

# Sex hormones and cardiovascular risk in men and women

## The Skaraborg Project

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Department of Public Health and Community  
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UNIVERSITY OF GOTHENBURG

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# ABSTRACT

The general aim of this thesis was to explore the associations between sex hormones and high blood pressure in men and women and to investigate their further role in the development of acute myocardial infarction particularly with regard to the large effect of type 2 diabetes, especially seen in women. Differences in levels of sex hormones and their specific effects in men and women might partially explain the differences in cardiovascular risk between men and women. Our specific aims were to investigate the relationship between sex hormones and high blood pressure as a major risk factor for cardiovascular disease, to investigate mechanisms that control the concentrations of sex hormone-binding globulin (SHBG), to investigate testosterone as a risk factor for AMI in men and women, to explore the modifying effect of type 2 diabetes on the outcome, and to investigate the association between sex hormones and cardiovascular disease, stroke and AMI, in men and women with or without type 2 diabetes.

This thesis included studies on 2 cohorts: 1. A population survey in the municipalities of Vara and Skövde (VSC) 2002-2005 (n=2816, aged 30-74 years, 50% female, participation rate 76%); 2. A population survey in the municipality of Skara (SC3) 1993-1994 (n=1109, aged 40-80+ years, 50% female, participation rate 79%).

Findings: Low concentrations of SHBG were associated with high blood pressure in men, whereas SHBG was independently associated with hypertension in postmenopausal women. We also found that insulin levels were independently associated with SHBG levels. Low testosterone levels in men with diabetes significantly predicted AMI independently of major cardiovascular risk factors. Endogenous estradiol concentrations were significantly associated with stroke risk in both sexes but with opposite relationships; estradiol was associated with reduced stroke risk in women, but with increased stroke risk in men.

In conclusion, concentrations of sex hormones predicted cardiovascular morbidity in both men and women, albeit differently. While testosterone was protective in men, estradiol and SHBG were protective in women. Moreover, SHBG seems to play an active role in the modulation of sex hormone effects, as it was found to be independently associated with hypertension. However, more studies are needed to explore the association of this globulin with diabetes and hypertension, in order to confirm our results suggesting a role of insulin in the control of SHBG.

Correspondingly, the effects of estradiol in men seem negative while the effects of testosterone in women were uncertain. Thus, in each sex the characteristic hormone supports health. Diabetes also modified the association between concentrations of sex hormones and CVD in both sexes. These modifications might at least partially explain the loss of cardiovascular protection in women when they develop type 2 diabetes.

**Keywords:** keyword1, keyword2, keyword3

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# ABSTRAKT

Det övergripande syftet med denna avhandling var att undersöka sambandet mellan könshormoner och högt blodtryck hos män och kvinnor samt att utforska deras roll i utvecklingen av akut hjärtinfarkt. Skillnader i nivåer av könshormoner och deras specifika effekter hos män och kvinnor skulle, åtminstone delvis, kunna förklara skillnaderna i kardiovaskulär risk mellan män och kvinnor. Våra specifika syften var att undersöka associationen mellan koncentrationer av könshormoner och högt blodtryck, att undersöka mekanismer som styr halterna av könshormonbindande globulin (SHBG), att undersöka testosteron som en riskfaktor för akut hjärtinfarkt respektive hjärt- och kärlsjukdom hos män och kvinnor, samt att utforska den modifierande effekten av typ 2 diabetes på dessa resultat.

Avhandlingen baseras på 2 kohorter: 1. En befolkningsundersökning i kommunerna Vara och Skövde 2002-2005 (n= 2816, i åldern 30-74 år, deltagandet 76 %), 2. En befolkningsundersökning i kommunen Skara 1993-1994 (n = 1109, i åldern 40 till 80+ år, deltagandet 79 %).

Låga halter av SHBG var förknippade med högt blodtryck hos män, medan detta samband hos kvinnor var begränsat till de som var äldre än 50 år. Vi fann också att insulinivån hade ett oberoende samband med SHBG för båda könen. Låga testosteronnivåer hos män med diabetes förutspådde akut hjärtinfarkt oberoende av vanliga kardiovaskulära riskfaktorer. Vi fann också att endogena koncentrationer av estradiol hade en signifikant betydelse för stroke hos båda könen: skyddande hos kvinnor men en riskfaktor för män.

Sammanfattningsvis förutspådde halter av könshormoner kardiovaskulär morbiditet hos både män och kvinnor, om än på olika sätt. Medan testosteron var skyddande hos män, var estradiol och SHBG skyddande i kvinnor. Dessutom verkar SHBG spela en aktiv roll i moduleringen av könshormonernas effekter. Studier som undersöker mekanismer bakom associationen mellan SHBG och diabetes/hypertoni behövs. Våra resultat tyder även att endogent insulin spelar roll för kontrollen av SHBG.

Slutligen, estradiol hade negativa effekter hos män medan effekterna av testosteron hos kvinnor var osäkra. Således var det karakteristiska hormonet för respektive kön kopplat till en bättre hälsa. Diabetes modifierade också sambandet mellan halter av könshormoner och hjärtkärlsjukdom hos båda könen. Dessa effekter kan åtminstone delvis förklara varför kvinnor förlorar sitt naturliga skydd mot hjärtsjukdom när de insjuknar i typ 2 -diabetes.

## LIST OF PAPERS

This thesis is based on the following papers, referred to in the text by their Roman numerals.

- I. Daka Bledar, Rosen Thord, Jansson Per Anders, Råstam Lennart, Larsson Charlotte A, Lindblad Ulf. Inverse association between serum insulin and sex hormone-binding globulin in a population survey in Sweden. *Endocr Connect* 2013;2(1):18-22
- II. Daka Bledar, Rosen Thord, Jansson Per Anders, Larsson Charlotte A, Råstam Lennart, Lindblad Ulf. Low sex hormone-binding globulin is associated with hypertension: A cross- sectional study in a Swedish population. *BMC Cardiovasc Disord* 2013;13:30.
- III. B. Daka, R.D. Langer, C.A. Larsson, T. Rosen, P. Jansson, L. Råstam, U. Lindblad. Low concentrations of testosterone predict acute myocardial infarction in men with type 2 diabetes mellitus. (Submitted).
- IV. B. Daka, U. Lindblad, V Dandolu, R.D. Langer. Concentrations of estradiol and testosterone predict cardiovascular morbidity in men and women. A prospective study in Sweden. Manuscript.

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# ABBREVIATIONS

CVD	CardioVascular Diseases
SHBG	Sex Hormone-Binding Globulin
DHEA	Dehydroepiandrosterone
SWAN	<i>Study</i> of Women's Health Across the Nation
WHI	Women's Health Initiative
AMI	Acute Myocardial Infarction
BMI	Body Mass Index
HT	Hypertension
SC	Skara Cohort
VSC	Vara Skövde Cohort
DM	Diabetes Mellitus

# 1 INTRODUCTION

Cardiovascular disease (CVD) is the major cause of death in developed countries [1]. Cardiovascular mortality and morbidity differs greatly between men and women. Women seem to be protected and are affected by these diseases more rarely and later in life compared to men [2]. Hormonal differences between men and women may, speculatively, play a role in these discrepancies regarding cardiovascular morbidity and mortality. In light of such hormonal differences, we would expect testosterone to have an adverse effect or estrogen to have a protective effect or both. Surprisingly, the majority of studies suggest a protective effect of testosterone in men [3-5] while the effects of estrogen in women are not so clear [6-8]. Moreover, the sex-specific cardiovascular protection in women seems to disappear when they develop type 2 diabetes. In fact, women with type 2 diabetes have age-specific cardiovascular risk that is very similar to the risk in men. Thus, two key questions are: 1) How much of the differences in risk can be explained by the effects of sex hormones? 2) Is there any other factor that modulates the effects of sex hormones or that may obscure these effects?

## 1.1 Sex hormones, production and physiology

### 1.1.1 Men

The production of sex hormones starts with as illustrated in *Figure 1*. In men, sex hormones are produced in the testis, and the main hormone is testosterone. Testosterone is then converted to the more potent hormone dihydro-testosterone. Aromatase converts part of testosterone to estradiol in testis, brain, bones, adipose tissue, and the activity increases with age,

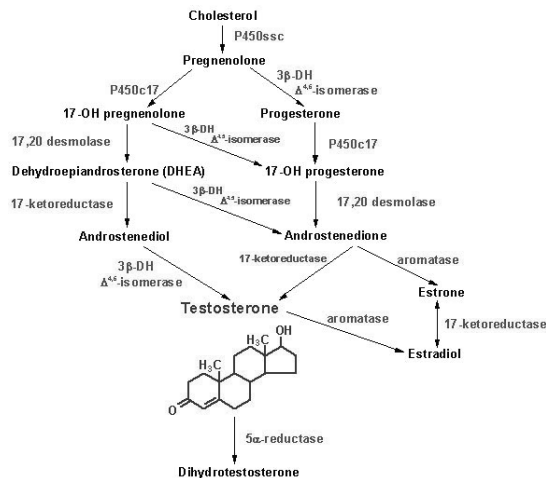


Figure 1. Production of sex hormones

obesity and hyperinsulinemia [9]. Testosterone and estradiol are liposoluble hormones and circulate bound to albumin or sex hormone-binding globulin (SHBG) (*Figure 2*).

The free fraction of testosterone together with the fraction bound to serum albumin constitutes the bioavailable fraction. However, it is unknown whether the fraction bound to SHBG is bioactive or whether it is only a reservoir of sex hormones in the circulating blood stream. Dehydroepiandrosterone (DHEA) is a pre-hormone produced in the adrenals and converted into sex hormones in the target tissues by local enzymes. DHEA is responsible for about 50% of active sex hormones in men aged 60 years or older [10]. DHEA and sulphated-DHEA serve as reservoirs of sex

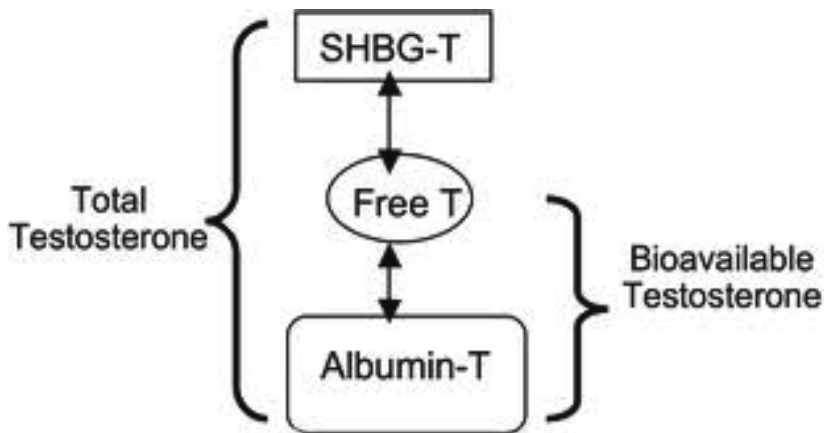


Figure 2. The major part of testosterone circulates bound to SHBG and albumin

hormone in the circulation as their concentrations are high, and both forms are easily converted into active sex hormones. Whether sulphated-DHEA has an independent effect in the body is still a subject of controversy. Sex hormones are deactivated by glucuronidation into hydrosoluble metabolites. The action of testosterone and estradiol is mediated by the androgen and the estrogen receptors, which are both intracellular receptors. The androgen receptor is responsible for the anabolic effects. In bones and in the brain, testosterone is converted into estradiol, and the activation of estrogen [11]

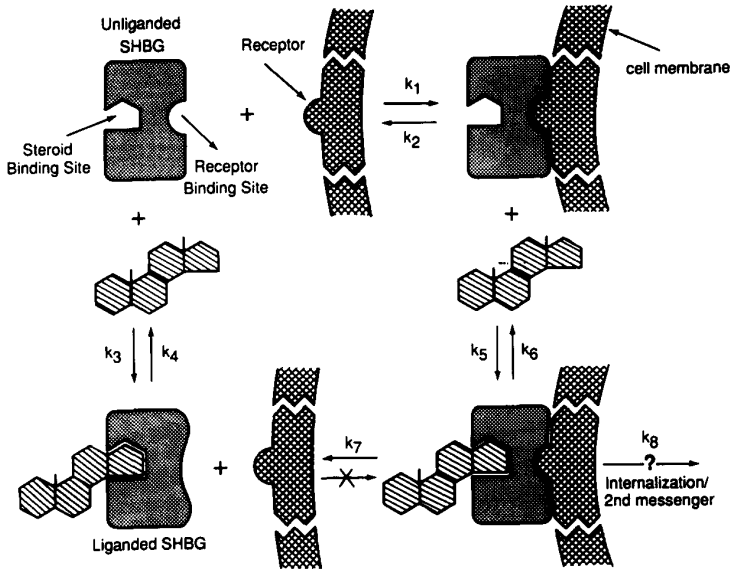


Figure 3. Model of receptor-SHBG-steroid system.

receptors is responsible for the mineralization and the closure of the epiphysis. In the brain, estradiol is responsible for sexual behaviour.

SHBG is the major carrier of sex-hormones in the blood stream. SHBG is mainly produced in liver. In men the levels of SHBG increase with aging [12] and in women they decrease during the menopause and then remain constant [13, 14]. Hypothyroidism and the metabolic syndrome are associated with low concentrations of SHBG and hyperthyroidism, and anorexia is related to higher concentrations of SHBG [15-18]. Estradiol also seems to induce production of SHBG in the liver, whereas no such effect is seen from testosterone. Although glucose seems to inhibit the production of SHBG, the effects of insulin are debated [19]. Although SHBG has been considered as a transport protein, there is growing evidence showing a more active role for this protein in the modulation of the effects of sex hormones. In fact, receptors for SHBG in the membrane of prostatic tissue have been shown [20], and these receptors seem to activate an increase of c-AMP concentrations if the receptor is activated by the complex SHBG-Hormone [21, 22] (Figure 3).

The combined effect of the testosterone decrease and the SHBG increase in aging men results in a dramatic attenuation of the physiological effects of

testosterone. This decrease is, however, gradual and slow in contrast to the situation in women where there is a relatively steep decline in estrogen production coinciding with the onset of menopause.

### 1.1.2 Women

Similar to testosterone in men, the production of estradiol in women starts from cholesterol (*Figure 1*). In fertile women, the major part of estradiol is produced in granulosa cells of ovaries and involves the conversion of androstenedione into estrone, and the further conversion of the latter into estradiol. Another form of estradiol production is through the aromatization of testosterone in adipose tissue. Adrenal glandules also produce estradiol in minor quantities. The concentrations of estradiol vary during the ovarian cycle and are involved in ovulation (*Figure 4*). In postmenopausal women, there is a cessation of estradiol production in the ovaries, and the major part of estradiol is then produced by aromatization of circulating androgens. The levels of estrogen in postmenopausal women are comparable to the levels of estrogen in men. However, there are large sex-differences in the levels of testosterone during the entire lifespan, with 10 times higher testosterone levels in men compared to women. Even if the levels of testosterone tend to decrease in women after menopause, the decrease is attenuated as the production of androgens in stromal tissue of ovaries continues after the menopause [23], and the production of androgens in the adrenals seems not to be influenced by the menopause. DHEA and sulphated-DHEA decrease

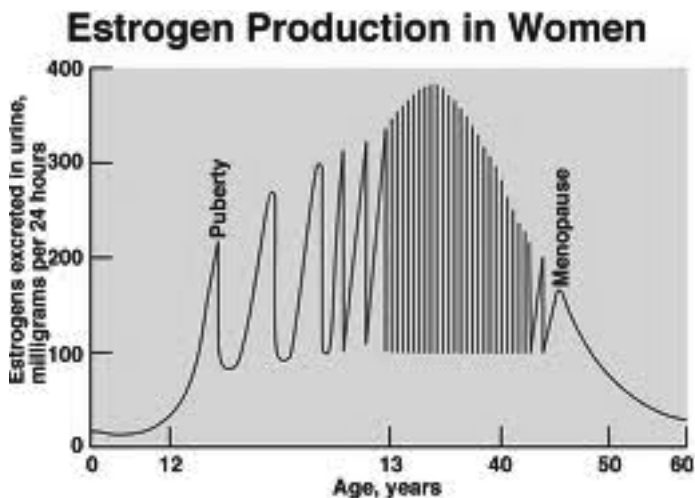


Figure 4. Estrogen production in women during lifespan.

gradually in women and this decrease is reflected in testosterone levels. The levels of DHEAS during the menopause are only 50% of those at ages 25-30. Similar to men, only 2% of estradiol circulates free and is responsible for the hormonal effects [24]. In aging women, the levels of SHBG do not increase as they do in men. The levels of SHBG in women are higher than in men after puberty, probably because of the inductive effect of estradiol in the liver. These levels decrease in the menopause and are then almost constant in older women.

## *1.2 Sex hormones and cardiovascular risk*

### **1.2.1 Men**

#### *Testosterone*

Androgens have previously been considered to decrease glucose tolerance, induce hyperinsulinemia, and increase cardiovascular risk in women as well as in men. In contrast to these notions, observational studies in men have shown an association between insulin resistance, diabetes and low s-testosterone [25, 26]. The association with metabolic syndrome and diabetes seems to be bidirectional. On the one hand, the expansion of the adipose tissue in obesity increases the activity of aromatase in the adipose tissue, resulting in a decrease of testosterone levels [17, 18]. On the other hand, low testosterone concentrations diminish insulin sensitivity and cause obesity in castrated men [27]. Low s-testosterone has also been associated with lower HDL levels in men [28], and there are studies that show beneficial effects of testosterone on arterial stiffness [29]. In several studies, low levels of endogenous testosterone have been associated with hypertension [30], and this relationship seems to be independent of other factors related to the metabolic syndrome [31]. Although these observations suggest a more direct effect of testosterone on the vessels, the mechanisms are still unknown. It has been speculated that testosterone receptors in the vessels have a vasodilator effect in coronary vessels [32]. To our knowledge, this observation has not been confirmed in other studies and is based on findings in arteries with advanced atherosclerosis. Thus, the effect in normal vessels is still unknown. The modulating effect of testosterone [33] in inflammation might explain the effects of testosterone in vessels. Inflammation per se is related to an increase in peripheral resistance and hypertension [34]. Several prospective observational studies in elderly men have shown a strong association between low levels of testosterone and cardiovascular mortality [35]. Moreover, castrated men have significantly higher rates of CVD [36-38]. However, observations of the effects of testosterone in middle-aged men

with regard to cardiovascular mortality and morbidity are lacking. The absence of evidence might be attributable to lack of statistical power due to the rarity of these events in a younger population. Another biological reason for findings only in older populations might be differences in the effect of testosterone in older and younger men, respectively. Aging in the endothelial tissue might be a cause of vulnerability to cardiovascular disease, in that the role of testosterone may become more important for the endothelial function in older than in younger individuals with intact endothelium. Type 2 diabetes is associated with early vascular aging [39, 40]. Therefore, analyses comparing the effects of testosterone in men with and without diabetes, respectively, would represent a good way of modelling the differing effects of testosterone on cardiovascular endpoints in younger and older men.

Testosterone replacement therapy in men is used if symptoms of hypogonadism are associated with lower levels of testosterone [41, 42]. Although a good effect is observed in the metabolic syndrome [43], randomized controlled trials with hard endpoints in individuals with high risk are still lacking.

#### *Estradiol*

Testosterone is the most important source of estradiol in men [11]. Although the activity of aromatase increases with age, the concentration of estradiol remains almost unchanged as testosterone levels decrease. Estradiol is important in men in the process of the closure of the epiphysis, the control of the function of pituitary production of luteinizing hormone and in sexual behaviour. The effect of estradiol with regard to cardiovascular risk in men is uncertain. Epidemiological prospective studies investigating the effects of estradiol concentrations in the intima media have yielded contrary results [44-46]. Also, one prospective study has shown that high levels of endogenous estradiol levels predict stroke [47]. In the mid-1960s the Coronary Drug Project tested two doses of exogenous estrogen for the prevention of heart disease in men. Both estrogen arms were terminated early due to an excess of adverse events [48]. Similarly, men receiving estrogen for prostate cancer have been shown to have an increased risk of CVD [49]. Although epidemiological studies show in general an adverse effect of exogenous estradiol and an uncertain effect of endogenous estradiol, the mechanisms underlying these associations remain unexplained.

#### *Sex hormone binding globulin (SHBG)*

Epidemiological studies have shown an association between low levels of SHBG and the metabolic syndrome and diabetes mellitus, respectively [17, 18, 25, 50]. In fact, high levels of glucose in subjects with metabolic syndrome inhibit the production of SHBG [19]. In 2009, Ding et al. could

show that in both men and women low concentration of SHBG predicted type 2 diabetes mellitus [51]. The paper also reported that genetic variants associated with lower levels of SHBG production could also predict diabetes. The same observation was made in another cohort of only men, where SHBG, but not testosterone, predicted type 2 diabetes [52]. These results combined with the presence of membranous receptors for SHBG indicate that this globulin plays a more active role in the modulation of sex-hormone effects and in glucose metabolism in general. Moreover, a recent study in post-menopausal women has shown an association between SHBG and the incidence of hypertension [50]. Taken together, these findings suggest an important role for SHBG as a predictor of cardiovascular disease. Only a few studies have investigated the predictive value of SHBG for CVD-risk, and the results are ambiguous due to contradictory findings [53, 54].

## 1.2.2 Women

### *Testosterone*

Sex-specific differences have been reported in the association between testosterone and the metabolic syndrome, with high levels of testosterone are shown in women and low levels of testosterone are shown in men [25, 31]. Although serum concentrations of testosterone seem to be a risk factor in women with regard to the metabolic syndrome, the association between testosterone levels and cardiovascular disease is somewhat more complicated [55]. The increase of CVD-rate in women with Poly Cystic Ovary Syndrome has not been as consistent as expected [56]. Recent studies show an association between low levels of testosterone and atherosclerotic disease in women [57-60], raising the possibility that testosterone may have beneficial effects on the heart or suggesting a U-shaped association with suboptimal effects at both extremes.

### *Estradiol*

Levels of serum estradiol decrease dramatically during and after menopause. This decrease, combined with a large increase in the risk of CVD in women after menopause [61] has been the basis for the theory that estradiol might be protective against CVD in women. Randomized trials with estradiol have shown that estradiol has beneficial effects on the lipid status [62]. Among women near menopause enrolled in the SWAN Study, lower estradiol levels were associated with poorer arterial health as reflected by larger carotid artery interadventitial diameter [61]. In the Women's Health Initiative (WHI) the clinical trial of estrogen-only treatment found a reduction in coronary artery disease in women aged 50 to 59, but no difference in coronary artery



disease in the entire group of women aged 50 to 79 who were, on average, 12.5 years postmenopausal [7]. For treatment with estrogen plus progestin, the WHI found no benefit for coronary artery disease overall, but a non-significant reduction in rates for women within 10 years of menopause [63]. However, stroke events were more frequent in the intervention arm [64] suggesting different effects in the cerebrovascular diseases and in coronary disease. A change in the functional response of estrogen receptors after menopause can be the reason for the differing action of estradiol in aged women. The Three-City study [6], a French cohort of women >65 years of age, found an association between high levels of endogenous estradiol and higher risk for CVD in women who did not use exogenous hormones, with stroke being the most common event. Thus, emerging evidence suggests that estrogen associations with CVD may change with age for women without exogenous hormone exposure so that a protective association early in menopause gives way to increased risk associated with changes in arterial architecture.

#### Sex hormone-binding globulin

In similarity with the effects seen in men, low levels of SHBG predict diabetes, and in postmenopausal women, low levels have been associated with higher incidence of hypertension[50, 51]. However, studies regarding the association between SHBG levels and risk of CVD morbidity and mortality are still lacking.

## 2 AIMS

### 2.1 *General Aims*

The general aim of this thesis was to explore the association between sex hormones and high blood pressure in men and women, and to investigate their further role in the development of cardiovascular disease. Moreover, this thesis aims to investigate differences in levels of sex hormones and their specific effects in men and women, and whether these differences might partially explain the differences in cardiovascular risk between men and women.

### 2.2 *Specific aims*

1. To investigate mechanisms that control the concentrations of SHBG.
2. To investigate the associations between sex hormones and high blood pressure in men and women.
3. To investigate testosterone as a risk factor for cardiovascular disease in men and women, and to explore the modifying effect of type 2 diabetes on this outcome.
4. To investigate the risk of cardiovascular disease associated with concentrations of estradiol in men and women with or without type 2 diabetes.

## 3 SUBJECTS AND METHODS

### 3.1 Cohorts in the Skaraborg project

#### *The Skaraborg Hypertension Project*

The Skaraborg Hypertension Project was launched in 1977 in the county of Skaraborg as a 5-year trial to improve blood pressure control, and to reduce the risk of acute myocardial infarction (AMI) and acute stroke in the community[65]. The prognosis of hypertension based on participants in the program was evaluated by follow-up through 1987[66]. About half of the population lives in the countryside and the other half in urban areas and a high percentage are Swedish-born. Skara was one of the municipalities included in the project.

#### Skaraborg Project 1977-2013

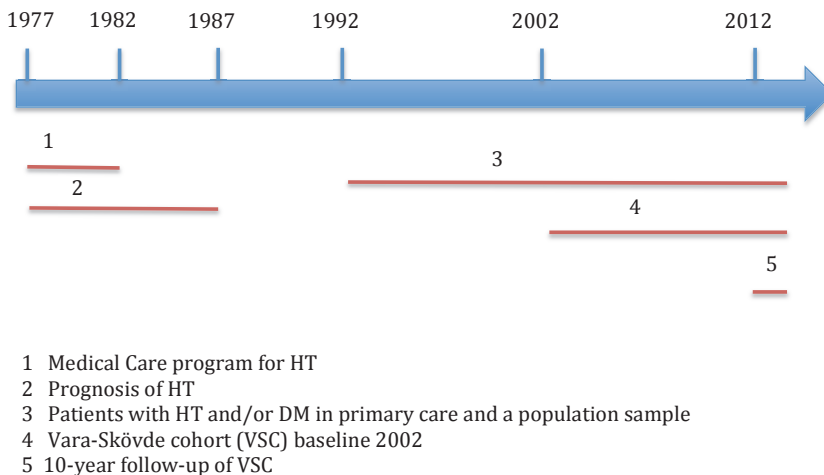


Figure 5. Cohorts from Skaraborg project between 1977-2013

#### *The Skara Patient and Population Cohorts*

After the medical care program Skara was well prepared for further population-based studies on hypertension and diabetes. Thus, in 1992-1993 all patients in primary care with these conditions were surveyed. In 1993-

1994 a corresponding population study was conducted as an age-stratified random sample drawn from the population census register. Subjects aged 40 or over were invited to the Health Care Centre for a health examination. Among those aged 80 or more, 100 male and 100 female subjects were randomly selected and invited, as were 150 male and 150 female subjects from each ten-year age category between 40 and 79 years. In total 1400 subjects were invited to participate in the study and of these, 1109 (79%) completed the study visit[67].

### *The Vara-Skövde Cohort*

The next phase of the project was focused on the early development of high blood pressure and impaired glucose metabolism, in order to gain more insight into their potential prevention. For this purpose, it was necessary to study a younger population to collect more information on lifestyles and metabolism, and thus, the Vara-Skövde cohort was formed. Vara is a small municipality with approximately 16 000 inhabitants. Ninety-five per cent of the residents are Swedish born and many are farmers. Skövde is the largest town in Skaraborg, with approximately 50 000 residents, of which 90 per cent are Swedish born. Skövde is more urbanized than Vara and has a more developed infrastructure, with a hospital and a university. Compared with national data, the cohort has a higher mean body mass index (BMI) and a higher rate of subjects born in Sweden.

From the population census register, random samples, stratified by gender and five-year age groups, were generated from the total number of all individuals between 30 and 74 years residing in each municipality. There were no exclusion criteria, and subjects between 30 and 50 years of age were intentionally over-sampled (three-fold), as compared to subjects over 50 years. Subjects who provided written consent to participate in the study visited the study nurse, completed the questionnaires, and gave venous blood. The final Vara-Skövde cohort consisted of 1811 subjects who fulfilled all requirements for participation from the Vara population (81% participation rate) and 1005 subjects from the Skövde population (70% participation rate) [68].

All participants provided signed informed consent prior to enrolment, and the Regional Ethical Review Board in Gothenburg, Sweden, approved the study.

## 3.2 Subjects

### *Paper I, II and IV*

*Paper I, II.* These studies were based on the Vara-Skövde cohort with 2816 participants. All participants with valid SHBG analyses were included in the study. After exclusion of subjects without such analyses for SHBG, 2,782 subjects remained.

### *Paper IV*

The last study was also based on the Vara-Skövde cohort. The hormone assays were successful for testosterone in 2671 (95%) individuals, for estradiol in 2777 (99%) individuals and for SHBG in 2782 (99%) individuals. The associations between sex hormones and outcomes were analysed separately and the participants in each analyses were restricted to those with valid results for that specific hormone. All analyses were stratified by sex and age (less than 50, vs. 50 or above).

### *Paper III*

This study was based on the 1109 subjects from the population survey in 1993-1994. After excluding 64 patients with previous AMI, 1045 subjects remained for data analyses. Analyses were stratified for sex and diabetes status.

### 3.3 Procedures

#### *Skara (Paper III)*

After an overnight fast (10h), participants were seen by specially educated and trained nurses. Participants gave verbal informed consent, and the nurses measured body weight and height with the participants wearing light clothing without shoes. Blood pressure was taken in a supine position and venous blood samples were drawn. The study nurses collected information regarding medical history and on-going medication with a special focus on treatment for hypertension and diabetes. All participants also answered a questionnaire regarding smoking habits, alcohol consumption, and physical activity [67].

#### *Vara-Skövde (Paper I, II and IV)*

Participants were seen in the morning after an overnight fast (10h) and signed an informed consent form before venous blood samples were drawn. An oral glucose tolerance test was performed with an intake of a 75 g standard glucose load. During the two-hour wait for the final blood drawing, participants filled out a validated lifestyle questionnaire [67]. Participants also filled out questionnaires regarding psychosocial health, stress, quality of life, and civil- and socioeconomic status. Approximately two weeks after the first visit the participants came for a second examination by the nurses. Body weight and height were measured while participants were wearing light clothing and without shoes. They also had their blood pressure taken and provided detailed information on medical history and on-going medication.

#### *Medical History*

*In both cohorts*, the information regarding medical history was collected at baseline according to predefined criteria. The variables included were acute myocardial infarction, angina pectoris, heart failure, acute stroke, intermittent claudication, diabetes mellitus, and smoking habits.

#### *Physical Examination*

*In both cohorts*, systolic and diastolic blood pressure in the right brachial artery was measured in a supine position after 5 minutes rest, as well as in a standing position after 1 minutes rest, and recorded to the nearest 2 mm Hg. Tricuff™ for automatic adjustment of cuff size to arm circumference was used and heart rate was registered simultaneously with blood pressure [69]. A pillow supported the arm to be at heart level. Participants were weighed

while wearing light clothing on a calibrated scale to the nearest 0.1 kg. Height was measured without shoes to the nearest cm. Waist circumference was measured between the lowest rib margin and iliac crest and hip circumference at the largest circumference between waist and thighs. Waist-hip ratio was defined as the ratio of waist to hip circumference. Both waist-hip ratio and waist circumference were used to estimate abdominal obesity. Body mass index was calculated by the body weight in kilograms divided by the square of the height in meters ( $\text{kg}/\text{m}^2$ ), and obesity was defined as body mass index  $\geq 30 \text{ kg}/\text{m}^2$  [70]. Waist circumference  $>88 \text{ cm}$  in women and  $>102 \text{ cm}$  in men was defined as abdominal obesity [71].

### *Laboratory Examinations*

*In both cohorts*, standard laboratory tests were used for serum cholesterol, fasting triglycerides, fasting blood glucose in Skara Cohort and fasting plasma glucose in Vara and Skövde Cohort, and also HbA1c for subjects with diabetes. Tests for blood glucose and serum triglycerides were performed in a non-fasting state in insulin-treated patients with diabetes, as fasting tests were considered unsuitable for these patients. The analyses of fasting blood glucose were performed using a modified glucose dehydrogenase method from Hemocue (Hemocue AB, Ängelholm)[72].

Both glucose and HbA1c were analysed by the laboratory at the local hospital (Kärnsjukhuset, Skövde). Serum samples for further tests were frozen immediately at  $-82^\circ\text{C}$  and analysed later for lipids at the Department of Clinical Chemistry, Skåne University Hospital, Lund. Serum insulin was also analysed later at the Wallenberg Laboratory (Malmö University Hospital), using an enzyme linked immunosorbent assay (ELISA) with  $<0.3\%$  cross-reactivity for proinsulin using a kit from DAKO Diagnostics Ltd [73, 74]. Insulin resistance was estimated based on the Homeostasis Model Assessment of insulin resistance (HOMA-ir): fasting insulin  $\times$  fasting blood glucose / 22.5 [74, 75].

### *Questionnaires*

*In the Skara cohort*, leisure time physical activity was measured based on four answer alternatives to the question “How physically active are you during your leisure time?”. The question referred to the past year and the answer alternatives were: 1) Sedentary leisure time: reading, watching television, stamp collecting or other sedentary activity; 2) Light leisure time physical activity: walking, cycling, or other physical activity under at least four hours per week; 3) Moderate leisure time physical activity: running, Swimming, tennis, aerobic, heavier gardening, or similar physical activity

during at least 2 hours a week; 4) Heavy training or competitive sport: heavy training or competitions in running, skiing, swimming, football, etc, which is performed regularly and several times a week [76].

Alcohol consumption was assessed by questions concerning the number of days during the past 30 days during which the subjects had consumed beer, wine, and strong liquor, respectively. Each of these questions was followed by questions concerning how many cans, glasses, and/or bottles that were normally consumed on such days. The quantity of alcohol in gram consumed per week was then calculated by multiplying the number of days of alcohol consumption by the number of grams of alcohol contained in the consumed alcoholic beverage [77].

Smoking habits were investigated by the question “Do you smoke?”. The answer alternatives were: 1) No, I have never smoked; 2) No, I have smoked but have given it up; 3) Yes, I smoke.

*In the Vara-Skövde cohort*, leisure time physical activity, alcohol consumption, and smoking were measured by the same questionnaire as that utilized in the Skara studies.

Educational level was assessed by a question with 10 alternatives ranging from primary school to PhD-exams. General self-rated health was defined based on five answer alternatives (from “very good” to “very poor”) to the question “How would you rate your current health status in general?”[78]

### *Diagnostic procedures*

*In the Skara cohort*, subjects without known hypertension were followed up with two further blood pressure measurements (1-2 weeks between each measurement) if the initial diastolic blood pressure was  $\geq 90$  mmHg. In accordance with national and international guidelines at the time, all three DBP had to be at least 90 mmHg, for a new diagnosis of hypertension to be confirmed[79].

In subjects without a diagnosis of diabetes, further examinations of fasting blood glucose were performed (1-2 weeks between measurement) if the initial fasting glucose value was  $>5.5$  mmol/L. Diagnosis of diabetes was confirmed after two fasting blood glucose values of  $\geq 6.7$  mmol/L. If the second fasting blood glucose test showed a value between 5.6 and 6.6 mmol/L, an oral glucose tolerance test was performed [80], and if the 2-h glucose value was  $\geq 11.1$  mmol/L, a diagnosis of diabetes was confirmed. Differentiation between type 1 and type 2 diabetes was based on clinical



criteria: i.e., age at onset, body weight, symptoms at initial stage, tendency of ketosis, the need for early insulin treatment, and in some cases C- peptide.

*Vara-Skövde (Paper II-IV)*

If the initial diastolic blood pressure was  $\geq 90$  mmHg or the initial systolic blood pressure was  $\geq 140$ , in accordance with international standards [81, 82], subjects without a diagnosis of hypertension were followed up with two further blood pressure measurements (1-2 weeks between each measurement) All three consecutive measurements of the diastolic blood pressure had to be  $\geq 90$  mmHg and/or the systolic blood pressures had to be  $\geq 140$  mmHg to confirm the diagnosis of hypertension.

Diagnosis of diabetes was confirmed after two fasting plasma glucose values of  $\geq 7.0$  mmol/L (1-2 weeks between measurements), or after one 2-h plasma glucose value of  $\geq 11.1$  mmol/L in an oral glucose tolerance test [83]. Differentiation between type 1 and type 2 diabetes was based on clinical criteria: i.e. age at onset, weight, symptoms at initial stage, tendency of ketosis, treatment, and in some cases C-peptide.

### *3.4 Measurements of sex hormones concentrations*

In both cohorts, total testosterone was analysed according to UniCel<sup>TM</sup> DxI 800 Beckman Access ® Immunoassay System Main Instrument DxI-1 in Malmö (Skåne University Hospital) [84]. The technique is fair in the estimation of testosterone levels in men [85] but poor regarding the estimation of testosterone in women [86]. In order to estimate the bioavailable fraction of testosterone and estradiol, in the Vara and Skövde cohorts, SHBG and estradiol were measured at Unilabs at Skaraborg Hospital in Skövde with results expressed in nmol/L [84]. In 19 cases with SHBG levels reported to be higher than 180 nmol/L but not further specified, we assumed them to be 180 nmol/L. We calculated the free testosterone (FT) according to previous studies [87, 88]. The measurements of SHBG and estradiol in the Skara cohort were computed later using an Electro-Chemio-Luminiscence-Immunoassay technique[89].

### 3.5 Outcomes

#### Paper I

The outcome in the paper I is the concentration of SHBG. The measurements of SHBG in the Vara and Skövde cohort were performed according to validated methods [89, 90].

#### Paper II

Definition: In adults hypertension is defined as a systolic and/or a diastolic

JNC 6 Category		JNC 7 Category	
	SBP/DBP		
<b>Optimal</b>	< 120/80	→	<b>Normal</b>
<b>Normal</b>	120–129/80–84	→	<b>Prehypertension</b>
<b>Borderline</b>	130–139/85–89	→	
<b>Hypertension</b>	≥ 140/90	→	<b>Hypertension</b>
Stage 1	140–159/90–99	→	Stage 1
Stage 2	160–179/100–109	→	Stage 2
Stage 3	≥ 180/110	→	

Sources: The sixth report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure. *Arch Intern Med* 1997;157:2413–46.

The seventh report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure. *JAMA* 2003;289:2560–2571.

Figure 6. Definition of hypertension according to JNC7.

blood pressure measurement consistently higher than 139 mmHg systolic and/or higher than 89 mmHg diastolic[91]. Recent international hypertension guidelines have also created categories below the hypertensive range to indicate a continuum of risk with higher blood pressures in the normal range [92-94]. In order to avoid misclassifying, a trained nurse performed repeated measurements of blood pressure. In our cohort the diagnosis of hypertension was based on clinical information regarding patients already diagnosed and under treatment and on three consecutive measurements if the blood pressure was higher than 140/90. Each measurement of blood pressure included two separate measurements within 1 minute after 5 minutes of rest.

### *Paper III*

Definition: In the current definition [95], AMI is defined if one of the following criteria satisfies the diagnosis for an acute, evolving or recent MI:

1) Typical rise and gradual fall (troponin) or more rapid rise and fall (CK-MB) of biochemical markers of myocardial necrosis with at least one of the following:

- a) ischemic symptoms
- b) development of pathologic Q waves on the ECG;
- c) ECG changes indicative of ischemia (ST segment elevation or depression); or
- d) coronary artery intervention (e.g., coronary angio-plasty).

2) Pathologic findings of an acute MI

According to those diagnostic criteria all such events were identified by record linkage with the Swedish Cause of Death and Hospital Discharge Registers, which is a valid alternative to revised hospital discharge and death certificates [96, 97]. The outcomes considered in this study were nonfatal and fatal events of AMI (ICD-8 and -9: 410; ICD-10: I21)[98]. All participants were followed from the baseline examination until a first cardiovascular event or death, or otherwise until December 31, 2011.

### *Paper IV*

The outcomes investigated in this study were AMI, Stroke and CVD. CVD was defined when at least one of the events was present: AMI, Stroke, coronary heart disease, cardiac heart failure or atrial fibrillation.

According to WHO [99] stroke is defined as a neurological deficit of cerebrovascular cause that persists beyond 24 hours or is interrupted by death within 24 hours. Stroke was defined as nonfatal or fatal cerebral infarction or intra-cerebral haemorrhage including ICD-10 codes I61, I63 and I64 [100].

Coronary artery disease is a narrowing or blockage of the arteries and vessels that provide oxygen and nutrients to the heart. The resulting blockage restricts blood flow to the heart. When the blood flow is completely cut off, the result is an AMI. According to this, coronary heart disease was defined as nonfatal myocardial infarction (ICD-10 code I21), percutaneous coronary intervention and/or coronary artery bypass grafting, while fatal CHD was defined as ICD-10 codes I21-I23, I25[98].

Atrial fibrillation is a supraventricular tachyarrhythmia characterized by uncoordinated atrial activation with consequent deterioration of atrial mechanical function. According to this, atrial fibrillation was defined as ICD-10 code I48[101].

Congestive heart failure occurs when the heart is unable to provide sufficient pump action to maintain blood flow to meet the needs of the body, and CHF was defined as ICD-10 code I50 [102]. All events were retrieved by data linkage with the Swedish Cause of Death and Hospital Discharge Registers, which is a reliable validated alternative to revised hospital discharge and death certificates [96, 97]. All participants were followed from the baseline examination until a first cardiovascular event or death, or otherwise until December 31, 2011.

### 3.6 *Statistical methods*

#### Paper I

General linear models were used to investigate the differences in SHBG levels between subjects with type 1 diabetes, type 2 diabetes, and those without diabetes, respectively. The association between serum insulin levels and SHBG concentrations was studied with linear regression after excluding subjects on treatment with exogenous insulin and/or on hormonal replacement therapy. All statistical tests were two-sided and significance was accepted if  $p < 0.05$ . SPSS Statistics Version 20 for Mac was used for all statistical calculations.

#### Paper II

The association between SHBG, sex hormones and blood pressure was investigated in linear regression models after exclusion of subjects treated with blood pressure lowering drugs. Logistic regression analyses were performed to estimate the association between hypertension and sex hormones (SHBG). Associations were expressed as regression coefficients (B) and odds ratios (OR), respectively, both with 95% confidence intervals (CI). General linear models were used to estimate differences (CI) between continuous variables. Theoretical multivariate models were used to estimate the role of possible confounders on the investigated association. All analyses were two-sided, and significance was accepted if  $p < 0.05$ . All analyses were performed using SPSS Statistics for Mac. In order to evaluate the strength of the association for each possible risk factor for hypertension, we standardized the variables in consideration using the formula  $SV = V/SD$ , where SV is the standardized variable, V is the value of the variable and SD is the standard deviation. We then ranked their association with hypertension by the regression coefficient  $\beta$  in the logistic regression analyses.

#### Paper III

Schoenfeld proportional hazards were used to determine the feasibility of Cox regression analysis. Cox Proportional Hazards Regression was employed to investigate the associations between levels of sex hormones at baseline and outcomes. Multivariate models were used to assess interactions and to estimate the roles of possible confounders. Stratified analyses for Type 2 Diabetes Mellitus were computed to investigate possible effect modification by diabetes. All analyses were two-sided, and  $p < 0.05$  was used as level of statistical significance. All analyses were performed using SPSS Statistics for Mac, version 20.

## Paper IV

The three sex hormones (SHBG, estradiol and testosterone) were normally distributed in men. In women the distributions of all three sex hormones were highly skewed, and excluding outliers did not normalize them. Accordingly, log-10 transformations were calculated for these measurements in women. Moreover, in women, every model was adjusted for hormonal therapy. Kaplan-Meier survival curves for tertiles of sex hormones for each sex-age group were analysed to decide the feasibility of analysis with Cox regressions. Cox Proportional Hazards Regression was employed to investigate the associations between sex hormones at baseline and outcomes. Theoretical multivariate models were used to assess interactions and to estimate the roles of possible confounders. All highly skewed covariates were transformed by log-10. All analyses were two-sided, and  $p < 0.05$  was used to assess statistical significance. Interactions were deemed significant if  $p < 0.15$  and factors demonstrating interaction were stratified. In the age group above 50, stratified analyses for diabetes were computed to investigate possible effect modification by diabetes. All analyses were performed using SPSS Statistics for Mac, version 20.

Sex hormones and cardiovascular risk in men and women

Paper	Design	Subjects	Exposure	Outcome	Statistical methods
I	Cross-sectional	Vara Skövde Cohort	Insulin	Concentrations of SHBG	Linear regression, General linear models
II	Cross-sectional	Vara Skövde Cohort	SHBG	Hypertension and blood pressure	Logistic regression, Linear regression
III	Cohort study	Skara Population Cohort	Testosterone	Acute myocardial infarction	Kaplan Meier Proportional hazards models
IV	Cohort study	Vara Skövde Cohort	Testosterone, Estradiol, SHBG	Acute myocardial infarction, Stroke, Cardiovascular disease.	Kaplan Meier Proportional hazards models
Summary of designs, subjects and methods used in the respective papers.					



## 4 RESULTS AND DISCUSSION

### 4.1 Paper I

#### *Results*

Baseline characteristics of this cohort have been reported elsewhere [103]. Serum concentrations of SHBG were inversely associated with BMI, fasting plasma glucose, HOMA-IR and with serum triglycerides in men and women, respectively (all  $p < 0.001$ ).

Sex stratified analyses with General Linear Model showed that SHBG levels were higher in subjects with T1D than in subjects without diabetes (men:  $\delta = 15.1$  nmol/l,  $P < 0.001$ ; women:  $\delta = 72.9$  nmol/l,  $P < 0.001$ ) or those with T2D (men:  $\delta = 15.9$  nmol/l,  $P < 0.001$ ; women:  $\delta = 71.1$  nmol/l,  $P < 0.001$ ) (*Figure 7*). The difference was still statistically significant after adjustments for age, BMI, fasting glucose and triglycerides ( $p < 0.001$ ). In both men and women, subjects with type 2 diabetes had lower levels of SHBG compared to subjects without diabetes in the age-adjusted analyses. The differences remained after making further adjustments for BMI or triglycerides or both together. These differences were, however, not longer significant when fasting plasma glucose was included as a covariate (*data not shown*).

In the linear models, we found a strong association between concentrations of SHBG and fasting insulin in subjects without exogenous insulin, as presented in *Table 1*. Fasting plasma insulin was significantly and inversely associated with SHBG concentrations independent of age, BMI and fasting glucose levels in both men and women. The association was stronger in women as it remained significant when triglycerides were also included in the model, whereas the association was no longer significant in men (*data not shown*).

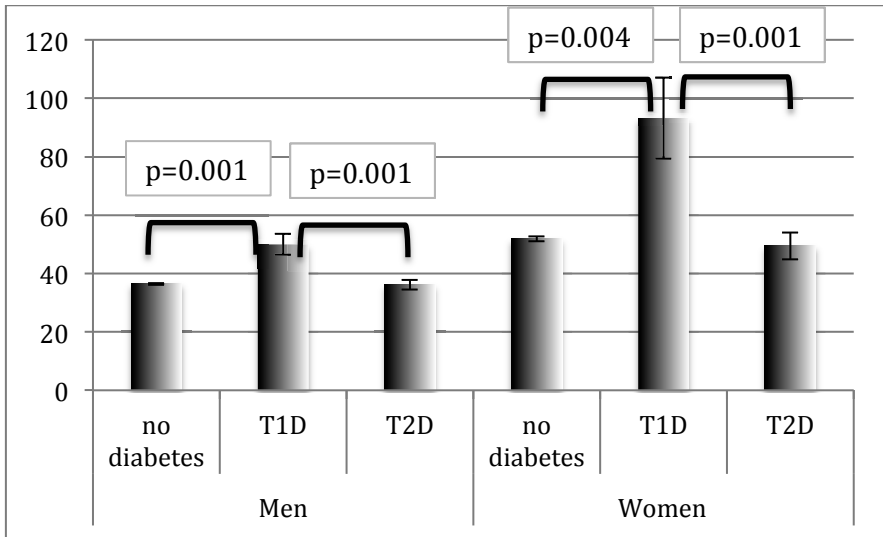


Figure 7. SHBG-levels in healthy controls, Type 1 and Type 2 diabetes patients for both genders. General linear models were used, with the results adjusted for age, BMI, fasting glucose and free testosterone.

Table 1. The association between sex hormone-binding globulin (SHBG) and fasting insulin in men (n=1,361) and women (n=1,002) without diabetes.

	$\beta$	p
<b>Men</b>		
<i>Adjusted for age</i>	-0.251	<0.001
<i>Adjusted for age and BMI</i>	-0.090	0.001
<i>Adjusted for age, BMI, F-PG</i>	-0.062	0.022
<i>Adjusted for age, BMI, F-PG, FT</i>	-0.040	0.303
<b>Women</b>		
<i>Adjusted for age</i>	-0.368	<0.001
<i>Adjusted for age and BMI</i>	-0.197	<0.001
<i>Adjusted for age, BMI, F-PG</i>	-0.176	<0.001
<i>Adjusted for age, BMI, F-PG, FT</i>	-0.149	<0.001

Multivariate linear regression analysis of the association between SHBG and insulin. BMI= Body mass index, FPG= fasting plasma glucose, FT= Free testosterone,  $\beta$ = standardized regression coefficient. Subjects exposed to exogenous insulin or other sex-hormone therapy (preventive pills or hormone replacement therapy) were excluded from these analyses.

## *Discussion*

In this study it was shown that subjects with T1D had significantly higher levels of SHBG than subjects without diabetes or with T2D and that concentrations of SHBG were strongly and independently associated with the concentrations of fasting serum insulin. Taken together, these findings suggest an inhibitory effect of insulin in the production of SHBG by the liver.

Our results are consistent with those from previous smaller studies [52, 104, 105], including those with only men and with *in-vitro* models [19, 106]. In accordance with other studies, a strong association was found between fasting serum insulin, triglycerides and SHBG levels. Low SHBG levels seem to be a marker for the metabolic syndrome in both men and women. In subjects with type 2 diabetes, the levels of SHBG were lower than in subjects without diabetes, but the association became insignificant after the adjustment for fasting glucose levels. In our study population the association between SHBG and insulin was significant and inverse in both men and women. While strong and robust in women, the association was somewhat weaker in men and became non-significant after simultaneously adjusting for age, BMI, fasting plasma glucose and free testosterone. The substantial reduction in the regression coefficient of serum insulin after adjusting for free testosterone suggests a minor and non-significant inhibitory effect of insulin additional to free testosterone with regard to the control of SHBG production in men. However, the association remained significant and strong in women. In addition, the changes observed in the regression coefficient during adjustments for BMI and fasting plasma glucose suggest an active regulatory role by these variables in the control of SHBG levels.

In conclusion, these results suggest an inhibitory effect of insulin on SHBG production. The effect of insulin on SHBG production is of particular interest, as low SHBG levels can predict the development of T2D. Although the mechanisms behind this phenomenon are still unknown, these results contribute to the understanding of the regulation of SHBG concentration.

## 4.2 Paper II

### Results

The characteristics of the study population are presented in *Table 2*. In men, SHBG increased linearly with age, but not in women. A strong negative association between SHBG and BMI, fasting plasma glucose, HOMA-IR and TG was found in both men and women and the same was true for systolic and diastolic blood pressure.

After excluding subjects under medication for hypertension (n=271 (10%)), linear regression analyses showed a significant association between SHBG and both diastolic (diastolic blood pressure:  $\beta = -0.143$   $p < 0.001$ ) and systolic blood pressure (systolic blood pressure  $\beta = -0.114$   $p < 0.001$ ). The association remained significant after adjusting for age, BMI, HOMA-IR, triglycerides, HDL and CRP (diastolic blood pressure:  $\beta = -0.113$   $p < 0.001$ ; systolic blood pressure  $\beta = -0.093$   $p = 0.001$ ). Age adjusted analyses with GLM showed that, in men, SHBG decreased significantly with the severity of high blood pressure. In women, however, the SHBG was almost constant in categories NT and prehypertension 1-3. Hypertensive women had significantly lower concentrations of SHBG when compared with all other categories ( $p < 0.001$ ) (*Figure 8*).

In the logistic regression analyses between quartiles of SHBG and hypertension in women, the odds for hypertension were higher for the lowest quartile (OR=4.2,  $p < 0.001$ ). Differences in the association were observed after stratifying for age (women  $< 50$  OR=2.8, CI 1.14-7.22,  $p = 0.025$ , women  $\geq 50$  OR=4.8, CI 2.61-8.88,  $p < 0.001$ ). For women  $\geq 50$  years, the association remained significant even when the model included information about history of stroke and diabetes in the equation ( $p$ -trend=0.043). In men an association was found between quartiles of SHBG and hypertension (OR=2.2  $p = 0.007$ ). However, this association was no longer significant after adjustment for BMI.

Logistic regression with standardized age, HOMA-IR, BMI and SHBG was used in order to evaluate the strength of the association of these measures with hypertension. The association between SHBG and hypertension was stronger than the association between HOMA-IR and hypertension in men, but not in women. The interaction between age, SHBG and hypertension was investigated in a logistic regression model, but no significant interaction was found.

Table 2. Phenotypic characterization of sex hormone-binding globulin in a Swedish population of men and women.						
	<b>Men n=1,385</b>					
		<i>Quartiles for SHBG</i>				<i>p-trend</i>
	<i>All</i>	Q1	Q2	Q3	Q4	
SHBG	33±14	17.8	26.7	35.1	50.8	
Age	47.8±11.8	42.2	45.6	48.8	54.4	<0.001
BMI	26.9±3.6	28.9	27.3	26.5	24.9	<0.001
Fasting insulin	6.7±5.2	8.9	7.0	6.2	4.9	<0.001
Fasting glucose	5.5±1.1	5.8	5.7	5.5	5.4	<0.001
HOMA-IR	1.7±1.6	2.3	1.8	1.5	1.1	<0.001
Hs-CRP	2.4±5.8	3.0	2.3	1.9	3.3	0.038
LDL	3.4±0.9	3.4	3.4	3.4	3.3	0.327
HDL	1.2±0.3	1.1	1.2	1.2	1.3	<0.001
Triglycerides	1.5±0.9	1.9	1.5	1.3	1.1	<0.001
Systolic BP	123±16	126	124	123	123	<0.001
Diastolic BP	72±10	73.8	71.7	71.8	70.6	<0.001
T-Testosterone	14.3±4.4	11.2	13.4	15.2	17.5	<0.001
Estradiol	127±56	125	128	124	132	0.026
FAI	48.5±17.3	64.5	50.5	43.4	35.3	<0.001
	<b>Women n=1,397</b>					
SHBG	55±31	26.2	41.1	55.6	92.6	
Age	47.7±11.7	46.9	47.3	49.0	47.7	0.033
BMI	26.8±5.3	30.1	27.2	26.5	24.9	<0.001
Fasting insulin	6.2±4.4	8.5	6.2	5.1	4.7	<0.001
Fasting glucose	5.3±1.1	5.7	5.4	5.1	5.1	<0.001
HOMA-IR	1.5±1.3	2.2	1.5	1.2	1.1	<0.001
Hs-CRP	2.7±4.6	4.1	2.5	2.2	2.3	0.097
LDL	3.1±0.9	3.2	3.1	3.2	3.2	0.287
HDL	1.4±0.3	1.2	1.4	1.5	1.5	<0.001
Triglycerides	1.2±0.6	1.4	1.1	1.0	1.1	0.004
Systolic BP	119±18	123	118	117	119	0.008
Diastolic BP	69±10	70.0	68.4	67.8	68.2	0.007
T-Testosterone	1.3±1.3	1.3	1.2	1.2	1.3	0.853
Estradiol	320±396	255	328	337	363	<0.001
FAI	3.1±5.8	5.7	2.9	2.4	1.4	<0.001
SHBG= sex hormone-binding globulin, Q1 is the lowest quartile and Q4 is the highest. BMI= Body mass index, HOMA-IR= homeostatic model assessment insulin resistance, hs-CRP= high sensitive c-reactive protein, LDL= low density lipoprotein, HDL= high density protein, BP= blood pressure, FAI= free androgen index. General linear models to estimate mean values and differences between quartiles were used. The significance was estimated with p-value <0.05.						

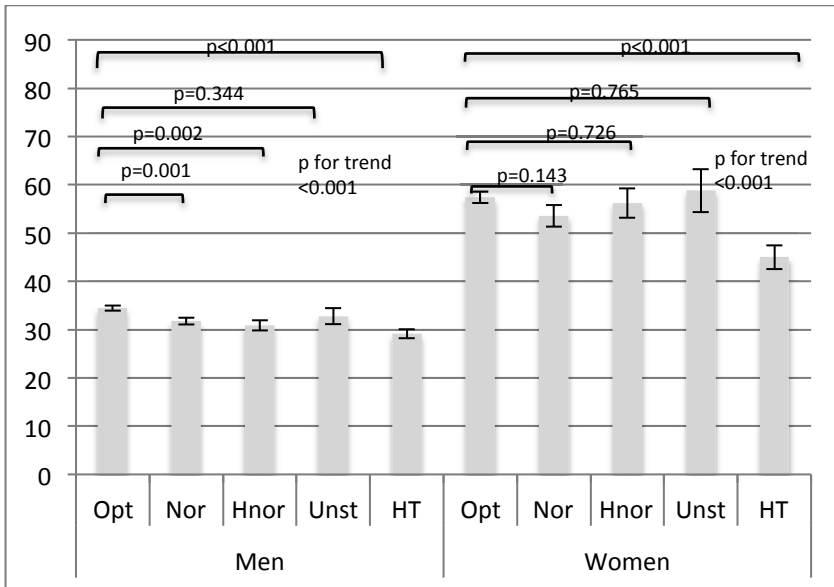


Figure 8. Comparison of serum concentrations of sex hormone-binding globulin (y-axis) between different blood pressure categories. JNC7 blood pressure categories; Opt=normal optimal BP <120/80mm Hg, Nor= normal BP <130/85 mm Hg, Hnor= normal high BP <140/90 mm Hg, Unst= unstable blood pressure. Hypertension was defined as known documented diagnosis for high blood pressure, or by three consecutive BP reading  $\geq 140/90$  mm Hg (systolic and/or diastolic). When the BP exceeded these limits only once or twice, the BP was categorized as unstable.

## *Discussion*

In this cross-sectional study, a strong inverse association between blood pressure and SHBG was observed. After the menopause, women with hypertension had significantly lower SHBG concentrations than women without hypertension. In men, we observed an inverse association between systolic blood pressure and SHBG. These findings were significant even after adjusting for major risk factors for hypertension such as age, BMI, diabetes and insulin resistance, indicating thus that low concentrations of SHBG may have independent negative effects on blood pressure control.

The novelty of this study was a detailed analysis of the factors that could influence the association between SHBG and hypertension. Although there are some reports regarding the association between SHBG and hypertension in men, very few reports concern similar studies in women. In a prospective study in men aimed at investigating the role of sex hormones in hypertension, Khaw *et al.* [107] observed that total testosterone but not SHBG was associated with hypertension. However, in another cross-sectional study in men conducted in Tromsø [108], an association between SHBG and systolic blood pressure was found even after adjusting for BMI and alcohol consumption. That study found lower concentrations of SHBG in hypertensive men even after adjusting for age and BMI, in accordance with our results in women [108].

Some gender differences were, however, observed in our study (*Figure 6*). In accordance with Khaw *et al.* [107], we found that levels of SHBG decreased in a linear fashion in men when the blood pressure increased. In contrast, the decrease in SHBG in women was observed first when they had developed hypertension. As the association between SHBG and hypertension in women was stronger over the age of 50, we suggest that these gender differences can at least in part be attributable to the effect of menopause in women. Total testosterone but not free testosterone was associated with hypertension in our population. We cannot confirm the vasodilative effects of free testosterone that were shown by Webb *et al.* [32]. A recent study has shown that SHBG but not testosterone is associated with increasing blood pressure. [109]. Lakshman *et al.* [52] speculate that a modulatory effect of SHBG exists that enhances the effects of testosterone. It must be emphasized that in a small study of men with hypertension, a strong association was found between SHBG and renin, which may be another pathway for direct effects of SHBG on the control of blood pressure [110]. Whether this association occurs in the blood vessel and whether it influences the blood pressure would need to be confirmed by studies utilizing a different design.



In accordance with previous studies, we found a positive linear association between SHBG and age in men, while in women the association was inverse before 50 years of age [111, 112]. The mechanisms underlying this gender duality are not fully understood, but it may be speculated that the decrease in testosterone levels is a possible factor. However, this effect does not seem to be a determinant of increasing SHBG concentrations in aging men according to De Ronde *et al* [50].

*In conclusion*, these results show a strong association between SHBG and blood pressure independent of the components of metabolic syndrome and inflammation. This association might be explained by a direct effect of SHBG in endothelial cells through the receptor for SHBG. Confirmation of these results by others may lead to the emergence of new opportunities for the development of therapies for lowering blood pressure.

## 4.3 Paper 3

### *Results*

The characteristics of the population at baseline are presented in Table 1 Appendix 3. Men had significantly higher diastolic blood pressure and Homa-ir than women, while their prevalence of hypertension, type 2 diabetes, smoking, and AMI was non-significantly higher than in women at baseline. Men with type 2 diabetes had significantly lower testosterone concentrations but no differences in the levels of SHBG were observed at baseline. In both men and women, the baseline prevalence of hypertension and previous AMI was higher in subjects with type 2 diabetes. However, there were fewer smokers among subjects with diabetes.

The mean follow-up time was  $14.1 \pm 5.3$  years. During that period 74 AMI events occurred in men, and 58 occurred in women. The event rate in the whole population was 10.3 per 1000 person years in men and 6.9 per 1000 person years in women. Of all the events, 10 AMIs in men and 9 in women occurred in people with diabetes. The event rate in subjects with type 2 diabetes was 13.8 per 1000 person years in men and 13.3 in women. Kaplan-Meyer analyses in men (*Figure 9*) showed that persons in the highest quartile of testosterone had a tendency towards better survival in the total cohort, while analyses stratified for type 2 diabetes showed a significantly lower rate of AMI in the highest quartile of testosterone compared with the three lowest quartiles in men with type 2 diabetes. Although a similar tendency towards better survival in the highest testosterone quartile was observed in men without diabetes, the findings in that group were not statistically significant.

Cox models were used to evaluate the association of concentrations of testosterone at baseline with AMI. In the model including all men regardless of diabetes status, the association between testosterone and AMI was marginally significant in the age-adjusted analysis (HR=0.950 95%CI 0.90-1.00,  $p=0.050$ ). When men with type 2 diabetes were analysed separately, a strong association was found in the age-adjusted model, which was further strengthened in the fully adjusted model (HR=0.754 95% CI 0.61-0.92  $p=0.006$ ). Models using free testosterone showed similar results (*Table 3*). An interaction term assessing the relationship between testosterone and type 2 diabetes was marginally significant ( $p=0.051$ ).

In women, trends were similar, although the results were generally not statistically significant. One model assessing the relationship between free testosterone and AMI in all women with full covariate adjustment was marginally significant (HR=0.722, 95% CI 0.52-1.00,  $p=0.046$ ) (*Appendix 3*)

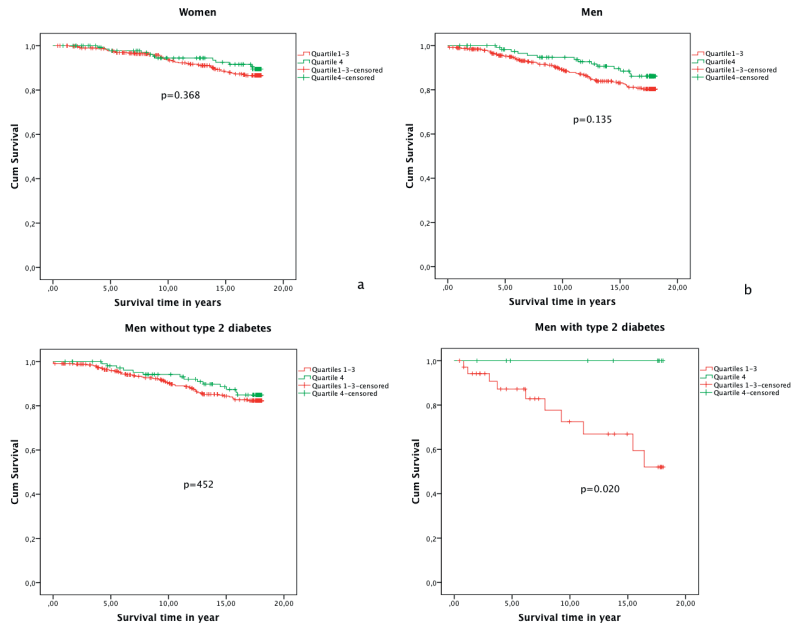


Figure 9. Kaplan-Meiers curves showing differences in survival between subjects in the highest quartile of serum testosterone (green) and the other three quartiles combined (red). Data stratified by sex (above), and in men also by diabetes status (below).

Table 3. Cox regressions analyses to investigate the association between testosterone and AMI.

	<u>Men</u>			<u>Women</u>		
	HR	CI	p	HR	CI	P
T-T	<i>All subjects</i>					
	<i>Adjusted for age</i>					
	0.950	0.90-1.00	0.050	0.720	0.45-1.12	0.142
	<i>Adjusted for age, estradiol and SHBG</i>					
	0.954	0.89-1.02	0.187	0.702	0.45-1.10	0.120
	<i>Adjusted for age, WHR, smoking, physical activities, LDL, SBP</i>					
	0.949	0.90-1.00	0.069	0.696	0.45-1.08	0.104
	<i>Adjusted for age, WHR, smoking, physical activities, LDL, SBP, SHBG, estradiol</i>					
	0.936	0.87-1.01	0.082	0.655	0.42-1.02	0.063
	<i>Subjects with type 2 diabetes</i>					
	<i>Adjusted for age</i>					
	0.828	0.71-0.97	0.022	0.759	0.24-2.39	0.638
	<i>Adjusted for age, estradiol and SHBG</i>					
	0.783	0.64-0.95	0.015	0.757	0.22-2.67	0.665
	<i>Adjusted for age, WHR, smoking, physical activities, LDL, SBP</i>					
	0.754	0.61-0.92	0.006	0.647	0.18-2.28	0.498
	<i>Adjusted for age, WHR, smoking, physical activities, LDL, SBP, SHBG, estradiol</i>					
	0.698	0.52-0.93	0.016	0.715	0.18-2.91	0.639

HR= hazard ratio by 1 mmol/L-change in total testosterone concentration and by 0.01 mmol/L change in free testosterone. CI= 95% of confidence interval. T-T= total testosterone, WHR=waist hip ration, LDL= low density lipoprotein, SBP= Systolic blood pressure, SHBG= sex hormone-binding globulin.

## *Discussion*

In this study, a strong and independent association between concentrations of testosterone and AMI was observed in men with type 2 diabetes. There was also a trend of an inverse association between serum testosterone and AMI in the cohort overall. The association in men was stronger than the association in women, and it remained significant after adjustment for age. The association was strongest among men with type 2 diabetes, where it remained significant after adjustment for age and factors within the metabolic syndrome. Previous studies in elderly men have shown an association between low levels of testosterone and higher risk for CVD [35, 113]; however, prospective studies in younger men are lacking. The cardiovascular impact of low testosterone in this group might be difficult to detect because of the low event rate, but another explanation might be that levels of testosterone are more important in aged blood vessels. In fact, in our study, the major impact of testosterone was observed among subjects with type 2 diabetes where early vascular aging is observed [40].

Low testosterone concentrations have previously been associated with the metabolic syndrome [114], and there is evidence supporting an inverse relationship between testosterone levels and the risk of developing type 2 diabetes and obesity [114]. These variables were considered in the multivariate analyses. However, the inverse relationship between testosterone and incident AMI in men with T2DM remained significant after the adjustment for age and metabolic risk factors, suggesting that other mechanisms may be involved. One possibility is an effect on the coronary perfusion through actions on the artery wall. Fukui et al have reported that low concentrations of endogenous androgens were associated with increased artery stiffness in men with type 2 diabetes [115]. A similar result was reported by Akishita et al. [29] in a study of the association between endothelial function and testosterone levels. Moreover, Web et al [32] have shown a vasoactive effect of testosterone in men with coronary disease, supporting the idea that testosterone might have an independent vasoprotective effect.

In the present study, we found that in women, free testosterone had a greater impact on the risk of AMI than total testosterone. This may be an artefact of the laboratory methods used.

*In conclusion*, the measurement of testosterone in men with type 2 diabetes may help in the assessment of their cardiovascular risk. Larger studies estimating the effects of low testosterone levels in men with diabetes in relation to AMI are needed.

## 4.4 Paper 4

### Results

Baseline characteristics and events registered during  $8.1 \pm 1.1$  years follow up for men and women are presented in *Table 4*. In men aged 50 years and older, there was a positive relationship between estradiol and stroke events. This association was independent of age, WHR, alcohol intake, smoking, hypertension, cholesterol, HDL-cholesterol, Hs-CRP and physical activity (HR=1.20 CI 1.08-1.32). This association was similar in men with and without diabetes. No significant associations were found between estradiol concentrations and AMI or CVD in men. Stratified analyses for sex and diabetes were conducted to evaluate the association between testosterone and AMI (*Table 5*). This association was strong and significant in men with diabetes (HR=0.82 CI 0.69-0.98), but not in men without diabetes (HR=1.00 CI 0.97-1.03). A similar pattern was observed for bioavailable testosterone. No significant associations were found between testosterone and stroke or CVD in men. There was also a positive association between SHBG and CVD in men aged 50 years and older. This association remained significant with adjustment for age, WHR, alcohol intake, smoking, hypertension, cholesterol, HDL-cholesterol, Hs-CRP and physical activity. The association with SHBG was not modified by diabetes. No significant associations were found between SHBG and AMI or stroke in men.

In women, distributions of the levels of all 3 sex hormones were highly skewed. Accordingly, we transformed these measures to log<sub>10</sub> to normalize the distributions. For AMI and CVD, Kaplan-Meier graphs suggested a J-shaped relationship with testosterone. However, further analyses using log-rank and Cox-regression with the second tertile as the reference group failed to substantiate these relationships. In women older than 50 years there was an inverse association between estradiol and incident stroke. This association became significant with adjustment for age, HRT, WHR, alcohol intake, smoking, hypertension, cholesterol, HDL-cholesterol, Hs-CRP and physical activity (HR=0.28 CI 0.08-0.96). An equivalent association was found between free estradiol and stroke in the fully adjusted model. There were no significant associations between estradiol levels and AMI or CVD. A similar, but weaker, inverse relationship was found between SHBG and CVD. This association was no longer significant when diabetes was included in the model (*Table 4*). Stratified analyses showed a strong association between SHBG and CVD in women without diabetes (HR=0.11 CI 0.02-0.63) but no association in those with diabetes (HR=4.18 CI 0.24-71.62).

Table 4. Baseline characteristics of Vara and Skövde Cohort.

	Men n=1,385	Women n=1,397	P
	Mean $\pm$ SD	Mean $\pm$ SD	
Age	47.8 $\pm$ 11.8	47.7 $\pm$ 11.7	0.773
BMI	26.9 $\pm$ 3.6	26.8 $\pm$ 5.3	0.821
Fasting insulin	6.7 $\pm$ 5.2	6.2 $\pm$ 4.4	0.002
Fasting glucose	5.5 $\pm$ 1.1	5.3 $\pm$ 1.1	<0.001
HOMA-IR	1.7 $\pm$ 1.6	1.5 $\pm$ 1.3	<0.001
Hs-CRP	2.4 $\pm$ 5.8	2.7 $\pm$ 4.6	0.090
LDL	3.4 $\pm$ 0.9	3.1 $\pm$ 0.9	<0.001
HDL	1.2 $\pm$ 0.3	1.4 $\pm$ 0.3	<0.001
Triglycerides	1.5 $\pm$ 0.9	1.2 $\pm$ 0.6	<0.001
Systolic BP	123 $\pm$ 16	119 $\pm$ 18	<0.001
Diastolic BP	72 $\pm$ 10	69 $\pm$ 10	<0.001
<i>Hormones</i>			
Total Testosterone	14.3 $\pm$ 4.4	1.3 $\pm$ 1.3	<0.001
Estradiol	127 $\pm$ 56	320 $\pm$ 396	<0.001
SHBG	33 $\pm$ 14	55 $\pm$ 31	<0.001
	<u>N/%</u>	<u>N/%</u>	
Hypertension	205(14.8)	202(14.7)	0.928
Type 2 diabetes	75(5.4)	62(4.4)	0.128
Smoking	212(15.3)	286(20.5)	<0.001
<i>Endpoints</i>			
AMI	47(3.4)	18(1.2)	<0.001
Stroke	27(2.0)	25(1.7)	0.749
CVD	126(9.0)	65(4.7)	<0.001
<i>CHD</i>	69(5.0)	24(1.7)	<0.001
<i>AF</i>	59(4.3)	27(1.9)	0.001
<i>HF</i>	12(0.9)	4(0.3)	0.055

Data are means and ( $\pm$ -SD) or n and (%). BMI=Body mass index, HOMA-IR=homeostatic model assessment for insulin resistance, Hs-CRP= high sensitive c-reactive protein, LDL= low density lipoprotein, HDL=High density lipoprotein, BP=blood pressure, SHBG=sex hormone binding globulin, AMI= Acute myocardial infarction, CVD=Cardiovascular disease, CHD=coronary heart disease, AF=Atrial fibrillation, HF=Heart failure. More than one endpoint may occur in the same subject.

Table 5. Cox regression analyses of the association between sex hormones and cardiovascular outcomes in men and women, respectively.							
<b>Men</b>				<b>Women</b>			
<i>No diabetes</i>		<i>Diabetes</i>		<i>No diabetes</i>		<i>Diabetes</i>	
<b>HR</b>	<b>CI</b>	<b>HR</b>	<b>CI</b>	<b>HR</b>	<b>CI</b>	<b>HR</b>	<b>CI</b>
<b>Testosterone and AMI</b>							
<i>Adjusted for age</i>							
0.95	0.87-1.04	0.88	0.78-0.99	1.33	N/A	1.38	N/A
<i>Adjusted for age, waist hip ratio, smoking and alcohol intake</i>							
0.98	0.89-1.08	0.87	0.75-0.99	1.19	N/A	1.14	N/A
<i>Adjusted as in model above +hypertension, cholesterol, HDL, CRP and PA</i>							
1.00	0.91-1.10	0.82	0.69-0.98	1.47	N/A	N/A	N/A
<b>Estradiol and Stroke</b>							
<i>Adjusted for age</i>							
1.17	1.06-1.29	1.14	0.91-1.44	0.40	0.11-1.45	N/A	N/A
<i>Adjusted for age, waist hip ratio, smoking and alcohol intake</i>							
1.16	1.05-1.27	1.41	0.97-2.03	0.54	0.14-2.07	N/A	N/A
<i>Adjusted as in model above +hypertension, cholesterol, HDL, CRP and PA</i>							
1.17	1.05-1.31	N/A	N/A	0.53	0.14-2.03	N/A	N/A
<b>Sex hormone binding globulin and CVD</b>							
<i>Model 1 Adjusted for age</i>							
1.01	0.99-1.02	1.00	0.98-1.03	0.11	0.03-0.47	N/A	N/A
<i>Adjusted for age, waist hip ratio, smoking and alcohol intake</i>							
1.02	1.00-1.03	1.01	0.98-1.04	0.12	0.02-0.58	N/A	N/A
<i>Adjusted as in model above +hypertension, cholesterol, HDL, CRP and PA</i>							
1.02	1.00-1.04	1.01	0.98-1.04	0.11	0.02-0.63	N/A	N/A
HR= hazard ratio for changes in concentrations of testosterone by 1mmol/L, estradiol by 10mmol/L and, SHBG by 1mmol/L in men and 1 unit of logarithm in women, CI= confidence interval, HDL= high density lipoprotein, CRP= c-reactive protein, PA= physical activity, N/A= not applicable.							



## Discussion

All significant associations were found solely in subjects aged 50 years and older. We found opposite, yet strong and independent associations between concentrations of estradiol and stroke in men and women. While higher concentrations of estradiol were protective in women, they were a risk factor in men. SHBG levels predicted CVD and, similarly to the findings for estradiol, the direction of the association was opposite by sex with higher concentrations of SHBG associated with risk in men and protection in women. We found effect modification by diabetes mellitus with low levels of testosterone associated with AMI in men with diabetes, but not in men without. Diabetes was also an effect modifier in women, as high concentrations of SHBG were protective in women without diabetes. Importantly, this protective association was lost in women with diabetes.

Cardiovascular effects of estradiol concentrations in men have been studied in two population studies with opposite conclusions [44, 45]. Tivesten et al [45] found a strong association between high levels of estradiol and carotid artery intima-medial thickness. On the other hand, Arnlov et al. [44] found that estradiol protects against new CVD in elderly men. The thickness of intima media in carotid artery is more related to stroke than to coronary events, but is still a strong intermediate endpoint for generalized atherosclerosis [116]. In our study we did not find any association between concentrations of estradiol and CVD overall, AMI or CHD. However, we found a strong association between estradiol concentrations and stroke. This suggests differences in effects of estradiol in cerebrovascular vessels compared to coronary vessels.

Previous epidemiological studies investigating the associations between estradiol and CVD in women have reached different results and the predictive value of estradiol concentrations on future cardiovascular events is unclear [8]. Similar to the SWAN-study we found that high levels of estradiol are protective in post-menopausal women [117, 118]. These similarities may reflect the age of the cohorts, since the ages of our cohort and the SWAN study were similar. In contrast, the Three-city study, which followed a much older population, reported increased risk [6]. These results suggest that there might be age differences in the effect of the estradiol, with estradiol seemingly protective against CVD in early menopause, but associated with increased risk in elderly women. This is also consistent with the findings of a protective effect of estrogen alone for women aged 50 to 59, and no increased risk with estrogen plus progestin for women within 10 years of menopause, in the WHI clinical trials [63]. These findings are consistent with the possibility that low levels of estrogen sustained for a decade or more result in vascular changes, such as increased intima-medial

thickness and atheroma burden, that ultimately result in increased risk associated with the actions mediated by estradiol. Currently, adequate clinical trial or cohort data that might answer the question of whether initiating estrogen treatment near menopause and continuing it through subsequent decades is associated with reduced CVD risk is lacking.

In this thesis an effect modification of diabetes is shown regarding the association between low testosterone and cardiovascular events. Specifically, in men aged 50 and older with type 2 diabetes, low concentrations of testosterone predicted AMI. This association was not found in men without diabetes. However, effects of testosterone in the vessels can differ depending on the health of the endothelium. Diabetes is associated with early vascular aging [40], such that the effects of low testosterone concentrations become harmful in the presence of endothelial dysfunction. The measurement of testosterone in men with diabetes may thus provide important information for cardiovascular risk assessment. Trials of testosterone treatment in men older than 50 years with diabetes may be warranted, as there is rationale for protection. Appropriate designs would also provide further information about potential mechanisms for testosterone effects on the risk of AMI.

Recent studies have shown an association between SHBG and type 2 diabetes. Furthermore, low concentrations of SHBG have been associated with hypertension and the cumulative incidence of hypertension [109, 119]. However, evidence regarding the association between SHBG and CVD is still limited, and the results have been discordant [53, 54]. In our study the association of SHBG with CVD was strong, and a sub-analysis showed that atrial fibrillation was the component with the strongest association. A previous study has shown a strong association between metabolic syndrome and atrial fibrillation [120]. To further investigate this association we adjusted our analysis for components of the metabolic syndrome. Even if concentrations of SHBG seem to predict hypertension and diabetes, the underlying mechanisms are still unknown. The presence of receptors of SHBG that modulate the effects expressed by sex-hormones has been shown [121]. A recent study could also show the presence of intracellular protein *megalyn* that transports the complex SHBG-steroid inside the cell, confirming that the SHBG is not only a carrier protein but also modulates the effects of sex hormones [122]. In our study, the protective effect of high levels of SHBG was lost in women with diabetes, although this effect modification was not observed for estradiol. This difference in associations did not seem related to study power and thus suggests the need for further investigation of SHBG-specific mechanisms.

## 5 GENERAL DISCUSSION

In this thesis the association between concentration of sex hormones and cardiovascular diseases were explored in population-based samples. In paper I, exploring the factors that influence concentrations of SHBG, a strong association between insulin levels and SHBG was found. In paper II, a strong and independent association between sex hormone-binding globulin and hypertension was found. Although the association was present in both men and in women, the strongest association was observed in women aged 50 years or older, suggesting that menopause may play an important role. In paper III, exploring the association between testosterone and AMI, a significant and independent association between concentrations of testosterone at baseline and the incidence of AMI in the Skara population cohort was found only in men with type 2 diabetes. These findings were confirmed in men with diabetes in the Vara-Skövde Cohort in paper IV. In paper IV, a strong association between estradiol and stroke was found in men and women. A sex difference was found in this association, as high concentrations of estradiol in men were predictive for stroke, while they protected against stroke in women. Moreover, SHBG seemed to have a general protective effect regarding cardiovascular disease in women. In this respect, diabetes modified the outcome, as the protective effect was strong only in women without diabetes.

Ever since the large sex-differences in cardiovascular disease observed in earlier epidemiological studies, sex hormone concentrations have been investigated as possible biological mediators of these differences. However, the results have been contradictory, and consensus is still lacking regarding the effects of these hormones in cardiovascular disease in men and women, and regarding the cardiovascular effect of exogenous replacement therapy of these hormones. It is still unknown whether the concentrations of these hormones are more critical in special subgroups of patients. As cardiovascular disease is strongly related to age, the majority of studies regarding the association between sex hormones and cardiovascular morbidity have been done in elderly cohorts. However, whether the effects of sex hormones are constant over time or whether they change with vascular aging is not known yet. These knowledge gaps provided the background for the subgroup analyses conducted in this thesis, in an attempt to answer these questions. Thus, the investigations in Paper III and IV defined men with diabetes as a group of patients where the levels of testosterone were critical for the risk of AMI. In both cohorts the relative risk for AMI decreased with increasing concentrations of endogenous testosterone at baseline, suggesting

a protective role of testosterone in men with type 2 diabetes, albeit a similar effect observed in men without type 2 diabetes was not significant. The reason why the observation was seen only in men with diabetes, and why only for AMI is currently unknown but deserves further investigation. Type 2 diabetes has been associated with an early aging of the vascular wall [40]. In the aged endothelium, a rapid decrease in sex hormones may cause a dysfunction resulting in increasing coagulability. Observations in intima media thickness in men with diabetes have shown a strong relationship between low levels of testosterone and the thickness of the intima media [123]. This observation suggests a protective effect of testosterone[123], which may be mediated by an effect of testosterone on insulin resistance [124, 125], on modulation of inflammation [126] and on the concentrations of serum lipids [127, 128]. In contrast with these positive effects of testosterone on improving health, a recent observational study in the US showed that hypogonadal men on testosterone replacement therapy had a higher incidence of cardiovascular events than controls[129]. The retrospective design of the study, however, could not address possible selection bias, as individuals with a higher cardiovascular risk might have been more likely to be treated with testosterone. Since the results of this thesis are in accordance with previous studies showing the protective effects of testosterone, it remains to be explored whether the use of testosterone in men with diabetes improves the prognosis for cardiovascular disease in this group of patients. Results of prior observational studies of the association between estradiol and cardiovascular events have been contradictory. Although studies of stroke risk in women in peri-menopause have observed a protective role of estradiol, studies in women aged 70 or older have shown a deleterious effect of estradiol. It seems that aging has a modifying effect on the association between estradiol and cardiovascular disease in women. The cohort investigated in this thesis was younger, and events in subjects younger than 50 at base line were scarce. However, in both men and women a strong association between stroke events and estradiol levels was observed. The results show a sex-duality in this association, as high concentrations of estradiol were protective in women, but deleterious in men. Estradiol levels in men reflect an increase of aromatase activity with aging and obesity. High age and obesity are two known risk factors for hypertension and stroke; however, the association in men was still significant after adjustments for both these factors. These findings are in accordance with other studies that found a strong association between concentrations of estradiol and an increase in the intima media thickness in men [44, 45]. Estradiol seems thus to have negative effects on the cardiovascular risk and endothelium function in men.

In the Vara-Skövde cohort SHBG levels were associated with higher risk for cardiovascular disease. While low concentrations of SHBG have been associated with higher risk for cardiovascular disease in both men and women, a sex duality was observed in our cohort. Thus, high concentrations in women were protective. However, this was confirmed only in women without diabetes suggesting that the protective effect in women is lost when they are affected with type 2 diabetes. Type 2 diabetes in women can have a modifying effect on the protective role of sex hormones that could explain at least partially the loss of protection regarding AMI observed in women with type 2 diabetes[130]. The young population and the low event rates might implicate low statistical power, and a type 2 error cannot be excluded.

In contrast, in paper 2 a strong association between SHBG and blood pressure was found in both men and women. The association had the same direction in both men and women, and no sex duality was observed in the association between SHBG and blood pressure. While the concentrations of SHBG predicted diabetes in both men and women in two different cohorts [51, 52], the mechanisms behind this association are unknown. Experimental studies have shown that the presence of membranous receptors for SHBG is capable of altering concentrations of c-AMP when bound to their ligands. The hypothesis is that SHBG essentially modulates the effects of sex hormones by influencing their intracellular transport [122]. These physiologic mechanisms of SHBG are still to be understood in detail, but could potentially explain how SHBG can predict type 2 diabetes and hypertension. These findings, however, are in accordance with the findings in the paper 4 and the higher risk for cardiovascular disease shown in women with lower concentrations of SHBG. The association between high levels of SHBG and higher risk of CVD in men needs further investigation, as it was unexpected. As SHBG is related to cardiovascular risk and the effect and function of sex hormones, it would be important to know which mechanisms control the concentrations of SHBG. Genetic variants have been associated with different concentrations of SHBG [131, 132]. Hormonal factors have also been associated with higher concentrations of SHBG[15-18]. Moreover, obesity is associated with low levels of SHBG and anorexia is associated with higher levels [15-18]. Whether glucose or insulin controls levels of SHBG has been an area of debate. The findings presented in paper I indicated, however, that endogenous insulin is independently associated with concentrations of SHBG, providing further information regarding mechanisms of control of SHBG concentrations.

*In conclusion*, concentrations of sex hormones predicted cardiovascular morbidity in both men and women, albeit differently according to sex. While

testosterone was protective in men, estradiol and SHBG were protective in women. Correspondingly, the effects of estradiol in men seem negative while the effects of testosterone in women were uncertain. Thus, in each sex the characteristic hormone supports health. Diabetes also modified the association between concentration of sex hormones and CVD in both sexes. These modifications might explain at least partially the loss of the cardiovascular protection in women when they contract diabetes. There may be a potential for replacement therapy using testosterone in men with diabetes.

## 6 METHODOLOGICAL CONSIDERATIONS

### *General considerations*

This thesis was based on large population samples with high participation rates. The participation rate was 79% in the Skara population cohort and 76% in the Vara and Skövde cohort. This high participation rate combined with register based random selection enhances the representativeness of the study sample. However, a minor selection bias is possible as nonparticipants tend to have different characteristics and generally have a higher burden of cardiovascular comorbidity [133]. Comprehensive characterization of the cohorts regarding health status, lifestyle factors and biological markers at baseline provided the opportunity to investigate the associations between sex hormones and cardiovascular outcomes as well as possible confounders of these associations. The sample size provided adequate power to evaluate clinically meaningful associations. Although these analyses are post-hoc, a stratified design for subjects older and younger than 50 years of age in Vara-Skövde cohort and for type 2 diabetes was planned from the beginning in order to avoid accidental findings and to investigate an effect modifying of diabetes in both men and women. It should also be acknowledged that the cross-sectional design of the study in papers I and II do not allow the establishment of causality regarding the association between SHBG and insulin and between SHBG and hypertension.

### *Outcomes*

The outcome in Paper I was SHBG. The method used is valid [84]. The measurement of SHBG concentrations were successful in 2782 subjects and permitted further analyses for the original hypothesis.

The outcome in Paper II was hypertension and blood pressure. The measurement of this outcome was based on repeated measurements. The diagnosis of hypertension was based on international criteria [134] only if the subject had 3 repetitive blood pressure measurements  $\geq 140/90$  mm Hg and/or already was diagnosed and treated for hypertension.

The outcome in Paper III was acute myocardial infarction. The outcome was defined using record linkage with the Swedish national mortality register and the National inpatient register. The method has been validated previously [98] and the use of register data regarding this outcome is common.

In Paper IV, 3 outcomes were considered: AMI, CVD and Stroke. While the use of AMI based on registries has been validated in previous publications [98], no recent publications appear to exist regarding the validity of the use of registries regarding stroke diagnosis. Two different studies in 1993 and 2000 [135, 136] showed an unsatisfactory correlation between the diagnosis stroke in the registers and the validation by revised hospital discharges. However, better diagnostic instruments and their frequent use in the last decade should have improved the quality of the register. The majority of register-based studies use the Swedish national mortality register and the National inpatient register [137-140] and we also chose to do so. Further studies to elucidate the validity in using these registers are needed.

In Papers III and IV the number of the events was lower than expected in the planning phase of these cohorts. The lack of events was attributable to the decrease of the incidence in the cardiovascular disease in the last decades [141]. The fact that these studies were based on a small number of events may have increased the risk for type two error. Nevertheless, there is a risk of overestimating the effects with Cox regression analyses on small samples [142]. Accordingly, the analyses of sex hormones were done in two cohorts and the results were compared between cohorts for the assessment of their consistency.

### *Exposure*

Blood samples were collected in the morning, thereby avoiding influences of circadian variations in the hormonal concentrations. The diagnosis of diabetes was accurate and in accordance with WHO recommendation [143].

A potential weakness of this study is the lack of information with regard to menopausal status. Still, the use of 50 years as a cut-off point for menopause is supported by findings from previous studies [144, 145]. The comprehensive medical history permitted identification of individuals using hormonal therapy and the analysis of them separately. Analyses restricted to the participants below the age of 50 had limited power due to the small number of events. Future analyses with longer follow-up in this subpopulation will permit investigation of the role of sex steroids in the development of the diseases.

Another limitation in this thesis is the low accuracy of the measurements of testosterone in the low range of the distribution, which concerns especially women [146, 147]. Moreover there is a circadian variation of concentrations of sex hormones and SHBG which probably attenuates our findings [148]. The small number of men with type 2 diabetes in Papers III and IV is another



weakness in this thesis, as there is a risk of overestimating effects in subgroup analyses [142]. However, the consistency of these results across strata, and the significant findings despite the small sample size in two different cohorts suggest a strong association.

## 7 CONCLUSION

### 7.1 *General conclusions*

Strong associations between sex hormones and cardiovascular risk were observed in both men and women. While testosterone was protective in men, estradiol levels seemed protective in women. Diabetes modified the effect of sex hormones in both men and women. While the protective effect of testosterone in men for acute myocardial infarct was observable in cases with diabetes, the presence of diabetes in women seemed to cancel the protective effects of SHBG regarding CVD. These differences in the effects of sex hormones observed in women with diabetes can, at least partially, explain the elimination of the protection against acute myocardial infarction in these women.

### 7.2 *Specific conclusions*

1. There is an inverse association between SHBG and endogenous insulin, independent of age, BMI, and plasma glucose.
2. There is a strong association between SHBG and blood pressure independent of metabolic factors in both men and women.
3. Low concentrations of testosterone are associated with higher risk for AMI in men with type 2 diabetes. Although the association was present in men without diabetes and in women it was weaker and statistically not significant.
4. Estradiol concentrations are inversely associated with the risk for stroke in women but are positively associated with the risk for stroke in men.
5. A duality in the effects of SHBG was also observed. High levels of SHBG were protective in women, especially in those without diabetes, whereas in men high levels of SHBG were related to higher risk for CVD.

## 8 FUTURE PERSPECTIVES

Apart from the physiological effects in the reproductive tissues, sex hormones have been associated with aging, and low levels of the representative sex hormone in each sex have been associated with deterioration of endothelial function. In Papers III and IV it was shown that the measurements of testosterone in men with type 2 diabetes may help in the assessment of their cardiovascular risk. These results suggest a potential benefit of testosterone replacement therapy in men with diabetes, although this needs to be replicated in other populations and tested in randomized controlled trials.

Together with the initiation of a 10-year follow-up of the Vara-Skövde cohort, these cohorts provide excellent opportunities for longitudinal studies. With this platform, it will be possible to study the factors that influence changes in sex hormones. Moreover, it will be possible to study how these changes in sex hormones influence the risk of cardiovascular disease and quality of life. Finally, the follow up of these cohorts as they progress from middle to older age will provide the opportunity to observe whether age is a modifying factor in the association between sex hormones and cardiovascular disease.

In Paper IV, stroke events were less frequent in women with high endogenous estradiol. These findings are in accordance with findings in the SWAN study, suggesting that endogenous estradiol has a protective effect in women. Our results, taken in context of findings for CVD from other studies such as WHI, support the possibility of different effects with age of estradiol in the menopause, as estradiol may be protective in the beginning of the menopause but harmful in the later menopause. The repeated measurement of estradiol levels in this population should provide a good possibility for the investigation of this hypothesis.

In Paper II, an independent association of SHBG with blood pressure levels was observed. These findings are in accordance with other observations suggesting a more active role for sex-hormone binding globulin in the action of sex hormones. The confirmation of these observations by other studies might open up a new field for the development of therapies for lowering blood pressure. In Paper I an inhibitory effect of insulin on the SHBG production was found. These results will contribute to the understanding of the regulation of SHBG concentration.

Finally, this study revealed large differences in how sex hormone concentrations are related to cardiovascular disease and to risk factors in men and women, suggesting that sex hormones have a key position in the understanding of the differences in the cardiovascular disease in men and women. More research concerning the endothelial effects of sex hormones may have an important impact on future cardiovascular risk assessment and treatment recommendations.

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