in organisms. The presumed functions of SA compiled by
Schauer et al. [132] include stabilisation of the conformation of glycoproteins and cellular membrane which assist in cell-cell recognition and interaction and serving as chemical messengers in tissue and body fluids, impacting transmembrane transportation mechanisms, affecting the function of membrane receptor molecules by developing binding sites for ligands, antibodies, enzymes, microbes or by blocking them.

The normal total sialic acid level in serum/plasma is 0.52-0.73 mg/L [133]. Some reports have documented slightly elevated serum SA concentrations with aging. Such findings might be explained by a higher frequency of individuals with sub-clinical disease among the elderly [133]. In a small sub-cohort, from the same cohort as used in Papers III and IV in this thesis, elevated SA levels have been reported in young male smokers but not in smoking women [134]. Elevated SA levels have been observed in association with several malignancies, and SA levels correlate positively with the degree of metastasis and may therefore be useful in monitoring treatment [135]. Some findings also suggest that SA levels could be elevated in cancer patients before the occurrence of clinical symptoms [136]. The mechanisms underlying the elevated SA concentrations in different disease states are not clear. Certainly, acute-phase protein response is one reason for elevated values [130]; however, the non-specificity of serum SA limits its clinical usefulness.

**Sialic acid and CVD**

Elevation in serum SA concentrations has been described in association with dyslipidaemia [137]. Elevated SA levels have previously also been observed to predict CVD in parts of the same cohort as in Papers III and IV [138, 139] in this thesis and in other cohorts [140]. SA has also been significantly elevated in patients with acute myocardial infarction [141]. Moreover, it has been shown that serum SA levels correlates with carotid atherosclerosis independent of major CVD risk factors [142].

The biomedical mechanism behind the correlation between SA and CVD is unknown. SA might reflect the existence or the activity of an atherosclerotic process and, furthermore, of increased thrombogenic activity as related to the raised fibrinogen levels, which has also been correlated with SA levels [141].

**Sialic acid and diabetes**

Crook et al. have shown that serum SA level is raised in patients with type 2 diabetes mellitus [143]. There is also a positive association between sialic acid and CHD in men with type 2 diabetes [144]. For microvascular complications, cross-sectional studies have shown a correlation with elevated
SA concentrations in patients with type 1 and type 2 diabetes and retinopathy [145] or albuminuria [146, 147] compared with people without theses complications. The cross-sectional analyses of the EURODIAB study showed elevated SA concentrations in patients with type 1 diabetes and retinopathy (men), neuropathy (men) and albuminuria (men and women) [148].
Aims
In this thesis the focus has been on conventional (smoking, blood pressure), as well as on some unconventional (the adipocyte-derived protein adiponectin as well as the inflammatory marker sialic acid) risk factors or markers for prediction of future cardiovascular events or hospital-treated diabetes based on endpoint data derived from national registers.

Specific aims
The aims of the subsequent four papers have been to:

1) To examine to what degree smoking habits modify the risk for cardiovascular morbidity and mortality in relation to systolic blood pressure levels in middle-aged men from a defined population (Paper I).
2) To investigate the predictive role of adiponectin for risk of first fatal or non-fatal cardiovascular event in middle-aged men, and in addition to investigate cross-sectional associations between adiponectin levels and markers of glucose metabolism (Paper II).
3) To examine whether increasing pulse pressure and increasing levels of sialic acid both increase the long-term risk of cardiovascular events and if their effects act in synergism in a population of men and women aged 50 years and above (Paper III).
4) To investigate the association of sialic acid with long-term risk of incident diabetes mellitus and related complications resulting in hospitalization in a defined population of men and women over a wide age range (Paper IV).
Subjects and Methods

Malmö Preventive Project

*Subjects in papers I and II*

Data from the Malmö Preventive Project (MPP) was used in papers I and II. The MPP was a large-scale, population-based, preventive case-finding programme for detecting cardiovascular risk factors, alcohol abuse and breast cancer in the general population. It was conducted in Malmö (250,000 inhabitants), the third largest city in Sweden, between 1974 and 1992 [149]. Birth cohorts were invited through a personal letter of invitation to participate in the project (men born in the years 1921, 1926 -1942, 1944, 1946 and 1948-9 and women born in 1926, 1928, 1930 -1936, 1938, 1941-42 and 1949). All together 22,444 men and 10,902 women attended [150, 151] the baseline examination with an overall attendance rate of 71% (range 64-78%). All participants underwent a baseline health examination, including a physical examination and a panel of laboratory tests. Additionally, every participant filled out a self-administered questionnaire, which included questions on sleep disturbance, family history, lifestyle (smoking, alcohol consumption and physical activity) social background characteristics and subjective health. Various interventions such as lifestyle modifications or drug therapy were offered to about 20% of the screened subjects because of cardiovascular risk factors or over-consumption of alcohol [149]. Because of longer follow-up in men (Paper I) and since oral glucose tolerance test (OGTT) was only performed in men, only data for men has been analysed in Papers I and II, figure 2.
Physical examination
After the participants received verbal information about the study, their weight (kg) and height (m) were measured while they wore light indoor clothing and their body mass index (BMI) was calculated (kg/m²). Blood pressure (mmHg) and heart rate (beats/min) were measured twice in the supine position after a 10-min rest by use of a sphygmomanometer with a modifiable cuff width and a chronometer. Following that a mean figure was recorded.

Laboratory methods
Blood samples were drawn from each individual (after overnight fasting). Serum total cholesterol, triglycerides and fasting blood glucose were analysed, using routine methods at the Department of Clinical Chemistry, Malmö University Hospital. Whole blood was stored in a biobank for later genetic analysis [152]. An OGTT, 75 gram of glucose and multiple deter-
determinations of blood glucose levels at 0, 20, 40, 60, 120 minutes, was performed in a subgroup of 3885 men, born in a pre-specified year and living in Malmö; they were not selected by any other criteria. Blood glucose was measured using a hexokinase method [150] and plasma insulin levels measured in mIU/L using a non-specific radio immunoassay [153]. Intra- and inter-assay coefficients of variation were 5 and 8% respectively.

Plasma adiponectin (mg/L) was determined by an in-house (Aarhus University Hospital, Denmark) time-resolved immunofluorometric assay (TR-IFMA), based on a method using two monoclonal antibodies and recombinant human adiponectin (R&D Systems, Abingdon, UK) [154]. The intra-assay coefficient of variation was less than 5% and the inter-assay CV was less than 10%.

**Follow-up procedure in Papers I and II**

Using the unique 10-digit personal identification number assigned to each Swedish citizen, we linked our cohort with local and national registers provided by the Swedish Board of Health and Welfare. The study participants discussed in Paper I were followed-up until the end of 1996 for total mortality, cause-specific mortality, non-fatal ischemic heart disease, and non-fatal stroke (ICD 9 numbers: 410-414 and 430-438).

The study participants discussed in Paper II were followed-up until the end of 2004 in national registers (Swedish Board on Health and Welfare) for fatal and non-fatal coronary events and strokes (ICD 8th and 9th versions 410-414 and 430-436, and ICD 10th versions: I20 -I25 and I60 -I64).
Värmland Health Survey

Subjects in Papers III and IV
The Swedish National Board of Health decided in 1961 to undertake a general health survey in the county of Värmland and also in parts of the county of Gävleborg in Sweden, the so called Värmland Health Survey (VHS), figure 3.

The survey was conducted between 1962 and 1965 in connection with the mass-screening programme of miniature photo fluorography (x-ray) of the chest [155-157]. The aim of the survey was to perform a chemical mass-screening to identify pre-symptomatic disease, especially different types of CVD, malignancies and inflammatory conditions. Improved laboratory techniques and automated laboratory methods were used for screening. Simple measurements of some physical variables were also performed. The

Figure 3. Map of Sweden
examination included a questionnaire with questions on previous hypertension, albuminuria, diabetes and anaemia or any infection during the previous three weeks. No information on diet, smoking habits, alcohol consumption or number of pregnancies was collected.

In three districts of Värmland (Arvika, Hagfors and Karlstad) and in one district of Gävleborg, (Hofors-Torsåker), inhabitants 25 years of age or older were invited to participate in the survey. Despite the age limit, a small number of individuals younger than 25 years of age also participated. All together 97,273 subjects underwent the screening, (~76% attendance in Värmland and 86% in Gästrikland), figure 4.

The large amount of data collected has previously been described and used in two different projects for studying the role of sialic acid in prediction of CVD by Lindberg et al [138, 139] and the role of cholesterol in prediction of cancer by Törnberg et al. [158, 159].

Since PP progressively increases after the age of 50 [160], the analyses discussed in Paper III were carried out in individuals at the age of 50 years or older at the time of screening (n=45,889). No data on emigration during the follow-up period was available. To minimize the risk of possible emigrants to contribute with too long follow-up time, individuals were followed-up long-term until their 81st birthday. Because no data on previous CVD or usage of cardiovascular drugs were available at screening, we excluded individuals with incident CVD events during the first five years of follow-up, to minimize the potential influence of pre-existing cardiovascular disease and co-morbidity. We excluded individuals with extreme data on blood pressure levels, SA or cholesterol, defined as outliers (>75th centile +3 x interquartile range) or (<25th centile -3 x interquartile range), and individuals with missing data on systolic blood pressure, diastolic blood pressure, sialic acid, cholesterol and socio-economic position, leaving 18,429 men and 19,414 women in the study.

For the same reasons as given in Paper III, individuals in Paper IV were followed-up until their 81st birthay. Because no data on existing diabetes or pervious drug usage were available at the screening, we excluded individuals with incident diabetes events or diabetes associated complications during the first five years of follow-up. We excluded individuals with extreme data on blood pressure levels, SA, cholesterol, aspartate aminotransferase (AST) and alanine aminotransferase (ALT), and individuals with missing data on systolic blood pressure, diastolic blood pressure, SA, cholesterol and socio-economic position, leaving 42,639 men and 44,396 women in the study.
Physical examination
The screening included measurement of blood pressure in the sitting position which was measured to the nearest 10 mmHg with a sphygmomanometer by use of an appropriate cuff, reflecting a simplified screening approach from the early 1960’s. Height (m) and weight (kg) were measured.

Laboratory methods
Venous blood was drawn for a chemical test batter. No requirement was made for fasting. All serum samples were chilled and sent by night-train in specially made boxes to Stockholm where they were analysed at the automated analytical laboratory (MEKALAB) in Stockholm, operated by Gun-
The SA concentration was analysed by the Svennerholm method [161]. The modified Liebermann-Burchard method described by Zak et al. [162] was used for the analyses of total cholesterol. AST and ALT were analysed by a method described by Reitman and Frankel and expressed in Karmen units (U) [163]. Calibration with standard serum was made after each 100 samples. No information is available from the chemical laboratory about method errors.

Follow-up procedures in Papers III and IV
With the use of unique 10-digit personal identification number, we linked our cohort with national registers provided by the Swedish Board of Health and Welfare. Subjects had been followed until end of 2005 for first fatal and non-fatal coronary events, stroke and other atherosclerosis-related disease manifestations (International Classification of Disease: ICD 7th version; 420-445, 454 and 450-453, 8th and 9th versions 410-414 and 440-445 and ICD 10th versions: I10-I14, I60-I69 and I70-I73) and for first fatal or non-fatal diabetes event treated in hospital, as diagnosed either as diabetes mellitus type 1, diabetes mellitus type 2 or as any diabetes-related complication (ICD 7th version; 260, 8th and 9th versions 250 and ICD 10th versions: E10-E11 and E13-E14). We also linked our cohort with data from the Swedish population and housing census in 1960 (“Folk- och Bostadsräkningen”, FoB, Statistics Sweden) to obtain socio-economic data. Subjects were stratified into social categories based on occupation: (a) non-manual workers at higher level; (b) non-manual workers at intermediate level; (c) non-manual workers at lower level; (d) farmers; (e) skilled and unskilled workers; and (f) others.

Ethics
Studies I and II were approved by the Regional Ethics Committee in Lund (Dnr: DNR: 85/2004) and studies III and IV was approved by the Regional Ethics Committee in Stockholm (Dnr: 2008/356-32).
Statistical methods
This thesis presents four epidemiological studies, all conducted in Sweden.

Paper I
In the first paper subjects were divided into quintiles (Q1-Q5) of mean systolic blood pressure. Subjects with treated hypertension (tHT) were excluded and studied separately. Age-adjusted rates of first cardiovascular events (fatal or non-fatal) were calculated using direct standardization and presented as age-adjusted morbidity/10,000 person-years rates with 95% confidence intervals. In each quintile (Q1-Q5) and the tHT-group separately, subjects were studied according to smoking status (non-smokers and smokers) for mortality and morbidity risk; the results were presented as risk ratios (RR) with 95% confidence intervals (95% CI). Similar analyses were then first made between current-, former- and never-smokers and secondly between non-smokers and the two categories of current smokers (≤ or > 10 cigarettes/day). In these analyses no further adjustments were carried out for other risk factors.

Paper II
In this paper subjects were divided into quintiles, wherein subjects in Q1 had the lowest adiponectin levels and those in Q5 had the highest. The “area under the curve” (AUC) for glucose levels during an oral glucose tolerance test was calculated and the association between adiponectin levels and glucose metabolism (glucose at 120 minutes during OGTT, AUC-glucose, and HOMA-IR index) was analysed separately using multiple regression analyses. Models were adjusted for BMI, SBP and total fasting cholesterol as well as triglyceride levels. The homoeostasis model assessment of insulin resistance (HOMA-IR) index was calculated as fasting glucose x insulin/22.5.

The association between baseline adiponectin and CVD risk was analysed by Cox proportional-hazards regression. The first fatal or non-fatal cardiovascular event was used and, as almost 50% of the subjects were current smokers, we decided to stratify for smoking [105]. Regressions were performed separately for stroke and CHD and then combined as CVD. Stepwise adjustment was made for BMI, SBP, total fasting serum cholesterol, fasting serum triglycerides (log-transformed), and AUC-glucose during OGTT. Quintile 5 was used as reference group. Results are given as hazard ratios for decreasing adiponectin levels after each step of adjustment. Kaplan-Meier plots were drawn for the associations between quintiles of adiponectin and risk of CVD events after adjustment for covariates.
Paper III
In Paper III, we started with investigating the biological interaction, as defined by Rothman [164], between gender and pulse pressure by calculating the relative excess risk due to interaction (RERI) using the method outlined by Andersson et al. [165]. The same method was used to investigate the biological interaction between gender and sialic acid. The lowest and highest tertiles for SA and PP were used for these analyses. No biological interaction was observed between gender and SA but since we had noticed an interaction between gender and PP, we performed further analysis separately for men and women. To describe the prediction of SA and PP on absolute risk of the composite of first coronary events, stroke, and atherosclerotic events, we created four different sub-cohorts for each gender. The participant were divided into groups of SA and PP below median (SA-/PP-), SA above and PP below median (SA+/PP-), SA below and PP above (SA-/PP+) and SA above and PP above median (SA+/PP+). Unadjusted Kaplan-Meier curves were used to present the absolute risk of the composite of first coronary events, stroke and atherosclerotic events in these groups. Using the long-rank test we tested for differences in absolute risk between the reference group (SA-/PP-) and the other three groups. To illustrate the magnitude of the differences on the relative hazard scale we used a Cox-proportional hazards model using SA/PP group as explanatory variable, both in unadjusted and adjusted analysis, adjusting for potential confounders (age at screening, BMI, cholesterol, with or without mean arterial pressure (MAP), and socio-economic status).

Finally we used adjusted (for age at screening, BMI, cholesterol, with or without MAP and socio-economic status) Cox regression analyses separately for men and women to analyse the effect of SA and PP on the risk of the composite of first coronary events, stroke, and atherosclerotic events. Results are presented as hazard ratios with 95% confidence intervals.

Paper IV
Initially, we investigated the biological interaction between gender and SA by calculating the relative excess risk due to interaction (RERI). In these analyses SA was divided into two groups of values, below and above the median level. Since we observed an interaction between gender and SA, we performed further analyses separately for men and women.

To describe the association of SA levels with first recorded diabetes-related event (based on hospitalization), we created four different subgroups. For each gender we constructed two groups of SA dichotomised at or above median and below median as: SA+/male, SA-/male; and SA+/female, SA-/female. Unadjusted event-free survival curves were used to
present the absolute risk of incident cases of diabetes mellitus or related complications. We tested for differences in absolute risk between SA+/male vs. SA-/male and SA+/female vs. SA-/female using the log-rank test combined with univariate Cox regression analyses to estimate the effect size of the difference on the relative hazard scale.

Finally, we performed Cox regression analyses with adjustment for age at screening, BMI, cholesterol, AST, ALT and socio-economic status to analyse the independent effect of SA on the risk of diabetes-related hospitalizations.
Results

Paper I
The baseline characteristics of subjects belonging to the quintiles Q1–Q5 of SBP, as well as the tHT group, are presented in in Table 1 in Paper I. As expected, the tHT group showed the highest mean age and the highest age-adjusted cardiovascular morbidity rate in spite of treatment.

In our comparison between smokers (n=7848) and present non-smokers, consisting of ex-smokers (n=4012) and never-smokers (n=4216), we found, that both cardiovascular morbidity and mortality are almost twice as common in smokers compared to non-smokers. For each quintile, the morbidity relative risk (RR) between these two groups, are 1.9 (95%CI: 1.5–2.4), 2.1 (1.8–2.5), 2.3 (1.8–2.9), 1.8 (1.5–2.1), and 1.7 (1.5–2.0), compared to non-smokers (reference), in relation to SBP (Q1–Q5). In tHTs the RR was 1.4 (1.1–1.8). Corresponding mortality ratios for each quintile were RR 1.8 (1.4–2.3), 2.5 (2.1–3.0), 2.7 (2.0–3.6), 2.2 (1.9–2.7), 2.5 (2.1–2.9), and 1.8 (1.3–2.5) in the tHT group. For both cardiovascular morbidity and mortality outcomes the risk ratio decreases somewhat in the tHT group.

No difference in risk of cardiovascular morbidity was found for never-smokers (n=4216) as compared to ex-smokers (n=4012), see figure 4 in Paper I. In an effort to learn whether cardiovascular morbidity was dependent on the quantity of cigarettes smoked each day, almost no difference was found between the two categories of smokers (<= or > 10 cigarettes/day) up to the fourth quintile. The difference increased in the fifth quintile (P < 0.005).

Paper II
Since the study subjects were recruited from the same age cohort, the mean age in our five quintiles was approximately 47 years in all. Details of baseline characteristics are presented in table 1 of Paper II. Mean values of age, weight, BMI, diastolic blood pressure, fasting serum triglycerides and different markers of glucose metabolism decreased significantly from Q1 to Q5.

In the multiple regressions analyses, cross-sectional associations between plasma total adiponectin and fasting plasma glucose, p-glucose at 120 minutes during OGTT, AUC-glucose and HOMA-IR index were all statistically significant. The total follow-up period for the entire cohort was 90,949 person-years. The total numbers of CVD events were 244, 214, 202, 209 and 219 in Q1-Q5.
In neither non-smokers nor in current smokers a significant relationship was observed across the quintiles in prediction of stroke, coronary events or total cardiovascular events. The only significant change in hazard ratio was observed in smokers for stroke in Q4 compared with Q5, but this could be an effect of multiple testing.

Paper III

The mean age of the cohort was 59.5 years and the mean BMI was 25.9 kg/m². The mean and median value of sialic acid was 700.7 mg/L (SD 81.8) and 700 mg/L, while the mean and median of pulse pressure was 70 mmHg (SD 18.4) and 70 mmHg in men, respectively. In women the mean and median of sialic acid was 714.4 mg/L (SD 82.5) and 700 mg/L, and the mean and median of pulse pressure was 76.4 mmHg (SD 19.6) and 70 mmHg, respectively. Details of the baseline characteristics are presented in table 1 of Paper III.

Since a significant RERI-estimate for the interaction between gender and PP and was observed, the following analyses were performed separately for men and women.

The total number of incident CVD events in men and women respectively were 3,641 (based on 299,102 person-years at risk) and 3,227 (based on 341,976 person-years at risk). In unadjusted models with the SA-/PP-group used as reference, in men the risk of morbidity was significantly higher for SA+/PP+ (hazard ratio (HR) 1.54, 95% CI: 1.41 to 1.69, p<0.0001) and for SA+/PP- (HR 1.26, 1.15 to 1.38, p<0.0001) and for SA-/PP+ (HR 1.29, 1.18 to 1.42, p<0.0001). In women, compared to the SA-/PP- group (reference), the risk was significantly higher for SA+/PP+ (HR 1.82, 1.65-2.00, p<0.0001) and for SA+/PP- (HR 1.26, 1.14 to 1.39, p<0.0001) and for SA-/PP+ (HR 1.54, 1.38 to 1.71, P<0.0001). When adjusting for age, BMI, cholesterol and socio-economic status the corresponding hazard ratios decreased but still remained significant for both men and women. After further adjustment for MAP, hazard ratios decreased even more in the sub-groups with PP-levels above median, Table 1.
Table 1. Relative risks of first cardiovascular event estimated by Cox regression analysis* (with 95% confidence intervals) according to SA/PP group belonging †

<table>
<thead>
<tr>
<th></th>
<th>Men</th>
<th>Women</th>
</tr>
</thead>
<tbody>
<tr>
<td>SA-/PP-</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>SA+/PP+</td>
<td>1.34 (1.22 to 1.47) P&lt;0.0001</td>
<td>1.49 (1.35-1.64) P&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>1.02 (0.92-1.13) P=0.73</td>
<td>1.10 (1.00-1.24) P=0.06</td>
</tr>
<tr>
<td>SA+/PP-</td>
<td>1.21 (1.10 to 1.33) P&lt;0.0001</td>
<td>1.18 (1.06 to 1.30) P=0.002</td>
</tr>
<tr>
<td></td>
<td>1.19 (1.09-1.31) P&lt;0.0001</td>
<td>1.14 (1.03-1.26) P=0.01</td>
</tr>
<tr>
<td>SA-/PP+</td>
<td>1.18 (1.07 to 1.30) P=0.001</td>
<td>1.35 (1.21 to 1.51) P&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>0.92 (0.84-1.02) P=0.12</td>
<td>1.04 (0.93-1.17) P=1.04</td>
</tr>
</tbody>
</table>

*All models adjusted for age, BMI, cholesterol and socio-economic position.
† SA+: Sialic-acid level above median, SA-: Sialic acid level below median, PP+: Pulse pressure value above median, PP-: Pulse pressure below median.
** Hazard ratios after further adjustment for mean arterial pressure (MAP)

The separate contributions to the relative risk of CVD from SA and PP as well as the interaction between the two variables were tested. The highest tertile of each variable (SA and PP) was tested. The relative excess risk due to interaction between SA and PP did not reach significance and the point estimates were generally small. In men, the interaction between SA tertile 3 and PP tertile 3 resulted in contribution to the relative risk of 0.02 (95% CI: -0.23 to 0.27) while the corresponding figure for women was 0.17 (95% CI: -0.63 to 0.97).

Our multivariable adjusted Cox-regression analyses were adjusted for age at screening, BMI, cholesterol, and socio-economic status. In both men and women PP and SA were observed to be significant risk factors for CVD, independently of each other. In men the hazard ratio for change of one standard deviation of PP was HR 1.10 (95% CI: 1.06 to 1.14, p<0.0001). However, after further adjustment for MAP the HR for PP
decreased to 0.92 (95% CI: 0.88 to 0.96, p<0.0001). The corresponding figures for SA were HR 1.10 (95% CI: 1.06 to 1.13, p<0.0001) and after further adjustment for MAP 1.09 (95% CI: 1.05 to 1.13, p<0.0001). For women the corresponding figures for PP were HR 1.20 (95% CI: 1.15 to 1.24, p<0.0001), and after adjustment for MAP 1.09 (95% CI: 1.05 to 1.13, p<0.0001). For SA the corresponding figures were HR 1.11 (95% CI: 1.07 to 1.15, p<0.0001) and after adjustment for MAP 1.09 (95% CI: 1.05-1.13, p<0.0001).

**Paper IV**

Baseline characteristics are described in table 1 of Paper IV. The mean age of the cohort was 47.2 years (SD 13.0) and the mean BMI was 25.0 kg/m² (3.4). The mean and median values of SA were 688.7 mg/L (77.9) and 680.0 mg/L in men. In women the corresponding figures were 694.5 mg/L (79.5) and 680.0 mg/L, respectively. The total number of incident hospitalizations for diabetes or related complications in men and women were 3445 (28.2/10,000 person-years at risk) and 3273 (23.4/10,000 person-years at risk), respectively. The incidence of diabetes or related complications was the highest in the SA+ group, for both men and women.

A significant negative RERI-estimate of -0.24 (95% CI: -0.36 to -0.12) for the biological interaction between gender and SA was observed. The increased risk in men with SA levels at or above the median was less than an additive effect. Therefore the following analyses were performed separately for men and women.

The unadjusted event-free survival curves during follow-up according to the four SA/gender subgroups are shown in figure 5.
a: SA below median in women  
b: SA below median in men  
c: SA at or above median in women  
d: SA at or above median in men

**Figure 5.** Unadjusted event-free survival without hospital-treated diabetes mellitus and its complications according to four SA/gender sub-groups, after dichotomisation at median of SA.

In unadjusted models, the diabetes risk was significantly higher for the SA+/male sub-group with hazard ratio HR 1.40 (95% CI: 1.31 to 1.50, p<0.0001) compared to the SA-/male subgroup. Also in women the risk was significantly higher for SA+/female subgroup compared to SA-/female subgroup, HR 1.85 (95% CI: 1.72 to 1.99, p<0.0001). Thus the relative intra-gender risk was higher in women than in men with above median SA levels, and with non-overlapping confidence intervals between genders. However, after adjustment for available covariates (age, BMI, SBP, cholesterol, AST, ALT and socio-economic status), the intra-gender comparison of the SA+/ SA- groups showed no significantly increased risk of diabetes associated with elevated SA.

Multivariable-adjusted survival analyses were performed separately for the entire group of men and of women. After adjustment for covariates, SA
remained a significant risk factor for incident diabetes-related hospitalizations. Hazard ratios per one standard deviation of SA were HR 1.12 (95% CI: 1.08 to 1.17, p<0.0001) in men and 1.17 (95% CI: 1.13 to 1.22, p<0.0001) in women.
Discussion

General discussion

The Framingham Heart Study, has become the worldwide standard for cardiovascular epidemiology based on screening [166]. Begun in 1948 it is among the longest running, most comprehensive multi-generational studies available in the world. Such seminal findings as the risk effects of smoking and high cholesterol on heart disease came from the Framingham Heart Study [167]. It has served as a model for several other observational studies. In Sweden a number of prospective cohort studies have been undertaken during the past 50 years. In Gothenburg all men born in 1913 on dates divisible by 3 were included in one study. Thus, 973 men were invited, and 855 were examined in 1963 at age 50 (attendance rate 88%). Further examinations were made at age 54, 60 and 67 [168-170]. Younger cohorts of 50-year-old men (i.e., men born in 1923, 1933, and 1943) were later examined every 10th year [171, 172]. In 1970, all men born from 1920 to 1924 and residing in Uppsala were invited to a health survey in which 82% (n = 2,322) participated [173]. These and other population-based screening studies, such as the Göteborg Primary Preventive Study [174, 175], the Västerbotten Study [176, 177], the Skaraborg Project [178], and the MONICA studies in western [179] and northern [180, 181] Sweden, have contributed substantially to the understanding of CVD epidemiology and the distribution of corresponding risk factors in Sweden.

Both the MPP and the VHS are prospective cohort studies using baseline health screening data. In the MPP the aim was to identify individuals at high risk for CVD and in the VHS the aim was to identify pre-symptomatic disease by chemical mass-screening. Both studies have contributed to the understanding of CVD risk factors. However, screening for CVD risk factors among healthy populations has not proven to be cost-effective and today Swedish authorities do not recommend it [150, 182].

Several associations have been tested in this thesis. In observational studies, an association between a modifiable risk factor or exposure of interest and an outcome may not be causal. It may be due to confounding factors that affect both an individual’s phenotype and the outcome. Although we can measure known confounders, we can never be certain that all confounders have been identified and therefore cannot interpret an estimate of association from an observational study as a causal effect. Mendelian randomization methodology uses genetic variants which are associated with the phenotype to estimate a causal effect [183]. For example, persistent inflammation has been implicated in the pathogenesis of CHD. C-reactive
protein is the most extensively studied systemic marker of inflammation [67]. Observational epidemiological studies have shown that CRP concentration is log linearly related to risk of subsequent CHD, though this association depends considerably on the levels of conventional risk factors. CRP binds to low density lipoproteins [184] and is present in atherosclerotic plaques. Randomised trials of interventions specific to CRP, however, have not yet been carried out in relation to vascular disease outcomes. In the absence of such trials, focused genetic studies can be used to help evaluate causality. To investigate whether long-term average (“usual”) concentration of CRP is causally relevant to coronary heart disease “mendelian randomisation” can be used. This procedure is based on Mendel’s second law, which states that alleles of different genes assort independently of one another during gamete formation. Consequently, mendelian randomisation analyses are based on Mendel’s observation that inheritance of one trait should be independent of inheritance of other traits [185]. For the causal assessment of CRP, a mendelian randomisation analysis should reduce confounding, provided the genetic variants used as proxies for concentration of CRP are unrelated to conventional vascular risk factors and other disease markers. Such studies should also avoid distortions caused by factors occurring later in life (such as the onset of disease) because genetic variants are fixed at conception [185]. Hence, mendelian randomisation analyses should confer certain design advantages akin to those in randomised trials. Using the principle of mendelian randomisation, one recent study indicated that genetically raised concentrations of C reactive protein are unrelated to conventional risk factors and risk of CHD. These results suggested that C-reactive protein concentration is unlikely to have even a modest causal role in CHD etiology [186]. The authors of that study suggest that studies seeking to test the inflammation hypothesis in coronary heart disease should examine inflammatory mediators other than CRP. In fact, applying the principle of mendelian randomisation Hingorani et al. have recently shown that the interleukin 6-receptor signalling seems to have a causal role in development of coronary heart disease [102].

When applied to other risk factors in coronary heart disease, mendelian randomisation has previously confirmed the causal relevance of low density lipoprotein cholesterol [187], increased the likelihood of causality for Lp(a) lipoprotein [188], and reduced the likelihood of causality for fibrinogen [189] and the protective role of high-density lipoprotein [190].

**Follow-up procedure**

For the purpose of the present studies, data from the Swedish National Inpatient Register (IPR; in Swedish: “Slutenvårdsregistret”), and cause-
specific death registers were linked to our two cohorts (MPP, VHS) through the personal identity number, which is unique for all Swedish citizens. The IPR, also called the Hospital Discharge Register, was established in 1964 and has a complete national coverage since 1987. From 1984 to 1986, coverage was complete for 19 of 24 Swedish counties, which represents 85% of the population. Currently, more than 99% of all somatic and psychiatric hospital discharges are registered in the IPR. Diagnoses in the IPR are coded according to the Swedish international classification of disease (ICD) system, first introduced in 1964 (adapted from the WHO ICD classification system). A previous validation of the IPR by the National Board of Health and Welfare showed that 85-95% of all diagnoses in the IPR are valid [191]. In Malmö, where the MPP was conducted, registration in IPR begun in 1970 with complete coverage.

Specific discussion

MPP, Papers I and II

In Paper I it was shown that the risk of cardiovascular morbidity (events) and total mortality in middle-aged men increases with increasing levels of systolic blood pressure and that smoking habits further influence this risk. We found, not surprisingly, that both cardiovascular morbidity and mortality are almost twice as common in smokers compared to non-smokers. Treated hypertensive patients were at increased risk in spite of antihypertensive drugs treatment offered during the 1970s and 1980s, when the health screening in Malmö was carried out. The antihypertensive drugs used during this period were dominated by selective beta-blockers and thiazide diuretics [192].

The effect of smoking on CVD risk is well known and has been described earlier. Our findings are in accordance with data from several other epidemiological studies. Evidence has unequivocally confirmed that active smoking is a risk factor for cardiovascular disease (CVD) and the leading cause of preventable death [193]. Two-thirds of sudden cardiac deaths due to acute coronary thrombosis occur in cigarette smokers [194] and smoking is associated with a 50% increase in the risk of stroke.

The increased risk in treated hypertensives has also been observed by another Swedish group where middle-aged men had 50% more myocardial infarctions, a doubled mortality rate from coronary heart disease and an increase of all-cause mortality by a third, compared with normotensives [195]. Our data are in consentient with this study. However, it is important to point out the overlapping confidence intervals in our compari-
son of cardiovascular mortality in non-smokers vs. smokers in treated hypertensives (figure 2, Paper I).

We could not find any difference in cardiovascular morbidity for never-smokers as compared to ex-smokers. There is a large body of evidence from prospective cohort studies regarding the beneficial effect of smoking cessation on CHD mortality risk [196]. However, the magnitude of the effect and the time required to achieve beneficial results are unclear. Some studies suggest that about 10 years after smoking cessation, CHD mortality risk is reduced to that of people who have never smoked [197, 198]. In the MPP, three questions were asked on previous smoking: “Did you quit smoking during the last year?”; “Did you quit smoking during the past 1-5 years?”; and “Did you quit smoking for more than 5 years ago?”. In the current study all participants were studied as ex-smokers. A 50-year follow-up of British physicians demonstrated that, among ex-smokers, the age of quitting has a major impact on survival prospects; those who quit between 35-44 years of age had the same survival rates as those who had never smoked [199]. Also in accordance with our study evidence from the Inter-Heart study [64] has highlighted the harm caused by even low consumption (1-5 cigarettes a day).

In Paper II we found significant associations between plasma total adiponectin and markers of glucose metabolism (fasting plasma glucose, p-glucose at 120 minutes during OGTT, AUC-glucose during OGTT and HOMA-IR index). As mentioned previously, total adiponectin has been shown to be strongly associated with insulin resistance [106-108] and is affected positively by physical activity which increases insulin sensitivity. Our findings are in accordance with these studies.

We could not, however, observe any significant relationships across the quintiles of adiponectin in prediction of stroke, coronary events or total cardiovascular events, either in smokers or in current smokers. Results from other studies evaluating the association between total adiponectin and CVD are also inconclusive [125, 200-203]. In 2005, Rothenbacher et al reported a gradual reduction in the odds ratio for CHD with increasing serum adiponectin levels in a cross-sectional case-control study comprising patients with angiographically confirmed stable CHD [204]. However, after adjustment for HDL cholesterol, the predictive value of adiponectin became insignificant and the authors suggested that the vasoprotective actions of adiponectin are mediated in part by its beneficial effects on HDL cholesterol. A nested case-control study in men, reported that a doubling in plasma adiponectin was associated with an approximately 20 to 50% reduction in the risk for CHD after multivariate adjustment, which included lipids [121]. Another study failed to confirm this relationship in females, a
finding which made the authors speculate that the effect of adiponectin varied in males and females [202]. Adiponectin has also been inversely associated to the severity of CAD [205]. Another prospective study from Sweden [206] reported that low levels of total adiponectin represented a risk factor for CHD in 832 elderly (70-year-old) men, followed-up for 10.5 years, a risk that was independent of conventional risk factors and insulin sensitivity, as determined by the hyperinsulinemic-euglycemic clamp test. However, it remains unclear as to what degree selective survival bias in these elderly men may have influenced the results.

Longitudinal data concerning HMW-adiponectin and cardiovascular risk have been reported. A decreased HMW-adiponectin level has been found to be an independent and stronger risk marker than total adiponectin, for progression to type 2 diabetes mellitus [207]. It has also been reported that HMW-adiponectin is a better predictor of cardiovascular events than total adiponectin [208]. However, results from other studies are contradictory [127, 128].

Papers I and II have several limitations in common. In Paper I only age-adjusted rates have been presented and several confounders were not adjusted for. In individuals with hypertension other risk factors such as diabetes, hypercholesterolemia, an increased BMI or waist-to-hip ratio are very common and should have been accounted for. As shown in an observational study from Gothenburg, Sweden, diabetes is common in treated hypertensive patients, and carries a high risk for cardiovascular complications and mortality [209]. Another limitation is the lack of individual drug treatment data. It would be interesting to compare the effect of smoking habits in treated hypertensives with different treatment strategies.

In both Paper I and Paper II the prevalence of risk factors, for example smoking, changed drastically during the observation period. We lack information about what happened to the screened subjects during the long follow-up period of more than 20 years. Many of these subjects were cared for by the local health care system when risk factors were found to be high and preventive drug therapy was initiated, as was the case for lifestyle interventions offered to men with impaired glucose tolerance [210]. The general health of the screened population was somewhat better in the participants (70%) compared with that of non-participants (30%), which also increased the differences in long-term cardiovascular event rates [150]. Another limitation is that we had to rely upon self-reported data on lifestyle. The impact of risk factors also reflects possible treatment options in the 1970’s and 1980’s before the era of statin therapy for hyperlipidaemia and a more extensive antihypertensive drug treatment approach during recent years.
Another limitation in Paper II is that we only had access to analysis of total adiponectin levels (and not HMW-adiponectin) because of the laboratory methodology used.

**VHS, Papers III and IV**

In Paper III, because of a significant but small estimate of biological interaction between gender and PP we performed our analysis separately for men and women. In this study brachial pulse pressure was used as a surrogate of arterial stiffness. The advantage of brachial PP as a marker of risk is that it can be determined simply by clinical or even home-based measurement. However, certain caveats have to be borne in mind while using PP as a measure of aortic stiffness. First, at least two other elements contribute to PP, cardiac output and pulse wave reflection. Second, the physiological amplification that occurs as the arterial pressure wave propagates peripherally tends to confound PP as a measure of central stiffness. However, the amplification becomes less pronounced in older subjects and this somewhat redeems the limitation of PP measurement in the elderly. The discrepancy between peripherally measured BP and central arterial pressure can be as significant as 20 mmHg in different individuals who have similar recording made from the brachial artery [211, 212].

Arterial stiffness has been demonstrated to be a predictor for increased risk for stroke, coronary artery disease and heart failure independent from blood pressure levels [160, 213]. Atherosclerosis, being considered in part as an inflammatory disease, and inflammation were represented in the third paper by the marker sialic acid. In this large, observational cohort study of men and women aged 50 or above we have shown that individuals belonging to the SA+/PP+ subgroup (with both sialic acid and pulse pressure levels above median) had the highest relative risk of CVD compared to all other SA/PP groups. However, after further adjustment for MAP the risk induced by higher levels of PP above the median decreased. From a physiologic viewpoint blood pressure consists of one static (MAP) and one dynamic (PP) component oscillating around the static component. MAP is determined by peripheral resistance in the microcirculation and PP by the stiffness of large arteries in the macrocirculation [214]. However, both are highly dependent of the SBP and the DBP and our adjustment for MAP may be statistically incorrect and an example of over-adjustment.

Based on our interaction analysis (RERI) and our survival analyses we have noticed that the excess risks contributed by SA and PP were not synergistic and that both variables increased the risk independently of each other. This can be explained by the fact that atherosclerosis in part is an inflammatory condition associated with increased levels of inflammatory
markers such as orosmucoid, α1-antitrypsin, haptoglobin, ceruloplasmin, fibrinogen, complement proteins, TNFα and IL-6 levels, where sialic acid could represent one additional marker.

In Paper IV we were able to show a small significant negative RERI estimate for the interaction between gender and SA and thus the following analyses were performed separately for men and women.

In unadjusted models the diabetes risk was significantly higher for subgroups with SA above the median level in both men and women and the relative intra-gender risk was higher in women than in men. However, after adjustment for available covariates (age, BMI, systolic blood pressure, cholesterol, AST, ALT and socio-economic status) the intra-gender comparison of the SA+/SA- groups showed no significantly increased risk of diabetes associated with elevated SA. In multivariable-adjusted survival analyses for the entire group of men and women separately SA remained a significant risk factor for incident diabetes-related hospitalizations in both men and women.

As mentioned previously, elevated inflammatory markers have been independently associated with risk of developing type 2 diabetes [88, 89]. The association with CRP may be stronger in women than men but this needs further investigation [90]. Several other acute phase response protein markers such as fibrinogen, orosomucoid and low serum albumin have also been linked to risk of incident type 2 diabetes. However, using the principle of mendelian randomisation, Timpson et al. have shown that CRP appears not to be causally related to insulin resistance [215]. In another study from 2010, also using mendelian randomisation, no causal relationship between CRP and obesity were observed [216]. Presently the alternative hypothesis that insulin resistance, obesity and diabetes drive inflammatory perturbances, which subsequently do not, or only to a small degree, contribute to further glucose metabolic impairment cannot be rejected [217].

Our findings in Paper IV are in accordance with findings in a subgroup analysis of the ARIC study (based on only 610 individuals) [218]. Schmidt e. al. also reported a significant association between SA and prospective risk of new-onset type 2 diabetes. They also found that the association between SA and incident diabetes only changed marginally after adjustment for elevated fasting insulin levels, a surrogate marker for insulin resistance. The strengths of our study include the large size of the cohort, the long follow-up period, and also the unique use of endpoint data (hospitalizations) from national registers of high quality.

In Papers III and IV, the historical cohort of the Värmland Health Survey was used and we are thereby restricted by the type of medical thinking
during the screening procedure in the early 1960’s. For example, at the time of the baseline investigation no data were collected on lifestyle habits such as smoking, but it is known that a large proportion of the male population at the time consisted of smokers (approximately 50%) and a lower proportion of women were smokers [219]. Another important limitation is that blood pressure was only measured to the nearest 10 mmHg. However, we believe that this is compensated for by the large size of the study when only mean values are used. We also lack data on glucose levels. Another limitation of the study is the lack of data on concomitant disease at baseline. We have tried to overcome this bias by excluding the first five years of follow-up in order to select mostly healthy individuals at baseline.

In Värmland (VHS), registration in IPR was not initiated completely until 1987 and we are confident that data from previous years on non-fatal events treated in hospitals are lacking to a substantial degree. However, as the study cohort is one of the largest in the world of its kind and has a remarkably long follow-up time, we believe these data to be of interest. The complete registration since 1987 can explain the higher slope in our morbidity and survival curves in Paper III (figure 1) and Paper IV (figure 1) after approximately 20 years of follow-up. On the other hand, since the national cause-of-death register, based on diagnoses from death certificates, in its present form is complete from 1961 we think that almost all fatal events should be covered by national registers. We believe these data to be of interest, since according to official statistics, the county of Värmland has been a high-risk area for CVD for many decades. In fact, the population of Värmland still suffers the highest coronary mortality rates in Sweden with currently an age-adjusted mortality rate of 194.7 deaths per 100,000 inhabitants compared to the mean value of 157.4 for Sweden. The corresponding figures for stroke is 103.4 (second highest in Sweden) compared to 80.9 in the general population (National Board of Health and Welfare Statistics). We therefore regard this cohort to consist of individuals at medium or high cardiovascular risk, recruited during a historical period with less common treatment of hypertension in general, and no treatment of lipids or promotion of smoking cessation.

A further important aspect is that modern drug therapy for risk factor control was little used (e.g., antihypertensive drugs and anti-diabetes drugs except for insulin), or not at all (lipid-lowering drugs) at the time of the screening examination. This rendered this cohort suitable for studying the natural course of disease manifestations, at least during initial decades. Other strengths of the studies in this thesis include the large size of the cohort, the long follow-up period, and the unique use of endpoint data from national registers of high quality. The lack of data on smoking habits...
and lifestyle reflects the lack, at the time of screening, of clinical awareness of this important risk factor.

**Gender aspects**

Because of lack of adequate data, only data on men were analysed in Papers I and II.

In Papers III and IV, however, we observed biological interactions between gender and PP as well as between gender and SA. This interaction between gender and pulse pressure has previously been reported. A greater increase in aortic stiffness with age among women, particularly following menopause has been described [220, 221]. Circulating oestrogens do have a regulating effect on several metabolic factors, such as lipids, inflammatory markers, and the coagulation system. The logical consequent hypothesis that replacing endogenous estrogens by exogenous estrogens in postmenopausal women would decrease CVD risk, which was supported by many observational studies, could not be proved in large randomized trials [222]. In contrast, per oral hormone therapy has been shown to increase CVD event rate in older (60 years) postmenopausal women and its use is not recommended for the primary and secondary prevention of CVD [223].

In our unadjusted analysis in Paper IV, the relative intra-gender risk for incident hospitalisation due to diabetes or its complication was higher in women than in men with SA levels above median, and with non-overlapping confidence intervals between genders. A greater adverse influence of diabetes on blood pressure, lipids and systemic inflammation in women compared with men may contribute to this greater relative risk of incident hospitalisation due to diabetes. A greater relative risk of CHD in women than in men with diabetes has been described [224]. Another observational study has reported that women with diabetes and previous myocardial infarction carry a substantial cardiovascular burden, which probably explain their increased morbidity and mortality [225].
Conclusions

Paper I
Cardiovascular morbidity and total mortality increased with increasing blood pressure levels in middle-aged men. This risk is further increased in smokers. The observed increased risk in smokers may reflect a more hazardous risk-factor profile in smokers. Furthermore, the remaining high risk in treated hypertensive patients calls for a more focused action to influence and improve the entire risk-factor profile.

Paper II
Total plasma adiponectin level is not associated with increased risk of future coronary events or stroke in middle-aged men, after full adjustment for conventional and metabolic risk factors. However, an inverse and independent cross-sectional association between circulating adiponectin levels and markers of glucose metabolism and insulin sensitivity was observed.

Paper III
Elevated pulse pressure and sialic acid both contribute to risk of future cardiovascular events independent of BMI, cholesterol and socio-economic position. However, their risk effects are not synergistic. After further adjustment for mean arterial pressure the risk induced by higher levels of PP decreased.

Paper IV
Elevated sialic acid, as a marker of systemic inflammation, contributes to increased risk of incident hospital-treated diabetes and related complications, independent of selected risk factors and socio-economic position.
Future perspectives

An on-going challenge is to develop screening strategies that can identify individuals at risk for cardiovascular events well before symptoms appear. The interest in cardiovascular biomarkers in primary prevention has increased dramatically in the past decades. In 2007, Morrow and de Lemos outlined three criteria for evaluating novel biomarkers: (a) ease of measurement; (b) addition of information; and (c) effect of management [226]. We believe that we have added information regarding total adiponectin and sialic-acid as risk markers for future CVD events.

Epidemiological studies can help us to describe correlations, identify risk factors and project future event risks in defined populations. On the other hand, correlations do not imply causation and cohort studies are by definition only descriptive. Intervention trials are therefore needed to establish whether risk relationships described in epidemiological studies can be influenced and reduced. For example, several large-scale have raised questions regarding the benefit of intensive glucose-lowering treatment among elderly subjects with type 2 diabetes and individuals who have had the disease for a long time [227]. The effect of multiple risk factor treatment and lifestyle intervention has, however, been more promising [51]. Other studies using the principle of mendelian randomisation have questioned the protective role of HDL cholesterol and the causal risk role of CRP and fibrinogen in the development of coronary heart disease.

The best way to dissect out causality in the complex field of inflammatory pathways is to intervene with novel anti-inflammatory agents. Several agents are currently being tested (such as salsalate [228-230] and more focused anti-inflammatory agents such as IL-1 pathways blockers [231]). Total adiponectin has been associated with changes in glucose metabolism. However one recent genetic study [232] failed to find any association with type 2 diabetes. The authors suggest large-scale and well-powered mendelian randomisation studies to examine this association. To our knowledge no such study for the evaluation of total adiponectin or HMW-adiponectin has been conducted so far. The association with CVD should also be studied by mendelian randomisation. Frozen blood samples are still available in the MPP and in the future such analyses could be possible.

We have linked our data in the VHS with the national Multi-Generation Register (MGR), containing extensive data on parents and siblings of Swedish citizens going back to the early 1930’s (Statistics Sweden, Örebro). Thus in the future it will be possible to describe the family risk burden of CVD and diabetes for development of end-points in the next generation,
which will be of interest in this high risk area of Värmland. This approach has previously been used in the MPP to show separate effects of maternal versus paternal influences on CVD risk in offspring [233].
Titel: Riskfaktorer för hjärt/kärl-sjukdomar och sjukhusvårdad diabetes


Material och Metod: Två stora populationsstudier med långa uppföljningstider har använts: Malmö Preventive Project (MPP) med 22444 individer användes i delarbete I medan en subgrupp med 3885 individer användes i delarbete II. Värmland Health Survey (VHS) användes i delarbete III med 37843 individer och i delarbete IV med 87035 individer.


Sammanfattning: Dessa undersökningensfynd bidrar till förståelsen av samspelet mellan hemodynamiska riskfaktorer, konventionella riskfaktorer, cirkulerande biomarkörer och HKS-risk. Våra fynd kan vara viktiga i formandet av framtidens förebyggande strategier.

Nyckelord: Adiponektin, blodtryck, diabetes, hjärtkärlsjukdom, inflammation, pulstryk, rökning, sialinsyra
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