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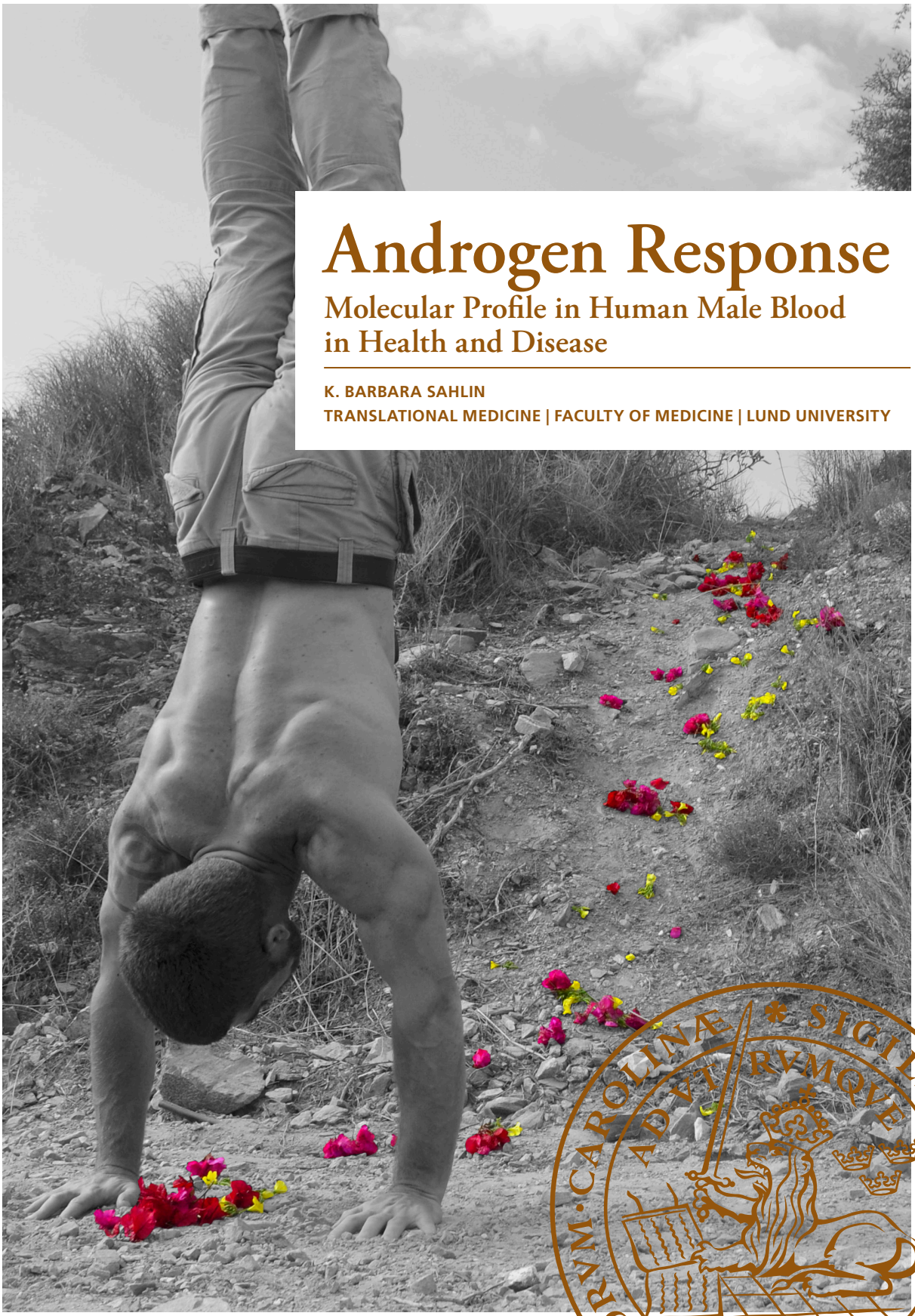

Androgen Response

Molecular Profile in Human Male Blood in Health and Disease

K. BARBARA SAHLIN
TRANSLATIONAL MEDICINE | FACULTY OF MEDICINE | LUND UNIVERSITY

Androgen Response

Molecular Profile in Human Male Blood in Health and Disease

Testosterone is an anabolic hormone with many important functions in men. Low testosterone is associated with common lifestyle diseases, such as metabolic syndrome. This thesis brings some novel insights in the early effects of testosterone deficiency in men and identifies candidate markers of testosterone activity.

K. BARBARA SAHLIN embarked her inquisitive academic journey by studying Physiotherapy at Lund University, where she decided to pursue her PhD. Currently, she works with cardiological rehabilitation at the hospital in Landskrona. She enjoys teaching university students and the international community that comes with academia. Her ambition is to investigate the link between exercise, hormones and lifestyle diseases.

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"Let us choose for ourselves our path in life,
and let us try to strew that path with flowers."

Emilie du Chatelet

Androgen Response

Molecular Profile in Human Male Blood in Health and Disease

K. Barbara Sahlin

DOCTORAL DISSERTATION

by due permission of the Faculty of Medicine, Lund University, Sweden.
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Faculty opponent

Professor Ole Nørregaard Jensen
Department of Biochemistry and Molecular Biology
University of Southern Denmark

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Abstract

Testosterone deficiency, hypogonadism, is a common condition in the aging male population with the prevalence estimated up to 30% between the ages of 46-89 years. Additionally, low testosterone increases the risk of diseases, such as diabetes type 2 and metabolic syndrome. The current method to evaluate testosterone levels in patients does not accurately reflect androgen activity, particularly when it comes to the cut off level of what is considered to be low testosterone.

The overall aim of the thesis was to elucidate the androgen response and identify novel markers of androgen activity by studying the downstream effects measured in human blood. The aim was also to study the link between testosterone deficiency and comorbidities. This thesis investigated the molecular profile by use of proteomics and metabolomics, as well as analysis of amino acids and common markers measured in the hospital. Firstly, a designed human model with healthy young men with induced testosterone deprivation followed by testosterone supplementation was studied. Secondly, a cohort of infertile men including common comorbidities was evaluated to test the novel protein markers of androgen activity and investigate the link between testosterone and comorbidities.

The present thesis provides orthogonal evidence that short-term effects of testosterone deficiency increases circulating amino acid levels, which suggests an early protein breakdown. This is supported by the increase in liver enzymes involved in gluconeogenesis as well as the increase of urea among others. Three candidate protein markers of androgen activity were identified, of which two markers significantly changed in those infertile patients with an impaired response to testosterone as defined by CAG-repeat length of the androgen receptor. Additionally, the protein markers also change in the patient cohort according to certain comorbidities. Some of these markers are also identified in previous studies in prediction of the future risk of development of metabolic disease. The present thesis provides novel insights on the molecular changes provoked by short-term testosterone deficiency, some of which are confirmed in a patient cohort of infertile men.

Key words Testosterone, Androgen, Hypogonadism, Proteomics, Metabolomics, Human Blood

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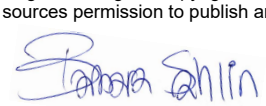
Signature  Date 2022-01-13

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To my family,
social and biological

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Studies included in the thesis

I. Sahlin KB*, Pla I*, Sanchez A, Pawłowski K, Leijonhufvud I, Appelqvist R, Marko-Varga G, Giwercman A, Malm J. Short-term effect of pharmacologically induced alterations in testosterone levels on common blood biomarkers in a controlled healthy human model. Scand J Clin Lab Invest. 2020, 80(1):25-31.

II. Pla I, Sahlin KB, Pawłowski K, Appelqvist R, Marko-Varga G, Sanchez A, Malm J. A pilot proteomic study reveals different protein profiles related to testosterone and gonadotropin changes in a short-term controlled healthy human cohort. J Proteomics. 2020, 30;220:103768.

III. Sahlin KB, Pla I, de Siqueira Guedes J, Pawłowski K, Appelqvist R, Marko-Varga G, Barbosa Domont G, Sousa Nogueira F, Giwercman A, Sanchez A, et al. Short-term effect of induced alterations in testosterone levels on fasting plasma amino acid levels in healthy young men. Life 2021, 11, 1276.

IV. de Siqueira Guedes J, Pla I, Sahlin KB, Monnerat G, Appelqvist R, Marko-Varga G, Giwercman A, Barbosa Domont G, Sanchez A, Sousa Nogueira F, Malm J. Testosterone depletion and replacement in a healthy human cohort promotes metabolic changes: a controlled and pharmacologically induced study. *Manuscript in writing.*

V. Giwercman A*, Sahlin KB*, Pla I, Pawłowski K, Fehniger C, Lundberg Giwercman Y, Leijonhufvud Y, Appelqvist R, Marko-Varga G, Sanchez A, Malm J. Novel protein markers of androgen activity in humans: proteomic study of plasma from young chemically castrated men. *Submitted to eLife November 2021.*

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- Almeida N, Rodriguez J, Pla Parada I, Perez-Riverol Y, Woldmar N, Kim Y, Oskolas H, Betancourt L, Valdés JG, Sahlin KB, Pizzatti L, Szasz AM, Kárpáti S, Appelqvist R, Malm J, Barbosa Domont G, Sousa Nogueira F, Marko-Varga G, Sanchez A. Mapping the melanoma plasma proteome (MPP) using single-shot proteomics interfaced with the WiMT database. Cancers 2021, 13, 6224.

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Abstract

Testosterone deficiency, hypogonadism, is a common condition in the aging male population with the prevalence estimated up to 30% between the ages of 46-89 years. Additionally, low testosterone increases the risk of diseases, such as diabetes type 2 and metabolic syndrome. The current method to evaluate testosterone levels in patients does not accurately reflect androgen activity, particularly when it comes to the cut off level of what is considered to be low testosterone.

The overall aim of the thesis was to elucidate the androgen response and identify novel markers of androgen activity by studying the downstream effects measured in human blood. The aim was also to study the link between testosterone deficiency and comorbidities. This thesis investigated the molecular profile by use of proteomics and metabolomics, as well as analysis of amino acids and common markers measured in the hospital. Firstly, a designed human model with healthy young men with induced testosterone deprivation followed by testosterone supplementation was studied. Secondly, a cohort of infertile men including common comorbidities was evaluated to test the novel protein markers of androgen activity and investigate the link between testosterone and comorbidities.

The present thesis provides orthogonal evidence that short-term effects of testosterone deficiency increases circulating amino acid levels, which suggests an early protein breakdown. This is supported by the increase in liver enzymes involved in gluconeogenesis as well as the increase of urea among others. Three candidate protein markers of androgen activity were identified, of which two markers significantly changed in those infertile patients with an impaired response to testosterone as defined by CAG-repeat length of the androgen receptor. Additionally, the protein markers also change in the patient cohort according to certain comorbidities. Some of these markers are also identified in previous studies in prediction of the future risk of development of metabolic disease. The present thesis provides novel insights on the molecular changes provoked by short-term testosterone deficiency, some of which are confirmed in a patient cohort of infertile men.

Abbreviations

ALAT	alanine aminotransferase
ANOVA	analysis of variance
ASAT	aspartate aminotransferase
apoA1	apolipoprotein A1
apoB	apolipoprotein B
AR	androgen receptor
BMI	body mass index
CVRLP	cardiovascular risk lipid profile
DM2	diabetes mellitus type 2
FDR	false discovery rate
FSH	follicular stimulating hormone
GnRH	gonadotropin releasing hormone
HDL	high density lipid
HPG	hypothalamo-pituitary gonadal
IR	insulin resistance
LBD	low bone mineral density
LDL	low density lipid
LH	luteinizing hormone
MetS	metabolic syndrome
MMA	multi marker algorithm
MS	mass spectrometry
SHBG	sexual hormone globulin hormone
TSH	thyroid stimulating hormone
PSA	prostate specific antigen

Introduction

Physiology of Testosterone

In men, testosterone is secreted by the testis and circulates in protein bound form (~ 99.5%) usually binding to sexual hormone globulin hormone (SHBG), leaving a very limited amount of testosterone free for biological action. Sufficient amounts of the reproductive hormone is crucial during growth and puberty to achieve peak musculoskeletal health and reproductive function. Testosterone is an androgen that is essential for male health, such as body composition and other organs. In adulthood, testosterone is necessary in order to preserve health [1–4].

Testosterone is a steroid hormone, which can freely enter cells as it is fat soluble [5] and binds to the androgen receptor (AR) in the cytoplasm. The ligand bound AR then translocates to the nucleus, where it binds to hormone response elements and influences the cellular mRNA production [6]. Furthermore, testosterone can irreversibly be converted into two different hormones: dihydrotestosterone and estrogen. Dihydrotestosterone binds with a higher affinity to the AR compared to testosterone. Yet, it has not been comparatively as well associated with androgen related disease [2]. Estrogen is a female sex hormone, which is important for male fertility and bone density among others [7]. Additionally, testosterone is important in females with physiological actions mediated by aromatization to estrogen throughout the body [8]. Hyperandrogenism, or androgen excess, is a contributing factor in the development of polycystic ovarian syndrome, which has a prevalence of 6-15% in women [9].

Reproduction

Testosterone is secreted from Leydig cells in the testis, which is regulated by the Hypothalamic-Pituitary Gonadal (HPG) axis displayed in **Figure 1**. Gonadotropin releasing hormone (GnRH) is released from the hypothalamus in the brain and stimulates the production and release of luteinizing hormone (LH) and follicular stimulating hormone (FSH) in the closely situated anterior part of the pituitary gland. In turn, FSH binds to the Sertoli cells in the testis and stimulates the production of sperm and the release of inhibin. Whereas, LH stimulates the Leydig cells in the testis to produce and release testosterone. Both inhibin and testosterone

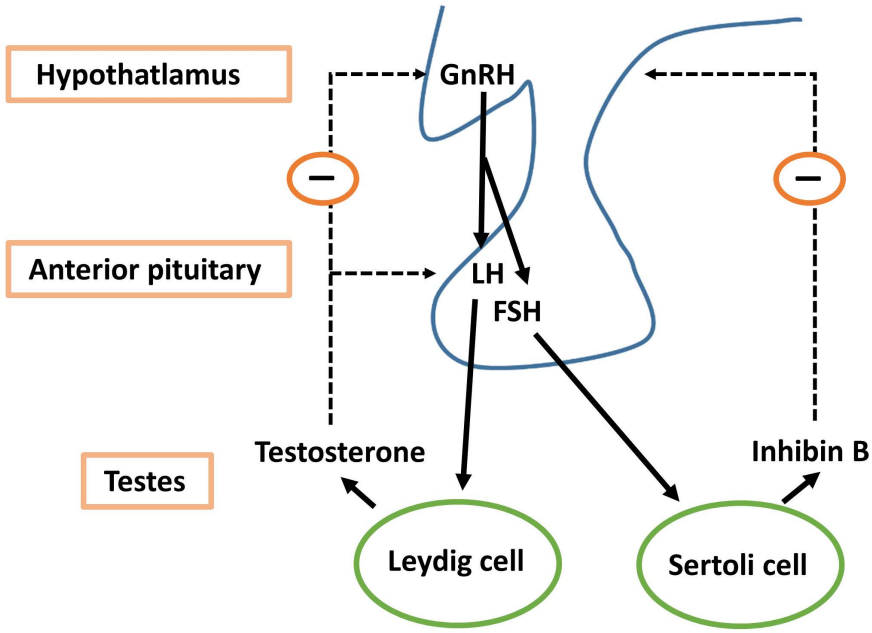

Figure 1. The hypogonadal pituitary gonadal axis feedback loop.
Gonadotroping releasing hormone (GnRH) is realeased from the hypothalamus in the brain, which stimulates the production and release of luteinizing hormone (LH) and follicular stimulating hormone (FSH). In the testis, LH stimulates the Leydig cells to produce and release testosterone, while FSH acts on the Sertoli cells to release inhibin B. Testosterone inhibits both in the hypothamalus and the anterior pituitary, while inhibin B inhibits the hypothalamus.

are involved in a negative feedback loop that decreases the production of the other hormones in the axis, in which inhibin hinders the production of LH and FSH, while testosterone inhibits all three of the upstream hormones. Thus, the axis is, as many other bodily systems, intricately constructed to maintain homeostasis. The main production of testosterone takes place in the testis via Leydig cells. The rest of testosterone production takes place in other tissues, such as adrenal tissue and muscle tissue [10,11].

Metabolism

Testosterone is also a metabolic hormone and has a highly complex metabolic effect on the liver, fat and muscle tissues [12–14]. Metabolic functions of testosterone include to increase glucose transport, facilitate insulin signalling, increase glycogen synthesis in skeletal muscle [14], as well as reduce protein loss and hepatic urea synthesis [15]. Testosterone stimulates muscle protein synthesis and intramuscular amino acid uptake, which improves the net protein balance. Testosterone increases

calcium channels in muscle cells and increases the number of motorneurons [16]. The AR is also upregulated by testosterone in muscle cells, myonuclei and satellite cells [10]. Additionally, both short- and long-term effects of testosterone supplementation suggest an anabolic response [17].

In healthy men, there is a balance throughout the diurnal cycle between catabolic (tissue breakdown) and anabolic (tissue repair) processes in order to maintain muscle mass. Muscle tissue is the main catabolic target to increase the amino acid pool during energy shortage, due to fasting or exercise (see **Figure 2**) [12,17]. The amino acids can then be utilized for energy production via entry to the citric acid cycle. This process is called gluconeogenesis and mainly takes place in the liver [18].

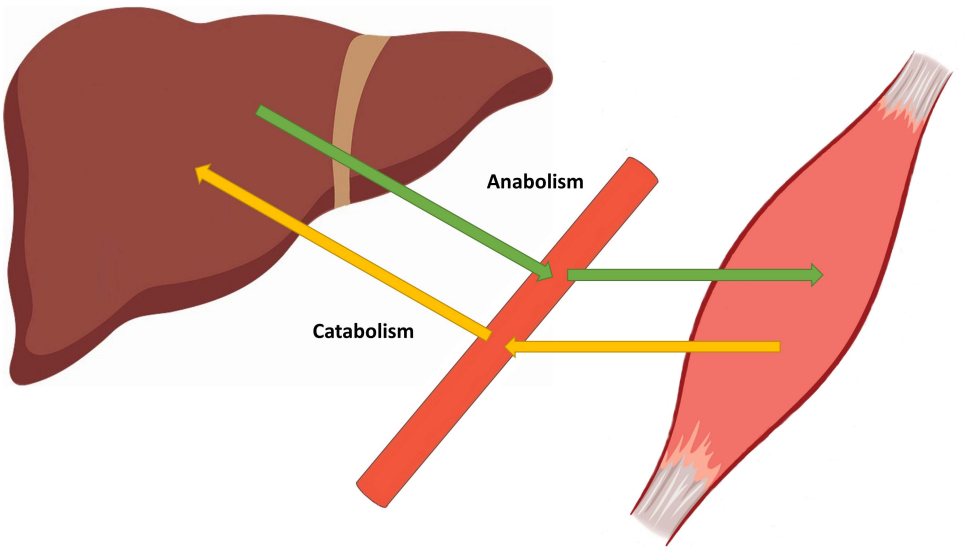

Figure 2. The flux of amino acids in the anabolic and catabolic state.
Amino acids in the anabolic state are made available in the blood stream via diet and liver, and absorbed by other tissues such as muscle. During the catabolic state, there is a need to free up amino acids for energy production, which is mainly provided by muscle breakdown. Amino acids then enter the blood stream and are absorbed by the liver for gluconeogenesis.

Aging

Testosterone declines at a slow and constant rate. Age-related testosterone decline beyond 35-40 years is associated with an annual decrease of 1-3% circulating testosterone levels, of which 2-3% decrease in bioavailable or free testosterone [19]. Increase in SHBG with age leads to less bioavailable testosterone and testosterone levels have been reported to be decreasing globally [20–23].

As men age there is a relative increase in adipose tissue and inflammation, as well as a decrease in muscle tissue and physical activity [24–26]. Additionally, the prevalence of obesity is increasing in Sweden and is now at 57% of males 16 years of age and older [27]. Adipose tissue is endocrinologically active, such as converting testosterone into estrogen with aromatase. Obesity among other factors contribute to accelerated testosterone decline, which, in turn, contributes to the development of late-onset hypogonadism [25]. Actually, epidemiological studies propose that obesity is the best predictor of testosterone deficiency in men [28].

Kinetic Boost

In response to exercise, there is an acute increase in testosterone [11] and cortisol, of which the latter is considered a catabolic effect favouring protein breakdown. The balance between these two hormones determine protein turnover of muscle mass and, thereby, muscle strength [16]. Exercise is referred to as a polypill as it can be used to treat a large variety of diseases, including diabetes mellitus type 2 (DM2), cardiovascular disease and even cancer [29]. This is due to exercise inducing a lot of physiological changes, such as processes pertaining to blood sugar balance, vascular changes and anti-inflammation [26,29].

Increased oxygen consumption occurs with exercise, often leading to temporarily decreased oxygen in skeletal muscle. This activates the hypoxia-inducible factor 1, which improves oxygen supply to the muscle and its metabolic capacity. Hypoxia-inducible factor 1 increase in response exercise [30]. However, hypoxia also has a negative effect on male fertility, such as by disrupting spermatogenesis [31]. Testosterone may reduce oxidative damage in Leydig cells in cell lines [32]. The mRNA for the AR is increased by exercise in muscle cells and testosterone also functions to upregulate AR in muscle cells [33]. Additionally, androgens regulate physical activity in male rodents, implicating a behavioural effect. However, this has not been reproduced in men possibly due to limitations in methods [24].

Pathophysiology of Testosterone

Deficiency

Testosterone deficiency, also known as hypogonadism, displays unspecific clinical manifestations. There are different types of hypogonadism: primary, secondary and mixed. Primary hypogonadism is characterized by an increase in luteinizing hormone (LH) and follicular stimulation hormone (FSH), due to the decrease in testosterone production by the testis, which could, for instance, be the consequence of radiation treatment. In secondary hypogonadism, LH and FSH are low, which

could be the consequence of a pituitary adenoma [34,35]. Mixed or late onset hypogonadism may involve aging and systemic disease, and phenotypically is a combination of primary and secondary hypogonadism [21,36].

Symptoms of late onset hypogonadism may include sexual symptoms: reduced sexual desire and activity, erectile dysfunction, decreased spontaneous erections, reduced testicular volume, and infertility. Other symptoms are loss of body hair, decreased bone mineral density, and hot flashes. Less specific signs include decreased energy, depressed mood, poor concentration, mild anemia, reduced muscle mass, increased body fat, and lower physical performance [20,21].

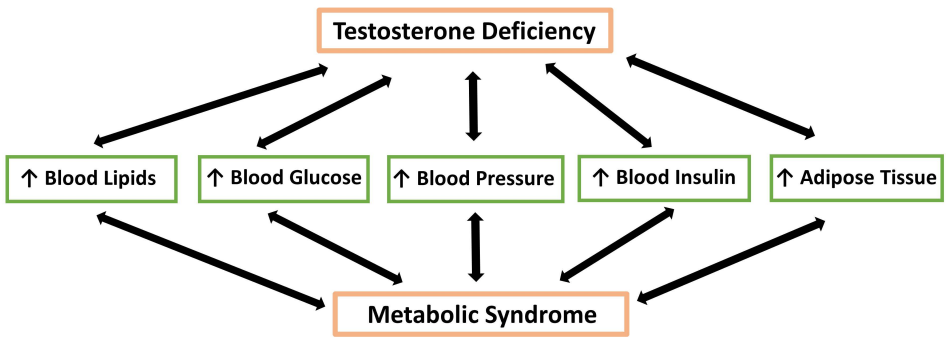

Figure 3. The similar clinical symtoms that are shared between testosteron deficiency and metabolic syndrome. Testosterone deficiency negatively impacts on healthy in many ways. Particularly, an increase in blood lipids, glucose, insulin, blood pressure and adipose tissue seem to have a bidirectional relationship with testosterone deficiency as well as metabolic syndrome.

Pathophysiological changes observed in hypogonadism include decreased insulin sensitivity and high density lipids (HDL), as well as increased triglycerides and cholesterol. These catalyze the development of other diseases, such as metabolic syndrome. In fact, testosterone deficiency and metabolic syndrome have very similar sequelae, which can be viewed in **Figure 3**. The pathological overlap in clinical manifestation includes an increase blood pressure, increase in adipose tissue and an increase in blood glucose, insulin and lipids [37]. These symptoms can all be reduced or reversed by exercise [29]. Additionally, decreased testosterone levels is associated with an increased all-cause mortality [20].

Diagnosis of Testosterone Deficiency

Hypogonadism is diagnosed based on the presence of low serum testosterone levels combined with clinical symptoms, which are highly unspecific [20,21]. Commonly, total testosterone is measured in a fasting morning condition by automated chemiluminescent methods using antibodies [38]; however, the association between testosterone levels and its response is affected by the concentration of binding proteins, body mass index (BMI), certain diseases (such as DM2), AR sensitivity, and more [39,40].

Hypogonadism is a common condition with an estimated prevalence of up to 30% in males from 46 to 89 years old [41]. However, it is difficult to determine the prevalence partly due to the limited diagnostic guidelines [21]. In general, a total testosterone concentration below 8 nmol/L indicates an insufficient hormone level, while levels above 12 nmol/L are considered normal [42]. Hence, there is a "grey zone" between 8 and 12 nmol/L, in which patients can neither be assigned low nor normal testosterone levels. Additionally, patients may also be misclassified due to the level of free testosterone is not being measured, which would account for binding proteins directly affecting the amount of bioavailable testosterone. Obese men often display relatively low SHBG levels, which may lead to a low concentration of total testosterone despite an unaffected androgen activity [43].

Pharmacological interventions

Gonadotropin Releasing Hormone agonist

GnRH agonists were developed to treat patients with hormone dependent prostate cancer when testosterone suppression is warranted. The medication functions to downregulate the GnRH receptors, which depletes all of the homrones included in the HPG-axis. This results in a chemical castration. A side effect of GnRH agonists is an initial increase of testosterone levels called a "flare," which increases the risk of tumor growth [44].

Gondotropin Releasing Hormone antagonist

GnRH antagonists are another alternative to use in hormone dependent prostate cancer. GnRH antagonists also suppresses the HPG to achieve chemical castration. However, this method avoids the initial testosterone flare and is considered to be a safer treatment option [44,45].

Both GnRH antagosnists and agonists have been associated with risk factors common with testosterone deficiency, such as decreasing insulin sensitivity and lean body mass, as well as increasing subcutaneous fat and HDL and LDL cholesterol. GnRH agonists compared to antagonists are associated with an increased risk of mortality and cardiovascular disease [44,46].

Testosterone Supplementation

Testosterone supplementation is a highly discussed topic, because it contributes to increase in muscle mass and strength [24], as well as decreased fat mass and insulin resistance [37]. This could potentially benefit a high number of men, particularly because as muscle weakness, obesity and DM2 are increasingly common with older age [26,47]. However, a study found an increased risk of cardiovascular adverse events upon testosterone supplementation in elderly men [48]. This combined with a marked increase in prescriptions has sparked a debate regarding the safety of testosterone supplementation [49].

-Omics as a Method for Blood Biomarker Discovery

Blood Components

Human blood contains thousands of proteins and is, thus, the most comprehensive version of the human proteome. Blood is a sample of choice because the collection is minimally invasive and it contains all tissue proteins due to tissue leakage. However, there are also major challenges in using blood for biomarker discovery as it has a large dynamic range of ten orders in magnitude in concentration, in which albumin accounts for 55% of plasma protein. There are potentially many proteins in blood that could hold potential as disease biomarkers, and a relatively few biomarkers are currently used for disease diagnosis in patients [50].

Clinical Blood Samples

The two types of blood samples most widely used are plasma and serum. In both sample types, the blood sample is centrifuged to yield layers of blood, in which erythrocytes are in the bottom layer, the buffy coat is a thin middle layer consisting of leukocytes and platelets, and the top layer is either serum or plasma depending on the tube in which blood is collected. The difference between the sample types is that in serum, the clotting factors have reacted and fallen out of the sample, such as prothrombin and fibrinogen [50].

-Omics

"Omics" is a field of study that aims to identify, characterize, and quantify all biological molecules that are involved in the structure, function and dynamics of a cell, tissue or organism. There are several different types of “omics”, including proteomics and metabolomics. Moreover, in proteomics, proteins are studied as related to their biochemical properties and functional roles, and how their quantities,

modifications and structures change during growth and in response to internal and external stimuli. Additionally, metabolomics studies all chemical processes involving metabolites or metabolite profiles, including metabolites in a biological cell, tissue, organ, or organism, which are the end products of cellular processes [51].

Mass Spectrometry

Mass Spectrometry (MS) is a technology with a wide application utilized to measure compounds. It is an indispensable tool for proteomic and metabolomic studies due to the flexibility of the instrumentation to measure compounds based on mass-to-charge, m/z. The instrument is comprised of three main parts: ionization source, mass analyzer and detector [52]. In the proteomic experiments, protein reduction and alkylation of disulfide bonds are performed to achieve a better sequence

Figure 4. Overview of the sample preparation for and analysis by mass spectrometry in proteomics.
Plasma or serum proteins are reduced, alkylated and digested into peptides. The sample is then separated by liquid chromatography prior to ionization into gas phase and entry to the mass spectrometer (MS). The MS detects the mass-to-charge or the peptide, followed by peptide fragmentation and a new detection of mass-to-charge. For discovery MS, the MS data is matched with libraries to enable protein identification. In targeted MS, the data is handled manually in order to verify the identifications.

coverage by MS [53]. This process is followed by an enzymatic digested by trypsin into peptides, which are then detected by MS [54]. The samples are commonly separated by liquid chromatography (LC) in order to reduce sample complexity prior to ionization and detection by MS [55]. Electron Spray Ionization (ESI) is the method of ionization that was used throughout the omic papers included in the thesis. With ESI, ionized droplets change from liquid to gas phase by high voltage prior to entry to the MS [56]. Inside the MS, the first spectra, MS1, on the precursor ions are separated in a mass anlayzer based on m/z and selected for fragmentation. This is followed by a collision, in which the selected peptides are fragmented into product ions and are analysed again to produce MS2 spectra. In untargeted MS, these spectra and m/z are matched to library spectra for identification [52]. An overview of this process can be seen in **Figure 4**.

In order to identify novel biomarkers by MS, discovery mode is used. Discovery MS is an unbiased approach, in which, theoretically, all the compounds may be measured in the sample [52]. There are two different options to embark on discovery MS, either by data independent acquisition (DIA) or data dependant acquisition (DDA). DIA entails that all of the peaks are measured in MS2 within set windows, whereas in DDA the top *n* peaks are measured in MS2 [57]. Additionally, in order to confirm findings and achieve more accurate concentrations of the candidate biomarkers, a targeted approach can be applied. In targeted MS, scheduled methods using multiple reaction monitoring (MRM) will probe the samples for selected peptides for MS2 measurement [58]. Additionally, heavy labelled selected peptides were spiked at known concentrations in the targeted experiments to improve quantification [59].

Main objective

The overall aim of the thesis was to elucidate the androgen response and identify novel markers of androgen activity by studying the downstream effects measured in human blood. The aim was also to study the link between testosterone deficiency and comorbidities. This thesis was conducted by investigating the molecular profile by use of proteomics and metabolomics, as well as analysis of amino acids and common markers measured in the hospital. Firstly, a designed human model with healthy young men with induced testosterone deprivation followed by testosterone supplementation was studied. Secondly, a cohort of infertile men including common comorbidities was evaluated to test the novel protein markers of androgen activity and investigate the link with comorbidities.

Specific objectives

Paper I: The aim was to gain insight into early testosterone effects on blood biomarkers routinely used in healthcare in the healthy human model.

Paper II: The aim was to detect the early testosterone driven influence in plasma proteome in the healthy human model.

Paper III: The aim was to determine the early testosterone effect on fasting plasma amino acids in the healthy human model.

Paper IV: The aim was to describe the metabolic change in plasma in the healthy human model.

Paper V: The aim was to identify novel protein markers of testosterone activity in the healthy human model and assess the proteins predictive value in patients with hypogonadism and comorbidities.

Material and Methods

Study design

An overview of the study design can be viewed in **Figure 5**, which was aimed to measure analytes in human blood. The first task was to determine whether the pharmacologically induced sexual hormones change as expected in the human model and if the shift in biomarkers measured at clinical chemistry could provide any additional biological information, Paper I. The next study, Paper II, was an explorative proteomic analysis performed on pooled plasma in order to see if there

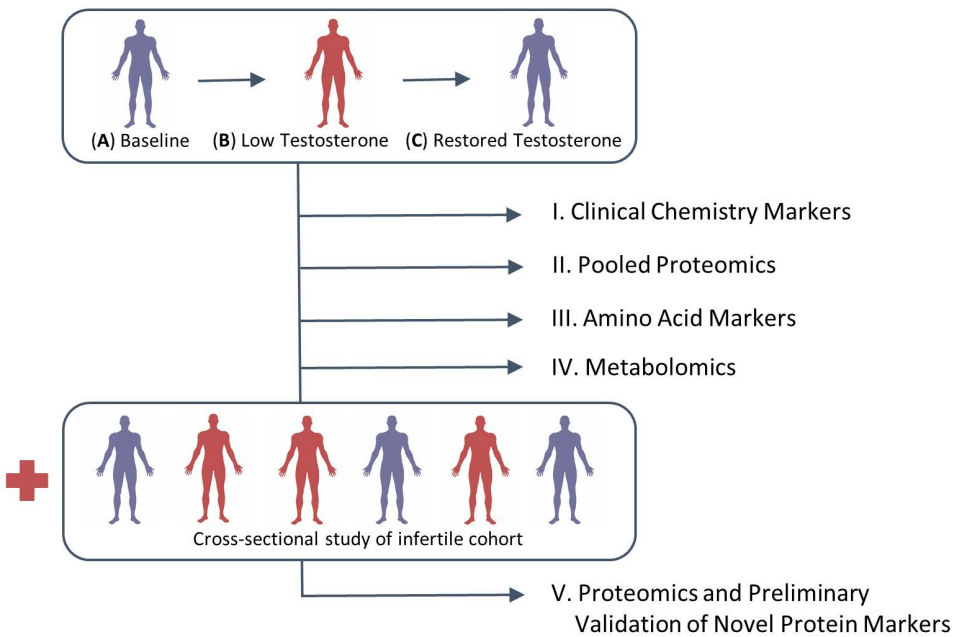

Figure 5. Overview of the study design of the thesis.
The designed human model consisted of healthy young males (n = 30) undergoing a treatment regimen with the purpose of suppressing endogenous testosterone with subsequent restoration by testosterone supplementation. Blood samples from each time point were subjected to different types of analyses resulting in five articles. The methods used were I) routine makers measured a clinical chemistry, II) pilot proteomic study with one pool per time point, III) routine amino acids measured at clinical chemistry, IV) metabolomic study, and V) proteomic study including a preliminary validation of novel protein markers in a cohort of patients suffering from infertility.

were proteomic shifts mediated by changes in testosterone levels. Because the data indicated that testosterone changes induced metabolic shifts, plasma amino acids were analyzed in the human model in Paper III. The following study, Paper IV, applied an unbiased metabolomics approach to reveal more about the metabolic changes mediated by testosterone in the human model. In Paper V, more substantial proteomic analysis was performed on individual samples from the human model and some candidate protein markers were identified. These findings were also probed in blood samples from an infertile cohort.

Study population

Two study populations were utilized in the thesis: the first one was based on healthy human volunteers recruited for the thesis and the other was based on a previously collected cohort of infertile patients.

Healthy human model

Thirty healthy young males were recruited in order to study the testosterone effect on the blood proteome and metabolome in a clean system. Blood was collected at three time points: baseline, low testosterone and restored testosterone. After the first blood collection, the subjects received an injection of a gonadotropin releasing hormone antagonist (240 mg Degeralix®, Ferring Pharmaceuticals, Saint-Prex, Switzerland). After two weeks, the second blood collection was completed, after which the subjects received an injection of synthetic testosterone (1000 mg testosterone undecanoate, Nebido®, Bayer AG, Leverkusen, Germany). The last blood collection was performed three weeks later. Thus, the duration of the human model was five weeks.

Infertile cohort

The cohort was previously collected for a study on hypogonadism on infertile males. It consisted of serum samples and clinical data of 213 infertile men and 223 age-matched controls [60]. Eighty-five subjects were randomly selected, of which 10 patients were excluded. Seven subjects presented Klinefelter syndrome, one did not have a testosterone value recorded, and two were statistical outliers. The clinical data that accompanied the samples revealed that some subjects exhibited comorbidities. The comorbidities defined were metabolic syndrome (MetS), insulin resistance (IR), cardiovascular risk lipid profile (CVRLP), diabetes mellitus type 2 (DM2), and low bone mineral density (LBD).

Blood processing

Vials of blood collected from the subjects in the human model were sent to the clinical chemistry lab (Paper I) at Skåne University Hospital in Malmö, Sweden, and to Lund University, Sweden, for storage in a semiautomated biobank [61]. A simple overview of the blood processing can be viewed in **Figure 6**. Samples were aliquoted into 192 vials per subject and time point and stored at – 80 °C. The plasma in the biobank was subsequently used for different experiments included in Papers II-V.

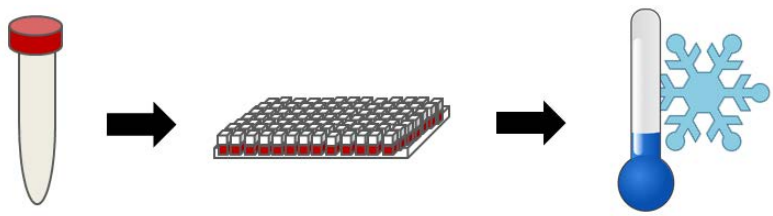

Figure 6. Schematic of the sample processing in the biobank.
Blood samples were aliquoted by a semiautomated robot yielding 192 vials per subject. The samples were stored at -80 °C and the cycle time from sample withdrawal to freezer was 2 hours to maintain sample integrity.

The serum samples from the infertile cohort (Paper V) were stored in a clinical biobank at the Reproductive Medicine Centre, Skåne University Hospital in Malmö, Sweden. The following sections are brief descriptions of the methods. Further details can be found in the respective paper (Papers I-V). In general, samples were randomized prior to sample preparation and analysis. To prepare for statistical analysis, the data is log2 transformation with subtraction of the median per analyte. Unless otherwise specified, significance was set at $p < 0.05$. *P*-values were adjusted to control the false discovery rate (FDR), of which 5% was accepted.

For the omic papers using DDA (II, IV,V), raw data was put into a program with a workflow that aligns the MS chromatographic peaks, matches, and compares parent ions with fragment ions for analyte identification in the samples based on data in library repositories.

Paper I: Clinical Chemistry Markers

Hematological analysis and assessment of clinical chemistry biomarkers from the healthy human model were analyzed at the Clinical Chemistry Laboratory at Skåne University Hospital in Malmö, Sweden, where the biomarkers were measured by automated analytical devices. The statistical analyses included descriptive analysis, checking for outliers, significance paired t-tests (p-value < 0.0167 after controlling for FDR), and 95% confidence interval of the mean of the differences to corroborate significance.

Paper II: Proteomic Pool

One pool per time point consisting of all subjects (n=30), resulting in three pools in total from the human model. Briefly, plasma proteins were prepared prior to MS analysis. Proteins were reduced and alkylated, followed by enzymatic digestion with trypsin. LC-MS/MS was performed on an Easy-nLC 1000 pump and a Q-Exactive Plus™ MS with a top 10 DDA method, label-free.

The corresponding gene ontology annotations and pathways were mapped to each protein to perform a functional enrichment analysis, in which fold changes between A-B and B-C were considered for analysis. Two probability distribution plots were made per comparison (A-B and B-C) and differences with less than 5 % of probability (higher ratios) were considered significant.

Paper III: Amino Acid Analysis

In Paper III, the plasma amino acids from the subjects in the human model was analysed automatically at the Clinical Chemistry Laboratory at Skåne university hospital in Malmö, Sweden. For the statistical analysis, the differential expression between time points were evaluated depending on their distribution (paired ANOVA or Freidman rank sum test followed by post hoc tests). As a complementary analysis, we used the 95% CI of the mean of the differences between time points to confirm previous analyses. Selection of the amino acids that reflect the effects of testosterone changes on fasting plasma amino acid levels was also performed by stepwise binomial regression analysis.

Paper IV: Metabolomics

Plasma proteins were precipitated and removed from the samples and an isotopically labeled internal standard was added. Samples were analyzed by LC-MS/MS with Dionex Ultimate 3000 UHPLC and Q-Exactive Plus™ in both positive and negative mode, scanning in full MS mode (m/z 70-800) with top-10 DDA. Statitisical analysis included a Tukey post hoc test was performed after the paired ANOVA to determine specific differences between time-points.

Paper V: Proteomics and Preliminary Validation of Protein Markers

Plasma from the healthy human model were analyzed by an LC coupled to a Q-Exactive Plus™. The scanning was in full MS mode (m/z 70-800) with DDA label-free. MRM analysis of both the healthy human model and the cohort of infertile males. Samples were analyzed on a TSQ Quantiva mass spectrometer with scheduled 5 minute windows for each selected peptide. The raw data were imported to Skyline v3.5 software for data analysis.

Differentially expressed proteins were analyzed by a paired ANOVA followed by a pairwise t-test (two-tails and paired). Candidate markers were tested by receiver operating characteristic analysis and included as predictors in a stepwise logistic regression (method: backward) to select the best combination of markers that predict the odds of being low testosterone. Included in the analysis was a new variable called Multi Marker Algorithm (MMA) that was derived from the predicted log-odds (of being low testosterone) obtained from a binomial logistic regression analysis.

Data collection and statistics for the patient cohort

Blood samples from the infertile males were previously analyzed at the Clinical Chemistry Laboratory at Skåne University Hospital in Malmö, Sweden. The following markers were measured total testosterone, SHBG, free testosterone, LH, FSH, estrogen, total cholesterol, LDL, HDL, free triglycerides, glucose, and hemoglobin A1c. Also, Homeostatic Model Assessment of Insulin Resistance and dual-energy x-ray absorptiometry were measured. CAG-repeat length from the AR was measured by DNA extraction from leukocytes in the blood.

The normal distribution of the background characteristics were evaluated by Kolmogorov-Smirnov test. The intensities of the candidate biomarkers were Log2 transformed. The MMA variable was utilized to predict the odds of testosterone

deficiency or related comorbidities. The cohort was divided into three patient groups based on the total testosterone concentration and the markers ability to distinguish between the defined patient groups was assessed by receiving operating characteristic analysis. Associations between candidate markers and three groups of infertile men was evaluated defined based on the CAG repeat length of the AR.

Results

The Human Model

Reported Side Effects

Regarding the subjects perceived symptoms during the study, no adverse or unexpected side effects were reported. Six subjects did not experience any side effects during the course of the study, while seven participants had side effects after both treatments. The most common complaints reported were decreased libido (n = 15), tiredness (n = 7), depressive symptoms (n = 6), irritation around the site of injection (n = 5), and hot flashes post testosterone injection (n = 8).

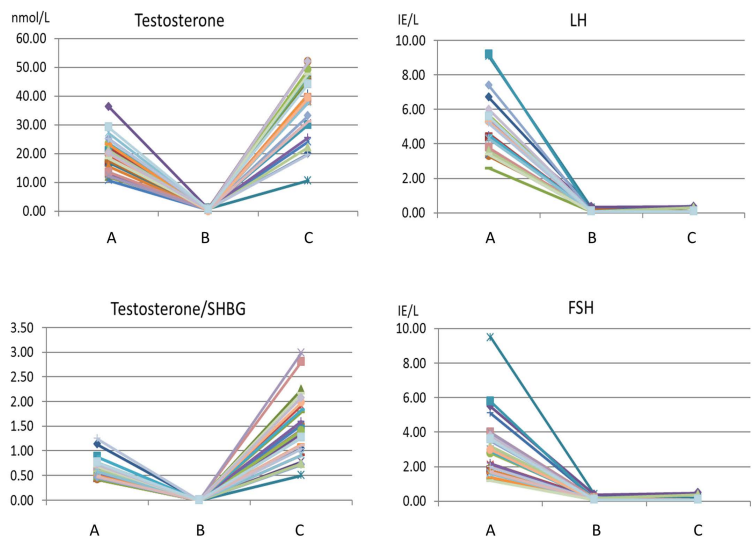

Figure 7. Individual changes in reproductive hormones in the healthy human model
Measurements from three different time points: (A) baseline, (B) low testosterone and (C) restored testosterone. The lines represent the volunteers' (n=29), which are assigned a unique color respectively. The reproductive hormones measured are total testosterone, testosterone/sexual hormone binding globulin, luteinizing hormone (LH) and follicular stimulating hormone (FSH). At time point C, LH and FSH remain low, while testosterone has been supplemented.

Reproductive Hormones

The human model yielded three different time points with predicted changes in hormonal levels, based on how the medications are used clinically. Thus, testosterone changes across the time points are A) baseline, B) low testosterone and C) restored testosterone. The other reproductive hormones LH and FSH are suppressed in time point B and remain suppressed in time point C. All but two subjects restored testosterone levels (C compared to A). In general, testosterone and testosterone/SHBG restored to higher levels than baseline. The inter-individual variation (each line representing a subject) was the greatest in time point C in terms of testosterone levels and in time point A for LH and FSH (see **Figure 7**).

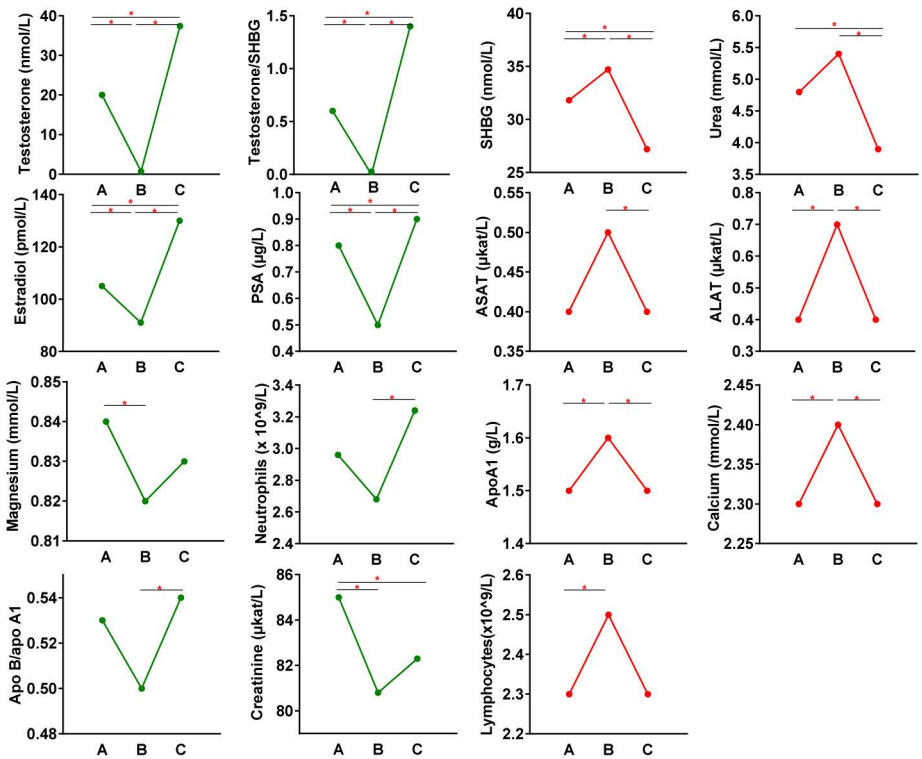

Figure 8. Association patterns of the biomarkers exhibiting at least one statistically significant change between time points in the healthy human model.

The lines indicating the biomarker levels are colored depending on if they change in the same direction as testosterone (green) or if they change in the opposite manner (red). The markers in green are testosterone, testosterone/SHBG, estradiol, PSA, magnesium, neutrophils, apoB/apoA1, and creatinine. Red markers are SHBG, urea, ASAT, ALAT, apoA1, calcium and lymphocytes. The time points are (A) baseline, (B) low testosterone and (C) restored testosterone. * *p*-value < 0.0167.

Clinical Chemistry Markers

Four markers exhibited significant changes in all three time point comparisons (B-A, C-B, and C-A), which were testosterone, testosterone/SHBG, estradiol and PSA. Markers that significantly changed between A-B and B-C included ALAT, apo A1 and calcium. Markers that presented a positive or negative alteration with testosterone between A-B and B-C, of which at least one of these changes was of statistical significance, included SHBG, estradiol, PSA, ASAT, ALAT, urea, magnesium, calcium, creatinine, apoA1, apoB/apoA1, neutrophils, and lymphocytes. The behavior of these biomarkers is presented in **Figure 8**, where biomarkers in green signify that the change is in the same direction as the testosterone changes (n = 6) and red biomarkers behavior in the opposite direction to testosterone changes (n = 7).

Proteomic Pool

In general, the global protein difference between the time points shows that time point B has more divergent expression compared to time points A and C. The 2D enrichment analysis divided predominant enriched biological processes that changed between time points into four quadrants (Qs), in which Qs II and IV capture the processes that change upon testosterone restoration and Qs I and III contain the processes affected by LH and FSH (see **Figure 9**). Processes that were testosterone dependent include gluconeogenesis and glycolysis (Q IV), as well as insulin-like growth factor I and II binding and angiogenesis (Q II).

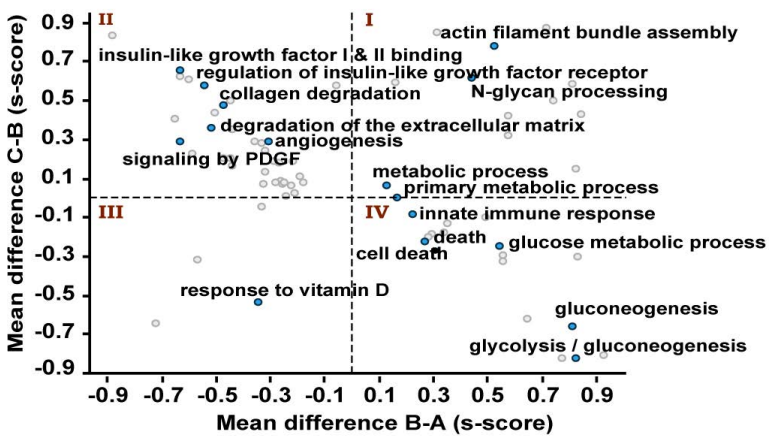

Figure 9. 2D annotation enrichment analysis in the healthy human model
The biological processes are enriched for fold-change in B-A versus fold-change C-B. The processes that are in quadrant (Q) II and Q IV are associated with changes in testosterone, whereas the other processes (Q I and Q III) are related to the changes in LH and FSH.

Amino Acid Markers

Nine out of 23 amino acids significantly changed with testosterone and displayed an inverse behavior, specifically aspargine, histidine, leucine, lysine, methionine, phenylalanine, tryptophan, tyrosine, and valine as seen in **Figure 10**. The combination of phenylalanine and tyrosine best explain the testosterone changes in the human model. They were significant ($p < 0.05$) 77% and 50 % of the times respectively in the stepwise binomial logistic regression.

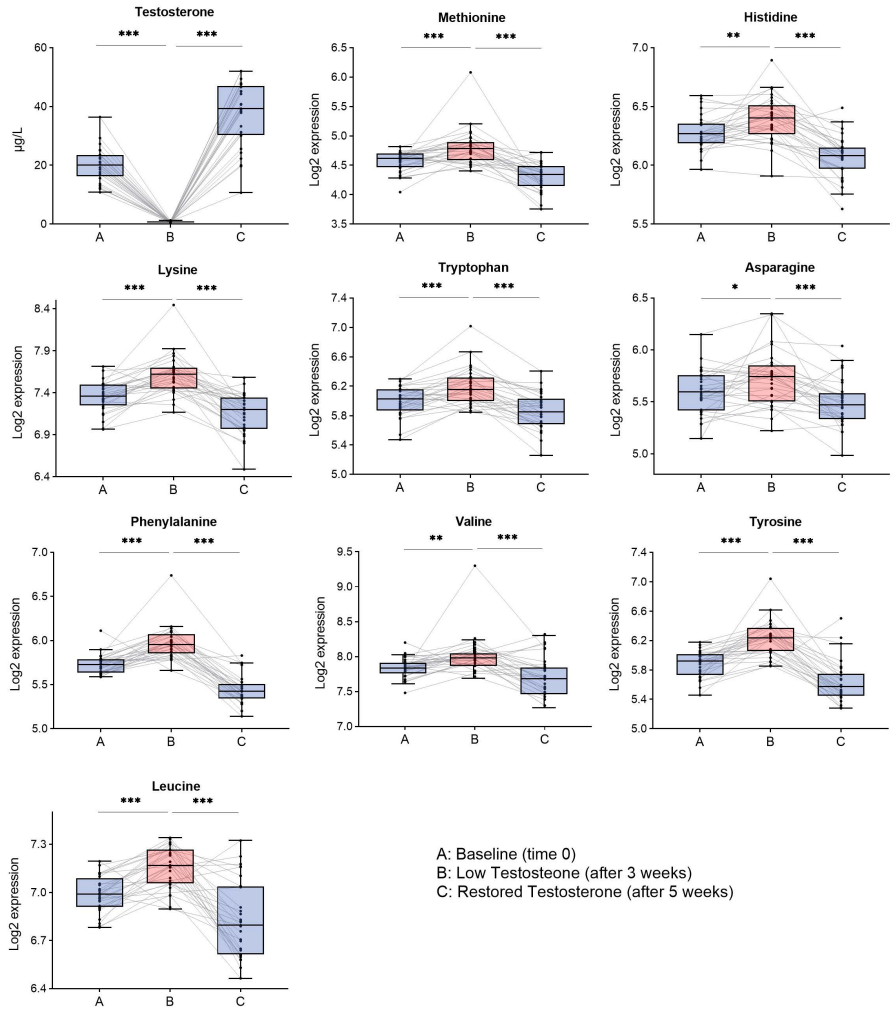

Figure 10. Box plots of the amino acids with at least one change in B-A or C-B in the healthy humna model
Nine amino acids were negatively influenced by T: aspargine, histidine, leucine, lysine, methionine, phenylalanine, tryptophan, tyrosine, and valine, meaning that they are increased with low testosterone and restored upon testoterone supplementation. * *p*-value < 0.05, ** p < 0.01, *** p < 0.001.

Metabolomics

The metabolic results suggest an enrichment in mainly carnitine synthesis, lipid oxidation and amino acid metabolism. Additionally, in **Figure 11**, metabolites that significantly change (p-value < 0.05) between at least A-B or B-C comparison are carnitine metabolites, carnitine, tyrosine and tryptophan, as well as creatinine and uric acid. Carnitine metabolites, creatinine and uric acid change in the same direction as testosterone, whereas carnitine, tyrosine and tryptophan change in the opposite direction.

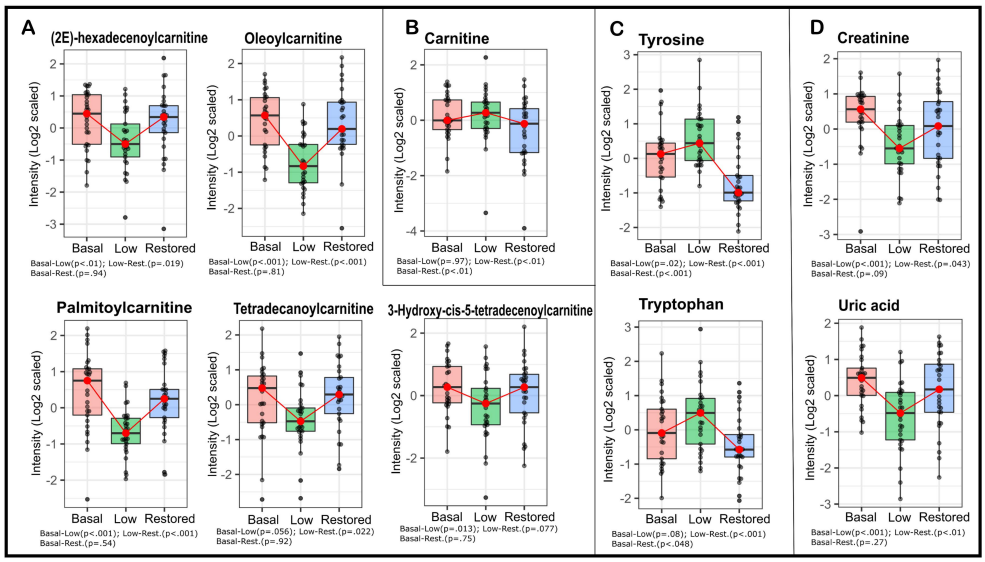

Figure 11. Box plots of metabolites influenced by testosterone in the healthy human model
Metabolites levels across the time points where pink is basal or baseline, green is low testosterone and blue is restored testosterone. Below the boxplots are the *p*-values between time points comparisons, in which not all comparisons are significant (*p*-value < 0.01). A) The metabolites of lipid oxidation follow the same behavior as testosterone between the time points. B) Carnitine shows a tendency to behave in the opposite manner to testosterone. C) the amino acids tyrosine and tryptophan increase significantly with low testosterone, and D) Creatinine and uric acid both decrease (A-B) and increase (B-C) significantly in the human model, following the same behavior as testosterone.

Proteomics and Preliminary Protein Markers

Thirty-one out of 676 proteins were significantly associated with testosterone levels (post hoc paired t-test, adjacent *p*-value < 0.05), see **Figure 12a**. The majority of proteins (28/31) were able to distinguish between low (B) and normal testosterone levels (A and C) using receiving operating characteristic analysis (*p*-value < 0.05). Two proteins were selected as preliminary protein markers based on significance level with the best area under the curve values were 4-hydroxyphenylpyruvate

dioxygenase (4HPPD) (AUC[95% CI]: 87[77-96]) and insulin-like growth factor-binding protein 6 (IGFBP6) (AUC[95% CI]: 84[75-91]). Also, fructose-bisphosphate aldolase (ALDOB) (AUC[95% CI]: 75[63-86]) was selected due it the relatively high score combined with the fact that it is and expressed in liver. Boxplots of the normalized expression of the candidate protein markers across the time points can be viewed in **Figure 12b**. The proteins that best explain the odds of having low testosterone in the human model is the combination of 4HPPD and IGFPB6. Therefore, these proteins were combined to create new variable called multi marker algorithm (MMA). The three proteins and MMA resulted as possible candidate markers to diagnose impaired biological androgen activity (**Figure 12c**, p-value < 0.05).

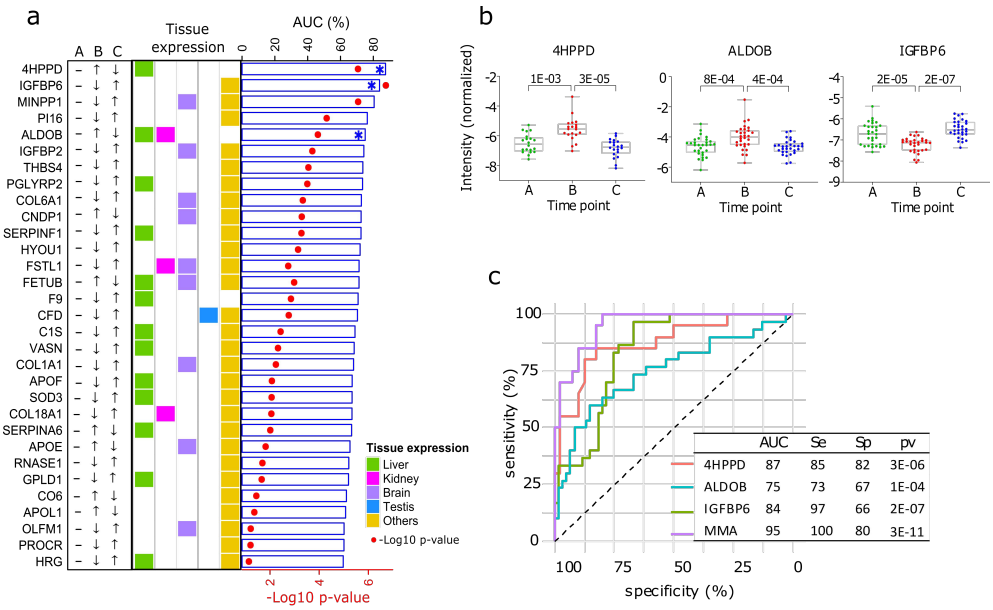

Figure 12. Proteins influenced by testosterone in the human model by recieveing operating characteristic

a) Top 31 significant proteins selected in the healthy human model (p-value < 0.01). The arrows indicate the direction of change in protein expression in the different conditions. The tissue with highest expression of each protein is indicated in colors. The area under the curve from the receiving operating characteristic analysis is displayed in the bar chart. b) Boxplots of the top three significant proteins proposed as candidate biomarkers of the different time points: 4-hydroxyphenylpyruvate dioxygenase (4HPPD), insulin-like growth factor-binding protein 6 (IGFBP6) and fructose-bisphosphate aldolase (ALDOB). The adjusted p-values are specified on top of the comparative horizontal lines. c) receiving operating characteristic of the analytes proposed as biomarker candidates, including the combination of 4HPPD and IGFBP6 called multi marker algorithm (MMA).

The Infertile Cohort

Candidate Protein Markers and Testosterone

As seen in **Figure 13**, the potential candidate protein markers yield significant findings in the patient cohort as well, where the cohort was divided into three groups based on testosterone levels: low testosterone, borderline testosterone, and normal testosterone. The candidate protein markers behave as expected across the groups based on the previous observation in the healthy human model. IGFBP6 was the only marker with a positive association to testosterone levels.

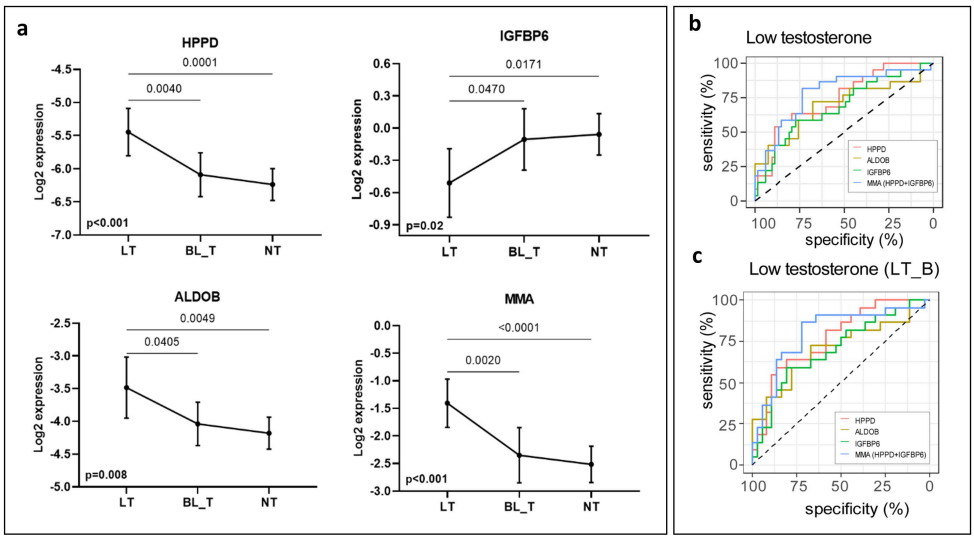

Figure 13. Novel protein markers distinguish between different testosterone levels in infertile patients (n = 75). a) Patients divided in three groups: low testosterone (LT) ≤ 8 nmol/L (n=22), border line testosterone (BL_T) between 8 and 12 nmol/L (n=17) and normal testosterone (NT) > 12 nmol/L (n=36). Each group is represented by the mean and its 95% CI. Adjusted p-values are specified and horizontal lines indicate significant differences between groups. b) the markers ability to distinguish the LT patients from the rest of the patitnets by recieveing operating characteristic analysis and c) the same analysis except for the exclusion of LT_B patients from the cohort. MMA is the combination of the proteins 4HPPD and IGFBP6.

Candidate Protein Markers and Comorbidities

In **Figure 14**, 4HPPD and ALDOB as well as MMA significantly distinguished DM2 and MetS, which was significantly improved compared to the ability of testosterone to distinguish the diseases (p-value < 0.01 in DM2 comparing individual markers v testosterone and p-value < 0.05 for MetS). For CVRLP and IR

there were no significant findings. In LBD, 4HPPD and MMA were significant, but were not significantly better compared to testosterone.

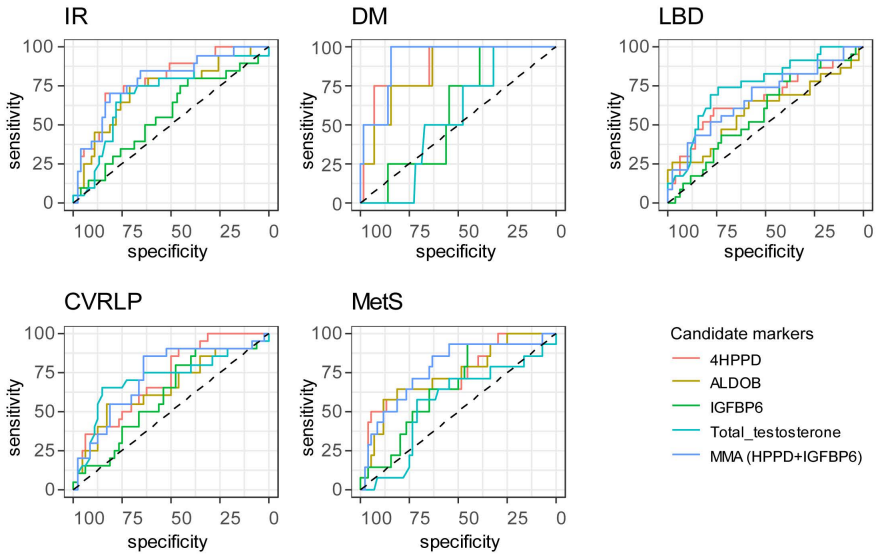

Figure 14. Novel markers ability to distinguish the presence of comorbiditiest. Five comorbidities were included in the receiving operating characteristic analysis: insulin resistance (IR), diabetes mellitus type 2 (DM2), low bone density (LBD), cardiovascular risk lipid profile (CVRLP), and metabolic syndrome (MetS).

Candidate Protein Markers and Androgen Receptor CAG-repeat

In **Figure 15**, 4HPPD and ALDOB had significant overall change between the groups based on CAG-length ($p < 0.05$ and $p < 0.01$ respectively). The most significant difference was between the reference and long CAG repeat ($p < 0.01$). Significant changes were not observed for the marker IGFBP6.

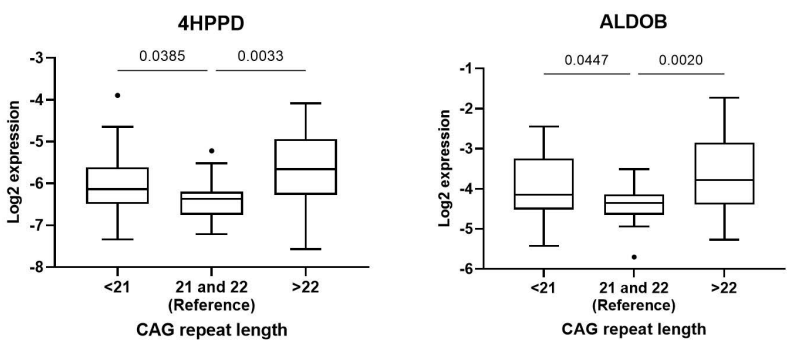

Figure 15. 4HPPD and ALDOB and association with androgen receptor CAG <21 (n=26), and CAG<22 (n=30). Reference is defined as CAG = 21 and 22 (n=18).

Discussion

Collectively, the results indicate for the first time in healthy men that early effects of testosterone deficiency provokes metabolic changes involved in protein catabolism that are detectable in blood. Novel potential biomarkers of testosterone deficiency are also presented in the studies, including tyrosine, phenylalanine, 4HPPD, ALDOB, and IGBP6, as well as MMA. The proteins 4HPPD and ALDOB also indicate to be testosterone dependent in the cohort of infertile males. Additionally, these proteins change with testosterone related comorbidities as the proteins achieved high scores in the receiving operating analysis for MetS and DM2, compared to no comorbidity and achieved higher scores than testosterone. 4HPPD and ALDOB also correlated significantly with men with either a short and a long CAG repeat length compared to men with reference length. The risk of male infertility significantly increases when the CAG repeat length is either short or long [62]. This is in agreement with our results, as 4HPPD and ALDOB were elevated in patients presenting with a long CAG repeat, indicating a decreased androgen activity.

4HPPD is an enzyme involved in tyrosine catabolism in the liver, regulating along with hormones the rate of protein turnover [63,64]. Phenylalanine is an essential amino acid that can only be broken down in the liver and tyrosine is sometimes considered a semi-essential amino acid as it needs phenylalanine for endogenous production in an irreversible reaction. This makes them both good candidates to study the net protein degradation [65,66]. They are precursors to catecholamines and monoamines. Catecholamines are involved in processes related to nerve conduction, blood circulation and hormone regulation, whereas monoamines regulate psychomotor function, cardiovascular, hormone secretion, and body temperature among others [67,68]. Tyrosine was significantly increased in the metabolomic study as well, strengthening the findings. In a study on hypogonadal men, tyrosine was upregulated [13]. Both tyrosine and phenylalanine were good predictors of future diabetes prior to disease onset in males in a longitudinal study [69], as well as elevated levels of isoleucine, tyrosine and phenylalanine in combination showed over a fivefold higher risk in the subjects in the top quartile of future DM2 [70]. Other studies have also found a link between branched chain amino acids and DM2 [71–73]. Additionally, hypogonadal patients with DM2 receiving testosterone replacement therapy became less insulin resistant [74].

IGFBP6 is secreted to the blood stream and functions to bind and inhibit insulin-like growth factors, in which cell proliferation and vitality is reduced. The protein is expressed in most tissues and the serum levels increase with age [64,75]. A study with a similar setup to our human model, found that IGFBP6 was significantly increased with synthetic testosterone in a healthy cohort of males who were chemically castrated at baseline [76]. Another study found that androgens significantly upregulated IGFBP6 in boys [77]. Serum levels of IGFBP6 increase during a maximal exercise test in elite rowers preceding the significant increase in growth hormone, which has a suggested synergistic effect to testosterone. IGFBP6 was the only IGFBP to significantly change in the study [10,78].

ALDOB is a glycolytic enzyme under dietary control and it is predominantly expressed in the liver and kidney. ALDOB is low in resting and fasting states, and increases upon fructose ingestion [79]. In the development of hyperglycemia, ALDOB is upregulated in β-cells in the pancreas, suggesting that the protein may play a role in the impairment of insulin secretion [80]. In the present thesis, ALDOB was found to be increased with low testosterone levels, suggesting that there is a higher metabolic activity to produce glucose. This could be due to reduced insulin sensitivity.

Increased catabolism or gluconeogenesis with testosterone deficiency is also supported by the increase of ALAT, ASAT and urea in the low testosterone state, in which ALAT has been found to be upregulated in males with insulin resistance [15,81]. Increased gluconeogenesis was in line with the findings from the proteomic pool study and the metabolic study. The metabolomic analysis suggests that low testosterone reduces lipid oxidation, as seen in the decrease of the carnitine metabolites involved in mitochondiral β-oxidation of fatty acids. Carnitine is a key protein involved in lipid oxidation, in which it transports the fatty acids into the mitochondria for β–oxidation. Carnitine is mainly stored in muscle cells, which rely on supply via diet or form the liver and kidney as muscle cells are unable to synthesize carnitine [82]. Thus, an elevation of carnitine could be associated with an increased protein breakdown of muscle tissue.

Creatinine was found to be significantly decreased with low testosterone in both the metabolomic study and the clinical chemistry study, which suggests that an early effect of low testosterone increases protein breakdown. The decrease in creatinine is associated with an increased renal clearance; however, in healthy men with normal renal function serum creatinine is directly and positively related to muscle mass [83,84]. The metabolomic results also suggest that uric acid is significantly affected by testosterone with a positive association. Although there is contradictory evidence, a study on patients with kidney transplants found a positive association between muscle mass and uric acid levels [85]. In general, an increased level of uric acid seems to contribute to the development of metabolic disease [86]. More research is needed in order to elucidate the connection between muscle mass and uric acid.

The causality in the literature between testosterone and metabolic syndrome is unclear and may be bidirectional [87,88]. However, the evidence in the present thesis supports that low testosterone contributes to the pathological development of metabolic disease. It would be of high interest to study how the novel markers from the present thesis may be influenced by exercise with or without the combination of testosterone replacement therapy. Additionally, how these markers may change in men with metabolic disease undergoing an exercise program, as the effects of exercise reverses the symptoms that contributes to the development of MetS [29].

Some evidence suggests a positive effect of exercise on testosterone levels; however, data are inconsistent [89]. Furthermore, a randomized controlled trial on hypogonadal men receiving testosterone replacement therapy found that the group that was submitted to a 12-week exercise program significantly increased testosterone levels and improved in their erectile dysfunction compared to the control group both during and after treatment cessation [90]. A clinical issue is that once testosterone supplementation has been initiated, it can be difficult to stop the treatment due to issues with withdrawal [91]. Thus, further understanding on the exercise benefits to boost testosterone levels could aid in the treatment and cessation of testosterone supplementation.

Additionally, high intensity exercise has a negative effect on testosterone levels [89] and exercise hypogonadism, or functional hypothalamic hypogonadism, can occur in highly trained or over-trained athletes [92]. The male athlete triad is a condition characterized by energy shortage due to high energy demands that are not sufficiently met through diet, functional hypothalamic hypogonadism and low bone mineral density. It is more common in certain types of sports, such as long-distance running or sports with a focus on weight class [93]. A study investigating the prevalence of male athlete triad in runners found that roughly 30% were at moderate risk and 5% at high risk by the triad cumulative risk assessment. The runners also had a higher prevalence of low bone mineral density compared to the normal population [94]. A possible explanation for the detrimental effects on both testosterone levels and fertility caused by high intensity exercise is by exercise-induced oxidative stress, which causes increased cellular damage [89]. It is, therefore, important to get the right exercise dosage for optimal promotion of male health and fertility.

Findings from the present thesis could provide new insights to improve doping screening, which is the most widely used performance appearance enhancing drug in sports, both at elite and recreational levels [95,96]. An epidemiological review found that the lifetime prevalence of steroid abuse in males is 6.4% [97]. Hence, steroids have been abused by millions of men, and the main proportion was suggested to be recreational athletes [98].

Steroid abuse carries serious health risks including hematological, metabolic and cardiovascular risks. Other side effects include liver and kidney damage, as well as

negative psychological effects, such as aggressive behavior and psychotic symptoms. The doses often significantly exceed doses that are clinically prescribed by 5-20 times. This, combined with the fact that abusers seldom come forward, hinders research advancements regarding the negative impacts of steroid abuse on health [99,100]. The high doses of steroids prevent the endogenous production of testosterone by inhibiting GnRH and LH in the HPG-axis, which may result in temporary or even chronic hypogonadism upon the termination of abuse. The withdrawal symptoms are, thus, similar to testosterone deficiency such as decreased libido, sexual dysfunction and depression [101]. These symptoms often cause relapse among abusers [91]. Thus, steroid abuse is relatively common in the male population and of clinical value to study more closely the detrimental effects of doping.

The strengths and limitations in the present thesis relate to the subjects, sample storage, sample preparation, and analysis. The main strengths in the present thesis is the depth of study in the healthy human model by use of different methods. The changes detected are induced by testosterone activity and different methods were applied increasing the reliability of the results obtained. Additionally, some of the results found in the human model were confirmed in a patient population of infertile men.

A strength of the designed human model is that the reproductive hormones are controlled in healthy volunteers to induce low testosterone. This enables the study of causality with testosterone deficiency. However, a limitation of the model is that in order to pharmacologically reduce testosterone, the reduction of LH and FSH levels are also induced. In order to control for the influence of the gonadotropin in the human model emphasis was placed on markers that followed the behavior of testosterone in both A-B and B-C, because there was no change in LH and FSH in B-C.

The time frame of the human model is short, possibly limiting the detection of the full scale of physiological effects of testosterone deficiency. The fact that the volunteers exercise habits were not registered or accounted for is another limitation as effects have previously been reported [65,102–107]. However, one of these longitudinal studies found that amino acids, such as tyrosine and phenylalanine, were not affected by the training period studied [105]. As in most cases, the power of study could have been improved with a larger number of volunteers, although, because the model is paired, the sample size was large enough to produce significant changes. Comparatively the sample size is large for the type of study conducted [13,108,109].

The current method to diagnose hypogonadism is in need of improvement as the symptoms are unspecific and testosterone activity are affected by different causes, such as BMI and AR sensitivity [39,40]. In Paper V, the receiving operating analysis yielded significant results for the candidate protein markers and suggested to be

more accurate in determining testosterone related activity in the patients compared to testosterone level itself.

By using MS, the search for molecules of testosterone response and candidate markers was performed with an explorative or open approach, meaning that the samples are probed for the existing analytes without being limited to previous findings. For the proteomic experiments, a strength is the depth of analysis achieved related to protein abundance in plasma, in which 450 proteins were identified in the same concentration range as 87% of biomarkers approved by the Food and Drug Administration [50]. This was possible due to sample depletion of the most abundant proteins, which otherwise would suppress the detection of proteins of interest due to the presence of high abundant proteins. This could also affect the metabolite identification in the metabolomic study, in which the sample was not depleted but the proteins were removed from the sample prior to detection by LC-MS.

Conclusions

- Short-term testosterone suppression in healthy young males provokes small, yet significant changes in biomarkers used clinically to monitor disease. Because the volunteers are without no other metabolic disturbance, the findings seem to be driven by testosterone.
- The pooled proteomic experiment yielded changes in protein content between time points that suggests that short-term testosterone deficiency provokes metabolic change.
- Several amino acid levels increase significantly in the healthy human model, which indicates that low testosterone increases protein breakdown.
- The metabolomic experiment suggests that early effects of testosterone deficiency include decreased lipid metabolism and increased protein breakdown in the human model.
- The proteins identified as novel markers are able to discern between low and normal testosterone in both the human model and in infertile patients.
- The novel protein markers also shift in relation to comorbidities associated with testosterone deficiency suggesting that these markers more accurately reflect the androgen activity.
- The protein markers changed according to CAG repeat length on the AR gene, which supports that the markers are related to androgen response.
- The novel markers identified in this thesis may serve as a tool to better precise testosterone deficiency, as well as to detect patients at risk for metabolic pathology associated with testosterone. The markers may also provide insight in which patients are in need of treatment and a tool to evaluate treatment outcome and monitoring.
- Collectively, this thesis has provided further understanding of testosterone activity in males in both health and disease. Further studies are needed in order to evaluate the clinical value of the novel findings.

Future perspectives

- Metabolomic analysis of the infertile cohort, similar to the one performed on the healthy human model is both a viable and interesting option.
- Test the markers identified in the present thesis in patients with DM2, preferably in a longitudinal setting to get paired data on before and after disease onset. This would also be valuable for cardiac disease and MetS.
- Investigate how the proposed markers in the present thesis behave in women, particularly in women with polycystic ovarian syndrome, as it is a condition characterized by high testosterone with a high comorbidity with DM2.
- Investigate in a paired model if the markers change after gastric bypass surgery as this procedure facilitates weight loss in obese patients.
- Investigate if any of the markers proposed could aid in earlier identification of delayed puberty to enable earlier initiation of treatment.
- Study long-term effects of testosterone deficiency in prostate cancer patients in order to improve care and the results may also provide new insights for long-term effects of testosterone deficient males.
- Investigate what the exercise induced molecular effects and how they relate to testosterone, both in healthy people, cardiac patients, DM2, and MetS. Additionally, identify the optimal dosage of exercise in order to normalize testosterone levels or facilitate the effects of testosterone replacement.
- Study the harmful effects of steroid abuse to improve identification and care.
- Learn more about the risks and debut of both female and male athlete triad in both adolescence and adult, and develop screening methods.
- Study the effects of testosterone supplementation or depletion in hormonal therapy in transsexual men or transsexual women, respectively.

Populärvetenskaplig sammanfattning (Popularized Summary in Swedish)

Testosteron är ett viktigt hormon hos män där det exempelvis upprätthåller muskelmassa och minskar risken för övervikt. Produktionen av testosteron minskar med åldern, vilket bidrar till muskelförsvagning. Åldrarnde är också kopplat till ökad risk för övervikt och inaktivitet. Muskelnedbrytning sker även vid metabola sjukdomar så som metabolt syndrom, ett tillstånd förknippat med ökad insulinresistens. Bevisligen är testosteronbrist kopplat till för tidig död bland annat på grund av en ökad risk att drabbas av vanliga livstidssjukdomar.

Testosteronmätning avspeglar inte fullt ut hormonets effekt i kroppen, vilket gör att en man med ett normalt testosteronvärde kan ha flertal symtom av testosteronbrist, medan en man med lågt testosteron kanske inte känner av detta alls. För att förbättra mätningen av testosteronets verkan i kroppen fokuserar avhandlingsarbetet istället på vad som sker i kroppen genom att mäta hur olika markörer i blodet påverkas av testosteron. Alla molekyler i kroppen läcker i viss mån ut till blodomloppet, varför detta används som material för att hitta markörer för testosteroneffekt.

Målet med arbetet är att kartlägga hur testosteronbrist påverkar kroppens ämnesomsättningen och medverkar till uppkomst av diverse sjukdomar. Målet är även att undersöka ifall några blodmarkörer bättre kan avspegla testosteronets effekt i kroppen. Detta genomförs genom att unga friska deltagare donerar blod under tre olika tillstånd: normal testosteronnivå, testosteronbrist och normaliserad testosteron. Blodet analyseras med hjälp av högteknologiska instrument för att mäta olika markörer i blodet vid de olika tillstånden för att kunna hitta tesosteronberoende markörer. Vissa potentiella blodmarkörer testas därefter i en patientgrupp som består av infertila män varav vissa också har metabola sjukdomar. Resultaten visar att markörerna som hittats hos de friska unga männen ger liknande resultat i patientgruppen, och ökar kunskapen om hur testosteron påverkar ämnesomsättningen och muskelmassan. Fler experiment behöver genomföras med ett stort antal försökspersoner för att säkerställa resultaten innan markörerna eventuellt kan börja användas på klinik för att underlätta diagnosticering och/eller bedöma risken för patienter att drabbas av följdsjukdomar.

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