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# TESTOSTERONE - OF IMPORTANCE IN PATIENTS WITH DYSGLYCEMIA AND CARDIOVASCULAR DISEASE?

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To my family

*“Science never solves a problem  
without creating ten more”*

George Bernard Shaw (1856-1950)

Department of Medicine, Division of Cardiology  
Karolinska Institutet, Stockholm, Sweden

# Testosterone - of importance in patients with dysglycemia and cardiovascular disease?

by

**Anne Wang**

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## ABSTRACT

**Background:** Testosterone has been associated with cardiovascular (CV) health in men and women with or without diabetes. There are however conflicting results which warrant further investigations to understand if testosterone is important for prognosis, in particular in relation to diabetes and cardiovascular disease (CVD). To gain further insight, several aspects such as when and which testosterone fraction to assess as well as genetic variations in the androgen receptor are of interest to study.

**Aims:** To study the role of testosterone in men and women with different levels of dysglycemia and CVD by investigating:

1. the dynamics of testosterone levels up to a year following an acute myocardial infarction (AMI) in men with and without dysglycemia
2. the relation between the androgen receptor gene CAG repeat length, testosterone levels and prognosis
3. the prognostic implications of total testosterone, free testosterone and the binding protein sex hormone-binding globulin (SHBG) in patients with AMI compared to healthy controls as well as in men and women with dysglycemia and high CV risk

**Methods:** **Studies I and II** were based on data from the GAMI study, a prospective cohort study of patients with AMI providing blood samples at four time points up to a year post-infarction, and healthy, age-matched controls. Study participants were classified as having normal (NGT) or abnormal glucose tolerance (AGT) based on oral glucose tolerance tests and followed for about 11 years for CV events, and CV and all-cause mortality; **Study I** comprised male patients (n=123) and controls (n=124), **Study II** comprised male patients (n=121) with blood samples available for DNA analyses. **Studies III and IV** were based on a biomarker substudy of ORIGIN which was a large, multicenter randomized controlled trial following patients with dysglycemia and high CV risk for about six years for CV events and all-cause mortality. **Study III** comprised all male patients (n=5 553) and **Study IV** all female patients (n=2 848) in the biomarker substudy.

**Results:** In **Study I**, median testosterone levels were lower immediately after an AMI compared to controls at baseline (243 ng/dl vs. 380 ng/dl;  $p<0.01$ ) but increased at discharge, three months and 12 months to 311, 345 and 357 ng/dl respectively. Patients with AGT had the lowest levels at the first timepoint (230 ng/dl). Total and free testosterone did not predict CV events or all-cause mortality in men with AMI but CV and all-cause mortality in controls.

In **Study II**, contrary to the hypothesis, there was no correlation between CAG repeat length and testosterone and moreover CAG repeat length did not predict CV events or all-cause mortality.

In **Study III**, total and free testosterone did not predict prognosis in Cox regression analyses by one standard deviation increment but low free testosterone ( $\leq 7$  ng/dl) was associated with increased all-cause mortality. Additionally, increasing SHBG was related to a higher risk of CV events (HR 1.07; 95% CI 1.00–1.14;  $p<0.03$ ) and all-cause mortality (HR 1.13; 95% CI 1.06–1.21;  $p<0.01$ ).

Finally, in **Study IV**, total and free testosterone did not predict any outcomes in women but SHBG was related to increased all-cause mortality (HR 1.14; 95% CI 1.05–1.24;  $p<0.01$ ).

**Conclusions:** Low testosterone was common in patients hospitalized with AMI, in particular in those with AGT, but increased over time indicating that samples taken in close proximity to AMI should be interpreted with caution. In contrast to healthy controls where low total and free testosterone was predictive of prognosis, only free testosterone  $\leq 7$  ng/dl was associated with all-cause mortality in patients. This suggests that testosterone may be a mediator in CVD and prognosis rather than a traditional risk factor. The potential importance of CAG repeat length in this context was not confirmed. Interestingly, SHBG was an independent predictor for CV events and all-cause mortality in men and for all-cause mortality in women with dysglycemia. This warrants further study to clarify whether the actions of SHBG are mediated through an impact on the distribution of testosterone or if SHBG is a direct prognostic marker.

## SAMMANFATTNING

**Bakgrund:** Testosteron tycks vara kopplat till kardiovaskulär sjukdom hos män och kvinnor med eller utan diabetes. Tillgängliga data är dock bristfälliga med många kunskapsluckor och ytterligare studier behövs för att förstå om testosteron är viktigt ur en prognostisk synvinkel, särskilt i relation till kardiovaskulär sjukdom och diabetes. Flera aspekter såsom när och vilken fraktion av testosteron som ska mätas samt genetiska variationer i androgenreceptorn är av intresse att studera.

**Mål:** Att studera testosteronets roll hos män och kvinnor med olika nivåer av dysglykemi och kardiovaskulär sjukdom genom att undersöka:

1. dynamiken av testosteronnivåer upp till ett år efter en hjärtinfarkt hos män med eller utan dysglykemi
2. kopplingen mellan antal CAG repetitioner i androgenreptorgen och testosteronnivåer samt prognos.
3. totalt och fritt testosteron samt SHBGs prognostiska betydelse hos patienter med hjärtinfarkt jämfört med hos friska kontroller och hos män och kvinnor med dysglykemi och hög kardiovaskulär risk.

**Metoder:** **Studie I** och **II** baserades på GAMI-studien, en prospektiv kohortstudie av hjärtinfarktspatienter som lämnade blodprover vid fyra tillfällen upp till ett år efter infarkten samt friska, åldersmatchade kontroller. Deltagarna klassificerades med hjälp av ett glukosbelastningstest som att ha normal (NGT) eller abnormal glukostolerans (AGT) och följdes i ca 11 år vad avser kardiovaskulära händelser och kardiovaskulär och total dödlighet. **Studie I** inkluderade manliga patienter (n=123) och kontroller (n=124), **Studie II** inkluderade manliga patienter (n=121) med prover tillgängliga för DNA analys. **Studie III** och **IV** baserades på den biokemiska substudien av ORIGIN som var en stor, multicenter, randomiserad kontrollerad studie som följde patienter med dysglykemi och hög risk för kardiovaskulär sjukdom i sex år med avseende på kardiovaskulära händelser och total dödlighet. **Studie III** inkluderade alla manliga patienter (n=5 553) och **Studie IV** alla kvinnliga patienter (n=2 848) i substudien.

**Resultat:** I **Studie I** var medianvärdet av testosteron lägre omedelbart efter en hjärtinfarkt jämfört med det hos kontroller (243 ng/dl jfrt. med 380 ng/dl;  $p<0.01$ ) men steg vid utskrivning, tre månader och 12 månader till 311, 345 och 357 ng/dl. Patienter med AGT hade lägst nivåer vid den första tidpunkten (230 ng/dl). Totalt och fritt testosteron predikerade inte kardiovaskulära händelser eller dödlighet hos patienterna med hjärtinfarkt men kardiovaskulär och total dödlighet hos kontrollerna.

I **Studie II** förelåg ingen korrelation mellan antal CAG repetitioner och testosteron. Antalet CAG repetitioner var ej kopplat till vare sig kardiovaskulära händelser eller total dödlighet.

I **Studie III** predikerade varken en standarddeviations ökning av totalt eller fritt testosteron prognos i Cox regressionsanalyser, men lågt fritt testosteron ( $\leq 7$  ng/dl) var kopplat till högre risk för total dödlighet. Dessutom var stigande SHBG kopplat till ökad risk för kardiovaskulära händelser (HR 1.07; 95% CI 1.00–1.14;  $p<0.03$ ) och total dödlighet. (HR 1.13; 95% CI 1.06–1.21;  $p<0.01$ ).

I **Studie IV** predikerade varken totalt eller fritt testosteron kardiovaskulära händelser eller total dödlighet hos kvinnor men högre SHBG var kopplat till ökad dödlighet (HR 1.14; 95% CI 1.05-1.24;  $p<0.01$ ).

**Slutsatser:** Lågt testosteron var vanligt hos patienter med akut hjärtinfarkt, särskilt hos de med AGT, men steg över tid. Prover tagna i nära anslutning till en hjärtinfarkt bör därför bedömas med försiktighet. I motsats till kontrollerna där både lågt totalt och fritt testosteron predikerade prognos var endast fritt testosteron  $\leq 7$  ng/dl kopplat till ökad mortalitet hos patienter. Detta talar för att testosteron kan vara en mediator för kardiovaskulär sjukdom och prognos snarare än en riskfaktor. En faktor av potentiellt intresse i detta sammanhang, CAG repetitioner, verkade ej vara viktig hos män med hjärtinfarkt. Å andra sidan var SHBG en oberoende prediktor för kardiovaskulära händelser och total dödlighet hos män och för dödlighet hos kvinnor med dysglykemi. Huruvida SHBGs effekter förmedlas via dess koppling till testosteron eller om SHBG är en direkt prognostisk markör är oklart varför fler studier behövs för ökad förståelse kring bakomliggande mekanismer.

## LIST OF SCIENTIFIC PAPERS

- I. Wang A, Arver S, Flanagan J, Gyberg V, Näsman P, Ritsinger V, Mellbin L  
**Dynamics of testosterone levels in patients with newly detected glucose abnormalities and acute myocardial infarction**  
Diab Vasc Dis Research 2018;Nov;15(6):511-518.  
doi: 10.1177/1479164118802543
- II. Wang A, Flanagan J, Arver S, Norhammar A, Näsman P, Rydén L, Mellbin L  
**Androgen receptor polymorphism, testosterone levels and prognosis in patients with acute myocardial infarction**  
Submitted manuscript
- III. Wang A, Arver S, Boman K, Gerstein HC, Lee SF, Hess S, Rydén L, Mellbin L  
**Testosterone, sex hormone-binding globulin and risk of cardiovascular events: A report from the Outcome Reduction with an Initial Glargine Intervention trial**  
Eur J Prev Cardiol. 2019 May;26(8):847-854.  
doi: 10.1177/2047487318819142.
- IV. Wang A, Gerstein HC, Lee SF, Hess S, Paré G, Rydén L, Mellbin L  
**Prognostic impact of testosterone and sex hormone-binding globulin in dysglycemic women at high cardiovascular risk: A report from the Outcome Reduction with an Initial Glargine Intervention trial**  
Submitted manuscript



## **ABBREVIATIONS**

ACS	Acute Coronary Syndrome
AGT	Abnormal Glucose Tolerance
AMI	Acute Myocardial Infarction
BMI	Body Mass Index
CAD	Coronary Artery Disease
CAG	Cytosine-Adenosine-Guanine
CI	Confidence Interval
CRP	C-reactive Protein
CV	Cardiovascular
CVD	Cardiovascular Disease
DHT	Dihydrotestosterone
DM	Diabetes Mellitus
DNA	Deoxyribonucleic Acid
FSH	Follicle Stimulating Hormone
FT	Free Testosterone
GAMI	Glucose in Acute Myocardial Infarction
GnRH	Gonadotropin Releasing Hormone
HbA1c	Hemoglobin A1c
HOMA-IR	Homeostasis Model Assessment-estimated Insulin Resistance
HPT	Hypothalamic-Pituitary-Testicular
HR	Hazard ratio
IFG	Impaired Fasting Glucose
IGT	Impaired Glucose Tolerance
LC-MS/MS	Liquid Chromatography-tandem Mass Spectrometry
LDL	Low Density Lipoprotein
LH	Luteinizing Hormone
NGT	Normal Glucose Tolerance
OGTT	Oral Glucose Tolerance Test
ORIGIN	Outcome Reduction with an Initial Glargine Intervention
PCOS	Polycystic Ovary Syndrome
PCR	Polymerase Chain Reaction
SD	Standard deviation
SHBG	Sex Hormone-Binding Globulin
T2DM	Type 2 Diabetes Mellitus
TT	Total Testosterone
WHO	World Health Organization

## INTRODUCTION

### Testosterone

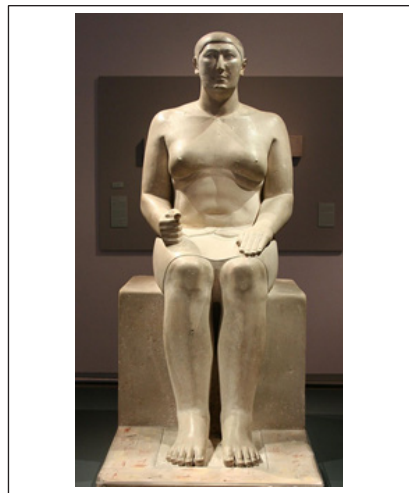
#### *History*

In the archeological Roemer-Pelizaeus Museum in Hildesheim, Germany, the statue of Hemiuni, the closest advisor to pharaoh Kheops and the architect of the pyramid of Giza (fl. 2570 BC) is exhibited (Figure 1). Contrary to the more idealized presentations of males from the time period of Ancient Egypt as well-built, athletic men, Hemiunu is portrayed as an obese man with no beard and bilateral gynecomastia. The plaque on Hemiunu's statue outlines his professional achievements, but it does not mention family as was customary at the time, suggesting he could have been infertile. The statue of Hemiunu is believed to be one of the first images of a hypogonadal patient, mirroring testosterone deficiency (1).

Over time, the basic elements of the testes were discovered and described in terms of sperm and fertilization, but how endocrine function from the testes could present itself in physiological appearances was unknown. In the middle of the 19<sup>th</sup> century, Arnold Adolph Berthold (1803–1861) discovered that following the castration of four cockerels, there was a loss of interest in hens (2). This was reversed in the two cockerels receiving an ectopic testicular transplantation. Berthold concluded that “the testes act upon the blood, and the blood acts upon the organism as a whole”. These findings were confirmed in other animals studies in the early 20<sup>th</sup> century, initiating a search for the testicular substance acting in the blood (2).

Simultaneous to the animal experiments, studies in the field of steroid hormone biochemistry was emerging following the discovery of the steroid ring structure. In the 1930s, the German biochemist Adolf Butenandt (1903–1995) isolated the androgenic steroid androsterone from 15 000 liters

of urine provided by young policemen and published the chemical synthesis of testosterone (3). Butenandt was awarded the Nobel prize in Chemistry in 1939 “for his work on sex hormones” together with Leopold Ruzicka (1887-1976) who was awarded the prize “for his work on polymethylenes and higher terpenes” that served as a basis for his discoveries on testosterone synthesis. Not long thereafter, different forms of testosterone became clinically available as injections for the treatment of hypogonadism and trials using testosterone therapy were initiated (4). In studies of castrate and eunuchoid patients, testosterone therapy was shown to have beneficial effects on peripheral vascular disease (5). Furthermore, case reports and a small clinical study (n=46) showed promising results of testosterone therapy in patients with angina pectoris (6). This was followed by a somewhat larger study in the 1940s in which 100 consecutive patients with angina pectoris were prescribed testosterone and followed for months to years with regards to their chest discomfort (7). In total, 91 of the



**Figure 1. The statue of Hemiunu.** Hemiunu was the closest advisor to the pharaoh and the architect of the pyramid of Giza (fl. 2570 BC).

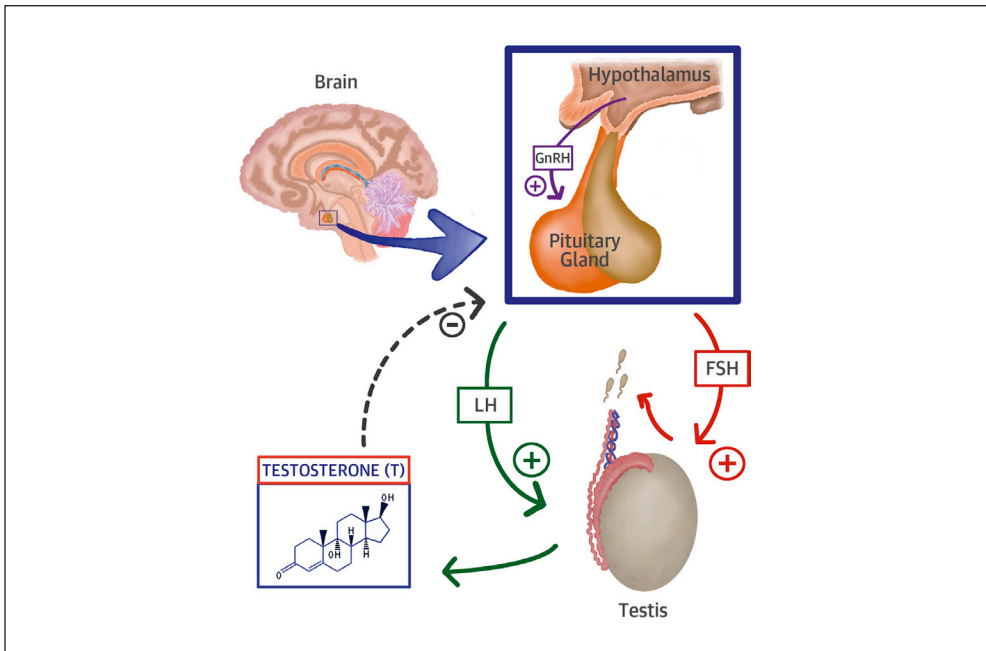
<https://commons.wikimedia.org/wiki/File:Statue-of-Hemiun.jpg>. Reproduced under the Creative Commons Attribution-Share Alike 3.0 Unported license

100 patients reported moderate to marked improvement. It was concluded that “Testosterone propionate, properly used, not only reduces the frequency of attacks of angina pectoris, but decreases their severity when they do occur. Further time will be required for the evaluation of results before it can be determined definitely whether or not this treatment will prolong the life of the patient.”

The role of endogenous and exogenous testosterone in the cardiovascular (CV) system and the impact on CV risk factors such as dysglycemia has been studied since with inconsistent results.

### *Regulation and transport of testosterone*

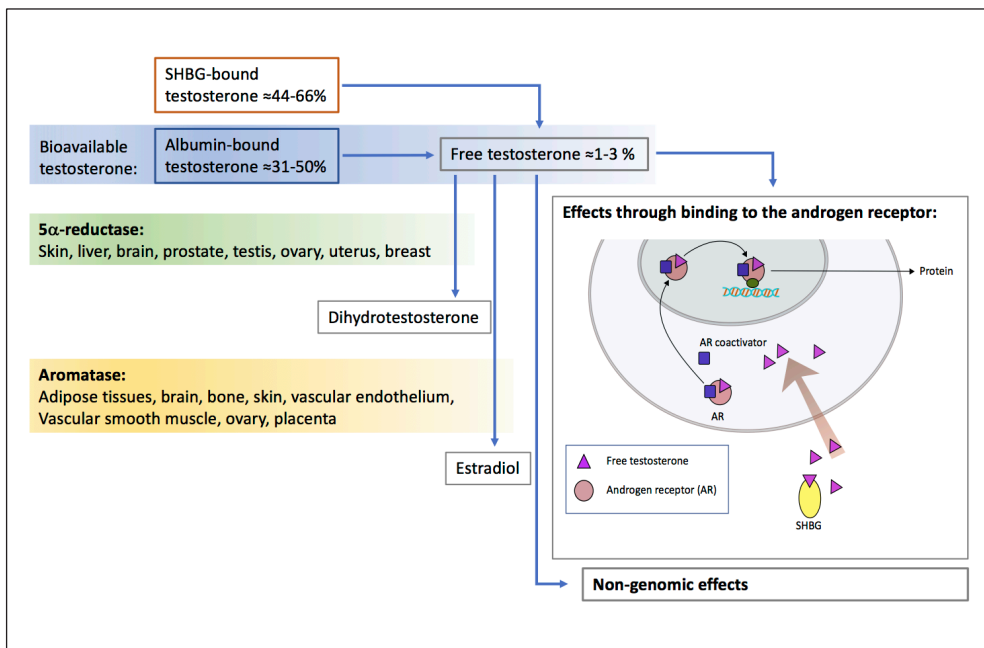
Testosterone is a steroid hormone which plays an essential role in sexual and cognitive function as well as body development in men (8). The synthesis and release of testosterone into the blood from the Leydig cells in the testes is regulated by the hypothalamic-pituitary-testicular (HPT)-axis and secretion of luteinizing hormone (LH) (Figure 2) (9). Apart from that LH regulates the Leydig cell function and testosterone levels, several other endocrine pathways such as the insulin-like growth factor 1, thyroid hormones and glucocorticoids are believed to affect the regulation of testosterone (10, 11). Moreover, the HPT-axis is sensitive to stress and acute illness is associated with a decrease in testosterone levels (12).



**Figure 2. Hypothalamic-pituitary-testicular (HPT) axis.** Reproduced with permission from Elsevier (26). When the levels of plasma testosterone are low, the HPT axis responds by increasing the release of gonadotropin releasing hormone (GnRH) from the hypothalamus and luteinizing hormone (LH) from the pituitary gland which stimulates the testes to produce and release more testosterone (9). When levels are restored or elevated above normal levels, the HPT axis responds instead by decreasing stimulating hormones which result in lower plasma testosterone levels, thereby completing a negative feedback loop. LH stimulates Leydig cells to synthesize testosterone and follicle stimulating hormone (FSH) stimulates Sertoli Cells to increase spermatogenesis.

Testosterone is also an important hormone in women, exerting reproductive and non-reproductive effects as well as acting as a precursor for estradiol synthesis. Levels vary during the menstrual cycle with a peak mid-cycle and remain high during the latter phase of the cycle (13). Testosterone is in part produced by the ovaries and in part produced by peripheral conversion of pre-androsterone from the ovaries and the adrenal gland (1). The regulation of testosterone in women is not well-known, but androgen production from the ovaries is possibly regulated by the LH levels.

Testosterone is transported mainly in three fractions in blood in men as well as in women: one part bound to sex hormone binding globulin (SHBG) with high affinity, one part bound to albumin with low affinity and one small part as unbound free testosterone (Figure 3) (14). The proportion transported by the different binding proteins varies slightly between men and women with a higher proportion SHBG-bound testosterone in women (14, 15). Free testosterone is the fraction that diffuses into target cells and exerts androgenic effects (9, 16). The albumin-bound fraction together with free testosterone is called bioavailable testosterone.



**Figure 3. Different fractions of testosterone and mechanisms of action.** Testosterone circulates in blood bound to SHBG, albumin and as an unbound, free fraction. The free fraction can enter the cell by different mechanisms, the “free hormone hypothesis” outlined in the figure (16, 19). Free testosterone diffuses across the cell membrane and binds to the androgen receptor. After binding, the androgen receptor induces dimerization which facilitates the binding to a specific sequence of DNA, known as the hormone response element. It regulates transcription of specific androgen-responsive genes which in turn promotes synthesis of proteins. Free testosterone can also be converted to dihydrotestosterone or estradiol by conversion of enzymes, which are located in various parts of the body.

SHBG is a homodimeric glycoprotein, composed of two identical subunits of polypeptide chains to which androgens can bind (1, 16, 17). It is produced in the liver and the production is inhibited by inflammatory factors such as hepatic lipids, tumor necrosis factor- $\alpha$ , interleukin 1 and also by high insulin levels. On the other hand, thyroid hormones may e.g. stimulate SHBG production. Several conditions such as diabetes, obesity, hypo- and hyperthyroidism, kidney disease, liver disease, use of glucocorticoids and androgenic steroids are associated with altered SHBG levels (16, 18). SHBG binds testosterone with high affinity, determining the bound and free fractions of circulating testosterone and it has been hypothesized that SHBG possibly regulates the bioavailability of testosterone.

Studies investigating the molecular interactions between testosterone and SHBG suggest that testosterone binds to the two binding sites of SHBG in an equivalent, non-allosteric way, and this has served as a basis for formulas used to calculate free testosterone such as the Södergård formula and the more commonly used Vermeulen formula (20, 21). However, Zakharov et al. recently suggested another mechanism in which a multistep, dynamic process of testosterone binding to the SHBG molecule takes place. The binding of testosterone to one site of the SHBG dimer leads to an allosteric interaction between the two binding sites, resulting in testosterone binding into the second site with a different affinity (22). Calculation of free testosterone by means of Zakharov's model is considered to provide more accurate results than previous models (22, 23).

#### *Mechanisms of testosterone actions*

Testosterone exerts effects (Table 1) primarily by binding to the intracellular androgen receptor. Different mechanisms by which testosterone enters the cell have been suggested whereof the free hormone hypothesis has been depicted in Figure 3. Androgen receptors are found in many tissues, including typical androgen-dependent organs such as prostate, epididymis, testes and muscles but also the kidneys, spleen and heart (24, 25). Testosterone is also converted to other metabolites such as estradiol through activation by aromatase and dihydrotestosterone (DHT) through activation by 5 $\alpha$ -reductase (Figure 3).

Target organ	Effects
Bone	Maintain bone density, bone growth, bone marrow production of red blood cells
Brain	Cognitive function, memory, libido, emotions
Kidneys	Stimulates erythropoetin responsible for red blood cell production
Muscle	Increase muscle mass and strength
Skin	Hair growth
Reproductive organs	Sperm production, growth of reproductive organs, erectile function
Glucose metabolism (27, 28)	Insulin sensitivity
Cardiovascular system* (29)	Vasodilatation, decrease in atherosclerotic plaque volume, shorter QTc interval, protection from ischemic injury

\*Associations determined in experimental animal models.

The androgen receptor gene, located on chromosome X, has different functional domains comprising a transactivating, a DNA-binding and a hormone-binding domain respectively. The transactivating domain contains a variable number of cytosine-adenosine-guanine (CAG) repeat which encodes a polyglutamine stretch of variable length in the receptor protein (19, 30). In a European population, the mean length of the CAG repeat in men is 21-22 (31-33). The peripheral effects of testosterone are dependent on a functional androgen receptor and it has been suggested that the number of CAG repeat is inversely correlated to the transcriptional activity of the androgen receptor and thereby the androgen effects in target tissues (30). Most androgenic effects work through activation of DNA transcription after binding to the androgen receptor, but there are also non-genomic effects by interaction with cellular membrane receptors although this area is not as well-studied (34).

In women, testosterone exerts effects either by binding to the androgen receptor or by non-genomic effects as described for men. It also exerts effects by conversion to estradiol and binding to estrogen receptors (13). Testosterone affects bone metabolism, cognitive health, gynecological health and sexual function in women. Recently it was also suggested that testosterone, as in men, may have effects on the CV and metabolic systems (35).

*Low testosterone levels in men*

Hypogonadism is defined as low levels of total testosterone accompanied by related symptoms as listed in Table 2 (18). The threshold for low total testosterone varies considerably between laboratories and assays but is usually defined as <300 ng/dl or <8-12 nmol/l (18, 23). There is an even higher variability in the threshold for low free testosterone and laboratories are recommended to establish their own lower limit based on equilibrium dialysis and/or calculations. The lower limit usually ranges between 5-9 ng/dl or 0.2-0.3 nmol/L. Measurement or calculation of free testosterone is recommended in conditions affecting SHBG as already outlined. Current guidelines discourage testing for testosterone deficiency in men recovering from acute illness since testosterone levels are known to be affected by stress. Still, it is not clearly described when it is appropriate to assess testosterone levels following an acute illness (23).

**Table 2.** Symptoms and signs typical for testosterone deficiency in men according to the Endocrine Society Clinical Practice Guidelines (18, 23).

<b>Symptoms and signs</b>	
More specific	Less specific
Incomplete or delayed sexual development	Decreased energy, self-confidence
Diminished libido	Depressed mood
Erectile dysfunction	Poor concentration and memory
Hot flushes	Trouble with sleep, increased fatigue
Gynecomastia	Reduced muscle strength
Small (<6 ml) or shrinking testes	Increased body fat, body mass index
Loss of body hair	Mild anemia
Height loss, low bone mineral density	
Inability to father children, low/zero sperm count	

The HPT-axis serves as a basis for hypogonadism classification (8). Primary hypogonadism is caused by testicular failure while secondary hypogonadism is caused by hypothalamic or pituitary failure. Testosterone replacement therapy initiated when the criteria for hypogonadism are fulfilled aims at normalizing the testosterone levels, ameliorate symptoms and increase the feeling of well-being (18, 23). There are different ways to administer testosterone including intramuscular injections at different intervals, transdermal patches, topical gels and oral drugs (36).

The prevalence of low testosterone levels/androgen deficiency varies in different populations and seems to increase with age. Androgen deficiency, defined as low total or free testosterone in combination with symptoms, was studied in males in the longitudinal Massachusetts Male Aging Study and reported as 6% at baseline and 12% after a follow-up of nine years (37). In the Baltimore Longitudinal Study of Aging, low testosterone levels ( $\leq 325$  ng/dl) were prevalent in 20% of men between 60-69 years and 30% of those between 70-79 (38). In another study of male American veterans (mean age=70 years) a larger proportion (34%) had testosterone levels below 300 ng/dl (39). Even if the difference in prevalence may at least partly be explained by use of different thresholds, the divergent data underlines the need for further studies.

#### *Low testosterone levels in women*

Testosterone levels are lower in women than in men even if described reference ranges varies between different study populations (around 0.3-3.2 nmol/L or 9-92 ng/dl) (1, 40). Circulating levels of testosterone decline with age but they do not fall specifically at menopause (41). Other causes for decreasing testosterone levels include oophorectomy or hysterectomy and treatment with estrogen or corticosteroids. Contrary to men, there is no clearly defined condition caused by androgen deficiency (such as hypogonadism in men) (42). Moreover, there is no testosterone level below which the level in women is defined as low. The guidelines on Androgen Therapy in Women by the Endocrine Society have therefore recommended against diagnosing healthy women with an androgen deficiency syndrome (42). Information on symptoms related to low testosterone levels and also the prognostic implication is sparse. Some studies have shown correlations between low testosterone and SHBG levels with decreased bone mineral density, impaired cognitive function, depressive symptoms, insulin resistance and surrogate markers of CVD similar to the effects in men (13, 35, 42). These observations do, however, need to be confirmed in larger cohorts.

## **Cardiovascular disease and dysglycemia**

In this thesis, testosterone is studied in the context of CVD and diabetes, two conditions that are associated with increased mortality per se but also in this regard interlinked. Much is known about the background of these conditions as well as their prognostic implications but there are still gaps in knowledge attracting interest to further explore the role of testosterone.

#### *Cardiovascular disease*

CVD is the leading cause of mortality worldwide, accounting for 31% of global deaths in 2016 (43). In Europe, CVD was the reason for 45% of all deaths in 2017 with age-standardized rates greater in males than in females (44). CVD is also the most common cause of premature death (age <70 years) in European males whereas in females cancer is a more common cause

of death (45). The number of people living with CVD is steadily increasing due to longer life expectancy combined with a decreasing mortality not the least after myocardial infarction. Apart from improved treatment of coronary artery disease (CAD) and a subsequently better survival, other reasons for the increasing incidence of CVD are related to dietary habits and a more sedentary lifestyle leading to an accumulation of CV risk factors such as obesity, hypertension and dysglycemia (46).

CVD is defined as a group of disorders affecting the coronary, cerebral and peripheral arteries, which are often caused by atherosclerosis (47). Atherosclerosis can lead to thickening and stiffening of the arterial walls, which may impair the blood flow due to luminal obstruction (48, 49). If the obstruction causes a mismatch between oxygen supply and oxygen demand in the myocardial tissue, it may lead to ischemia presenting as stable angina pectoris or an acute coronary syndrome (ACS) (48). ACS relates to a critical phase of CAD and describes a spectrum of clinical manifestations characterized by chest pain, release of troponins and presence of electrocardiographic changes. The different manifestations are defined as unstable angina, non-ST-elevation myocardial infarction and ST-elevation myocardial infarction (48), the last two defined as an acute myocardial infarction (AMI).

Several factors may contribute to the development and progression of atherosclerotic heart disease such as inflammation, dyslipidemia and hyperglycemia (50). Male sex has long been considered a risk factor for CVD since the prevalence in CAD is generally higher in men than in women as well as the CV mortality rate (51). However, there are gaps in knowledge regarding through which mechanisms the risk factors act. Hormones may contribute and androgens are of particular interest given the gender differences already described.

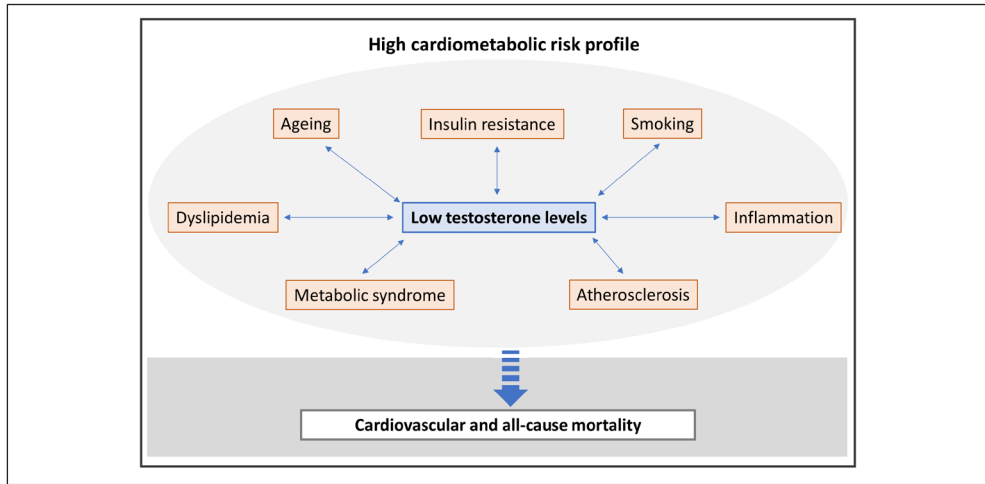
### *Testosterone and cardiovascular disease in men*

In addition to its effects on reproductive organs, testosterone has been linked to CV surrogate markers such as dyslipidemia, insulin resistance and inflammation (Figure 4) (29). It has also been hypothesized that testosterone may be directly associated with the development of CVD. Indeed, testosterone levels are reported as lower in men with CAD than in those without. The prevalence of low testosterone in men with CAD ranges widely, between 17 and 43% (52-55).

Low levels of testosterone does not only seem to be common among men with CVD, they have also been related to impaired CV prognosis and increased CV and all-cause mortality, in men with established CVD (55) and in the general population (15, 26, 29). In the European Prospective Investigation into Cancer in Norfolk (EPIC-Norfolk) study low total testosterone was related to increased risk of CV and all-cause mortality in 2 314 men followed for seven years (56). Furthermore, the Osteoporotic Fractures in Men (MrOS) study in which elderly men (n=2 416) were followed for five years found that total testosterone in the highest quartile was associated with a lower risk of major CV events compared to the lower quartiles (57). While a majority of the observational studies have reported an inverse correlation between testosterone levels and CV prognosis and all-cause mortality, other investigations reported on the opposite relation or no relation at all (58-60). A meta-analysis from 2010 comprising 19 studies on testosterone and CVD showed no association between testosterone and CVD in middle-aged men and only a weak association in elderly men (61). Shortly thereafter another meta-analysis was published in 2011, comprising 11 community-based studies of



men with all-cause mortality (n=16 184) and seven studies with CV mortality (n=11 831) as the primary endpoint (whereof six studies were the same as in the previous analysis). This meta-analysis showed that low total testosterone was related to both all-cause and CV mortality with a stronger relation in older men (62). The conflicting results in observational studies including the two meta-analyses may be related to differences in baseline comorbidities or differences in analytic methods and thresholds. Furthermore uncertainties in when testosterone should be assessed following an acute illness, in the light of that levels decrease following critical conditions, is unclear.



**Figure 4. Association between low testosterone and cardiovascular risk factors.** The association between low testosterone levels and CV risk factors have been suggested to be potential mediators by which testosterone is related to cardiovascular and all-cause mortality. Adapted from Gencer et al. with permission (29).

### Dysglycemia

Dysglycemia is a term used to describe abnormal blood glucose levels, such as type 2 diabetes mellitus type 2 (T2DM) and prediabetes (comprising impaired glucose tolerance test (IGT) and impaired fasting glucose (IFG)). Diabetes is defined as a group of disorders characterized by hyperglycemia as a result of deficient insulin production, secretion and/or action (63, 64). The characteristics are outlined in Table 3. T2DM is the most common type accounting for >90% of all people with diabetes.

Diagnosis	Characteristics
Type 1 diabetes mellitus	Autoimmune destruction of pancreatic $\beta$ -cells leading to an absolute insulin deficiency.
Type 2 diabetes mellitus	Deficient insulin secretion in the context of increasing insulin resistance.
Gestational Diabetes	Hyperglycemia developed during pregnancy, probably due to increased insulin resistance.
Other specific types of diabetes	Genetic defects in $\beta$ -cells, disease in the exocrine pancreas, surgery of the pancreas, drug induced diabetes.
Prediabetes	Hyperglycemic conditions at risk of developing diabetes and CVD

Diabetes, and more specifically T2DM, is increasing rapidly around the world, in part explained by increased longevity but also dietary changes and physical inactivity (66). According to the International Diabetes Federation, it is the seventh most common cause of global mortality. Approximately 463 million adults (20-79 years) had known diabetes in 2019 with an estimated increase to 700 million by 2045 (66). In addition, about 374 million people have IGT. The risk of developing T2DM in individuals with IGT or IFG five years after receiving diagnosis is 26 and 50% respectively (67). It has been estimated that in the absence of any interventions, 90% of the people with prediabetes will progress to T2DM 20 years after the diagnosis (68).

The diagnosis of dysglycemia is determined by using one or more of four diagnostic tests; fasting plasma glucose, two-hour post load glucose from an oral glucose tolerance test (OGTT), glycated haemoglobin (HbA1c) or random plasma glucose (Table 4).

**Table 4. Diagnostic criteria and classification of dysglycemia** according to the WHO guidelines from 2006 and 2011 (69, 70).

	Diagnostic criteria			
	Fasting glucose (mmol/L)*	2h-plasma glucose (mmol/L)*	HbA1c (mmol/mol)*	Random plasma glucose (mmol/L)
Diabetes Mellitus	≥7.0	≥11.1**	≥48	Symptoms + ≥11.1
Impaired glucose tolerance	<7.0	7.8-11.0**	-	
Impaired fasting glucose	6.1-6.9	-	-	

\*should be repeated twice and may be measured in venous or capillary blood. \*\*denotes cut-off for venous blood samples. Fasting glucose should be measured from after eight hours of fasting. Two hour plasma glucose should be measured from two hours after ingesting a standardized 75 g glucose load.

People with T2DM are, compared to those free from this condition, more prone to develop CVD such as AMI and have a more dismal prognosis if they do (71-73). About half of the mortality among patients with T2DM is related to CVD (66, 74). Among patients with AMI, approximately 20-25% have known diabetes and of those without known dysglycemia, one third is diagnosed with diabetes and one third with IGT when tested with OGTT (72). There are still gaps in knowledge regarding how dysglycemia is related to CVD (75, 76). Hyperglycaemia and/or traditional CV risk factors such as obesity and hypertension cannot provide a full explanation. Hence, other factors related to glucometabolic disorders may be of importance in this aspect and further investigations of potential risk factors and/or risk markers may explain the relation between dysglycemia and CVD.

#### *Testosterone and dysglycemia in men*

As outlined in Table 1 testosterone is potentially involved in glucose metabolism. Low levels have been associated with insulin resistance in observational studies and the relation is likely bidirectional (Figure 4) (77). Testosterone levels are lower in men with T2DM compared to their healthy counterparts and the prevalence of low testosterone is higher (25-40% vs

12-34%) in those with T2DM (77-79). In a cross-sectional study comprising 355 men with T2DM, 20% had total testosterone levels  $\leq 8$  nmol/L, 31% had levels between 8.1-12 nmol/L (borderline deficiency), and 50% had low free testosterone levels ( $< 0.26$  nmol/L) (80). The reduction in testosterone has been partly attributed to altered SHBG levels in patients with diabetes (81, 82). However, free testosterone is also considerably lower in males with T2DM (83, 84) suggesting that the reduction is not entirely related to SHBG levels.

Men with T2DM and low testosterone seems to have a more dismal prognosis than those with normal testosterone. Muraleedharan et al showed that in 581 men with T2DM of whom 40% had total testosterone  $\leq 300$  ng/dl, low testosterone was related to increased all-cause mortality (85). In an observational cohort study by Tint et al, low levels of free testosterone and high levels of SHBG predicted all-cause mortality during 7.6 years of follow-up in men with T2DM (n=531) (86).

### *Testosterone, cardiovascular disease and dysglycemia in women*

Investigations of sex hormones in women have traditionally put estrogen in focus. As described above, testosterone also plays a pivotal role in females both as a precursor for estrogen biosynthesis and for exerting androgen effects (13, 87). Whether androgens also affect the CV system in women have not been clearly established and it is far less studied than in men. Studies have been conducted in women with polycystic ovarian syndrome (PCOS), a condition typically characterized by hyperandrogenism (high testosterone levels) and hyperinsulinemia. For example, an observational study comprising 21 470 women with PCOS followed for a median of five years found an increased risk of T2DM (88).

Differences in sex hormones between men and women with T2DM have been described, for example showing that high testosterone in women but low testosterone in men was associated with higher risk of T2DM (89). In a study following women with PCOS for 31 years, there was no difference in morbidity or mortality from CAD compared to the control group (90). Similar to findings in men there are observational studies showing that low levels of testosterone are associated with CVD and all-cause mortality in women (87, 91, 92). Evidently, the relationship between testosterone and CVD in women is complex and even more so in hyperinsulinemic conditions e.g. PCOS. To what extent testosterone is associated with an impaired survival in a dysglycemic population remains to be clarified.

## **Gaps in knowledge**

In summary, available data favours the assumption that there is an association between testosterone, CVD and dysglycemia both in men and women. The results are however inconsistent, especially in individuals already afflicted with CVD and dysglycemia. The dynamics of testosterone levels, prevalence of low testosterone and prognostic implications of testosterone in men and women with different stages of dysglycemia and CVD need further clarification. Analyses of different fractions of testosterone, i.e. total and free testosterone and the binding protein SHBG, as well as the CAG repeat length which is associated with androgen receptor responsiveness are of particular interest. Such studies have the prerequisite to add a piece to the puzzle explaining this complex relationship and possibly be helpful in the search for high-risk individuals.

## **AIMS**

The overall aim was to study the role of testosterone in men and women with different levels of dysglycemia and CVD by investigating:

- 1) the dynamics of testosterone levels up to a year following an AMI in men with and without dysglycemia (**Study I**)
- 2) the relation between CAG repeat length, testosterone levels and prognosis (**Study II**)
- 3) the prognostic implications of total testosterone, free testosterone and SHBG in patients with AMI compared to healthy controls and in men and women with dysglycemia and high CV risk (**Studies I, III and IV**)

## MATERIAL AND METHODS

This thesis is based on four different patient populations derived from a cohort study and a clinical trial as summarized in Table 5.

<b>Table 5.</b> Overview of the present study cohorts that form the basis of this thesis.				
<b>Study</b>	<b>I</b>	<b>II</b>	<b>III</b>	<b>IV</b>
	<b>Original studies</b>			
<b>Cohort name</b>	GAMI		ORIGIN	
<b>Original study design</b>	Observational		Randomized controlled trial, Biomarker substudy	
<b>Number of study subjects</b>	Patients=181 Controls=184		12 537 whereof 8 494 participated in the biomarker substudy	
	<b>Present studies</b>			
<b>Present study design</b>	Observational	Observational	Observational	Observational
<b>Number of study subjects</b>	Patients: 123 males Controls: 124 males	Patients: 121 males	5 553 males	2 848 females
<b>Mean age at inclusion (years)</b>	Patients: 61 Controls: 63	61	64	64
<b>Recruitment years</b>	1998-2002*	1998-2000**	2003-2005	2003-2005
<b>Dysglycemia category (%)</b>	Patients: 36% normal, 64% IGT or DM Controls: 62% normal, 38% IGT or DM	35% normal, 65% IGT or DM	80% known DM 7% newly detected DM 13% newly detected IGT/IFG	84% known DM 5% newly detected DM 11% newly detected IGT/IFG
<b>Median follow-up period (years)</b>	Patients: 11.6 Controls: 10.4	11.6	6.2	6.1
<b>Outcomes</b>	CV events, CV mortality, all-cause mortality	CV events, CV mortality, all-cause mortality	CV events, all-cause mortality	CV events, all-cause mortality
<b>Number of CV events/mortality</b>	Patients: CV events 50 CV mortality 25 Controls: CV events 27 CV mortality 12	CV events 50 CV mortality 25	CV events 1 028	CV events 377
<b>Number of all-cause deaths</b>	Patients: 38 Controls: 21	37	951	389

\*Recruitment years for patients and controls. \*\*Recruitment years for patients only. CV: cardiovascular; DM: diabetes mellitus; GAMI: Glucose in Acute Myocardial Infarction study; IFG: impaired fasting glucose; IGT: impaired glucose tolerance; ORIGIN: Outcome Reduction with an Initial Glargine Intervention trial.

## Study populations and design

### Studies I and II

#### *Hypotheses*

In male patients with AMI with or without dysglycemia:

#### **Study I**

- 1) Low testosterone levels are common
- 2) The testosterone levels vary at different time points after the AMI
- 3) The testosterone levels have a prognostic implication.

#### **Study II**

- 1) CAG repeat length is associated with the testosterone levels and cardiometabolic risk factors
- 2) CAG repeat length has prognostic implications

#### *Study cohorts*

**Studies I and II** are based on the observational Glucose Tolerance in Patients with Acute Myocardial Infarction (GAMI) study (72). GAMI comprised 181 AMI patients (males 69%) without previously diagnosed diabetes and admission capillary glucose <11.1 mmol/L who, outside weekends and holidays, were admitted to the coronary care units at Karolinska University Hospital Solna and Västerås Hospital 1998 - 2000. People >80 years of age and those with a serum creatinine  $\geq 200$   $\mu\text{mol}$  were excluded. Patients had fasting blood samples drawn at four time points: on the day after admission, at hospital discharge and three and 12 months thereafter. An OGTT was performed before hospital discharge in all but 13 patients (2 patients died during the hospital stay; 7 due to severe illness, 2 refused to participate, 2 due to technical problems).

Age- and gender matched controls (n=185; males 69%) without diabetes and CVD (apart from well-controlled hypertension) were randomly selected and recruited from the population in the recruitment areas between 2001-2002. Five age- and gender matched subjects for each patient were randomly selected as possible controls. Control subjects had blood samples drawn and performed an OGTT at one occasion (96).

Blood samples were stored at  $-70^{\circ}\text{C}$  pending analyses. **Study I** comprised all male study participants with blood samples available for testosterone analyses at baseline (123 patients and 124 controls). **Study II** comprised all male patients with frozen whole blood samples available for DNA extraction (n=122).

#### *Follow-up and outcomes*

Patients and controls were followed until December 31 2011, resulting in a median follow-up time of 11.6 and 10.4 years respectively (97). The primary endpoint was a composite of major CV events (first occurrence of CV death i.e. death from AMI, stroke, aortic dissection or sudden death without obvious reasons; nonfatal AMI; nonfatal stroke; severe heart necessitating hospitalization). The two secondary endpoints were CV mortality and all-cause mortality. Information on CV events was prospectively collected using hospital records for diagnosis and medical interventions. Information on mortality was collected from the Swedish National Death Registry and the cause of death in the death certificates were validated against available hospital records by the study investigators. In total, one patient and one control were lost to follow-up.

## Studies III-IV

### *Hypotheses*

In individuals with dysglycemia and high CV risk:

### **Study III**

Low levels of total and free testosterone as well as SHBG levels are associated with CV events and all-cause mortality in males.

### **Study IV**

Low levels of total and free testosterone as well as SHBG are associated with CV events and all-cause mortality in females.

### *Study cohorts*

**Studies III** and **IV** are based on the randomized Outcome Reduction with an Initial Glargine Intervention (ORIGIN) trial (94, 98). Individuals  $\geq 50$  years with newly detected IFG, IGT or diabetes based on an OGTT or known T2DM on stable therapy without or with one oral glucose-lowering medication and high CV risk were recruited from 578 clinical sites in 40 countries during September 2003 to December 2005. In total, 12 537 participants were enrolled and randomized in a 2x2 factorial design to either insulin glargine (Gla-100) targeting a fasting plasma glucose  $\leq 95$  mg/dl (5.3 mmol/L) or standard care, and omega 3 fatty acids or placebo.

High CV risk was defined as confirmed evidence of at least one of

1. Prior AMI, stroke or revascularization
2. Angina with documented ischemia
3. Morning urinary albumin/creatinine ratio  $>30\mu\text{g}/\text{mg}$
4. Evidence of left ventricular hypertrophy
5.  $\geq 50\%$  stenosis of a coronary, carotid, or lower extremity artery documented angiographically
6. An ankle/brachial index  $<0.9$

Main exclusion criterion was use of, indication for or intolerance to insulin or omega 3 fatty acids. Patients provided medical history, filled out questionnaires (Figure 5) and fasting blood samples were drawn at the baseline visit.

A total of 8 494 (68%) of the ORIGIN participants consented to the collection and storage of blood samples for future studies (99). These were obtained at baseline after an overnight fast, divided into aliquots and subsequently stored in nitrogen vapour-cooled tanks at  $-160^{\circ}\text{C}$ . After completion of the ORIGIN trial, coded aliquots of serum were transported to Myriad RBM Inc (Austin, Texas, USA) for further analyses.

**Study III** comprised all male participants ( $n=5\ 553$ ) and **Study IV** all female participants ( $n=2\ 848$ ) from the biomarker substudy regardless of treatment allocation.

Follow-up

Patients were followed for a median of 6.2 years in **Study III** and 6.1 years in **Study IV**. The primary outcome in **Studies III and IV** was a composite of major CV events (incident CV death and nonfatal AMI) while all-cause mortality served as a secondary outcome. In addition, an expanded primary outcome including a revascularization procedure or hospitalization for heart failure was studied in **Study III**. All outcomes were ascertained at every visit based on provided information and supporting documentation as well as adjudicated by an adjudication committee whose members were unaware of participant allocation. The primary outcome was known in 12 443 (99%) of the participants in the main study (98).

Participant ID#   Initials   
Centre # Participant # F M L

**FOR WOMEN ONLY (please ask the following)**

1. Have you ever been diagnosed with diabetes during a pregnancy?

No

Yes → How was this treated?  Diet alone  Pills  Insulin → Did the diabetes go away after the pregnancy?  No  Yes

2. Have you ever been diagnosed with pre-eclampsia (pregnancy induced hypertension)?  No  Yes

3. Have you ever been diagnosed with eclampsia (pregnancy induced severe hypertension)?  No  Yes

4. Have your menstrual periods stopped permanently?

Unknown

No → What contraception are you currently using?

Not sexually active  None  Tubal ligation  Other (specify) \_\_\_\_\_

Oral contraceptives (birth control pill)  IUD  Barrier method  Partner Vasectomy/sterile

Yes → At what age did they stop?  years

→ Why did they stop? (check one)

Menopause  Radiation/Chemotherapy  Hysterectomy  Other (specify) \_\_\_\_\_

5. Have you ever used female hormone replacements for reasons other than birth control?

Unknown

No

Yes → How long?  years → Are you still taking them?  No → stopped:  years  Yes

→ Which type of replacement have you used the most?

Estrogen alone  Estrogen + Progesterone  Unknown

	No	Yes	Age at Surgery
6. Have you had a hysterectomy (removal of the uterus)?	<input type="checkbox"/>	<input type="checkbox"/>	<input type="text"/> <input type="text"/>
7. Have you had a bilateral oophorectomy (removal of both ovaries)?	<input type="checkbox"/>	<input type="checkbox"/>	<input type="text"/> <input type="text"/>
8. Have you ever tried unsuccessfully for a full year or more to become pregnant?	<input type="checkbox"/>	<input type="checkbox"/>	
9. Have you ever used fertility drugs?	<input type="checkbox"/>	<input type="checkbox"/>	
10. Between the ages of 18 and 45 have you ever had 6 or less menstrual cycles per year? (not including pregnancy)	<input type="checkbox"/>	<input type="checkbox"/>	
11. How many children have you given birth to? <input type="text"/> <input type="text"/>			<input type="text"/> <input type="text"/>
(If one or more) → a) Age at which your 1st child was born?			<input type="text"/> <input type="text"/>
b) Age at which your last child was born?			<input type="text"/> <input type="text"/>

**Figure 5.** Questionnaire for women in the ORIGIN trial.



## Definitions

### *Dysglycemia*

In **Studies I and II**, diabetes and IGT were defined according to the WHO definition from 1998 based on an OGTT with 75 g of glucose in 200 ml water (93). Patients were categorized as having normal glucose tolerance (NGT: 2-hour post-load glucose <7.8 mmol/L) or abnormal glucose tolerance (AGT) which comprised diabetes (2-hour post-load glucose >11.0 mmol/L) and IGT (2-hour post-load glucose 7.8-11.0 mmol/L).

In **Studies III and IV**, dysglycemia was defined as IFG (FPG 6.1-6.9 mmol/L), IGT (2-hour post-load glucose 7.8-11.0 mmol/L) or newly detected diabetes (2-hour post-load glucose >11.0 mmol/L) after an OGTT with 75 g oral glucose load or known T2DM on stable therapy without or with one oral glucose lowering medications for  $\geq 3$  months (94).

### *Low testosterone levels*

Low total testosterone levels were defined as  $\leq 300$  ng/dl (=10.4 nmol/L) in **Studies I-III**, in line with guidelines from the Endocrine Society published in 2010 (18). Since there is no established cut-off for free testosterone, the threshold used in **Studies I-III** was  $\leq 7$  ng/dl (=0.2 nmol/L), based on <2.5<sup>th</sup> percentile in a community-based sample of men (95).

### *Free testosterone*

Free testosterone was calculated using the Vermeulen formula, assuming an albumin constant set at 43 g/L (Figure 6) (20).

#### **How to calculate free testosterone using the Vermeulen formula:**

1. Convert everything to mol/L.  
Testosterone (ng/dl) \* 0.03470 / 1 \* 10<sup>9</sup> = testosterone (mol/L) = [T]
2. SHBG (nmol/L) / 1 \* 10<sup>9</sup> = SHBG (mol/L) = [SHBG]
3. There are three constants in the equation.
  - Association constant for albumin: 6.24 \* 10<sup>-4</sup> mol/L
  - K<sub>at</sub> which is the association constant of albumin binding to T: 3.6 \* 10<sup>4</sup> L/mol
  - K<sub>i</sub> which is the association constant of SHBG binding to T: 1 \* 10<sup>9</sup> mol/L

4. The formula looks like this.

$$[\text{FT}] = \frac{-b + \sqrt{b^2 + 4a[\text{T}]}}{2a}$$

Start by calculating a and b.

$$a = K_{at} + K_i + (K_{at} * K_i)([\text{SHBG}] + [\text{albumin}] - [\text{T}])$$

$$b = 1 + K_i[\text{SHBG}] + K_{at}[\text{albumin}] - ((K_{at} + K_i)[\text{T}])$$

5. Insert a and b into the formula.
6. Convert free testosterone in mol/L to ng/dl = [FT] x 1 \* 10<sup>9</sup> / 0.0347

**Figure 6.** Free testosterone calculation by means of the Vermeulen formula.

## Laboratory investigations

### Studies I-II

#### *Oral glucose tolerance test*

A standardized OGTT with 75 g of glucose dissolved in 200 ml of water was performed during stable conditions (day 4 or 5) following a 12 hour overnight fasting at the local hospital and for controls at the baseline visit. The glucose levels were measured at 0, 60 and 120 minutes.

#### *Testosterone*

Testosterone was extracted from serum using solid phase extraction and determined by high performance liquid chromatography-mass spectrometry. The linear range was 1.0–1000 ng/dl ( $r \geq 0.9995$ ) with lower limit of sensitivity at 1ng/dl and the inter-assay coefficient of variation was <7%. The analyses were performed at Brigham Research Assay Core at Brigham and Women's Hospital in Boston, USA.

#### *LH and SHBG*

LH and SHBG were assessed using solid phase sandwich enzyme-linked immunosorbent assays (ELISA), Human LH ELISA (BQ Kits, San Diego, CA, USA) and Human SHBG Quantikine ELISA (R&D system, Abingdon, UK). The sensitivity for the LH assay was 1 mIU/ml and intra- and inter-assay coefficient of variation was 5.0% and 8.4%, respectively. The sensitivity for the SHBG assay was 0.5 nmol/l and intra- and inter-assay coefficient of variation was 4.9% and 9.9% respectively.

#### *CAG repeat length*

Genomic DNA was extracted using QIAamp DNA Mini Kit from peripheral whole blood samples and the CAG repeat length was amplified from genomic DNA using PCR. For amplification, the following published primers flanking the CAG repeats were used: 5'-FAM6-TCC AGA ATC TGT TCC AGA GCG TGC -3' and 5'- GCT GTG AAG GTT GCT GTT CCT CAT-3' (100). PCR was performed on a GeneAMP 9700 thermocycler (Applied Biosystems), PCR-FAM amplicons were resolved with capillary electrophoresis and thereafter identified using an ABI 3730 Genetic Analyzer (Applied Biosystems). By use of GeneScan™- 500LIZ® Size standards (Applied Biosystems) with GeneMapper Software (Applied Biosystems) the CAG repeat length was determined. The analyses for LH, SHBG and CAG repeat length were performed at ANOVA (Center for Andrology, Sexual Medicine and Transmedicine), Karolinska University Hospital, Stockholm, Sweden.

### Studies III-IV

#### *Testosterone, LH, SHBG*

Sex hormones were measured as part of a prespecified panel by means of the Human Discovery Multi-Analyte Profile (MAP) 250+ panel on the LUMINEX 100/200 platforms. After careful blinded scrutiny of the results, 237 biomarkers from 8 401 study subjects were available for analysis including total testosterone, LH and SHBG with inter-run coefficients of variation of 7, 6 and 14% respectively. Analyses were performed at Myriad RBM Inc. (Austin, Texas, USA).

## Statistical analyses

Analyses were performed using SAS statistical software, version 9.4 (**Studies I-II, IV**) and version 9.2 (**Study III**). The nominal level of significance for all analyses was a two-sided p-value of  $<0.05$ .

### *Descriptive statistics*

In **Study I**, continuous variables were presented as median and interquartile ranges and categorical variables as numbers and percentages. Differences between groups, e.g. patients vs. controls or AGT vs. NGT, were compared using Wilcoxon two-sample test for continuous variables (e.g. testosterone levels) and chi-square test for dichotomous variables (e.g. prevalence of low testosterone).

In **Study II**, the relation between CAG repeat and testosterone as well as selected CV risk factors, which were based on clinical relevance and previous studies and include e.g. different measures of dysglycemia, BMI and CRP, were assessed using Pearson's correlation coefficient. Additionally, CAG repeat length was dichotomized according to the median level as  $>20$  vs.  $\leq 20$  in the baseline table and analyses on testosterone levels over time by CAG group.

In **Studies III-IV**, continuous variables were presented as mean and standard deviation (SD) and categorical variables as numbers and percentages.

Comparison between groups with lower vs. higher hormone levels was assessed using *t*-test for continuous variables and chi-square test for dichotomous variables.

### *Survival analyses*

In **Studies I-II**, the relationship between testosterone and CAG respectively with outcomes were assessed using Cox proportional hazards regression in univariate and multivariate models, presented as hazard ratio (HR) and 95% confidence interval (CI). In **Study I**, analyses by one standard deviation (SD) increment from testosterone samples at the day after hospital admission for patients and at the baseline visit for controls were performed. The SD was calculated separately for patients and controls. Adjustments were made for smoking, body mass index (BMI), SHBG and 2 hour-post glucose load both separately and combined. The covariates were selected based on previous studies and clinical relevance (77, 101). Model A was unadjusted and Model B was adjusted for smoking, BMI, SHBG and 2-hour post-load glucose. In **Study II**, Cox analyses for CAG repeat length was carried out by one unit increment and by CAG group, in unadjusted (Model A) and age-adjusted analyses (Model B).

In **Studies III-IV**, the prognostic ability of sex hormones (testosterone, free testosterone and SHBG) was tested using Cox proportional hazard regression as well as illustrated with Kaplan-Meier curves. HR were estimated for one SD increment and by higher vs. lower levels (according to total and free testosterone threshold and SHBG median level) in **Study III**. In multivariate models the association between respective hormone and events were adjusted for age in Model A and for age, LH levels, previous CVD, previous DM, use of metformin, use of statins, systolic blood pressure, HbA1c, low density lipoprotein (LDL) cholesterol, BMI and smoking in Model B. The covariates were chosen based on previous data and clinical relevance (1, 15).

HRs of total testosterone, free testosterone and SHBG for prognosis by one SD increment were estimated in **Study IV**. Multivariate adjustments were performed in several models. In Model A the respective hormone was adjusted for age. Model B included Model A (age) plus LH levels, previous CVD, previous DM, use of metformin, use of statins, systolic blood pressure, HbA1c, LDL-cholesterol, BMI and smoking. Model C included Model B plus menopause with or without hormone therapy, thyroid hormone treatment, alcohol consumption, ethnicity and obstructive sleep apnea. Tests for interaction between total testosterone, free testosterone, SHBG, outcomes and study allocation were performed in **Studies III and IV**.

## **Ethical approvals**

All studies agree with the Declaration of Helsinki. **Study I** had been previously approved by the Regional Ethical Board in Stockholm and an additional ethical approval was obtained for **Study II**. **Studies III and IV** were approved by the local ethics committee at each study site. Patients provided written, informed consent prior to participation in the main studies.

## RESULTS

### Study I

#### Baseline characteristics

The study cohort comprised 123 male patients and 124 male controls. As outlined in Table 6, patients had more comorbidities and were more often prescribed cardioprotective drugs. Capillary blood glucose, HbA1c and creatinine were higher in patients than controls. Glucose categorization at discharge disclosed AGT in 75 patients (64%) compared to 47 (38%) in the control group.

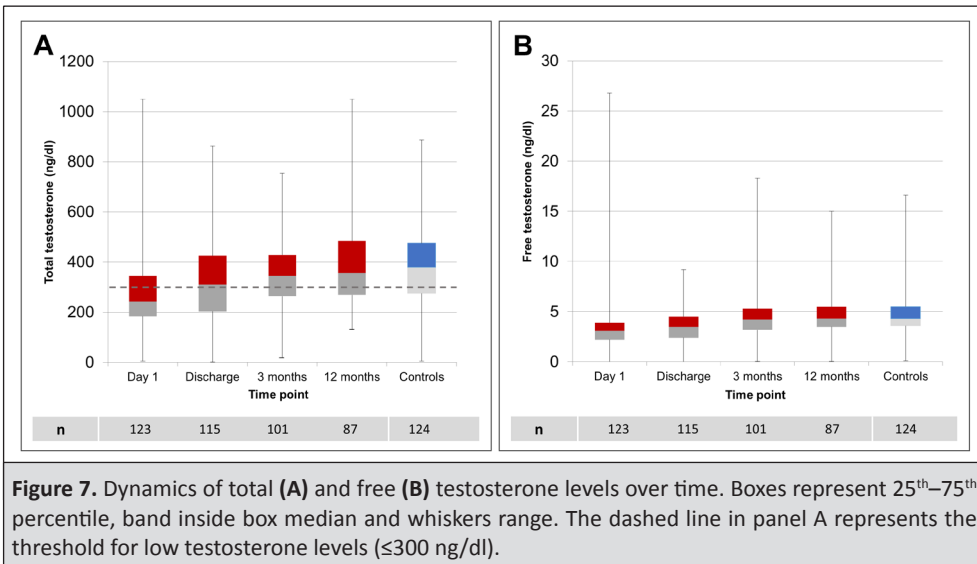
<b>Table 6.</b> Selected baseline characteristics of the study cohort for <b>Studies I and II.</b>				
Continuous variables presented as median (IQR) and categorical variables presented as n (%).				
	Study I		Study II	
	Total cohort (n=247)		Patients (n=121)	
	Patients n=123	Controls n=124	CAG ≤20 n=68	CAG >20 n=53
<b>Clinical characteristics</b>				
Age (years)	61 (55-71)	63 (56-73)	61 (56-68)	62 (56-73)
Current smokers	39 (32)*	13 (10)*	19 (28)	19 (36)
BMI (kg/m <sup>2</sup> )	26 (24-29)	26 (24-29)	26 (24-29)	26 (24-28)
Family history T2DM	21 (18)	16 (13)	13 (19)	8 (16)
Family history IHD	60 (50)*	32 (26)*	38 (57)	22 (42)
<b>Previous disorders</b>				
MI	27 (22)*	0 (0)*	16 (24)	10 (19)
Hypertension	33 (27)*	20 (16)*	21 (31)	11 (21)
Hyperlipidemia	18 (15)*	6 (5)*	10 (15)	7 (13)
<b>Pharmacological treatment</b>				
ACE Inhibitor	10 (8)	9 (7)	19 (28)	15 (28)
β-Blocker	36 (29)*	13 (10)*	7 (10)	3 (6)
Statins	13 (11)*	3 (2)*	7 (10)	5 (9)
<b>Biochemical characteristics at baseline</b>				
Capillary blood glucose	6.2 (5.6-7.4)*	5.0 (4.6-5.5)*	6.0 (5.4-7.0)*	7.1 (5.9-7.8)*
HbA1c (%)	4.9 (4.5-5.3)*	4.6 (4.3-5.0)*	4.9 (4.6-5.3)	4.9 (3.8-4.9)
Creatinine (μmol/l)	96 (86-107)*	84 (78-94)*	95 (86-106)	99 (85-111)
NGT	43 (36)*	77 (62)*	25 (38)	16 (32)
Testosterone (ng/dl)	243 (184-346)*	380 (276-476)*	226 (177-334)	265 (207-383)
Free testosterone (ng/dl)	3.1 (2.2-3.9)*	4.3 (3.6-5.5)*	2.6 (2.0-3.5)	3.2 (2.6-4.3)
SHBG (nmol/L)	70 (52-93)	71 (51-91)	67 (51-93)	72 (53-92)
LH (mIU/ml)	2.1 (1.4-3.7)	1.9 (1.1-3.3)	1.9 (1.1-3.1)	2.0 (1.3-3.5)
Prevalence of low testosterone	79 (64)*	35 (28)*	47 (69)	30 (57)

\* denotes a significant difference (p<0.05) between groups.  
Abbreviations: ACE: angiotensin-converting enzyme; BMI: body mass index; IHD: ischemic heart disease; LH: Luteinizing hormone; MI: myocardial infarction; N/A: Not applicable; NGT: Normal Glucose Tolerance; SHBG: Sex hormone-binding globulin; T2DM: type 2 diabetes

### Testosterone levels over time

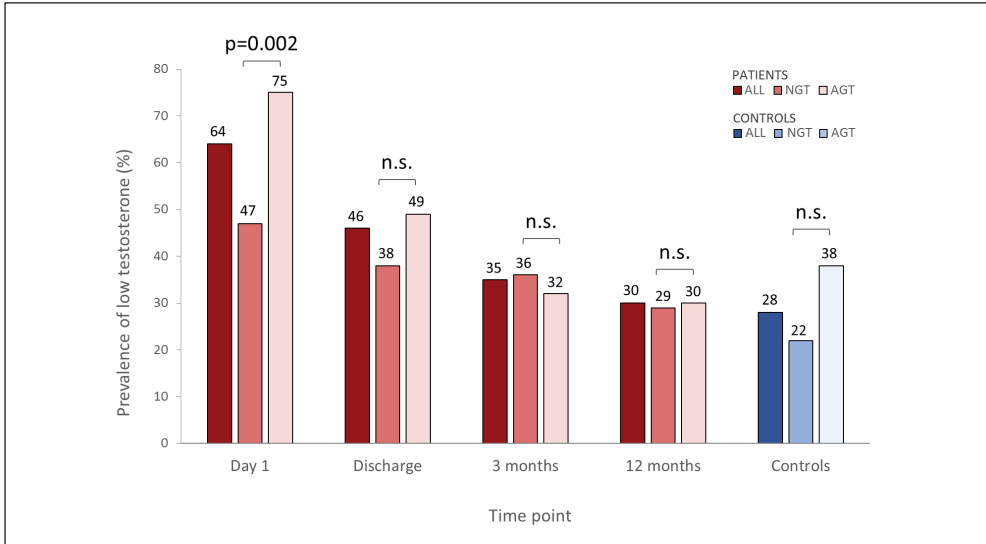
Total testosterone levels on the day after admission were lower in patients than in controls (243 ng/dl vs. 380 ng/dl;  $p < 0.01$ ) but the levels had increased at the time for hospital discharge to a median testosterone of 311 ng/dl and further to 345 ng/dl after three and 357 ng/dl after 12 months (Figure 7A). The levels of free testosterone were lower at admission (3.1 ng/dl) but increased to a similar level (3.5, 4.2, 4.3 ng/dl respectively) as that observed among controls (4.3 ng/dl) over time (Figure 7B).

The total testosterone/LH ratio (TT/LH) was 115.7 in the patient and 200 in the control cohort at the first time point. At the following time points TT/LH was 163.2, 156.8 and 162.3 respectively. The free testosterone/LH ratio (FT/LH) in patients was 1.5, 1.8, 1.9 and 1.9 at the different time points compared to 2.3 in controls.



Sixty four percent of the patients had low total testosterone levels ( $\leq 300$  ng/dl) on the day after admission compared to 28% of the controls at baseline ( $p < 0.001$ ; Figure 8). The proportion of patients with low total testosterone decreased over time from 46% at hospital discharge, to 35% after three and to 30% after 12 months, a prevalence similar to that of the controls. In subgroup analyses, the prevalence of low testosterone was higher in patients with AGT compared to NGT at the first time point ( $p = 0.002$ ). The difference between testosterone levels and prevalence of low testosterone in the two glucose tolerance categories had diminished at the time for discharge and thereafter. There was no significant difference between AGT vs. NGT group among controls ( $p = 0.05$ ).

Total testosterone levels were lower in patients with AGT at the first time point, but this difference became attenuated over time (Table 7). Among controls, there was no difference in testosterone levels between the different glucose categories. Free testosterone levels did not differ between patients or controls with AGT and NGT at any of the time points.



**Figure 8.** Prevalence of low testosterone ( $\leq 300$  ng/dl) over time in all patients and by glucose category. NGT: normal glucose tolerance; AGT: abnormal glucose tolerance. n.s.: not significant. \* denotes significant difference between the NGT and AGT group.

**Table 7.** Total and free testosterone levels (ng/dl) for patients and controls by glucose category. The testosterone levels are presented as median and interquartile range (IQR). P-value denotes significance level in testosterone between NGT/AGT.

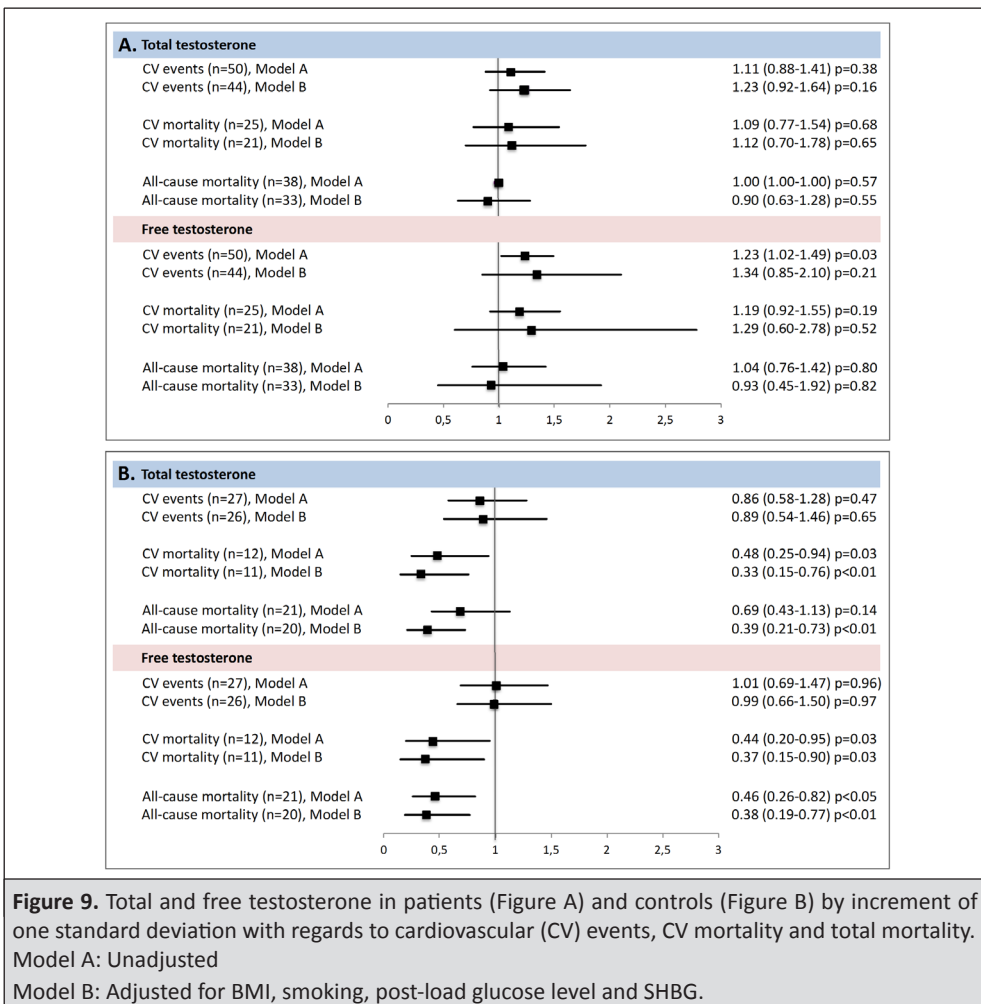
	Patients				Controls
	Day after admission (n=118)	Discharge (n=113)	3 months (n=98)	12 months (n=84)	Baseline (n=124)
	NGT n=43 AGT n=75	NGT n=42 AGT n=71	NGT n=36 AGT n=62	NGT n=34 AGT n=50	NGT n=77 AGT n=47
<b>Total testosterone</b>					
<b>NGT</b>	308 (199-387)	349 (236-497)	342 (231-452)	363 (270-505)	386 (315-481)
<b>AGT</b>	230 (178-301)	306 (188-408)	352 (284-428)	357 (269-450)	341 (266-441)
<b>p</b>	0.04	0.09	0.81	0.79	0.15
<b>Free testosterone</b>					
<b>NGT</b>	3.2 (2.2-4.2)	3.6 (2.8-4.5)	4.2 (3.2-5.5)	4.5 (3.4-5.6)	4.3 (3.7-5.5)
<b>AGT</b>	3.0 (2.1-3.6)	3.4 (2.0-4.6)	4.2 (3.2-5.0)	4.4 (3.5-5.4)	4.1 (3.2-5.2)
<b>p</b>	0.18	0.35	1.00	0.69	0.28

Abbreviations: AGT: Abnormal glucose tolerance; NGT: Normal glucose tolerance.

*Survival analyses*

Patients and controls were followed for a median time of 11.6 and 10.4 years respectively. In patients, an increment by one SD of total testosterone at admission (SD=163.4 ng/dl; Figure 9A) did not predict any of the studied outcomes. In unadjusted analyses (Model A), one SD increment of free testosterone (2.93 ng/dl) predicted the primary CV endpoint in patients (HR 1.23; 95% CI 1.02-1.49; p=0.03). This association remained after univariate adjustments for post-load glucose but not following univariate adjustments for SHBG, BMI and smoking separately or in the multivariate model (Model B).

In controls, an increment by one SD of total testosterone (SD=153.1 ng/dl) was associated with reduced risk of CV death by 52% (HR 0.48; 95% CI 0.25-0.94; p=0.03) but did not influence the other endpoints in unadjusted analyses (Figure 9B). Total testosterone remained a significant predictor for CV and total mortality after multivariate adjustments (Model B). Furthermore, free testosterone in controls (SD=1.99 ng/dl) was associated with lower CV and total mortality, in unadjusted (Model A) as well as adjusted analyses (Model B) (Figure 9B).



**Figure 9.** Total and free testosterone in patients (Figure A) and controls (Figure B) by increment of one standard deviation with regards to cardiovascular (CV) events, CV mortality and total mortality. Model A: Unadjusted  
Model B: Adjusted for BMI, smoking, post-load glucose level and SHBG.



## Study II

### Baseline characteristics

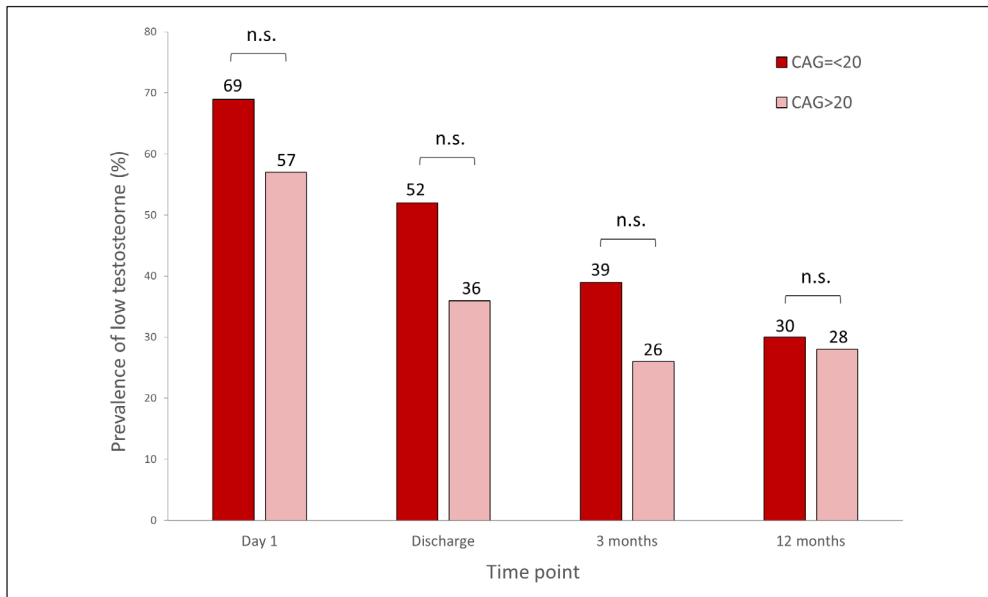
In total 121 patients had samples available for CAG characterization. In Table 6 baseline characteristics are presented for the study cohort dichotomized into those below or above the median CAG level (CAG  $\leq$ 20 and CAG  $>$ 20). The CAG  $\leq$ 20 group had lower capillary blood glucose at admission compared to the CAG  $>$ 20 group (6.0 vs 7.1 mmol/L;  $p=0.0016$ ), otherwise the two groups were similar in all the described aspects including age, previous medical history and testosterone levels.

### CAG repeat, testosterone and cardiovascular risk factors

The median CAG repeat length was 20 (range 13-26). There was no significant difference in median testosterone levels or in the prevalence of low testosterone between the CAG  $\leq$ 20 and CAG  $>$ 20 groups as outlined in Table 8 and Figure 10.

**Table 8.** Sex hormone levels (ng/dl) in relation to CAG repeat group in the study population. P-value denotes significance level in testosterone between CAG group.

Total testosterone (ng/dl)				
	Day after admission (n=121)	Discharge (n=113)	3 months (n=99)	12 months (n=85)
<b>CAG <math>\leq</math>20</b>	226 (177-334)	269 (204-415)	345 (261-427)	352 (270-437)
<b>CAG <math>&gt;</math>20</b>	265 (207-383)	369 (212-471)	359 (298-456)	393 (287-512)
<b>p</b>	0.12	0.08	0.29	0.13



**Figure 10.** Prevalence of low testosterone ( $\leq 300$  ng/dl) over time in patients with CAG  $\leq 20$  and  $> 20$ . n.s.=not significant difference.

CAG repeat length in the cohort as a whole did not correlate to the CV risk markers studied, i.e. capillary blood glucose, HbA1c, glucose category, CRP or creatinine and not to the sex hormones i.e. testosterone, free testosterone, SHBG or LH (Table 9).

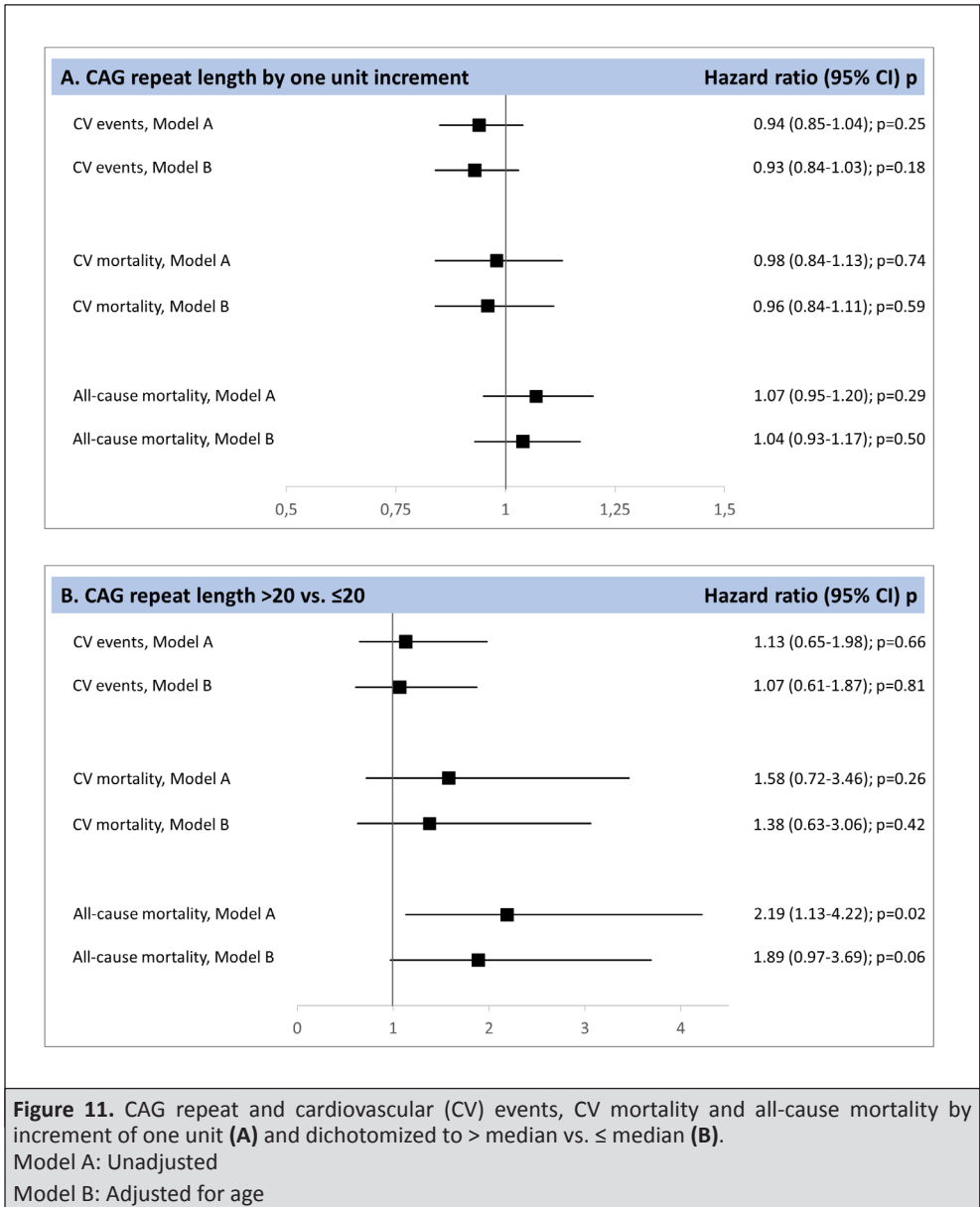
**Table 9.** Pearson's Correlation for the association between CAG repeat length and cardiometabolic risk factors.

CAG repeat length		
Variables	r <sup>2</sup>	p
BMI (kg/m <sup>2</sup> )	-0.06	0.50
CRP (mg/L)	0.12	0.20
Capillary blood glucose (mmol/L)	0.13	0.16
HbA1c (%)	-0.05	0.57
Creatinine (μmol/L)	0.03	0.77
Normal glucose tolerance (%)	-0.002	0.99
Testosterone (ng/dl)	0.14	0.12
Free testosterone (ng/dl)	0.07	0.41
Prevalence of low testosterone (%)	0.10	0.27
SHBG (nmol/L)	0.13	0.17
LH (mIU/ml)	-0.02	0.82

BMI: Body Mass Index; CRP: C-reactive protein; HbA1c: Glycated haemoglobin; LH: Luteinizing hormone; SHBG: Sex hormone-binding globulin

### *Survival analyses*

Patients experienced 50 CV events and 37 all-cause deaths whereof 25 (68%) died from CV causes during the follow-up of 11.6 years. More patients in the CAG >20 group died from all causes (42 vs. 22%; p=0.02) while there was no difference in proportion of events between the two groups for CV events and mortality. CAG repeat length was not associated with any of the endpoints in unadjusted and age-adjusted Cox analyses by one unit increment (Figure 11A). When analyzed as a below/equal to vs above median (=20), CAG repeat length was not associated with any CV outcomes. CAG >20 was related to increased all-cause mortality in unadjusted analyses but this association was attenuated in the age-adjusted analyses (Figure 11B).



### Study III

#### Baseline characteristics

The cohort in **Study III** comprised 5 553 men (mean age 63.5 years) whereof 80% with previously diagnosed diabetes and the remainder with newly detected diabetes, IFG or IGT (Table 10). There was a high prevalence of previous CVD (including myocardial infarction, stroke and coronary, carotid or peripheral artery revascularization) and hypertension.

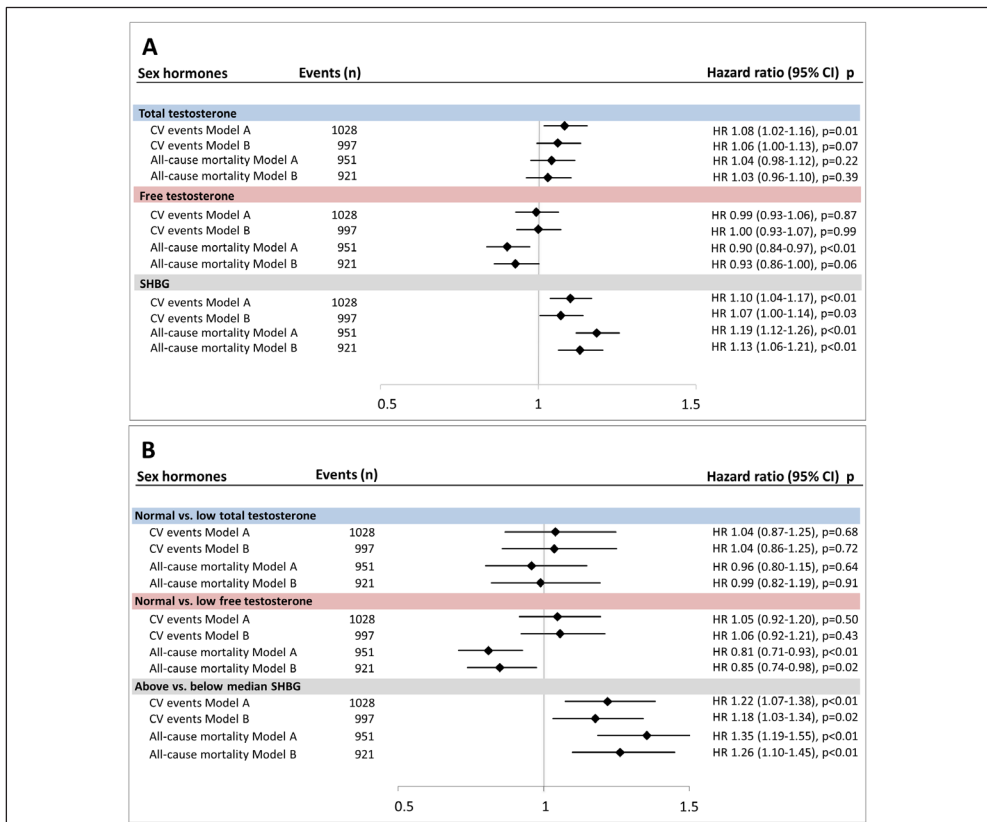
**Table 10.** Selected baseline characteristics of the study cohort in Studies III-IV. Continuous variables are presented as mean (SD) and categorical variables as n (%).

	STUDY III	STUDY IV
	Males n=5 553	Females n=2 848
<b>Clinical characteristics</b>		
Age (years)	63.5 (7.9)	64 (8)
Known diabetes	4441 (80.0)	2399 (84.2)
Newly detected diabetes	400 (7.2)	150 (5.3)
Newly detected IFG/IGT	710 (12.8)	298 (10.5)
Previous cardiovascular disease	3763 (67.8)	1228 (43.1)
Myocardial infarction	2397 (43.2)	640 (22.5)
Hypertension	4219 (76.0)	2419 (84.9)
Smoker	754 (13.6)	296 (10.4)
Menopause	N/A	2086 (73.2)
<b>Pharmacological treatment</b>		
Metformin	1431 (25.8)	886 (31.1)
Sulfonylurea	1613 (29.0)	810 (28.4)
Other glucose lowering drug	118 (2.1)	51 (1.8)
No glucose lowering drug	2394 (43.1)	1101 (38.7)
Statins	3375 (60.8)	1241 (43.6)
ACE/ARB inhibitors	3836 (69.1)	1957 (68.7)
Beta-blockers	3160 (56.9)	1366 (48.0)
<b>Biochemical characteristics</b>		
BMI	29.6 (4.7)	31.1 (6.2)
eGFR (ml/min/1.73m <sup>2</sup> )	79.0 (21.4)	74.5 (22.6)
HbA1C (DCCT %; mmol/mol)	6.5 (0.9); 48	6.6 (1.0); 49
Cholesterol (mmol/L)	4.7 (1.1)	5.2 (1.2)
LDL-cholesterol (mmol/L)	2.8 (1.0)	3.1 (1.1)
Total testosterone (ng/dl)	416.6 (109.2)	122.6 (76.6)
Low total testosterone	701 (12.6)	N/A
Free testosterone (ng/dl)	8.4 (3.2)	2.2 (1.7)
Low free testosterone	2035 (36.6)	N/A
SHBG (nmol/L)	35 (25–47)*	44.6 (24.7)
LH (mIU/ml)	2.8 (1.6)	8.4 (4.1)
* Presented as median (IQR). ACE/ARB: angiotensin-converting enzyme/angiotensin receptor II blocker; BMI: body mass index; eGFR: estimated glomerular filtration; HbA1c: glycated haemoglobin; HDL: high-density lipoprotein; IFG: impaired fasting glucose; IGT: impaired glucose tolerance; LDL: low-density lipoprotein; LH: luteinizing hormone; N/A: Not applicable; SHBG: sex hormone-binding globulin.		

Frequently prescribed pharmacological treatments included statins and angiotensin-converting enzyme/angiotensin receptor II blocker (ACE/ARB) inhibitors. The mean (SD) of total and free testosterone levels were 416.6 (109.2) ng/dl and 8.4 (3.2) ng/dl, and 13% had low total testosterone  $\leq 300$  ng/dl and 37% low free testosterone ( $\leq 7$  ng/dl). The median (IQR) SHBG was 35 (25–47) nmol/L. Individuals with SHBG levels above median had lower free testosterone levels (6.7 vs 10.0 ng/dl;  $p < 0.001$ ) and higher LH level (3.0 vs 2.5 mIU/ml;  $p < 0.001$ ).

*Survival analyses*

During a median of 6.2 years follow-up, 1 028 patients experienced a CV event and 951 patients died. As outlined in Figure 12A, neither total nor free testosterone were significant predictors of events in the multivariable, adjusted models (Model B). An increment of SHBG by one SD did, however, predict CV events and all-cause mortality in age- and multivariable, adjusted models. In categorical analyses, total testosterone did not predict any of the outcomes. Normal compared to low levels of free testosterone was associated with a reduced risk of all-cause mortality in models A and B (Figure 12B). SHBG above median was associated with an increased risk for CV events as well as all-cause mortality compared to levels at or below median (Figures 12B and 13). There was no significant interaction between study allocation and total testosterone, free testosterone and SHBG (data not shown). In additional analyses of the expanded primary outcome, including revascularization and heart failure hospitalizations, none of the sex hormones were significant predictors (data not shown).

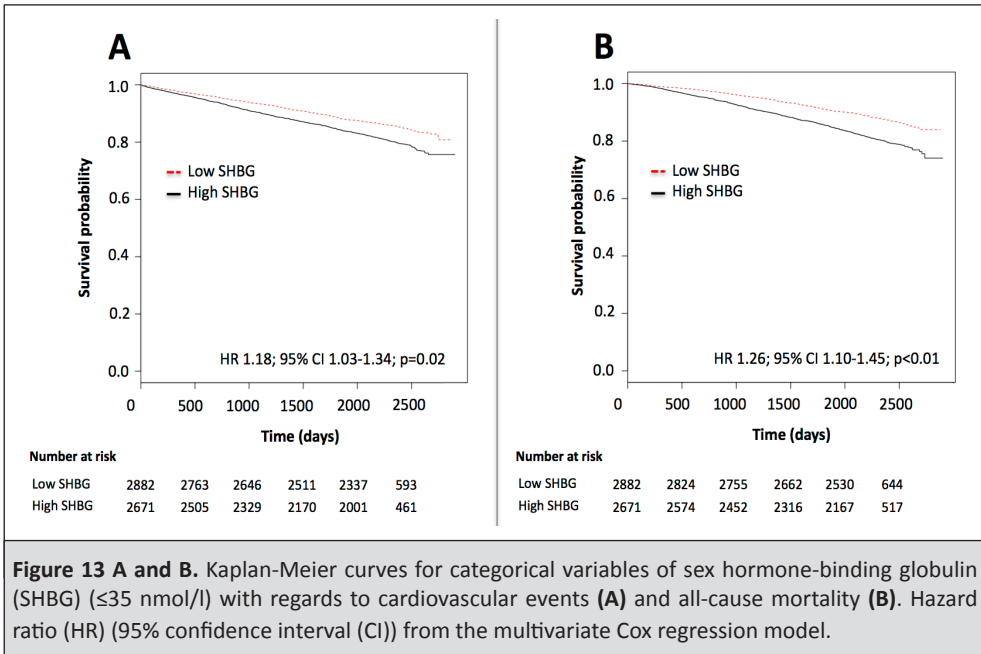


**Figure 12.** Total testosterone, free testosterone and sex hormone-binding globulin (SHBG) with regards to cardiovascular (CV) events and all-cause mortality in males by an increment of one standard deviation (A) and dichotomized as normal vs. low levels (low testosterone  $\leq 300$  ng/dl, low free testosterone  $\leq 7$  ng/dl and SHBG  $\leq 35$  nmol/L) (B).

CI: confidence interval.

Model A: adjusted for age.

Model B: adjusted for age, luteinizing hormone levels, previous cardiovascular disease, previous diabetes diagnosis, use of metformin, use of statins, systolic blood pressure, glycated haemoglobin, low-density lipoprotein cholesterol, body mass index and smoking.



## Study IV

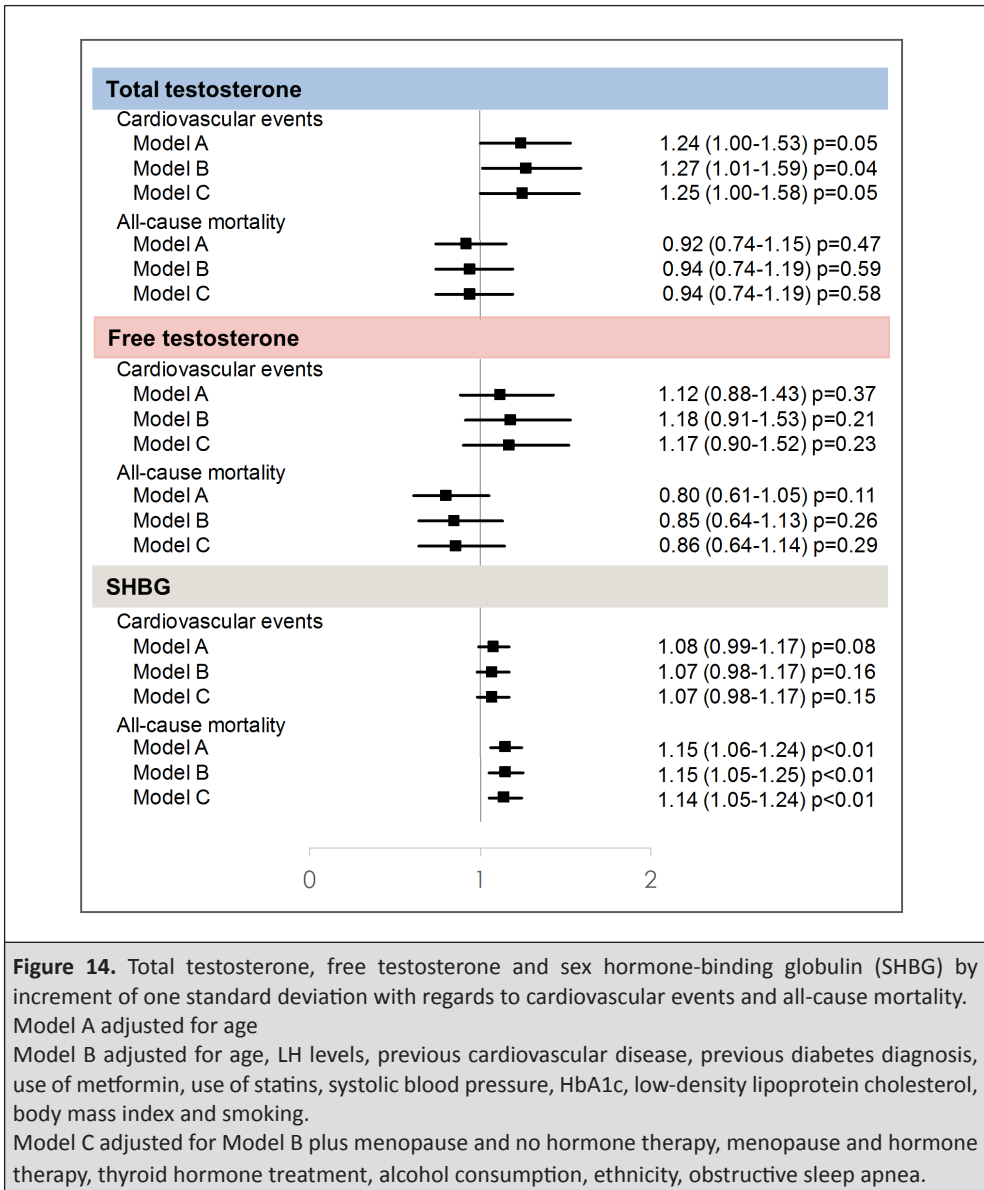
### Baseline characteristics

A total of 2 848 women of mean age 64 years were included in the study cohort whereof 73% were post-menopausal. Known diabetes was found in 84% and newly detected diabetes, IFG or IGT in 16% of the total cohort. Previous CVD was reported by 43%, previous myocardial infarction by 23% and hypertension by 85%. Cardioprotective drugs including ACE/ARB-inhibitors and statins were frequently used as outlined in Table 10.

The mean total and free testosterone levels were 122.6 ng/dl and 2.2 ng/dl respectively. The median SHBG was 39 nmol/L.

### Survival analyses

There were 377 CV events and 389 all-cause deaths during a median follow-up period of 6.1 years. While neither total nor free testosterone were significant predictors of future events, an increment by one SD SHBG was associated with all-cause mortality even after adjustments (HR 1.14; 95% CI 1.05-1.24;  $p < 0.01$ ) (Figure 14). There was no significant interaction between sex hormones, outcomes and study allocation.



**Figure 14.** Total testosterone, free testosterone and sex hormone-binding globulin (SHBG) by increment of one standard deviation with regards to cardiovascular events and all-cause mortality. Model A adjusted for age  
 Model B adjusted for age, LH levels, previous cardiovascular disease, previous diabetes diagnosis, use of metformin, use of statins, systolic blood pressure, HbA1c, low-density lipoprotein cholesterol, body mass index and smoking.  
 Model C adjusted for Model B plus menopause and no hormone therapy, menopause and hormone therapy, thyroid hormone treatment, alcohol consumption, ethnicity, obstructive sleep apnea.

## DISCUSSION

### Summary of the main findings

CVD and diabetes are major contributors to the global health burden. By studying testosterone, this thesis aimed at advancing the understanding of why patients with diabetes have an impaired CV prognosis. In summary, low testosterone was common in patients hospitalized with AMI, in particular in those with AGT, but the levels increased over time. However, and in contrast to healthy controls where low testosterone was predictive of prognosis, there was no clear association between the testosterone level and the prognosis in people already afflicted with CAD. However, the relation seems to be complex as both low free testosterone ( $\leq 7$  ng/dl) and increasing levels of SHBG were associated with prognosis in men with dysglycemia and high risk for CVD. SHBG but not testosterone was related to prognosis in women. A variable of potential interest in the relation between testosterone and CVD, CAG repeat length, did not seem to be of importance in men with AMI.

### Low testosterone in cardiovascular disease and dysglycemia

In **Study I**, investigating the dynamics of testosterone over time, low total testosterone levels were found in 64% of the patients on the day after admission for an AMI. After three and 12 months, this prevalence (35 and 30%) was similar to that in the control population (28%). In **Study III**, the proportion with low total testosterone was lower, 13%. One of the possible explanations to the higher prevalence of low testosterone during the first time point in **Study I** is that the samples were acquired during the early phase of an AMI. As described in the introduction, the Endocrine Society guidelines recommend against diagnosing testosterone deficiency in men recovering from acute illness since the levels may be influenced by stress (18, 23). Similar to the findings in **Study I**, previous investigations of the dynamics of testosterone in small groups of AMI patients ( $n=13-30$ ) showed a reduction of testosterone in the acute phase (102, 103). Less is known about the levels after discharge. In a case-control study by Pesonen et al. including 264 males with ACS, a high prevalence of low testosterone levels (around 44%) was shown within five days of hospitalization (60). In similarity to the findings in **Study I**, the levels had increased to comparable levels as measured in controls 11 months after the acute event. A clear definition of an appropriate time for accurate testosterone assessment following acute illness is missing in available guidelines. The observations from **Study I** suggest that testosterone may be assessed about three months after an AMI, but the results should be validated in another cohort before a strong recommendation is made.

The reasons for decreased testosterone levels during ACS is not fully understood but acute critical illness is known to affect the HPT-axis leading to a fall in testosterone (12). LH concentrations may be normal or slightly elevated in the setting of acute illness, which also suggest a peripheral resistance during stress. LH levels in patients in **Study I** were rather constant at all time points whereas testosterone levels varied. Other factors that may contribute to low testosterone levels are comorbidities increasing the susceptibility to stress such as diabetes. Interestingly, during the acute phase of the AMI in **Study I**, the prevalence of low testosterone was higher in those with dysglycemia compared to those without (75% vs. 47%). However, the difference in testosterone levels between dysglycemic and normoglycemic individuals was attenuated over time. The findings could relate to dysregulation of the HPT-



axis following insulin resistance, which is augmented during the early stage of an AMI (77, 104). In animal and in-vitro studies, insulin action and responsiveness have been related to the function of the HPT-axis (79, 105, 106). Furthermore insulin resistance has been associated with changes in Leydig cell function, such as a decreased responsiveness to LH resulting in a reduced production and release of testosterone (107). Thus, it may be hypothesized that patients with dysglycemia have lower testosterone levels in the acute phase since their HPT-axis is more sensitive to acute illness due to insulin resistance.

The prevalence of low testosterone varies greatly in different observational studies, as outlined in Table 11, and this may at least partially be attributed to differences in comorbidities such as CVD and diabetes. In **Study I**, the prevalence of low testosterone remained quite high even in the more stable phase of CAD in comparison to **Study III**. The presence of established CAD could partially explain this higher prevalence, since although patients were at high CV risk in **Study III**, only 43% had a history of AMI. Furthermore, all men in **Study III** had dysglycemia. This alters the SHBG levels, which may confound the prevalence of a low total testosterone. For this reason, the international Endocrine Society have recommended measurement of free testosterone in individuals with diabetes to assess androgen status more accurately. Indeed, the prevalence of low free testosterone was markedly higher than that of total testosterone (37 vs. 13%) in **Study III**. In line with these results, the prevalence of low total testosterone levels was 20-31% in 355 men with T2DM and higher, 50%, with low free testosterone (80). These findings underline the importance of assessing free testosterone in individuals with dysglycemia as recommended by guidelines.

#### *Different threshold for low testosterone and assay methods*

Another potential explanation for the diverging prevalence of low testosterone in different studies is the thresholds (Table 11). In contrast to the prevalence after 12 months (30%) in **Study I**, the prevalence of low total testosterone was around 17% in a prospective study investigating 930 men with stable CAD, i.e. similar to the prevalence (13%) in **Study III (55)**. The cut-off in that study was however lower (<234 ng/dl) than in **Studies I and III** (≤300 ng/dl) illustrating that many different aspects have to be accounted for and therefore preclude direct comparisons between trials. On the other hand, men in the control group in **Study I** had a higher prevalence of low testosterone (28%) than men in **Study III**. The same cut-off but different assay methods was used as described below and this could also contribute to a higher prevalence in **Study I**.

Several studies (Table 11) dichotomize testosterone according to cut-offs provided by guidelines for diagnosing hypogonadism or reference ranges provided by their respective laboratory whereas other categorize testosterone into tertiles and quartiles. Attempts have been made in effort to clarify the cut-off level for total and free testosterone. In 2011, the 2.5<sup>th</sup> percentile (by convention used to define the lower limit of the reference range) for total testosterone was 348 ng/dl and for free testosterone 7 ng/dl in a community-based sample of 1 893 men (95). A more recent study from 2017, aiming at providing a reference range generalizable to a wider population, was based on four cohorts from Europe and the United States. Based on testosterone measurements performed in healthy, young, non-obese men, the lower limit of total testosterone (2.5<sup>th</sup> percentile) in each cohort which was calibrated into a harmonized reference range was 264 ng/dl (108). This cut-off was adopted by the most recent Endocrine Society guidelines for total testosterone and applies to when Centers

for Disease Control and Prevention certified assays are used (23). If such certified assays are not available, the use of reference ranges established by the laboratory in question is recommended (18, 23). A harmonized reference range for free testosterone has not been established but the cut-off recommended in the guidelines is 5-9 ng/dl (0.2-0.3 nmol/L) (18). For this thesis, the threshold  $\leq 300$  ng/dl for total testosterone was used based on available guidelines (from 2010) at the time of performing **Studies I-III**. This threshold is frequently used in other studies (18, 26). For free testosterone, the cut-off in the community-based study from 2011 mentioned above ( $\leq 7$  ng/dl) was used.

**Table 11.** Prevalence of low testosterone in men, threshold used and assay method of testosterone in observational studies.

	Cohort	Assay method	Prevalence of low testosterone
Araujo et al. (37)	Population-based cohort (n=1691)	Radioimmunoassay	6% at baseline* 12% after 9 years follow-up
Harman et al. (38)	Population-based cohort (n=890)	Radioimmunoassay	20-30% with TT $\leq 325$ ng/dl
Kapoor et al. (80)	Men with T2DM (n=355)	ELISA	20% with TT <231 ng/dl 31% with TT 231-346 ng/dl 50% with FT <7 ng/dl
Malkin et al. (55)	Men with CAD (n=930)	ELISA	17% with TT <234 ng/dl 21% with bio-T <75 ng/dl
Shores et al. (109)	Population-based cohort (n=1032)	LC/MS	25% with TT $\leq 278$ ng/dl
Hamilton et al. (110)	Men with T2DM (n=788)	LC/MS	16% with TT <231 ng/dl 30% with TT <288 ng/dl
Pesonen et al. (60)	Men with ACS (n=264) and healthy controls (n=238)	Radioimmunoassay	44% with TT $\leq 266$ ng/dl
Gencer et al. (111)	Men with ACS (n=1054)	Electrochemi-luminescence assay	41% with TT $\leq 300$ ng/dl

Bio-T: Bioavailable testosterone; CAD: Coronary artery disease; ELISA: Enzyme-linked immunosorbent assay; FT: Free testosterone; LC/MS: Liquid chromatography/Mass spectrometry; SHBG: Sex hormone-binding globulin; T2DM; Type 2 diabetes mellitus; TT: Total testosterone.

\* Defined as low TT (<200 ng/dl) + symptoms of androgen deficiency or low-normal TT (200-400 ng/dl) + low FT (<8.9 ng/dl) + symptoms of androgen deficiency.

Finally, the discrepancy between studies could be related to the different assays used to analyze testosterone. There are two main methods currently in use; 1) liquid chromatography-tandem mass spectrometry (LC-MS/MS) which is considered the gold standard and 2) different types of immunoassays (1). In **Study I**, testosterone analyses were performed by LC-MS/MS whereas multiplex analyses (a kind of immunoassay measuring a large number of analytes) were used in **Study III**. Due to analytical difficulties and requirement of trained personnel, the LC-MS/MS method is used less often in research and clinical practice (Table 11) and different types of immunoassay are more frequently found in laboratories around the world even if there is a high inter-assay variability and inaccuracy compared to LC-MS/MS. This could potentially contribute to the prevalence of low testosterone in **Study III** being slightly less than expected in a population with dysglycemia and CVD.

## Prognostic implications of sex hormones

### *Testosterone and SHBG in men*

Total testosterone was not a significant predictor of prognosis in men with AMI or at high CV risk and with different levels of dysglycemia (**Studies I and III**) but free testosterone below threshold ( $\leq 7$  ng/dl) was associated with increased all-cause mortality in men from the ORIGIN biomarker substudy (**Study III**). This suggests that total testosterone is of questionable importance as a prognostic marker in men already afflicted with CVD and dysglycemia, in particular in the setting of acute illness where low testosterone may rather be a marker of general disease. It may be argued that the findings in **Study I** relate to the relatively low number of participants but similar results have been reported in a larger cohort of patients with ACS. In 1 054 men hospitalized for ACS, total testosterone in blood samples drawn during angiography was low ( $\leq 300$  ng/dl) in 41% of the men and did not predict prognosis (111). On the other hand, men with stable CAD ( $n=930$ ) and low bioavailable testosterone levels had an impaired CV survival during seven years follow-up compared to those with normal levels (55). Evidently, the association between testosterone and CAD seems to be different in acute and stable conditions.

The relation between testosterone and prognosis is still not fully understood, especially in populations with a high proportion of dysglycemia as in **Studies I and III**. What further complicates the interpretation is that several studies have shown impaired prognosis in groups both with the lowest and the highest levels, suggesting a U-shaped rather than a linear relation between testosterone and prognosis (29, 110, 112). Moreover, the divergent results could relate to the fraction of testosterone investigated. As suggested by Oskui et al, low free or bioavailable testosterone have generally presented more consistent prognostic information than total testosterone, potentially due to the biologically inactive parts of total testosterone confounding the impact on prognosis (15). Indeed, low levels of free and bioavailable testosterone and/or low SHBG have been associated with impaired CV and all-cause survival in men with CVD or T2DM (55, 86, 113-116). In comparison, free testosterone  $\leq 7$  ng/dl and increasing SHBG levels were related to a reduced risk of CV events and all-cause mortality in **Study III**. The mechanism behind the association is not clear. One possible explanation relates to the hypothesis that only free testosterone is biologically active (rather than the total volume in plasma) and SHBG which binds testosterone with a high affinity modulates the level of free testosterone available (16, 117). Thus, high SHBG could indicate low levels of free testosterone in plasma. However, as testosterone levels in blood strive to reach equilibrium, it could be that while increasing SHBG can lead to a decrease of free testosterone, this triggers the feedback mechanism of the HPT-axis to secrete more LH stimulating the testosterone production until levels are restored (16). Interestingly, the association between SHBG and outcomes remained significant after adjustment of LH levels in **Study III**. This suggests that SHBG may have independent and unknown effects on the CV system. For instance, given that SHBG production is inhibited by high insulin levels as described in the introduction (9), insulin could be a possible pathophysiological link between SHBG, dysglycemia and CVD. The results in **Study III** underline that SHBG should be considered when evaluating androgen status in men with dysglycemia and CVD.

In contrast to the lack of prognostic impact of total testosterone in men with established CAD or high CV risk in **Studies I and III**, both total and free testosterone predicted CV and all-cause mortality in controls in **Study I**. As described in the introduction, low testosterone

levels have been associated with increased CV events and all-cause mortality in some, but not all, population-based studies (56-58, 61, 62, 118-120). This suggests that testosterone might be a more important prognostic marker in healthy individuals compared to those already afflicted with disease where other, traditional risk factors are of greater importance. Testosterone may be a mediator in CVD and prognosis rather than a traditional risk factor. If testosterone have direct effects on the CV and metabolic systems or if it is a marker of underlying disease is not clear but the effects of testosterone on several CV risk factors, such as dysglycemia, dyslipidemia, inflammation and the metabolic syndrome as well as atherosclerosis have been suggested as part of a potential link between testosterone and CVD (Figure 4) (121). For instance, epidemiological studies have shown that men with low testosterone have an increased risk of developing diabetes (89, 122) and a randomized clinical trial showed improving fasting glycemic control and reduced homeostatic model of insulin resistance in men with diabetes prescribed testosterone therapy (27). Whether low testosterone causes T2DM or vice versa or if it is merely a marker of a hyperglycemic and insulin resistant condition is not known but there is likely a bidirectional relationship.

The present findings showed an association between testosterone and prognosis in men without CVD but do not support a relation between low testosterone and CVD in men with such conditions. There are, to the best of our knowledge, no available results from randomized controlled trials investigating the impact of testosterone therapy on CV events to date. Interestingly, despite the lack of a clear association between endogenous testosterone and CV prognosis, testosterone therapy in men with CAD have shown effects on exercise-induced ischemia, myocardial perfusion, peripheral vascular resistance, cardiac electrophysiology and cardiac output in mechanistic studies (123-125). Further studies, preferably randomized controlled clinical trials, are warranted to clarify this relationship.

#### *Testosterone and SHBG in women*

As expected, testosterone levels were lower in females (**Study IV**) than in males (**Study III**) in the ORIGIN biomarker substudy. They were, however, higher (123 ng/dl) than anticipations based on previous studies (126). This may be related to the assay method as already discussed. Another potential reason is the high prevalence of dysglycemia in the present population. In women, diabetes is associated with high testosterone levels (89). In a study by Wehr et al, women were categorized according to quartiles of free testosterone. In the first/lowest quartile, 24% had diabetes and mean total testosterone was 40 ng/dl. In the fourth/highest quartile, 47% had diabetes and mean total testosterone was 121 ng/dl, similar to the mean level found in **Study IV** (123 ng/dl).

In **Study IV**, neither total nor free testosterone were associated with any of the outcomes. The results are in line with some observational studies in which there was no association between testosterone and prognosis (127, 128). But, as described in the introduction, there are contradictory findings i.e. some investigations with an association between low testosterone and increased CV risk (91, 92) and others indicating that high testosterone is deleterious for CV health (126, 129). As for men, the discrepant results may relate to comorbidities but also the hormonal fractions studied and the assays used. Moreover, the possibility of a U or J-shaped relation needs to be considered, e.g. as shown in a population-based study comprising elderly women (mean age=75 years) where participants both in the lowest and highest quartiles had a higher prevalence of coronary heart disease than those in the second

quartile (130). It has been hypothesized that changes in estrogens and androgens during menopause may mediate the increased risk of CVD in post-menopausal women (131, 132). However, this was not shown for testosterone in women with dysglycemia and high CV risk in **Study III** and estrogens were not studied.

In contrast to testosterone, low levels of SHBG were associated with decreased risk of all-cause mortality in women (**Study IV**). This association is less well-studied in women than in men. While some studies suggest a U-shaped relationship similar to that for testosterone (92, 133) other investigations did not show any prognostic relationship at all (127, 128, 134, 135). In a subgroup analysis of the Multi-Ethnic Study of Atherosclerosis (n=2 834 postmenopausal women) comprising women not on hormonal therapy (n=1 934), high levels of SHBG and low levels of free testosterone and estradiol were related to an increased risk of coronary heart disease. As described above for men, the effects of SHBG in **Study IV** could be testosterone-related but the lack of predictive ability of testosterone indicates that other, testosterone independent pathways may be more important, possibly through insulin levels as described above for men. More studies on sex hormones and CVD are needed to clarify this relationship, and testosterone should not be investigated without considering SHBG levels.

## CAG repeat length and testosterone

**Study II** hypothesized that CAG repeat length would be associated with testosterone levels, but it did not correlate with testosterone measured at the four time points up to a year following an AMI and was not correlated to CV risk factors either.

As described in the introduction, CAG repeat length is thought to be inversely correlated with transcriptional activity of the androgen receptor, thus influencing androgen responsiveness and possibly explaining phenotype variability beyond what can be deduced from testosterone measurements (136). Shorter CAG repeat length has been associated with an increased risk of incident low testosterone in German men during follow-up for five years (137). On the other hand, there was no difference in testosterone levels in people with  $CAG \leq$  or  $>21$  in a nested case-control study comprising 172 Norwegian men which supports the results from **Study II** (138). The Norwegian study also demonstrated that men with more components of the metabolic syndrome had shorter CAG repeat length. This could explain the slightly lower median CAG (=20) in **Study II** given the high proportion of dysglycemia compared to the expected mean of a Caucasian population (=21-22) (1). Shorter CAG repeat length have previously been associated with both a protective CV risk profile such as low body fat mass and plasma insulin (139) as well as a worse risk profile with low HDL-levels, metabolic syndrome, higher fasting glucose, HbA1c and reduced endothelial response to ischemia as measured by vascular ultrasound (138, 140). This could not be verified in **Study II**, and furthermore there was no relation between CAG repeat length and cardiometabolic risk factors in the German study either (137).

A study of 474 men with T2DM showed that high testosterone levels were associated with decreased mortality in those with longer CAG repeat length while an increased mortality was seen in those with shorter CAG repeat length. The authors suggested that CAG repeat length modulates the prognostic capacity of testosterone (141). Another hypothesis in **Study II** was thus that CAG repeat length would be associated with CV outcomes. Although more patients

with CAG repeat length above median died from all-cause mortality, it was nominally but not significantly associated with all-cause death in age-adjusted analyses and not associated with CV events at all. Due to limited number of events, subgroup analyses with low or normal testosterone levels were not performed. In line with these study results, CAG repeat length was not related to any increased risk of CVD or myocardial infarction in a case-control study in 544 men (142). While this corroborates the results of **Study II**, the lack of association could also be related to the limited sample size.

If low testosterone in those with short CAG repeat length is a consequence of a more sensitive androgen receptor and not in itself related to testosterone deficient symptoms, this could confound associations seen in observational studies and partly explain the diverging results.

## Methodological considerations

### *Strengths*

This thesis is based on data from the prospective cohort study GAMI (**Studies I and II**) and the biochemical substudy from the clinical trial ORIGIN (**Studies III and IV**). The GAMI and ORIGIN study populations represent patients with different levels of dysglycemia including prediabetes (IGT/IFG) and diabetes as well as different stages of CVD.

GAMI consists of a well-defined, consecutive cohort of patients with AMI and a well-matched control group. The follow-up period was very long (11 years) with few patients lost to follow-up. Events were carefully collected, ascertained and adjudicated by the investigators using direct information from patients, hospital records and national registries. Testosterone was measured using LC/MS which is considered gold standard.

ORIGIN was a large, multicenter, randomized controlled trial. Follow-up time was six years, outcome data was determined by an adjudication committee and the primary outcome was known in 99% (n=12 443) of all patients. The biomarker substudy was based on a standardized collection, processing and storage of the blood samples. Moreover, routine clinical and biochemical measurements were available in all consenting participants.

### *Limitations*

The number of patients in **Studies I and II** was limited making subgroup analyses, e.g. as tertiles or quartiles, difficult due to lack of statistical power. Another limitation was that some patients did not have testosterone levels measured at each time point due to technical problems or their condition. Moreover although testosterone was measured at several time points, the prognostic analyses were based on a single measurement in patients and controls. In **Studies III and IV**, testosterone levels were only obtained at one occasion though guidelines recommend measurement at two different occasions due to day-to-day variations. However, a single sample may be sufficient for epidemiological analyses (143). Information on testosterone treatment was not gathered for **Studies I-III**. Testosterone was measured using multiplex analyses, a kind of immunoassay, which as mentioned previously have a higher variability and inaccuracy compared to LC-MS/MS. Since this is of particular importance at low hormone levels, it is an important aspect to consider in women. The mean testosterone was indeed higher in the cohort of **Study IV** compared to previous studies which could be attributed to the use of the immunoassay. However, immunoassay has been used to measure testosterone in many previous studies in females (91, 126, 144). Moreover, although

the prevalence of PCOS as well as treatment with testosterone was likely low, information on this was not gathered and could have contributed to the higher testosterone levels observed.

Another limitation in all studies was that free testosterone was not measured but calculated by means of the Vermeulen algorithm. The estimate of free testosterone is important, especially in individuals with dysglycemia where SHBG levels may be altered, as is the case with the present study cohorts. Free testosterone can be measured using equilibrium dialysis which is considered the reference method, however the method is technically demanding and not widely available (16). Therefore, the Endocrine Society recommended in their 2010 guidelines the use of calculated free testosterone which is considered to provide satisfactory estimates if adequate assay methods are not available (18). As described in the introduction, in addition to the frequently used formula by Vermeulen et al., Zakharov et al. recently suggested another formula based on a different theory of binding mechanisms between testosterone and SHBG (22, 23). This was mentioned in the 2018 guidelines by the Endocrine Society, recommending measurement of free testosterone by equilibrium dialysis first of all and if this is unavailable, calculation by means of the Vermeulen or Zakharov formulas underlining that the latter, allosteric model, is more accurate and provides estimates close to values measured using equilibrium dialysis (23). Unfortunately, the Zakharov formula was not available at the time when **Study I** was conducted. A benefit of using the Vermeulen formula is that the study results are easier to compare with previous studies since currently it is the most frequently used formula.

Moreover, testosterone was dichotomized in **Studies I-III** according to the Endocrine Society guidelines to reflect the thresholds used in clinical practice, where low testosterone levels are diagnosed based on specific cut-off levels. Dichotomization makes the results easier to transfer to clinical practice, but also presents a simplified view of the continuous variable of testosterone. As previously discussed, there could be a U-shaped relationship between testosterone and the studied outcomes which suggest that for study purposes, the use of quartiles or cubic splines might provide more accurate estimates. However, these data could be more difficult to translate into clinically useful conclusions.

## **Ethical considerations**

Testosterone levels were investigated in populations that are usually not targeted for sampling of testosterone and the prevalence of low testosterone levels was quite high (13-30%). Therefore, an important question is whether screening for testosterone levels in the absence of symptoms should be offered in such patients in clinical practice. Such screening cannot be advocated until several ethical considerations have been discussed. The Wilson-Jungner screening criteria outline a number of conditions that should be fulfilled before general screening can be accepted (145, 146). First of all, low testosterone levels without symptoms can not be considered an important health problem given current data. Furthermore, the natural history of low testosterone, and the potential link to CVD is not fully understood. Although there are tests available to detect low testosterone levels, there are issues with assay methods, diverging thresholds for total testosterone and the lack of a harmonized reference range for free testosterone. Contemporary guidelines do in general not recommend treatment for men with low testosterone levels in the absence of symptoms related to testosterone deficiency. Thus, screening for low testosterone levels cannot be recommended in clinical practice from an ethical point of view.

## Future perspectives

Testosterone and the prognosis in individuals with dysglycemia and CVD were investigated in observational studies in which SHBG in particular but low free testosterone as well were potential prognostic markers and of importance to consider in a pathophysiological perspective when trying to understand the link between dysglycemia and CVD.

The increased CV risk in dysglycemic individuals has been studied previously and traditional risk factors cannot fully explain this relation. Interestingly, increase in the homeostasis model assessment - estimated insulin resistance (HOMA-IR) has been related to impaired CV survival in patients with T2DM (147). SHBG has been closely linked to T2DM in observational and Mendelian randomization analyses and its synthesis in the liver is inhibited by high insulin levels (16, 81, 148). Thus during the progress of dysglycemia, high insulin levels in the early phase could result in low SHBG levels and as insulin levels decrease over time SHBG levels might start to increase. One hypothesis is therefore that SHBG could be a marker for insulin status and through this possibly the progress of diabetes which could have prognostic implications. Future studies need to clarify the link between SHBG and insulin by investigating SHBG, HOMA-IR and insulin levels with CV events to assess if and why SHBG can identify dysglycemic individuals at high risk.

Observational data are somewhat inconsistent on the relationship between endogenous testosterone and CVD. Furthermore, in retrospective and clinical studies, concerns have been raised about the safety of testosterone therapy (149-152). However, a recent meta-analysis comprising 39 RCTs and 10 observational studies found no association between testosterone therapy and CV events (153). To close the gaps in knowledge and increase the understanding about testosterone and its potential cardiac effects, it is essential to study this further in clinical trials. Several small randomized controlled trials in men with CAD have reported on potential beneficial cardiac effects of testosterone (15, 29, 125). For example, short-term testosterone therapy by one-time injection as well as transdermal administration up to 12 weeks have shown improvements in exercise-induced myocardial ischemia (123, 154). Mechanistic studies have suggested effects on coronary blood flow and improved myocardial function as part of the explanation. Interestingly, in a randomized, controlled cross-over study comprising men with hypogonadism and CAD (n=22), oral testosterone substitution for eight weeks showed beneficial effects on myocardial perfusion in areas supplied by unobstructed coronary arteries but not in obstructed areas (124). Future studies should investigate longer-term testosterone therapy in men with CVD, preferably administered by intramuscular injection because of better compliance and absorption to assure raising low testosterone levels above threshold. Moreover, the use of cardiac magnetic resonance imaging could be of benefit for a more detailed assessment of myocardial perfusion, vascular function and CV physiology. This would shed some light over the possible pathophysiological mechanisms between testosterone and CVD. Finally, a randomized controlled trial on testosterone therapy and CV events is essential to confirm whether it is safe to administer from a CV perspective and if there is an impact of testosterone substitution on prognosis.



## **CONCLUSIONS**

- 1) Testosterone levels decrease following an AMI, with the lowest levels in men with dysglycemia. They increase to levels similar to those in healthy controls when measured one-year post-infarction. The initial reduction may be a reaction to stress due to AMI. Testosterone samples taken in close proximity to an AMI should be interpreted with caution.
- 2) CAG repeat length was not correlated to testosterone levels and did not predict future CV events in men with AMI.
- 3) Testosterone predicted CV and all-cause mortality in healthy controls but not in patients with AMI. On the other hand, free testosterone  $\leq 7$  ng/dl was associated with all-cause mortality in men with dysglycemia and high CV risk. This suggests that testosterone may be a mediator in CVD and prognosis rather than a traditional risk factor.
- 4) SHBG was an independent predictor of CV events and all-cause mortality in men and for all-cause mortality in women with dysglycemia and high CV risk. Further studies are warranted to explore whether this action is mediated through an impact on the distribution of testosterone or if SHBG is a direct prognostic marker.

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