SEX STEROID METABOLISM AND BODY COMPOSITION

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ABSTRACT

Background: The bioactive androgens testosterone (T) and dihydrotestosterone (DHT) regulate bone and fat mass in men. The effects of androgens are largely determined by the rate of their synthesis and inactivation. Irreversible conjugation of androgens or androgen metabolites by UDP glucuronosyltransferases (UGTs) into water-soluble glucuronidated androgen metabolites plays an important role in the inactivation of androgens and thereby in the regulation of local intracellular androgen levels.

Aims: To *in vivo* characterize genetic variations associated with substrate-specific glucuronidation of androgens/androgen metabolites and to explore the impact of androgen metabolites and polymorphisms associated with glucuronidation of androgens/androgen metabolites as predictors of bone and fat mass.

Methods: Three candidate polymorphisms in enzymes, proposed from *in vitro* studies to be involved in glucuronidation of androgens (UGT2B7, UGT2B15 and UGT2B17), and androgens/glucuronidated androgen metabolites, measured by mass spectrometry, were analyzed in two well-characterized population-based cohorts of young adult and elderly Swedish subjects.

Results: We demonstrated *in vivo* that the UGT2B7 H^{268}Y, UGT2B15 D^{85}Y and UGT2B17 deletion polymorphisms are functional or in linkage with functional polymorphisms. We provided *in vivo* evidence for substrate-specific glucuronidation of androgens by the three UGT2B enzymes. Both UGT2B15 and UGT2B17 were involved in the glucuronidation of the androgen metabolite 5α-androstane-3α,17β-diol (3α-diol) into 3α-diol-17glucuronide (17G), while only UGT2B17 had the capacity to directly glucuronidate T. The urinary T to epiT ratio, commonly used in antidoping test programs, was strongly associated with the UGT2B17 deletion polymorphism. The glucuronidation of DHT was partly dependent on UGT2B17. UGT2B7 was involved in the glucuronidation of 3α-diol into 3α-diol-3glucuronide (3G).

Importantly, the glucuronidated androgen metabolites 3G and 17G associated more strongly with bone mineral density (BMD) than the bioactive androgens. The UGT2B7 H^{268}Y polymorphism associated with cortical bone size. Young adult men homozygous for the UGT2B7 Y-allele had larger cortical bone size than individuals homozygous for the H-allele.

The glucuronidated androgen metabolite 17G, and especially the 17G/DHT ratio, were directly related to fat mass and metabolic risk factors. The 17G/DHT ratio explained a substantial part of the variance of total body fat mass in young adult and elderly men (12% and 15%, respectively). The UGT2B15 D^{85}Y and UGT2B17 deletion polymorphisms associated with fat mass and metabolic risk factors. Subjects homozygous for the UGT2B17 deletion or the UGT2B15 Y-allele had increased amount of fat.

Conclusions: The present findings indicate that analyses of specific glucuronidated androgen metabolites might provide additional information for prediction of the risk of osteoporosis and metabolic diseases. Genetic variations in enzymes responsible for the glucuronidation of androgens result in altered levels of glucuronidated androgen metabolites in serum and probably also of androgen levels in androgen-dependent tissues. Some of these genetic variations associate with bone and/or fat mass.

Keywords: UDP glucuronosyltransferases, polymorphisms, androgens, glucuronidated androgen metabolites, fat mass, bone, metabolic risk factors, population study

KÖNSHORMONSMETABOLISM OCH KROPPSSAMMANSÄTTNING

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SVENSK SAMMANFATTNING

Bakgrund: De aktiva androgenerna testosteron (T) och dihydrotestosteron (DHT) reglerar ben och fettmassa hos män. Androgeners effekter bestäms i hög grad av deras syntes och inaktivering hastighet. Irreversibel konjugering av androgener eller androgenmetaboliter av UDP glukuronosyltranferaser (UGTs) till mer vattenlösliga androgenmetaboliter spelar en viktig roll i inaktiveringen av androgener och därmed också i regleringen av androgennivåer lokalt.

Syfte: Att *in vivo* karaktärisera genetiska variationer associerade med substratspecifik glukuronidering av androgener/androgenmetaboliter och att utforska androgenmetaboliters roll samt polymorfier, associerade med glukuronidering av androgener/androgenmetaboliter, som prediktorer för ben och fettmassa.

Metoder: Tre kandidatpolymorfier i enzymer föreslagna från studier *in vitro* att vara involverade i glukuronideringen av androgener (UGT2B7, UGT2B15 och UGT2B17), och androgener/glukuroniderade androgenmetaboliter, mätta med masspektrometri, analyserades i två välkaraktäriserade populationsbaserade kohorter bestående av unga och äldre svenska individer.

Resultat: Vi visar *in vivo* att UGT2B7 $H^{268}Y$ polymorfin, UGT2B15 $D^{85}Y$ polymorfin och UGT2B17 deletionspolymorfin är funktionella eller kopplade till funktionella polymorfier. Vi ger bevis *in vivo* för att dessa tre UGT2B enzymer utför substratspecifik glukuronidering av androgener. Både UGT2B15 och UGT2B17 var involverade i glukuronideringen av androgenmetaboliten 5α-androstane-3α,17β-diol (3α-diol) till 3α-diol-17glukuroniden (17G), medan endast UGT2B17 hade kapaciteten att direkt glukuronidera T. Ratiot mellan T och epiT i urin, vanligen använt i antidoping tester, var starkt associerat med UGT2B17. UGT2B7 var involverat i glukuronideringen av 3α-diol till 3α-diol-3glukuroniden (3G).

Viktigt nog visade sig de glukuroniderade androgenmetaboliterna 17G och 3G vara starkare associerade till mineralinnehållet i ben (BMD) än de aktiva androgenerna. UGT2B7 $H^{268}Y$ polymorfin var associerad till kortikal benstorlek. Unga individer homozygota för Y-allelen av UGT2B7 hade större kortikal benstorlek än individer homozygota för H-allelen.

Den glukuroniderade androgenmetaboliten 17G, och speciellt 17G/DHT ratiot, var direkt relaterade till fettmassa och metabola riskfaktorer. 17G/DHT ratiot förklarade en väsentlig del av variationen i mängden totalt kroppsfett hos unga och äldre män (12%, respektive 15%). UGT2B15 $D^{85}Y$ polymorfin och UGT2B17 deletionspolymorfin var associerade med fettmassa och metabola riskfaktorer. Individer homozygota för Y-allelen av UGT2B15 eller deletionen av UGT2B17 hade ökad fettmassa.

Slutsatser: Dessa fynd indikerar att analyser av specifika glukuroniderade androgenmetaboliter kan ge ytterligare information för att prediktera risken för osteoporos och metabola sjukdomar. Genetiska variationer i enzymer ansvariga för glukuronideringen av androgener resulterar i förändrade nivåer av glukuroniderade androgenmetaboliter i serum och troligtvis också av androgennivåer i androgenkänsliga vävnader. Några av dessa genetiska variationer var associerade med ben och fettmassa.

Nyckelord: UDP glukuronosyltranferaser, polymorfier, androgener, glukuroniderade androgenmetaboliter, fettmassa, benmassa, metabola riskfaktorer, populationsstudie

LIST OF PUBLICATIONS

This thesis is based on the following articles, which will be referred to by their roman numerals.

I. **The uridine diphosphate glucuronosyltransferase 2B15 D^{85}Y and the 2B17 deletion polymorphisms predict the glucuronidation pattern of androgens and fat mass in men.**
Swanson C, Mellström D, Lorentzon M, Vandenput L, Jakobsson J, Rane A, Karlsson M, Ljunggren Ö, Smith U, Eriksson AL, Bélanger A, Labrie F, Ohlsson C.
J Clin Endocrinol Metab. 2007 Dec;92(12):4878-82

II. **Sex steroid levels and cortical bone size in young men are associated with a uridine diphosphate glucuronosyltransferase 2B7 polymorphism (H^{268}Y).**
Swanson C, Lorentzon M, Vandenput L, Labrie F, Rane A, Jakobsson J, Chouinard S, Bélanger A, Ohlsson C.
J Clin Endocrinol Metab. 2007 Sep;92(9):3697-704

III. **Serum levels of specific glucuronidated androgen metabolites predict BMD and prostate volume in elderly men.**
Vandenput L, Labrie F, Mellström D, Swanson C, Knutsson T, Peeker R, Ljunggren Ö, Orwoll E, Eriksson AL, Damber JE, Ohlsson C.
J Bone Miner Res. 2007 Feb;22(2):220-7

IV. **Androgens and glucuronidated androgen metabolites are associated with metabolic risk factors in men.**
Vandenput L, Mellström D, Lorentzon M, Swanson C, Karlsson MK, Brandberg J, Lönn L, Orwoll E, Smith U, Labrie F, Ljunggren Ö, Tivesten Å, Ohlsson C.
J Clin Endocrinol Metab. 2007 Nov;92(11):4130-7

CONTENTS

LIST OF ABBREVIATIONS

aBMD	areal BMD
A-dione	androstanedione
ADT	androsterone
ADTG	androsterone-glucuronide
ANOVA	analysis of variance
AR	androgen receptor
ARE	androgen responsive element
BMD	bone mineral density
BMI	body mass index
CNV	copy number variation
COMT	catechol-O-methyltransferase
CYP450	cytochrome P450
D	aspartate
DHEA	dehydroepiandrosterone
DHEAS	dehydroepiandrosterone sulfate
DHT	dihydrotestosterone
DHTG	dihydrotestosterone-glucuronide
DNA	deoxribonucleic acid
DXA	dual-energy X-ray absortiometry
E1	estrone
E2	estradiol
epiT	epitestosterone
ER	estrogen receptor
ERE	estrogen responsive element
FSH	follicle stimulating hormone
FT	free testosterone
GC-MS	gas chromatography-mass spectrometry
GH	growth hormone
Gn-RH	gonadotrophin-releasing hormone
GOOD	Gothenburg Osteoporosis and Obesity Determinant
GPR30	G protein-coupled receptor 30
GWA	genome-wide association
H	histidine
HDL	high density lipoprotein
HOMA	homeostasis model assessment
HSD	hydroxysteroid dehydrogenase

IGF-I	insulin-like growth factor-I
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LD	linkage disequilibrium
LH	luteinizing hormone
MrOS	Osteoporotic Fractures in Men
OR	odds ratio
PCR	polymerase chain reaction
pQCT	peripheral quantitative computed tomography
RIA	radioimmunoassay
RNA	ribonucleic acid
SD	standard deviation
SEM	standard error of the mean
SHBG	sex hormone binding globulin
SNP	single nucleotide polymorphism
SULT	sulfotranferase
T	testosterone
TG	testosterone-glucuronide
TRUS	transrectal ultrasound
UDP	uridine diphosphate
UGT	UDP glucuronosyltransferase
vBMD	volumetric BMD
WHO	World Health Organization
Y	tyrosine
17G	5α-androstane-3α,17β-diol-17glucuronide
3G	5α -androstane-3α,17β-diol-3glucuronide
3α-diol	5α-androstane-3α,17β-diol
4-dione	androstenedione
5-diol	5-androstene-3β,17β-diol

INTRODUCTION

GENERAL INTRODUCTION

Sex steroids are known to play an important role in the regulation of bone and fat mass in men. Androgens are secreted primarily from the testes, but are also made locally in peripheral target tissues from adrenal-derived androgen precursors. This local transformation is dependent on the expression of metabolizing enzymes in each tissue. Degradation of androgens into androgen metabolites terminates the androgenic signal. The UDP glucuronosyltransferases (UGTs) are responsible for the conjugation of androgens. These proteins are also present locally within the peripheral tissues. This means that the synthesis and degradation of androgens can take place in the same cell in which they exert their action, without diffusion into the circulation. This limits the interpretation of serum levels of androgens. Instead, measuring androgen metabolites might be a better indication of true intracellular androgen levels. Recently, a mass spectrometry (MS) technique was developed capable of distinguishing between the different glucuronidated androgen metabolites. With the access to two large well characterized cohorts of Swedish men and the MS technique, we have investigated the role of genetic variations in the UGT genes and the role of the androgen metabolites for bone and fat mass, including metabolic risk factors. A better understanding of the metabolism of androgens and the genetic variations involved could result in improved diagnostic markers or treatment strategies for androgen-dependent disorders.

GENETICS

The genetic code – DNA to protein

The essential attributes of the gene were defined by Mendel more than a century ago. Mendel discovered that different characteristics pass unchanged from parent to off-spring in a predictable manner. Later, it was found that the information inherited was located in the deoxribonucleic acid (DNA) molecule, which resides in the nucleus of the cell (1, 2). DNA is like a cellular library that contains all the information required for development, function and phenotype of all animals and plants.

DNA consists of two polynucleotide strands that wound together to form a helix-spiral (1) that contains three structures; a nitrogenous base, a sugar and a phosphate

group. Two sugar-phosphate groups make up the back bone on the outside of the helix, and the bases project toward the inside. Hydrogen bonds and van der Waals interactions between the stacked base pairs contribute to the stability of the DNA structure (1, 2). There are four bases in the DNA; adenine (A), thymine (T), cytosine (C) and guanine (G) (1), where A always pairs with T and C always pairs with G. It is the sequence of the bases that make up the genetic code. DNA is organized in pairs of chromosomes, one which is inherited from the mother and one from the father (2). Humans have 23 pairs, with 22 pairs of autosomes (non-sex chromosome) and one pair of sex chromosomes. Women's sex chromosome pair is XX, while men have XY.

In the process of transcription, information from the DNA is copied into ribonucleic acid (RNA). RNA is very similar to DNA, but instead of the base thymine it has uracil (U) and instead of the sugar deoxyribose it has ribose (1). Transcription starts when a RNA polymerase binds to the start codon of a gene. Transcription factors are recruited to help the process, regulating the amount of RNA synthesized and controlling tissue specific expression. After the mRNA is transcribed, it is spliced. Coding parts (exons) are fused together while non-coding parts (introns) are spliced away. The RNA is then translated to a protein (2).

Genetic variations

Mutations change the sequence of the DNA and can become a permanent part of the genetic information. If the mutation has a minor allele frequency of >1%, it is called a polymorphism. Single nucleotide polymorphisms (SNPs), where one nucleotide/base is changed, is the most common type (2). SNPs appearing in the coding part of the DNA can either change the base without resulting in a change of the amino acid it codes for, or it can affect the amino acid sequence and subsequently the protein. There are also polymorphisms that are caused by insertions, deletions or duplications of stretches of DNA sequences, so called copy number variations (CNVs), which change the copy number of a specific allele (3). Another kind of polymorphism is the tandem repeat segments, which consist of a segment repeated one or more times after each other. It should be noted, that SNPs found in the introns of the DNA still might have an effect for example on mRNA stability and for correct splicing. Also, SNPs found in the exons of the DNA that do not change the amino acid sequence, can have similar effects (4). In the human population, SNPs occur about once every 1000 bases (5, 6). These common polymorphisms constitute 90% of the variation in the population.

Since one allele is inherited from the mother and one from the father (2), we have two alleles of each SNP. For example for the UGT2B7 D^{85}Y polymorphism, where a G is changed to a T, some individuals will inherit G from both the mother and the father or T from both. These individuals are homozygous for this allele (GG or TT, respectively). Some will inherit a G from one parent and a T from the other parent, and are heterozygous (GT) for this allele.

SNPs that lie close to each other and in regions with little recombination are inherited together in block-like structures, so called haplotypes. Although, theoretically, many combinations of the SNPs could occur, only a few haplotypes are found (7). This leads to SNPs that are genetically linked to each other; they are said to be in linkage disequilibrium (LD) (5). When LD is equal to 1, the SNPs are said to be in perfect LD (8). By genotyping only a few carefully chosen tag SNPs in the haplotype, it is possible to predict the information from the remaining SNPs in that haplotype. For example, of the 10 million SNPs in the population, only 500 000 tag SNPs are suggested to be needed to be genotyped to provide information of >90% of common SNP variation (5, 7).

In 2002, a consortium was formed, the so called HapMap Project, with the aim to characterize SNP frequencies and LD patterns across the human genome (5, 7, 9). By developing a haplotype map of the human genome, using samples from 270 individuals from Europe, Asia and West Africa, the common patterns of variations are described and tag SNPs are identified. The project had genotyped 3 million SNPs by 2007 (10). The results from HapMap and other researchers are published in a public database (dbSNP) (11). So far, 14 700 000 SNPs have been entered and of these, 6 600 000 have been validated by at least one second entry. Each SNP is given an identification number (rs-number) (9, 11).

Studying genetic variations

There are different ways to study genetic regulation of a disease. *Association studies*, used for Papers I and II in this thesis, typically involve identifying a polymorphism in a candidate gene and relating the genotype of individuals to e.g. BMD in a population study (12). Alternatively, in case-control studies, a group of cases affected by a disease and a group of controls are genotyped for a polymorphism and the allele frequencies are compared. Association studies of candidate genes/SNPs are hypothesis-driven. They are relatively simple to perform, although a large homogenous sample size (cohort) is usually needed (13).

Association studies work the best when detecting common genetic variants, of modest effect, contributing together to a multi gene disease like osteoporosis (14).

Recently (15, 16), it has become possible to do *genome-wide association (GWA) studies* where closely spaced tag SNPs are analyzed and can give information about most of the genetic variation. The genotyped SNPs must be spaced sufficiently dense to be in LD with most of the variants that are not genotyped. It is estimated that around 500 000 SNPs are needed for GWA studies (15, 17). As for association studies, GWA studies work the best when detecting common genetic variants, of modest effect, contributing together to a multi gene disease like osteoporosis (18) and obesity (19). GWA studies are hypothesis-free. Although GWA studies can be very informative, they do have limitations. For example, the effects of rare variant (20) might be missed (21). In addition, the multiple testing performed in GWA studies requires large materials to reach the genome wide statistical significance level of $p<5*10^{-8}$ (7).

Linkage analysis studies in humans are widely used to identify genes contributing to rare monogenic diseases. Genetic markers (SNPs or microsatellites) evenly spread over the genome are analyzed in sets of families with affected individuals. After identifying regions of interest, a denser mapping is performed of these areas and the specific gene, or mutation related to the disease, can be found. A disadvantage is that it is difficult to identify genes that have modest effect even when the sample size is very large (13, 22).

In recent years, an explosion of association, linkage and GWA studies have been performed to find connections between diseases and genetic markers. For example, for obesity, 28 genetic loci were described in 1997 (12). Eight years later, in 2005, the number of locus suggested to be involved in the genetics behind obesity had increased to more than 600 (23).

SEX STEROIDS

Sex steroids include androgens such as testosterone (T) and dihydrotestosterone (DHT) as well as estrogens such as estradiol (E2) and estrone (E1) (Fig. 1). DHT and E2 possess the highest affinity for the androgen receptor (AR) and estrogen (ER) receptors, respectively (24). Sex steroid precursors include androstenedione, dehydroepiandrosterone (DHEA) and DHEA sulfate (DHEAS) (Fig. 1), which do not substantially bind to the AR (25). Progesterone is sometimes included as a third class of sex steroids. Serum levels of sex steroids are influenced by both genetic and
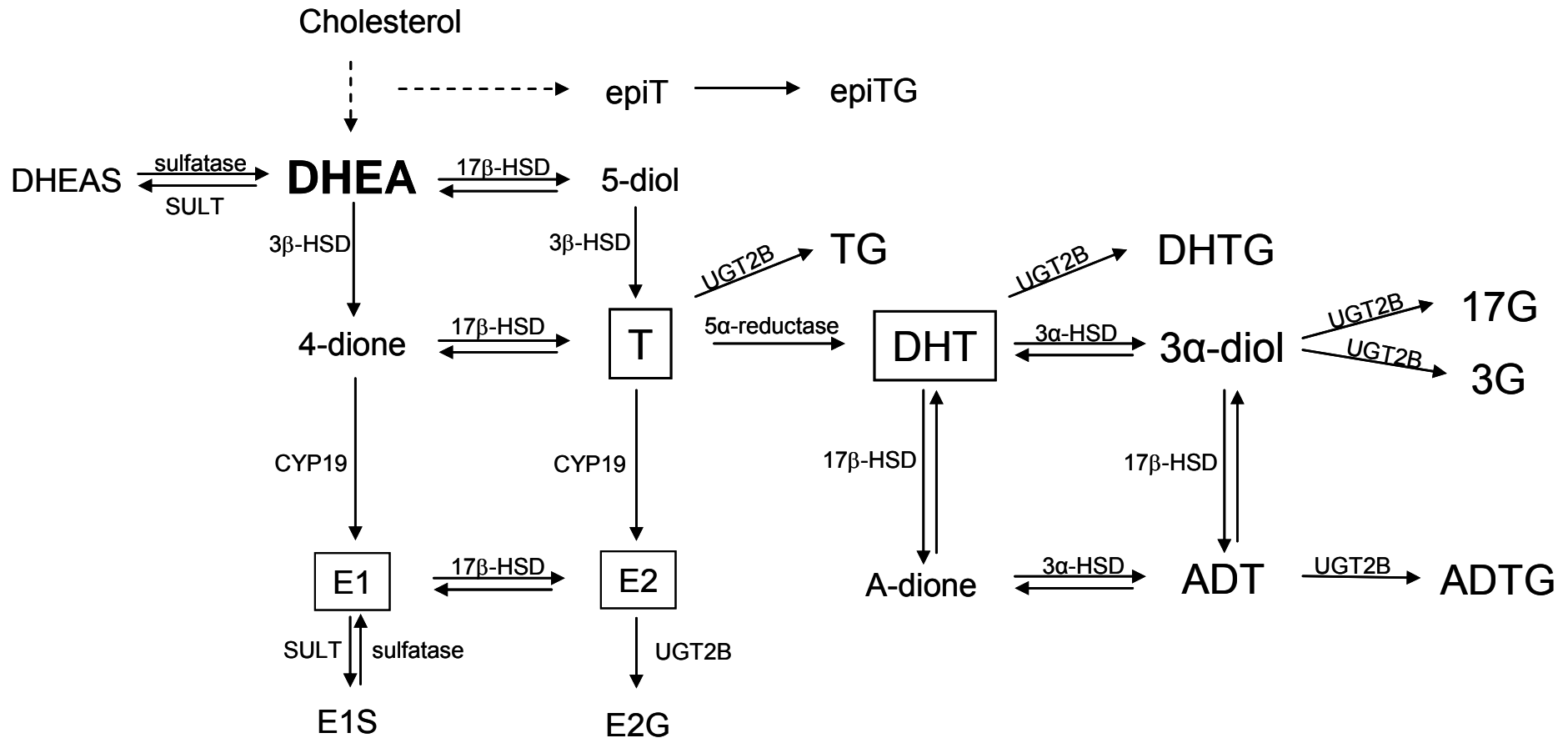
Fig. 1. *Synthesis and metabolism of sex steroids in peripheral intracrine tissues. Enzymes involved are indicated. Boxed sex steroids are active and bind the AR/ER receptors. General scheme based on previous studies.*

Abbreviations: DHEA = dehydroepiandrosterone, DHEAS = dehydroepiandrosterone sulfate, T = testosterone, DHT= dihydrotestosterone, epiT = epitestosterone, 4-dione = androstenedione, A-dione = androstanedione, E2 = estradiol, E1 = estrone, ADT = androsterone, E2G = estradiol glucuronide, E1S = estrone sulfate, TG = T-17glucuronide, DHTG = DHT-17glucuronide, epiTG = epiT-glucuronide, ADTG = androsterone-3glucuronide, 5-diol = 5-androstene-3β,17β-diol, 3α-diol = 5α-androstane-3α,17β-diol, 3G = 3α-diol-3glucuronide, 17G = 3α-diol-17glucuronide, HSD = hydroxysteroid dehydrogenase, CYP19 = aromatase, SULT = sulfotransferase, UGT2B = UDP glucuronosyltranferase family 2B.

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environmental factors (26, 27).

Synthesis of sex steroids

All sex steroids are derived from cholesterol. DHEA is a very important prohormone secreted by the adrenals (28). Members of the cytochrome P450 (CYP450), 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-HSD enzyme families catalyze the various steps of sex steroid formation (28, 29) (Fig. 1). Aromatase (CYP19) catalyzes the aromatization of androgens to estrogens and is the rate-limiting enzyme in the biosynthesis of estrogens (29). All the enzymes required for transforming DHEA into androgens and/or estrogens are expressed in a cell-specific manner in the peripheral tissues (30-32). This means that androgens and estrogens can be produced locally. This new field of endocrinology is called *intracrinology*.

Degradation of androgens

Androgens can be degraded either into phase I metabolites (reversible) or further into phase II metabolites (irreversible). Only T and DHT have the ability to activate the AR (25, 33) and affect gene transcription.

Phase I metabolism

Degradation of sex steroids occurs in the liver and in peripheral tissues. Androgens are mainly metabolized by 3α/β-HSD and 17β-HSD isoforms to metabolites with essentially no androgenic activity (e.g. androsterone (ADT) and 5α-androstane-3α,17β-diol (3α-diol)) (33, 34), (Fig. 1). Most androgen dependent tissues synthesize HSD isoforms capable of back-converting the phase I metabolites into DHT (35), suggesting that this is a mechanism to regulates local androgenic levels. The expression of 17β-HSD type 2 and 3α-HSD type 3 transcripts have been detected in all androgen target tissues studied so far (36-38).

Phase II metabolism

Phase I metabolites can be glucuronidated by uridine-diphosphate (UDP) glucuronosyltransferases (UGTs). This is an irreversible step which leads to a complete inactivation of the androgen, thus regulating intracellular hormone levels. The major phase II metabolites are 3α-diol-17glucuronide (17G), 3α-diol-3glucuronide (3G), and ADT-glucuronide (ADTG) (Fig. 1). Androgens can also be sulfated by sulphotransferases, but in contrast to glucuronidation sulfonation is reversible (39-42).

UGT enzymes

It is well-established that UGTs are responsible for the glucuronidation of androgens and their metabolites in humans (30, 33, 43, 44). The UGT enzymes are detoxification enzymes that transfer a glucuronosyl group from UDP-glucuronic acid to its substrate. This converts the substrate to a polar, water-soluble, less toxic conjugate that can diffuse into the circulation, and be further excreted through the kidneys. When the glucuronidated conjugates are released in the circulation, they can be measured. *In vitro*, it has been seen that the different enzymes have different substrate specificities (33, 45).

Based upon homology of the primary structure, the UGT family is categorized into two subfamilies, UGT1 and UGT2 (46, 47). There are nine functional proteins in the UGT1A1 family. UGT1A1, A3, A4, A5, A6 and A9 are expressed in the liver, while UGT1A7, A8 and A10 are expressed in the gastrointestinal tract (48). The UGT1A family glucuronidates mainly synthetic substances and pollutants and serves as the first line of metabolic defense (49). Some members of the UGT1A family can glucuronidate estrogens (UGT1A1) and catecholestrogens (UGT1A3) (50, 51) but they have no significant activity toward androgens (33, 38, 52).

Each UGT2 enzyme is encoded by a separate gene. Subfamily UGT2 is further divided into family UGT2A and UGT2B. Each subfamily has a high percentage of amino acid sequence homology (33). There are only two members of the UGT2A family, which are found in the olfactory system and conjugate odorant molecules (48). The human *UGT2B* genes are clustered on chromosome 4q13–21.1 (33, 38) and encode seven functional enzymes. Of the functional UGT2B enzymes, there are three which are reported to glucuronidate androgen with high capacity, namely UGT2B7, 2B15 and 2B17 (33, 52). These three UGT enzymes are currently thought to be the major enzymes responsible for conjugating of all androgens in humans.

Expression and specificity of the androgen conjugating UGT2B enzymes
UGT2B7

UGT2B7 has been found in the intestine, liver, kidney, skin, brain, uterus and mammary gland, but not in prostate or adipose tissue (52). UGT2B7 conjugates the 3-position of 3α-diol but not the 17-position. ADT, with a hydroxyl group at the 3-position is also a good substrate for UGT2B7, whereas DHT and T, which only have a hydroxyl group at the 17-position are both poor substrates for UGT2B7 (33).

The efficiency of the UGT2B7 enzyme toward 3α-diol is almost tenfold higher than for ADT (52) (Fig 2). However, UGT2B15 and UGT2B17 also glucuronidate 3α-diol, but at the 17-hydroxy position, and these two enzymes seem to be more efficient than UGT2B7 (53) (Fig. 2). The UGT2B7 enzyme also has the capacity to glucuronidate glucocorticoids and mineralocorticoids (54-56).
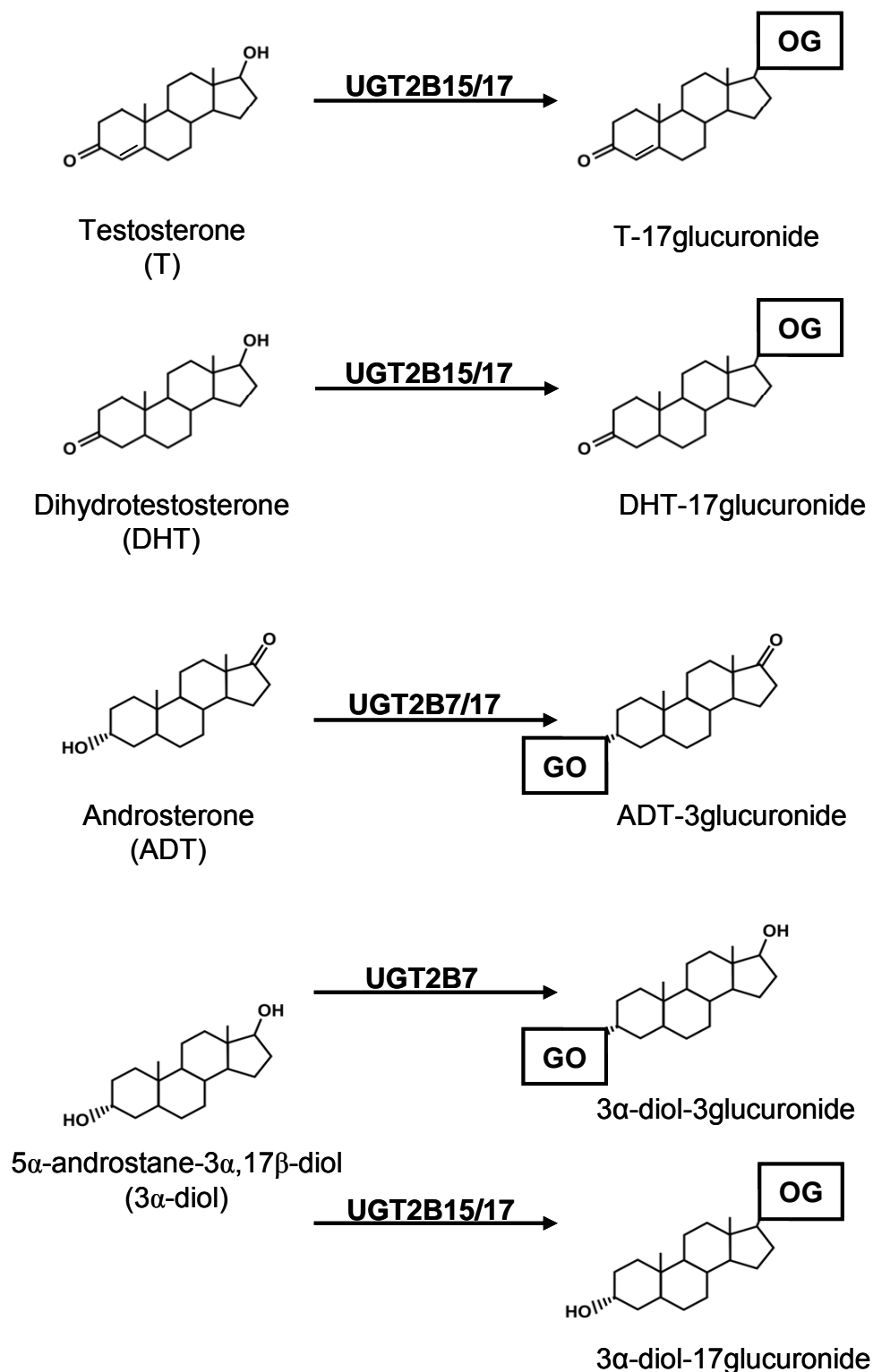

Fig. 2. *Specificity of UGT2B7, UGTB15 and UGTB17 for testosterone, dihydrotestosterone, androsterone and 5α-androstane-3α,17β-diol (based on in vitro data). The G represents glucuronidation.*

There is a C to T polymorphism at nucleotide 802, which changes the amino acid histidine (H268) to a tyrosine (Y268) in the *UGT2B7* gene (57). No difference in activity between the two variants was seen *in vitro* (57). Paper II investigated this polymorphism in relation to the glucuronidation pattern and bone mass in young adult men.

UGT2B15

Expression of UGT2B15 has been found in the liver, kidney, skin, mammary gland, uterus and prostate. As the only androgen conjugating UGT enzyme, UGT2B15 is also expressed in adipose tissue (58). UGT2B15 conjugates at the 17-hydroxy position and therefore has the capacity to glucuronidate 3α-diol and DHT with high and moderate efficiency, respectively (52) (Fig. 2). *In vitro* studies have indicated that T is also glucuronidated by UGT2B15. UGT2B15 can glucuronidate catecholestrogens and hydroxyestrones as well, but to a much lower efficiency than androgens (33).

There is a G to T polymorphism in the *UGT2B15* gene, resulting in an aspartate (D85) to a tyrosine (Y85) amino acid change at position 85 (47). Previous *in vitro* studies have given inconsistent results, describing both increased (59) and decreased (60) glucuronidation activity for the D85 variant compared to the Y85 variant. Several studies investigated the association between the D[85]Y polymorphism and prostate cancer (60-63), but with conflicting results. Paper I investigated this polymorphism in relation to the glucuronidation pattern and fat mass in young adult and elderly men.

UGT2B17

UGT2B17 has been isolated in the liver, kidney, skin, mammary gland, uterus and prostate (44). The sequence homology between UGT2B15 and UGT2B17 is high (96%), but UGT2B17 has broader specificity (44). From *in vitro* studies it was determined that UGT2B17 can glucudonidate both at the 3-hydroxy position and at the 17-hydroxy position, which means that it can glucuronidate 3α-diol and DHT as well as ADT (Fig. 2). UGT2B17 has the highest capacity to conjugate DHT, followed by T. Compared to UGT2B15, UGT2B17 is 6-10-fold more active toward DHT and T (52). UGT2B17 is reported to glucuronidate ADT and is considered the major ADT-conjugating enzyme (33). The efficiency of UGT2B17 to conjugate 3α-diol is in the range as for UGT2B7 and UGT2B15, however, incubation of 3α-diol with cells expressing UGT2B17 only results in the production of 3α-diol-17-glucuronide, indicating a major difference in specificity between the enzymes (33, 44).

A 150kb deletion polymorphism spanning the whole *UGT2B17* gene has been described (64, 65). It was shown that the deletion is strongly associated with urinary T levels and the urinary T to epiT ratio, commonly used in antidoping programs (66). The deletion was also studied in relation to prostate cancer, two confirming an association (60, 67), while two large population-based studies could not see such an association (68, 69). Paper I investigated this polymorphism in relation to the glucuronidation pattern and fat mass in young adult and elderly men.

Degradation of estrogens

Estrogens can be reversibly converted to catecholestrogens by CYP450 enzymes (70). Sulfonation is another way to convert estrogens and these estrogen sulfates are more water soluble (71) and represent a form of storage that acts as precursors of E2 and E1. Glucuronidation of estrogens is irreversible and leads to complete inactivation (40) (Fig. 1).

Mechanisms of action of sex steroids and their receptors

The AR and ERs belong to the nuclear receptor family and are DNA-binding proteins. There are different pathways through which sex steroids can exert their action. Androgens bind to the AR while estrogens bind to the estrogen receptors (ERα and β). Androgens can exert their effects either directly via the AR or by binding the ER after aromatization to E2.

The *classical direct genomic pathway* involves direct binding of the sex steroid to the receptors. After binding of the ligand, the receptor conformation is altered, the receptor dimerizes with another receptor and enters the nucleus of the cell. Following recruitment of co-factors, the receptor complex binds to androgen responsive elements (AREs), or estrogen responsive elements (EREs), present in the promoter of the genes (72, 73).

The *non-classical indirect genomic pathway* includes binding of a steroid to the receptor. However, instead of DNA binding, the receptor interacts directly with transcription factors, which in turn bind to DNA and regulate transcription. Thus, this pathway involves gene regulation by indirect DNA binding (72, 74).

The *non-genomic pathway with rapid effect* involves the activation of a yet not identified receptor, possibly attached to the cell membrane. For estrogens, G protein-coupled receptor 30 (GPR30) as well as ERα/β have been suggested as a mediators

of the rapid effects (72, 75). The signaling cascade involves second messengers and the response occurs within seconds or minutes without involving gene regulation (72).

Finally, the *ligand-independent pathway* involves activation by phosphorylation of the receptors or associated coregulators in the absence of a ligand. This pathway involves gene regulation (72).

Binding of sex steroids to plasma proteins

T and E2 circulate in the plasma in large bound to plasma proteins (76). T and E2 bind albumin in a nonspecific manner and to sex hormone binding globulin (SHBG) in a specific and stronger manner (77). Only a small percentage of the circulationg levels of T and E2 are unbound and this constitutes the free fraction. The fraction bound to albumin, plus the free fraction, is considered the bioavailable fraction (or non-SHBG bound fraction) (78). When bound to SHBG, the sex steroid is prevented from entering the cell since the complex is too large to cross the capillary barrier. In men, but not in women, there is a marked increase in SHBG levels with age (79) which means that the levels of bioactive sex steroids decrease, although the total levels of sex steroids might stay the same (77).

Calculation of serum levels of sex steroid

Free, or bioavailable, sex steroid levels in serum can be measured or calculated theoretically. Using a method described by Vermeulen et al. (78) and van den Beld et al. (77) free T (FT) can be calculated using mass action equations. The method is taking the concentration of total T, total E2, and SHBG into account while assuming a fixed albumin concentration of 43g/liter.

INTRACRINOLOGY

As mentioned in the general introduction, *intracrinology* is a rather new concept in endocrine physiology (30, 80). After several reports of lack of consistency between the serum levels of sex steroids and the incidence of disease like obesity, prostate cancer and breast cancer (81-85), the clinical significance of measurements of total as well as free sex steroids in serum was in doubt. Labrie et al. suggested that the lack of correlation could be related to that the majority of androgens are made locally in the peripheral target tissues from the inactive precursor DHEA of adrenal origin (30, 83, 86). All the enzymes responsible for both the synthesis and the degradation of androgens are present locally within the peripheral cells. This means that the androgens made locally do not only originate from T in the circulation (Fig. 3) and therefore it is reasonable to expect that measurement of the serum levels of T is of questionable significance (87). The androgens made locally exert their action in the same cell in which their synthesis take place, with only minimal release of active androgen into the circulation (30) (Fig. 3).
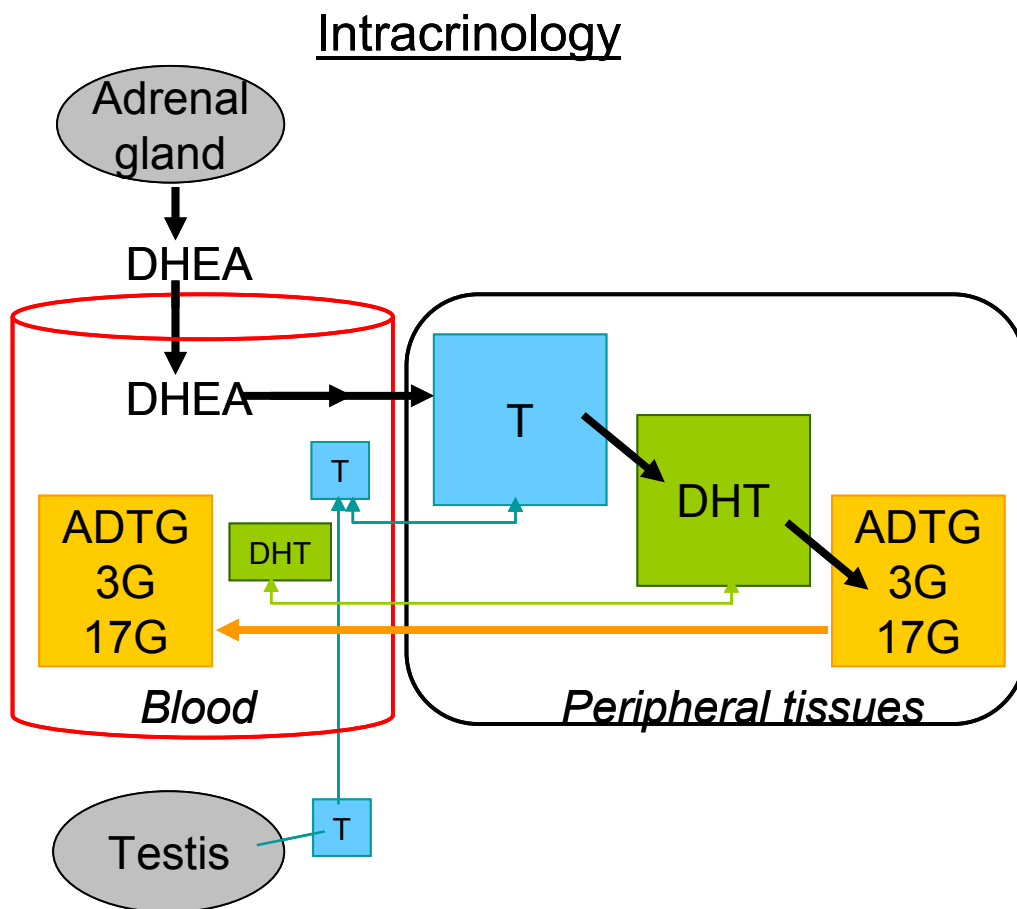

Fig. 3. Schematic figure of the principle of intracrinology and the formation of glucuronidated androgen metabolites in humans. Thickness of the arrows corresponds to the diffusion rate of the prohormone/androgen/glucuronidated androgen metabolite.

Instead, glucuronidated metabolites (3G, 17G and ADTG) in the circulation resulting from the local degradation of androgens, can all be measured before their elimination by the kidneys, and have been proposed to be better indicators of local intratissular androgen activity (Fig. 3) (see Paper III and IV). We can now, for the first time, measure and distinguish these three major glucuronidated metabolites of androgens in serum.

Testosterone/epitestosterone ratio

In addition to being a better indicator of intracellular androgen levels, measurements of serum levels of glucuronidated androgen metabolites, and urine levels of androgens ($>$99% glucuronidated (88)), are also found to be more useful when it comes to other areas than disease. For example, the urine levels of T divided by the urine levels of epitestosterone (epiT) is now a commonly used ratio in international antidoping test programs (66). EpiT is the 17α epimer of T and not a metabolite of T, and has no known physiological function (89). T in urine is mostly found in its glucuronidated form (90). A deletion polymorphism in the *UGT2B17* gene was studied in relation to the T/epiT ratio (66). The study indicated that individuals homozygous for the UGT2B17 deletion had no or negligible amounts of urinary T (66). Jakobsson et al. also found that there were serious interethnic differences in urine T levels between Caucasian and Asian men. This was in accordance with the fact that the UGT2B17 deletion genotype was more common in Asian men than in Caucasians (10% in Caucasians, 65% in Asians) (66). This opens up the possibility for an explanation of the differences in androgen levels previously seen between Asian and Caucasian men (91, 92). One can speculate that changes in the ability to synthesize and degrade androgens may account for other interethnic differences when it comes to association between androgens, androgen metabolites and disease.

THE SKELETON

The human skeleton is comprised of 213 bones and offers support to the body, protects vital organs and serves as the main reservoir of calcium. The skeleton also serves as an attachment for muscles, supporting motion and is a rich source of growth factors and cytokines.

The adult bone consists mainly (~70%) of inorganic material, and 95% of this is made up of hydroxyapatite. About 20% of the skeleton is organic material, and 98% of this is type I collagen and other proteins such as osteocalcin, bone sialoproteins and

osteonectin. The remaining 2% of the organic fraction is made up of bone cells; osteoblasts of mesenchymal origin responsible for bone formation, osteoclasts of hematopoietic origin responsible for bone resorption and osteocytes, which represents the terminal differentiation stage of the osteblasts, involved in the support of the bone structure and metabolic functions. The remaining 5-8% of the bone consists of water and lipids (93).
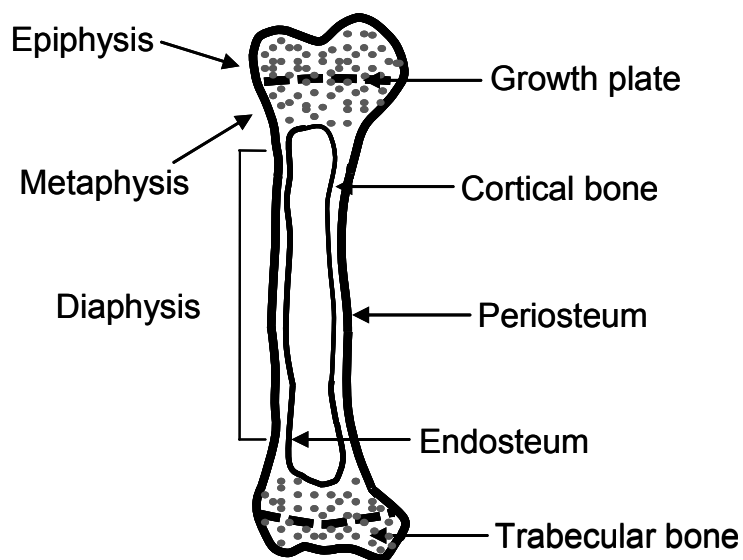

Fig 4. Schematic view of a longitudinal section of a long bone.

There are two main types of bones; flat bones such as the skull and long bones such as the femur and tibia. The long bones are built up like a tube (diaphysis) which flares at the ends. The diaphysis is mainly made up of cortical bone while the ends mainly consist of trabecular bone. The growth plate is found in the middle of the wider ends and the area below the growth plate is called metaphysis, while the area above the growth plate is called the epiphysis (Fig. 4).

The adult skeleton consists of both cortical bone and trabecular bone, but the relative proportions vary between different sites. Cortical bone is mainly found on the outside of the long bones, and serves as a mechanical and protective layer. The vertebrae and the pelvis, as well as the metaphysis of the long bones, are mainly made up by trabecular bone which is generally considered to be more metabolically active and is therefore more sensitive to stimuli such as hormones or drugs. All outer bone surfaces are covered by a fibrous sheath, called the periosteum, containing blood vessels, nerves, osteoblasts and osteoclasts. The inner surfaces of the long bones are covered by a membranous sheath, the endosteum, containing blood vessels, osteoblasts and osteoclasts. The endosteum covers the surface of the trabecular bone as well (93) (Fig. 4).

Bone growth

Adult stature and skeletal maturation are significantly influenced by genetic factors, up to 70% has been suggested to be under genetic control, but nutritional and hormonal factors are also important. Prepubertal growth is a relatively stable process mainly governed by the thyroid hormones and the growth hormone (GH)/insulin-like growth factor (IGF)-I axis (94, 95). As puberty approaches and continues, there is a sudden acceleration in growth velocity; the pubertal growth spurt. On average, girls enter and complete each stage of puberty earlier than boys. The shorter duration of prepubertal growth contributes to that girls, in general, are shorter than boys (94). Also, men have greater peak bone mass than women mainly due to increased periosteal expansion during sexual maturation, which give men greater bone size (longer and wider bones) (96). The timing of puberty has been reported to predict both cortical and trabecular volumetric BMD (vBMD) in young adult men (97).

Puberty is triggered by an increased pulsatile secretion of gonadotrophin-releasing hormone (Gn-RH) by the hypothalamus, leading to increased secretion of gonadotropins (luteinizing hormone (LH) and follicle stimulating hormone (FSH)) from the pituitary, which in turn leads to an increased production of sex steroids by the gonads (98). The increase in serum E2 leads to further increased IGF-I levels and together, the changed levels of GH, IGF-I and E2 support the pubertal growth spurt (99). In both sexes, closure of the epiphyseal growth plate is induced by E2 (96), thus E2 both initiates and ends the pubertal growth spurt (99). For about four years after puberty, the high levels of GH and IGF-I are maintained, but then decreases gradually, although the sex steroids remain at adult levels (99).

Previously it was believed that androgens were responsible for bone growth in males, while estrogens were responsible for bone growth in females. However, the importance of E2 for growth also in males was understood after case reports of a man homozygous for a lack-of-function deletion in the ERα gene (100) and of men with complete aromatase deficiency (101, 102). These men were tall and had unfused epiphyses and marked osteopenia. The phenotype of the aromatase-deficient men was reversed when treated with E2 (101). On the other hand, males with androgen insensitivity had reduced BMD, which leads to the conclusion that both estrogens and androgens are important for optimal bone growth and mineral accrual (103).

Age-related bone loss

Age-related changes of the skeleton include a large decrease in BMD at the spine, femur neck, distal radius and tibia. The decrease is smaller in men than in women. Aging is associated with an increased periosteal circumference of the bone. Although periosteal apposition continues through life, the endosteal resorption increases with age, leading to a net decrease in cortical area and thickness for both men and women (104). However, men add about 3-fold more bone in the periosteal apposition process during life than do women, which leads to that women have less strong bones (99).

Both observational and prospective studies in men have, in general, shown that serum E2 is a stronger predictor of BMD than serum T (77, 79, 105-113). Furthermore, in an interventional study by Falahati-Nini et al. using E2 or T treatment in aging men with eliminated endogenous E2 and T, it was shown that E2 inhibits bone resorption, whereas both E2 and T are important for maintaining bone formation (114). This finding might explain why BMD is more strongly associated with serum E2 than with serum T in men. Recently, in a study by Mellström et al. (115), using mass spectrometry (MS) to measure sex steroids, it was shown that low serum E2, low serum T, and high serum SHBG levels associated with increased risk of fractures.

OSTEOPOROSIS IN MEN

Osteoporosis is a skeletal disorder, characterized by low bone mass and disturbed microarchitecture of the bone. The disease has traditionally been considered a problem for postmenopausal women. However, osteoporosis is an important health problem for both genders. Fractures represent the primary clinical consequence of osteoporosis, and the risk of an osteoporosis related fracture in Sweden, at the age of 50, is around 40% for women, and 20% for men (116). Considering the risks, it seems like men are partly protected against osteoporosis and osteoporosis-related fractures (117). Therefore, studying the male skeleton may lead to new ideas for treatment and prevention of osteoporosis in both men and women.

OBESITY

Obesity is caused by a long-term imbalance between energy intake and energy expenditure, leading to excessive fat accumulation (118). Obesity has reached epidemic proportions globally and the World Health Organization (WHO) has stated it as a major risk factor for a number of chronic diseases, including diabetes, cardiovascular diseases and cancer (118). In Sweden, about 10-15% of the

population is obese (119). This is still low in an international perspective; globally, 400 million adults are obese and 1.6 billion adults are overweight or obese. Overweight and obesity are generally defined using the body mass index (BMI; body weight in kilograms/height in meters squared; kg/m^2). Individuals with normal weight have a BMI of 18.5-25, overweight individuals have a BMI >25 and obese individuals have a BMI >30 (118). Obesity is caused both by environmental and genetic factors. For example, studies have attributed at least 50% of the variation in BMI to genetic factors (120-124). The 2005 human obesity gene map (23) includes >600 loci including data from single-gene mutations in mouse models of obesity, non-syndromic human obesity cases due to single-gene mutations, obesity-related Mendelian disorders that have been mapped, transgenic and KO mice models, and genome-wide scans, and genes or markers that have been shown to be associated or linked with an obesity phenotype. The large number of genes and loci described in the obesity gene map is a good indication of the complexity of the task of identifying genes associated with the susceptibility to obesity. The most recent GWA studies have discovered 15 new loci associated to BMI (19, 125-127). The function of these candidate genes in associated regions supports the notion of a role for the hypothalamus in the genetics of obesity. At present, 1% of obesity is proposed to be explained by the current observed genetic variation determined using GWA studies (125).

Obesity has been suggested to be associated to increased bone mass (128-130). However, these studies did not control for the mechanical loading effects of body weight on bone mass. When this was accounted for, the positive correlation between fat mass and bone mass previously seen was in some studies inverted to a negative correlation (131).

Research in recent years has identified adipose tissue not only as a storage place for excess energy, but also as a highly active endocrine organ secreting several hormones and peptides. The identification of factors involved in obesity is important to be able to better understand the disease. Distribution of body fat differs according to gender. Men tend to accumulate fat in the abdomen (visceral area) whereas women tend to accumulate fat in the gluteal-femoral region (132). Sex steroid levels are altered in upper body obesity (133). For example, abdominal obesity (134-136) and visceral fat accumulation (137) in men were in general associated to lower serum levels of T. Serum levels of 3α-diol-glucudonides were reported to be positively associated with adiposity and visceral fat accumulation (138). Weight gain increased the serum levels of 3α-diol-glucudonides (139), while weight loss decreased the 3α-diol-glucudonides levels (140).

Type 2 diabetes mellitus

Obesity is a major determinant of the incidence of type 2 diabetes. Type 2 diabetes is a heterogeneous disorder that occurs with increasing frequency with age and increased body weight. The disease is associated with insulin resistance (141).

The metabolic syndrome

Obesity is a major risk factor for the metabolic syndrome. The metabolic syndrome is defined by a cluster of risk factors, highly associated with development of type 2 diabetes and cardiovascular disease. The term metabolic syndrome first appeared in 1923 (142). The risk factors have been reevaluated several times and include abdominal obesity, insulin resistance, high plasma triglycerides, low HDL cholesterol and/or hypertension (143). Reduced plasma levels of T are associated with increased number of features of the metabolic syndrome in men (144-147).

AIMS OF THE THESIS

The specific aims of this thesis were:

To characterize genetic variations associated with substrate-specific glucuronidation of androgens/androgen metabolites:

- Polymorphisms in enzymes (UGT2B15, UGT2B17 and UGT2B7), which have been shown *in vitro* to be involved in glucuronidation of androgens, will be validated in large population-based cohorts (Paper I and II).

To explore the impact of androgen metabolites and polymorphisms associated with glucuronidation of androgens/androgen metabolites on bone mass:

- The role of androgen metabolites as predictors of BMD (Paper III).
- The role of polymorphisms associated with glucuronidation of androgens/androgen metabolites as determinants of bone mass (Paper II).

To explore the impact of androgen metabolites and polymorphisms associated with glucuronidation of androgens/androgen metabolites on fat mass and metabolic risk factors:

- The role of androgen metabolites as predictors of fat mass and metabolic risk factors (Paper IV).
- The role of polymorphisms associated with glucuronidation of androgens/androgen metabolites as determinants of fat mass (Paper I).

METHODOLOGICAL CONSIDERATIONS

HUMAN COHORTS

Male osteoporosis is less studied than female osteoporosis. Therefore, two male cohorts were initiated, one with young adult men from Gothenburg and one international multicenter study with elderly men from Sweden, USA and Hong Kong. The purpose in young adult men was to determine both genetic and environmental predictors of peak bone mass while the purpose in elderly men was to establish the diagnostic criteria of osteoporosis for men. WHO previously established such a criteria only for women.

Thus, we first measured the glucuronidated androgen metabolites in both male cohorts. We have now initiated studies where we measure the glucuronidated androgen metabolites also in females. This thesis does not include the female studies.

Variables	COHORT	
	GOOD	*MrOS Gothenburg*
Number of subjects	1068	1001
Age (years)	18.9 ± 0.6	75.3 ± 3.2
Smokers (%)	8.7	8.0
Height (cm)	181.4 ± 6.7	175.7 ± 6.4
Weight (kg)	73.8 ± 11.9	80.9 ±12.2
BMI (kg/m^2)	22.4 ± 3.2	26.2 ± 3.5

Table 1. *Characteristics of the study subjects in the GOOD and MrOS studies. Values are given as means ± SD.*

The GOOD study

The *G*othenburg *O*steoporosis and *O*besity *D*eterminants (*GOOD*) study was initiated to determine environmental and genetic factors involved in the regulation of bone and fat mass (148). Study subjects were randomly identified using national population registers, contacted by telephone, and invited to participate. Altogether, 1068 men (aged 18.9 ± 0.6 years) were included in the study. Except for the age limits (subjects had to be >18 and <20 years of age), there was no exclusion criterion. The participation rate among contacted candidates was 48.6%. Informed consent was

obtained from all participants. A standardized questionnaire gathered information on present and former physical activity (PA), nutritional intake, smoking status, fracture history and fracture history in the subject's family was collected. Blood and urine samples were collected from all study participants. Bone properties and body composition were investigated using DXA and pQCT (148).

The GOOD study is used in Paper I, II and IV.

Due to careful phenotyping, the population-based recruitment, the narrow age range, and the relatively large number of study subjects, the GOOD study is one of its' kind.

The MrOS study

The Osteoporotic Fractures in Men (MrOS) study in Sweden is part of an international study including elderly men in Sweden (n=3014), Hong Kong ($\cong$2000) and the United States ($\cong$6000). MrOS Sweden is a population-based study with three study centers; Göteborg=1010, Malmö=1005, and Uppsala=999. Men aged 69–80 were randomly identified using national population registers, contacted and invited to participate. To be eligible for the study, the subjects had to be able to walk without aids, understand and fill out the study questionnaire in Swedish and have no bilateral hip prosthesis. There were no other exclusion criteria. An informed consent was obtained from all study participants. Of those who were invited, 45 % agreed to participate in the study (109). A standardized questionnaire gathered information on current PA, nutritional intake, smoking status, and fracture history was collected. Bone properties and body composition were investigated using DXA. Blood and urine samples were collected from all study participants.

Papers I and IV used the Göteborg cohort of MrOS Sweden. Paper III was written while the glucuronidated androgen metabolites were being measured. Therefore, a sub sample (n=631) that was first measured of the Göteborg part of the MrOS Sweden study was used in this study. The average age of the subjects in this sub-sample was slightly higher than in the whole Göteborg cohort (75.9 versus 75.3 years of age, p<0.05) while their height (175.4 cm versus 175.7 cm) and body weight (80.9 kg versus 81.0 kg) were similar as in the whole Göteborg cohort.

The large number of study subjects and the population-based nature of the MrOS study make it a unique cohort.

IN VIVO IMAGING TECHNIQUES

Dual-energy X-ray absortiometry (DXA)

DXA is a widely used non-invasive technique for investigation of bone and body composition in humans as well as animals. The current criteria for the diagnosis of osteoporosis are based on this technique by measuring BMD.

The DXA can distinguish between soft tissues and bone since the emitted X-ray is divided into two different levels by a filter, and different tissues absorb the energy to different degrees; a dual-energy spectrum is created. Sensors detect the amount of energy absorbed when the X-ray passes through the body. The use of two energies allows bone mineral to be assessed independently of soft tissue (Fig. 5). The radiation dose is rather low, less than 1/10 of the dose of a chest X-ray.

Fig. 5. DXA scan of whole body.

DXA measurements can provide the area, the bone mineral content (BMC) and BMD for individual bones as well as the whole body of the study subject. One drawback when using DXA for the determination of bone mass is that it is two-dimensional, thus only accounts for changes in length and width. The BMD determined is therefore an *areal*-BMD (aBMD; g/cm^2). This quantity is the amount of bone mineral per area unit and not the true vBMD (g/cm^3) which is the amount of mineral per volume unit. This means that a thicker bone will inevitably have a higher aBMD than a thinner bone. Fat and lean mass can also be separately measured by DXA.

In Paper I and IV, the DXA technique was used to measure fat mass, lean mass and percent body fat of the whole body and fat mass of the trunk, as well as upper and lower extremities. Central fat mass was calculated as trunk fat/total body fat x 100. In Paper III, DXA was used to assess aBMD of the whole body, total hip, femoral trochanter, femoral neck, and lumbar spine.

Peripheral quantitative computed tomography (pQCT)

pQCT is a useful technique for the measurement of bone, fat and muscle in humans as well as animals. The radiation dose is somewhat higher than for DXA, but is considered safe since radiation to the central body is low.
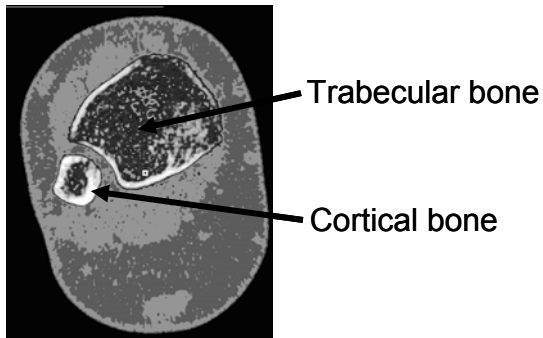
Fig. 6. *pQCT of the metaphysis of the distal tibia.*

The technique is based on a rotating X-ray source which moves to fixed positions around the arm or leg that is being measured. A computer processes local attenuation data from each position and produces an image which represents a section of the body part being measured. The pQCT is superior to the DXA technique since it allows cortical and trabecular bone to be studied separately (Fig. 6). In addition, it is a 3D-technique, making it possible to measure true vBMD and the bone dimensions.

The diaphysis of bone consists almost only of cortical bone and here periosteal (outer) and endosteal (inner) circumference can be measured and accordingly cortical bone mineral content, cortical thickness, cortical cross-sectional area, cortical BMC, and cortical vBMD can be determined (Paper II). The metaphysis consists almost only of trabecular bone and here trabecular vBMD can be measured in the central part of the bone. The growth plate is used as a reference point in determining where to place the scan along the longitudinal axis.

The pQCT technique is also successfully used for the precise measurement of cross-sectional areas of deep and subcutaneous adipose tissue at any site of the body (149). We used pQCT to determine the cross-sectional subcutaneous adipose tissue of the legs and arms (Paper IV).

Abdominal computed tomography (CT)

This technique uses the same principle as the pQCT described above. It is used to determine the cross-sectional adipose areas of the abdomen, including subcutaneous fat, deep subcutaneous fat, intra-abdominal fat, intra-and retroperitoneal fat. Liver attenuation, which is inversely associated with the amount of fat in the liver, is also measured. The study subject lies in a recumbent position and the adipose tissue areas are measured at the fourth lumbar vertebra level (Paper IV).

Measurement of prostate volume

Prostate volume is measured with transrectal ultrasound (TRUS), using an ultrasonic device (Paper III). The patient lies on his left side with flexed hip and knee joints. Three prostatic diameters are measured; transverse, anterior-posterior, and craniocaudal. Prostate volume is calculated using the formula for prolate ellipsoid, as described by Collins et al. (150).

DETERMINATION OF GENETIC VARIATIONS

DNA isolation

In both cohorts, genomic DNA was isolated from whole blood using commercial kits.

Genetic polymorphism analysis

Allelic discrimination

The development in SNP genotyping has been very rapid and there are several techniques that offer high throughput at a low cost. In Paper I and II, allelic discrimination was used for genotyping the UGT2B15 D^{85}Y and the UGT2B7 H^{268}Y SNP, respectively.

The allelic discrimination technique involves designing specific primers for the DNA area of interest. Site specific probes are also designed, one for each allele of the SNP. Each probe is labeled with a reporter dye (VIC or FAM). The probes are also labeled with a quencher, which prevents the fluorescent dye to emit light. When the reaction starts, the primers and probes bind to the area which they are designed for. While moving along the DNA, the polymerase in the PCR reaction encounters the probe and by its 5' nuclease activity cleaves its quencher. The probe is permitted to emit light, which is recorded. The genotype of the study sample is determined based on the relationship between the fluorescence of the two reporter dyes. This is easily visualized by an allelic discrimination scatterplot. FAM light appears on the Y scale and VIC light on the X scale (Fig. 7). Samples are run on plates with 384 wells.
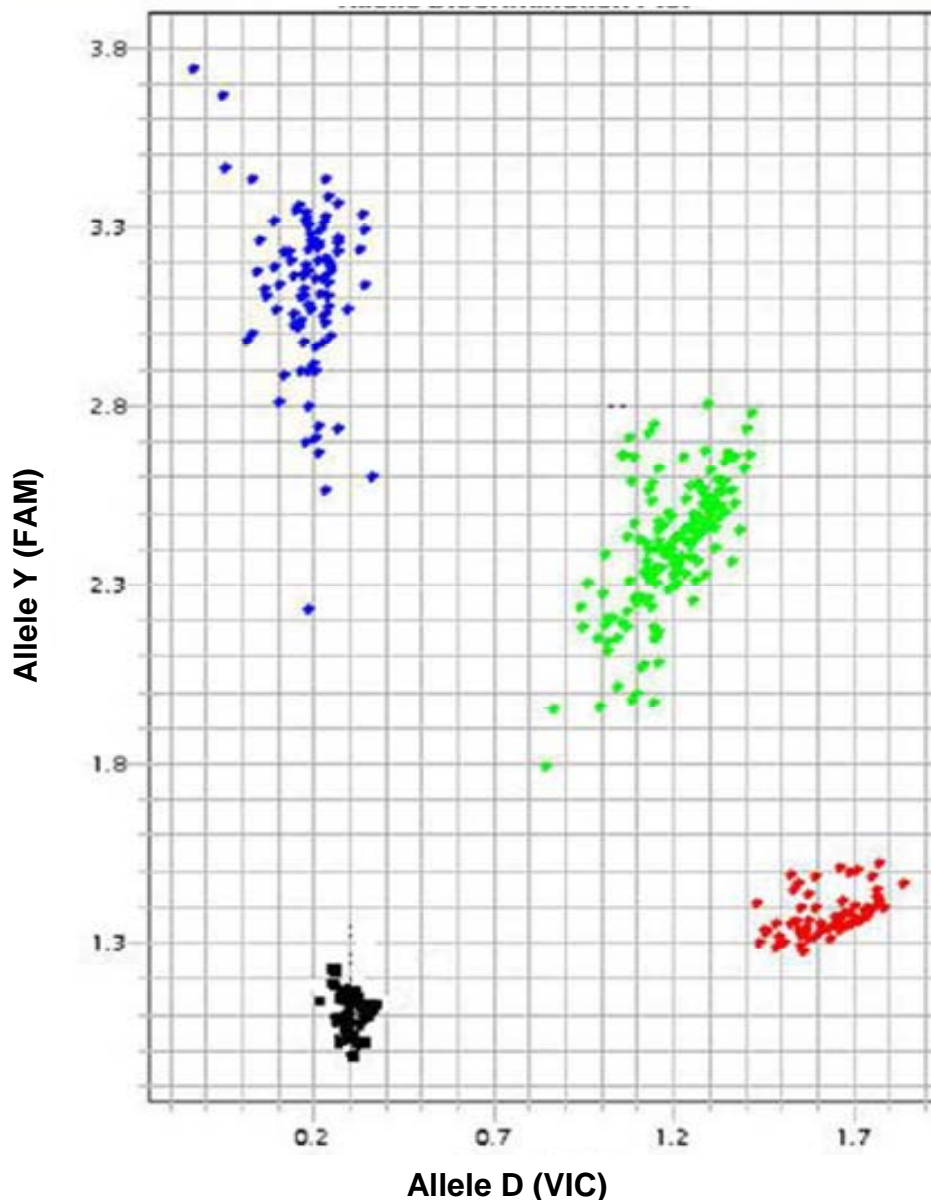
Fig. 7. *An allelic discrimination plot of individuals genotyped for the UGT2B15 D^{85}Y SNP. Blue dots represent the YY subjects, green dots represent the DY subjects and red dots represent the DD subjects. Black dots are negative control samples (water).*

Fragment Analysis

Since the UGT2B17 polymorphism is a deletion, the allelic discrimination technique cannot be used. Instead, a polymerase chain reaction (PCR) followed by fragment analysis was used to identify the *UGT2B17* gene, while a PCR followed by separation on gel was used to identify the deletion. Primers and probe were designed based on those from Wilson et al. (64).

Briefly, the size of the *UGT2B17* gene PCR fragment was determined by fragment analysis on a capillary instrument, after addition of a fragment marker and size standard, and then further analyzed through the computer software Gene Mapper. The presence or the absence of the gene was confirmed. For identification of the deletion, a separate PCR was run and the product from this deletion PCR was manually analyzed on an agarose gel containing ethidium bromide and visualized under UV fluorescence.

Using this methodology, the whole MrOS Gothenburg cohort in Paper I was genotyped for the UGT2B17 deletion polymorphism. Due to massive manual workload (PCR product separation on gel for the deletion detection), this polymorphism was only analyzed in a randomized sub-sample (n=615) of the GOOD cohort; only those not found having the *UGT2B17* gene in the initial PCR and fragment analysis were chosen for the UGT2B17 homozygous deletion allele identification by an additional PCR followed by gel separation.

Thus, for the MrOS Gothenburg cohort the genotype for the UGT2B17 polymorphism is given as homozygous for deletion, homozygous for the gene (wild type) or heterozygous, while for the individuals in the GOOD cohort we give the genotype as homozygous for the deletion or homozygous for the gene together with heterozygous individuals. Importantly, none of the parameters investigated showed any difference between the subjects heterozygous for the gene and homozygous for the gene in the MrOS Gothenburg cohort.

SERUM AND URINE MEASUREMENTS

Mass spectrometry (MS)

The validated and highly-specific MS technique was used in our studies to measure unconjugated sex steroid precursors (DHEA, DHEAS, 4-dione, 5-diol), sex steroids (T, DHT, E1, E2) and conjugated androgen metabolites (3G, 17G, ADTG) in serum (paper I-IV) as well as conjugated androgens in urine (TG, DHTG, epiT) and E1 sulfate (E1S) (paper I, II). The MS technique is more specific than radioimmunoassays (RIAs) due to identification based on mass-to-charge ratio.

Gas chromatography-mass spectrometry (GC-MS)

In our studies, the GC-MS technique was used to measure the conjugated steroids in urine and the unconjugated steroids in serum. All urinary values are expressed as the

unconjugated (typically less than 1% of the glucuronide fraction (88)) plus the glucuronidated fraction.

Briefly, the procedure of extracting conjugated steroids from urine consists of an initial addition of an internal standard (methylT) to 2 ml of sample, followed by enzymatic hydrolysis with β-glucuronidase to remove the conjugates. After extraction, the hydrolyzed steroids are derivatized to improve sensitivity. The derivatized steroids are then analyzed by GC-MS (66).

Steroids in serum are first removed from their binding protein (albumin, SHBG). After addition of an internal standard to 0.74 ml of serum sample, the steroids are extracted to the organic phase and purified over a column to avoid interference. After extraction and ionization by soft negative ionization, which decreases the fragmentation and also increases sensitivity further, the steroids are analyzed by GC-MS (87).

Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

We used LC-MS/MS to measure serum levels of the glucuronidated androgen metabolites as well as sulphated estrogens and sulphated sex steroid precursors. After initial addition of citric acid buffer to stabilize the glucuronide, an internal standard is added to 0.5 ml of serum sample. The steroids are purified and separated on a column and injected to the LC-MS/MS, no derivatization is required. Ionization is accomplished by negative electrospray ionization to remove fluid and transform the COOH group in the glucuronide into COO^- group (87).

Serum/plasma assays

Commercial immunoassay-based techniques were used to measure the total fraction of T and SHBG as well as LH, FSH, IGF-I and leptin and the samples were run in duplicates. Free T and bioavailable T were calculated according to the method described by Vermeulen et al (78) and van den Beld et al. (77), using mass action equations, taking the concentration of total T and SHBG into account and assuming a fixed albumin concentration of 43g/liter. The measured and calculated levels of free T (FT) and bioactive T are highly correlated (78). Serum lipid analysis was performed on a Konelab autoanalyzer. Total cholesterol and triglyceride levels were determined in fasting serum. HDL was determined after precipitation of ApoB-containing lipoproteins with magnesium sulphate and dextran sulphate. LDL was calculated using Friedewald's formula. ApoB and ApoA1 were determined by immunoprecipitation.

Fasting serum insulin was measured with an immunometric method based on chemiluminescence technology. Fasting plasma glucose was quantified by an enzymatic method. Homeostatis model assessment (HOMA) index was calculated as the product of the fasting serum insulin level (microU/ml) and fasting plasma glucose level (mmol/l), divided by 22.5.

STATISTICS

In all papers, standardized β-values were used and values were given as means ± standard deviation (SD).

Hardy-Weinberg equilibrium was tested using χ^2 analysis (Papers I-II). When comparing continuous variables, such as serum and urine measurements between the different genotypes, ANOVA, followed by Tukey´s post hoc test, was used (Paper I and II). The independent predictive value of the polymorphisms for fat mass and serum parameters was investigated by linear regression, including age (Paper I), and for cortical bone parameters, height, weight, age, smoking, calcium intake, and physical activity as covariates (Paper II). To analyze any differences in activity between the two variants of the UGT2B7 enzyme in the enzymatic assay (Paper II), a Student t-test was used.

Correlation analysis in all four papers was performed with Pearson's correlation after log transformation of non-normally distributed variables. Linear regression models were used to analyze the predictive value of androgens and androgen metabolites for BMD, using height, weight, age, calcium intake, physical activity and smoking as covariates (Paper III). For prostate volume, height, weight and age were used as covariates (Paper III). Age was used as a covariate in the linear regression model to investigate the predictive value for DHT and 17G for fat mass, insulin resistance and serum lipids (Paper IV). Logistic regression analysis was used to calculate odds ratios (per SD, 95% confidence intervals given), and determine the predictive value of 17G/DHT ratio for overweight, obesity, insulin resistance and prevalent diabetes. Subjects with diabetes were excluded from regression analysis regarding insulin resistance (Paper IV).

RESULTS AND COMMENTS

PAPER I

UGT2B15 and UGT2B17 genotype, glucuronidation pattern of androgens and body composition

To investigate the glucuronidation pattern of UGT2B15 and UGT2B17 as well as the associations between UGT2B15 and UGT2B17 genotypes and serum levels of glucuronidated androgen metabolites and fat mass in young adult and elderly men, we used the GOOD study (n=1068) and the MrOS study (n=1001). The androgens and glucuronidated androgen metabolites were measured by GC-MS and LC-MS/MS, respectively. Body composition was measured by DXA.

Results

- There was no linkage between the UGT2B15 and UGT2B17 polymorphisms.
- The UGT2B15 $D^{85}Y$ and UGT2B17 deletion polymorphisms were associated to serum levels of 17G, in both young adult and elderly men.
- Urine levels of T and DHT were associated to the UGT2B17 deletion but not with the UGT2B15 $D^{85}Y$ polymorphism.
- Subjects with the UGT2B15 YY genotype had lower serum levels of 17G (-33% in elderly men, -33% in young adult men, p<0.001) and the UGT2B17 del/del genotype had lower serum levels of 17G (-37%, p<0.001), and urine levels of TG (-93%, p<0.001) and DHTG (-43%, p<0.015; by ANOVA).
- Linear regression analysis adjusted for age, indicated that the UGT2B17 polymorphism was associated to BMI, body weight, total body fat and trunk fat in elderly men (p<0.05), while the UGT2B15 polymorphism was associated with fat parameter in both elderly and young adult men (p<0.05).
- Subjects with the UGT2B15 YY genotype and the UGT2B17 del/del genotype had higher BMI and increased amount of fat.
- The UGT2B17 deletion polymorphism was a predictor of insulin resistance (HOMA index) and serum levels of insulin in the elderly cohort.

In conclusion, both the UGT2B15 $D^{85}Y$ and UGT2B17 deletion polymorphisms are associated with serum levels of 17G. The UGT2B17 deletion polymorphism but not the UGT2B15 $D^{85}Y$ polymorphism is associated with urine levels of T and DHT.

Importantly, only UGT2B17 has the capacity to glucuronidate T. Both the UGT2B15 D^{85}Y and UGT2B17 deletion polymorphisms are associated to fat mass and metabolic risk factors in men.

PAPER II

UGT2B7 genotype, glucuronidation pattern of androgens and bone mass

To determine the glucuronidation pattern by UGT2B7 and the association between the UGT2B7 H^{268}Y polymorphism genotypes and serum levels of androgens/androgen metabolites and bone parameters in young adult men, we used the GOOD study. The androgens and glucuronidated androgen metabolites were measured by GC-MS and LC-MS/MS, respectively. Bone mass and structure were assessed by pQCT.

Results

- The UGT2B7 H^{268}Y polymorphism was associated to serum levels of the sex steroids T, DHT and E2 as well as the metabolites 3G and 17G.
- Subjects with the UGT2B7 YY genotype had higher serum levels of T and DHT and also of 3G and 17G (p<0.05).
- Linear regression analysis found the UGT2B7 H^{268}Y polymorphism to be an independent predictor of cortical bone parameters, such as periosteal and endosteal circumference (p<0.01, adjusted for height, weight, age, smoking, calcium intake, physical activity). The association remained significant after addition of E2 and FT to the regression model.
- Subjects homozygous for the UGT2B7 Y allele had larger cortical bone size, reflected by larger periosteal circumference and higher cortical moment of inertia (p<0.05).

In conclusion, the UGT2B7 H^{268}Y polymorphism is associated to serum levels of T and DHT and to the glucuronidation pattern of 3G. The UGT2B7 H^{268}Y polymorphism is a predictor of cortical bone size in young adult men.

PAPER III

Androgen metabolites and bone mass

To investigate the role of androgens and androgen metabolites as predictors for bone mass in elderly men, a randomized subsample of the MrOS Gothenburg study was used (n=631). The androgens and glucuronidated androgen metabolites were measured by GC-MS and LC-MS/MS, respectively. Bone parameters were measured by DXA.

Results

- The androgen metabolites 3G and 17G were stronger predictors of BMD than the bioactive androgens.
- Linear regression analyses showed that 17G and 3G were independent predictors of the different BMD sites (adjusted for age, height, weight, calcium intake, physical activity and smoking).
- The sum of 3G+17G explained a larger part of the variance (2.8%) in BMD than the metabolites alone (3G=2.1%, 17G=2.0%).
- 17G was also a positive predictor of prostate volume (n=159; Pearson's correlation, r=0.21) and the metabolite explained 4.5% of the variance.
- Linear regression analyses showed that 17G, but not 3G or ADTG, was an independent predictor of prostate volume (adjusted for age, height, weight; β=0.19, $p<0.05$).

In conclusion, the androgen metabolites 17G and 3G are associated to BMD in elderly men, and are stronger predictors of BMD than the bioactive androgens. The metabolite 17G is also a predictor of prostate volume.

PAPER IV

Androgen metabolites, fat mass and metabolic risk factors

To investigate the role of serum levels of androgens and androgen metabolites as predictors for fat mass and metabolic risk factors in young adult and elderly men, the GOOD study and MrOS study were used. Fat mass was measured by DXA in both cohorts. In addition, in the GOOD study, fat mass in arm and leg was measured by pQCT and cross-sectional adipose tissue was measured by abdominal CT. Leptin,

insulin, glucose and lipids were measured. The androgens and glucuronidated androgen metabolites were analyzed by GC-MS and LC-MS/MS, respectively.

Results

- Inverse rather strong associations between DHT and T and body weight, BMI, serum leptin and all measurements of total body as well as region-specific fat deposits were found in both young adult and elderly men.
- DHT, but not T, was found to be independently and negatively associated with fat mass in both young adult and elderly men.
- A clear positive association was found between 17G and most fat deposits and central fat distribution in both young adult and elderly men.
- DHT was negatively, while 17G was positively correlated with serum insulin and HOMA index in elderly men. 17G was also negatively associated with HDL cholesterol and ApoA1 and positively with triglycerides.
- When including both DHT and 17G in a linear regression model, a larger part of the variance in total body fat (14%) was explained in both young adult and elderly men, in comparison to analyzing DHT and 17G separately.
- The 17G/DHT ratio was rather strongly associated with all fat deposits in both young adult (r=0.22-0.36) and elderly men (r=0.24-0.42), explaining 12% and 15% of the variance in total body fat, respectively.
- The 17G/DHT ratio was rather strongly associated to HOMA-index (r =0.32), measured in elderly men, explaining 10% of the variance. The correlation remained significant after adjustment for age and percent total body fat.
- The 17G/DHT ratio was strongly associated with overweight and obesity in young adult and elderly men (OR=2.06-4.45 per SD increase), and with insulin resistance and diabetes in elderly men (OR=1.48-1.96 per SD increase) (logistic regression analysis).

In conclusion, 17G, and especially the 17G/DHT ratio, were found to be strongly correlated with fat mass, fat distribution and metabolic risk factors in men.

DISCUSSION

Androgens are important for many physiological processes. In addition to binding of these hormones to the AR, the synthesis and inactivation are also important in the action of androgens. Genetic variation has a large impact on the androgen disposition. We have shown that genetic polymorphisms considerably influence the serum and urinary androgen and androgen metabolite levels. These changes in serum levels could affect androgen-dependent tissues such as bone, prostate and fat.

Genes and polymorphisms involved in substrate-specific glucuronidation of androgens/androgen metabolites

Androgens are metabolized in two steps, phase I and phase II (Fig. 8). Phase I includes the reversible metabolism of DHT into 3α-diol and ADT, while phase II metabolism includes the irreversible conjugation of the phase I metabolites into more water-soluble compounds. Androgen metabolites are mostly conjugated to glucuronides, with 3α-diol being transformed into 3G and 17G, and ADT into ADTG (Fig. 8).
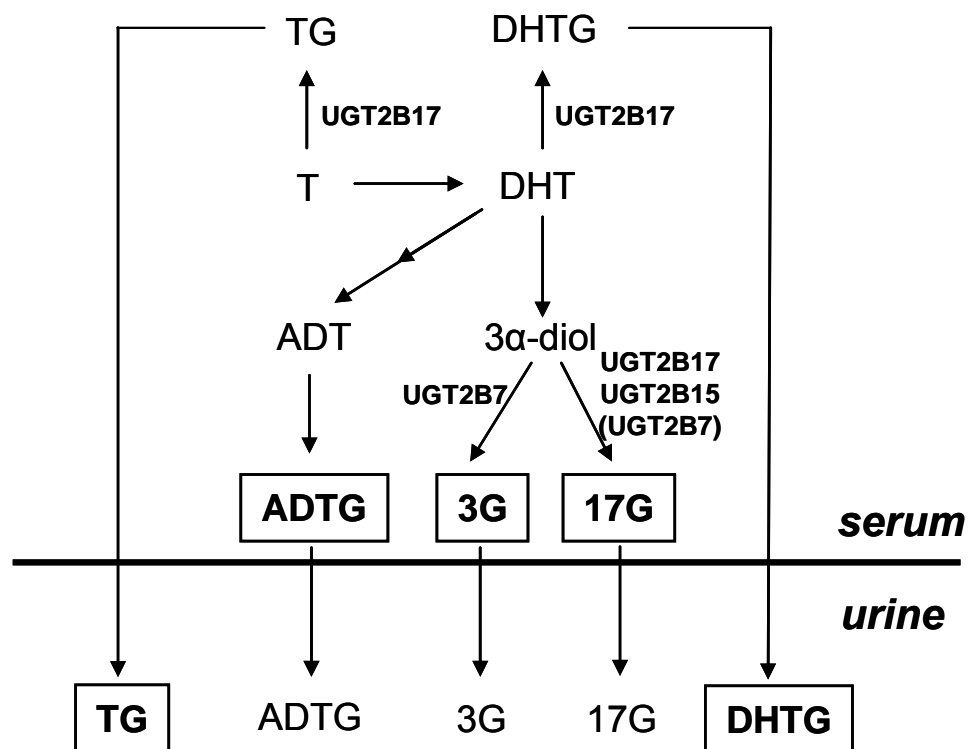
Fig. 8. The glucuronidated androgen metabolites in serum and urine. UGT2B7, UGT2B15 and UGT2B17 glucuronidation based on in vivo analyses of polymorphisms in these genes, as reported in this thesis, is indicated. Boxed glucuronidated metabolites are measured by LC-MS/MS or GC-MS.

Alternatively, the bioactive androgens T and DHT can be inactivated directly by glucuronidation (Fig. 8). This phase II glucuronidation is catalyzed by the UGT2B7, UGT2B15 and UGT2B17 enzymes. The glucuronidation process is substrate-specific and probably also tissue-specific since the different UGT enzymes show a tissue-specific expression (44, 52, 58). The relative enzymatic activity of the UGT enzymes in each tissue can give rise to changes in androgen levels within the tissue.

By *in vitro* studies (33, 52), it has been determined that the androgens T and DHT and the androgen metabolites 3α-diol and ADT are glucuronidated by the enzymes UGT2B7, UGT2B15 and UGT2B17 into TG, DHTG, 17G, 3G and ADTG (Fig. 8). In this thesis, I have investigated the *in vivo* specificity and effects of the UGT enzymes in two large, well characterized human cohorts of young adult and elderly men. These two cohorts were also used to study the effect of polymorphisms in the *UGT2B7, UGT2B15* and *UGT2B17* genes. We have measured the glucuronidation products of androgens and androgen metabolites with the very specific LC-MS/MS and GC-MS techniques that can distinguish 3G from 17G in serum. The glucuronidated androgen metabolites 3G, 17G, and ADTG are measured directly in the serum, while the glucuronidated forms of T and DHT (TG and DHTG) in serum are measured indirectly in urine (Fig. 8). In urine, most (>99%) of the T and DHT molecules are glucuronidated (88).

Both the UGT2B7 H^{268}Y and the UGT2B15 D^{85}Y polymorphism result in an amino acid change. The amino acid change in the UGT2B7 H^{268}Y polymorphism takes place where the substrate binds to the enzyme. The change in the protein chain could result in altered enzyme activity. When studying associations between the UGT2B15 D^{85}Y and the UGT2B7 H^{268}Y polymorphisms and glucuronidated products, no association found could either mean that the polymorphism is not functional or unimportant for the reaction studied, or that the UGT enzyme itself is not important for the reaction. An association would suggest that the polymorphism is functional or in linkage with another functional polymorphism, and that the gene is important for the reaction studied. However, the UGT2B17 polymorphism is a deletion, and subjects homozygous for the deletion express no enzyme. Therefore, an association between the UGT2B17 deletion polymorphism and a glucuronidated androgen/androgen metabolite demonstrates that the UGT2B17 enzyme is required for the reaction while no association demonstrates that the enzyme is not required for the reaction.

Glucuronidation of T: We show that UGT2B17 has a key role for glucuronidation of T. Urine levels of glucuronidated T (Fig. 8) were only associated with the UGT2B17

polymorphism, and not to the UGT2B7 or UGT2B15 polymorphism (Paper I-II), which confirms previous *in vitro* data (33, 52). This suggests that UGT2B17 is the only enzyme capable of the glucuronidation of T, and in subjects homozygous for the UGT2B17 deletion, no glucuronidation of T will take place. The importance of UGT2B17 has previously been demonstrated in subjects homozygous for the UGT2B17 deletion polymorphism. These subjects lack the UGT2B17 enzyme and have a seriously impaired ability to glucuronidate T, as shown by negligible amounts of glucuronidated T in the urine (66) (Fig. 8). Administration of T to subjects homozygous for the UGT2B17 deletion also resulted in extremely low urine levels of glucuronidated T compared to subjects with one or two alleles of the UGT2B17 gene (151). The T/epiT ratio, a value introduced as a criterion for T abuse with increased ratio indicating the possible abuse of androgens, was shown to be largely affected by the UGT2B17 deletion polymorphism in a small sub-sample of young adult men included in the GOOD study (151). Using the complete GOOD cohort of young adult men and the large MrOS cohort of elderly men, we showed that subjects homozygous for the deletion had extremely low T/epiT ratio (Fig. 9) (Paper I), suggesting that these subjects can abuse T with hardly any risk of detection.
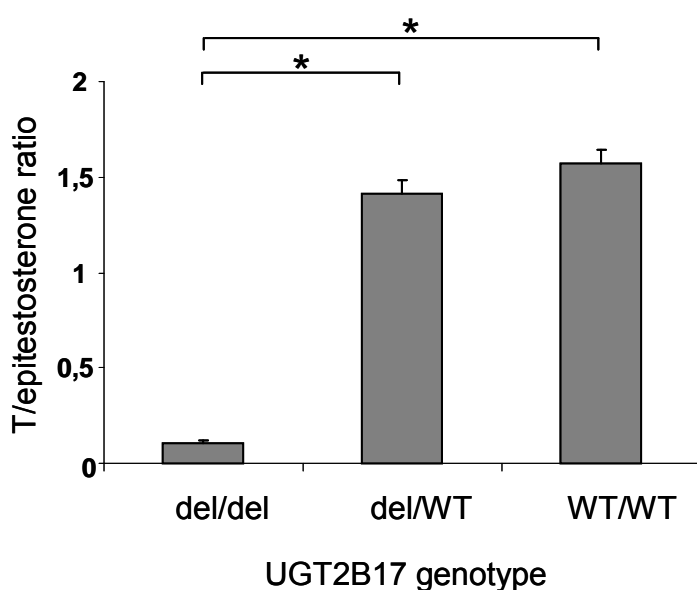

Fig. 9. *The UGT2B17 deletion polymorphism as predictor of the T/epitestosterone ratio in elderly men (Paper I). Values are given as means ± SEM.* $* = p < 0.001.$

In addition to this, the deletion polymorphism in the *UGT2B17* gene is much more common in Asian (66.7%) than in Caucasian (9.3%) populations (66), and the UGT2B17 expression level differs remarkably between Asians and Caucasians (152). This has led to the suggestion that the T/epiT cut off ratio for doping should be individual-based instead of population-based (153). Increased understanding of androgen elimination by glucuronidation, and its associated genetic variation, is important for improving the interpretation of doping test results and the development of new antidoping tests.

UGT2B17 has also been reported to be important for other clinical aspects of androgen action such as fracture risk. In a large Chinese study in elderly men, studying genome-wide copy number variation, osteoporotic fracture risk was found to be associated with copy number of the *UGT2B17* gene, with an increased risk seen for subjects with one or two alleles of UGT2B17 (18). UGT2B17 has also been studied in relation to prostate cancer, showing either an increased risk for subjects lacking one or two alleles of the UGT2B17 gene (67, 154) or no association (68), possibly suggesting a protective effect for subjects carrying the *UGT2B17* gene. The del/del genotype is more common among Asians (66), and since del/del subjects were shown to have increased risk for prostate cancer, prostate cancer should be more common among Asian men if the UGT2B17 deletion polymorphism is involved. The prostate cancer incidence does differ between ethnic groups, but Asians have the lowest incidence (155). The genetic variation in UGT2B17 does therefore not seem to explain the ethnic incidence differences of prostate cancer.

Glucuronidation of DHT: Regarding glucuronidation of DHT, urine levels of DHT (mostly glucuronidated; Fig. 8) were only associated with the UGT2B17 polymorphism, which confirms previous *in vitro* data (33). No association was seen with the UGT2B15 $D^{85}Y$ polymorphism, although UGT2B15 previously was described *in vitro* to have low capacity to glucuronidate DHT (52). The reason for the difference between low activity found *in vitro*, and no activity found in our *in vivo* study, could either be that the UGT2B15 $D^{85}Y$ polymorphism does not affect the activity of the enzyme, or that UGT2B15 enzyme is not important for glucuronidation of DHT *in vivo*. A previous *in vivo* study, using part of the GOOD study, reported that individuals homozygous for the UGT2B17 deletion had significantly lower levels of DHT in urine (66), and we can now confirm this also in elderly men (Paper I). The presence of the UGT2B17 deletion reduces the ability to glucuronidate DHT (-43%), which explains the lower levels of DHT found in the del/del individuals. In contrast to the glucuronidation of T, where a 93% reduction of urine levels was seen for subjects lacking the *UGT2B17* gene, the reduced glucuronidation of DHT for subjects homozygous for the UGT2B17 deletion was less pronounced, indicating that for the glucuronidation of DHT other enzymes besides the UGT2B17 enzyme are involved.

17-glucuronidation of 3α-diol: In our *in vivo* study, we saw that the glucuronidation of 3α-diol into 17G was clearly affected by both the UGT2B15 $D^{85}Y$ and the UGT2B17 deletion polymorphisms in both young adult and elderly men (Paper I). As expected, subjects homozygous for the UGT2B17 deletion had lower levels of 17G. The UGT2B15 YY subjects showed lower 17G levels in serum, which made us conclude that subjects homozygous for the Y variant have a less efficient enzyme.

The UGT2B15 and UGT2B17 data is in accordance with previous *in vitro* data regarding enzyme specificity (33, 52). However, the Y85 variant of UGT2B15 has *in vitro* been seen to have a higher Vmax (58), which was interpreted as a more efficient enzyme, but our *in vivo* data suggests that the Y variant corresponds to the least efficient enzyme.

In young adult men (Paper II), the glucuronidation of 3α-diol into 17G was also associated with the UGT2B7 polymorphism with elevated levels of 17G seen for subjects homozygous for the Y allele. But, in our enzymatic assay, we did not find any conjugation of 3α-diol into 17G. In addition to elevated 17G levels in the UGT2B7 YY subjects, we observed elevated serum levels of T and DHT. Since glucuronidation of 3α-diol into 17G by UGT2B7 has not been reported in previous *in vitro* studies, it is plausible that the unexpected 17G data for UGT2B7 might be a reflection of the higher levels of serum T and DHT detected in YY subjects. Finally, when comparing the effect of the different UGT polymorphisms on 17G formation (Papers I-II), the UGT2B15 and UGT2B17 showed larger differences between the high efficiency allele and the low efficiency allele than UGT2B7 (UGT2B15: 50% DD over YY in young adult men, UGT2B17: 27% Del/WT + WT/WT over del/del in young adult men and UGT2B7: 11% YY over HH in young adult men). In addition, when the UGT2B7 H^{268}Y, UGT2B15 D^{85}Y and UGT2B17 deletion polymorphisms were included as covariates in the same linear regression model, it was the UGT2B15 D^{85}Y and the UGT2B17 deletion polymorphisms, and not the UGT2B7 H^{268}Y polymorphism, which were independent predictors of 17G. We therefore suggest that UGT2B15 D^{85}Y and UGT2B17 deletion polymorphisms affect the 17β-glucuronidation of 3α-diol into 17G to a larger extent than the UGT2B7 H^{268}Y polymorphism (Fig. 8).

3-glucuronidation of 3α-diol: The 3α-glucuronidation of 3α-diol into 3G (Fig. 8) was only associated with the UGT2B7 polymorphism in young adult men, and not to the UGT2B15 D^{85}Y or the UGT2B17 deletion polymorphisms (Paper I-II). In our study, subjects with the UGT2B7 YY genotype had elevated 3G levels, whereas it was previously reported that the UGT2B7 H^{268}Y polymorphism did not show any inter-genotypic activity differences for its substrates *in vitro* (57). This finding emphasizes the importance of *in vivo* studies. These data supports previous *in vitro* findings that 3G is a good substrate for UGT2B7 (52). As mentioned above for 17G, UGT2B7 YY subjects had elevated serum levels of T and DHT. We cannot rule out that the elevated 3G levels seen are also a consequence of these elevated serum levels of T and DHT, as hypothesized for 17G. Still, we show for the first time that the UGT2B7 H^{268}Y polymorphism is associated with serum levels of 3G *in vivo*.

Glucuronidaton of ADT: The glucuronidation of ADT into ADTG has been shown *in vitro* to be performed by UGT2B17 (33) and UGT2B7 (52). We do not see this in our human cohorts (Fig. 8) (Papers I-II), which are the first studies to investigate the ADT glucuronidation pattern in humans in relation to the UGT2B7 polymorphism. Actually, none of the polymorphisms in the UGT2B enzymes investigated affected serum levels of ADTG *in vivo* (Papers I-II), suggesting that the UGT2B15 $D^{85}Y$ and the UGT2B7 $H^{268}Y$ polymorphisms, do not change the activity or expression of the enzymes considerably, or that the UGT2B15 and UGT2B7 enzymes are not required for ADT glucuronidation *in vivo*. Since the UGT2B17 polymorphism is a deletion, we can establish that the UGT2B17 is not required for a normal ADT glucuronidation. In our *in vitro* enzymatic assay, however, we found conjugation of ADT into ADTG by UGT2B7 (Paper II), with higher levels seen for the Y variant. Again, this clearly emphasizes the importance of *in vivo* studies.

In conclusion, the results in Papers I and II clearly show that the UGT2B7 $H^{268}Y$, UGT2B15 $D^{85}Y$ and UGT2B17 deletion polymorphisms are functional or in linkage with other functional polymorphism, and we also show the specificity of the enzymes *in vivo* (Fig. 8). The above results show that the glucuronidation of 3α-diol into 17G is mainly performed by UGT2B15 and UGT2B17, while glucuronidation of T is specifically and only performed by UGT2B17. Glucuronidation of DHT is partly performed by UGT2B17. Our data indicate the glucuronidation of 3α-diol into 3G is performed by UGT2B7 (Fig. 8). Moreover, our findings indicate that polymorphisms in UGT2B7, UGT2B15 and UGT2B17 may influence the local levels of T and DHT at the tissue levels (Papers I-II) which may in turn affect tissue-specific androgen activity.

In the following studies, we investigated the possible functional consequences in bone and fat of differences in androgen metabolism and if the different UGT2B polymorphisms associated with bone and fat mass.

Androgen metabolites and bone mass

In paper III, the glucuronidated androgen metabolites 3G and 17G were found to be stronger predictors of BMD than the bioactive androgens in elderly men. We suggest that measuring the glucuronidated androgen metabolites in serum might be a better indicator of local androgen activity than measuring serum levels of androgens. The poor predictability of serum T is for example seen in castrated men, where castration reduces the concentration of DHT in serum with 90-95%, while serum levels of the glucuronidated metabolites ADTG and 3α-diol-G are only reduced by 50-70% (82,

156, 157). Mellström et al. have previously shown that FT, measured by RIA, was a significant, but not very strong, predictor of BMD in the same MrOS cohort (109). Weak associations with BMD (77, 79, 109, 158, 159) and BMD loss (107) in men have also been found in other studies. Two recent studies, using the MS technique, have shown that serum T levels are inversely associated with fracture risk (115, 160). However, the glucuronidated androgen metabolites were not measured in these two latter studies. In our study in elderly men in Paper III, we measured the glucuronidated androgen metabolites with tandem mass spectrometry and showed that actually the glucuronidated androgen metabolites, and not FT or T, were predictors of BMD, with the sum of 3G and 17G explaining 2.8% of the variance in BMD. Notably, the 2.8% of variance in bone mass explained by the glucuronidated androgen metabolites is not much. Nevertheless, 3G+17G were still better markers of BMD than the bioactive androgens. This suggests that the glucuronidated androgen metabolites are more valid estimates of androgenic activity in bone than the androgens. Low BMD is considered the best established risk factor for osteoporotic fractures (160, 161). Future studies should investigate whether 3G+17G is a new better predictor also for fractures compared to the androgens. Studies to determine the predictive value of glucuronidated androgen metabolites for fracture risk are ongoing in our research group.

To determine if the predictive role of glucuronidated androgen metabolites for androgen action is specific for bone, we investigated the associations in another androgen-sensitive tissue. Interestingly, serum levels of 17G were associated with prostate volume whereas the bioactive androgens showed no significant associations. The lack of association between serum T and prostate volume is supported by others (162, 163). Future studies should investigate if 17G can be used as a predictor of prostate cancer. Such studies are ongoing in our research group.

In conclusion, Paper III shows that serum levels of specific glucuronidated androgen metabolites are stronger predictors of BMD than the bioactive androgens in elderly men. BMD was predicted by both 3G and 17G levels, which indicates that the androgens made locally by intracellular mechanisms are of importance for the regulation of BMD, supporting the idea of intracrinology.

Polymorphisms in glucuronidation enzymes and bone mass

Polymorphisms in genes encoding the enzymes involved in the synthesis and degradation of androgens can affect the expression levels of sex steroids such as T, DHT and E2 locally in peripheral tissues, e.g. bone tissue, and thereby affect bone

mass. We and others have studied polymorphisms that affect the synthesis of sex steroids. For example, we have shown that a repeat polymorphisms in aromatase, the key enzyme for conversion of T to E2, affected areal BMD and cortical bone size (164). This polymorphism was also reported to be associated with peak bone mass (165), and with BMD (166) and BMD loss in elderly men (113). Other studies have examined polymorphisms that influence the degradation of sex steroids. For example, we observed that a polymorphism in catechol-O-methyltransferase (COMT), a key enzyme in the degradation of estrogens, was correlated to BMD in young adult men (167) and to life time fracture risk in elderly men (168). This association has also been seen in women (169). A polymorphism in the SULT1A1 enzyme, involved in the catabolism of estrogens, was also associated to increased BMD (170).

This thesis examined the polymorphisms in the enzymes responsible for the degradation of androgens by glucuronidation and their relation to bone mass. Our study in Paper II, which showed that the UGT2B7 $H^{268}Y$ polymorphism is associated to cortical bone size in young adult men, is the first study that has associated a bone phenotype to genetic alterations in a glucuronidation enzyme. The UGT2B7 polymorphism was an independent predictor of cortical bone size in both the weight-bearing tibia and nonweight-bearing radius as a result of an impact on periosteal circumference. In addition, the polymorphism was associated to serum T levels (Paper II). Androgens increase cortical bone size in men (148, 171). During puberty, boys acquire bigger bone size probably resulting from the high androgen levels, and this confers reduced fracture risk compared to women (172). Since the YY subjects had both higher T levels and a larger periosteal circumference, we investigated if the impact of the UGT2B7 polymorphism on cortical bone size was indirectly mediated through the affected levels of T. However, inclusion of young adult serum T levels in the regression model had no substantial impact on the association seen, indicating a possible effect of the UGT2B7 polymorphism on T levels during earlier pubertal growth could be of greater importance. Alternatively, possibly high local levels of T in bone, that cannot be measured, could account for this effect. It remains unknown if UGT2B7 is expressed in bone, and this would be interesting to study. In contrast to the UGT2B7 $H^{268}Y$ polymorphism, the UGT2B15 $D^{85}Y$ and the UGT2B17 deletion polymorphism did not significantly associate with bone mass (data not shown).

Androgens and fat mass

It has been widely recognized that serum levels of T are negatively associated to fat mass (137, 138, 173-178). The inverse relationship between T and fat mass could

either depend on that fat mass decreases the gonadotropins LH and FSH, produced by the pituitary, which in turn would lead to low T levels, or that low levels of T could give rise to increased fat mass. A modest decrease of serum T is normal in elderly men, but men with lower than normal T levels, due to failure of the testes to produce adequate T levels, are considered hypogonadal (179). Men are considered to have lower than normal T levels when the mid-morning serum samples of total levels of T are between 8 to 11 nmol/l or under (25, 179-182). There is a diurnal variation in serum T levels in men (183), but it declines with age. Some suggest measuring LH and FSH since secondary hypogonadism (so called hypogonadotropic hypogonadism) is a common cause of androgen deficiency in older men (179). Hypogonadal men show a variety of sometimes vague symptoms including a decreased sense of well-being, decreased BMD, muscle mass, strength, energy, libido and sexual activity, and increased sweating, mood swings and fat mass (25, 179, 184). Since T therapy can reestablish the physiological concentration of serum T and relieve symptoms such therapy should be considered in men with definite symptoms in conjunction with low serum T levels (25, 179, 180). However, the long-term effects have not been studied and therefore treatment with T remains controversial (25, 179, 184). Alternatively, DHEA replacement could be considered, but the studies so far (185-190) did not show any convincing evidence for beneficial effects thus far.

In overweight and obese men, total T and FT levels are significantly lower compared to nonobese men (191). The effect of increasing BMI on circulating T is more pronounced compared to that of age (191), indicating that obesity impairs the hypothalamic/pituitary function independent of age. Some authors hypothesize that low T levels may be the cause, rather than the consequence, of obesity (135, 192). Also, men with acquired hypogonadism get increased fat mass (193), whereas T therapy has been reported to reduce fat mass in hypogonadal men (194).

Although the more potent bioactive androgen DHT has also been described as a negative predictor of fat mass (195), the independent impact of DHT on fat mass has not been studied. In Paper IV, serum levels of T and DHT were correlated and statistical analyses indicated that it is actually DHT, and not T, that was a negative independent predictor of fat mass in men (Paper IV). In relation to this, perhaps also serum DHT levels should be measured and considered when investigating the relationship between androgens and fat mass in men. With increased longevity and the increased amount of obese men, the number of older men with T deficiency will increase substantially over the next decades (25, 196). We also propose, given the relationship between DHT and fat mass and the impact of obesity on the diagnosis of

hypogonadism, that a threshold for serum DHT might be considered when diagnosing hypogonadism.

Androgen metabolites and fat mass

Degradation of androgens can take place in adipose tissue, since all the enzymes required for androgen metabolism are found in adipose tissue (197-199). Within the peripheral fat tissue, androgens can be either aromatized into E2 or metabolized into androgen metabolites which in turn can be glucuronidated to form glucuronidated androgen metabolites (Fig. 1). This is supported by the finding that UGT2B15 is expressed in adipose tissue (58, 200). However, UGT2B17 is not expressed (33).

Increased metabolism of DHT has been detected in visceral adipose tissue in comparison to subcutaneous fat (201) and increased levels of 3α-diol have been detected in obese patients (138). It has previously been seen in a small study by Tchernof et al. (138), that 3α-diol-glucuronides were positively correlated to visceral adipose tissue accumulation in young men supporting the idea that local androgen conversion may be related to adipose tissue. However, our study is the first study where the 3α-diol metabolites 3G and 17G have been measured and distinguished from each other. The glucuronidated androgen metabolites 3G and 17G were both found to be associated with total body fat, trunk fat and central fat distribution in both young adult and elderly men (Paper IV). When both 3G and 17G were included in the same regression model, we discovered that it was 17G that was independently positively associated to both total and regional fat deposits. This finding suggests that 3α-diol is specifically glucuronidated into 17G in adipose tissue, which means that there is a tissue-specific glucuronidation pattern of 3α-diol. From our cross sectional association study in Paper IV, we cannot determine whether the glucuronidation of 3α-diol is directly involved in the regulation of the amount of fat or whether the increased amount of fat results in an increased total capacity for 17-glucuronidation of 3α-diol, which in turn would result in increased levels of 17G and decreased levels of DHT in serum.

Metabolic risk factors include not only fat mass (especially abdominal fat mass) but also insulin resistance, intra-hepatic fat, hypertension, diabetes and unfavorable lipid profile (202). In our study, DHT was independently negatively, while 17G was positively, associated with insulin resistance, as measured by HOMA index (Paper IV). 17G was strongly associated with a disturbed lipid profile as shown by the negative association with HDL cholesterol and ApoA1, and the positive association with serum triglycerides. Liver attenuation, which is inversely associated to the

amount of intra-hepatic fat, was positively associated to DHT, while negatively associated with 17G.

It is important to note that the relationship between metabolic risk factors and DHT and 17G, respectively, is inverse, with DHT being negatively, and 17G being positively, associated with body weight, BMI, insulin resistance, serum leptin levels and triglycerides. Therefore, it is interesting to study the 17G/DHT ratio in this respect.

The 17G/DHT ratio and fat mass

The concept of the 17G/DHT ratio can be somewhat difficult to understand. First we saw that DHT was negatively, and 17G was positively, associated to fat mass (Paper IV). The connection between DHT and 17G is straightforward, since 17G is the metabolite of DHT, via 3α-diol. The ratio is a new approach that could be regarded as a measurement of the total activity of enzymes involved in the conversion of DHT into 17G.

The 17G/DHT ratio was strongly associated to total body fat and central fat distribution, calculated as trunk fat/total body fat, in both young adult and elderly men, explaining a substantial part of the variance of e.g. central fat distribution (Paper IV). In the young adult men we measured the different abdominal fat depots by CT. We show that the 17G/DHT ratio was associated to abdominal fat distribution, with stronger association seen for visceral than for subcutaneous fat depots. Based on these associations, we suggest that the visceral fat depots might be the main site for the conversion of DHT into 17G, again a sign of tissue-specificity for the conjugation of DHT. We hypothesize that there might be a difference between the DHT conjugation in central and peripheral fat depots, and that it specifically is the central visceral fat that is responsible for the conjugation of DHT into 17G. This idea is supported by the finding that several metabolic risk parameters associated to the 17G/DHT ratio. Importantly, several of these associations remained significant after adjustment for BMI (Paper IV). Taken together, we propose that the 17G/DHT ratio could be regarded as a novel metabolic risk factor in men.

If the amount of fat would determine the metabolism of DHT, increased fat mass would lead to increased conjugation of DHT and subsequently increased 17G levels in serum. If true, adipose tissue would be primarily responsible for the 17β-glucuronidation of 3α-diol. On the other hand, it cannot be excluded that the degree of local DHT inactivation (reflected by the 17G/DHT ratio) affects local DHT levels in

fat and/or other tissues (i.e. hypothalamus) of importance for the regulation of fat mass and that the local tissue DHT in turn regulates fat mass.

Polymorphisms and fat mass

Several measures of fat mass, including total body fat, trunk and arm fat, were associated with the UGT2B15 D^{85}Y polymorphism in young adult and elderly men (Paper I). In addition, the UGT2B17 deletion polymorphisms was associated with fat parameters, including total body fat, trunk fat, arm fat and central fat distribution in elderly men (Paper I). Since the UGT2B17 deletion polymorphism did not associate with fat mass in young adult men, we suggested that the effect of the polymorphisms on fat mass might be age-dependent. In elderly men, the UGT2B17 deletion polymorphism, but not the UGT2B15 D^{85}Y polymorphism, was a negative predictor of metabolic risk factors, such as serum levels of insulin and HOMA index. Since UGT2B15 is expressed in adipose tissue (58, 200) we hypothesize that a significant amount of the UGT2B15 action occurs in adipose tissue. Obviously, the polymorphism by itself might affect local androgens and their metabolites and secondarily fat mass. Since no studies have reported UGT2B17 expression in adipose tissue, it probably acts through another pathway.

We show in Paper I that the UGT2B15 D^{85}Y and the UGT2B17 deletion polymorphisms are associated with glucuronidated androgen metabolites, fat mass and metabolic risk factors. Possible mechanisms include that affected androgens and/or their metabolites have an effect locally on the adipose tissue where they are produced, which is supported by the concept of intracrinology. An effect on the feedback and serum levels of T, DHT or E2 is less plausible, since the serum levels of these sex steroids were not affected. Although less probable one cannot exclude the possibility that the androgen metabolites by themselves might regulate fat mass. Further studies are clearly required to increase the understanding of the mechanism behind the association seen between the UGT2B15 and UGT2B17 polymorphisms and fat mass. In contrast, the UGT2B7 H^{268}Y did not significantly associate with fat mass.

In conclusion, we determined that the UGT2B15 D^{85}Y and the UGT2B17 deletion polymorphisms were not only associated with glucuronidation of androgens and their metabolites, but also fat mass (Paper I). Therefore, we find it likely that UGT2B15, which is expressed in adipose tissue, acts locally, while the mechanism behind the association with UGT2B17 is less clear.

SUMMARY

- The UGT2B7 H^{268}Y, UGT2B15 D^{85}Y and UGT2B17 deletion polymorphisms are functional or in linkage with a functional polymorphism. UGT2B15 and UGT2B17 are involved in the glucuronidation of 3α-diol into 17G, while only UGT2B17 has the capacity to glucuronidate T. The glucuronidation of DHT is partly performed by UGT2B17. UGT2B7 is involved in the glucuronidation of 3α-diol into 3G (Fig. 8).

- Androgen metabolites, namely 3G and 17G, are more strongly associated to BMD than the bioactive androgens in elderly men.

- The UGT2B7 H^{268}Y polymorphism, but not the UGT2B15 D^{85}Y or the UGT2B17 deletion polymorphisms, is associated with cortical bone size in young adult men.

- The androgen metabolite 17G, and especially the 17G/DHT ratio, are directly related to fat mass and metabolic risk factors. The 17G/DHT ratio explained a substantial part of the variance of total body fat for young adult and elderly men (12% and 15%, respectively).

- The UGT2B15 D^{85}Y and UGT2B17 deletion polymorphisms are associated to fat mass and metabolic risk factors.

CONCLUDING REMARKS

Advances in a new area of endocrinology, called intracrinology, have followed the cloning and characterization of enzymes involved in sex steroid metabolism. The concept of intracrinology has emphasized the added value of measuring the phase II metabolites of androgens in serum, instead of only the bioactive androgens, since androgens made locally do not significantly diffuse into the circulation. With mass spectrometry it is possible to measure all the different metabolites of DHT in serum and urine. Since we have shown that the metabolites are stronger predictors of BMD and prostate volume in men than the bioactive androgens, we suggest that when assessing androgenic activity in man, the androgen metabolites and not only the bioactive androgens might be valuable to measure. In addition, the glucuronidated androgen metabolites might be used in the clinic as novel markers for changes in bone, fat and prostate tissue. The associations in this thesis will not change doctors' practice immediately, but can be expected over time to lead to improved understanding of mechanisms involved in for example male osteoporosis and/or obesity. Also, modulating the glucuronidation process by drugs might be a new future target for treating osteoporosis and metabolic risk factors. Due to the different substrate-and tissue-specificity of the enzymes, different effects might be obtained through modulating the different glucuronidation enzymes.

The effects of the genetic variations in glucuronidation enzymes may also be of importance from another clinical point of view. For example, the response to androgen therapy might depend on the subject's genetic background. In Caucasian men, the rate of prostate cancer is 2-40 times higher than in Asian men (155). It is interesting to note that the allele frequencies of some of the polymorphisms investigated in this thesis differ significantly between these two groups. The large genetic variation in androgen metabolizing enzymes within, and between, ethnic groups is not profoundly studied and future research will show the importance of this variation in health and disease. The polymorphisms might also be of importance for the pharmacokinetics of drugs since it has been seen that different variants of the polymorphisms can change the way drugs are metabolized (203-206). In this respect, genetic variations in enzymes involved in androgen metabolism are of great importance for the interpretation of doping test results. In the future, determining an athletes' UGT2B17 genotype might be performed to improve doping test sensitivity, making doping tests individual-based instead of population-based.

Future perspectives

In the future, studies related to the research in this thesis could be useful when developing new antidoping programs. This is especially true when it comes to research regarding UGT2B17 and the deletion polymorphism. It will be interesting to see how the glucuronidated androgen metabolites will be used in the clinic. Our results in this thesis indicate that the metabolites found in serum reflect the androgenic activity within the peripheral tissues better than serum levels of the bioactive androgens themselves. One might speculate that glucuronidated androgen metabolites might be useful as clinical markers of patients with risk for developing osteoporosis and prostate abnormalities. In addition to this, the glucuronidated androgen metabolites could possibly be used to indicate a patient's risk to develop the metabolic syndrome.

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