COMT GENOTYPE, SEX STEROIDS AND BONE PHENOTYPE IN MAN AND MICE

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ABSTRACT

Sex steroids are of profound importance for several physiological processes including reproduction, growth, and maintenance of skeletal integrity. Serum levels of sex steroids are associated with bone mineral density (BMD) and have been shown to be predictive of fracture risk in older people. Sex steroid levels in serum, and also BMD and fracture risk, are under genetic control. Catechol-O-methyltransferase (COMT) is an important estrogen-degrading enzyme. In the COMT gene there is a single nucleotide polymorphism (SNP), COMT val108/158met, differentiating three levels of activity: high ($COMT^{HH}$), intermediate ($COMT^{HL}$), and low ($COMT^{LL}$), as a result of lower enzyme activity of the Met variant.

The aim of the studies in this thesis was to investigate the role of COMT val108/158met for serum levels of sex steroids, skeletal phenotype, and fracture risk. Four human cohorts and one mouse strain devoid of COMT activity (COMT KO) were used.

In girls in early puberty, $COMT^{LL}$ was found to be associated with higher estradiol (E2) levels, increased longitudinal and radial cortical bone growth, and an earlier pubertal development compared with $COMT^{HH}$. Girls with the $COMT^{LL}$ genotype were 5.4 cm taller on average than girls with $COMT^{HH}$. Regression models indicated that most of the associations with pubertal development and growth were mediated through elevated levels of E2. This is plausible, because in theory the $COMT^{LL}$ genotype would be associated with higher E2 levels due to impaired degradation of estrogens. Increased longitudinal and radial cortical bone growth was also seen in COMT KO mice, compared with their wild-type siblings.

In young adult men, COMT genotype was found to be associated with BMD and it was also found to be a modulator of the positive associations previously found in these young adult men between physical activity (PA) and BMD. In general, the association between PA and BMD was stronger in the $COMT^{LL}$ genotype than in the $COMT^{HH}$ genotype. In elderly men, COMT genotype was associated with an increased risk of self-reported fractures during their lifetime. In addition, $COMT^{LL}$ was found to be associated with increased E2 levels in middle-aged men and a decreased risk of myocardial infarction (MI) in middle-aged men and women combined.

In conclusion, the findings in this thesis indicate that COMT may be implicated in several physiological processes including the regulation of timing of puberty and growth in young girls and female mice, bone phenotype in young adult men, fracture risk in elderly men, the incidence of MI in middle-aged individuals, and serum E2 levels in middle-aged men.

COMT-GENOTYP, KÖNSHORMONER OCH BENFENOTYP HOS MÄNNISKA OCH MUS

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Könshormoner har stor betydelse för många processer i kroppen, såsom fortplantning, tillväxt och skelettets bevarande vid högre åldrar. Könshormonnivåer i blodet är relaterade till bentäthet (BMD) och har visat sig kunna förutsäga frakturrisk hos äldre. Både könshormonnivåer i blod, BMD och frakturrisk påverkas av ärftliga faktorer. Katekol-O-metyltransferas (COMT) är ett enzym som deltar i nedbrytningen av östrogen. I COMT-genen finns en enbaspolymorfi (SNP) som resulterar i ett aminosyrabyte från valin till metionin (COMT val108/158met). Metioninvarianten ($COMT^{L}$) har en lägre enzymaktivitet än valinvarianten ($COMT^{H}$). Följaktligen finns hos människa tre olika aktivitetsnivåer när det gäller COMT – hög ($COMT^{HH}$), mellan ($COMT^{HL}$) och låg ($COMT^{LL}$).

Syftet med arbetena i den här avhandlingen har varit att studera betydelsen av COMT val108/158met för nivåer av könshormoner i blodet, skelettets egenskaper och frakturrisk. Fyra olika kohorter, och en musstam som saknar COMT (COMT KO) har använts.

Hos flickor som befann sig i de tidigaste faserna av puberteten sågs hos dem med $COMT^{LL}$ högre östradiol (E2) nivåer, en ökad längdtillväxt, en ökad radiell kortikal bentillväxt samt en tidigare pubertetsutveckling jämfört med flickorna som var av $COMT^{HH}$ genotyp. Flickor med $COMT^{LL}$ var i genomsnitt 5,4 cm längre än flickor med $COMT^{HH}$. Regressionsanalyser tydde på att det mesta av sambandet mellan COMT-genotyp, pubertetsutveckling och tillväxt förmedlades via förhöjda nivåer av E2. Detta verkar rimligt eftersom $COMT^{LL}$-genotypen teoretiskt sett borde vara associerad med högre E2 nivåer på grund av en försämrad östrogennedbrytning. Ökad längdtillväxt och ökad radiell bentillväxt sågs också hos möss som saknade COMT.

Hos unga män sågs samband mellan $COMT^{LL}$ och en lägre BMD, men inte med nivåer av könshormoner i blodet. COMT-genotyp påverkade också de positiva samband som tidigare setts mellan fysisk aktivitet och BMD hos de unga männen. På det hela taget var sambanden mellan fysisk aktivitet och BMD starkare hos $COMT^{LL}$ än hos $COMT^{HH}$. Bland äldre män sågs samband mellan $COMT^{LL}$ och en högre risk för frakturer, när information om sådana inhämtats från frågeformulär ifyllda av studiedeltagarna. Dessutom fanns samband mellan $COMT^{LL}$ och högre E2 nivåer hos medelålders män, och en minskad risk för hjärtinfarkt hos personer i medelåldern.

Sammanfattningsvis tyder reusltaten i den här avhandlingen på att COMT kan vara inblandat i flera fysiologiska processer såsom reglering av pubertetsstart och tillväxt hos unga flickor och honmöss, benfenotyp hos unga men, frakturrisk hos äldre män samt risken för hjärtinfarkt hos individer i medelåldern, och E2 nivåer hos medelålders män.

LIST OF PUBLICATIONS

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

I. Association between the low activity genotype of catechol-O-methyltransferase and myocardial infarction in a hypertensive population.
Eriksson AL, Skrtic S, Niklason A, Hultén LM, Wiklund O, Hedner T, Ohlsson C
European Heart Journal 2004 Mar; 25(5):386-91

II. The COMT val158met polymorphism is associated with peak BMD in men.
Lorentzon M, Eriksson AL, Mellström D, Ohlsson C
Journal of Bone and Mineral Research 2004 Dec; 19(12):2005-11

III. The COMT val158met polymorphism is associated with early pubertal development, height and cortical bone mass in girls.
Eriksson AL, Suuriniemi M, Mahonen A, Cheng S, Ohlsson C
Pediatric Research 2005 Jul; 58(1):71-7

IV. Association between physical activity and BMD in young men is modulated by catechol-O-methyltransferase (COMT) genotype: the GOOD study.
Lorentzon M, Eriksson AL, Nilsson S, Mellström D, Ohlsson C
Journal of Bone and Mineral Research 2007 Aug; 22(8):1165-72

V. The COMT val158met polymorphism is associated with prevalent fractures in Swedish men.
Eriksson AL, Mellström D, Lorentzon M, Orwoll ES, Redlund-Johnell I, Grundberg E, Holmberg A, Ljunggren Ö, Karlsson MK, Ohlsson C
Bone. 2008 Jan; 42(1):107-12

VI. Catechol-*O*-methyltransferase is a physiological regulator of bone growth and cortical bone dimensions in female mice.
Eriksson AL, Forsberg MM, Karayiorgou M, Gogos JA, Männistö PT, Ohlsson C
Manuscript

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LIST OF ABBREVIATIONS

AAM	age at menarche
aBMD	areal BMD
ANOVA	one-way analysis of variance
ADT	androsterone
AR	androgen receptor
ARE	androgen response element
BMC	bone mineral content
BMD	bone mineral density
Calex	Calcium and Exercise
CAPPP	captopril prevention project
CI	confidence interval
COMT	catechol-*O*-methyltransferase
$COMT^{H}$	COMT high activity
$COMT^{L}$	COMT low activity
COMT KO	*comt* disrupted mice
CVD	cardiovascular disease
CYP450	cytochrome p450
DHEA	dehydroepiandrostenedione
DHT	dihydrotestossterone
DNA	deoxyribonucleic acid
DXA	dual X-ray absorptiometry
E1	estrone
E2	estradiol
ERα/β	estrogen receptor α/β
ERE	estrogen response element
fE2	free estradiol
fT	free testosterone
GC-MS	gas chromatography/mass spectrometry
GH	growth hormone
GOOD	Gothenburg Osteoporosis and Obesity Determinants Study
GWA	genome-wide association study
HRT	hormone replacement therapy
HSD	hydroxysteroid dehydrogenase
IGF-1	insulin-like growth factor 1
LD	linkage disequilibrium
MB-COMT	membrane-bound COMT
Met	methionine
MI	myocardial infarction
MrOS Sweden	Osteoporotic Fractures in Men

nsSNP	non-synonymous SNP
OR	odds ratio
PA	physical activity
PCR	polymerase chain reaction
pQCT	peripheral quantitative computerized tomography
RNA	ribonucleic acid
S-COMT	soluble COMT
SHBG	sex hormone binding globulin
SNP	single nucleotide polymorphism
T	testosterone
UGT	uridine diphosphate glucurunosyltransferas
Val	valine
vBMD	volumetric BMD
WT	wild type

2ME2	2-methoxyestradiol
2ME1	2-methoxyestrone
2OHE1	2-hydroxyestrone
3α-DIOL	androstane-3α, 17β-diol
4ME2	4-methoxyestradiol

INTRODUCTION

GENERAL INTRODUCTION

In addition to being responsible for reproductive functions and the development of secondary sex characteristics in males and females, sex steroids are also involved in numerous physiological and pathophysiological processes in mammals, including growth and maintenance of skeletal integrity. Osteoporosis is a clearly sex steroid-dependent disorder involving low bone mass and increased skeletal fragility, leading to an increased risk of fractures. Fractures cause substantial morbidity and mortality, and constitute a major health problem. Levels of sex steroids can be measured in serum and have been shown to be predictive of fracture risk. Sex steroids are synthesized not only in the gonads but also in peripheral tissues, where they exert effects in the same cells in which their synthesis took place. Only small fractions of the peripherally synthesized sex steroids reach the circulation, and hence serum measurements poorly reflect the activity of peripherally synthesized steroids. Sex steroid levels and sex steroid-related disorders are under genetic influence. The mechanisms that are responsible for this influence have been poorly understood. A better understanding of these genetic mechanisms could give better risk estimates and help to improve prevention and treatment strategies, e.g. targeting of new drugs. Genes involved in the synthesis, degradation, and effects (i.e. receptor genes) of sex steroids are candidate genes for serum levels of sex steroids and for sex steroid related phenotypes and disorders. One such gene is the *COMT* gene which codes for catechol-*O*-methyltransferase, a protein involved in the degradation of estrogens.

MOLECULAR GENETICS

From DNA to protein

In the middle of the nineteenth century, Gregor Mendel discovered that traits can be inherited in units from parent to offspring. Later it was found that instructions for this heredity, and for the development and function of all animals and plants, reside within the deoxyribonucleic acid (DNA), which is located in the cell nucleus. DNA is made up of two long polymers running in opposite directions to each other. The polymers consist of units called nucleotides, which are made up of three joined structures: a nitrogenous base, a sugar, and a phosphate group. There are four types of bases; adenine (A), thymine (T), guanine (G), and cytosine (C). The polymers are connected to each other by pairing of these bases. T will always pair with A, and G will always pair with C. It is the sequence of the bases that makes up the genetic code, and it is thus very much the key to who we are. DNA is organized in pairs of chromosomes. One chromosome of each pair comes from the

mother and one from the father. Humans have 23 pairs; 22 pairs of autosomes (non-sex chromosomes) and one pair of sex chromosomes. Women have two X chromosomes and men have one X and one Y chromosome.

The genome is made up of all the DNA in the cell nucleus. In humans, the genome consists of approximately 3.1 billion base pairs. There are approximately 20,000 – 25,000 human genes, and they are estimated to make up < 2% of the base pairs in the human genome (1). Genes code for proteins, which are chains of amino acids, and they are the fundamentals of human life. The parts of the genome that do not code for proteins are called non-coding regions. Less is understood about these regions, but they are known to be of importance for the regulation of genes.

Transcription is the first step in the synthesis of proteins encoded by genes. In this process a complementary strand of ribonucleic acid (RNA) is synthesized from one of the DNA strands. RNA is a nucleic acid very similar to DNA, but it differs in that instead of thymine (T) it has the base uracil (U). In order for transcription to begin, a protein called RNA polymerase binds to the promoter, which is a DNA segment located just before the gene to be transcribed. In the meantime transcription factors are recruited; these modulate the transcription process, regulate the amount of RNA synthesized, and control the tissue-specific expression of genes. After the gene has been transcribed, the resultant RNA is spliced. This means that sequences within the gene that do not code for protein (introns) are removed and the coding sequences (exons) are joined together. The RNA is then translated into protein. During translation, the RNA is read in triplets by the protein building machinery. Thus, three consecutive nucleotides in the RNA, a so-called codon, code for one amino acid. There are 20 different amino acids, but there are many more possible combinations of nucleotides in triplets, so several different triplets can code for the same amino acid. Some codons are stop codons, which means that when they appear in the RNA the translation process is finished for that protein.

Genetic variation

It has previously been estimated that any two human genomes are about 99.9% identical, Recently, this has been challenged by the discovery of the so called copy number variations (CNVs), and we are probably a little less identical than 99.9% although at present the exact figure is not known (2). The remainder of the DNA that is not identical is what accounts for the heritable variation among individuals.

Differences between human genomes are due to changes in the DNA sequence, called mutations. A mutation that has a minor allele frequency of > 1% is called a polymorphism. Common polymorphisms include single nucleotide polymorphisms (SNPs), tandem

repeated segments (minisatellites, 0.1–20 kb; microsatellites 2–100 nucleotides), and segmental deletions/insertions/duplications including CNVs. The most common polymorphism is the SNP, which is a variation occurring when a single nucleotide, A, T, C, or G, differs between individuals, or between paired chromosomes in an individual. For example, in individual A a certain sequence might read TG**A**CT while in individual B the same sequence might read TG**G**CT. In this case, there is an SNP with two alleles: A and G. SNPs are estimated to occur every 1,000 basepairs (3). Because we carry one copy of each chromosome from each of our parents, we also have two alleles of each SNP. In the case of the A/G SNP, some individuals will inherit an A from both parents. They are *homozygous* for the A allele and they have the AA genotype. Others will inherit an A from one parent and a G from the other parent. They are *heterozygous* and have the AG genotype. Those who inherit a G from both parents are homozygous for the G allele and have the GG genotype.

The Single Nucleotide Polymorphism Database (dbSNP) is a public-domain archive into which newly discovered SNPs can be entered. The latest build of the database (April 2008) includes more than 14,700,000 human SNPs out of which nearly 6,600,000 have been validated by at least one more submission. Each SNP in dbSNP is given a reference (rs-number, for example rs4680). Some of the SNPs have a strong impact on phenotypic characteristics and disease susceptibility, and are the origins of many rare monogenic disorders (4). In contrast to the monogenic disorders, the common disorders such as osteoporosis and cardiovascular disease, that affect large numbers of individuals, are complex polygenic disorders. This means that probably a relatively large number of genes is involved, each with a small effect. Moreover, gene-gene and gene-environment interactions are likely to be of importance. This is valid also for many phenotypic traits such as bone mineral density (BMD) (5, 6). Most of the SNPs, however, probably do not contribute to phenotypic characteristics or disease susceptibility, and a central goal in genetic studies is to pinpoint the DNA variations that contribute most significantly to population variation in each trait.

SNPs can be located within either coding or non-coding regions of the genome. If present in a coding region of a gene, an SNP may give rise to a codon coding for an amino acid that differs from the original, so that there is a change in the protein sequence. This can lead to functional consequences and may affect factors such as protein stability, ligand binding and posttranscriptional modification (7). An SNP may also introduce a stop codon, which leads to a premature termination of translation. These SNPs are called non-synonymous (nsSNPs), or missense variants.

Because there is redundancy in that several different codons code for the same amino acid, an SNP in a coding region does not necessarily result in a change in amino acid. These

SNPs are said to be synonymous or silent. However, it has been shown that synonymous SNPs can affect splicing, mRNA stability, and folding of the protein (4, 8).

The vast majority of SNPs are located in non-coding regions (in introns or in the DNA sequences surrounding the genes). These can affect regulatory sequences such as promoters, enhancers, and silencers of gene transcription, transcription factor binding sites (9), or microRNA (10).

A certain combination of alleles on a chromosome is called a haplotype. Alleles situated within a short distance of each other tend to be inherited together from parent to offspring. When this happens, the alleles are said to be genetically linked with each other. Linkage disequilibrium (LD) is the non-random associations of alleles at two or more loci. This describes a situation in which some combinations of alleles occur more (or less) frequently in a population than would be expected from a random formation of haplotypes from alleles based on their frequencies. The degree of non-random associations between SNPs at different loci is measured by the degree of LD. There are several ways in which to describe LD. D' and r^2 are common measures and when D'=1 or r^2= 1 the loci are said to be in perfect LD (11).

Studying genetic variation

In the identification of genes involved in rare monogenic disorders such as cystic fibrosis, the genetic *linkage study* has been very successful. No previous knowledge about the biology of the disease is needed and the approach is hypothesis-free. In sets of families where individuals affected by the disease of interest are found, genetic markers (microsatellites, SNPs) evenly spread throughout the genome are analyzed. Regions suspected of having some relation with the disease are pinpointed, and through a more dense mapping of these regions the specific gene and the specific mutation responsible for the disease can be found. However, in the case of complex polygenic disorders this method has not proven to be very successful.

In contrast to the linkage study, the *association study* is based on previous knowledge and hypotheses regarding genes that could be involved in the pathogenesis of a disease or a phenotypic trait. Due to limitations in genotyping technology, the early association studies included one or two SNPs while recent studies have involved hundreds of SNPs in many genes. In case-control studies a group of cases affected by the disease and a group of controls are genotyped and the allele frequencies are compared, as exemplified by the study of MI patients in Paper I in this thesis. Alternatively, a homogenous group (cohort) of individuals can be genotyped and the association between specific alleles of the candidate SNP and a continuous trait such as serum estradiol levels or BMD, are

calculated. Papers II, III and IV in this thesis are examples of this. If the cohort is large enough and a disease or an event of interest is common enough, the cohort can also be used for the study of the associations between this disease and an SNP. An example of this is Paper V in this thesis where the MrOS Sweden cohort was used for the study of occurrence of fractures. In most areas of research, association studies have yielded conflicting results. This could be due to small sample sizes, heterogenous populations, non-standardized phenotyping, and/or variations in study design. There is probably also significant publication bias, which means that studies showing an association are more likely to be published than studies not showing an association.

The development of genotyping technologies has been very rapid. Array-based chips make it possible to genotype more than 1,000,000 SNPs in large cohorts in just a few weeks. This has allowed the introduction of *genome-wide association studies* (GWAS). GWAS use dense SNP maps that cover the human genome to look for differences in allele frequency between cases and controls or associations between an allele or a genotype and a phenotype. GWAs are similar to the linkage studies in that they are not hypothesis-driven, but they differ in the number of genetic markers analyzed. When associations have been found in GWAs, the next step is to try to confirm these in other cohorts (12).

Because of genetic linkage, an association found between an SNP and a phenotype or a disease does not necessarily mean that that particular SNP is implicated functionally in the disease or phenotype. It may well be that it is just linked to a genetic variation with functional significance.

SEX STEROIDS

Androgens (e.g. testosterone (T), dihydrotestosterone (DHT)) and estrogens (e.g. estrone (E1), and estradiol (E2)) constitute the sex steroids. DHT is more potent than T and E2 is more potent than E1. Sometimes progesterone is also included as a third class of sex steroids, distinct from androgens and estrogens. Serum levels of sex steroids are influenced by both genetic and environmental factors (13, 14).

Synthesis and degradation

Synthesis of sex steroids

All the sex steroids are derived from cholesterol. Specific synthetic machinery, including members of the cytochrome P450 (CYP450), 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-HSD families, catalyzes the various steps of sex steroid formation. Aromatase,

which is encoded by the CYP19 gene, catalyzes the aromatization of androgens to estrogens and is the rate-limiting enzyme in the biosynthesis of estrogens (15).

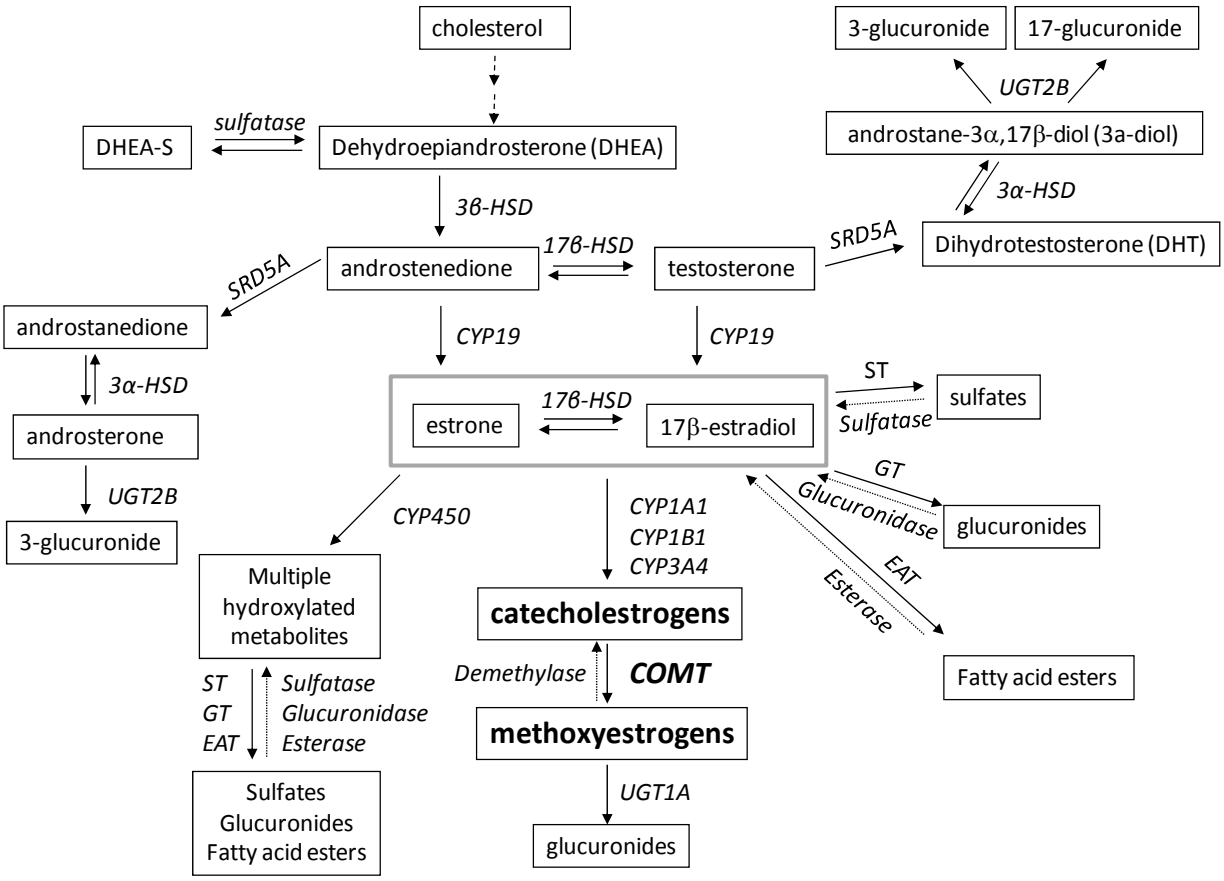

Fig 1. Synthesis and metabolism of sex steroids. ST=sulfotransferase. GT=glucurunosyltransferase, EAT=estrogen acyltransferase for fatty acid formation, HSD=hydroxysteroid dehydrogenase, SRD5A=steroid 5alpha reductase

Degradation of androgens

Degradation of sex steroids takes place in the liver and in the peripheral tissues. Androgens are mostly metabolised by the phase I enzymes 3α-, 3β- and 17β-HSD to compounds with essentially no androgenic activity (e.g. androsterone (ADT) and androstane-3α,17β-diol (3α-DIOL)). In most tissues there are HSD isoforms capable of back-converting these metabolites, so this is probably one of the mechanisms by which the tissues regulate their local levels of androgens. Most phase I metabolites are subsequently glucuronidated (phase II reaction) by members of the UDP-glucuronosyltransferase (UGT) family, and then excreted in the urine. UGT2B7, UGT2B15, and UGT2B17 are thought to be the major isoenzymes that conjugate androgens in humans (16).

Oxidative metabolism of estrogens

Estrogens undergo extensive oxidative metabolism. E2 is readily converted to E1. Back-transformation to E2 occurs but is slower, and in many cases the first step in the metabolism of E2 is oxidation to E1 (17). The major enzymes responsible for the subsequent oxidative metabolism are members of the CYP450 family, many of which show selective catalytic activity for regio-specific hydroxylation. The 16α-hydroxylation and formation of catechol estrogens (2- or 4-hydroxylation), are the best characterized pathways, but hydroxylation at other sites also occurs (18, 19). 16α-hydroxylated estrogens retain estrogenic activity and activate estrogen receptors (ERs). 16α-hydroxyestrone is equivalent to E2 in uterotropic potency in some studies, and it can be excreted in the urine or degraded further to 16α-hydroxyestradiol (estriol). Estriol is used clinically to treat vaginal atrophy and urinary tract infections in postmenopausal women. When constantly present in target organs estriol has a potency similar to that of E2, but the half-life of the binding to ERs is much shorter and its potency is classified as low when used clinically. Estriol is considered to be a terminal product of estrogen metabolism (20, 21).

2-hydroxylation takes place mainly in the liver but also in the peripheral tissues (22). CYP1A2 and CYP3A4 are the major enzymes responsible for this reaction. The peripheral tissues are the main site of 4-hydroxylation, with CYP1B1 being the major enzyme (19). 2-hydroxylated (2-OH) metabolites can bind to the ERs but have a reduced receptor affinity and hormonal potency compared to the parent substances. 2-hydroxyestrone (2OHE1) has been shown to partially antagonize the growth-stimulatory effects of E2 in MCF-7 breast cancer cells. Several physiological functions of 2-OH metabolites have been proposed, but they do not possess carcinogenic activity (22). 4-OH metabolites, on the other hand, have been associated with cancer development in studies on both humans and animals. It has been proposed that both receptor-mediated and non-receptor-mediated pathways are involved. 4-OH metabolites have an affinity for ERs and a hormonal potency similar to that of the parent hormone (22). Moreover, 4-OH metabolites can be converted to estrogen quinones. These are capable of forming stable depurinating DNA adducts that may ultimately lead to cancer development (23).

O-methylation and methoxyestradiols

Catecholestrogens are rather short-lived compounds that can be rapidly methylated by the catechol-*O*-methyltranferase (COMT) enzyme to form 2- and 4-methoxyestrogens. Some demethylation of methoxyestrogens occurs releasing cathecolestrogens *de novo.* 2-methoxyestradiol (2ME2) and 4-methoxyestradiol (4ME2) bind to ERs but with a very much lower affinity than E2 (24, 25). 2ME2 has unique biological effects, but most of them seem to be independent of ERs (26, 27). It has been shown that non-uterotrophic

doses of 2ME2 inhibit ovariectomy-induced bone loss (28), as well as longitudinal bone growth in rats (29). It has also been reported that 2ME2 possesses anti-atherogenic effects in mice (30). In addition, 2ME2 has antiangiogenic activity *in vitro* and *in vivo* and shows strong anti-proliferative activity in a variety of human cancer cell lines (31). It is currently being evaluated in multiple tumor types in phase II clinical trials (26). 2ME2 is to a large extent oxidized in the 17-position to form 2-methoxyestrone (2ME1), which is at least 10 times less active than 2ME2 (32). Both 2ME2 and 2ME1 are glucuronidated by members of the UGT1A family (33). Whether or not 4-methoxyestradiol possesses unique biological properties is not known at present.

Conjugation of estrogens

Hydroxylated and *O*-methylated metabolites, as well as the mother compounds E2 and E1, can be conjugated to glucuronides and sulphates. These conjugated metabolites do not possess estrogenic activity and are excreted in the urine. They can also be enzymatically deconjugated to release biologically active substances *de novo*. Estrogens can also be converted to fatty acid esters, which do not have estrogenic activity. These are very lipophilic substances and reside mainly in fatty tissues. They serve as a reservoir as de-esterification can occur and active hormone is released (22).

Intracrinology

Sex steroids are synthesized in the gonads. In primates including humans, the sex steroid precursor dehydroepiandrosterone (DHEA) and its sulfate DHEA-S, are also synthesized and secreted in large amounts from the adrenals. Cells in a wide range of tissues possess the synthetic machinery necessary to convert these precursors to androstenedione (4-dione) and then into potent androgens and estrogens. Degradation of locally formed sex steroids also occurs in the peripheral target cells. Thus, the target tissues can regulate their local steroid environment. This pathway is of wide significance. For instance, it has been reported that nearly 100% of estrogen synthesis after menopause occurs peripherally. In men, the contribution of peripherally synthesized steroids is smaller but still very significant. For example, 50% of androgens in the prostate are made locally. Adrenal secretion of DHEA and DHEA-S reaches peak values between the ages of 20 and 30. Thereafter, levels of DHEA and DHEA-S decline. At 70 years of age, serum levels of DHEA and DHEA-S are approximately 20% of their peak values, and at ages 85–90 as little of 5% of peak values may remain. The reduced amount of precursors available leads to a substantial fall in the formation of androgens and estrogens in the peripheral tissues (34).

Effects of sex steroids

There is growing evidence to suggest that there are several distinct pathways by which sex steroids and their receptors may regulate biological processes

The classical direct genomic pathway

This pathway involves binding of sex steroids to receptors. At present, there is one known androgen receptor (AR) and two known estrogen receptors (ERα and ERβ), which are all members of the nuclear hormone receptor superfamily. Androgens can exert their effects either directly via the AR, or, after aromatization to E2, via the ERs. Sex steroids act as ligand-activated transcription factors. After binding of a ligand, the receptor undergoes a conformational change, dimerizes with another receptor, and moves into the cell nucleus. Cofactors are recruited and the receptor complex binds to estrogen response elements (EREs) or androgen response elements (AREs) in target genes, whereby transcription is regulated. Depending on the ligand (e.g. endogenous hormone, synthetic hormone, or antagonist) different conformational changes occur and different cofactors are recruited, resulting in distinct effects on transcription. This is the most studied and best understood pathway (35, 36).

The non-classical indirect genomic pathway

After binding of a steroid to the receptor, it can interact with other transcription factors, which in turn bind to the DNA and regulate transcription. Thus, this pathway involves gene regulation by indirect DNA binding (35-37).

The non-genomic pathway with rapid effect

This mechanism involves activation of a receptor, possibly associated with the cell membrane. It might either be a classical sex steroid receptor, or another as yet unknown receptor, or some other structure such as an ion channel. In the case of estrogens, G protein-coupled receptor 30 (GPR30) has been proposed to be a mediator of the rapid effects. This initiates signaling cascades via second messengers, resulting in rapid physiological responses (ion channels and nitric oxide) without involving gene regulation. These rapid effects occur within seconds or minutes after addition of E2 or AR. (35, 36)

The ligand-independent pathway

Signaling through this pathway occurs when growth factor signaling leads to activation of kinases that may phosphorylate and thereby activate receptors or associatied coregulators in the absence of ligand. This pathway involves gene regulation (35, 36).

Binding to plasma proteins

T and E2 are transported in the blood, bound to plasma proteins. The most important proteins are albumin, to which T and E2 are bound in an unspecific manner, and sex hormone binding globulin (SHBG), to which they are bound in a specific way. Only a small proportion (a few per cent) of total T and E2 in the circulation is not bound to plasma proteins, and this constitutes the free fraction. The fraction bound to albumin plus the free fraction is considered to be the biologically active fraction, or non-SHBG-bound fraction (38). This is because T and E2 have relatively high binding affinities for SHBG, and SHBG is too large to cross the capillary barrier. Thus, SHBG-bound sex steroids are prevented from entering target cells. This is slightly more complicated at the cellular level in specific tissues, but the non-SHBG-bound fraction has been shown to be more strongly correlated to muscle mass, strength, and BMD than total levels of sex steroids (39, 40). Serum levels of SHBG are influenced by nutritional, hormonal and metabolic factors (41). In men, but not in women, there is a marked increase in SHBG levels with age, and as a result of this levels of bioactive sex steroids decrease much more than total levels of sex steroids (42). SHBG levels are under genetic influence, and it has been estimated from twin studies that as much as 60% of inter-individual variation in SHBG levels can be accounted for by genetic factors (13, 14). We have previously shown that rs1777941, which is a G/AN SNP located in the promoter region of the SHBG gene, is an independent predictor of SHBG levels in young adult (the Gothenburg Osteoporosis and Obesity Determinants (GOOD) study) as well as in elderly (the Osteoporotic Fractures in Men (MrOS Sweden) study) Swedish men. Carriers of the GG genotype had 24.6% and 22.2% higher SHBG levels than carriers of the AA genotype, in GOOD and MrOS respectively. Interestingly, carriers of the GG genotype also had higher levels of T and glucuronidated androgen metabolites (43).

Serum levels of free or bioavailable sex steroids can be estimated either through measurements of the free or bioavailable fraction in serum, or by the use of theoretical calculations. A method for calculation of free T (fT) based on mass-action equations, taking the concentrations of total T, total E2, and SHBG into account, and assuming a fixed albumin concentration of 43 g/l, correlates very well with direct measurement of fT using equilibrium dialysis. Moreover, levels of fT and bioactive T, determined both by direct methods and by calculations, are highly correlated (38).

COMT

Catechol-*O*-methyltransferase (COMT) catalyzes the transfer of a methyl group from S-adenosyl-L-methionine (AdoMet) to one of the hydroxyl groups in a catechol substrate in the presence of Mg^{2+}. Important substrates for COMT in mammals include the catecholamines, the catecholestrogens, the xenobiotics, and a multitude of drugs (44).

COMT is encoded by one single gene located on chromosome 22 (at 22q11.21). Through the use of alternative translation initiation sites and promoters, two COMT proteins are formed: soluble COMT (S-COMT) and membrane-bound COMT (MB-COMT) (45). In humans, S-COMT and MB-COMT contain 221 and 271 amino acids respectively, and the differences between S-COMT and MB-COMT reside within the N termini. COMT has been found in a wide range of human tissues such as liver, kidney, gastrointestinal tract, spleen, pancreas, lung, eye, brain, heart, and erythrocytes (22). COMT is also expressed in the hypothalamus and the pituitary (46), as well as in the ovary (47). Recently, it was found that COMT is expressed in osteoblastic cell lines, indicating that it is also expressed in bone cells *in vivo* (48). In most tissues the majority of COMT present is S-COMT, but in the brain 70% of total COMT is MB-COMT (49).

In codon 4 of the human COMT gene, there is a functional G to A single nucleotide polymorphism. This results in a valine to methionine amino acid substitution at codon 108 (in S-COMT) or 158 (in MB-COMT), (COMT val108/158met, rs4680), giving three levels of activity - high ($COMT^{HH}$), intermediate ($COMT^{HL}$) and low ($COMT^{LL}$), (50, 51), as a result of thermolability of the Met variant, even at 37º C. The activity of COMT has been reported to fluctuate by about 40% due to this polymorphism (52), while in earlier studies as much as a 2-4 fold difference in enzyme activity was reported by some groups (53, 54). This could be due to the use of different methodologies.

The frequency of the A allele (methionine, $COMT^{L}$) differs in populations of different ethnic origin. For example, the A-allele frequency was found to be 0.18 in a population of Han Chinese and in a Finnish population it was found to 0.58 (55). In a recent study of a Dutch population, the A allele frequency was reported to be 0.55 (48)

Because of the involvement of COMT in the metabolism of estrogens, and because of the functional nature of the val108/158met polymorphism, it is a candidate SNP for hormone related phenotypes and disorders. COMT val108/158met has already been investigated in more than 40 studies in relation to breast cancer, but the results have been conflicting (56-58).

COMT metabolizes catecholamines in glial cells and postsynaptic neurons (44). Peripherally, COMT genotype does not appear to be of importance for catecholamine

levels because neuronal uptake and degradation by the monoamine oxidase (MAO) enzyme compensate for pharmacological inhibition of COMT (44). In contrast, in the brain there is accumulating evidence that COMT plays a significant role in dopamine (DA) metabolism in the prefrontal cortex (59). In an autopsy, study Akil et al. found increased levels of tyrosine hydroxylase (TH) mRNA levels, which is an indicator of DA synthesis, in $COMT^{HH}$ individuals (60). DA is involved in cognitive function, and promising results were initially presented on the association between COMT genotype and cognitive function. Even so, a recent meta-analysis investigating several measures of cognition could only find an association with one (i.e. IQ), the effect size being rather modest (61). Associations between COMT val108/158 met and various psychiatric disorders have also been investigated in a large number of studies, mostly with conflicting results (62).

THE SKELETON

The skeleton serves the purpose of offering support to the body, of protecting the inner organs, and of acting as an attachment for the muscles. It is also a reservoir of calcium and phosphate ions.

About 70% of the bone mass is composed of inorganic material, and 95% of this is hydroxyapatite. Organic material makes up about 20% of the bone mass and 98% of this is type I collagen and other proteins such as osteocalcin, bone sialoprotein, and osteonectin. The remainder of the organic fraction consists of cells. Osteoblasts, osteoclasts, and osteocytes are the major cell types in the bone. Osteoblasts are of mesenchymal origin and responsible for bone formation. Osteoclasts are of hematopoietic origin and responsible for bone resorption. Osteocytes represent the terminal differentiation stage of the osteoblasts and they are involved in the support of bone structure and metabolic functions. Five to eight per cent of the bone mass is water.

Anatomically there are two main types of bone: flat bones such as the scull, mandible, and scapula and long bones such as the femur, tibia, and radius. Long bones have the shape of a hollow tube (shaft or diaphysis) which widens at the ends to form the metaphyses and the epiphyses. The growth plate is the border between the former two.

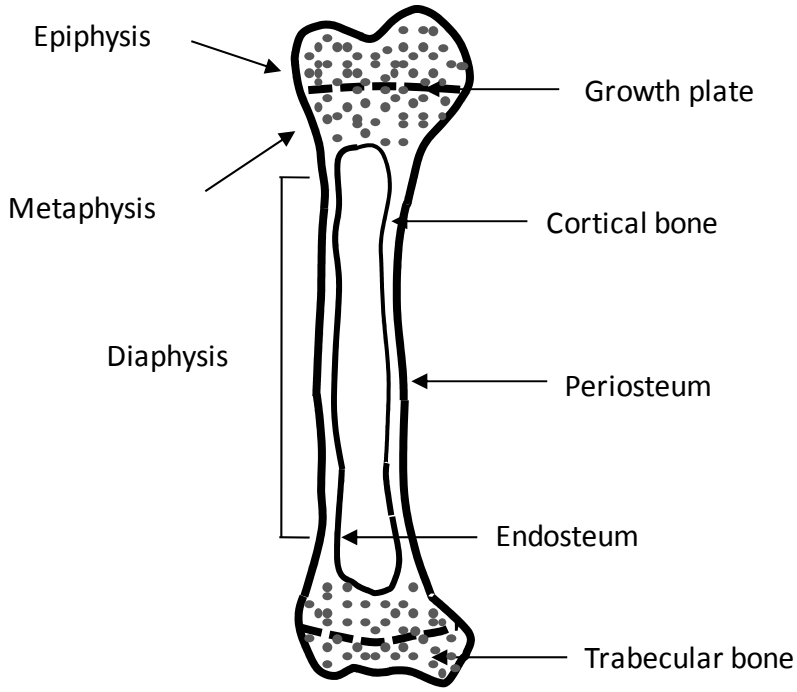

Fig 2. Schematic view of a longitudinal section through a long bone.

Two biologically different types of bone can be distinguished: cortical (compact) bone and trabecular (spongy) bone. Cortical bone is found mainly on the outside of the long bones and makes up 80% of the skeleton. Cortical bone mainly has a mechanical and protective role. The trabecular bone makes up 20% of the bone mass. It is found mainly in the vertebrae and the pelvis, and also in the metaphyses of the long bones. Due to its spongy appearance, the surface area is very large and the trabecular bone is much more active metabolically than the cortical bone. As a consequence of this, trabecular bone is generally more sensitive to external stimuli such as medications than cortical bone. On the outer surface, bones have a fibrous sheath called the periosteum, which contains blood vessels that nourish the bone, nerve fibers and bone cells. On the inner surface there is also a fibrous sheath called the endosteum. It contains blood vessels and bone cells (63).

Bone growth

Growth and maturation of children and adolescents are governed by nutritional, hormonal, and genetic factors, which work independently or together. Thyroid hormones and the growth hormone/insulin like growth factor (GH/IGF) axis are the main hormones that control prepubertal growth, which is a rather stable process (64). On the other hand, at the start of puberty there is a rapid increase in growth velocity: the pubertal growth spurt. With the onset of puberty, the hormonal regulation of growth becomes increasingly more complex. Reactivation of secretion of gonadotrophin-releasing hormone (GnRH) from the hypothalamus is mandatory for the initiation of puberty (65). This leads to a progressively

increased secretion of gonadotrophins (LH, FSH) from the pituitary, which in turn results in elevated levels of gonadal steroid hormones. These gonadal steroids are responsible for the sexual maturation and the development of secondary sex characteristics, and they also play a major role in pubertal growth, either in concert with or independent of other hormones. For example, estrogens stimulate the secretion of GH (66), which in turn promotes the secretion of IGF-I both locally in bone and in the liver (67).

In humans the pubertal growth period is crucial for bone accretion. Previous studies have shown that areal BMD (aBMD) increases by 40-50% during puberty (68-70). Peak bone mass is the maximum bone mass attained during growth. It is also an important determinant of developing osteoporosis later in life, because at any time bone mass and BMD are functions of peak bone mass and age-related bone loss. Peak bone mass is also influenced by genetic factors; some studies have suggested that as much as 70% of the interindividual differences are due to genetic factors (6, 71, 72).

Bone growth at puberty is both longitudinal and radial. Longitudinal growth is accomplished throughout endochondral bone formation at the growth plates. E2 stimulates longitudinal growth during puberty and is also necessary for the final closure of the epiphyseal growth plates, and thus the cessation of longitudinal growth, in both males and females (73, 74). Radial growth is accomplished through periosteal apposition, and subsequent endosteal resorption. Men gain more bone than women during puberty and several studies have shown that greater periosteal expansion in men than in women accounts for this sex-based difference (75). During early puberty the increase in bone size predominates in both boys and girls, whereas there is very little increase in trabecular and cortical volumetric BMD (vBMD). The increase in vBMD, which results from accrual of bone mineral, comes in the later stages of puberty (76, 77).

Boys have two more years of prepubertal growth, because of their later puberty than girls, and their pubertal growth spurt lasts for 4 years rather than the 3 years it lasts in females (78). As a result of this, adult males generally have longer and wider bones than adult females. Previously it was believed that androgens are responsible for bone growth in males and that estrogens are responsible for bone growth in females. However, the importance of E2 for growth also in males was understood after the presentation of a case report of a man who was homozygous for a lack-of-function mutation in the ERα gene (74), and case reports of men with complete aromatase deficiency. These men had unfused epiphyses and marked osteopenia (79, 80). In the case of aromatase deficiency, a normal male skeletal phenotype results after treatment with E2 (80). Data from males with androgen insensitivity indicate that lack of androgens leads to a reduced BMD, and it seems reasonable that both estrogens and androgens are needed for optimal bone growth and mineral accrual (81). Most studies have shown that at the age of peak bone mass, there is no association between serum E2 levels and aBMD of the spine, which is considered to

be an estrogen sensitive bone compartment (82, 83). Moreover, in the GOOD study, free estradiol (fE2) was not found to be associated with trabecular vBMD of the radius or the tibia, but fE2 was positively associated with cortical vBMD. Free T was found to be associated with measures of size such as cortical cross sectional area, periosteal circumference, and periosteal circumference (82).

Age-related bone loss

It has been estimated that with advancing age, men lose up to 1% of their BMD per year (84, 85). Previously it was thought that bone loss begins at menopause in women and even later in life in men (78). This was because at that time only cross-sectional studies using dual X-ray absorptiometry (DXA), which cannot discriminate between trabecular and cortical bone and is unable to give relevant information on changes in bone size and geometry, had been performed. This was also in line with the prevailing idea that loss of estrogens after menopause in women and age-related factors in men were the major causes of age-dependent bone loss. However, recently this notion has been challenged. In a longitudinal study by Nordström et al, peak aBMD of the proximal femur in young men was attained at the age of 19 years, and immediately after that there was a substantial loss of BMD (0.02 g/cm^2 per year) in the following five years (86).

The introduction of new technologies such as quantitative computerized tomography (QCT) has also given us a better understanding of age related bone loss. It is now believed that trabecular bone loss starts in early adulthood in both women and men. In a semi longitudinal study by Riggs et al., 37% and 42% of total life trabecular bone loss (lumbar spine, distal radius and distal tibia) in women and men, respectively, occurred before the age of 50. In men, the rate of trabecular bone loss peaked at around the age of 35-40 (87). Women have a phase of accelerated trabecular bone loss around menopause. Loss of cortical vBMD is, on the other hand very slow in young adulthood and accelerates in mid-life, in men possibly even later (87, 88).

Periosteal apposition continues throughout life. On the endosteal side the cortex is resorbed and, because resorption is greater than apposition, the net result is a decrease in cortical area. This leads to an outward displacement of the cortex, which, because of mechanical laws, makes the bone stronger and more resistant to bending forces. This partially compensates for the loss of bone strength resulting from the reduction in cortical area (88, 89).

In men, serum levels of sex steroids decrease slightly with ageing, but, more importantly, serum SHBG levels more than double in men from young adulthood to old age. As a consequence of this, there is a marked reduction in levels of fT and fE2 during this time

(42). In numerous cross-sectional studies using DXA, it has been shown clearly that fE2 is an independent predictor of BMD in men of varying ages, while conflicting results regarding T have been shown; thus its role has been less clear (39, 42, 90, 91). However, we recently showed in our large cohort of elderly men (n=2,908, MrOS Sweden study) that both fT and FE2 are independent predictors of BMD in elderly men (92). Longitudinal studies have also demonstrated that there is a negative association between age-related bone loss and fE2 (93, 94). In a longitudinal study using QCT, Riggs et al. found that the late loss of cortical vBMD (at ≥ 50 years of age) was negatively associated with fE2, while the late trabecular loss was negatively associated with both fE2 and fT. For the early trabecular loss, however, no associations were found with the levels of sex steroids, but there were suggestions of an involvement of the IGF-I system (87).

Finally, we have shown that the glucuronidated androgen metabolites androstane-3α,17β-diol-3glucuronide (3G) and androstane-3α,17β-diol-17glucuronide (17G) are stronger predictors of BMD than testosterone in a sub-sample from the MrOS Sweden study (n = 631), which lends support to the notion that intracrinology is of importance for bone health and that measurements of serum levels of sex steroids are insufficient when trying to understand the regulation of bone metabolism by sex steroids (95).

Osteoporosis and fractures

Fracture incidence has a bimodal pattern with two peaks. The first peak occurs in childhood and adolescence, and the second one occurs in old age (96). There are data to suggest that there is an inverse correlation between childhood fractures and BMD (97). It has been shown that before the age of 50 years, men have more fractures than women. This is probably related to differences in the kinds of trauma that affect men rather than women, such as those from more extreme sports activities, fights, and work-related injuries. After the age of 50, women have more fractures than men but there is an increase in fracture incidence with advancing age in men also (81).

Osteoporosis is a skeletal disorder characterized by low bone mass and microarchitectural deterioration of bone, resulting in an increase in bone fragility and susceptibility to fractures (98). Traditionally, it has been considered a disease of postmenopausal and elderly women, but it is now well recognized that osteoporosis is a major health problem in both sexes. Fractures represent the primary clinical consequence of osteoporosis. In Sweden, the lifetime risk of a hip, spine, or forearm fracture (which are common osteoporosis related fractures), at the age of 50, is 46% for women and 22% for men (99). Fractures are associated with increased morbidity and mortality. This is especially true for hip fractures. Men have poorer outcome after a hip fracture than women. It has been

estimated that 1-year mortality in men after a hip fracture is 30-35%, and that 50% may need institutionalized care (81).

BMD is an important predictor of fractures, but it should be kept in mind that the risk of sustaining a fracture is dependent on both bone strength and the amount of force applied to the bone. Thus, elderly individuals with unintentional falls are more likely to have fractures than individuals who do not fall, and risk factors for falls such as poor vision or certain medications are also risk factors for fractures.

Regarding sex steroids and fracture risk, data from prospective studies have been conflicting: either showing independent associations with E2 (100), or T (101), or neither of them (102). This could be due to lack of power, or due to the use of immunoassay-based techniques for measurement of sex steroids. Recently, however, it was found that in the MrOS Sweden cohort both fT and fE2 were associated with incident fractures, but only fE2 was an independent predictor. The inverse relationship between fE2 and fractures was nonlinear with a strong relationship at 0.27 pg/ml, corresponding to E2 levels of 16 pg/ml (103). This supports the concept of a threshold E2 level for skeletal health in men which has been proposed by others (40, 93). Interestingly, SHBG levels were independently positively associated with fractures in MrOS Sweden. The number of fractures in this study was relatively high (n = 209), and follow-up of all study subjects was complete. Moreover, sex steroid levels were measured with the gas chromatography/mass spectrometry (GC-MS) technique, which is not associated with questionable specificity at lower concentrations (as described for previously used immunoassay-based techniques) (103).

MYOCARDIAL INFARCTION

Myocardial infarction (MI) is most often caused by complete epicardial coronary artery occlusion from plaques vulnerable to erosion or rupture (104). This thrombotic process diminishes microcirculatory perfusion by reduced coronary artery flow through epicardial stenoses, as well as by distal embolisation of thrombi. Other causes of MI include coronary spasm, emboli, or dissection of the coronary arteries (105). In a minority of patients, angiographically normal coronary arteries are found despite there being elevated levels of biomarkers indicative of MI (106).

Even though there have been considerable improvements in risk factor reduction, prevention, and treatment in the last few decades, MI remains a major cause of morbidity and mortality worldwide (107, 108). Early pre-hospital ventricular fibrillation accounts for the majority of deaths in patients with acute MI (109). Heart failure, mechanical complications, and ventricular arrhythmias are common causes of death in hospitalized patients with MI (110, 111). After hospital discharge there can be an increased risk of

death due to heart failure, recurrent myocardial infarction, or sudden cardiac death (111-113). Thus, prevention of MI still remains an important issue.

MI is a complex disorder with a strong genetic basis (114). Many genes (each with a relatively small effect) are thought to be involved, and gene-gene and gene-environment interactions are probably of importance (5, 115). Identification of genetic risk factors could lead to better risk estimates and the possibility of better direct prevention and therapy (116). Moreover, elucidation of the genetic background could reveal pathways that might be of special interest regarding development of new strategies for prevention and treatment.

Cardiovascular disease, myocardial infarction, and sex steroids

Cardiovascular effects of estrogens are complex and include interactions with vascular endothelium, smooth muscle cells, coagulation factors, blood lipids, and platelet aggregation (117). In experimental studies, estrogens have been shown to protect mice of both genders from atherosclerosis (118). There are data suggesting that some of the atheroprotective effects seen may be mediated by the metabolite 2-methoxyestradiol (27, 30). In humans there have been conflicting results suggesting both protective and adverse effects of E2 in atherosclerotic disease and cardiovascular outcomes.

In general, women experience cardiovascular disease (CVD) 5-10 years later than men. It has been postulated that this may be related to cardiovascular protection from estrogens, a protection that is lost when estrogen levels fall after menopause (119). However, no break-point in female cardiovascular risk at the age of menopause has been identified (120), which is in contrast with other endpoints that are definitely estrogen-dependent such as breast cancer and BMD (36). Still, a large number of observational studies have shown positive effects on cardiovascular risk when estrogens are replaced pharmacologically after menopause (121, 122), but a later large randomized controlled trial indicated the opposite: a slightly increased risk of CVD in users of hormone replacement therapy (HRT) (123). More recent studies have shown divergent results in women of different ages, and one could thus speculate that the effects of estrogens are dependent on the stage of the atherosclerotic process with positive effects at the early stages and detrimental effects later on in the process (124). Dose, type of estrogen (17β-estradiol, conjugated estrogens), mode of administration (oral versus transdermal) and type of progestogen are matters still under debate meriting further investigation.

Conflicting data have been presented regarding associations between serum levels of sex hormones and cardiovascular disease in men, a fact that might be related to immunoassay-based techniques with questionable specificity at lower concentrations, study design and

inadequate power. Recently, a large study showed a lower incidence of CVD in men with higher E2 levels (125). Other studies have reported the opposite, with positive associations being found between E2 levels and progression of intima media thickness of the carotid artery (126) as well as peripheral arterial disease of the lower extremity (PAD) (127).

A single case report of a 31-year-old man homozygous for a disruptive mutation in ERα exists. This individual had early atherosclerosis and endothelial dysfunction (128, 129), indicating that a complete lack of ERα signaling has rather negative effects on the cardiovascular system.

Pharmacological treatment with estrogens has also been tried in men. An early study of administration of high doses of conjugated estrogens (5 mg/d) doubled the risk of MI in high-risk patients (130). A study involving administration of lower doses of estrogens in healthy elderly men showed positive effects on lipid profiles without affecting markers of thrombotic risk (131).

AIMS OF THE THESIS

The general aim of this thesis was to gain a better understanding of the implications of a genetically altered COMT activity for sex steroid serum levels and sex steroid related phenotypes.

The specific aims were to investigate the relationship between:

I) COMT and serum estradiol (E2) levels (papers I and III);

II) COMT and bone phenotype in females (papers III and VI);

III) COMT and bone phenotype in males (papers II, IV and V);

METHODOLOGICAL CONSIDERATIONS

HUMAN COHORTS

Table 1 Characteristics of the study subjects

	CAPPP	GOOD(1)	GOOD(2)	Calex	MrOS Sweden
Number of subjects	522	458	1068	246	2822
Age (years)	57.0 ± 6.6	19.0 ± 0.6	18.9 ± 0.6	11.2 ± 0.8	75.4 ± 3.2
Male sex (%)	74.1	100	100	0	100
Smokers (%)	37.4	9.2	8.7	-	8.4
Height (cm)	172.7 ± 8.3	181.1 ± 6.8	181.4 ± 6.8	145.6 ± 8.0	174.8 ± 6.5
Weight (kg)	82.1 ± 14.2	73.6 ± 12.2	73.8 ± 11.9	38.9 ± 8.4	80.7 ± 12.1
BMI (kg/m^2)	27.4 ± 4.0	22.4 ± 3.3	22.4 ± 3.2	18.2 ± 2.8	26.4 ± 3.6

GOOD(1) denotes the subpopulation of GOOD used in Paper II, GOOD(2) denotes the entire cohort used in Paper IV. BMI = body mass index. Values are given as mean ± SD.

CAPPP

The Captopril Prevention Project (CAPPP) was a prospective, randomized open trial comparing the ACE inhibitor captopril with diuretics and beta blockers in hypertensive patients in Sweden (n = 7,511) and Finland (n = 3,476) (132). For the purpose of our study on COMT genotype, a sub-population consisting of 522 patients was drawn from the Swedish cohort. Blood samples for DNA extraction were not available from the Finnish cohort.

In the CAPPP study, all possible myocardial infarctions were assessed by an endpoint committee, from which the treatment allocation was concealed. A diagnosis of acute MI required that at least two of the following criteria were met: central chest pain for more than 15 min, transient increase in serum concentrations of enzymes indicating myocardial damage; and electrocardiographic changes typical of myocardial infarction. In the case of a fatal MI, a statement of the diagnosis in hospital or necropsy reports was also valid. Compared with conventional treatment, captopril did not affect the risk for MI in the CAPPP study.

Mean duration of follow-up was 6.1 years and 256 individuals in the Swedish sample experienced at least one MI during this time period. Blood for DNA extraction was available to us from 174 patients with MI. Each patient with MI was matched for sex, age, and smoking status with two control subjects from CAPPP who had not suffered an MI during the time until the occurrence of the MI of their nested patient. Thus, 348 controls were drawn up. Due to the prospective nature of the study, and the well-characterized

cardiovascular endpoints and study subjects, we believe that our cohort is very suitable for studies on MI.

GOOD study

The Gothenburg Osteoporosis and Obesity Determinants (GOOD) study is a population based study with the aim of determining environmental and genetic factors of importance for bone and fat mass in young men. Men > 18 and < 20 years of age in the greater Gothenburg area were randomly identified using national population registers, contacted by telephone and asked to participate. Except for the age limits, there were no exclusion criteria. The participation rate among those contacted was 48.6%. Altogether, 1,068 (aged 18.9 ± 0.6 years) men were included in the study. Through a standardized questionnaire information on present and former physical activity (PA), nutritional intake, smoking status, fracture history and fracture history in the subject's family was collected. Bone properties and body composition were investigated using DXA and pQCT (133).

Paper II was written while the recruitment was still under way. Thus, the first 458 men to be enrolled are included in that study. In paper IV all 1068 study participants are included.

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

Calex

Calex (CALcium and EXercise) is a randomized intervention trial evaluating the effects of calcium, vitamin D, dairy products, and exercise on acquisition of bone mass during early puberty in girls. Inclusion criteria were no history of serious medical conditions, no history of medication known to affect bone metabolism, pubertal development at Tanner stage I–II (as determined by a public health nurse), age of 10–12 years, and a dietary calcium intake of less than the Finnish national recommendation of 900 mg/day. Recruitment was performed by teachers in 61 schools in the Jyväskylä area (corresponding to 96% of the schools in this area) in Finland. Out of 3,118 girls invited, 1,367 agreed to be screened for enrollment and participated in the screening process. The most common reason for not qualifying was a dietary daily calcium intake of > 900 mg/day (n=799, 58.4%). Two hundred ninety-six girls fulfilled the inclusion criteria. One hundred ninety-seven of these agreed to participate and were included in the study. In addition, 61 girls with a dietary calcium intake of > 900 mg/day were included leaving 258 girls available for baseline examination (134). DNA was not available from 11 of them and consequently 247 girls were included in the study in Paper III. Genotyping for COMT val108/158met was

successful in 246 girls. Careful phenotyping including both DXA and pQCT makes this cohort very suitable for the study of bone in young girls.

Of the individuals screened, 58.4% were excluded due to a calcium intake meeting the Finnish national recommendations (> 900 mg/day). Although 61 girls with a higher calcium intake were added, in terms of calcium intake our study subjects were not representative of the general population. However, although calcium is considered to be important for optimal bone acquisition, longitudinal studies of adolescents have generally shown that calcium intake in the lower range has little effect on long-term bone gain (135-137).

The MrOS Sweden study

Osteoporotic Fractures in Men (MrOS) Sweden is part of the international MrOS study, which includes men from Sweden (n = 3,014), HongKong (n ≈ 2000) and the United States (n ≈ 6000). MrOS Sweden is a population-based study with three study centers (Gothenburg, n = 1010, Malmö, n = 1005, and Uppsala, n = 999). Men 69–81 years of age were eligible for the study as long as they could walk without aids, were able to understand and fill out the study questionnaire in Swedish, and did not have bilateral hip prostheses. National population registers were used to select study candidates randomly, who were then contacted by telephone and asked to participate. Of those who were invited, 45 % of agreed to participate in the study (92). In the study on COMT val108/158met, subjects with successful genotyping and data on prevalent fractures and were included (n=2822).

Through a standardized questionnaire, information on current physical activity, nutritional intake, smoking status, and fracture history was collected. Bone properties and body composition were investigated using DXA.

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

ANIMALS

Comt disrupted mice (COMT KO)

Mice have been widely used in bone metabolic research during the past few years because of the relative ease with which they can be genetically modified. This has greatly increased our understanding of bone physiology.

COMT KO mice were originally generated by Gogos et al. (138). The mutated COMT allele was introduced into a mixed 129Sv/C57BL/6J genetic background, and by ten-generation backcrossing the mutation was introduced into a more homogenous C57BL/6J genetic background. The mouse population was regularly enriched using C57BL/6J males or females bred with COMT heterozygotes. Heterozygous male and female mice were bred to produce mice of all three genotypes. COMT KO mice are fertile and healthy, and under normal conditions they show only minor changes in catecholamine concentrations of the brain despite a full reduction of COMT dependent catecholamine metabolites (138-140).

In Paper VI, 70-day old female COMT KO mice (n = 8) and their wild-type (WT) siblings (n = 10) were used.

TECHNIQUES

Fig 3. DXA scan

Dual X-ray Absorptiometry (DXA)

DXA is a widely used non-invasive technique for investigation of bone and body composition in humans as well as in animals. In clinical practice it is the gold standard for evaluating BMD, and current criteria for the diagnosis of osteoporosis are based on DXA measurements.

Different tissues absorb energy to different degrees and this is the underlying principle of the DXA technique. From an X-ray source, a dual-energy spectrum is created. Sensors detect the amount of energy absorbed when each X-ray passes through the body. The use of two energies allows bone mineral to be assessed independently of soft-tissue inhomogeneities. Radiation dose is very low—less than 1/10 of the dose of a chest X-ray.

DXA measurements are two-dimensional, and only changes in length and width are accounted for. The BMD determined by DXA is thus an aBMD (g/cm^2). This quantity is the amount of bone mineral per unit area and is thus not the true volumetric BMD (vBMD) (g/cm^3). From this, it follows that a thicker bone will inevitably have a higher aBMD than a thinner bone. This is especially problematic when growing children are measured, or when age-related bone loss is being assessed. To compensate somewhat for this, a volume-corrected BMD (BMDvol) can be calculated according to the formula BMDvol = BMC/vol = aBMD [4/(π x width)] (141). From a DXA scan, information on bone area and bone mineral content (BMC) will also be available.

Peripheral Quantitative Computerized Tomography (pQCT)

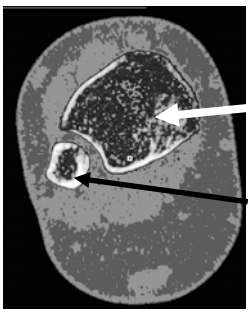

Fig 4. PQCT distal tibia

PQCT is a useful technique for the measurement of bone, fat and muscle in humans and animals. Radiation dose is slightly higher than for DXA, but radiation to the central body is extremely low. It has been considered safe, even in pediatric settings

PQCT 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 that body part. The pQCT can discriminate between cortical and trabecular bone, enabling these bone compartments to be studied separately. In the diaphysis, where almost only cortical bone exists, outer and inner circumferences (periosteal and endosteal) can be measured and accordingly cortical thickness, cortical area, cortical BMC, and cortical vBMD can be determined. In the metaphysis, trabecular vBMD can be determined. The growth plate is used as a reference point in determining where to place the scan along the longitudinal axis.

GENOTYPING

In all cohorts DNA has been extracted from whole blood using commercial kits.

Dynamic Allele Specific Hybridization (DASH)

The CAPPP, Calex and GOOD cohorts were genotyped using DASH. The key to DASH is dynamic heating and coincident monitoring of DNA denaturation.

Briefly a short DNA sequence (60-90 basepairs) covering the SNP of interest is amplified by polymerase chain reaction (PCR). One of the two PCR-primers is biotinylated. After completion of the PCR, the product is transferred to a 96 well streptavidin-coated microtiter plate. The biotinylated strand is bound to the microtiter plate, and the non-biotinylated strand is rinsed away with alkali. A short single stranded DNA sequence (a probe, 15-21 nucleotides), specific for one allele of the SNP, is hybridized to the target at low temperature. The double-stranded DNA thus formed interacts with a double-strand specific intercalating dye. Upon excitation, the dye emits fluorescence which is proportional to the amount of doublestranded DNA present. The sample is steadily heated

and eventually the probe-target complex denaturates. Fluorescence is continuously measured during the heating, and when denaturation occurs there is a rapid fall in fluorescence because the amount of double stranded DNA is reduced. One single-base mismatch between the target and the probe results in a dramatic lowering of melting temperature and this can easily be detected when fluorescence is measured.

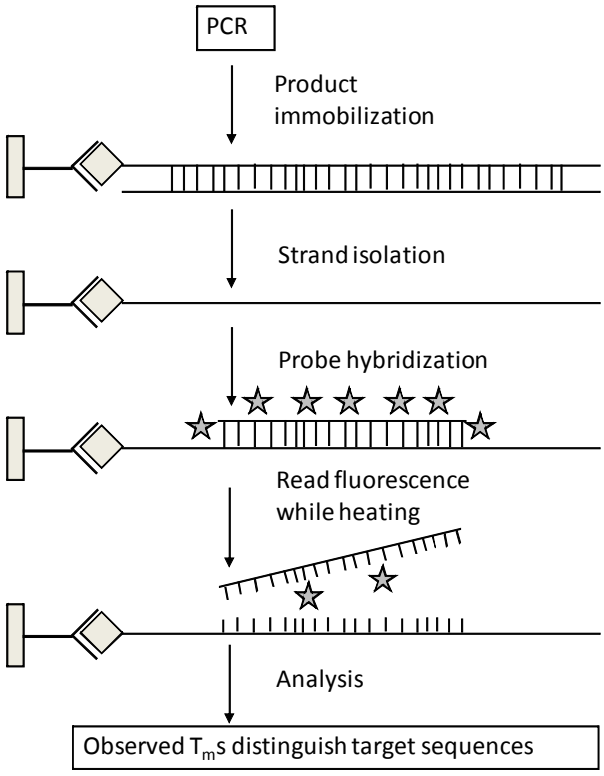

Figure 5. DASH-assay principle

To most readily interpret DASH results the first negative derivate of the fluorescence curves are used. This provides peak values directly related to the probe-target melting temperature (142).

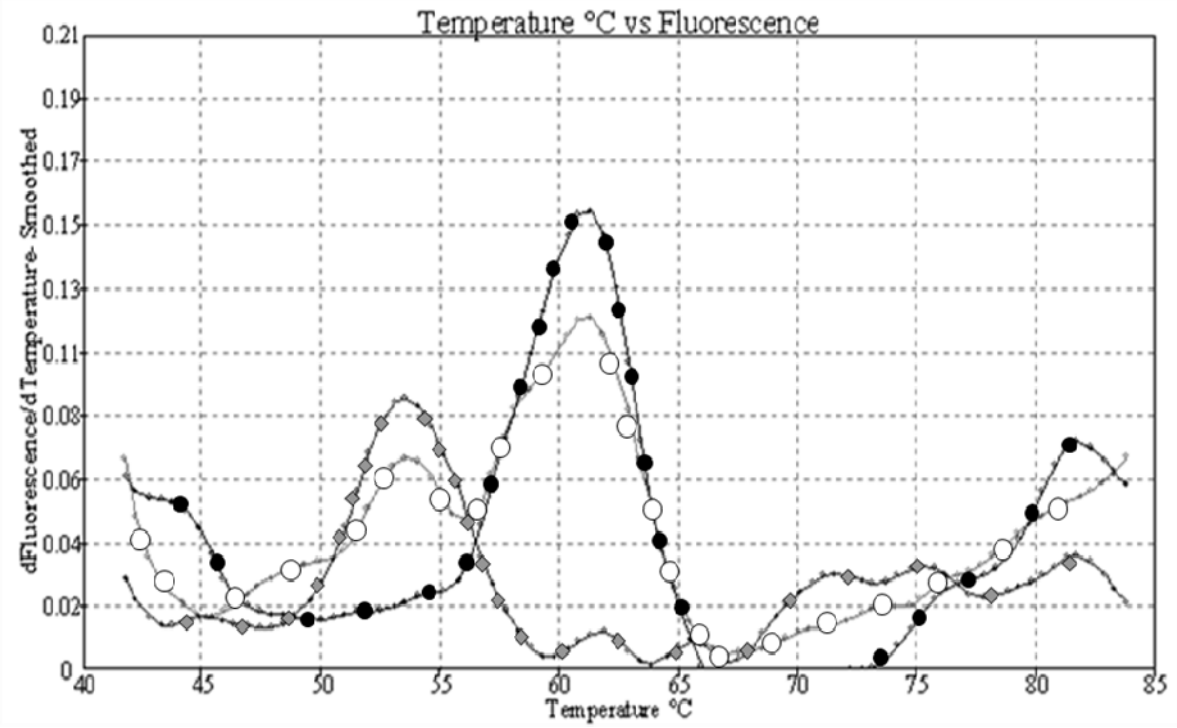

Fig 6. The negative first derivative of DASH fluorescence curves. (●) sample from an individual homozygous for the allele matching the probe, (◆) sample from an individual homozygous for the allele mismatching the probe, and (○) sample from a heterozygous individual.

TaqMan

The development in SNP genotyping technology has been very rapid, and the introduction of technologies with a higher throughput and lower costs made us leave the DASH and turn to other platforms such as the TaqMan, which was used for genotyping in MrOS Sweden (paper V).

Briefly, site specific probes are generated, one for each allele of the SNP. A quencher dye and two different reporter dyes (VIC and FAM) are attached to the probes – one reporter dye for each probe. The probe anneals to the DNA if its target of interest is present. During the PCR reaction, as the strand extends towards the probe, the probe is cleaved due to 5' nuclease activity of the DNA polymerase. This separates the reporter and the quencher dyes from each other and fluorescence of the reporter dye is recorded. Genotype of the sample is determined based on the relationship between fluorescence of the two reporter dyes.

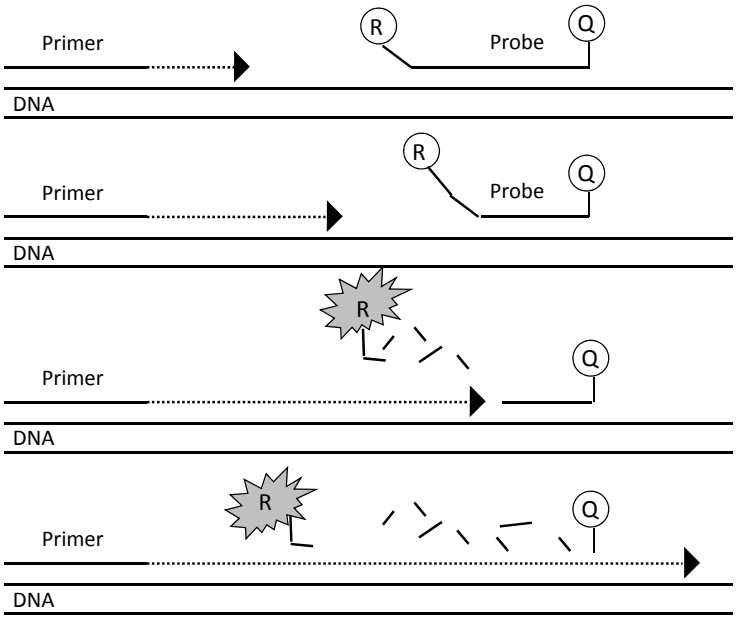

Fig 7. TaqMan assay principle

SERUM MEASUREMENTS

Immunoassay based techniques were used for measurements of sex steroids. Commercial kits were used and all samples were run in duplicates.

STATISTICS

In Paper I serum levels of sex steroids were compared between individuals with the different COMT genotypes using the Mann-Whitney *U* test. Crude and adjusted (adjusted for diabetes, cholesterol and triglycerides) odds ratios (ORs) with 95 % confidence intervals (CI) as estimates of the relative risk for myocardial infarction were calculated using conditional logistic regression. Patients and controls were matched for age, sex and smoking status, and this was taken into account in the regression models.

In Papers II-VI, continuous variables were compared between individuals with different COMT genotypes using one-way analysis of variance (ANOVA) and the independent samples t-test. Categorical variables were compared using Mantel Henszel test (Paper III) or the x^2 test. Linear regression analyses were used to investigate the independent contribution of COMT genotype to skeletal parameters. OR including 95 % CI for sustaining ≥ 1 fracture were calculated in Paper V. Covariates in the regression analyses included age, height, weight, smoking status, physical activity and calcium intake.

RESULTS

PAPER I

COMT genotype, serum levels of E2 in middle-aged men, and myocardial infarction

To investigate the associations between COMT genotype and serum E2 levels in middle-aged men, we used a sub-sample from a hypertensive cohort (CAPPP), consisting of patients with myocardial infarction (n = 174) and age- and sex-matched controls (n = 348). 74.1 % of study subjects were men.

Results

- Serum E2 levels and the E2/SHBG index were higher in men with the $COMT^{LL}$ genotype than in men in the combined $COMT^{HL+HH}$ group ($p < 0.006$). There were no association between COMT genotype and E2 levels in women
- In a comparison between all three genotypes ($COMT^{HH}$, $COMT^{HL}$, and $COMT^{LL}$), COMT genotype was associated with the risk of MI ($p < 0.05$)
- The frequency of the $COMT^{LL}$ genotype was 25.9% and 35.3% in patients with and without MI, respectively ($p = 0.032$). In subjects above the median age of 58 years, 22.0% of cases and 40.2% of controls were carriers of the $COMT^{LL}$ genotype
- In a conditional logistic regression analysis adjusted for sex, age, smoking, diabetes mellitus, cholesterol, and triglycerides the OR for MI in patients with $COMT^{LL}$ was 0.65 (95% CI 0.44–0.97). In subjects older than 58 years the OR was 0.42 (95% CI 0.22–0.80)
- The associations described between COMT genotype and MI in the whole cohort were no longer significant when serum E2 levels were included as covariates in the conditional logistic regression models.

In conclusion $COMT^{LL}$ was associated with increased serum E2 levels in men, and with a decreased risk of myocardial infarction in men and women combined, in this cohort of middle-aged hypertensive patients.

PAPER II

COMT genotype and bone parameters in young adult men

To investigate the associations between COMT genotype and bone phenotype in young adult men, we used a sub-sample from the GOOD cohort consisting of 458 individuals.

Results

- COMT genotype was associated with aBMD at the femur but not the spine (by one-way ANOVA). Values for $COMT^{HL}$ and $COMT^{HH}$ were very similar, and in the subsequent analyses these two genotypes were pooled into one group
- COMT genotype was found to be an independent predictor of aBMD in the total body and in the femur, but not in the spine, when this was investigated with regression models using physical activity, height, weight, age, and COMT genotype as covariates
- COMT genotype explained 1.5% of the variation in total femur aBMD using the above-mentioned regression model
- In the trochanter and the total femur, aBMD in $COMT^{LL}$ was 4.5% and 3.7% lower than in the combined $COMT^{HL/HH}$ group. BMC of the trochanter and the total femur was 7.6% and 4.7% lower in $COMT^{LL}$ than in $COMT^{HL/HH}$
- COMT genotype was an independent predictor of trabecular vBMD in the tibia, radius and fibula. Trabecular vBMD of the radius, and fibula in the $COMT^{LL}$ group was 5.3% and 7.4% lower, respectively, than in the $COMT^{HL/HH}$ group
- COMT genotype was also an independent predictor of cortical vBMD, cortical BMC, and cortical thickness, but not cortical cross-sectional area in the tibia.
- There were no associations between COMT genotype and hormone levels in this study.

In conclusion COMT genotype was found to be an independent predictor of BMD in this cohort of young adult men. The $COMT^{LL}$ genotype was associated with a lower BMD than the $COMT^{HL/HH}$ genotype.

Paper III

COMT genotype, E2 levels, and bone parameters in young girls.

To investigate the associations between COMT genotype, E2 levels, and bone parameters in girls the Calex cohort was used.

Results

- Girls with the $COMT^{LL}$ genotype were 5.4 cm taller than girls with the $COMT^{HH}$ genotype ($p < 0.001$)
- BMC and bone area, but not aBMD, measured by DXA were elevated in $COMT^{LL}$
- Cortical BMC of the tibia was increased in $COMT^{LL}$. This was due to an increased cortical area. Cortical vBMD was not associated with COMT genotype
- Cortical thickness was greater in $COMT^{LL}$ due to an increased periosteal circumference. There was also a slight increase in endosteal circumference

- Trabecular vBMD was not associated with COMT genotype
- Girls with $COMT^{LL}$ had more lean mass as measured by DXA, and an increased muscle area in the tibia as measured with pQCT
- Serum levels of fE2 and IGF-1 were higher in $COMT^{LL}$ than in $COMT^{HH}$
- Linear regression models indicated that the associations between COMT genotype and BMC of the total body and the femur and also cortical bone of the tibia were mediated via serum levels of fE2
- Linear regression models also indicated that the associations between COMT genotype and height were mediated partly by elevated levels of free E2
- Pubertal development as measured by Tanner staging was associated with COMT genotype and girls of the $COMT^{LL}$ genotype were more likely to be at Tanner stages II and III (= early puberty) than girls with the $COMT^{HH}$ genotype, who were more likely to be in Tanner stage I (= prepubertal).

In conclusion, COMT genotype is associated with free E2 levels, longitudinal and radial cortical bone growth, muscle area, and pubertal timing in pre-pubertal girls or girls in early puberty. The associations with radial cortical bone growth as well as some of the associations with longitudinal growth appear to be mediated via the increased levels of free E2.

Paper IV

The interaction between COMT and physical activity with respect to BMD in young adult men

To investigate the independent predictive role of PA and COMT with respect to BMD, multiple linear regression analysis was used, including age, height, weight, smoking and calcium intake, COMT genotype and amount of PA (hours per week) as covariates. To investigate the interactions between COMT genotype and PA, a general linear model was used. As previously reported, there was an association between PA (≥ 4 h/week) and BMD in the GOOD cohort (143). Subjects were thus divided into a low-PA (< 4h/week, n = 554) and a high-PA group (≥ 4 h/week, n = 514) in this study. These two groups were further subdivided into six subgroups based on COMT genotype.

- Both amount of PA and COMT genotype were found to be independent predictors of aBMD of the total femur, trochanter and neck, and trabecular vBMD. PA was an independent predictor of aBMD of the lumbar spine and the total body, and cortical bone size
- Significant interactions were found between COMT and amount of PA for aBMD at all sites, and trabecular vBMD of both the radius and the tibia

- The difference in BMD between high and low PA was generally greater in $COMT^{LL}$ than in $COMT^{HH}$ (lumbar spine aBMD: $COMT^{LL}$ 7.8% versus $COMT^{HH}$ 3.9%, $p = 0.04$, trabecular vBMD of the tibia: $COMT^{LL}$ 7.1% versus $COMT^{HH}$ 1.0%, $p < 0.01$)
- In the low-PA group, COMT genotype explained 2.2% of the variance in trabecular vBMD of the tibia ($p < 0.01$) and 1.8% of the variance in total femur aBMD ($p < 0.01$), while in the high PA group the corresponding figures were 0.1 % and 0.2 % (not significant).

In conclusion COMT genotype modulates the association between PA and aBMD and also between PA and trabecular vBMD. The difference in BMD between high- and low- PA groups was generally greater in $COMT^{LL}$ than in $COMT^{HH}$.

Paper V

The role of COMT for prevalent fractures in elderly men

To investigate the associations between COMT genotype and lifetime fracture risk, self-reported incidence of fractures in participants in the MrOS Sweden cohort was used.

- The number of individuals who had previously sustained ≥ 1 fracture during their life-time was associated with COMT genotype. Percentages for $COMT^{LL}$, $COMT^{HL}$ and $COMT^{HH}$ individuals were 37.2%, 35.7% and 30.4%, respectively
- Early fractures (≥ 1 fracture in ≤50 years) were more common in the combined $COMT^{LL+HL}$ group than for the $COMT^{HH}$ genotype. No significant associations were found with late fractures.
- Fractures of the non-weight-bearing skeleton were more common in the combined $COMT^{LL+HL}$ than in for $COMT^{HH}$ genotype. No significant associations were found with fractures of the weight bearing skeleton.
- No significant associations with BMD or hormone levels were found.

In conclusion the COMT genotype is associated with lifetime self-reported prevalence of fractures in Swedish men. The combined $COMT^{LL+HL}$ genotype is associated with an increased prevalence of fractures. This is mainly driven by early fractures and fractures of the non-weight bearing skeleton.

Paper VI

Role of COMT in the skeleton of female mice

To investigate the role of COMT in the skeleton of female mice, we used 70-day-old female COMT KO mice and their WT siblings.

- Femurs and tibias in young COMT KO mice were 3.6% and 4.4% longer respectively, than in their WT siblings

- Cortical thickness of the femur was found to be increased in COMT KO mice. This was due to a reduced endosteal circumference
- Cortical vBMD, but not trabecular vBMD, was increased in COMT KO mice.

In conclusion, young female COMT KO mice have a bone phenotype resembling that of young girls with the COMT^{LL} genotype (paper III).

DISCUSSION

SEX STEROIDS

Women and female mice

The association between E2 levels and COMT genotype in the early pubertal girls in Paper III is in line with what one would expect, because theoretically a reduced COMT activity leads to increased estrogen levels due to a reduced degradation. Our hypothesis is that as the secretion of E2 from the ovaries begins, the less efficient degradation caused by the $COMT^{L}$ allele will lead to a more rapid increase in E2 levels. This in turn would lead to earlier pubertal development, which is exactly what was seen in Paper III.

In the COMT KO mice, no statistically significant effect on E2 levels was found. Even so, there was a non-significant 10% increase in E2 levels in COMT KO and perhaps the power of our animal study was not sufficient for detection of small differences in E2 levels. However, in contrast to the young girls in Paper III, the mice in the study had reached sexual maturity. If the effect of COMT on E2 levels is age-dependent in females, and if this is valid also in mice, these mice might have been too old when they were sacrificed for an effect on E2 level to be detected.

In contrast to the situation in young girls, no association between COMT genotype and hormone levels in women was found in the CAPPP study. Although the relatively low number of women (n = 135) and the lack of information on menopausal status and HRT at the event of blood sampling makes this sub-sample of CAPPP less suitable for exploring COMT genotype and sex steroid levels in women, the same findings have been presented by others in both premenopausal (144, 145) and postmenopausal women (146-149). This might indicate that the effect of COMT on serum E2 levels is age-dependent, and that compensatory feedback mechanisms may attenuate the effects of a reduced COMT activity in adult women. In premenopausal women, there is a feedback system involving the hypothalamic-pituitary-gonadal axis, which fine-tunes E2 levels during the different phases of the menstrual cycle. In postmenopausal women, almost all of the circulating E2 is synthesized peripherally, and this feedback mechanism is inactive because the levels of E2 are too low (150). Thus, compensatory mechanisms probably reside within other systems in postmenopausal women. During premenarche, however, there is an auto-amplification of the hypothalamic-pituitary-gonadal axis (151), which could explain why COMT genotype was able to have such an influence on E2 in the young girls in Paper III.

Worda et a. administered 2 mg of estradiol valerate to postmenopausal women and found that the serum E2 levels three hours after administration were higher in women with the

$COMT^{LL}$ and the $COMT^{HL}$ genotypes than in women with the $COMT^{HH}$ genotype. There were no associations between COMT and endogenous E2 levels in these women (152). One could speculate that when single doses of E2 are administered, the feedback systems cannot compensate for different COMT activities. To my knowledge, there have been no publications on associations between E2 levels during long-term treatment with E2.

Thus, COMT is involved in the regulation of E2 levels in early pubertal girls, but it does not appear to be involved in the regulation of E2 levels in adult women. In association studies, a large number of other genes involved in the synthesis, degradation, and effects of sex steroids have been analyzed in adult women (153). Very few associations have been successfully replicated in other studies, but some promising results have been shown for CYP19 and E2 (153, 154).

Men

In contrast to the young girls in Paper III, the serum E2 levels of the young men in Paper II were not COMT-associated. One reason could be that the associations are age-dependent in both sexes and that if blood samples had been taken during pre- or early puberty, association would have been apparent in boys as well. However, there could also be sex-based differences. During puberty boys secret increasing amounts of T (155), which can be converted by aromatase to E2; this might make E2 levels less dependent on COMT in young males.

In the middle-aged men in the CAPPP study, the $COMT^{LL}$ genotype was associated with higher E2 levels, which is in line with the findings in the young girls in the Calex study. Average age at inclusion in CAPPP was 57.0 ± 6.6 years. On the other hand, in the slightly older men in the MrOS Sweden study (mean age 75.4 ± 3.2 years), no significant associations with E2 levels were found. The same findings of no associations were made in the Rotterdam study, a large study of 2,217 men who in terms of age were in between the study subjects of CAPPP and Mr OS (mean age 68 ± 8 years) (48). To my knowledge, this is the only publication apart from ours to investigate this issue. It remains unclear however why there is inconsistency between the results of CAPPP study and those of the other two studies. MrOS Sweden and the Rotterdam study were both population based studies, while all study subjects in the CAPPP study had hypertension. Other differences between the cohorts include number of smokers (CAPPP 37.4%, Rotterdam study 21% and MrOS Sweden 8.4%) and diabetes incidence. However, it is not clear why COMT genotype would be associated with E2 levels in subjects with an increased risk of cardiovascular disease only. Still, the findings in the CAPPP study were rather robust with 12.7% higher E2 levels in $COMT^{LL}$, and this was highly significant ($p = 0.006$). Also, the same pattern was found in both cases and controls.

Data on the effect of variations in other sex steroid-related genes on serum E2 levels in men have so far been rather scarce. The $H^{268}Y$ polymorphism of uridine diphosphate glucuronosyltransferase 2B7 (UGT2B7), which glucuronidates sex steroids and their metabolites, has been shown to be associated with E2 levels in young adult men (156). In a recent study by Peter et al., a haplotype of the CYP19 gene was shown to be associated with E2 levels in a rather large cohort of men (n = 834) (157). This is in line with recent findings from our group. We performed a screening for the impact of 604 SNPs in 50 important sex steroid-related genes on serum sex steroid levels in the GOOD study. We found the rs2470152 in intron 1 of the CYP19 gene (an A/G SNP) to be most significantly associated with E2 levels ($p=2 \times 10^{-6}$). We confirmed our results both in the MrOS Sweden study (n = 2,568) and the MrOS US study (n = 1,922). In all cohorts combined rs2470152 was clearly associated with both E2 ($p = 2 \times 10^{-14}$) and E1 ($p = 8 \times 10^{-19}$) levels. The GG genotype had 8–13 % higher E2 levels and 10–16 % higher E1 levels than the AA genotype. Interestingly, the GG genotype was also associated with an increased lumbar spine BMD and a reduced number of self-reported previous fractures (own unpublished data).

COMT genotype was not associated with T levels in any of the studies in this thesis. The most studied polymorphism with regard to T levels in men is the CAG microsatellite in exon 1 of the AR (158-160). The study by Peter et al also found a haplotype in the CYP 19 gene to be associated with T levels in men (157). Furthermore, we have shown that the rs1777941 SNP of the SHBG promoter is associated with a 16% difference in T levels in the MrOS Sweden study (43).

BONE

Bone growth and pubertal development in females

Puberty is characterized by longitudinal and radial bone growth, and sexual development. In girls, increasing secretion of E2 from the ovaries underlies these changes. In the Calex study (Paper III), we showed that all of the above mentioned parameters were associated with COMT genotype.

To our knowledge, the average difference in height of 5.4 cm between individuals of the $COMT^{LL}$ and $COMT^{HH}$ genotype is the largest difference in height between genotypes of an SNP ever demonstrated. During early puberty, height increases by 0.5–1 cm per month. In this case that would mean that the COMT polymorphism causes a 6–12- month difference in pubertal timing between the $COMT^{LL}$ and the $COMT^{HH}$ genotypes. Interestingly, the COMT KO mice in paper VI had longer femurs than their wild-type

siblings, further supporting the notion that COMT is involved in the regulation of longitudinal growth during puberty.

In the Calex study, COMT genotype was associated with measures of size (bone area, cortical cross-sectional area, cortical thickness, and periosteal circumference), but not with measures of density (aBMD, cortical and trabecular vBMD). This is in line with what one would expect at these early Tanner stages from a polymorphism involved in the regulation of pubertal timing. During early puberty, the increase in size predominates whereas there is very little increase in trabecular and cortical vBMD. The increase in BMD is confined to the later stages of puberty (76, 77). Hence, in this mixture of pre- and early pubertal girls, individuals of the $COMT^{LL}$ genotype were more likely to have entered puberty (Tanner stage II) and they were thus also more likely to have started their pubertal growth spurt– including both longitudinal and radial growth. In fact, at follow-up two years later cortical vBMD was higher in $COMT^{LL}$ individuals than in $COMT^{HH}$ individuals, indicating that $COMT^{LL}$ but not $COMT^{HH}$ had entered the phase of increasing cortical vBMD (own unpublished data). Interestingly, there was an increased cortical area also in the COMT KO mice in Paper VI, indicating that COMT is involved in radial cortical growth during puberty in female mice also. The mice were 70 days old, which corresponds to a more advanced stage of puberty than that of the girls. Cortical vBMD was also increased in the COMT KO mice. This might indicate that just as in humans, there is an earlier accumulation of bone if COMT activity is decreased or absent.

It is not known whether the bone phenotype of the COMT KO mice is estrogen-dependent, as was the case in the young girls, because no statistically significant differences in E2 levels were detected. One possibility is that E2 levels were affected earlier in life, and that the affected bone phenotype reflects events that took place earlier on in life.

An easy and reliable measure of pubertal timing in girls is age at menarche (AAM). A recent twin study suggested that the heritable component for AAM is > 50% (161). Several candidate genes have been associated with AAM in prospective studies, including ERα (162) and SHBG (163). There have also been retrospective studies, (which, of course, are subject to recall bias) showing associations with CYP19 (164) and interestingly, also COMT (152). Recently, a few genome-wide linkage studies were published; in two of them, significant associations with 22q11, which is where the COMT gene resides, and age at menarche have been found (48, 165, 166).

It is not known at present whether the COMT genotype will be associated with bone phenotype in the girls in the Calex study, when they have attained peak bone mass. A late AAM has been found to be a risk factor for low BMD (167-169) and fractures (170, 171) in postmenopausal women; in premenopausal women, an early AAM has been associated with a higher BMD (172, 173). One could thus expect the $COMT^{LL}$ girls to be better off in

terms of BMD than the $COMT^{HH}$ girls at the age of peak bone mass. However, there have been three publications investigating associations between COMT genotype in adult women, all postmenopausal. There was no association between COMT genotype and BMD in any of them, (48 , 149, 174), but in one of the studies there was an increased loss of radius BMD in $COMT^{LL}$ (149).

BMD in young adult men

The finding in the GOOD study of a lower BMD in men with the $COMT^{LL}$ genotype might at first seem a bit surprising. Theoretically, a low COMT activity would lead to high estrogen levels, and estrogens are considered to be beneficial for the skeleton, while in this study COMT genotype was found to be associated with lower aBMD of the femur, lower trabecular vBMD of the radius and the tibia, and lower cortical vBMD of the tibia. However, in the GOOD study there were associations between serum E2 levels and cortical bone only, so the other associations seen between COMT genotype and BMD must either be independent of serum E2 levels, or reflect events that took place earlier in life.

In addition, sex steroid levels in serum do not necessarily reflect hormonal concentrations in the target tissues, where synthesis and degradation can occur with little leakage of steroids to plasma (175). Many of the enzymes necessary for sex hormone synthesis and metabolism, including aromatase, are expressed in bone tissue (176). COMT is expressed in osteoblastic cell lines, indicating that it is also expressed in bone cells *in vivo* (48). The estrogen metabolite 2OHE1 binds to ERα. In MCF7 cancer cell lines 2OHE1 has been shown to possess antiestrogenic activity, perhaps due to interference with the binding of E2 to ERα. Moreover, E2 downregulates expression of COMT *in vitro* (177). It is not known whether this has a physiological effect, but one could speculate that peripheral effects of the $COMT^{LL}$ genotype could be amplified by this mechanism and that a low COMT activity would lead to increased levels of 2OHE1 which would reduce the effect of estrogens in the bone tissue. It is also possible that during puberty there is a slower degradation of estrogens in the periphery in individuals with the $COMT^{LL}$ genotype, resulting in high E2 levels in the bone tissue. In men this might have a negative impact with a feminized skeleton leading to a lower BMD.

There were no associations with aBMD of the lumbar spine, indicating that COMT is of importance for the appendicular but not for the axial skeleton. BMD is a polygenic trait and the genes regulating BMD of the appendicular and the axial skeleton respectively are not necessarily the same (178, 179).

Associations between peak bone mass in males and some other candidate genes including parathyroid hormone-related protein (PTHrP) (180), low-density lipoprotein receptor-

related protein 5 (LRP5) (181), parathyroid hormone receptor type 1 gene (PTHR1) (182), CYP19 (183), Methylene Tetrahydrofolate Reductase (MTHFR) (184), vitamin D receptor (VDR) (185) and ERα (186) have been found. These studies are often hampered by limited sample size, and in many studies measurements of BMD have been performed using DXA only. Moreover, most of the findings have not yet been replicated. Thus, our knowledge is still far from complete as to which genes are involved in the accretion of peak bone mass.

BMD and fractures in elderly men

In the elderly men in the MrOS Sweden study (Paper V) COMT genotype was associated with lifetime prevalence of self-reported fractures. The $COMT^{L}$ allele ($COMT^{LL+HL}$) conferred a higher risk of fracture than the $COMT^{H}$ allele, which is in line with the findings from the GOOD study in Paper II of a lower BMD in the $COMT^{LL}$ genotype. However, in the MrOS Sweden study there were no associations between COMT genotype and BMD. The BMD at younger ages is not known in MrOS Sweden, but one could speculate that the higher incidence of fracture in the $COMT^{L}$ allele reflects a lower BMD in that genotype at younger ages. There were no associations between COMT genotype and vertebral fractures, a location in the skeleton where no associations with COMT genotype were found in the GOOD study.

It is known from twin and family studies that there is a high heritability for peak bone mass (187, 188), while the role of genetics in age-related bone loss is less clear (189, 190). It is also quite possible that the genes responsible for peak bone mass are not the same as those responsible for bone loss. This might explain why there were associations between COMT and BMD in the younger men but not in the elderly men. It has also been shown that genetic loci strongly associated with BMD are not necessarily associated with fractures, and vice versa (178).

A recent publication from the Rotterdam study showing a higher incidence of osteoporotic fractures in the $COMT^{LL+HL}$ group has strengthened the notion that COMT may be involved in fracture risk. It is of interest to notice that also in the Rotterdam study there were no associations between COMT genotype, and serum E2 levels, BMD and prevalent vertebral fractures (48).

It cannot be excluded that some of the associations between COMT genotype and bone phenotype in men are mediated through effects on catecholamines. Data from animal studies have indicated that the sympathetic nervous system has a catabolic effect on bone; and the $COMT^{L}$ allele, which was associated with a low bone mass and an increased risk of fractures, would in theory be associated with an increased tone in the sympathetic

nervous system (191). However, COMT does not appear to be of importance for peripheral removal of catecholamines (44).

A number of polymorphisms in candidate genes have been investigated in regard to BMD, bone size, osteoporosis, and/or fractures in the elderly. The majority of studies have been performed on women but lately the number of studies involving males has increased. For most of the genes the results have been inconsistent. However, meta-analyses have indicated that some of these genes do indeed take part in the genetic regulation of bone properties and fractures, although individually each polymorphism only accounts for a small contribution. Some of the most studied candidate genes are those for the Vitamin D receptor (VDR) (192), Collagen type IαI (COLIA1) (193), ERα (194) and low-density lipoprotein receptor–related protein 5 (LRP5) (195).

Lately, the whole-genome approach has been exploited in a small number of studies in the bone and osteoporosis field (178, 196, 197). There was inconsistency in the results between studies, but in two studies associations were found for the osteoprotegerin (TNFRSF11B) gene, which has been considered a candidate gene for osteoporosis. Genes for ERα, LRP5, and receptor activator of nuclear factor-κB ligand (RANKL) were previous candidate genes that reached statistical significance in one of the studies (178, 197). New loci not previously considered to be involved in bone regulation were also identified (196).

Interactions between COMT genotype and physical activity

In numerous papers it has been shown that physical activity helps one to build strong bones, especially during childhood and adolescence. It has been proposed that this is because when mechanical forces are applied to the bone, these are sensed by a mechanism termed the mechanostat–which as a result of the strain regulates bone formation and resorption to adapt the skeleton to the forces applied to it. The largest forces applied to the bone come from muscle contractions (198). Estrogens have been proposed to modulate the mechanostat. Using intact and ovariectomized rats on earth and on the orbiting space station Westerlind et al. showed that estrogens govern the rate of bone turnover, but the greatest effect on the balance between bone formation and resorption is exerted by mechanical loading (199). It has also been shown that mice lacking ERα have a less effective adaptive response to mechanical loading compared with WT mice (200).

There are two ways in which the findings in Paper IV of an interaction between COMT and PA with respect to BMD can be interpreted. Either the $COMT^{LL}$ individuals have more to gain by PA in terms of an increased BMD, or subjects with a high degree of PA have a maximal response to mechanical loading that cannot be modulated further by COMT

genotype, whereas subjects with a low degree of PA and submaximal response to maximal loading are clearly affected by the COMT polymorphism.

Although gene-environment interactions are considered to be of importance for many phenotypic characteristics, and in the pathogenesis of complex disorders the number of reports of this type of interaction regarding sex steroid-related genes and bone phenotype are still few. Two papers have reported interactions between ERα genotype and PA (201, 202).

MYOCARDIAL INFARCTION

The mechanism underlying the protective effect of the $COMT^{LL}$ genotype for MI in the CAPPP study (Paper I) is not clear. The effects of estrogens in the cardiovascular system are complex. Association studies and also pharmacological trials in men have yielded conflicting results, but one might speculate that the affected E2 levels are involved in the protective effect. When E2 levels were included in regression models, the associations between MI and COMT genotype in Paper I were no longer significant, which could indicate that E2 is involved in the protective effect.

It has been shown that 2ME2 mediates many of the cardioprotective effects of estrogens in mice, by an ER-independent mechanism and that in mice lacking COMT, 2OHE1 cannot be converted to 2ME2 (203). Thus, individuals with the $COMT^{LL}$ genotype could be expected to have a higher incidence of MI due to decreased levels of 2ME2, and that was not the case in our study. It should be kept in mind though that it has proven difficult to translate findings regarding E2 and the cardiovascular system from mice to man.

It is also possible that an affected degradation of catecholamines is involved in the associations between COMT and MI. Theoretically, the $COMT^{LL}$ genotype would involve a slower catecholamine metabolism. However, neuronal uptake and the degradation by the MAO enzyme compensate for blockade of COMT; thus peripheral clearance of catecholamines is not affected by pharmacological COMT inhibitors (44), and an effect mediated by catecholamines seems less likely.

Results from several recent genomewide association studies (GWAs) (204, 205) and subsequent replication studies (206, 207) have shown strong associations between a locus on chromosome 9p21.3 and coronary artery disease. However, MI is thought to be a polygenic disease and in the work by Samani et al. (205), 126 SNPs in 76 genes previously reported in the literature to be associated with MI or coronary artery disease were specifically investigated, in addition to the whole-genome approach. Two case-control studies including a total of 2801 cases and 4582 controls were used. Most of the candidate

SNPs were not represented on the gene chip array used in the GWA study (GeneChip Human Mapping 500K Array Set) and SNPs that were in complete or near complete linkage disequilibrium with the candidate SNPs were selected from the chips. Interestingly, both SNPs substituting COMT val108/158met (rs4646312 (D'=1, r^2=0.738) and rs4633 (D'=1, r^2=0.967)) were associated with CAD in the first cohort, OR and 95% CI for the allele corresponding to $COMT^L$ being being 0.90 (0.82-0.98) and 0.87 (0.80-0.95) respectively. In the other cohort rs4646312 was of borderline significance (0.90 (0.79-1.01)). Another relatively large case-control study (cases n=811, controls n=650) analyzed 85 genetic variants previously reported to be associated with MI. In that study, the association between $COMT^{LL}$ and a decreased risk of MI could not be replicated, and as a matter of fact that was true for all but one of the 85 variants analyzed (208).

CONCLUSION

The genetic regulation of multifactorial phenotypes and disorders is complex. Many genes are involved, and although the field of genetics is evolving with an ever increasing speed, there is still a lot to be discovered before a more comprehensive understanding of heredity can be accomplished. The results of the papers presented in this thesis indicate that the COMT val108/158met polymorphism is one of the contributors in this complex regulation, and that it may be implicated in physiological processes including the regulation of pubertal timing and growth in young girls and female mice, bone phenotype in young adult men, fracture risk in elderly men, incidence of MI in middle-aged individuals and serum E2 levels in middle-aged men.

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