Determinants of Plasma Androgen and Estrogen Levels in Men

The work presented in this thesis was conducted at the department of Internal Medicine of the Erasmus Medical Center Rotterdam and at the department of Endocrinology of the VU Medical Center Amsterdam

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Determinants of Plasma Androgen and Estrogen Levels in Men

Determinanten van plasma androgeen en oestrogeenspiegels bij de man

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Aim of the thesis

The steroid hormone testosterone is responsible for the development of the primary and secondary male sex characteristics such as male pattern hair growth, deepening of the voice and increased lean body mass. Testosterone is produced in the testicular Leydig cells in response to stimulation by pituitary derived luteinizing hormone (LH). In its turn the pituitary LH secretion is regulated by the hypothalamus. Testosterone will feed back onto the pituitary and hypothalamus thereby allowing the hypothalamo-pituitary-testicular (HPG) axis to maintain the plasma testosterone concentration within close limits. The serum testosterone concentration is considered normal when within the reference range as supplied by the laboratory. The lower limit of this reference range represents the 2.5 percentile of testosterone levels of a group of apparently healthy men. However, testosterone levels may vary considerably between individuals.

Older men may have signs and symptoms reminiscent of testosterone deficiency such as lack of libido, erectile dysfunction and lower bone and lean body mass. In older men the mean testosterone concentration in blood is lower compared to young men. The question is whether the above-mentioned symptoms truly represent testosterone deficiency. For an adequate answer to this question a better understanding of the determinants of the serum testosterone level in men is necessary in order to better differentiate between normal and abnormal levels in a specific individual.

In the body testosterone is converted to estradiol. In the past ten years it has become evident that estradiol is responsible for a number of the effects formerly attributed to testosterone. Estradiol has an important role in gaining and maintaining bone mass, closing of the epiphyses and the feedback on gonadotropin release by the pituitary. Since estradiol may be a determinant of the circulating testosterone concentrations but may also be involved in the development of the clinical syndrome associated with androgen deficiency, evaluation of estradiol levels in men seems appropriate. However, the interpretation of estradiol levels in men is probably even more difficult than the interpretation of testosterone concentrations. Only little is known about the determinants of the estradiol serum concentration in men, its interaction with testosterone and the minimal tissue level needed to prevent symptoms of estrogen deficiency.

Therefore, the aim of the present thesis was to gain more insight into the determinants of the testosterone and estradiol concentrations in men. This information may be helpful when interpreting the serum testosterone and estradiol concentrations of men with symptoms reminiscent of androgen deficiency.

Chapter 1

The importance of oestrogens in males

For a long time oestrogens in the human male have been regarded as a mere by-product of testosterone synthesis. Only since the description of an oestrogen-resistant man (1) has it been fully realized that oestrogens play an import role in bone homeostasis, cardiovascular health and pituitary–gonadal interactions in men. Due to a disruptive homozygous oestrogen receptor alpha (ERα) mutation, this man was virtually insensitive to oestrogens which lead to osteoporosis, unfused epiphyses resulting in linear growth into adulthood, increased gonadotrophin levels and evidence of premature atherosclerosis (2) and endothelial dysfunction (3). Additionally, there was low viability of sperm and glucose intolerance with acanthosis nigrans. Five adult aromatase-deficient men showed, besides undetectable oestrogen levels, low bone mass, unfused epiphyses, increased gonadotrophins (4;5) and an unfavourable lipid profile (6-8). Earlier, Korach (9) described similar observations in ERα knockout mice. Heterozygous mice exhibited no obvious phenotypic abnormalities. Homozygous male mice proved to be infertile, showing smaller testes with dysmorphic seminiferous tubules and a sperm count of less than 10% compared to normal mice. Finally, bone mineral density was 20–25% lower in male and female mutants compared to wild-type animals. These reports indicate that oestrogens also play a central role in several metabolic processes in men. The interrelation between testosterone and oestrogen biosynthesis makes it difficult to separate their respective biological effects. Therefore, it is not unlikely that biological effects formerly attributed to testosterone actually represent effects of oestrogens. In light of the observations in oestrogen-resistant or -deficient males, and because of the wealth of evidence for the role of oestrogens in the (patho)physiology of bone and lipid metabolism, cardiovascular disease and the hypothalamo–pituitary–gonadal axis in women, this review will discuss these areas, focusing on the clinical importance of oestrogens in males.

Oestrogen synthesis and plasma concentrations

Oestradiol is the most potent oestrogen produced in the body. It is synthesized from testosterone or oestrone via the aromatase- or 17β-hydroxysteroid dehydrogenase (17β-HSD) enzymes, respectively (figure 1).

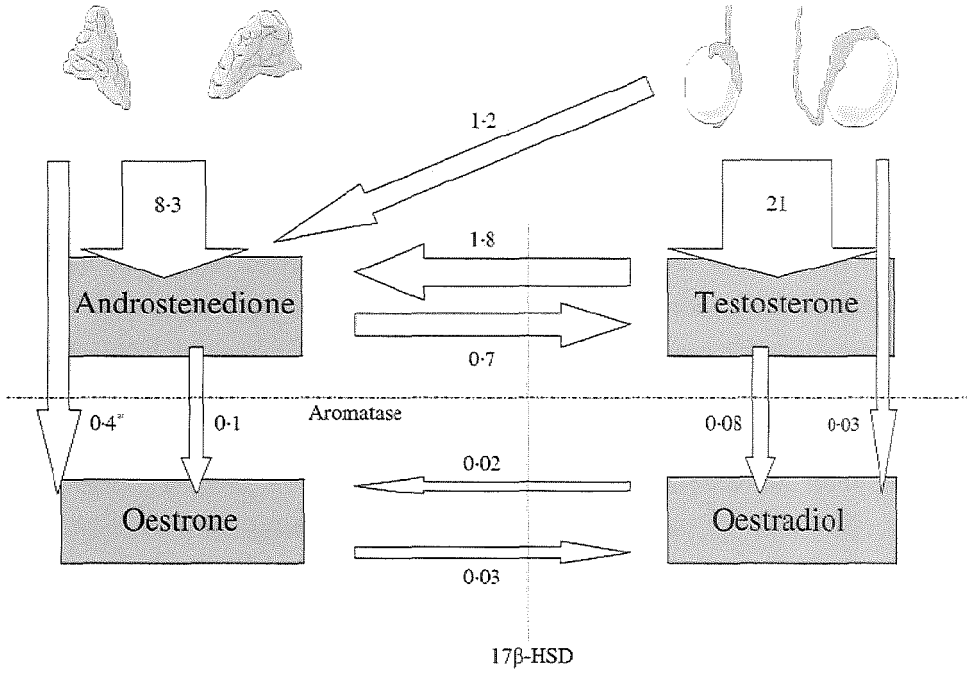
The total oestradiol production rate in the human male has been estimated to be 35-45 μg (0·130–0·165 μmol) per day, of which approximately 15-20% is directly produced by the testes (10;12). Roughly 60% of circulating oestradiol is derived from peripheral aromatization of circulating testosterone and 20% is the product of peripheral conversion of oestrone (10). Oestrone is the product of peripheral aromatization of androstenedione which is partly

produced by the adrenal glands and partly derived from peripheral conversion of testosterone. Oestrone can also be directly secreted by the adrenals (13). The testes contribute more to the total amount of circulating oestradiol than the adrenal glands. Therefore suppression of adrenal steroid synthesis using dexamethasone leads to moderately decreased oestradiol levels (14), whereas orchiectomy leads to a more dramatic suppression of plasma oestradiol concentrations (15;16). One of the largest studies on peripheral levels of oestradiol in 1640 men aged 38–70 showed a mean total serum oestradiol concentration of 110 ± 54 pmol/l (mean ± SD; (17). These concentrations are comparable to those in women in the early follicular phase of the menstrual cycle. Oestrogen concentrations in postmenopausal women decrease to levels significantly lower than in men of the same age. The plasma concentrations of testosterone and androstenedione tend to decrease with advancing age in men (18-21). Oestradiol levels in men decrease mildly after the age of 30 (19), remain constant until the age of 75 (22;23) and decrease significantly after that age (21;23). Circadian variation of oestradiol levels has hardly been investigated but is theoretically likely as levels of both testosterone and androstenedione show a circadian rhythm, being highest early in the morning. (24) found a nadir of serum oestradiol concentrations between 24·00 and 02·00 h, and a zenith between 15·00 and 17·00 h in six healthy men (mean age 33 years). Surprisingly, the oestradiol circadian rhythm in this study was in counterphase with the testosterone circadian rhythm. (25) measured oestradiol concentrations at 08·30 and 17·00 h in eight young men (mean age 34) and 10 elderly men (mean age 73) but found no significant difference between morning and afternoon values. The variation in oestradiol levels through the day in men is slight and in no way comparable to the dramatic changes in plasma levels during the menstrual cycle in women. This means that a single measurement of oestradiol in peripheral blood will give a good indication of the oestrogen exposure in men. Clinical syndromes characterized by altered peripheral concentrations of total oestradiol have been summarized in table 1.

Binding proteins and the free hormone hypothesis

Sex Hormone Binding Globulin (SHBG) is a protein consisting of 373 amino acids for which the gene is located on chromosome 13. The circulating fraction of SHBG is primarily produced by the liver. In plasma SHBG circulates as a homodimer exposing two binding sites for steroid hormones. SHBG binds dihydrotestosterone, testosterone and oestradiol with high affinity (26). Albumin can also bind sex hormones although the binding affinity is much lower. However, due to the high concentrations of albumin in plasma a substantial proportion

Figure 1: Sources of circulating estrogens in men. Figures indicate production in μmol/24 hr (data derived from (10;11))



*combined production from adrenal secretion and peripheral conversion of DHEA and DHEAS.

of circulating testosterone and oestradiol is bound to albumin. As a result only 2 to 3% of circulating estradiol and 1-2 % of circulating testosterone is free, most hormone is bound to albumin or SHBG (26). There is some controversy whether or not the protein bound hormone can leave the bloodstream to interact with the intracellular hormone receptor. According to the free hormone transport hypothesis only free, unbound, hormone can diffuse freely into tissues. The bound proportion of the hormone acts as a reservoir of hormone from which the free hormone pool can be continuously replenished (27). Therefore, in tissues in which extensive metabolism of hormones takes place and the transit time through the capillaries is long enough, the tissue uptake can exceed the free amount of hormone. In humans, such a situation is probably only encountered in the liver (44). In other tissues the metabolism of steroid hormones is assumed to be low and the tissue uptake of hormone is therefore primarily dependent on the free hormone fraction (27). The free hormone hypothesis was first postulated by Recant and Riggs (45) shortly after the discovery of thyroid hormone binding

proteins. They observed that patients with low levels of binding proteins as a result of the nephrotic syndrome were euthyroid despite low thyroxin levels. Rats that lack albumin and SHBG show much lower plasma levels of total testosterone but normal levels of free testosterone and are phenotypically indistinguishable from wild type rats (46). These observations underline the limited effect of binding proteins on the level of free testosterone in males. As long as the hypothalamus-pituitary-gonad (HPG) axis is intact it will maintain the free testosterone level at a predefined level. The question arises whether this is also true when the HPG axis is dysfunctional and no longer able to compensate for changes in the free testosterone concentration. In men this question remains to be resolved. In male castrated rats, treated with testosterone pellets, infusion of humane SHBG resulted in an increase of circulating total testosterone and only small reductions in free testosterone levels (47). This probably relates to the inhibitive effect of SHBG on testosterone clearance. Although a direct effect of SHBG on the metabolic clearance rate of testosterone was also found in monkeys a substantial effect was only seen up to a certain level of SHBG (48). It has been assumed that at a certain level of SHBG, virtually all testosterone is bound to SHBG. Therefore, increasing the SHBG concentration above this level does not have a substantial effect on the free hormone fraction and thus on the metabolic clearance rate.

SHBG can have effects on both production and clearance of estradiol in men. Firstly, SHBG can, by binding testosterone, decrease the amount of precursor hormone for estradiol synthesis (49). Secondly, by binding estradiol, SHBG can inhibit its metabolic clearance rate in a similar way as described for tetosterone (50). In men, circulating oestradiol is probably only indirectly involved in the feedback regulation of the HPG axis activity (see below). Therefore, fluctuations in the free oestradiol concentration will not be directly compensated for by changes in HPA-axis activity. It is conceivable that, also in men with a normally functioning HPG axis, SHBG can have an effect on circulating levels of free oestradiol. Although SHBG has been viewed as a passive carrier protein, recently it was shown that SHBG bound hormone can be actively transported into the cell via an endocytic receptor called megalin (51). The importance of this endocytic uptake of steroid hormones in rodents was demonstrated by impaired descent of the testes into the scrotum in male megalin knockout mice. Whether SHBG dependent uptake of hormones is of clinical significance in humans remains to be determined.

Tabel 1: Conditions characterized by altered plasma estradiol concentrations in combination with high, normal or low plasma testosterone concentrations in men.

Estradiol level	Testosterone level	
high	high or normal	Exogenous testosterone (28)
		Androgen receptor antagonist (29)
		Estrogen receptor antagonist (30)
		Estrogen resistance (1)
	low	Obesity (31)
		Increased aromatase activity
		-hereditary (32)
		-sporadic (33)
		Klinefelters syndrome (34)
		Aromatase excess
		-hepatocellular carcinoma (35)
		-adrenocortical carcinoma (36)
		-testicular tumors (35)
		Hepatic cirrosis (37)
		Androsteendione excess
		-adrenocortical adenoma (38)
		-adrenocortical carcinoma (39)
		-glucocorticoid resistance (40)
low	high	Aromatase deficiency (6;7)
		Aromatase inhibition (41)
	low / normal	Hypogonadism (42)
		Glucocorticoid use (14)
		Estrogen receptor agonist use (diethylstilbestrol) (16)
		Nonaromatizable androgen use (43)

Aromatase, 17β-hydroxysteroid dehydrogenase and the concept of intracrinology

The aromatase enzyme, localized in the endoplasmic reticulum of the oestrogen-producing cell, is encoded by the *CYP*19 gene. This gene is a member of the *CYP* gene family, encoding a class of enzymes active in the hydroxylation of endogenous and exogenous substances. The *CYP*19 gene is localized on chromosome 15 and comprises nine coding exons and several untranslated exons upstream of codon II, named exons I_1–I_5. The finding of aromatase transcripts with specific 5'-termini in different tissues indicates that there are various tissue-specific promoters each using its own exon I. These promoters are under the influence of different transcription factors such as gonadotrophins (gonadal promotor II) and IL-6, IL-11

and TNF-α (adipose/bone promotor I.4; for review see (52). As exon I is not translated, these different transcripts are all translated to the same protein. Aromatase activity has not only been demonstrated in testes and ovaries but also in brain (53), fat tissue (54;55), muscle (55), hair (56), bone (57;58), vascular tissue (59;60) and placenta (61).

To date, six males with aromatase deficiency have been described: five adults (4-8) and one newborn (62). Oestradiol levels in these males were extremely low. All adult aromatase-deficient men demonstrated a remarkably low bone mass and unfused epiphyses leading to linear growth into adulthood and above average body length. Sexual and pubertal development in these men were normal. Testicular size in these five man ranged from micro to macroorchidism and the plasma testosterone levels varied roughly in accordance with testes size. LH levels were either normal or elevated. Four men were infertile (5-8), in one younger male fertility was not described (4). However two aromatase deficient men had a brother who also suffered from infertility despite a normal aromatase genotype suggesting an unrelated second condition (7;8). Once treated with oestradiol, epiphyses closed and bone mineral density (BMD) increased (4;5;7;63;64).

A boy showing increased extraglandular aromatase activity was described by Hemsell et al. (33). This prepubertal boy presented with gynaecomastia, accelerated growth and premature bone maturation due to excessive peripheral oestrogen synthesis of unknown origin. The measured conversion rate of androstenedione to oestrone was 50 times higher compared to the conversion rate in normal prepubertal boys. Berkovitz (32) presented a family in which gynaecomastia occurred in five boys at age 10–11. Fractional conversion rates of androstenedione to oestrone were 10 times the value found in normal prepubertal boys. The underlying genetic defect in these cases was not elucidated. A 17-year-old boy with the same syndrome was described by Bulun et al. (35). Sequencing of the aromatase gene in this case did not reveal mutations in the promoter or coding regions. Stratakis et al. described a family with aromatase excess syndrome in which the syndrome appeared to be caused by inappropriately high expression of an alternative first exon (65). Shozu et al. (66) described a father and his son and one unrelated patient with aromatase excess caused by a chromosomal rearrangement which placed the aromatase gene adjacent to cryptic promoters. As a result an inappropriate amount of aromatase was expressed in adipose tissue of the affected subjects. Increased androgen aromatization can also be caused by hepatocellular carcinoma (35), adrenocortical tumours (36) and testicular tumours (35;67). In these tumours, inappropriate amounts of the aromatase enzyme are expressed. Peripheral androgen aromatization is enhanced in subjects with increased body mass index (BMI; (31). Massively obese men show

markedly increased plasma oestradiol concentrations and low testosterone concentrations (68-70). After weight reduction these alterations can be reversed (71), underlining the importance of adipose tissue for peripheral aromatization of androgens. Elevated plasma oestradiol levels are also observed in men with cirrhosis of the liver despite decreased plasma testosterone levels (37;72). The metabolic clearance rate of oestrogens in liver cirrhosis patients seems to be unaltered (72). The observed hyperoestrogenism could therefore be caused by an unexplained increment of androgen aromatization. Klinefelter's syndrome also leads to relative hyperoestrogenism with a similar pattern of metabolic alterations as in liver cirrhosis patients (34). Bulun et al. demonstrated increased efficiency of peripheral conversion of androstenedione to oestrone with increasing age probably as a result of increased aromatase expression in adipose tissue (73).

17β-Hydroxysteroid dehydrogenase (17β-HSD, or 17-ketosteroid reductase) actually represents a group of enzymes of which, to date, 12 subtypes have been described (74). These enzymes are important in hormone metabolism because they catalyse the conversion of biologically inactive 17-keto-steroids (androstenedione and oestrone) into their active 17β-hydroxysteroid counterparts (testosterone and oestradiol) or vice versa. The enzymes are numbered in the order they were cloned. The subtypes are structurally quite different and differ in localization and substrate preference. The type 1 isoform for instance preferentially metabolizes oestrone to oestradiol and is found in the human ovary and placenta. The type 2 isoform preferentially converts oestradiol and testosterone to oestrone and androstenedione, respectively. This subtype is abundantly expressed in all kinds of tissue and it has been suggested that by oxidizing androgens and oestrogens to their less active forms it protects these tissues from steroid actions (75). The type 3 isoform is predominantly expressed in the testes (76). The clinical syndrome of 17β-HSD type 3 deficiency is frequently described and consists of male pseudohermaphroditism due to deficient testicular testosterone biosynthesis. The clinical picture can be indistinguishable from the androgen insensitivity syndrome but patients can be identified by low testosterone/androstenedione ratios in peripheral blood and by mutation analysis (77). Other 17β-HSD subtypes are expressed in all kinds of tissues including osteoblasts (58;74;78-80) for review).

As described above, both aromatase and the 17β-HSD enzymes are important mediators of androgen and oestrogen metabolism. The presence of these enzymes in the gonads is essential for adequate sex steroid synthesis in men and women. The extragonadal localization of these enzymes, however, also implies a local role in steroid hormone metabolism whereby plasma-derived sex steroids can locally be converted into more or less active hormones, a process

earlier named intracrinology (81). A demonstration of this phenomenon was given by Janssen et al. (58) in a human osteoblast cell line in which both aromatase and 17β-HSD were present. Administration of testosterone resulted in local production of both androstenedione and oestradiol and, interestingly, both aromatase and 17β-HSD reductive activity declined with differentiation. This shows that local tissue concentrations of androgens and oestrogens are not only dependent on peripheral blood hormone levels but also on local conversion by aromatase and 17β-HSD isoenzymes. In men, but also in postmenopausal women, peripheral conversion of androgens is of vital importance for oestrogen formation. This suggests that in men and postmenopausal women peripheral steroid levels cannot always be relied upon as a measure of local androgenic or oestrogenic activity (82).

The oestrogen receptors

The oestrogen receptors (ERs) are members of the steroid receptor family. All members of this family share similar characteristics. These include a separate hormone binding domain, a high-affinity DNA binding domain, a tendency to form dimers and an enhanced affinity for the cell nucleus in the presence of bound hormone. To date, two ER subtypes have been cloned, called ERα and ERβ (83). The two subtypes are highly homologous in DNA and ligand binding domains but overall homology is only 55%. The binding affinity of ERα for oestradiol is somewhat higher compared to that of ERβ (kDa 0·1 nM *vs.* 0·4 nM). Both subtypes are widely distributed throughout the body. ER mRNA or ER immunoreactivity of both subtypes has been demonstrated in the prostate (84), osteoblasts (85;86), cartilage (87;88), adipose tissue (89), brain (90) including pituitary (91), and testis (92). Both ER subtypes have been knocked out in male mice. The different phenotypes are summarized in table 2 and compared with phenotypic characteristics of the aromatase knockout mouse. The observations presented in table 2 suggest an important most likely indirect role for ERα-bound oestrogen in spermatogenesis. Studies by Hess (93) indicated that the efferent ductules of ERα knockout mice fail to resorb luminal fluids leading to fluid accumulation in the seminiferous tubules, eventually resulting in tubular atrophy and infertility (see also (94) for review). The increased levels of LH and testosterone in the ERα knockout mice are most likely the result of decreased negative feedback of oestradiol on the pituitary gland and/or hypothalamus, thus implying that this effect is at least partly mediated by ERα. The effect of oestradiol on male bone seems to be mediated by ERα, although both ER subtypes are present in human osteoblasts (85;86;95). ERα knockout mice and aromatase knockout mice show similar alterations in bone length and bone density. Heterozygous ERα knockout mice are

Tabel 2: fenotypes of ERα, ERβ and aromatase male knockout mice (96-98;102;110-116)

	ERα knockout	ERβ knockout	Aromatase knockout
fertility	absent	normal	decrease during life
sperm	low count, subnormal motility, unsuccessful *in vitro* fertilization	normal	disrupted spermatogenesis at older age
testes	reduced size, dysmorphic seminiferous tubules	normal	normal
epididymis / efferent ductules	dilated efferent ductules	normal	normal
prostate	normal	signs of epithelial hyperplasia	normal
seminal vesicles	increased volume	normal	increased volume
sexual behaviour	normal mounts, reduced intromissions, no ejaculation	normal	reduced mounting
serum estradiol	elevated	normal	low
serum testosterone	elevated	not described	elevated
LH	elevated	not described	elevated
bone	25% lower density, growth arrest of long bones	normal	decreased trabecular bone volume, decreased femur length

phenotypically normal and show unaffected fertility. Crosses of the heterozygous mice resulted in live birth of offspring containing the traditional Mendelian distribution of genotypes. It is not known to what extent the observations in rodents can be translated to humans. To date, only one homozygous ERα-deficient man has been described with phenotypic abnormalities similar to those observed in ERα knockout mice. These remarkable symptoms cannot easily be overlooked and homozygous-disruptive ERα mutations in the human male must therefore be rare. This indicates that ERα mutations in humans are somehow disadvantageous in early life or fetal development. The prevalence of heterozygous ERα mutations in humans is unknown.

ERβ knockout mice are phenotypically hardly affected and prevalence of mutations in this receptor subtype in the human population can be more widespread. This, however, remains to be determined. Although Krege *et al.* (96) and Weihua *et al.* (97) found signs of prostate and bladder epithelial hyperplasia in older ERβ knockout mice this was not confirmed by others

(98). However, recently more evidence was found for a antiproliferative role for ERβ in the mouse prostate (99). It was hypothesized that a metabolite of dihydrotestosterone, 5α-androstane-3ß,17ß-diol, acts as a natural ligand for ERβ thereby inhibiting prostate cancer cell formation (100). Experimental evidence in favour of this hypothesis has been described very recently (101). An in depth discussion on ER and ER-subtypes is beyond the scope of this article but can be appreciated elsewhere (102-104).

Oestrogens, the hypothalamus–pituitary-gonadal axis and the brain

It is well known that testosterone inhibits gonadotrophin release from the pituitary (28;105;106) either by direct effects or by suppression of hypothalamic luteinizing hormone releasing hormone (LHRH) release. The question arises whether aromatization to oestradiol is involved in this feedback mechanism. Several observations indicate that androgens can influence gonadotrophin release without being aromatized first. Dihydrotestosterone, a nonaromatizable androgen, can suppress gonadotrophin secretion at supraphysiologic doses (28;107-109) and

androgen-resistant genetic males have elevated gonadotrophin levels despite normal plasma oestradiol concentrations (117;118). Furthermore, administration of an androgen antagonist to men leads to increased gonadotrophin secretion (29;119;120). However, oestrogens administered at physiological or supraphysiological doses also have inhibitory effects on gonadotrophin release in men (28;107-109;121). This effect has been used for the treatment of androgen-dependent prostatic carcinoma. High doses of intramuscular or oral oestrogens suppressed gonadotrophin and testosterone levels dramatically. Administration of tamoxifen or clomiphene, which are oestrogen receptor antagonists at the pituitary and/or hypothalamic level, resulted in elevation of gonadotrophins, despite concomitant elevation of testosterone levels (30;122;123). Oestrogen insensitivity (1), aromatase deficiency (4;6;7) and administration of an aromatase inhibitor (41;105;106) all lead to elevated gonadotrophin levels despite normal or elevated serum testosterone concentrations. Furthermore, an experiment with the selective aromatase inhibitor anastrozole in both normal men and men suffering from idiopathic hypogonadotrophic hypogonadism indicated that oestrogens have an inhibitory effect on both hypothalamus and pituitary (124). These observations indicate that oestrogens and androgens have independent effects on gonadotrophin release in men. However, their relative influences under physiological conditions remain to be determined. In this context it is important to realize that oestradiol exerts its influence at picomolar levels, while the average serum testosterone concentration is in the nanomolar range. This combined

influence of oestradiol and testosterone on gonadotrophin secretion is important when evaluating hypogonadotrophic hypogonadism. Low oestradiol levels indicate a primary hypothalamic or pituitary disorder, while high oestradiol levels reflect a physiological suppression of gonadotrophins, prompting a search for the origin of this excess oestrogen. As is described below, oestrogens in men have bone-preserving capacities. Hypogonadism with oestrogen excess is therefore not necessarily associated with bone loss diminishing the need for androgen supplementation for this reason. An example of this condition is a man, evaluated for hypogonadotrophic hypogonadism, who had extremely high peripheral oestradiol levels as a result of overactive adrenals compensating for cortisol resistance (40). This man's bone mineral density was normal.

In addition, the limited observations described above in ERα knockout mice (110), aromatase knockout mice (114) and men with ERα (1) or aromatase (7) mutations suggest an intratesticular effect of oestrogens on spermatogenesis and fertility but their precise roles remain to be determined.

Aromatase (53) and ERα and -β (90) are present in human brain. The importance of oestrogens in male brain, however, is largely unclear. Increased expression of ERα and -β in the nucleus basalis of Meynert of male and female patients with Alzheimer's disease suggests a role for oestrogens in the aetiology of this disease. In women there is some evidence that endogenous oestrogens prevent cognitive decline (125;126) although estrogen use in postmenopausal women was not found to be associated with better cognitive functioning (127;128). In a cross-sectional study elderly men with higher endogenous oestradiol levels performed slightly worse on cognitive function tests (129). The aromatase-deficient (6-8) and oestrogen-resistant men (1) showed heterosexual preference and no evident cognitive abnormalities.

Experimental stroke studies in rats show that stroke-induced damage is less in intact female compared to male or ovariectomized animals and that oestrogen administration to male rats can decrease infarct-size. However, oestrogen replacement in postmenopausal women was associated with a significant increase in stroke, thrombosis and embolism (130). It appears therefore that the importance of oestrogens in both male and female brain, apart from the pituitary, is at this moment unclear.

Oestrogens and bone in men

Sex steroids are essential to maintain bone homeostasis in adults; hypogonadism in men is associated with loss of bone mass (131;132). Because hypogonadal men not only suffer from

androgen deficiency but also lose most of the substrate for oestrogen biosynthesis it is not easy to determine if loss of androgens or oestrogens is the cause of the observed negative effects on bone.

Androgen-resistant 46,XY phenotypic females show no abnormal growth pattern during puberty, but bone mass is generally lower relative to female age-matched controls, and much lower when compared to age-matched normal men (133-135). This difference can partly be explained by relative hypogonadism as a result of orchidectomy with insufficient oestrogen replacement in patients with the androgen insensitivity syndrome. However, even with sufficient hormone replacement therapy lumbar spine and, to a lesser extent, femoral neck bone mineral density is lower in androgen-insensitive males when compared to normal females and males. Male to female transsexuals treated with high-dose oestrogens and antiandrogens do not suffer bone loss despite markedly reduced testosterone levels (136;137). In most cross-sectional studies the plasma total testosterone concentration, after adjustment for age and weight, is not related to BMD at different sites of the skeleton (138-142). In some studies free androgen or free testosterone is related to total body BMD ((143;144), BMD at lumber spine (140), radius (140;145) or femur (139;140;143). In a prospective study in older men, serum testosterone levels were not associated with vertebral fracture risk (146). These observations suggest that androgens are not essential for bone development and maintenance in men.

In contrast, oestrogen insensitivity (1) and aromatase deficiency (4-8) cause decreased BMD in men, despite normal or elevated testosterone levels. In aromatase deficient men physiologic oestrogen replacement was associated with a dramatic increase in BMD (4;5;7;63;64). In cross-sectional studies plasma concentrations of total oestradiol (141;143;144;147) and bioavailable oestrogen (140;143;144;147) were independently related to BMD in men. Bioavailable oestradiol levels are negatively correlated with biochemical markers of bone resorption in men (144). Khosla et al. (148) showed that bone acquisition in younger men is related to total and bioavailable levels of oestrogens, but not of testosterone. Moreover, higher serum estrogens in men are significantly associated with lower rates of bone loss (148-150). Reports indicate that male idiopathic osteoporosis is associated with low peripheral oestradiol levels (151;152) and impaired ERα expression in bone (85). Older men with low serum oestradiol levels are at higher risk of vertebral fracture compared to age-matched men with high oestradiol levels (146). Recently, Falahati-Nini (153) described a interventional study in which an attempt was made to unravel the effects of oestradiol and testosterone on male bone. They rendered elderly men hypogonadal by administrating a GnRH agonist and an aromatase

inhibitor followed by supplementation with testosterone, oestradiol or a combination of the two hormones. Testosterone alone was not able to prevent bone resorption as measured by the excretion of deoxypyridinoline (Dpd) and N-telopeptide (NTx) in urine. Oestradiol alone decreased Dpd and NTx excretion significantly (as compared to non oestradiol non testosterone-treated individuals) but only combination treatment normalized Dpd and NTx excretion. Oestradiol was much more effective in increasing bone formation markers (osteocalcin and the amino-terminal propeptide of procollagen type I) compared to testosterone.

Oestrogen-deficient (5-8) or -resistant males (1) show abnormal growth patterns during puberty. There is no growth spurt induction and epiphyses do not fuse. This results in a larger than average body length. On the other hand, increased aromatase activity not only causes feminization but also leads to accelerated growth and premature closing of epiphyses (33). Administration of an aromatase inhibitor to boys with familial male limited precocious puberty due to an activating LH receptor mutation leads to near normalization of growth pattern despite testosterone levels in the adult range (154). This underlines the importance of oestradiol in growth spurt induction and epiphysial closure not only in girls but also in boys. Furthermore, oestradiol levels in prepubertal girls are eight times higher compared to those in prepubertal boys, probably explaining the more rapid epiphysial maturation in girls (155). Taken together, these observations suggest that oestrogens are of major importance for bone development and maintenance in men. The current paradigm for the roles of androgens and oestrogens in male bone metabolism is that in men both oestrogens and androgens are involved in bone formation whereas oestrogens are primarily responsible for bone maintenance and epiphysial closure (156;157). Androgens are primarily responsible for periosteal bone formation and thus contribute to the increased bone size in men (158) although this concept has been challenged recently (4;159).

Oestrogens, lipid metabolism and cardiovascular disease

Because the incidence of cardiovascular disease differs significantly between men and women the relationship between levels of sex steroids and lipid metabolism has been investigated in detail. In most cross-sectional studies in men, plasma levels of total or free testosterone are positively related to high density lipoprotein-cholesterol (HDL-c; (160-163), but negatively related to plasma triglyceride levels (161;163). Oestradiol is positively related to HDL-c but this correlation is lost when adjusted for plasma testosterone concentration (161). Associations between oestradiol and low density lipoprotein-cholesterol (LDL-c) are

conflicting; some studies found a negative correlation (164;165) but others found the opposite (162). Most data on the effect of supraphysiological concentrations of oestrogens on lipid profile in men are derived from studies on the treatment of prostatic carcinoma patients with oestrogen. The observed effects are largely independent of the dramatic decrease in testosterone concentration because the results strongly differ when compared to lipid levels in nonoestrogen-treated men after orchiectomy. Treatment with intramuscular and/or oral oestrogens leads to an elevation of HDL-c and triglycerides, while LDL-c and total cholesterol levels tend to decrease (16;166-168). Eriksson (169) demonstrated increased LDL synthesis but also a more pronounced LDL catabolism, ultimately resulting in decreased LDL-c concentrations after oestradiol administration. Higher biliary cholesterol secretion rates and higher prevalence of gallstones were also observed in oestrogen-treated men (169). Despite the apparently favourable lipid profile induced by administration of oestrogens to men, this treatment modality was abandoned because of increased cardiovascular morbidity and mortality (170-172). Indeed, a study investigating the effects of oestrogen administration to men after myocardial infarction was discontinued because of lack of efficacy. There were indications of a higher incidence of thromboembolism in the treatment group (173). More recent short-term low-dose administration of oestrogens to hypogonadal men, however, showed, besides a minor improvement of lipid profile, a small but significant reduction in blood pressure and a reduction of norepinephrine- and angiotensin II-induced vasoconstriction (174). Direct vasoactive effects of oestrogens in men were described earlier. Oestradiol was shown to induce relaxation of human coronary arteries *in vitro* (175). Intravenous conjugated oestrogens can attenuate the paradoxical acetylcholine and cold induced constriction of coronary arteries in men (176;177). These effects, however, were not observed after intracoronary infusion of oestradiol (178). Because of their rapidity these vasoactive effects of oestrogens are not believed to be mediated by the classical genomic route: oestradiol, bound to a membrane-associated ER, can directly activate kinase pathways leading to activation of endothelial nitric oxide synthase (179;180). As both ER and aromatase are present in male vascular tissue (59;60;181), these nongenomic actions of oestrogens might be of relevance in male vascular physiology.

Despite these observations, case–control studies in men do not show a relation between the plasma concentration of endogenous oestradiol and the extent of angiographically confirmed coronary artery disease (182-184), peripheral artery disease (185), stroke (186) or haemostatic indices (182). In addition, large prospective trials failed to relate plasma oestradiol levels to the incidence of myocardial infarction in men (187-190). In a cross-sectional study in very old

men low free testosterone levels and higher oestradiol levels were associated with a more rapid progression of carotid atherosclerosis (191) although this association failed to reach significance for oestradiol. In the oestrogen-resistant male (1), relatively low total cholesterol, LDL-c and HDL-c levels were found, and significant calcification in the left anterior descending artery was demonstrated by computed tomography (2). Two adult aromatase-deficient males showed high total cholesterol and LDL-c but low HDL-c (6;7). These levels partially improved after transdermal oestrogen administration (7). In two other aromatase deficient men cholesterol levels were normal (5;8). In three aromatase deficient men signs of insulin resistance were found (5;6;8). However, combined estrogen and antiandrogen administration to normal men resulted in a decrease of insulin sensitivity (192). These observation show that in men oestrogens may affect components of the metabolic syndrome although the impact of these effects in normal men remains to be determined.

Aspects of future research

The growing knowledge of the importance of oestrogens in men has induced numerous new lines of research. The role of oestrogens in the aetiopathogenesis of prostatic carcinoma is currently investigated. Other researchers focus on oestrogens and male fertility, cognition , bone or the metabolic syndrome. The mutual influences of androgens and oestrogens, the existence of two oestrogen receptors and the possibility of nongenomic effects of oestrogens preclude simple conclusions. Moreover, the tissue-specific regulation of aromatase activity by the specific promoters of the aromatase enzyme, the specific action and distribution of the 17β-hydroxysteroid dehydrogenase isoenzymes and the demonstration of ER mutations and polymorphisms indicate that serum concentrations of oestrogens cannot always be relied upon as indicators of intratissue oestrogen concentrations and hormone activity. Other exciting prospects are raised by selective oestrogen receptor modulators (SERMs). These substances have poorly understood tissue-specific agonistic or antagonistic actions on the ERs ((193) for review). Tamoxifen and raloxifene are currently prescribed to women because of their efficacy against breast cancer and postmenopausal bone loss. The current limited knowledge of the effect of oestrogens in males does not allow large-scale interventions with SERMs in men at present but small, short-term experiments were and will be conducted to gain more insights in the pharmacological effects of these substances.

Conclusions

Oestrogens play a major role in many metabolic processes in men. Not only are oestrogens important for growth spurt induction and epiphysial closure, they also help to achieve and maintain peak bone mass. Oestradiol has an androgen-independent inhibitory effect on gonadotrophin release, a minor effect on lipid metabolism and a largely undetermined effect on human male fertility. Additional research has to be performed to further specify the clinical use of this knowledge. Variations in genes encoding the aromatase and 17β-HSD enzymes or the oestrogen receptors may lead to alterations in bone metabolism, lipid metabolism, the hypothalamic–pituitary–gonad axis and fertility in men. The increased awareness of the impact of oestrogens in men will probably lead to the discovery of more subtle variations in oestradiol metabolism.

On the basis of present knowledge of the effects of oestrogens we advise to add oestrogen status to the work-up of premature osteoporosis, hypogonadotrophic hypogonadism and pubertas praecox in men.

Reference List

1. **Smith EP, Boyd J, Frank GR, Takahashi H, Cohen RM, Specker B, Williams TC, Lubahn DB, Korach KS** 1994 Estrogen resistance caused by a mutation in the estrogen-receptor gene in a man. N Engl J Med 331:1056-1061

2. **Sudhir K, Chou TM, Chatterjee K, Smith EP, Williams TC, Kane JP, Malloy MJ, Korach KS, Rubanyi GM** 1997 Premature coronary artery disease associated with a disruptive mutation in the estrogen receptor gene in a man. Circulation 96:3774-3777

3. **Sudhir K, Chou TM, Messina LM, Hutchison SJ, Korach KS, Chatterjee K, Rubanyi GM** 1997 Endothelial dysfunction in a man with disruptive mutation in oestrogen-receptor gene. Lancet 349:1146-1147

4. **Bouillon R, Bex M, Vanderschueren D, Boonen S** 2004 Estrogens are essential for male pubertal periosteal bone expansion. J Clin Endocrinol Metab 89:6025-6029

5. **Maffei L, Murata Y, Rochira V, Tubert G, Aranda C, Vazquez M, Clyne CD, Davis S, Simpson ER, Carani C** 2004 Dysmetabolic syndrome in a man with a novel mutation of the aromatase gene: effects of testosterone, alendronate, and estradiol treatment. J Clin Endocrinol Metab 89:61-70

6. **Morishima A, Grumbach MM, Simpson ER, Fisher C, Qin K** 1995 Aromatase deficiency in male and female siblings caused by a novel mutation and the physiological role of estrogens. J Clin Endocrinol Metab 80:3689-3698

7. **Carani C, Qin K, Simoni M, Faustini-Fustini M, Serpente S, Boyd J, Korach KS, Simpson ER** 1997 Effect of testosterone and estradiol in a man with aromatase deficiency. N Engl J Med 337:91-95

8. **Herrmann BL, Saller B, Janssen OE, Gocke P, Bockisch A, Sperling H, Mann K, Broecker M** 2002 Impact of estrogen replacement therapy in a male with congenital aromatase deficiency caused by a novel mutation in the CYP19 gene. J Clin Endocrinol Metab 87:5476-5484

9. **Korach KS** 1994 Insights from the study of animals lacking functional estrogen receptor. Science 266:1524-1527

10. **Baird DT, Horton R, Longcope C, Tait JF** 1969 Steroid dynamics under steady-state conditions. Recent Prog Horm Res 25:611-664

11. **Saez JM, Morera AM, Dazord A, Bertrand J** 1972 Adrenal and testicular contribution to plasma oestrogens. J Endocrinol 55:41-49

12. **MacDonald PC, Madden JD, Brenner PF, Wilson JD, Siiteri PK** 1979 Origin of estrogen in normal men and in women with testicular feminization. J Clin Endocrinol Metab 49:905-916

13. **Baird DT, Uno A, Melby JC** 1969 Adrenal secretion of androgens and oestrogens. J Endocrinol 45:135-136

14. **Veldhuis JD, Lizarralde G, Iranmanesh A** 1992 Divergent effects of short term glucocorticoid excess on the gonadotropic and somatotropic axes in normal men. J Clin Endocrinol Metab 74:96-102

15. **Bartsch W, Horst HJ, Becker H, Nehse G** 1977 Sex hormone binding globulin binding capacity, testosterone, 5alpha-dihydrotestosterone, oestradiol and prolactin in plasma of patients with prostatic carcinoma under various types of hormonal treatment. Acta Endocrinol (Copenh) 85:650-664

16. **Moorjani S, Dupont A, Labrie F, Lupien PJ, Gagne C, Brun D, Giguere M, Belanger A, Cusan L** 1988 Changes in plasma lipoproteins during various androgen suppression therapies in men with prostatic carcinoma: effects of orchiectomy, estrogen, and combination treatment with luteinizing hormone-releasing hormone agonist and flutamide. J Clin Endocrinol Metab 66:314-322

17. **Longcope C, Goldfield SR, Brambilla DJ, McKinlay J** 1990 Androgens, estrogens, and sex hormone-binding globulin in middle-aged men. J Clin Endocrinol Metab 71:1442-1446

18. **Vermeulen A** 1991 Clinical review 24: Androgens in the aging male. J Clin Endocrinol Metab 73:221-224

19. **Simon D, Preziosi P, Barrett-Connor E, Roger M, Saint-Paul M, Nahoul K, Papoz L** 1992 The influence of aging on plasma sex hormones in men: the Telecom Study. Am J Epidemiol 135:783-791

20. **Field AE, Colditz GA, Willett WC, Longcope C, McKinlay JB** 1994 The relation of smoking, age, relative weight, and dietary intake to serum adrenal steroids, sex hormones, and sex hormone-binding globulin in middle-aged men. J Clin Endocrinol Metab 79:1310-1316

21. **Ferrini RL, Barrett-Connor E** 1998 Sex hormones and age: a cross-sectional study of testosterone and estradiol and their bioavailable fractions in community-dwelling men. Am J Epidemiol 147:750-754

22. **Drafta D, Schindler AE, Stroe E, Neacsu E** 1982 Age-related changes of plasma steroids in normal adult males. J Steroid Biochem 17:683-687

23. **Zumoff B, Strain GW, Kream J, O'Connor J, Rosenfeld RS, Levin J, Fukushima DK** 1982 Age variation of the 24-hour mean plasma concentrations of androgens, estrogens, and gonadotropins in normal adult men. J Clin Endocrinol Metab 54:534-538

24. **Juneja HS, Karanth S, Dutt A, Parte P, Meherjee P** 1991 Diurnal variations and temporal coupling of bioactive and immunoactive luteinizing hormone, prolactin, testosterone and 17-beta-estradiol in adult men. Horm Res 35:89-94

25. **Murono EP, Nankin HR, Lin T, Osterman J** 1982 The aging Leydig cell V. Diurnal rhythms in aged men. Acta Endocrinol (Copenh) 99:619-623

26. **Dunn JF, Nisula BC, Rodbard D** 1981 Transport of steroid hormones: binding of 21 endogenous steroids to both testosterone-binding globulin and corticosteroid-binding globulin in human plasma. J Clin Endocrinol Metab 53:58-68

27. **Mendel CM** 1989 The free hormone hypothesis: a physiologically based mathematical model. Endocr Rev 10:232-274

28. **Winters SJ, Janick JJ, Loriaux DL, Sherins RJ** 1979 Studies on the role of sex steroids in the feedback control of gonadotropin concentrations in men. II. Use of the estrogen antagonist, clomiphene citrate. J Clin Endocrinol Metab 48:222-227

29. **Knuth UA, Hano R, Nieschlag E** 1984 Effect of flutamide or cyproterone acetate on pituitary and testicular hormones in normal men. J Clin Endocrinol Metab 59:963-969

30. **Sterzik K, Rosenbusch B, Mogck J, Heyden M, Lichtenberger K** 1993 Tamoxifen treatment of oligozoospermia: a re-evaluation of its effects including additional sperm function tests. Arch Gynecol Obstet 252:143-147

31. **Kley HK, Deselaers T, Peerenboom H, Kruskemper HL** 1980 Enhanced conversion of androstenedione to estrogens in obese males. J Clin Endocrinol Metab 51:1128-1132

32. **Berkovitz GD, Guerami A, Brown TR, MacDonald PC, Migeon CJ** 1985 Familial gynecomastia with increased extraglandular aromatization of plasma carbon19-steroids. J Clin Invest 75:1763-1769

33. **Hemsell DL, Edman CD, Marks JF, Siiteri PK, MacDonald PC** 1977 Massive extranglandular aromatization of plasma androstenedione resulting in feminization of a prepubertal boy. J Clin Invest 60:455-464

34. **Wang C, Baker HW, Burger HG, De Kretser DM, Hudson B** 1975 Hormonal studies in Klinefelter's syndrome. Clin Endocrinol (Oxf) 4:399-411

35. **Bulun SE, Noble LS, Takayama K, Michael MD, Agarwal V, Fisher C, Zhao Y, Hinshelwood MM, Ito Y, Simpson ER** 1997 Endocrine disorders associated with inappropriately high aromatase expression. J Steroid Biochem Mol Biol 61:133-139

36. **Young J, Bulun SE, Agarwal V, Couzinet B, Mendelson CR, Simpson ER, Schaison G** 1996 Aromatase expression in a feminizing adrenocortical tumor. J Clin Endocrinol Metab 81:3173-3176

37. **Gordon GG, Olivo J, Rafil F, Southren AL** 1975 Conversion of androgens to estrogens in cirrhosis of the liver. J Clin Endocrinol Metab 40:1018-1026

38. **Paja M, Diez S, Lucas T, Ojeda A, Salto L, Estrada J** 1994 Dexamethasone-suppressible feminizing adrenal adenoma. Postgrad Med J 70:584-588

39. **Boyar RM, Nogeire C, Fukushima D, Hellman L, Fishman J** 1977 Studies of the diurnal pattern of plasma corticosteroids and gonadotropins in two cases of feminizing adrenal carcinoma: measurements of estrogen and corticosteroid production. J Clin Endocrinol Metab 44:39-45

40. **Karl M, Lamberts SW, Koper JW, Katz DA, Huizenga NE, Kino T, Haddad BR, Hughes MR, Chrousos GP** 1996 Cushing's disease preceded by generalized glucocorticoid resistance: clinical consequences of a novel, dominant-negative glucocorticoid receptor mutation. Proc Assoc Am Physicians 108:296-307

41. **T'sjoen GG, Giagulli VA, Delva H, Crabbe P, De Bacquer D, Kaufman JM** 2005 Comparative Assessment in Young and Elderly Men of the Gonadotropin Response to Aromatase Inhibition. J Clin Endocrinol Metab

42. **Fiet J, Villette JM, Bertagna C, de Gery A, Hucher M, Husson JM, Raynaud JP** 1987 Plasma hormone levels before and after orchiectomy in prostate cancer patients. Prog Clin Biol Res 243A:33-44

43. **Hobbs CJ, Jones RE, Plymate SR** 1996 Nandrolone, a 19-nortestosterone, enhances insulin-independent glucose uptake in normal men. J Clin Endocrinol Metab 81:1582-1585

44. **Rivarola MA, Singleton RT, Migeon CJ** 1967 Splanchnic extraction and interconversion of testosterone and androstenedione in man. J Clin Invest 46:2095-2100

45. **RECANT L, RIGGS DS** 1952 Thyroid function in nephrosis. J Clin Invest 31:789-797

46. **Mendel CM, Murai JT, Siiteri PK, Monroe SE, Inoue M** 1989 Conservation of free but not total or non-sex-hormone-binding-globulin-bound testosterone in serum from Nagase analbuminemic rats. Endocrinology 124:3128-3130

47. **Damassa DA, Gustafson AW** 1988 Effects of chronic infusions of sex steroid-binding protein on the testosterone-mediated inhibition of gonadotropin secretion and maintenance of sex accessory glands in male rats. Endocrinology 123:1885-1892

48. **Petra PH, Stanczyk FZ, Namkung PC, Fritz MA, Novy MJ** 1985 Direct effect of sex steroid-binding protein (SBP) of plasma on the metabolic clearance rate of testosterone in the rhesus macaque. J Steroid Biochem 22:739-746

49. **Vermeulen A, Ando S** 1979 Metabolic clearance rate and interconversion of androgens and the influence of the free androgen fraction. J Clin Endocrinol Metab 48:320-326

50. **Plymate SR, Namkung PC, Metej LA, Petra PH** 1990 Direct effect of plasma sex hormone binding globulin (SHBG) on the metabolic clearance rate of 17 beta-estradiol in the primate. J Steroid Biochem 36:311-317

51. **Hammes A, Andreassen TK, Spoelgen R, Raila J, Hubner N, Schulz H, Metzger J, Schweigert FJ, Luppa PB, Nykjaer A, Willnow TE** 2005 Role of endocytosis in cellular uptake of sex steroids. Cell 122:751-762

52. **Simpson ER, Davis SR** 2001 Minireview: aromatase and the regulation of estrogen biosynthesis--some new perspectives. Endocrinology 142:4589-4594

53. **Stoffel-Wagner B, Watzka M, Schramm J, Bidlingmaier F, Klingmuller D** 1999 Expression of CYP19 (aromatase) mRNA in different areas of the human brain. J Steroid Biochem Mol Biol 70:237-241

54. **Schindler AE, Ebert A, Friedrich E** 1972 Conversion of androstenedione to estrone by human tissue. J Clin Endocrinol Metab 35:627-630

55. **Longcope C, Pratt JH, Schneider SH, Fineberg SE** 1978 Aromatization of androgens by muscle and adipose tissue in vivo. J Clin Endocrinol Metab 46:146-152

56. **Schweikert HU, Milewich L, Wilson JD** 1975 Aromatization of androstenedione by isolated human hairs. J Clin Endocrinol Metab 40:413-417

57. **Schweikert HU, Wolf L, Romalo G** 1995 Oestrogen formation from androstenedione in human bone. Clin Endocrinol (Oxf) 43:37-42

58. **Janssen JM, Bland R, Hewison M, Coughtrie MW, Sharp S, Arts J, Pols HA, van Leeuwen JP** 1999 Estradiol formation by human osteoblasts via multiple pathways: relation with osteoblast function. J Cell Biochem 75:528-537

59. **Harada N, Sasano H, Murakami H, Ohkuma T, Nagura H, Takagi Y** 1999 Localized expression of aromatase in human vascular tissues. Circ Res 84:1285-1291

60. **Murakami H, Harada N, Sasano H** 2001 Aromatase in atherosclerotic lesions of human aorta. J Steroid Biochem Mol Biol 79:67-74

61. **Thompson EA, Jr., Siiteri PK** 1974 The involvement of human placental microsomal cytochrome P-450 in aromatization. J Biol Chem 249:5373-5378

62. **Deladoey J, Fluck C, Bex M, Yoshimura N, Harada N, Mullis PE** 1999 Aromatase deficiency caused by a novel P450arom gene mutation: impact of absent estrogen production on serum gonadotropin concentration in a boy. J Clin Endocrinol Metab 84:4050-4054

63. **Bilezikian JP, Morishima A, Bell J, Grumbach MM** 1998 Increased bone mass as a result of estrogen therapy in a man with aromatase deficiency. N Engl J Med 339:599-603

64. **Herrmann BL, Janssen OE, Hahn S, Broecker-Preuss M, Mann K** 2005 Effects of estrogen replacement therapy on bone and glucose metabolism in a male with congenital aromatase deficiency. Horm Metab Res 37:178-183

65. **Stratakis CA, Vottero A, Brodie A, Kirschner LS, DeAtkine D, Lu Q, Yue W, Mitsiades CS, Flor AW, Chrousos GP** 1998 The aromatase excess syndrome is associated with feminization of both sexes and autosomal dominant transmission of aberrant P450 aromatase gene transcription. J Clin Endocrinol Metab 83:1348-1357

66. **Shozu M, Sebastian S, Takayama K, Hsu WT, Schultz RA, Neely K, Bryant M, Bulun SE** 2003 Estrogen excess associated with novel gain-of-function mutations affecting the aromatase gene. N Engl J Med 348:1855-1865

67. **Aiginger P, Kolbe H, Kuhbock J, Spona J, Geyer G** 1981 The endocrinology of testicular germinal cell tumors. Acta Endocrinol (Copenh) 97:419-426

68. **Kley HK, Edelmann P, Kruskemper HL** 1980 Relationship of plasma sex hormones to different parameters of obesity in male subjects. Metabolism 29:1041-1045

69. **Strain GW, Zumoff B, Kream J, Strain JJ, Deucher R, Rosenfeld RS, Levin J, Fukushima DK** 1982 Mild Hypogonadotropic hypogonadism in obese men. Metabolism 31:871-875

70. **Strain GW, Zumoff B, Miller LK, Rosner W, Levit C, Kalin M, Hershcopf RJ, Rosenfeld RS** 1988 Effect of massive weight loss on hypothalamic-pituitary-gonadal function in obese men. J Clin Endocrinol Metab 66:1019-1023

71. **Stanik S, Dornfeld LP, Maxwell MH, Viosca SP, Korenman SG** 1981 The effect of weight loss on reproductive hormones in obese men. J Clin Endocrinol Metab 53:828-832

72. **Longcope C, Pratt JH, Schneider S, Fineberg E** 1984 Estrogen and androgen dynamics in liver disease. J Endocrinol Invest 7:629-634

73. **Bulun SE, Simpson ER** 1994 Competitive reverse transcription-polymerase chain reaction analysis indicates that levels of aromatase cytochrome P450 transcripts in adipose tissue of buttocks, thighs, and abdomen of women increase with advancing age. J Clin Endocrinol Metab 78:428-432

74. **Mindnich R, Moller G, Adamski J** 2004 The role of 17 beta-hydroxysteroid dehydrogenases. Mol Cell Endocrinol 218:7-20

75. **Vihko P, Isomaa V, Ghosh D** 2001 Structure and function of 17beta-hydroxysteroid dehydrogenase type 1 and type 2. Mol Cell Endocrinol 171:71-76

76. **Geissler WM, Davis DL, Wu L, Bradshaw KD, Patel S, Mendonca BB, Elliston KO, Wilson JD, Russell DW, Andersson S** 1994 Male pseudohermaphroditism caused by mutations of testicular 17 beta-hydroxysteroid dehydrogenase 3. Nat Genet 7:34-39

77. **Boehmer AL, Brinkmann AO, Sandkuijl LA, Halley DJ, Niermeijer MF, Andersson S, de Jong FH, Kayserili H, de Vroede MA, Otten BJ, Rouwe CW, Mendonca BB, Rodrigues C, Bode HH, de Ruiter PE, Delemarre-van de Waal HA, Drop SL** 1999 17Beta-hydroxysteroid dehydrogenase-3 deficiency: diagnosis, phenotypic variability, population genetics, and worldwide distribution of ancient and de novo mutations. J Clin Endocrinol Metab 84:4713-4721

78. **Peltoketo H, Luu-The V, Simard J, Adamski J** 1999 17beta-hydroxysteroid dehydrogenase (HSD)/17-ketosteroid reductase (KSR) family; nomenclature and main characteristics of the 17HSD/KSR enzymes. J Mol Endocrinol 23:1-11

79. **Feix M, Wolf L, Schweikert HU** 2001 Distribution of 17beta-hydroxysteroid dehydrogenases in human osteoblast-like cells. Mol Cell Endocrinol 171:163-164

80. **Adamski J, Jakob FJ** 2001 A guide to 17beta-hydroxysteroid dehydrogenases. Mol Cell Endocrinol 171:1-4

81. **Labrie F** 1991 Intracrinology. Mol Cell Endocrinol 78:C113-C118

82. **Labrie F, Belanger A, Cusan L, Candas B** 1997 Physiological changes in dehydroepiandrosterone are not reflected by serum levels of active androgens and estrogens but of their metabolites: intracrinology. J Clin Endocrinol Metab 82:2403-2409

83. **Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson JA** 1996 Cloning of a novel receptor expressed in rat prostate and ovary. Proc Natl Acad Sci U S A 93:5925-5930

84. **Leav I, Lau KM, Adams JY, McNeal JE, Taplin ME, Wang J, Singh H, Ho SM** 2001 Comparative studies of the estrogen receptors beta and alpha and the androgen receptor in normal human prostate glands, dysplasia, and in primary and metastatic carcinoma. Am J Pathol 159:79-92

85. **Braidman I, Baris C, Wood L, Selby P, Adams J, Freemont A, Hoyland J** 2000 Preliminary evidence for impaired estrogen receptor-alpha protein expression in osteoblasts and osteocytes from men with idiopathic osteoporosis. Bone 26:423-427

86. **Braidman IP, Hainey L, Batra G, Selby PL, Saunders PT, Hoyland JA** 2001 Localization of estrogen receptor beta protein expression in adult human bone. J Bone Miner Res 16:214-220

87. **Nilsson LO, Boman A, Savendahl L, Grigelioniene G, Ohlsson C, Ritzen EM, Wroblewski J** 1999 Demonstration of estrogen receptor-beta immunoreactivity in human growth plate cartilage. J Clin Endocrinol Metab 84:370-373

88. **Ushiyama T, Ueyama H, Inoue K, Ohkubo I, Hukuda S** 1999 Expression of genes for estrogen receptors alpha and beta in human articular chondrocytes. Osteoarthritis Cartilage 7:560-566

89. **Pedersen SB, Bruun JM, Hube F, Kristensen K, Hauner H, Richelsen B** 2001 Demonstration of estrogen receptor subtypes alpha and beta in human adipose tissue: influences of adipose cell differentiation and fat depot localization. Mol Cell Endocrinol 182:27-37

90. **Ishunina TA, Swaab DF** 2001 Increased expression of estrogen receptor alpha and beta in the nucleus basalis of Meynert in Alzheimer's disease. Neurobiol Aging 22:417-426

91. **Shupnik MA, Pitt LK, Soh AY, Anderson A, Lopes MB, Laws ER, Jr.** 1998 Selective expression of estrogen receptor alpha and beta isoforms in human pituitary tumors. J Clin Endocrinol Metab 83:3965-3972

92. **Makinen S, Makela S, Weihua Z, Warner M, Rosenlund B, Salmi S, Hovatta O, Gustafsson JK** 2001 Localization of oestrogen receptors alpha and beta in human testis. Mol Hum Reprod 7:497-503

93. **Hess RA, Bunick D, Lee KH, Bahr J, Taylor JA, Korach KS, Lubahn DB** 1997 A role for oestrogens in the male reproductive system. Nature 390:509-512

94. **O'Donnell L, Robertson KM, Jones ME, Simpson ER** 2001 Estrogen and spermatogenesis. Endocr Rev 22:289-318

95. **Arts J, Kuiper GG, Janssen JM, Gustafsson JA, Lowik CW, Pols HA, van Leeuwen JP** 1997 Differential expression of estrogen receptors alpha and beta mRNA during differentiation of human osteoblast SV-HFO cells. Endocrinology 138:5067-5070

96. **Krege JH, Hodgin JB, Couse JF, Enmark E, Warner M, Mahler JF, Sar M, Korach KS, Gustafsson JA, Smithies O** 1998 Generation and reproductive phenotypes of mice lacking estrogen receptor beta. Proc Natl Acad Sci U S A 95:15677-15682

97. **Weihua Z, Makela S, Andersson LC, Salmi S, Saji S, Webster JI, Jensen EV, Nilsson S, Warner M, Gustafsson JA** 2001 A role for estrogen receptor beta in the regulation of growth of the ventral prostate. Proc Natl Acad Sci U S A 98:6330-6335

98. **Dupont S, Krust A, Gansmuller A, Dierich A, Chambon P, Mark M** 2000 Effect of single and compound knockouts of estrogen receptors alpha (ERalpha) and beta (ERbeta) on mouse reproductive phenotypes. Development 127:4277-4291

99. **Imamov O, Morani A, Shim GJ, Omoto Y, Thulin-Andersson C, Warner M, Gustafsson JA** 2004 Estrogen receptor beta regulates epithelial cellular differentiation in the mouse ventral prostate. Proc Natl Acad Sci U S A 101:9375-9380

100. **Imamov O, Lopatkin NA, Gustafsson JA** 2004 Estrogen receptor beta in prostate cancer. N Engl J Med 351:2773-2774

101. **Guerini V, Sau D, Scaccianoce E, Rusmini P, Ciana P, Maggi A, Martini PG, Katzenellenbogen BS, Martini L, Motta M, Poletti A** 2005 The androgen derivative 5alpha-androstane-3beta,17beta-diol

inhibits prostate cancer cell migration through activation of the estrogen receptor beta subtype. Cancer Res 65:5445-5453

102. **Couse JF, Korach KS** 1999 Estrogen receptor null mice: what have we learned and where will they lead us? Endocr Rev 20:358-417

103. **Nilsson S, Makela S, Treuter E, Tujague M, Thomsen J, Andersson G, Enmark E, Pettersson K, Warner M, Gustafsson JA** 2001 Mechanisms of estrogen action. Physiol Rev 81:1535-1565

104. **Koehler KF, Helguero LA, Haldosen LA, Warner M, Gustafsson JA** 2005 Reflections on the discovery and significance of estrogen receptor beta. Endocr Rev 26:465-478

105. **Marynick SP, Loriaux DL, Sherins RJ, Pita JC, Jr., Lipsett MB** 1979 Evidence that testosterone can suppress pituitary gonadotropin secretion independently of peripheral aromatization. J Clin Endocrinol Metab 49:396-398

106. **Finkelstein JS, Whitcomb RW, O'Dea LS, Longcope C, Schoenfeld DA, Crowley WF, Jr.** 1991 Sex steroid control of gonadotropin secretion in the human male. I. Effects of testosterone administration in normal and gonadotropin-releasing hormone-deficient men. J Clin Endocrinol Metab 73:609-620

107. **Stewart-Bentley M, Odell W, Horton R** 1974 The feedback control of luteinizing hormone in normal adult men. J Clin Endocrinol Metab 38:545-553

108. **Santen RJ** 1975 Is aromatization of testosterone to estradiol required for inhibition of luteinizing hormone secretion in men? J Clin Invest 56:1555-1563

109. **Veldhuis JD, Rogol AD, Samojlik E, Ertel NH** 1984 Role of endogenous opiates in the expression of negative feedback actions of androgen and estrogen on pulsatile properties of luteinizing hormone secretion in man. J Clin Invest 74:47-55

110. **Eddy EM, Washburn TF, Bunch DO, Goulding EH, Gladen BC, Lubahn DB, Korach KS** 1996 Targeted disruption of the estrogen receptor gene in male mice causes alteration of spermatogenesis and infertility. Endocrinology 137:4796-4805

111. **Honda S, Harada N, Ito S, Takagi Y, Maeda S** 1998 Disruption of sexual behavior in male aromatase-deficient mice lacking exons 1 and 2 of the cyp19 gene. Biochem Biophys Res Commun 252:445-449

112. **Fisher CR, Graves KH, Parlow AF, Simpson ER** 1998 Characterization of mice deficient in aromatase (ArKO) because of targeted disruption of the cyp19 gene. Proc Natl Acad Sci U S A 95:6965-6970

113. **Ogawa S, Chan J, Chester AE, Gustafsson JA, Korach KS, Pfaff DW** 1999 Survival of reproductive behaviors in estrogen receptor beta gene-deficient (betaERKO) male and female mice. Proc Natl Acad Sci U S A 96:12887-12892

114. **Robertson KM, O'Donnell L, Jones ME, Meachem SJ, Boon WC, Fisher CR, Graves KH, McLachlan RI, Simpson ER** 1999 Impairment of spermatogenesis in mice lacking a functional aromatase (cyp 19) gene. Proc Natl Acad Sci U S A 96:7986-7991

115. **Windahl SH, Vidal O, Andersson G, Gustafsson JA, Ohlsson C** 1999 Increased cortical bone mineral content but unchanged trabecular bone mineral density in female ERbeta(-/-) mice. J Clin Invest 104:895-901

116. **Oz OK, Zerwekh JE, Fisher C, Graves K, Nanu L, Millsaps R, Simpson ER** 2000 Bone has a sexually dimorphic response to aromatase deficiency. J Bone Miner Res 15:507-514

117. **Faiman C, Winter JS** 1974 The control of gonadotropin secretion in complete testicular feminization. J Clin Endocrinol Metab 39:631-638

118. **Lacroix A, McKenna TJ, Rabinowitz D** 1979 Sex steroid modulation of gonadotropins in normal men and in androgen insensitivity syndrome. J Clin Endocrinol Metab 48:235-240

119. **Veldhuis JD, Urban RJ, Dufau ML** 1994 Differential responses of biologically active luteinizing hormone secretion in older versus young men to interruption of androgen negative feedback. J Clin Endocrinol Metab 79:1763-1770

120. **Verhelst J, Denis L, Van Vliet P, Van Poppel H, Braeckman J, Van Cangh P, Mattelaer J, D'Hulster D, Mahler C** 1994 Endocrine profiles during administration of the new non-steroidal anti-androgen Casodex in prostate cancer. Clin Endocrinol (Oxf) 41:525-530

121. **Finkelstein JS, O'Dea LS, Whitcomb RW, Crowley WF, Jr.** 1991 Sex steroid control of gonadotropin secretion in the human male. II. Effects of estradiol administration in normal and gonadotropin-releasing hormone-deficient men. J Clin Endocrinol Metab 73:621-628

122. **Winters SJ, Troen P** 1985 Evidence for a role of endogenous estrogen in the hypothalamic control of gonadotropin secretion in men. J Clin Endocrinol Metab 61:842-845

123. **Dony JM, Smals AG, Rolland R, Fauser BC, Thomas CM** 1985 Effect of lower versus higher doses of tamoxifen on pituitary-gonadal function and sperm indices in oligozoospermic men. Andrologia 17:369-378

124. **Hayes FJ, Seminara SB, Decruz S, Boepple PA, Crowley WF, Jr.** 2000 Aromatase inhibition in the human male reveals a hypothalamic site of estrogen feedback. J Clin Endocrinol Metab 85:3027-3035

125. **Yaffe K, Lui LY, Grady D, Cauley J, Kramer J, Cummings SR** 2000 Cognitive decline in women in relation to non-protein-bound oestradiol concentrations. Lancet 356:708-712

126. **Cunningham CJ, Sinnott M, Denihan A, Rowan M, Walsh JB, O'Moore R, Coakley D, Coen RF, Lawler BA, O'Neill DD** 2001 Endogenous sex hormone levels in postmenopausal women with Alzheimer's disease. J Clin Endocrinol Metab 86:1099-1103

127. **Espeland MA, Rapp SR, Shumaker SA, Brunner R, Manson JE, Sherwin BB, Hsia J, Margolis KL, Hogan PE, Wallace R, Dailey M, Freeman R, Hays J** 2004 Conjugated equine estrogens and global cognitive function in postmenopausal women: Women's Health Initiative Memory Study. JAMA 291:2959-2968

128. **Shumaker SA, Legault C, Kuller L, Rapp SR, Thal L, Lane DS, Fillit H, Stefanick ML, Hendrix SL, Lewis CE, Masaki K, Coker LH** 2004 Conjugated equine estrogens and incidence of probable dementia and mild cognitive impairment in postmenopausal women: Women's Health Initiative Memory Study. JAMA 291:2947-2958

129. **Barrett-Connor E, Goodman-Gruen D, Patay B** 1999 Endogenous sex hormones and cognitive function in older men. J Clin Endocrinol Metab 84:3681-3685

130. **Anderson GL, Limacher M, Assaf AR, Bassford T, Beresford SA, Black H, Bonds D, Brunner R, Brzyski R, Caan B, Chlebowski R, Curb D, Gass M, Hays J, Heiss G, Hendrix S, Howard BV, Hsia J, Hubbell A, Jackson R, Johnson KC, Judd H, Kotchen JM, Kuller L, LaCroix AZ, Lane D, Langer RD, Lasser N, Lewis CE, Manson J, Margolis K, Ockene J, O'Sullivan MJ, Phillips L, Prentice RL, Ritenbaugh C, Robbins J, Rossouw JE, Sarto G, Stefanick ML, Van Horn L, Wactawski-Wende J, Wallace R, Wassertheil-Smoller S** 2004 Effects of conjugated equine estrogen in postmenopausal women with hysterectomy: the Women's Health Initiative randomized controlled trial. JAMA 291:1701-1712

131. **Stepan JJ, Lachman M, Zverina J, Pacovsky V, Baylink DJ** 1989 Castrated men exhibit bone loss: effect of calcitonin treatment on biochemical indices of bone remodeling. J Clin Endocrinol Metab 69:523-527

132. **Daniell HW** 1997 Osteoporosis after orchiectomy for prostate cancer. J Urol 157:439-444

133. **Soule SG, Conway G, Prelevic GM, Prentice M, Ginsburg J, Jacobs HS** 1995 Osteopenia as a feature of the androgen insensitivity syndrome. Clin Endocrinol (Oxf) 43:671-675

134. **Bertelloni S, Baroncelli GI, Federico G, Cappa M, Lala R, Saggese G** 1998 Altered bone mineral density in patients with complete androgen insensitivity syndrome. Horm Res 50:309-314

135. **Marcus R, Leary D, Schneider DL, Shane E, Favus M, Quigley CA** 2000 The contribution of testosterone to skeletal development and maintenance: lessons from the androgen insensitivity syndrome. J Clin Endocrinol Metab 85:1032-1037

136. **Lips P, Asscheman H, Uitewaal P, Netelenbos JC, Gooren L** 1989 The effect of cross-gender hormonal treatment on bone metabolism in male-to-female transsexuals. J Bone Miner Res 4:657-662

137. **Van Kesteren P, Lips P, Gooren LJ, Asscheman H, Megens J** 1998 Long-term follow-up of bone mineral density and bone metabolism in transsexuals treated with cross-sex hormones. Clin Endocrinol (Oxf) 48:347-354

138. **Meier DE, Orwoll ES, Keenan EJ, Fagerstrom RM** 1987 Marked decline in trabecular bone mineral content in healthy men with age: lack of association with sex steroid levels. J Am Geriatr Soc 35:189-197

139. **Murphy S, Khaw KT, Cassidy A, Compston JE** 1993 Sex hormones and bone mineral density in elderly men. Bone Miner 20:133-140

140. **Greendale GA, Edelstein S, Barrett-Connor E** 1997 Endogenous sex steroids and bone mineral density in older women and men: the Rancho Bernardo Study. J Bone Miner Res 12:1833-1843

141. **Slemenda CW, Longcope C, Zhou L, Hui SL, Peacock M, Johnston CC** 1997 Sex steroids and bone mass in older men. Positive associations with serum estrogens and negative associations with androgens. J Clin Invest 100:1755-1759

142. **Amin S, Zhang Y, Sawin CT, Evans SR, Hannan MT, Kiel DP, Wilson PW, Felson DT** 2000 Association of hypogonadism and estradiol levels with bone mineral density in elderly men from the Framingham study. Ann Intern Med 133:951-963

143. **van den Beld AW, de Jong FH, Grobbee DE, Pols HA, Lamberts SW** 2000 Measures of bioavailable serum testosterone and estradiol and their relationships with muscle strength, bone density, and body composition in elderly men. J Clin Endocrinol Metab 85:3276-3282

144. **Szulc P, Munoz F, Claustrat B, Garnero P, Marchand F, Duboeuf F, Delmas PD** 2001 Bioavailable estradiol may be an important determinant of osteoporosis in men: the MINOS study. J Clin Endocrinol Metab 86:192-199

145. **Kelly PJ, Pocock NA, Sambrook PN, Eisman JA** 1990 Dietary calcium, sex hormones, and bone mineral density in men. BMJ 300:1361-1364

146. **Barrett-Connor E, Mueller JE, von Muhlen DG, Laughlin GA, Schneider DL, Sartoris DJ** 2000 Low levels of estradiol are associated with vertebral fractures in older men, but not women: the Rancho Bernardo Study. J Clin Endocrinol Metab 85:219-223

147. **Khosla S, Melton LJ, III, Atkinson EJ, O'Fallon WM, Klee GG, Riggs BL** 1998 Relationship of serum sex steroid levels and bone turnover markers with bone mineral density in men and women: a key role for bioavailable estrogen. J Clin Endocrinol Metab 83:2266-2274

148. **Khosla S, Melton LJ, III, Atkinson EJ, O'Fallon WM** 2001 Relationship of serum sex steroid levels to longitudinal changes in bone density in young versus elderly men. J Clin Endocrinol Metab 86:3555-3561

149. **Van P, I, Goemaere S, Kaufman JM** 2003 Bioavailable estradiol and an aromatase gene polymorphism are determinants of bone mineral density changes in men over 70 years of age. J Clin Endocrinol Metab 88:3075-3081

150. **Gennari L, Merlotti D, Martini G, Gonnelli S, Franci B, Campagna S, Lucani B, Dal Canto N, Valenti R, Gennari C, Nuti R** 2003 Longitudinal association between sex hormone levels, bone loss, and bone turnover in elderly men. J Clin Endocrinol Metab 88:5327-5333

151. **Carlsen CG, Soerensen TH, Eriksen EF** 2000 Prevalence of low serum estradiol levels in male osteoporosis. Osteoporos Int 11:697-701

152. **Van P, I, Goemaere S, Zmierczak H, Kaufman JM** 2004 Perturbed sex steroid status in men with idiopathic osteoporosis and their sons. J Clin Endocrinol Metab 89:4949-4953

153. **Falahati-Nini A, Riggs BL, Atkinson EJ, O'Fallon WM, Eastell R, Khosla S** 2000 Relative contributions of testosterone and estrogen in regulating bone resorption and formation in normal elderly men. J Clin Invest 106:1553-1560

154. **Laue L, Jones J, Barnes KM, Cutler GB, Jr.** 1993 Treatment of familial male precocious puberty with spironolactone, testolactone, and deslorelin. J Clin Endocrinol Metab 76:151-155

155. **Cutler GB, Jr.** 1997 The role of estrogen in bone growth and maturation during childhood and adolescence. J Steroid Biochem Mol Biol 61:141-144

156. **Khosla S** 2004 Role of hormonal changes in the pathogenesis of osteoporosis in men. Calcif Tissue Int 75:110-113

157. **Gennari L, Nuti R, Bilezikian JP** 2004 Aromatase activity and bone homeostasis in men. J Clin Endocrinol Metab 89:5898-5907

158. **Seeman E** 2004 The growth and age-related origins of bone fragility in men. Calcif Tissue Int 75:100-109

159. **Vanderschueren D, Venken K, Ophoff J, Bouillon R, Boonen S** 2005 Sex Steroids and the Periosteum: Reconsidering the Role of Androgens and Estrogens in Periosteal Expansion. J Clin Endocrinol Metab

160. **Heller RF, Wheeler MJ, Micallef J, Miller NE, Lewis B** 1983 Relationship of high density lipoprotein cholesterol with total and free testosterone and sex hormone binding globulin. Acta Endocrinol (Copenh) 104:253-256

161. **Lichtenstein MJ, Yarnell JW, Elwood PC, Beswick AD, Sweetnam PM, Marks V, Teale D, Riad-Fahmy D** 1987 Sex hormones, insulin, lipids, and prevalent ischemic heart disease. Am J Epidemiol 126:647-657

162. **Khaw KT, Barrett-Connor E** 1991 Endogenous sex hormones, high density lipoprotein cholesterol, and other lipoprotein fractions in men. Arterioscler Thromb 11:489-494

163. **Haffner SM, Mykkanen L, Valdez RA, Katz MS** 1993 Relationship of sex hormones to lipids and lipoproteins in nondiabetic men. J Clin Endocrinol Metab 77:1610-1615

164. **Hamalainen E, Adlercreutz H, Ehnholm C, Puska P** 1986 Relationships of serum lipoproteins and apoproteins to sex hormones and to the binding capacity of sex hormone binding globulin in healthy Finnish men. Metabolism 35:535-541

165. **Stefanick ML, Williams PT, Krauss RM, Terry RB, Vranizan KM, Wood PD** 1987 Relationships of plasma estradiol, testosterone, and sex hormone-binding globulin with lipoproteins, apolipoproteins, and high density lipoprotein subfractions in men. J Clin Endocrinol Metab 64:723-729

166. **Wallentin L, Varenhorst E** 1981 Plasma lipoproteins during anti-androgen treatment by estrogens or orchidectomy in men with prostatic carcinoma. Horm Metab Res 13:293-297

167. **Bulusu NV, Lewis SB, Das S, Clayton WE, Jr.** 1982 Serum lipid changes after estrogen therapy in prostatic carcinoma. Urology 20:147-150

168. **Henriksson P, Angelin B, Berglund L** 1992 Hormonal regulation of serum Lp (a) levels. Opposite effects after estrogen treatment and orchidectomy in males with prostatic carcinoma. J Clin Invest 89:1166-1171

169. **Eriksson M, Berglund L, Rudling M, Henriksson P, Angelin B** 1989 Effects of estrogen on low density lipoprotein metabolism in males. Short-term and long-term studies during hormonal treatment of prostatic carcinoma. J Clin Invest 84:802-810

170. **Blackard CE, Doe RP, Mellinger GT, Byar DP** 1970 Incidence of cardiovascular disease and death in patients receiving diethylstilbestrol for carcinoma of the prostate. Cancer 26:249-256

171. **Henriksson P, Linde B, Edhag O** 1987 Deleterious effects of low-dose oestrogen therapy on coronary status in patients with prostatic cancer. Eur Heart J 8:779-784

172. **Mikkola AK, Ruutu ML, Aro JL, Rannikko SA, Salo JO** 1998 Parenteral polyoestradiol phosphate vs orchidectomy in the treatment of advanced prostatic cancer. Efficacy and cardiovascular complications: a 2-year follow-up report of a national, prospective prostatic cancer study. Finnprostate Group. Br J Urol 82:63-68

173. The Coronary Drug Project. Findings leading to discontinuation of the 2.5-mg day estrogen group. The coronary Drug Project Research Group 1973 JAMA 226:652-657

174. **Komesaroff PA, Fullerton M, Esler MD, Dart A, Jennings G, Sudhir K** 2001 Low-dose estrogen supplementation improves vascular function in hypogonadal men. Hypertension 38:1011-1016

175. **Chester AH, Jiang C, Borland JA, Yacoub MH, Collins P** 1995 Oestrogen relaxes human epicardial coronary arteries through non-endothelium-dependent mechanisms. Coron Artery Dis 6:417-422

176. **Blumenthal RS, Heldman AW, Brinker JA, Resar JR, Coombs VJ, Gloth ST, Gerstenblith G, Reis SE** 1997 Acute effects of conjugated estrogens on coronary blood flow response to acetylcholine in men. Am J Cardiol 80:1021-1024

177. **Reis SE, Holubkov R, Zell KA, Smith AJ, Cohen HA, Feldman MD, Blumenthal RS** 1998 Estrogen acutely abolishes abnormal cold-induced coronary constriction in men. Chest 114:1556-1561

178. **Collins P, Rosano GM, Sarrel PM, Ulrich L, Adamopoulos S, Beale CM, McNeill JG, Poole-Wilson PA** 1995 17 beta-Estradiol attenuates acetylcholine-induced coronary arterial constriction in women but not men with coronary heart disease. Circulation 92:24-30

179. **Chambliss KL, Yuhanna IS, Mineo C, Liu P, German Z, Sherman TS, Mendelsohn ME, Anderson RG, Shaul PW** 2000 Estrogen receptor alpha and endothelial nitric oxide synthase are organized into a functional signaling module in caveolae. Circ Res 87:E44-E52

180. **Mendelsohn ME** 2000 Nongenomic, ER-mediated activation of endothelial nitric oxide synthase: how does it work? What does it mean? Circ Res 87:956-960

181. **Kim-Schulze S, McGowan KA, Hubchak SC, Cid MC, Martin MB, Kleinman HK, Greene GL, Schnaper HW** 1996 Expression of an estrogen receptor by human coronary artery and umbilical vein endothelial cells. Circulation 94:1402-1407

182. **Small M, Lowe GD, Beastall GH, Beattie JM, McEachern M, Hutton I, Lorimer AR, Forbes CD** 1985 Serum oestradiol and ischaemic heart disease--relationship with myocardial infarction but not coronary atheroma or haemostasis. Q J Med 57:775-782

183. **Chute CG, Baron JA, Plymate SR, Kiel DP, Pavia AT, Lozner EC, O'Keefe T, MacDonald GJ** 1987 Sex hormones and coronary artery disease. Am J Med 83:853-859

184. **Goldberg RJ, Gore JM, Zive M, Brady P, Klaiber E, Broverman D, Ockene IS, Dalen JE** 1987 Serum estradiol and coronary artery disease. Am J Med 82:1-4

185. **Price JF, Lee AJ, Fowkes FG** 1997 Steroid sex hormones and peripheral arterial disease in the Edinburgh Artery Study. Steroids 62:789-794

186. **Taggart H, Sheridan B, Stout RW** 1980 Sex hormone levels in younger male stroke survivors. Atherosclerosis 35:123-125

187. **Cauley JA, Gutai JP, Kuller LH, Dai WS** 1987 Usefulness of sex steroid hormone levels in predicting coronary artery disease in men. Am J Cardiol 60:771-777

188. **Barrett-Connor E, Khaw KT** 1988 Endogenous sex hormones and cardiovascular disease in men. A prospective population-based study. Circulation 78:539-545

189. **Phillips GB, Yano K, Stemmermann GN** 1988 Serum sex hormone levels and myocardial infarction in the Honolulu Heart Program. Pitfalls in prospective studies on sex hormones. J Clin Epidemiol 41:1151-1156

190. **Eldrup E, Winkel P, Lindholm J, Bentzon MW, Jensen G, Schnohr P** 1989 Coronary risk factors, development of myocardial infarction, and plasma oestrogens: a prospective case-control study in men. J Intern Med 225:367-372

191. **Muller M, van den Beld AW, Bots ML, Grobbee DE, Lamberts SW, van der Schouw YT** 2004 Endogenous sex hormones and progression of carotid atherosclerosis in elderly men. Circulation 109:2074-2079

192. **Elbers JM, Giltay EJ, Teerlink T, Scheffer PG, Asscheman H, Seidell JC, Gooren LJ** 2003 Effects of sex steroids on components of the insulin resistance syndrome in transsexual subjects. Clin Endocrinol (Oxf) 58:562-571

193. **Riggs BL, Hartmann LC** 2003 Selective estrogen-receptor modulators -- mechanisms of action and application to clinical practice. N Engl J Med 348:618-629

Chapter 2

Associations of sex hormone binding globulin with non-SHBG bound levels of testosterone and estradiol in independently living men

Abstract

Results of *in vitro* experiments indicate that with increasing concentrations of sex hormone binding globulin (SHBG), testosterone (T) is preferentially bound to SHBG in comparison with estradiol (E2). In these studies the ratio of non-SHBG-bound-E2 to non-SHBG-T increased with increasing levels of SHBG. SHBG has consequently been regarded as an estrogen amplifier. In this cross-sectional study in 399 men aged between 40 and 80 years we tested whether higher levels of SHBG are associated with a higher estrogen/androgen ratio *in vivo*. The mean T level of these men was in the eugonadal range (536±152 ng/dl (18.6±5.26 nmol/L), mean ± SD). With increasing SHBG levels the non-SHBG-bound fraction of T decreased from 80% to 36% and that of E2 from 89% to 53%. Higher levels of SHBG were associated with higher levels of both total T (regression coefficient (β) after adjustment for age and BMI = 286±15.8, $p<0.001$) and total E2 (β=4.47±0.90, $p<0.001$). However, SHBG levels were negatively related with levels of non-SHBG-bound E2 (β=-1.78±0.69, $p<0.001$), whereas there was a positive association between levels of SHBG and non-SHBG-T (β=32.0±9.78, $p=0.001$). Furthermore we observed a negative relationship between SHBG levels and the estradiol/testosterone ratio of either total (β=-0.016±0.002, $p<0.001$) or non-SHBG bound (β=-0.011±0.002, $p<0.001$) hormone. Therefore, we conclude that in eugonadal men higher SHBG levels are associated with lower levels of non-SHBG-E2 but slightly higher levels of non-SHBG-T. This means that SHBG cannot be regarded as an estrogen amplifier in eugonadal men.

Introduction

Sex-Hormone Binding Globulin (SHBG), corticosteroid binding globulin (CBG) and albumin are important steroid hormone binding proteins in human plasma. Although recent evidence shows that SHBG can participate in signal transduction via its own membrane receptor (1;1), it is best known for its role as a binding protein of sex hormones in human plasma. In normal men and women between 40 and 65% of circulating testosterone (T) and between 20 and 40% of circulating estradiol (E2) is bound to SHBG (2). Binding of T to SHBG decreases its metabolic clearance rate and its conversion rate to androstenedione (3). Binding to SHBG also prevents bound hormone from diffusing out of the bloodstream thereby preventing hormone binding to the intracellular androgen or estrogen receptors. The non-SHBG bound fraction of hormone is, therefore, considered to be bioactive (free hormone hypothesis as reviewed in (4)).

T and E2 bind to the same binding site on SHBG but the binding affinity for T is higher than that for E2 (5). *In vitro* experiments show that with increasing levels of SHBG and stable levels of T and E2 the ratio of unbound E2 to unbound T increases (6). On the basis of the relatively greater decrease in the bioavailability of T compared to that of E2, SHBG has been regarded as an estrogen amplifier. This might provide an explanation for the gynaecomastia frequently observed in thyrotoxic men since thyrotoxicosis is associated with high concentrations of SHBG (7-9). An alternative explanation for this observation might be that levels of luteinizing hormone (LH) in these patients are increased, causing an increase in testicular E2 production (10), although others did not detect increased E2 production rates in hyperthyroidism (11;12). In healthy males there is a wide variation in SHBG concentrations. In cross sectional studies the plasma concentrations of T and SHBG are positively correlated (13). This correlation not only reflects the high binding affinity of SHBG for T, resulting in increased storage of the steroid, but may also be explained by the effect of SHBG levels on the bioavailability of T. Higher SHBG levels would then lead to lower levels of bioactive T, a decreased feedback signal on GnRH and thereby on LH secretion by the pituitary and a subsequent increase of T levels until a new setpoint is reached. This dependence of total T on variations in SHBG in men *in vivo* differs from the stable T levels in the *in vitro* experiments described above. It is, therefore, doubtful whether the conclusions drawn from these *in vitro* experiments apply to the *in vivo* situation. The aim of this study was to evaluate if the relationships between T, E2 and SHBG in healthy men support the conclusions based on the *in vitro* experiments.

Subjects and Methods

Subjects

The study is a cross-sectional, single center study of 400 independently living men aged 40 to 80 years. The study was originally designed to study the relationships between endogenous sex hormones and risk factors for, or manifestations of chronic diseases. The subjects were recruited by asking female participants of other studies conducted by the department whether they knew any man who might be interested in volunteering for the study. Invitation letters were sent to 770 female participants. Eventually 240 men volunteered for participation. Subsequently, names and addresses of a randomly selected male population aged 40-80 years were drawn from the municipal register of Utrecht, a large sized town in the middle part of the Netherlands. 1230 invitation letters were sent. From this group 390 men volunteered for participation.

From the 630 volunteers we excluded the subjects who did not live independently and subjects who were not physically or mentally able to visit the study center independently (n=16). No additional health related eligibility criteria, other than being physically and mentally able to visit the study center independently were used. Of the remaining 614 men 400 men were randomly selected to participate. To obtain equal numbers in each age-decade we sampled 100 men in each decade of age. One subject was excluded from analysis because of clear hypogonadism (total testosterone = 0.24 nmol/L). Data collection took place between March 2001 and April 2002.

All participants gave written informed consent before enrolment and the institutional review board of the Utrecht University Medical Center approved the study.

Height and weight were measured in the standing position without shoes. Body mass index (BMI) was calculated as the weight in kilograms divided by the square of the height in meters. Visceral and intra-abdominal fat were assessed using ultra-sound measurements (14;15). Ultrasonography was performed with an HDI 3000 (Philips Medical Systems, Eindhoven, The Netherlands) using a C 4-2 transducer. The distances between the posterior edge of the abdominal muscles and the lumbar spine or psoas muscles were measured using electronic callipers. For all images the transducer was placed on a straight line drawn between the left and right midpoint of lower rib and iliac crest. Distances were measured three times from three different angles: medial, left and right for intra-abdominal fat mass and medial for subcutaneous fat mass. Measurements were made at the end of quiet expiration, applying minimal pressure without displacement of intra-abdominal contents as observed by ultrasound

image. Visceral fat was measured as the distance between the skin and the linea alba and intra-abdominal fat as the distance between the peritoneum and lumbar spine.
Details on lifestyle and health of the subjects have been published earlier (16).

Laboratory measurements

Fasting blood samples were obtained by venapuncture. Cell free serum was immediately stored at –20C. T was measured after diethyl extraction using an in house radioimmunoassay employing a polyclonal antiT-antibody (AZG 3290, a gift from Dr. J.J. Pratt, Groningen, the Netherlands). The lower limit of detection of the assay was 0.24 nmol/L and inter-assay variation was 6.0; 5.4 and 8.6% at 2.1; 5.6 and 23 nmol/L respectively. SHBG was measured using an immunometric technique on an Immulite Analyser (Diagnostic Products Corporation, Los Angeles, California, USA). The lower limit of detection was 5 nmol/L and inter-assay variation was 6.1; 4.9 and 6.9% at 11.6; 36 and 93 nmol/L respectively. E2 was measured after diethylether extraction and Sephadex chromatography using an in house radioimmunoassay employing a polyclonal anti-E2 antibody. The lower limit of detection was 20 pmol/L and inter-assay variation was 10 and 3.1% at 81 and 660 pmol/L respectively. Non-SHBG bound T and E2 were calculated using the method described by Sodergard et al.(17) using a fixed plasma albumin concentration of 40 gr/l. The equations for these calculations are given in table 1.

Tabel 3: equations for the calculation of non-SHBG-T and non-SHBG-T according to Sodergard et al. (17)

non-SHBG-T (nmol/l)	$\{(k_{at}*[albumin]*[FT]/(1+k_{at}*[FT])\}+[FT]$
FT (nmol/l)	$\{-b+\sqrt{(b^2+4a[TT]}\}/2a$, in which
	$a=k_{at}+k_t+(k_{at}*k_t)([SHBG]+[albumin]-[T])$
	$b=1+k_t[SHBG]+k_{at}[albumin]-(k_{at}+k_t)[T]$
non-SHBG-E2 (pmol/l)	$[E2]-\{k_e*[SHBG]*FE2/(1+k_e*[FE2]+k_t*[FT])\}$
FE2 (pmol/l)	{-b-v(b2-4ac)}/2a, in which
	$a=(k_{at}*[albumin]+1)k_e$
	$b=([E2]*k_e)-(k_{ae}*[albumin]+1)(1+k_{t*}[FT])-(k_e*[SHBG])$
	$c=[E2]*(1+kt*[FT])$

k_{at}=association constant for binding of testosterone to albumin ($4.06*10^4$; L/mol)
k_t=association constant for binding of testosterone to SHBG ($5.97*10^8$; L/mol)
k_{ae}=association constant for binding of estradiol to albumin ($4.21*10^4$; L/mol)
k_e=association constant for binding of estradiol to SHBG ($3.14*10^8$; L/mol)
[FT]= plasma concentration of free (non-albumin-non-SHBG bound) testosterone (mol/L)
[FE2]= plasma concentration of free (non-albumin-non-SHBG bound) estradiol (mol/L)
[T]= plasma concentration of testosterone (mol/L)
[E2]= plasma concentration of estradiol (mol/L)

The association constants we used for the calculation of the binding of T (k_t) and E2 (k_e) to SHBG were $5.97x10^8$ and $3.14x10^8$ respectively (17). In the literature various estimates for these binding affinities have been calculated on basis of various methodologies. Values of $10x10^8$ (18), $16x10^8$ (2) or $19x10^8$ (6) have also been reported for k_t and values ranging from $3.14x10^8$ (17) to $6.8x10^8$ (2) have been reported for k_e. Changing these constants in the equations will obviously lead to changes in the calculated levels of unbound hormones and can influence the observed relationships between SHBG and the bioavailable levels of T and E2. Therefore we repeated the analyses after introducing alternative values for k_t and k_e in the equations.

Statistics

All calculations were performed using SPSS 11.0 software. Relations between SHBG and hormone levels were assessed using linear regression for continuous variables described as the linear regression coefficient (ß) using SHBG as the independent variable before and after adjustment for age and BMI. Because site specific differences in aromatase activity have been described (19) we tested whether adding visceral or abdominal fat mass to the regression analysis had any impact on the results. The linear regression coefficient β indicates the change of the dependent variable for every 1 nmol/L change in SHBG. Adjustments for age and BMI were made by adding these parameters as independent variables to the regression model. Adjustments were made because both age and BMI have been shown to be associated with levels of SHBG, T and E2 in men (13;16).

Results

The characteristics of the studied men are presented in table 2. The mean T level was in the eugonadal range. Mean values for abdominal and visceral fat mass as measured by ultrasound were 7.52±2.23 cm and 2.65±0.85 cm respectively.

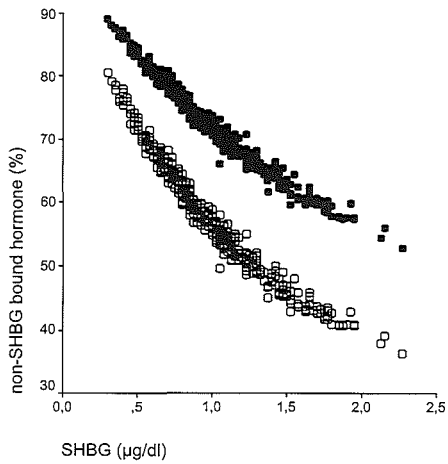
With increasing SHBG concentration the percentages of hormone not bound to SHBG decreased from 80 to 36 for T and from 89 to 53 for E2 (figure 1). The relationships between SHBG and T and non-SHBG T before and after adjustment for age and BMI are presented in table 3 and in figure 2. Higher levels of SHBG were strongly associated with higher levels of T (β=286±15.8, $p<0.001$). SHBG and non-SHBG-T were related only after adjustment for age and BMI (β=32.0±9.78, $p=0.001$).

Tabel 2: Characteristics of the 399 studied men (mean ±SD)

Age (yrs)	60.2±11.3
BMI (kg/m2)	26.3±3.48
SHBG (µg/dl)	1.01±0.36 (40.6±14.5)
T (ng/dl)	536±152 (18.6±5.26)
Non-SHBG-T (ng/dl)	300±75.4 (10.4±2.62)
E2 (pg/ml)	24.9±6.15 (91±23)
Non-SHBG-E2 (pg/ml)	17.9±4.60 (66±17)
E2 (pg/ml)/T (ng/dl)	0.05±0.02 (5.20±1.61)
Non-SHBG-E2 (pg/ml) /non-SHBG-T (ng/dl)	0.06±0.02 (6.57±1.94)

Systeme International units are given in *parentheses*

Figure 1: SHBG versus the percentages of non-SHBG bound testosterone (open symbols) and non-SHBG bound estradiol (closed symbols) in 399 men.



The relationships between plasma levels of SHBG and E2 and non-SHBG-E2 are presented in table 3 and in figure 3. High SHBG levels were associated with higher E2 levels (β=4.47±0.90, $p<0.001$) but with lower concentrations of non-SHBG-E2 (β=-1.78±0.69, $p=0.008$).

Finally the relationships between plasma levels of SHBG and the E2/T ratio and the non-SHBG-E2/non-SHBG-T ratio are presented in table 3 and figure 4. SHBG levels were

negatively related to both ratios (β= -0.016±0.002, p<0.001 and β= -0.011±0.002, P<0.001 respectively).

Adding abdominal or visceral fat mass to the regression analyses did not change the results. Introducing a k_t of $10x10^8$ into the equations of table 1 (according to the frequently used Vermeulen method (18))did not essentially change the relationship between SHBG and non-SHBG-T (β= -2.56±8.41, p=0.76 after adjustment for age and BMI). After introducing a k_e of $6.8x10^8$ the inverse relation between SHBG and non-SHBG-E2 was more pronounced (β= -3.76±0.57, p<0.001 after adjustment for age and BMI). Only after introducing the highest reported value for k_t ($19x10^8$ (6)) and the lowest reported value for k_e ($3.14x10^8$ (17)) the relationship between SHBG and the ratio of non-SHBG-E2 / non-SHBG-T appeared to be slightly but significantly positive (β= 0.011±0.004, p=0.01 after adjustment for age and BMI). However, it is questionable if it is allowed to combine these k-values, as they were obtained under different circumstances.

Discussion

In this study the relationship between SHBG levels and the E2/T ratio was studied in eugonadal healthy men. The concept of "SHBG as an estrogen amplifier"(6) is based on the observation that with stable T and E2 levels, an increase of SHBG will decrease unbound T more than unbound E2 resulting in an increase of the non-SHBG-E2 / non-SHBG –T ratio. However, in eugonadal men this theory does not apply because the hypothalamo-pituitary-gonadal (HPG) axis will respond to a decreasing level of non-SHBG-T with an increase in LH and T, assuming that non-SHBG-T is driving the feedback inhibition of the male HPG axis. The validity of this hypothesis is supported by our observation that increased levels of SHBG are associated with increased levels of total T, but are barely associated with the level of non-SHBG-T.

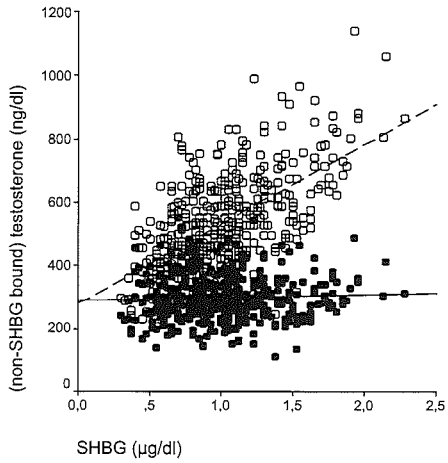
Levels of SHBG only show a modest positive association with total levels of E2, but are negatively related with those of non-SHBG-E2. As a result, a high concentration of SHBG is associated with a lower (non-SHBG bound) estrogen / androgen ratio and vice versa. Endogenous E2 can also have an effect on LH release by the pituitary (20;21). However, in contrast to T, E2 levels are not directly regulated by HPG axis activity. When bioavailable E2 levels decrease, this might lead to increased LH release by the pituitary with a resulting increase in testicular T production. Total E2 levels will only be increased if T is subsequently aromatized, the extent of which is influenced by parameters such as age and BMI.

Tabel 3: Linear regression coefficients (β) for the relationships of SHBG with T, non-SHBG-bound T, E2, non-SHBG-bound E2 and estrogen/androgen ratios before and after adjustment for age and BMI

	unadjusted		adjusted for age and BMI	
	β±SE	p	β±SE	p
T (ng/dl)	252±16.8	<0.001	286±15.8	<0.001
Non-SHBG-T (ng/dl)	10.2±10.4	0.33	32.0±9.78	0.001
E2 (pg/ml)	3.22±0.84	<0.001	4.47±0.90	<0.001
Non-SHBG-E2 (pg/ml)	-2.77±0.62	<0.001	-1.78±0.69	0.008
E2 (pg/ml)/T (ng/dl)	-0.016±0.002	<0.001	-0.016±0.002	<0.001
Non-SHBG-E2 (pg/ml)/non-SHBG-T (ng/dl)	-0.011±0.002	<0.001	-0.011±0.002	<0.001

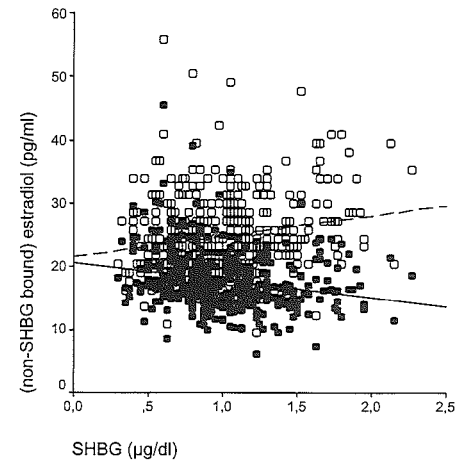
β : figure indicates change of the dependent variable for every 1 µg/dl change in SHBG (for conversion of (non-SHBG bound) T to nmol/L multiply by 0.0347; for conversion of (non-SHBG bound) E2 to pmol/L multiply by 3.67).

Figure 2: SHBG versus testosterone (open symbols) and non-SHBG bound testosterone (closed symbols) in 399 men.



The relationship between SHBG and testosterone is given by the formula [T] = 280+252[SHBG] and that between SHBG and non-SHBG testosterone by [non-SHBG-T] = 290+10.2[SHBG] (for conversion of (non-SHBG bound) T to nmol/L multiply by 0.0347; for conversion of SHBG to nmol/L multiply by 40).

Figure 3: SHBG versus estradiol (open symbols) and non-SHBG bound estradiol (closed symbols) in 399 men.



The relationship between SHBG and estradiol is given by the formula [E2] = 21.6+3.22[SHBG] and that between SHBG and non-SHBG estradiol by [non-SHBG-E2] = 20.7-2.77[SHBG] (for conversion of (non-SHBG bound) E2 to pmol/L multiply by 3.67; for conversion of SHBG to nmol/L multiply by 40).

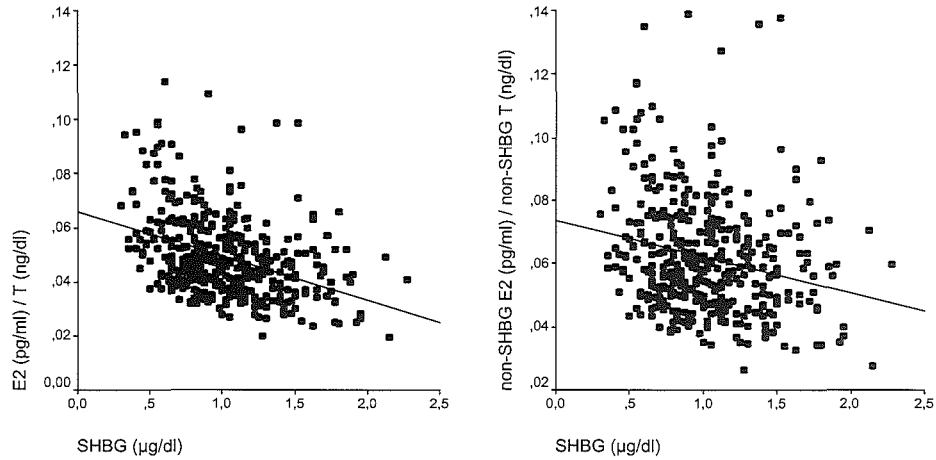
The regulation of peripheral E2 levels by the HPG axis is indirect and therefore probably not as tight as compared to T levels. The fact that an intact HPG axis appears to prevent the non-SHBG-T concentration to fall with increasing SHBG levels makes the *in vivo* situation in eugonadal men totally different from the *in vitro* situation where changes in hormone binding to SHBG do not evoke adaptations in the HPG axis. This lack of similarity between *in vivo* and *in vitro* conditions was already alluded to by Rosner (22) but, to our knowledge, was never formally tested.

Our findings in healthy men seem to conflict with conditions associated with high SHBG levels in men such as advanced age, liver disease, hyperthyroidism and estrogen administration (22). These conditions are associated with increased estrogen / androgen ratios and gynaecomastia (23;24) and they seem to confirm the concept of "SHBG as an estrogen amplifier". However, besides the altered SHBG levels, these conditions are also associated with altered gonadal function. Hypogonadism is frequently observed in liver cirrhosis patients (25;26). In hyperthyroid men lower levels of non-SHBG bound T are frequently (7-9) but not always (27;28) reported which suggests that the HPG axis in these men is not always able to fully compensate for the rise in SHBG concentration. Moreover, the increased estrogen/androgen ratio in hyperthyroid subjects might be caused by increased androgen aromatization (10;29). The age-associated increase in SHBG is not associated with an increase in T levels (30) which suggests that the HPG axis of older men is not capable of responding to a fall in T levels. Therefore it is likely that the relative hypogonadism and not the increased SHBG per se may explain the high estrogen/androgen ratio in these men.

The question of the clinical relevance of our observation arises. In the pathogenesis of gynaecomastia a high estrogen / androgen balance seems to be of importance (23;24). According to our results men with low levels of SHBG and a resulting high estrogen / androgen ratio would have a higher risk of developing gynecomastia, although this association has not been reported in the literature. Probably the changes in the estrogen / androgen ratio brought about by SHBG in eugonadal men are too subtle to cause gynaecomastia.

Our results show that high levels of SHBG are associated with lower levels of non-SHBG bound E2 but normal or even slightly higher levels of non-SHBG bound T. The decreased feedback inhibition of non-SHBG bound E2 on the release of LH by the pituitary probably explains the slightly positive relationship between levels of non-SHBG-T and SHBG.

Figure 4: SHBG versus the ratio of estradiol over testosterone (left) and the ratio of non-SHBG-E2 over non-SHBG-T (right) in 399 men.



The relationship between SHBG and estradiol / testosterone ratio is given by the formula E2/T = 0.066-0.016[SHBG] and that between SHBG and non-SHBG estradiol by non-SHBG-E2/non-SHBG-T = 0.074-0.011[SHBG] (for conversion of T to nmol/L multiply by 0.0347; for conversion of E2 to pmol/L multiply by 3.67; for conversion of SHBG to nmol/L multiply by 40).

It is well known that lower levels of non-SHBG bound E2 in men are associated with lower bone mineral density (31;32). Apparently even in eugonadal men elevated SHBG levels might contribute to estrogen deficiency and to conditions such as osteoporosis.

One might speculate that while passing through capillaries, a proportion of the bound hormone dissociates from SHBG and in fact becomes bioavailable. In that case the amount of bioavailable hormone might be underestimated when using the described equations for the calculation of the bioavailable fractions. Consequently, the amount of bioavailable E2 would be underestimated more in comparison to the amount of bioavailable T because of the weaker binding of E2 to SHBG. However, the validity of this hypothesis remains to be determined.

For the calculation of the levels of non-SHBG bound E2 and non-SHBG bound T we used the equations as described by Sodergard et al. (Table 1) (17) in which the association constants for the binding of T (k_t) and E2 (k_e) to SHBG are $5.97x10^8$ and $3.14x10^8$ respectively. In the literature alternative estimates for these binding affinities are reported (2;6;18). Use of a higher association constant in the equation will tilt the slope of the regression lines shown in figures 2 and 3 (right panels) slightly down and vice versa. Theoretically, combining a high k_t with a low k_e in the equations of Table 1 can result in a positive relation between SHBG and

the non-SHBG-E2 / non-SHBG-T ratio. However, when the combination of values as reported by Dunn et al. (2) and Burke et al. (6) were used this was not the case.
Although the subjects we studied were prone to health selection bias this does not undermine the conclusions of this study. In fact it contributed to the uniformity of the analyses because there were only a few hypogonadal subjects (based on T and non-SHBG bound T levels) in this group of men. On the other hand, it prevented us from doing separate analyses on data from eugonadal and hypogonadal men.

The conclusion of our study is that, in eugonadal men, higher SHBG levels are associated with lower levels of non-SHBG-E2 but unaltered or even slightly higher levels of non-SHBG-T. This means that SHBG cannot be regarded as an estrogen amplifier in eugonadal men.

Reference List

1. **Kahn SM, Hryb DJ, Nakhla AM, Romas NA, Rosner W** 2002 Sex hormone-binding globulin is synthesized in target cells. J Endocrinol 175:113-120

2. **Dunn JF, Nisula BC, Rodbard D** 1981 Transport of steroid hormones: binding of 21 endogenous steroids to both testosterone-binding globulin and corticosteroid-binding globulin in human plasma. J Clin Endocrinol Metab 53:58-68

3. **Vermeulen A, Ando S** 1979 Metabolic clearance rate and interconversion of androgens and the influence of the free androgen fraction. J Clin Endocrinol Metab 48:320-326

4. **Mendel CM** 1989 The free hormone hypothesis: a physiologically based mathematical model. Endocr Rev 10:232-274

5. **Rosner W** 1991 Plasma steroid-binding proteins. Endocrinol Metab Clin North Am 20:697-720

6. **Burke CW, Anderson DC** 1972 Sex-hormone-binding globulin is an oestrogen amplifier. Nature 240:38-40

7. **Ford HC, Cooke RR, Keightley EA, Feek CM** 1992 Serum levels of free and bound testosterone in hyperthyroidism. Clin Endocrinol (Oxf) 36:187-192

8. **Garden GA, Hannan CJ, Spencer CA, Plymate SR** 1988 The effect of sex hormone binding globulin (SHBG) on the pituitary testicular axis in hyperthyroid men. Steroids 52:385-386

9. **Abalovich M, Levalle O, Hermes R, Scaglia H, Aranda C, Zylbersztein C, Oneto A, Aquilano D, Gutierrez S** 1999 Hypothalamic-pituitary-testicular axis and seminal parameters in hyperthyroid males. Thyroid 9:857-863

10. **Ruder H, Corvol P, Mahoudeau JA, Ross GT, Lipsett MB** 1971 Effects of induced hyperthyroidism on steroid metabolism in man. J Clin Endocrinol Metab 33:382-387

11. **Ridgway EC, Longcope C, Maloof F** 1975 Metabolic clearance and blood production rates of estradiol in hyperthyroidism. J Clin Endocrinol Metab 41:491-497

12. **Ridgway EC, Maloof F, Longcope C** 1982 Androgen and oestrogen dynamics in hyperthyroidism. J Endocrinol 95:105-115

13. **Vermeulen A, Kaufman JM, Giagulli VA** 1996 Influence of some biological indexes on sex hormone-binding globulin and androgen levels in aging or obese males. J Clin Endocrinol Metab 81:1821-1826

14. **Stolk RP, Wink O, Zelissen PM, Meijer R, van Gils AP, Grobbee DE** 2001 Validity and reproducibility of ultrasonography for the measurement of intra-abdominal adipose tissue. Int J Obes Relat Metab Disord 25:1346-1351

15. **Tornaghi G, Raiteri R, Pozzato C, Rispoli A, Bramani M, Cipolat M, Craveri A** 1994 Anthropometric or ultrasonic measurements in assessment of visceral fat? A comparative study. Int J Obes Relat Metab Disord 18:771-775

16. **Muller M, den Tonkelaar, I, Thijssen JH, Grobbee DE, van der Schouw YT** 2003 Endogenous sex hormones in men aged 40-80 years. Eur J Endocrinol 149:583-589

17. **Sodergard R, Backstrom T, Shanbhag V, Carstensen H** 1982 Calculation of free and bound fractions of testosterone and estradiol-17 beta to human plasma proteins at body temperature. J Steroid Biochem 16:801-810

18. **Vermeulen A, Verdonck L, Kaufman JM** 1999 A critical evaluation of simple methods for the estimation of free testosterone in serum. J Clin Endocrinol Metab 84:3666-3672

19. **McTernan PG, Anderson LA, Anwar AJ, Eggo MC, Crocker J, Barnett AH, Stewart PM, Kumar S** 2002 Glucocorticoid regulation of p450 aromatase activity in human adipose tissue: gender and site differences. J Clin Endocrinol Metab 87:1327-1336

20. **Finkelstein JS, O'Dea LS, Whitcomb RW, Crowley WF, Jr.** 1991 Sex steroid control of gonadotropin secretion in the human male. II. Effects of estradiol administration in normal and gonadotropin-releasing hormone-deficient men. J Clin Endocrinol Metab 73:621-628

21. **Hayes FJ, Seminara SB, Decruz S, Boepple PA, Crowley WF, Jr.** 2000 Aromatase inhibition in the human male reveals a hypothalamic site of estrogen feedback. J Clin Endocrinol Metab 85:3027-3035

22. **Rosner W** 1990 The functions of corticosteroid-binding globulin and sex hormone-binding globulin: recent advances. Endocr Rev 11:80-91

23. **Ismail AA, Barth JH** 2001 Endocrinology of gynaecomastia. Ann Clin Biochem 38:596-607

24. **Glass AR** 1994 Gynecomastia. Endocrinol Metab Clin North Am 23:825-837

25. **Pignata S, Daniele B, Galati MG, Esposito G, Vallone P, Fiore F, Ricchi P, Pergola M** 1997 Oestradiol and testosterone blood levels in patients with viral cirrhosis and hepatocellular carcinoma. Eur J Gastroenterol Hepatol 9:283-286

26. **Nagasue N, Ogawa Y, Yukaya H, Ohta N, Ito A** 1985 Serum levels of estrogens and testosterone in cirrhotic men with and without hepatocellular carcinoma. Gastroenterology 88:768-772

27. **Hudson RW, Edwards AL** 1992 Testicular function in hyperthyroidism. J Androl 13:117-124

28. **Kidd GS, Glass AR, Vigersky RA** 1979 The hypothalamic-pituitary-testicular axis in thyrotoxicosis. J Clin Endocrinol Metab 48:798-802

29. **Chopra IJ** 1975 Gonadal steroids and gonadotropins in hyperthyroidism. Med Clin North Am 59:1109-1121

30. **Vermeulen A** 1991 Clinical review 24: Androgens in the aging male. J Clin Endocrinol Metab 73:221-224

31. **van den Beld AW, de Jong FH, Grobbee DE, Pols HA, Lamberts SW** 2000 Measures of bioavailable serum testosterone and estradiol and their relationships with muscle strength, bone density, and body composition in elderly men. J Clin Endocrinol Metab 85:3276-3282

32. **Szulc P, Munoz F, Claustrat B, Garnero P, Marchand F, Duboeuf F, Delmas PD** 2001 Bioavailable estradiol may be an important determinant of osteoporosis in men: the MINOS study. J Clin Endocrinol Metab 86:192-199

Chapter 3

Serum levels of Sex Hormone-Binding Globulin (SHBG) are not associated with lower levels of non-SHBG-bound testosterone in male newborns and healthy adult men

Abstract

It is generally accepted that Sex Hormone Binding Globulin (SHBG) decreases the bioavailability and activity of testosterone (T). In *in vitro* experiments increased levels of SHBG will be associated with decreased levels of non-SHBG bound testosterone (non-SHBG-T). However, *in vivo* SHBG can alter both production and clearance rates and thus plasma levels of T.

In order to study the effect of SHBG on the levels of non-SHBG-T *in vivo* in the presence of an active Hypothalamus-Pituitary-Gonadal (HPG) axis we conducted a cross sectional study in 400 healthy adult men with an age range of 40 to 80 years and in 106 newborn boys.

In both groups regression coefficients (β) and partial correlation coefficients (r) were calculated for the relationship between SHBG and T or non-SHBG-T. Adult men were divided into age groups per decade (40-50 yrs, 51-60 yrs, 61-70 yrs and 71-80 yrs) to study possible differences in the impact of SHBG on the level of non-SHBG-T throughout aging.

Higher levels of SHBG were associated with higher levels of total testosterone in neonates ($\beta=0.02\pm0.004$, $r=0.44$, $p<0.001$) but not with non-SHBG-T ($\beta=-0.001\pm0.001$, $r=0.05$, $p=0.52$). In adult men there was a significant age related increase in levels of SHBG and an age related decrease of both total and non-SHBG-T. Higher SHBG was strongly associated with higher total testosterone in all age groups ($\beta=0.26$, 0.26, 0.26 and 0.23 for 40-50 yrs, 51-60 yrs, 61-70 yrs and 71-80 yrs respectively, $p<0.001$ for all age groups). Higher SHBG was not or only slightly associated with higher non-SHBG-T ($\beta=0.02$ ($p=0.32$), $\beta=0.04$ ($p=0.03$), $\beta=0.04$ ($p=0.02$) and $\beta=0.02$ ($p=0.16$) for 40-50 yrs, 51-60 yrs, 61-70 yrs and 71-80 yrs respectively.

In contrast to general belief, SHBG levels barely influence levels of non-SHBG-bound testosterone both in male newborns and healthy adult men and that the influence, if any, is positive. Consequently the age related increase of SHBG does not account for the age related decline in non-SHBG-T in healthy adult men.

Introduction

Sex-Hormone Binding Globulin (SHBG), corticosteroid binding globulin (CBG) and albumin are important steroid hormone binding proteins in human plasma. In normal adult men approximately 44% of the circulating testosterone is specifically bound to SHBG, 50% is nonspecifically bound to albumin and 3.5% is bound to CBG implicating that only 2-3% is unbound or free (1). By binding testosterone SHBG decreases the metabolic clearance rate of testosterone (2;3) and decreases peripheral conversion of testosterone to androstenedione (2). To exert its genomic action testosterone must bind to an intracellular androgen receptor. SHBG withholds bound hormone to diffuse from the bloodstream as free hormone to this receptor. There is some controversy whether or not albumin-bound testosterone can dissociate freely from this carrier protein and enter tissues (4;5) however, non-SHBG bound testosterone (non-SHBG-T = free+albumin bound testosterone) and non-SHBG-non-albumin bound testosterone (=free testosterone) are extremely well correlated (6) and interchangeable in most cases.

The levels of SHBG vary widely between healthy men and depend on factors such as diet (7), body mass index (BMI), insulin levels and age (8). On the basis of simple mathematics a higher level of SHBG is expected to result in a lower level of non-SHBG-T. However, when the non-SHBG-T is considered bioactive variations in SHBG concentrations will cause a variable secretion of luteinizing hormone releasing hormone by the hypothalamus and luteinizing hormone (LH) secretion by the pituitary. Low levels of non-SHBG-T will then lead to increased LH and testicular testosterone production via a decreased negative feedback signal on the hypothalamus and pituitary. Estimation of the effect of SHBG on the levels of non-SHBG-T *in vivo* by simply applying the laws of mass action might thus be inappropriate in men with a functional hypothalamo-pituitary-gonadal (HPG) axis.

In healthy males two phases of life are characterized by high gonadal axis activity; the neonatal period and the period during and after puberty (9). In the first six months of life levels of both LH and testosterone are high, reaching levels in the low adult range (10). At the age of six months both LH and testosterone levels have decreased to very low levels only to increase at the start of puberty (11). In adult men testosterone levels remain high throughout life although aging is associated with a slight decrease of the levels of total serum testosterone and an increase of the levels of SHBG (12).

To study the effect of SHBG on the levels of non-SHBG-T *in vivo* we conducted a cross sectional study in 400 healthy adult men with an age range of 40 to 80 years and in 106 newborn boys.

Subjects and Methods

Neonates

Serum samples were collected from 113 boys with ages between 1 and 6 months who served as controls in a case-control study on cryptorchism and hypospadias. Controls had been randomly selected from the population of boys that visited Child Healthcare Centers (CHCs). In the Netherlands, CHCs are notified of live births within two days after registration in the municipal birth register. CHCs invite all parents to participate free of charge in the national preventive child healthcare programme, including growth monitoring and vaccination, in which 95% of all parents participate with their child. This study was approved by the ethical committee of the Erasmus Medical Center, Rotterdam. Six subjects were excluded for analysis because of missing data on SHBG (5) or age (1). Eventually 106 subjects were available for analysis.

Laboratory measurements in neonates

Testosterone levels were estimated using a non-extraction coated tube radioimmunoassay (Coat-a-Count, Diagnostic Products Corporation, Los Angeles, California, USA). The lower limit of detection of the assay was 0.1 nmol/l. Intra- and interassay coefficients of variation were below 9% for the concentration range measured in these samples. SHBG concentrations were measured using a chemoluminescence based immunometric method (Immulite 2000) from the same supplier. The lower limit of detection was 5 nmol/l Variations for this method were below 7%. Non-SHBG bound T was calculated using the method described by Sodergard et al (13) using a fixed albumin level of 40 g/l. The formulas for these calculations have been described earlier (14).

Adult subjects

The study is a cross-sectional, single center study in 400 independently living men aged 40 to 80 years. The study was originally designed to study the relationships between endogenous sex hormones and risk factors or manifestations of chronic diseases. The subjects were recruited in two ways. Firstly, by asking female participants of other studies conducted by the department whether they knew a possibly interested male volunteer. Invitation letters were sent to 770 female participants. Eventually 240 men volunteered for participation. Secondly, names and addresses of a randomly selected male population aged 40-80 years were drawn from the municipal register of Utrecht, a large sized town in the central part of the

Netherlands. 1230 invitation letters were send. From this group 390 men volunteered for participation.

The subjects who did not live independently or were not physically or mentally able to visit the study centre independently (n=16) were excluded. No additional health related eligibility criteria were used. Of the remaining 614 men, 400 men were randomly selected to participate. One subject was excluded from analysis because of clear hypogonadism (total testosterone = 0.24 nmol/L). To obtain equal numbers in each age-decade we sampled 100 men in each decade of age. Data collection took place between March 2001 and April 2002.

All participants gave written informed consent before enrolment and the institutional review board of the Utrecht University Medical Centre approved the study. Height and weight were measured in standing position without shoes. Body mass index (BMI) was calculated as the weight in kilograms divided by the square of height in meters. Details on lifestyle and health of the subjects have been published earlier (15).

Laboratory measurements in adult subjects

Fasting blood samples were obtained by venepuncture. Cell free serum was immediately stored at –20C. T was measured after diethylether extraction using an in house radio immunoassay employing a polyclonal antiT-antiserum (AZG 3290 Dr. J.J. Pratt, Groningen, the Netherlands). The lower limit of detection of the assay was 0.24 nmol/L and inter-assay variation was 6.0, 5.4, and 8.6% at 2.1, 5.6 and 23 nmol/L respectively. Results using this assay were comparable to those obtained with the assay used for estimation of T in neonates (r=0.91, n=37, slope of regression line 1.03, intercept on y-axis 0.37 nmol/l). SHBG was measured as described for the measurement in neonates. Non-SHBG bound T was calculated as described above.

Data analysis

All variables, except for age in neonates, were normally distributed. In neonates age was logarithmically transformed to obtain normality. Associations between SHBG, total and non-SHBG bound testosterone were assessed and tested for significance using linear regression analysis with SHBG as the independent variable and expressed as regression coefficient (β) or (partial) correlation coefficient (r). The linear regression coefficient β indicates the change of the dependent variable for every 1 nmol/L change in SHBG. With every regression analysis, residuals were checked for normality. In both groups analyses were repeated with adjustments for age (days) in neonates, and adjustments for age (years) and BMI in adult men.

Adjustments for age and BMI were made by adding these parameters as independent variables to the regression and correlation model. Adjustments were made because both age and BMI were shown to be associated with levels of SHBG and T in men (10;15). Adult men were studied as a group and after division into age groups per decade (40 to 50 yrs, 51 to 60 yrs, 61 to 70 yrs and 71 to 80 yrs). Effect modification by age was tested using a linear regression model in which BMI, age group, SHBG and an interaction term (SHBG*age group) were used as independent variables and total testosterone or non-SHBG-T as dependent variables. Analyses were done using SPSS 11.0 software.

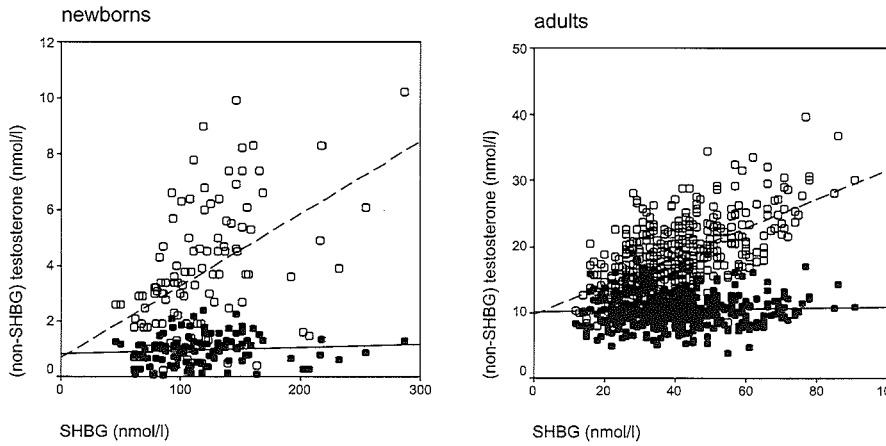
Results

The characteristics of newborns and adult men are presented in table 1. In newborns levels of SHBG were much higher and levels of both total and unbound testosterone were much lower compared to adults. Higher levels of SHBG were significantly associated with higher levels of total testosterone in neonates but levels of SHBG were not significantly associated with levels of non-SHBG-T (Unadjusted, for T: β=0.03±0.005, r =0.49, p<0.001; for non-SHBG-T : β=0.001±0.001, r = 0.09, p=0.35. After adjustment for age (days), for T: β=0.02±0.004, r =0.45, p<0.001; for non-SHBG-T : β=0.001±0.001, r =0.06, p=0.52). The relations between SHBG and total or unbound testosterone in neonates are depicted in figure 1. A similar pattern was seen in adult men (figure 1; Unadjusted, for T: β=0.22±0.02, r =0.59, p<0.001; for non-SHBG-T : β=0.01±0.01, r =0.05, p=0.35). After adjustment for age and BMI the slightly *positive* association between SHBG and non-SHBG-T was statistically significant (for T: β=0.25±0.01, r =0.68, p<0.001; for non-SHBG-T : β=0.03±0.01, r =0.15, p=0.01). The characteristics of adult men after division in age groups are presented in table 2. There was a significant age related increase in levels of SHBG and a significant age related decrease of both total and non-SHBG-T (p<0.001 for all variables). Relationships between SHBG and total or unbound testosterone after adjustments for age and BMI are presented in table 3 and figure 2. Higher levels of SHBG were strongly associated with higher levels of total testosterone in all age groups. Levels of SHBG were not or marginally positively associated with levels of non-SHBG-T. There was no modifying effect of age on the associations between SHBG and total testosterone (p for interaction = 0.36) or non-SHBG-T (p for interaction = 0.94).

tabel 4: Levels of (non-SHBG-bound) testosterone and SHBG in newborns and adult men (mean ±SD)

	newborns	**adult men**
n	106	399
age (days/years)	79.1±27.3	60.2±11.3
SHBG (nmol/L)	118±43	40.6±14.5
testosterone (nmol/L)	3.73±2.30	18.6±5.26
non-SHBG-T (nmol/L)	0.97±0.53	10.4±2.62

figure 1: SHBG versus total testosterone (open symbols) and non-SHBG-T (closed symbols) in newborn boys and adult men.



Tabel 5: Levels of (non-SHBG-bound) testosterone and SHBG in adult men by age decade (mean ±SD)

age group (yrs)	**40-50**	**51-60**	**61-70**	**71-80**
n	100	100	100	99
age (yrs)	45.0±3.12	56.2±2.89	65.4±2.77	74.4±2.69
SHBG(nmol/L) ***	34.7±13.7	38.0±13.3	43.6±15.0	46.1±13.2
testosterone (nmol/L) ***	20.2±5.59	18.5±5.22	17.9±5.53	17.8±4.32
non-SHBG-T (nmol/L) ***	12.2±2.75	10.6±2.37	9.57±2.42	9.29±1.79

*** = p<.001for differences between age groups

Discussion

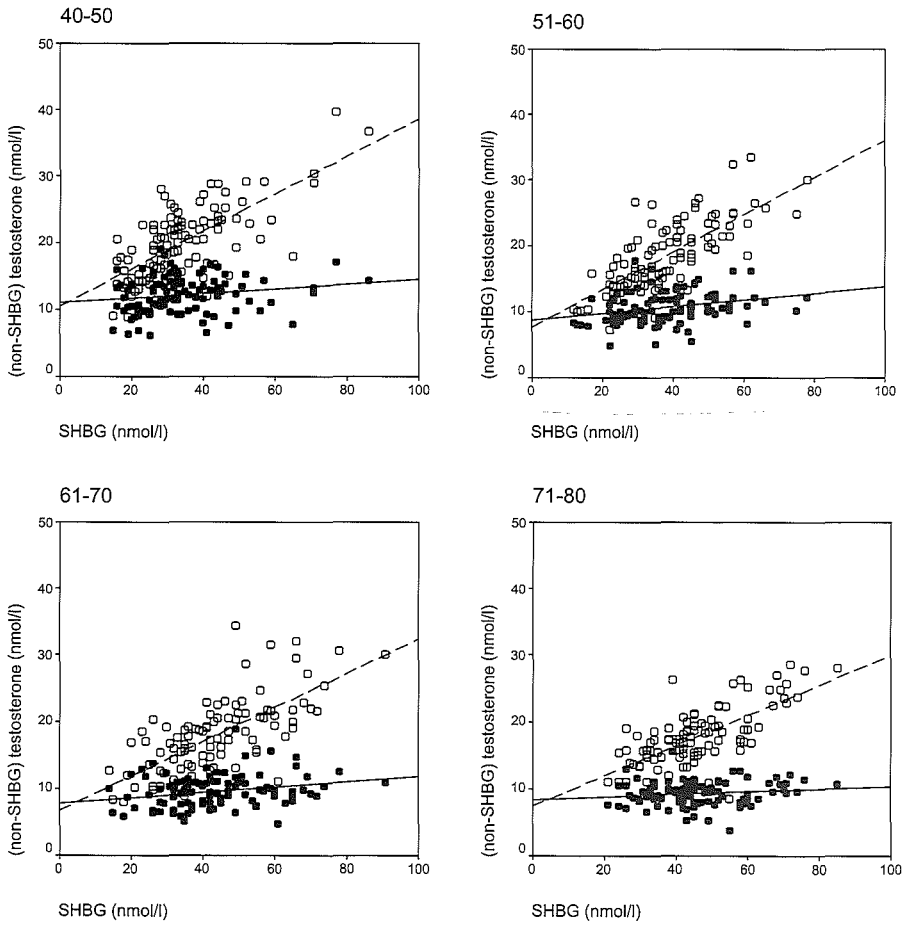
This study in a large number of neonates and men across a wide age range confirms that levels of SHBG can vary widely between individuals (8). Our results show that there is a strong and positive relation between SHBG and total testosterone levels in both newborn boys and adult men. Moreover there is no, or only a weak *positive* association between SHBG levels and levels of circulating non-SHBG-T. These associations are completely different compared to the relationships anticipated on the basis of mathematical models (1). In these models, SHBG does not have an effect on the level of total testosterone. However, *in vivo* SHBG can have an effect on clearance and might also affect production rates of testosterone. It can be hypothesized that the SHBG induced decreased bioavailability of testosterone will result in increased production of LH and subsequently testosterone until a new equilibrium is reached. SHBG decreases the metabolic clearance rate of testosterone in men and women (2;3). Higher levels of SHBG will thus be associated with higher levels of testosterone if the testosterone production rate does not change. However in pre-and postmenopausal women, in whom testosterone is not directly involved in the regulation of the HPG axis, plasma testosterone levels were shown to be negatively correlated with SHBG levels (16) which argues against an important effect of SHBG on testosterone levels by effects on testosterone clearance alone. These observations support the conclusion that non-SHBG-T and not total testosterone is driving HPG-axis activity in men. The fact that in male neonates SHBG is not negatively associated with non-SHBG-T suggests that during the first months of life the HPG axis is functional and sensitive to androgen feedback. This is supported by the observation that castration of neonatal monkeys results in elevated LH levels comparable to those found in castrated adults (17). In fact, androgen sensitivity appears to be a prerequisite for neonatal HPG axis activity as the postnatal hormone surge is absent in infants with androgen insensitivity (18). The absent relation between SHBG and non-SHBG-T in our subjects implies that the observed testosterone levels are not just the result of an unleashed HPG axis but are restrained by the feedback inhibition of non-SHBG-T. This is supported by the observation that LH and/or testosterone secretion in neonates can be further increased when stimulated with gonadotropin-releasing hormone or human chorionic gonadotropin (19). Although the HPG axis is operative in neonates, circulating (non-SHBG) T levels are much lower than in adults. It appears that the higher SHBG levels found in neonates are not responsible for the relatively low levels of non-SHBG-T. A different setpoint of the HPG axis in neonates might thus be postulated.

Tabel 6: multiple linear regression coefficients (β) and partial correlation coefficients (r) for the relation between SHBG, testosterone and non-SHBG-bound testosterone in adult men for the total population (n=399) and by age decade

	total population		age group							
			40-50		51-60		61-70		71-80	
	β±SE	r	β±SE	r	β±SE	r	β±SE	r	β±SE	r
T (nmol/L)	0.25±0.02	0.68***	0.26±0.03	0.64***	0.26±0.03	0.66***	0.26±0.03	0.69***	0.23±0.03	0.66***
non-SHBG-T (nmol/L)	0.03±0.01	0.15*	0.02±0.02	0.10NS	0.04±0.02	0.20*	0.04±0.02	0.24*	0.02±0.01	0.15NS

***= p<.001, *=p<.05, NS=not significant; all associations have been adjusted for age and BMI

Figure 2: SHBG versus total testosterone (open symbols) and non-SHBG-T (closed symbols) in four age groups of adult men.



Our study confirms the well-known increase of SHBG and slight decrease of total testosterone with increasing age in adult men (12). This age related increase of SHBG is generally believed to be an important factor in the pronounced decline of non-SHBG-T in aging men. However, in our cross-sectional analysis of different age groups we found that even in the oldest age group higher levels of SHBG are not associated with lower levels of non-SHBG-T. Therefore, the age related increase of SHBG does not appear to be responsible for the age related decline of non-SHBG-T.

Several studies indicate that with age the sensitivity to the negative feedback action of sex hormones increases (20-22). Also in our adult men there seems to be a change of the setpoint of the HPG axis to testosterone feedback with aging. The reason for this increased feedback sensitivity remains unknown. There are no arguments to believe that the expression of androgen action is stronger with age; the extent of SHBG suppression by androgens is similar in young and aged men (21).

Aging appears to be associated with combined hypothalamic and gonadal defects. However, the pituitary of elderly men responds adequately to stimulation by exogenous LHRH (23), but the Leydig cell responsiveness to LH is decreased (24). With aging, alterations in hypothalamic GnRH secretion are noted; decreased LH burst amplitude, increased LH burst frequency and a disordered LH burst pattern. Androgen deprivation reveals impaired GnRH secretory reserve in older men compared to young men (25). Our study shows that these defects of aging do not cause an inadequate adaptation of testosterone concentrations to variations in SHBG levels.

In neonates it appears that the HPG axis is even more sensitive to androgen feedback than the HPG axis of elderly men. The mechanism behind this is unknown. The response of the HPG axis to varying SHBG levels shows that the level of non-SHBG-T in newborns and adults is not solely a result of randomly combined SHBG and testosterone levels but the product of the HPG axis responding to negative and positive feedback signals. The precise nature and impact of these factors remain to be determined.

Our study has certain limitations. The adult volunteers for this study were all living independently. Especially in the highest age groups this might have led to a health selection bias.

The levels of testosterone in the neonates were low in comparison to adult men. Since commercial testosterone assays are designed to measure reliably in the adult range it is well known that they loose accuracy in the lower ranges (26). For several reasons we believe that our conclusions drawn from the neonatal data are justified. The method used in our study for

the determination of testosterone showed the closest correlation with values determined by liquid chromatography-tandem mass spectrometry (26). The intra- and interassay coefficients of variation were below 9% for the concentration range measured in the samples and the mean testosterone level in our study was similar to levels reported by others (10). The studied population was large. Moreover, this study was not designed to make statements about individual hormone levels but to analyse relations between parameters in a group of individuals. The finding of a highly significant association between levels of total testosterone and SHBG in both neonates and adult men underlines the validity of our estimates; if the estimates for the testosterone concentration in neonates were highly inaccurate such a relation would not have been found.

The approach used in this study allows us to make statements about the setpoint of the HPG axis of a group of men but cannot be translated to individuals. For instance, it is well known that all kinds of diseases are associated with an impaired function of the HPG axis (27). When the HPG axis is compromised not only by age but also by other factors the capability to adapt to testosterone binding by SHBG might be lost. In those cases SHBG may become a contributor to low non-SHBG-T levels in men.

The cross-sectional nature of this study inherently does not allow us to make definitive causative statements about the observed correlations between SHBG and sex hormones.

In conclusion the results of our study show that in male neonates and in healthy adult men levels of SHBG, if at all, barely contribute to variations in non-SHBG-T.

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Reference List

1. **Dunn JF, Nisula BC, Rodbard D** 1981 Transport of steroid hormones: binding of 21 endogenous steroids to both testosterone-binding globulin and corticosteroid-binding globulin in human plasma. J Clin Endocrinol Metab 53:58-68

2. **Vermeulen A, Ando S** 1979 Metabolic clearance rate and interconversion of androgens and the influence of the free androgen fraction. J Clin Endocrinol Metab 48:320-326

3. **Saez JM, Forest MG, Morera AM, Bertrand J** 1972 Metabolic clearance rate and blood production rate of testosterone and dihydrotestosterone in normal subjects, during pregnancy, and in hyperthyroidism. J Clin Invest 51:1226-1234

4. **Mendel CM** 1989 The free hormone hypothesis: a physiologically based mathematical model. Endocr Rev 10:232-274

5. **Manni A, Pardridge WM, Cefalu W, Nisula BC, Bardin CW, Santner SJ, Santen RJ** 1985 Bioavailability of albumin-bound testosterone. J Clin Endocrinol Metab 61:705-710

6. **Vermeulen A, Verdonck L, Kaufman JM** 1999 A critical evaluation of simple methods for the estimation of free testosterone in serum. J Clin Endocrinol Metab 84:3666-3672

7. **Longcope C, Feldman HA, McKinlay JB, Araujo AB** 2000 Diet and sex hormone-binding globulin. J Clin Endocrinol Metab 85:293-296

8. **Vermeulen A, Kaufman JM, Giagulli VA** 1996 Influence of some biological indexes on sex hormone-binding globulin and androgen levels in aging or obese males. J Clin Endocrinol Metab 81:1821-1826

9. **Mann DR, Fraser HM** 1996 The neonatal period: a critical interval in male primate development. J Endocrinol 149:191-197

10. **Andersson AM, Toppari J, Haavisto AM, Petersen JH, Simell T, Simell O, Skakkebaek NE** 1998 Longitudinal reproductive hormone profiles in infants: peak of inhibin B levels in infant boys exceeds levels in adult men. J Clin Endocrinol Metab 83:675-681

11. **Belgorosky A, Rivarola MA** 1986 Progressive decrease in serum sex hormone-binding globulin from infancy to late prepuberty in boys. J Clin Endocrinol Metab 63:510-512

12. **Harman SM, Metter EJ, Tobin JD, Pearson J, Blackman MR** 2001 Longitudinal effects of aging on serum total and free testosterone levels in healthy men. Baltimore Longitudinal Study of Aging. J Clin Endocrinol Metab 86:724-731

13. **Sodergard R, Backstrom T, Shanbhag V, Carstensen H** 1982 Calculation of free and bound fractions of testosterone and estradiol-17 beta to human plasma proteins at body temperature. J Steroid Biochem 16:801-810

14. **de Ronde W, van der Schouw YT, Muller M, Grobbee DE, Gooren LJ, Pols HA, de Jong FH** 2005 Associations of Sex-Hormone-Binding Globulin (SHBG) with Non-SHBG-Bound Levels of Testosterone and Estradiol in Independently Living Men. J Clin Endocrinol Metab 90:157-162

15. **Muller M, den Tonkelaar, I, Thijssen JH, Grobbee DE, van der Schouw YT** 2003 Endogenous sex hormones in men aged 40-80 years. Eur J Endocrinol 149:583-589

16. **Pasquali R, Vicennati V, Bertazzo D, Casimirri F, Pascal G, Tortelli O, Labate AM** 1997 Determinants of sex hormone-binding globulin blood concentrations in premenopausal and postmenopausal women with different estrogen status. Virgilio-Menopause-Health Group. Metabolism 46:5-9

17. **Plant TM** 1980 The effects of neonatal orchidectomy on the developmental pattern of gonadotropin secretion in the male rhesus monkey (Macaca mulatta). Endocrinology 106:1451-1454

18. **Bouvattier C, Carel JC, Lecointre C, David A, Sultan C, Bertrand AM, Morel Y, Chaussain JL** 2002 Postnatal changes of T, LH, and FSH in 46,XY infants with mutations in the AR gene. J Clin Endocrinol Metab 87:29-32

19. **Dunkel L, Perheentupa J, Tapanainen J, Leinonen P, Vihko R** 1984 Responsiveness of the pituitary-testicular axis to gonadotropin-releasing hormone and chorionic gonadotropin during the first week of life. Pediatr Res 18:1085-1087

20. **Deslypere JP, Kaufman JM, Vermeulen T, Vogelaers D, Vandalem JL, Vermeulen A** 1987 Influence of age on pulsatile luteinizing hormone release and responsiveness of the gonadotrophs to sex hormone feedback in men. J Clin Endocrinol Metab 64:68-73

21. **Winters SJ, Sherins RJ, Troen P** 1984 The gonadotropin-suppressive activity of androgen is increased in elderly men. Metabolism 33:1052-1059

22. **Winters SJ, Atkinson L** 1997 Serum LH concentrations in hypogonadal men during transdermal testosterone replacement through scrotal skin: further evidence that ageing enhances testosterone negative feedback. The Testoderm Study Group. Clin Endocrinol (Oxf) 47:317-322

23. **Kaufman JM, Giri M, Deslypere JM, Thomas G, Vermeulen A** 1991 Influence of age on the responsiveness of the gonadotrophs to luteinizing hormone-releasing hormone in males. J Clin Endocrinol Metab 72:1255-1260

24. **Mulligan T, Iranmanesh A, Veldhuis JD** 2001 Pulsatile iv infusion of recombinant human LH in leuprolide-suppressed men unmasks impoverished Leydig-cell secretory responsiveness to midphysiological LH drive in the aging male. J Clin Endocrinol Metab 86:5547-5553

25. **Veldhuis JD, Zwart A, Mulligan T, Iranmanesh A** 2001 Muting of androgen negative feedback unveils impoverished gonadotropin-releasing hormone/luteinizing hormone secretory reactivity in healthy older men. J Clin Endocrinol Metab 86:529-535

26. **Wang C, Catlin DH, Demers LM, Starcevic B, Swerdloff RS** 2004 Measurement of total serum testosterone in adult men: comparison of current laboratory methods versus liquid chromatography-tandem mass spectrometry. J Clin Endocrinol Metab 89:534-543

27. **Baker HW** 1998 Reproductive effects of nontesticular illness. Endocrinol Metab Clin North Am 27:831-850

Chapter 4

Calculation of bioavailable and free testosterone in men; a comparison of five published algorithms

Abstract

Estimating serum levels of free or bioavailable testosterone by calculation is cheap and appears to be uncomplicated.

We compared estimates obtained using five published algorithms, which were applied to samples from a cross-sectional, single centre study in 399 independently living men aged 40 to 80 years. Levels of bioavailable (bioT) and free testosterone (FT) were calculated using the algorithms described by Sodergard et al. ($bioT_S$ and FT_S), Vermeulen et al. ($bioT_V$ and FT_V), Emadi-Konjin et al. ($bioT_E$), Morris et al. ($bioT_M$) and Ly et al. (FT_L).

Mean bioavailable testosterone was highest for $bioT_S$ (10.4 nmol/l) and lowest for $bioT_E$ (3.87 nmol/l). Mean free testosterone was highest for FT_S (0.41 nmol/l), followed by FT_V (0.35 nmol/l) and FT_L (0.29 nmol/l). For bioavailable testosterone levels Pearson's coefficient of correlation was highest for the association between $bioT_S$ and $bioT_V$ ($r=0.98$) and lowest between $bioT_M$ and $bioT_E$ ($r=0.66$). FT_L was significantly associated with both FT_S ($r=0.96$) and FT_V ($r=0.88$). Pearson's coefficient of correlation for the association between FT_L and $bioT_M$ almost reached 1.0. Bland-Altman analysis showed large differences between the results of different algorithms. $BioT_M$, $bioT_E$, $bioT_V$ and FT_L were all significantly associated with SHBG levels.

Algorithms to calculate bioavailable or free testosterone cannot be transferred to other users unless a careful re-validation in the local setting is performed. Whenever the algorithm is applied by others without prior validation, one risks over- or underestimating free or bioavailable testosterone and potentially introduces confounding on the basis of SHBG levels.

Introduction

Sex-Hormone Binding Globulin (SHBG), corticosteroid binding globulin (CBG) and albumin are important steroid hormone binding proteins in human plasma. In normal adult men approximately 44% of the circulating testosterone is specifically bound to SHBG, 50% is nonspecifically bound to albumin and 3.5% is bound to CBG, implicating that only 2-3% is unbound or free (1). By binding testosterone SHBG decreases the metabolic clearance rate of testosterone (2;3) and decreases peripheral conversion of testosterone to androstenedione (2). To exert its genomic action testosterone must bind to an intracellular androgen receptor. The strong binding of testosterone to SHBG withholds bound hormone to diffuse from the bloodstream into the cell, although a very recent publication indicates that SHBG may be a necessary cofactor for cellular uptake of testosterone (4). There is some controversy whether or not albumin-bound testosterone can dissociate sufficiently fast from this carrier protein to enter tissues (5;6). However, levels of non-SHBG bound testosterone (bioT = free+albumin bound testosterone) and non-SHBG-non-albumin bound testosterone (=free testosterone) are extremely well correlated (7) and interchangeable in most cases.

The levels of SHBG vary widely between healthy men and depend on factors such as diet (8), body mass index (BMI), insulin levels and age (8-10). For a correct assessment of the bioactive fraction of testosterone measurement or calculation of free or bioavailable testosterone can be desirable. This particularly relates to aging men in which total testosterone levels tend to decrease whereas SHBG levels tend to increase (11). Whenever a diagnosis of hypogonadism in these men is suspected measurement of free or bioavailable testosterone is advocated (12).

Bioavailable testosterone can be measured ex vivo using the ammoniumsulphate precipitation technique as describe by Tremblay and Dube (13). The gold standard for free testosterone measurement still is the dialysis method (14) although a mass spectrometry based assessment of free testosterone in ultrafiltrates was recently proposed as a candidate reference method (15). However, both ammoniumsulphate precipitation and the dialysis technique are non-automated, time consuming and expensive techniques and therefore not routinely used in most laboratories. Alternatively, free testosterone levels can be measured using a direct radioimmunoassay but this assay has been criticized because of lack of accuracy (7;14-16).

The concentration of free and bioavailable testosterone can also be calculated using one of several published algorithms. The two most widely used equations for calculating bioavailable or free testosterone are those described by Vermeulen et al. (7) and Sodergard et al. (17). These algorithms assume that when the concentrations of total testosterone, SHBG

and albumin and the constants for the binding of testosterone to SHBG and albumin are known, free and bioavailable testosterone can be calculated. These calculations depend on a proper estimation of the association constant for binding of testosterone to SHBG (k_t) and albumin (k_{at}). However, based on various methodologies values for k_t of 5.97x 10E8 (17), 10x10E8 (7), 16x10E8 (1) or 19x10E8 (18) have been reported in the literature.
A more pragmatic approach has been to measure free or bioavailable testosterone in a limited number of samples and to create an algorithm based on these measurements to predict free or bioavailable testosterone in future samples using only total testosterone and SHBG concentrations. Recently three papers were published in which three new algorithms for estimating the concentration of free or bioavailable testosterone were proposed based on measured levels in large numbers of samples using a gold standard technique (19-21).
At least two algorithms have been placed on the internet as so called bioavailable testosterone calculators (www.issam.ch and www.him-link.com) making these algorithms readily available for distant users.
Calculated free or bioavailable testosterone levels are used widely in the endocrinological literature and most often the so called Vermeulen (14;22-27) and Sodergard(23;28-32) methods are used. In most publications no arguments are given for the choice of either constant in the calculation of free or bioavailable testosterone although, as described above, choosing a particular constant will obviously influence results of calculated free and bioavailable hormone levels and might thus influence results of analyses. Moreover, it is doubtful whether algorithms composed and validated in one laboratory can be applied to samples from an unrelated laboratory, using different assay techniques. The aim of this study therefore was to compare the results of five published algolrithms to calculate free or bioavailable testosterone when applied to samples from a cross-sectional, single centre study in 399 independently living men aged 40 to 80 years.

Subjects and Methods

The study is a cross-sectional, single centre study in 399 independently living men aged 40 to 80 years. Details on recruitment, lifestyle, health of the subjects and baseline characteristics have been described extensively (33). All participants gave written informed consent before enrolment and the institutional review board of the Utrecht University Medical Center approved the study.

Table1a: equation for the calculation of free and bioavailable testosterone according to Vermeulen et al.(7), Sodergard et al.(17) and Emadi-Konjin et al.(19)

bioT (mol/l)	$\{(k_{at}*[albumin]*[FT]/(1+k_{at}*[FT])\}+[FT]$
FT (mol/l)	$\{-b+\sqrt{(b^2+4a[TT]}\}/2a$, in which
	$a=k_{at}+k_t+(k_{at}*k_t)([SHBG]+[albumin]-[T])$
	$b=1+k_t[SHBG]+k_{at}[albumin]-(k_{at}+k_t)[T]$

k_{at}=association constant for binding of testosterone to albumin
k_t=association constant for binding of testosterone to SHBG
[FT]= plasma concentration of free (non-albumin-non-SHBG bound) testosterone (mol/L)
[T]= plasma concentration of testosterone (mol/L)

Table 1b: equation for the calculation of bioavailable testosterone according to Morris et al.(20)

bioT (nmol/l)	$e^{(-0.266+0.955*\ln[TT]-0.228*\ln[SHBG])}$

Table 1c: equation for the calculation of bioavailable testosterone according to Ly et al.(21)

freeT (pmol/l)	$-52.65+24.4[TT]-0.704[SHBG]-0.0782[TT]*[SHBG]-0.0584[TT]^2$

Laboratory measurements and calculations of free and bioavailable testosterone

Fasting blood samples were obtained by venapuncture. Cell free serum was immediately stored at –20C. T was measured after diethylether extraction using an in house radioimmunoassay employing a polyclonal antiT-antibody (AZG 3290, a gift from Dr. J.J. Pratt, Groningen, the Netherlands). The lower limit of detection of the assay was 0.24 nmol/L and inter-assay variation was 6.0; 5.4 and 8.6% at 2.1; 5.6 and 23 nmol/L respectively. SHBG was measured using an immunometric technique on an Immulite 2000 Analyser (Diagnostic Products Corporation, Los Angeles, California, USA). The lower limit of detection was 5 nmol/L and inter-assay variation was 6.1; 4.9 and 6.9% at 11.6; 36 and 93 nmol/L respectively. Bioavailable testosterone was calculated using the equations summarized in table 1 using a fixed plasma albumin concentration of 43 g/l. For the affinity constant for the binding of testosterone to SHBG or albumin either 5.97x 10E8 l/mol and 4.06 x 10E4 l/mol as proposed by Sodergard et al. (17) ($BioT_S$), 10 x 10E8 l/mol and 3.6 x 10E4 l/mol as described by Vermeulen et al. (7) ($BioT_V$) or 1.4 x 10E9 l/mol and 1.3 x 10E4 l/mol as described by Emadi-Konjin et al.(19) ($BioT_E$) were used (table 1a). Bioavailable testosterone was also calculated according to the algorithm presented by Morris et al.(20) ($BioT_M$) (table 1b). Free testosterone was calculated using the equations of Vermeulen (FT_V) and Sodergard (FT_S) (table 1a) and the equation as described by Ly et al.(21) (FT_L) (table 1c).

To evaluate the impact of a varying albumin concentration bioavailable testosterone was recalculated using a fixed plasma albumin concentration of 33 g/l, instead of 43 g/l for the Vermeulen, Sodergard and Emadi-Konjin algorithms only, since the albumin concentration is not entered in the Ly and Morris algorithms.

Data analysis

The association between the results of the calculations using all possible pairs of algorithms was assessed and expressed as Pearson's coefficient of correlation. The differences between the results of the calculations were quantified using the approach as described by Bland and Altman (34). Analyses were done using SPSS 11.0 software.

Results

Mean bioavailable and free testosterone as calculated by the different algorithms are presented in figures 1a and 1b.

As could be anticipated from the different association constants for the binding of testosterone to SHBG entered in the algorithm of table 1a, mean $bioT_S$ was highest, followed by mean $bioT_V$ and mean $bioT_E$. The result for mean $bioT_M$ was in between those for $bioT_V$ and $bioT_E$. The range for $bioT_M$ (%) covers only 15% points which implies that the impact of varying SHBG levels on the level of $bioT_M$ is limited. This is also indicated by the strong correlation between levels of total testosterone and $bioT_M$ levels (table 3). The ranges for bioT(%) calculated using the other algorithms varied between 41 and 47 %. For free testosterone, FT_S was highest followed by FT_V and FT_L.

Pearson's coefficients of correlation for the relationships between the results of the different algorithms are summarized in table 2. For the calculations of FT_V and FT_S an albumin concentration of 43 g/l was used. As a result, there was a fixed relation between FT and bioT. Thus, associations found between $bioT_S$ or $bioT_V$ and other variables are similar for FT_S and FT_V respectively. $BioT_V$ and $bioT_S$ appeared to be strongly correlated. The relationship between $bioT_M$ and $bioT_E$ was relatively weak. Whereas the relationship between $bioT_M$ and total testosterone was very strong; it was weak for $bioT_E$. There was also a striking difference between these two estimates and their association with SHBG levels; $bioT_M$ was positively

Figure 1a: bioavailable testosterone fractions as estimated by different algorithms

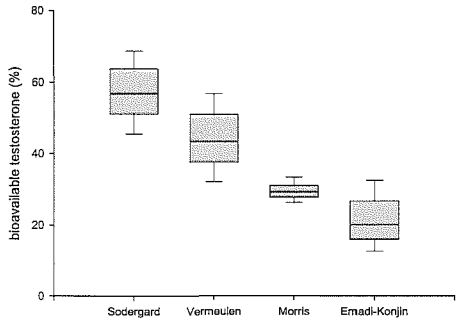


Figure 1b: free testosterone fractions as estimated by different algorithms

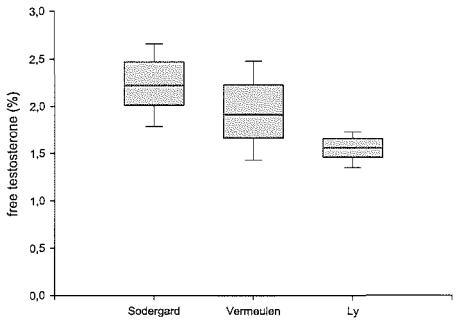


Table 2: Pearson's coefficients of correlation for the relationship between the results of various calculations

	Vermeulen	Morris	Emadi-Konji	Ly	Total testosterone	SHBG
Sodergard	0.98**	0.95**	0.84**	0.96**	0.82**	0.05
Vermeulen		0.87**	0.94**	0.88**	0.68**	-0.16**
Morris			0.66**	1.0**	0.95**	0.34**
Emadi-Konjin				0.67**	0.41**	-0.44**
Ly					0.94**	0.31**

** = $p<0.01$

associated, $bioT_E$ was negatively associated whereas $BioT_V$ and $bioT_S$ were not or only weakly related to the levels of SHBG. FT_L was strongly related to $bioT_M$, was also strongly associated with total testosterone and was positively associated with SHBG levels.
For the Vermeulen, Sodergard and Emadi-Konjin algorithms results were recalculated with a standard albumin concentration of 33 gr/l (instead of 43 gr/l) to evaluate the impact of varying albumin concentrations on the results. For al algorithms mean calculated bioavailable testosteron levels using the lower albumin concentration were slightly lower. The impact of varying albumin concentrations appeared to be stronger when the fractional bioavailable testosterone concentration was lower. Since the Sodergard algorithm resulted in the highest mean bioavailable testosterone fractions, this algorithm was least sensitive to variations in albumin concentration (percent difference = 7.2% of mean bioavailable testosterone, range

3.2-10.9%) followed by Vermeulen (9.1%, range 3.6-13.2%) and Emadi-Konjin (11.8%, range 4.2-14.9%).

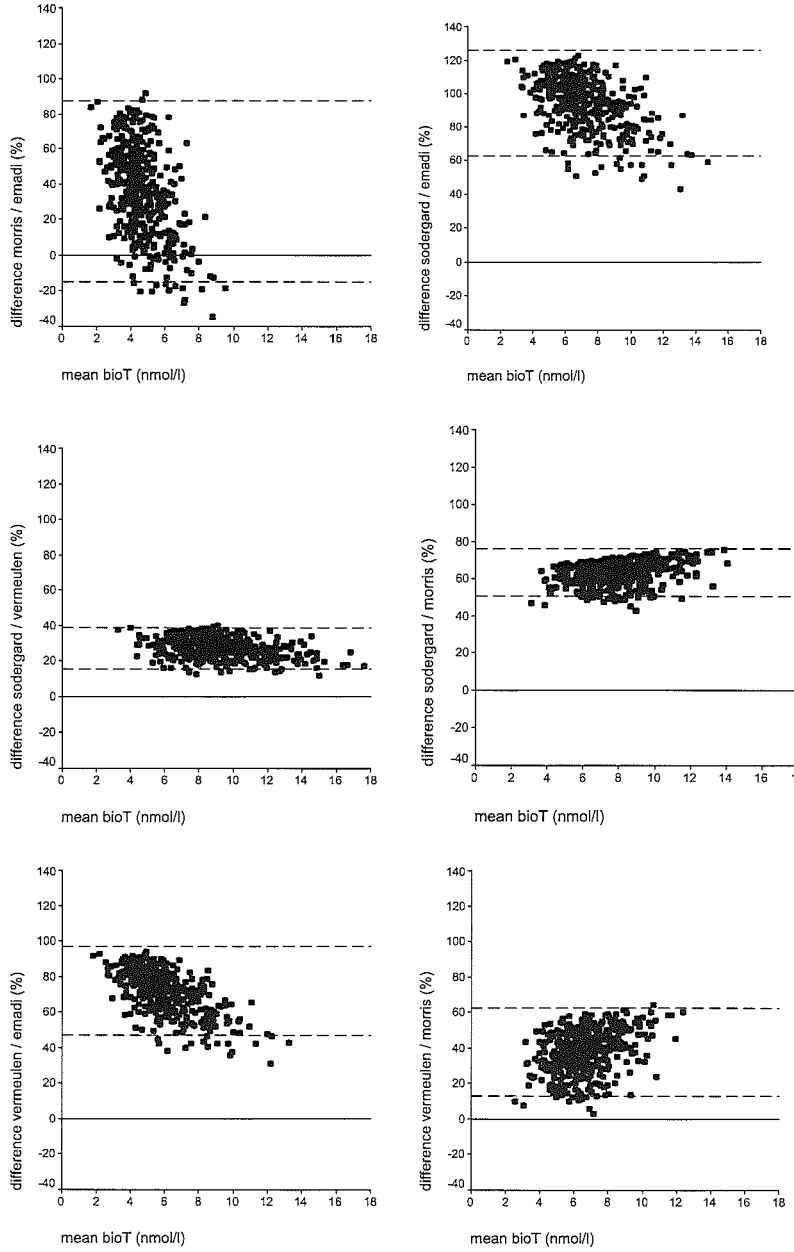
In figure 2 and 3 Bland-Altman plots are presented in which the results of the calculations using all pairs of algorithms are compared. Although differences between the results of the calculations were large they were smallest when $BioT_S$ and $bioT_V$ were compared. $BioT_S$ was estimated systematically higher as compared to $bioT_V$ with a mean difference of +27% of the mean bioavailable testosterone level. Both the Vermeulen and Sodergard algorithms provided systematically higher estimates as compared to the Emadi and Morris algorithms (mean difference 94% for $BioT_S$ versus $BioT_E$, 63% for $BioT_S$ versus $BioT_M$, 72% for $BioT_V$ versus $BioT_E$, and 38% $BioT_V$ versus $BioT_M$). The mean difference between $BioT_M$ versus $BioT_E$ was limited to 36%, however the difference between the methods ranged from –35% to +92%.

Both FT_S and FT_V gave higher estimates as compared to FT_L (mean difference 21% for FT_V versus FT_L and 35% for FT_S versus FT_L) but the ranges were much smaller for FT_S versus FT_L. $BioT_M$ and FT_L were extremely well correlated and the plots of their differences with $bioT_V$ and $bioT_S$ or FT_V and FT_S respectively look similar although not identical (figures 1 and 2).

Discussion

In the present study we compared the results of five published algorithms to calculate bioavailable or free testosterone levels in serum. Since these algorithms have been published or have been placed on the internet (www.issam.ch and www.him-link.com) by interest groups others may be tempted to apply these algorithms in combination with their own data. The approaches that have led to the Vermeulen and Sodergard algorithms on the one hand and the Emadi-Konjin, Ly and Morris calculations on the other hand are fundamentally different. The Vermeulen and Sodergard equations are based on the law of mass action in which estimates of total testosterone, SHBG and the values for the association constants for the binding of testosterone to albumin and SHBG are used to calculate bioavailable and free testosterone. The association constants for these algorithms were obtained experimentally and validated by comparing the results of the calculations with a gold standard technique. The consistency between the association constants and the results obtained with it add to the credibility of these algorithms. Emadi-Konjin et al. and Morris et al. first measured bioavailable testosterone by using ammonium-sulphate precipitation and used these results to create an algorithm to predict bioavailable testosterone levels in other samples using the

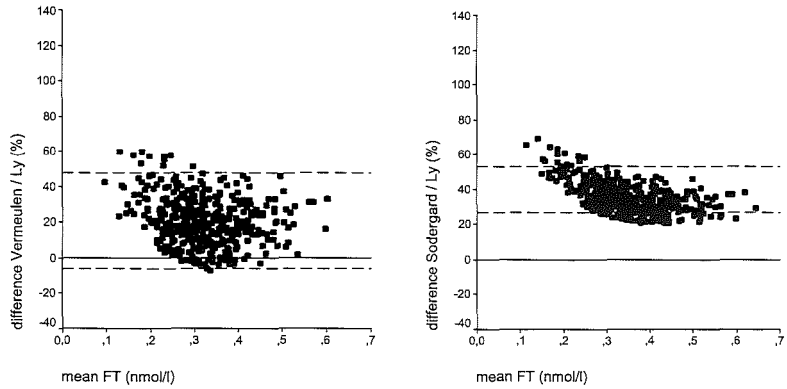
Figure 2: Plots of the percentage differences in calculated bioavailable testosterone levels against the average of the two applied algorithms. The solid line represents 0%, the dotted lines the 2 SD of the mean percentage difference.



concentration of total testosterone and SHBG. Emadi-Konjin et al. modified the equation also used by Vermeulen and Sodergard by altering the binding constants for the binding of testosterone to albumin and SHBG in order to obtain an optimal correlation with the results from their measurements. Morris et al. chose to create a regression equation based on their measurements. Ly et al. (21) first estimated free testosterone in almost 4000 samples using a ultrafiltration assay. Then they composed two regression models to predict free testosterone in these samples; one equation for total testosterone levels below 5 nmol/l and one for levels above this concentration. For our study we only used the latter equation since all subjects in our study had total testosterone levels above 5 nmol/l. In contrast to the Vermeulen and Sodergard method, the Morris, Ly and Emadi-Konjin algorithms solely rely on the accuracy of their measurements.

When using the Vermeulen and Sodergard equations the result of the calculation of free and bioavailable testosterone is only accurate when competition for the binding sites by other steroid hormones is limited. This implies that the calculation with either method will underestimate the level of bioavailable testosterone in the presence of supraphysiological levels of for instance estradiol or dihydrotestosterone. In clinical practice however, this problem is rarely encountered. Secondly, these equations assume that the association constants for binding of testosterone to SHBG (k_t) and albumin (k_{at}) are known. The fact that the Sodergard and Vermeulen equations differ in this regard shows that this is not the case. Thirdly, the results of the calculation of free and bioavailable testosterone with either algorithm depend on a proper determination of the concentration of total testosterone (also necessary for the measured free- or bioavailable testosterone levels using the dialysis, ultrafiltration or ammoniumsulphate precipitation techniques) and SHBG (35). Considerable differences do exist between the results of commercially available assays for testosterone (35-37) and SHBG (14;38). Since most commercial automated testosterone assays were shown to perform inaccurately in women and children (36;37), the results of calculated free and bioavailable testosterone using these results will be equally inaccurate. Differences in the estimation of the SHBG concentration will only influence the result of the calculated but not the measured free or bioavailable testosterone concentration. In the literature the so called Vermeulen and Sodergard methods appear to be used rather indiscriminately and proper validation of the calculated results is rarely described. In some studies the calculation as proposed by Sodergard is used and validated by referencing to validation experiments in which the Vermeulen calculation is used (23;32;39).

Figure 3: Plots of the percentage differences in calculated free testosterone levels against the average of the two applied algorithms. The solid line represents 0%, the dotted lines the 2 SD of the mean percentage difference.



This study shows that the results of the calculations based on different algorithms when applied to testosterone and SHBG levels measured in an unrelated laboratory are considerably different. $BioT_E$ and $BioT_M$, based on actual measurements using ammonium-sulphate precipitation, provide much lower estimates as compared to $BioT_S$ and $BioT_V$ which might indicate that these widely used algorithms systematically overestimate bioavailable testosterone levels. This is supported by validation experiments in which measured free testosterone was mostly lower when compared to calculated free testosterone using the Vermeulen equation (15;21;35;40). Also, Ly et al. (21) measured lower free testosterone with their ultrafiltration technique when compared to calculated levels using the Vermeulen or Sodergard algorithms.

Although both $BioT_M$ and $BioT_E$ are based on actual measurements of bioavailable testosterone the correlation between the results of the adapted algorithms is only 0.66 (Pearson's r) and the difference between the estimates ranges between –35% and +92% of the mean bioavailable testosterone level. As stated above, algorithms are not directly transferable to other users since they depend on the methods employed to measure total testosterone and SHBG. However, as is shown in table 4, the reference ranges of the assays used in the Morris and Emadi-Konjin studies were not very different from the results in our study which makes it unlikely that differences between assays can explain all variability as presented here. The lack of uniformity between the results of the Morris and Emadi-Konjin algorithms implies that either one of the methods or both are flawed. This is not entirely surprising knowing that accurate measurement of bioavailable testosterone using ammonium sulphate precipitation is

a demanding procedure. It relies on selectively and reproducibly precipitating all SHBG without precipitating albumin which, among others, requires a critical concentration of ammonium sulphate (41). The fact that $BioT_M$ is highly correlated with FT_L, the results of which are based on a different gold standard technique, argues in favor of the Morris algorithm. A positive finding of our study is that the two most widely used algorithms, those as described by Vermeulen and by Sodergard, are fairly concordant although the Sodergard algorithm provides systematically higher estimates.

$BioT_E$, $bioT_M$ and FT_L were significantly associated with SHBG levels (table 3). In healthy men neither a positive nor a negative relationship between SHBG and bioavailable testosterone is expected since the pituitary is assumed to increase its LH stimulation of the testes in order to compensate for higher SHBG levels, assuming that bioavailable or free testosterone truly represents the bioactive fraction of total testosterone. Therefore, a strong association between free or bioavailable testosterone levels and SHBG not only raises questions regarding the reliability of the estimates but also introduces fundamental problems when interpreting these estimates. Confounding of these results by SHBG can not be solved by establishing method specific reference ranges. Aging men, for instance, who generally have higher levels of SHBG as compared to young men, will be more prone to have low calculated bioavailable testosterone levels when applying the Emani-Konjin equation whereas the opposite is true when applying the Morris or Ly equations. Especially in older men, were an accurate estimation of free or bioavailable testosterone is needed for a proper diagnosis, dependency of these estimates on SHBG levels is highly undesirable. Neither Morris, nor Ly or Emadi-Konjin describe a coefficient of correlation for the relation between bioavailable or free testosterone levels and SHBG in their studies, while the absence of such a relation might have been used as an argument in favor of the validity of their results. In our study $BioT_S$ and $BioT_V$ were not or only weakly related to SHBG levels which might indicate that these estimates more closely resemble actual bioavailable testosterone levels as compared to estimates from other algorithms.

When applying the Morris or Ly equations, the range of (fractional) bioavailable or free testosterone is small which implies that over a wide range of SHBG levels, SHBG has little effect on absolute and fractional $bioT_M$ or FT_L levels. This is also reflected by the very high association between $bioT_M$ or FT_L and total testosterone levels. With such a high association it

Table 4: assay manufacturers and reference ranges of analyzed studies

	Emadi-Konjin	Morris	Ly	This study
Total testosterone (nmol/l)	Roche Elecsys 2010	DRG Instruments ELISA	DPC Immulite	In house RIA#
	7.3-28.1*	6.9-23.9*	5-240**	7.20-39.6**
SHBG (nmol/l)	DPC Immulite	DRG Instruments ELISA	DPC Immulite	DPC Immulite
	13-71*	15-100*	2.3-329**	12-91**

* reference ranges as supplied by manufacturer
** range in studied population
# this method yields results which are comparable to those obtained with the DPC Coat -a-Count radioimmunoassay (11)

is questionable whether calculated bioavailable or free testosterone levels have any additional value above the total testosterone measurements. This close association between total and (calculated) bioavailable or free testosterone levels was not found in the original study by Morris et al.(20) and was not described by Ly et al.(21).

In the Sodergard, Vermeulen and Emadi-Konjin algorithms the actual albumin concentration gains importance when fractional bioavailable testosterone concentrations are lower. Therefore, the actual albumin concentration should be added to the algorithm when gross abnormalities of the albumin level are expected (as in nephrotic syndrome or severe malnutrition) or when SHBG levels are high as in hyperthyroidism, women or aging men. Although the Morris, Ly and Emadi-Konjin algorithms were based primarily on measurements in male subjects (percentage males for Morris 100%, for Ly 87%, for Emadi 92%) we repeated the analyses in 415 premenopausal female patients of a tertiary fertility outpatient clinic and found Bland-Altman plots similar to those obtained in men (data not shown). In women the differences between the results of the algorithms were larger for all comparisons except for Morris/Vermeulen and Morris/Sodergard. This could be anticipated from the results found in men (figures 1 and 2); the results in men could be extrapolated in the very low bioavailable or free testosterone range. Differences between the results of the Sodergard, Vermeulen and Emadi-Konjin algorithms were larger due to the higher mean levels of SHBG in women; the difference between the results of these algorithms was, as could be expected, strongly associated with SHBG levels.

This study shows that appreciable differences exist between the results of several published algorithms to calculate bioavailable or free testosterone. We conclude that algorithms to calculate bioavailable or free testosterone are not simply transferable to samples from other laboratories unless careful re-validation in the local setting has been performed. Whenever the algorithm is applied by others without prior validation, one risks over- or underestimation of bioavailable or free testosterone and potentially introduces confounding by SHBG levels. The latter can be evaluated by quantifying the relationship between SHBG and calculated bioavailable or free testosterone levels or by adjusting the relationship between any variable and calculated bioavailable or free testosterone for SHBG.

Although these conclusions seem obvious, calculated levels of bioavailable and free testosterone are widely described in scientific journals and probably also used in clinical practice, mostly without prior in house validation of the calculated results. Without in house validation, the use of these algorithms is highly questionable.

Reference List

1. **Dunn JF, Nisula BC, Rodbard D** 1981 Transport of steroid hormones: binding of 21 endogenous steroids to both testosterone-binding globulin and corticosteroid-binding globulin in human plasma. J Clin Endocrinol Metab 53:58-68

2. **Vermeulen A, Ando S** 1979 Metabolic clearance rate and interconversion of androgens and the influence of the free androgen fraction. J Clin Endocrinol Metab 48:320-326

3. **Saez JM, Forest MG, Morera AM, Bertrand J** 1972 Metabolic clearance rate and blood production rate of testosterone and dihydrotestosterone in normal subjects, during pregnancy, and in hyperthyroidism. J Clin Invest 51:1226-1234

4. **Hammes A, Andreassen TK, Spoelgen R, Raila J, Hubner N, Schulz H, Metzger J, Schweigert FJ, Luppa PB, Nykjaer A, Willnow TE** 2005 Role of endocytosis in cellular uptake of sex steroids. Cell 122:751-762

5. **Mendel CM** 1989 The free hormone hypothesis: a physiologically based mathematical model. Endocr Rev 10:232-274

6. **Manni A, Pardridge WM, Cefalu W, Nisula BC, Bardin CW, Santner SJ, Santen RJ** 1985 Bioavailability of albumin-bound testosterone. J Clin Endocrinol Metab 61:705-710

7. **Vermeulen A, Verdonck L, Kaufman JM** 1999 A critical evaluation of simple methods for the estimation of free testosterone in serum. J Clin Endocrinol Metab 84:3666-3672

8. **Longcope C, Feldman HA, McKinlay JB, Araujo AB** 2000 Diet and sex hormone-binding globulin. J Clin Endocrinol Metab 85:293-296

9. **Muller M, den Tonkelaar, I, Thijssen JH, Grobbee DE, van der Schouw YT** 2003 Endogenous sex hormones in men aged 40-80 years. Eur J Endocrinol 149:583-589

10. **Vermeulen A, Kaufman JM, Giagulli VA** 1996 Influence of some biological indexes on sex hormone-binding globulin and androgen levels in aging or obese males. J Clin Endocrinol Metab 81:1821-1826

11. **de Ronde W, van der Schouw YT, Pierik FH, Pols HA, Muller M, Grobbee DE, Gooren LJ, Weber RF, de Jong FH** 2005 Serum levels of sex hormone-binding globulin (SHBG) are not associated with lower levels of non-SHBG-bound testosterone in male newborns and healthy adult men. Clin Endocrinol (Oxf) 62:498-503

12. **Nieschlag E, Swerdloff R, Behre HM, Gooren LJ, Kaufman JM, Legros JJ, Lunenfeld B, Morley JE, Schulman C, Wang C, Weidner W, Wu FC** 2005 Investigation, treatment and monitoring of late-onset hypogonadism in males: ISA, ISSAM, and EAU recommendations. Int J Androl 28:125-127

13. **Tremblay RR, Dube JY** 1974 Plasma concentrations of free and non-TeBG bound testosterone in women on oral contraceptives. Contraception 10:599-605

14. **Miller KK, Rosner W, Lee H, Hier J, Sesmilo G, Schoenfeld D, Neubauer G, Klibanski A** 2004 Measurement of free testosterone in normal women and women with androgen deficiency: comparison of methods. J Clin Endocrinol Metab 89:525-533

15. **Van Uytfanghe K, Stockl D, Kaufman JM, Fiers T, De Leenheer A, Thienpont LM** 2005 Validation of 5 routine assays for serum free testosterone with a candidate reference measurement procedure based on ultrafiltration and isotope dilution-gas chromatography-mass spectrometry. Clin Biochem 38:253-261

16. **Rosner W** 2001 An extraordinarily inaccurate assay for free testosterone is still with us. J Clin Endocrinol Metab 86:2903

17. **Sodergard R, Backstrom T, Shanbhag V, Carstensen H** 1982 Calculation of free and bound fractions of testosterone and estradiol-17 beta to human plasma proteins at body temperature. J Steroid Biochem 16:801-810

18. **Burke CW, Anderson DC** 1972 Sex-hormone-binding globulin is an oestrogen amplifier. Nature 240:38-40

19. **Emadi-Konjin P, Bain J, Bromberg IL** 2003 Evaluation of an algorithm for calculation of serum "bioavailable" testosterone (BAT). Clin Biochem 36:591-596

20. **Morris PD, Malkin CJ, Channer KS, Jones TH** 2004 A mathematical comparison of techniques to predict biologically available testosterone in a cohort of 1072 men. Eur J Endocrinol 151:241-249

21. **Ly LP, Handelsman DJ** 2005 Empirical estimation of free testosterone from testosterone and sex hormone-binding globulin immunoassays. Eur J Endocrinol 152:471-478

22. **Dhindsa S, Prabhakar S, Sethi M, Bandyopadhyay A, Chaudhuri A, Dandona P** 2004 Frequent occurrence of hypogonadotropic hypogonadism in type 2 diabetes. J Clin Endocrinol Metab 89:5462-5468

23. **Yildiz BO, Woods KS, Stanczyk F, Bartolucci A, Azziz R** 2004 Stability of adrenocortical steroidogenesis over time in healthy women and women with polycystic ovary syndrome. J Clin Endocrinol Metab 89:5558-5562

24. **Van Pottelbergh I, Goemaere S, Zmierczak H, Kaufman JM** 2004 Perturbed sex steroid status in men with idiopathic osteoporosis and their sons. J Clin Endocrinol Metab 89:4949-4953

25. **Bouillon R, Bex M, Vanderschueren D, Boonen S** 2004 Estrogens are essential for male pubertal periosteal bone expansion. J Clin Endocrinol Metab 89:6025-6029

26. **San Millan JL, Corton M, Villuendas G, Sancho J, Peral B, Escobar-Morreale HF** 2004 Association of the polycystic ovary syndrome with genomic variants related to insulin resistance, type 2 diabetes mellitus, and obesity. J Clin Endocrinol Metab 89:2640-2646

27. **Gu YQ, Tong JS, Ma DZ, Wang XH, Yuan D, Tang WH, Bremner WJ** 2004 Male hormonal contraception: effects of injections of testosterone undecanoate and depot medroxyprogesterone acetate at eight-week intervals in chinese men. J Clin Endocrinol Metab 89:2254-2262

28. **Rickenlund A, Eriksson MJ, Schenck-Gustafsson K, Hirschberg AL** 2004 Amenorrhea in Female Athletes is Associated with Endothelial Dysfunction and Unfavorable Lipid Profile. J Clin Endocrinol Metab

29. **Rickenlund A, Carlstrom K, Ekblom B, Brismar TB, Von Schoultz B, Hirschberg AL** 2004 Effects of oral contraceptives on body composition and physical performance in female athletes. J Clin Endocrinol Metab 89:4364-4370

30. **Goderie-Plomp HW, van der KM, de Ronde W, Hofman A, de Jong FH, Pols HA** 2004 Endogenous sex hormones, sex hormone-binding globulin, and the risk of incident vertebral fractures in elderly men and women: the Rotterdam Study. J Clin Endocrinol Metab 89:3261-3269

31. **Jakubowicz DJ, Essah PA, Seppala M, Jakubowicz S, Baillargeon JP, Koistinen R, Nestler JE** 2004 Reduced serum glycodelin and insulin-like growth factor-binding protein-1 in women with polycystic ovary syndrome during first trimester of pregnancy. J Clin Endocrinol Metab 89:833-839

32. **Araujo AB, O'donnell AB, Brambilla DJ, Simpson WB, Longcope C, Matsumoto AM, McKinlay JB** 2004 Prevalence and incidence of androgen deficiency in middle-aged and older men: estimates from the Massachusetts Male Aging Study. J Clin Endocrinol Metab 89:5920-5926

33. **de Ronde W, van der Schouw YT, Muller M, Grobbee DE, Gooren LJ, Pols HA, de Jong FH** 2005 Associations of Sex-Hormone-Binding Globulin (SHBG) with Non-SHBG-Bound Levels of Testosterone and Estradiol in Independently Living Men. J Clin Endocrinol Metab 90:157-162

34. **Bland JM, Altman DG** 1995 Comparing methods of measurement: why plotting difference against standard method is misleading. Lancet 346:1085-1087

35. **Rinaldi S, Geay A, Dechaud H, Biessy C, Zeleniuch-Jacquotte A, Akhmedkhanov A, Shore RE, Riboli E, Toniolo P, Kaaks R** 2002 Validity of free testosterone and free estradiol determinations in serum samples from postmenopausal women by theoretical calculations. Cancer Epidemiol Biomarkers Prev 11:1065-1071

36. **Wang C, Catlin DH, Demers LM, Starcevic B, Swerdloff RS** 2004 Measurement of total serum testosterone in adult men: comparison of current laboratory methods versus liquid chromatography-tandem mass spectrometry. J Clin Endocrinol Metab 89:534-543

37. **Taieb J, Mathian B, Millot F, Patricot MC, Mathieu E, Queyrel N, Lacroix I, Somma-Delpero C, Boudou P** 2003 Testosterone measured by 10 immunoassays and by isotope-dilution gas chromatography-mass spectrometry in sera from 116 men, women, and children. Clin Chem 49:1381-1395

38. **Bukowski C, Grigg MA, Longcope C** 2000 Sex hormone-binding globulin concentration: differences among commercially available methods. Clin Chem 46:1415-1416

39. **Mohr BA, Guay AT, O'donnell AB, McKinlay JB** 2005 Normal, bound and nonbound testosterone levels in normally ageing men: results from the Massachusetts Male Ageing Study. Clin Endocrinol (Oxf) 62:64-73

40. **Ross HA, Meuleman EJ, Sweep FC** 2005 A simple method for estimating equilibrium constants for serum testosterone binding resulting in an optimal free testosterone index for use in elderly men. Clin Chem Lab Med 43:613-616

41. **Davies R, Collier C, Raymond M, Heaton J, Clark A** 2002 Indirect measurement of bioavailable testosterone with the Bayer Immuno 1 system. Clin Chem 48:388-390

Chapter 5

A direct approach to the estimation of the origin of estrogens and androgens in elderly men by comparison with hormone levels in postmenopausal women

Abstract

The origin of oestrogens in men is only partly understood. From infusion studies with radioactively labelled hormones we know that oestradiol (E2) and oestrone (E1) are either directly secreted by the testes and adrenal glands or peripherally produced from testicular or adrenal androgens.

We determined E2, E1, androstenedione (A), testosterone (T) and dehydroepiandrosterone sulphate (DHEAS) in 292 elderly men and 367 postmenopausal women. We considered postmenopausal women as men without testes, assuming that the postmenopausal ovary is not endocrinologically active and that the testes do not contribute to circulating levels of DHEAS. Subjects were stratified according to DHEAS levels to adjust for differences in DHEAS levels between sexes. For men and women separately mean levels of E2, E1, A and T were calculated per DHEAS stratum. The relative direct and indirect contributions of the testes to steroid levels in men were calculated using the formula: ((Cm-Cf)/Cm)*100% in which Cm and Cf represent the mean concentrations of the steroid in men and women respectively.

The relative contribution (%) of the testes to hormone levels per DHEAS stratum (<2, 2-4, 4-6 and >6 μmol/L) respectively are: for E2; 72, 60, 52, 44, for E1: 54, 47, 35, 34, for A: 14, 4, 12, 0 and for T: 88, 88, 87, 83.

We conclude that in elderly men dependent on DHEAS levels, 44% to 72% of E2 and 34% to 54% of E1 originates directly or indirectly from the testes.

Introduction

Since the description of oestrogen resistant (1) and aromatase deficient men (2;3) it is known that oestrogens are important sex hormones in men, just as in women. In men, oestrogens have an important role in bone maintenance, growth spurt induction, closure of epiphyses and inhibition of gonadotrophin release (4). From observations in oestrogen receptor alpha (ERα) knockout mice it appears that oestrogens are also involved in the regulation of spermatogenesis (5). However, the origin of estrogens in men is not easily assessed. Oestrone (E1) and oestradiol (E2) are secreted by adrenal glands and testes (6;7). A substantial portion of circulating oestrogens however is derived from conversion of adrenal and testicular androgens in fat and muscle tissue by the aromatase enzyme (8;9). Finally, the 17β-hydroxysteroid dehydrogenases, found throughout the body, can convert E2 to E1 and testosterone (T) to androstenedione (A) and vice versa (10).

Several studies were performed to quantify the sources of E1 and E2 in men, mainly by using isotope-labeled steroid infusion techniques followed by measurement of radioactive steroids in urine (9) or in plasma (8). Interpretation of data obtained using these methods is hampered by several factors. When measuring in urine, production rates can only be reliably estimated after complete retrieval of infused radioactivity, which often is not the case. In addition interconversion of both products and precursors make it difficult to assess the importance of urinary data based on isotopic ratios in oestrogen conjugates. On the other hand, when measuring conversions in plasma, conversion rates are calculated on the basis of steady state levels of radioactive precursors and their products during infusion, which may take extremely long infusion times. Furthermore, interpretation of results of such techniques leans on the assumption that steroid infusion does not interfere with the metabolism of the endogenous steroid hormones and that labelling of the steroids does not influence the catabolism of that hormone. The complex nature of these techniques makes it difficult to examine large numbers of subjects. More importantly, these studies inform about production and interconversion rates but give no information about the sites of hormone production.

Comparing plasma hormone levels before and after castration or adrenalectomy can also give an indication of the contribution of testes and adrenal glands to the oestrogen pool. Bilateral adrenalectomy is a rare procedure and is mostly done to treat an endocrine disorder which means that the preoperative hormone levels can hardly be considered normal. Bilateral orchiectomy is an accepted treatment for hormone dependent prostatic carcinoma. Pre- and postoperative oestrogen measurements were only described by two groups (11;12) with different results. Suppression of adrenal steroid synthesis with exogenous glucocorticoids will

lead to suppression of endogenous oestrogen and T synthesis which will influence hypothalamus-pituitary-gonadal activity (13) and can therefore also lead to erroneous conclusions.

Considering the importance of oestrogens in men the aim of this study was to estimate the contribution of the adrenal glands and the testes to the circulating pool of E1, E2, T and A in a population based sample of elderly men by using a different, simple, non interventional approach. This approach has the advantage that results obtained are not distorted by exogenous hormone administration and that estimates can be made about the relative contribution of the various sites of hormone production.

Subjects and Methods

Subjects: All subjects were participants of the Rotterdam study, a population-based cohort study of determinants of chronic disabling diseases in the elderly. Details of this study have been described elsewhere (14). This study was approved by the medical ethics committee of the Erasmus Medical Center and all participants gave written informed consent. Subjects using exogenous hormones such as androgens, oestrogens or dehydroepiandrosterone (DHEA) were excluded from this study. This was tested by asking the participants for the use of exogenous hormones, and by examining the medication boxes brought by the subjects on request on the day of examination. A total of 665 men and 741 women, with an age range of 55 to 99 years were randomly selected for hormone measurements. Of these subjects 25 men and 22 women were excluded from analysis because of reported use of oral corticosteroids. Non-fasting blood samples were drawn at the baseline examination in the research centre between 08:30 h and 16:00 h; the time of blood collection was recorded. The mean sampling time was 11:22 for women and 11:25 for men. For the collection of plasma, blood was sampled in 5-ml tubes containing 0.5 ml sodiumcitrate solution. Cells were removed by centrifugation and the samples were stored at –80 °C until hormone measurements. The period of storage of frozen serum varied from 7.5 years to 12.5 years. Because of the relatively small volumes of serum available not all hormones could be determined in all samples. In 292 men and 367 women all hormone measurements could be performed. Baseline characteristics such as age, BMI and smoking in this subset were not different when compared to the overall group of subjects (data not shown).

Hormone measurements: Serum levels of T, A, E1, E2, DHEA-sulfate (DHEAS) and Sex Hormone Binding Globulin (SHBG) were estimated in 12 batches using coated tube or double antibody radioimmunoassays, purchased from Diagnostic Systems Laboratories (Webster,

Texas, USA). For E2 estimations, the ultrasensitive system was used. The results of these assays were compared to the results obtained using other commercial immunoassays, which in turn had been validated by comparison with in house immunoassays making use of steroid extraction and purification by column chromatography (T: (15); E2: (16); A: (17); E1: (18); DHEAS: (19)). The same procedure was used for SHBG, where the in house method used ammonium sulphate precipitation (20). Correlation coefficients varied between 0.925 for A and 0.980 for estradiol. Slopes of the regression lines were in between 0.89 for E2 and 1.22 for DHEAS. The sensitivities of the assays, defined as the value representing the blank + 2 times the standard deviation of the blank, varied between 4.8 pmol/l for E2 (Goderie-Plomp *et al.* 2004), 0.28 nmol/l for T , 5 nmol/l for SHBG and 0.05 μmol/l for DHEA sulphate. Steroids with the largest cross reactivities in the various assays were E1 (2.4%) and E2-3-glucuronide (2.6%) for E2, E1 sulphate (2.02%) and E2 (1.25 %) for E1, androsterone (0.33%) and 17-hydroxyprogesterone (0.25%) for A, 5α-dihydrostetosterone (5.8%) and A (2.3%) for T and DHEA (41%) and androsterone (7.3%) for DHEAS. Finally, further arguments supporting the validity of the estimation of E2 at the low level found in postmenopausal women using the present assay have been summarized by Goderie-Plomp *et al.* (21), who showed an increased risk for vertebral fractures in postmenopausal women with E2 levels in the lowest tertile. Because of the relatively small volumes of serum available, all values reported are single sample estimations. Intra-assay coefficients of variation, determined on basis of duplicate results of internal quality control (QC) serum pools with 3 different levels of each analyte, were below 15% for all assays, with the exception of E2 (18%) and E1 (21%). Since interassay variations were relatively large (between 20 and 30%, with the exception of T (19%) and SHBG (14%)) results of all batches were normalized by multiplying all concentrations within a batch with a factor, which equalized results for the internal quality control pools. This was considered justified because the results of these pools and the mean results for male and female sera in each assay batch showed very similar patterns (22).

To estimate the relative contributions of the testes and the adrenals to the plasma pool of oestrogens we considered postmenopausal women as men without testes. We assumed that the postmenopausal ovary is not endocrinologically active and that the testes do not contribute to circulating DHEAS levels. All subjects were stratified according to DHEAS levels (<2, 2-4, 4-6, >6 μmol/L) to adjust for differences in DHEAS levels between men and women. For men and women separately mean age and BMI and mean levels of E2, E1, A and T were calculated per DHEAS stratum. The relative contribution of the testes to hormone levels in

men was calculated for each stratum of DHEAS levels using the following formula: $(C_m-C_f)/C_m$ *100% in which C_m and C_f represent the mean concentrations of hormones in men and women respectively.

Statistics: All analyses were done using SPSS 9.0 software. A p-value <0.05 was considered significant. For all subgroups the distribution of the hormone levels was tested for normality using a Kolmogorov Smirnov test. Results are expressed as median, mean and Standard Deviation (SD) or Standard Error of the Mean (SEM). Differences between men and women for normally distributed variables were tested for significance using a Students t-test and for not normally distributed variables using a Mann-Whitney-U test. For men and women separately Spearman's correlation coefficients were calculated between the concentrations of the hormones studied, age and BMI.

To study the impact of sampling time on hormone levels, sampling time was divided into quartiles and mean levels of all hormones and SHBG were calculated per quartile for men and women separately. Using analysis of variance sampling time related differences in mean hormone levels were tested for significance.

Results

Table 1 summarizes the characteristics of the studied men and women. Men were slightly younger and had lower BMI compared to women. As expected T levels were much higher in men but also levels of E1 and E2 were respectively two and three times higher compared to those in postmenopausal women. The concentration of the adrenal androgens DHEAS and A were also higher in men. The mean concentration of SHBG was higher in women which resulted in a higher percentage of T and E2 bound to SHBG ($p<.001$ for all variables). After division in quartiles for sampling time, mean hormone and SHBG levels were not found to be significantly different between quartiles for men and women separately suggesting that differences in sampling time did not influence results (data not shown).

Tables 2 and 3 show Spearman's correlation coefficients for the relations between the levels of hormones, SHBG, age and BMI in men and women respectively. The levels of all hormones were weakly but significantly interrelated in both men and women. The correlations between SHBG and hormones were weak or absent. In both men and women older age was associated with significantly lower levels of DHEAS, A and E2 but higher levels of SHBG. Age and T were inversely associated in men only. Higher BMI was associated with lower SHBG in men and women. BMI was associated with higher oestrogen and T levels in women but not in men. In contrast higher BMI was weakly associated with lower T levels in men.

Table 1: Baseline characteristics of studied men and women (median / mean±SD)

	Men	Women
n	292	367
Age (yr)	68.7 / 69.7±8.06	71.2 / 71.6±8.91
BMI (kg/m^2)	25.8 / 25.8±3.00	26.4 / 26.6±3.95
E2 (pmol/L)	44.9 / 46.7 ±22.8	13.9 / 16.1 ±13.8
E1 (pmol/L)	85.5 / 88.3±42.9	41.5 / 43.0 ±31.1
A (nmol/L)	3.84 / 4.18±1.75	3.20 / 3.46 ±1.73
T (nmol/L)	11.3 / 11.3±4.01	1.22 / 1.31 ±0.80
DHEAS (µmol/L)	4.10 / 4.86±3.13	2.17 / 2.72 ±1.96
SHBG (nmol/L)	33.8 / 36.3 ±14.5	43.4 / 45.9 ±18.6

Table 2: Spearman's correlation coefficients between hormone levels, age and BMI in 292 men

	E1	A	T	SHBG	DHEAS	age	BMI
E2	.47***	.27***	.37***	-.08	.35***	-0.14**	0.06
E1		.18**	.28***	-.13*	.29***	-0.06	0.05
A			.32***	.14*	.30***	-0.15**	0.03
T				.12*	.28***	-0.26***	-0.12**
SHBG					-.07	0.22***	-0.23***
DHEAS						-0.32***	-0.04
age							-0.09*

*=p<.05, **=p<0.01, ***=p<0.001

Table 3: Spearman's correlation coefficients between hormone levels, age and BMI in 367 women

	E1	A	T	SHBG	DHEAS	age	BMI
E2	.47***	.42***	.42***	-.26**	.43***	-0.17***	0.24***
E1		.22***	.41***	-.34**	.31***	-0.04	0.28***
A			.53***	-.12*	.40***	-0.13**	0.01
T				-.17**	.30***	-0.07	0.15***
SHBG					-.22**	0.30***	-0.30***
DHEAS						-0.32***	0.03
age							0.07

*=p<.05, **=p<.01, ***=p<0.001

The mean levels of hormones per DHEAS stratum for men and women are given in table 4. Women were overrepresented in the lower DHEAS strata whereas men were overrepresented in the higher DHEAS strata. Hormone levels in all subgroups were normally distributed except for E2 in women in the DHEAS <2 category. In this subgroup there was an overrepresentation of very low E2 levels.

Mean age and BMI per DHEAS stratum for men and women are given in table 5. Both women and men in higher DHEAS subgroups were significantly younger ($p<.001$), but the mean ages of men and women per DHEAS stratum were similar. DHEAS and BMI were not significantly associated.

Figure 1 shows the calculated relative contributions of the testes and adrenal glands to the plasma levels of E2, E1, A and T in men, stratified according to DHEAS level. The relative contribution (%) of the testes to hormone levels per DHEAS stratum (<2, 2-4, 4-6 and >6 μmol/L) respectively are: for E2; 72, 60, 52, 44, for E1: 54, 47, 35, 34, for A: 14, 4, 12, 0 and for T: 88, 88, 87, 83.

Discussion

When postmenopausal women are considered as elderly men without testes it is possible to calculate the relative contribution of the adrenal glands and the testes to peripheral concentrations of sex steroids in elderly men using the formula given in the methods section. According to our calculations up to 56 % of E2, 66 % of E1 and 17 % of T can have its direct or indirect origin in the adrenal glands of elderly men (figure 1). The testes contribute only little to peripheral A levels (maximum 14%). These estimates are in agreement with earlier results from experiments using radio labeled hormone infusion techniques. MacDonald *et al.* (9) calculated that the daily urinary E2 excretion in men aged 26 to 35 years originates from direct secretion by the testes (13%) and from peripheral production from circulating T (38%) or A (49%). Urinary E1 is only peripherally produced via conversion of T (32%) or A (68%). The nature of their study however precludes conclusions about the production sites of T and A. Our study provides evidence that circulating T is mainly produced by the testes, while the adrenal glands are the main source of circulating A. Assuming that the conversion rates from androgens to oestrogens in young and elderly men are similar, the contribution by the testes to daily E2 and E1 production can be estimated to be 50% and 33% after adjustment for the small contribution of the testes and the adrenal glands to the A and T pools respectively.

Table 4: Hormone levels per DHEAS stratum in men and women (mean±SEM)

DHEAS stratum		n	DHEAS (µmol/L)	E2 (pmol/L)	E1 (pmol/L)	A (nmol/L)	T (nmol/L)
<2	men	36	1.38±.08	40.9±3.77	74.7±5.14	3.39±.17	9.45±.52
	women	167	1.19±.04	11.6±.93	34.5±1.97	2.92±.10	1.13±.05
2-4	men	107	2.93±.06	40.7±1.72	80.1±3.29	3.82±.13	10.7±.32
	women	121	2.87±.05	16.2±1.19	42.6±2.81	3.61±.13	1.30±.06
4-6	men	67	5.01±.07	48.9±3.41	90.6±5.98	4.51±.30	11.7±.59
	women	59	4.82±.07	23.6±1.54	58.9±4.23	3.98±.25	1.57±.10
>6	men	82	8.79±.32	55.5±2.34	103±5.33	4.72±.18	12.5±.45
	women	20	8.27±.30	31.2±3.87	68.2±8.95	5.47±.71	2.12±.24

Table 5: Age and BMI per DHEAS stratum in men and women (mean±SEM)

DHEAS		n	age	BMI
<2	men	36	74.6±1.62	25.8±.46
	women	164	74.3±.69	26.7±.31
2-4	men	107	71.0±.73	26.1±.26
	women	121	70.7±.72	26.5±.37
4-6	men	67	68.6±.91	25.9±.43
	women	59	68.1±1.2	26.6±.52
>6	men	82	66.7±.77	25.5±.35
	women	20	65.8±1.9	27.4±.93

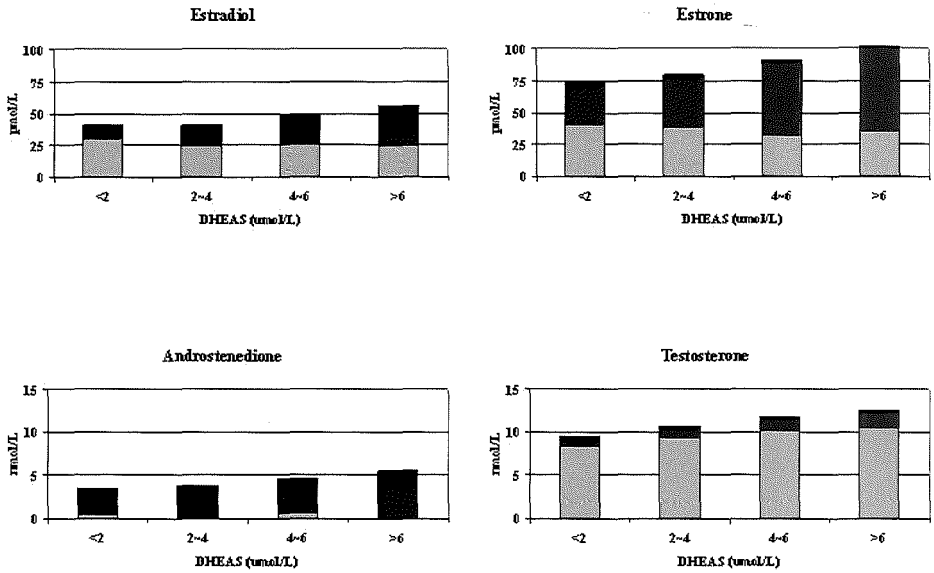
Our calculations are based on several assumptions. Firstly we assumed that the postmenopausal ovary is not endocrinologically active. This is supported by a report of Couzinet *et al.* (23) who found T and A to be below or close to the limit of detection of their assay in postmenopausal women with adrenal insufficiency. However, other reports indicate that the postmenopausal ovary can be an important source of androgens in women. Surgical castration of postmenopausal women resulted in a mean decrease of circulating testosterone levels of 46% (24) and 38% (25). The mean testosterone level in ovariectomized women was reported to be 40% lower when compared to concentrations in intact postmenopausal women (26). Pharmacological suppression of postmenopausal ovarian hormone synthesis also resulted in significantly lower peripheral testosterone levels (27;28). Moreover ovarian venous testosterone levels are higher than peripheral testosterone levels (25;29;30). This would mean that our model overestimates the adrenal contribution to peripheral sex steroid levels in men. No reliable data on the presence or absence of ovaries in our studied women was present and therefore we were not able to make proper adjustments. However, although

the androgen production of the postmenopausal ovary might be significant, it is small in comparison to the testicular production in men. Therefore adjustment for the ovarian contribution to circulating levels of T (38%) and E2 (6%) as reported by Sluijmer et al.(25) on basis of the formulas $(C_m-C_{f\text{-}38\%})/C_m$ for T and $(C_m-C_{f\text{-}6\%})/C_m$ for E2) will lead to a maximal increment of the testicular contribution to peripheral T and E2 concentrations of 7% and 3% respectively for every DHEAS stratum.

Although the testes produce no DHEAS but some A and DHEA (7) we assumed that the testes do not contribute to the plasma pool of DHEAS. However Stege *et al.* (31) found DHEAS levels 6 months after orchidectomy to be 17% lower compared to preoperative levels. In contrast Parker *et al.* (32) found no difference between pre- and postorchiedectomy DHEAS levels. If the testes would contribute to DHEAS levels, comparison of men and postmenopausal women after stratification for DHEAS will mean that the relative contribution of the adrenal glands to plasma concentrations of sex hormones is slightly overestimated. A testicular contribution to peripheral DHEAS levels of 17% as found by Stege *et al.* (31) for example will lead to a calculated maximal increase of testicular contribution in our model of 3% for E2, 3% for E1, 4% for A and 0% for T per DHEAS stratum ($DHEAS_{m\text{-}17\%}$ before stratification).

The women in our study population had significantly higher levels of SHBG. In normal men and women between 40 to 65% of circulating T and between 20 to 40% of circulating E2 is bound to SHBG. Binding of E1 and A to SHBG is less than 20% (33). SHBG decreases the metabolic clearance rate (MCR) of bound hormones (34). With similar production rates this will lead to higher plasma levels of T (and probably also of E2) in women compared to men. On top of that the conversion rate of T to A is linearly correlated with the non-SHBG bound fraction of T but not with total T (34). As can be seen in tables 2 and 3 higher SHBG levels are not or only weakly associated with higher levels of T or E2 in women and men. Considering the small difference in mean SHBG level between men and women (less than 10 nmol/l) and the weak associations between SHBG, E2 and T (tables 2 and 3), it is likely that the effects of SHBG on circulating levels of hormone are small. Nevertheless we cannot rule out that this has influenced our estimates.
It is unlikely that sex, either or not related to differences in SHBG levels, caused a difference in the metabolism of DHEA and DHEAS since the metabolic clearance rate of these hormones is reported to be similar in men and women (35;36).

Figure 1: DHEAS stratified calculated relative contributions of the testes (gray) and adrenals (black) to peripheral concentrations of E2, E1, A and T



Age is an important determinant of hormone levels in both men and women. As is evident from tables 2, 3 and 5 aging is associated with significantly lower DHEAS levels in both sexes, a phenomenon also reported by others (37). Stratification by DHEAS level therefore automatically results in stratification by age (table 5) and results obtained after stratification might thus be biased by age effects; comparison of men and women with similar DHEAS levels but totally different age might be inappropriate. However, age differences between men and women per DHEAS stratum were small and therefore not likely to influence the results of the calculations. Moreover age was not independently associated with levels of E2 or A and only weakly associated with higher E1 and lower T levels after adjustment for DHEAS level in both men and women (data not shown).

Adipose tissue is important for androgen aromatisation in men and women. Obesity is clearly associated with increased androgen aromatization (38;39). The percentage body fat is higher in women as compared to men and increases with age in both sexes (40;41). The reported age related increase in androgen aromatization might be attributed to this phenomenon (42). These

sex and age related changes in body composition might have biased our estimates. However, Longcope (43) estimated that adipose tissue accounts for only 10 to 15% of the extragonadal androgen aromatization and that muscle tissue is also an important source of peripheral oestrogen production. *In vitro* studies show that muscle tissue is almost equally effective in converting androgens to oestrogens (44). It is therefore questionable whether changes or differences in the fat mass/ muscle mass ratio will result in changes in aromatization rate. Moreover, older age appears to be associated with increased aromatase expression in adipose tissue (45;46) suggesting that not the age related change in body composition but the age related increase of aromatase expression might explain the increased androgen aromatization in older subjects. Whatever the cause, the androgen to oestrogen conversion rate appears to be determined by age and BMI. Since in our study age and BMI were similar for men and women in every DHEAS stratum (table 5) we believe that these parameters did not bias our estimates much.

The time of blood collection ranged from 08:30 h to 16:00 h but mean sampling times were similar for men and women. It is well known that in young men A and T but not DHEAS have a circadian rhythm (47;48) although the daily variation in hormone concentration appears to decrease with age (49;50). Because of the high mean age of our population and the large number of subjects we believe that differences in sampling time did not have a major influence on the results. This was confirmed by our observation that, after division in sampling time quartiles, mean hormone and SHBG levels between quartiles were not significantly different.

Both the steroid infusion techniques and our approach have their limitations as previously mentioned. The results obtained however are similar. Our results also show that the relative contribution of adrenal glands and testes to circulating levels of sex steroids is highly dependent on the hormone secreting activity of these glands. Although a correlation between DHEAS and T levels was found, the balance of these hormones can vary widely between individuals. Additionally the rate of conversion of androgens to oestrogens can vary dependent on the amount of fat mass (38;39), age (42;45;46), SHBG concentration (34) and liver disease (51). The large number of men in this study population makes the estimates relatively insensitive to interindividual variations in these variables. Another advantage of our approach is that it is non-interventional so that the delicate and complex equilibrium between

hormones is not disturbed. Finally, the population-based nature of the study makes the results applicable to a broad range of elderly men.

Conclusions

From our results we conclude that in elderly men testes and adrenal glands both contribute significantly to the circulating levels of oestrogens. The mean contribution of the testes to peripheral E2 and E1 levels in elderly men is 57% and 43% respectively, the remainder being of adrenal origin. When individual subjects are concerned these percentages are dependent on the individual balance between testicular and adrenal activity and interindividual differences in the conversion of androgens to oestrogens. With the growing body of evidence for the role of estrogens in males this study suggests that surgical or pharmacological suppression of gonadal or adrenal function in men can lower oestrogen levels significantly and can therefore contribute to changes in the response of oestrogen sensitive target tissues.

Reference List

1. **Smith EP, Boyd J, Frank GR, Takahashi H, Cohen RM, Specker B, Williams TC, Lubahn DB, Korach KS** 1994 Estrogen resistance caused by a mutation in the estrogen-receptor gene in a man. N Engl J Med 331:1056-1061

2. **Morishima A, Grumbach MM, Simpson ER, Fisher C, Qin K** 1995 Aromatase deficiency in male and female siblings caused by a novel mutation and the physiological role of estrogens. J Clin Endocrinol Metab 80:3689-3698

3. **Carani C, Qin K, Simoni M, Faustini-Fustini M, Serpente S, Boyd J, Korach KS, Simpson ER** 1997 Effect of testosterone and estradiol in a man with aromatase deficiency. N Engl J Med 337:91-95

4. **de Ronde W, Pols HA, van Leeuwen JP, de Jong FH** 2003 The importance of oestrogens in males. Clin Endocrinol (Oxf) 58:529-542

5. **O'Donnell L, Robertson KM, Jones ME, Simpson ER** 2001 Estrogen and spermatogenesis. Endocr Rev 22:289-318

6. **Saez JM, Morera AM, Dazord A, Bertrand J** 1972 Adrenal and testicular contribution to plasma oestrogens. J Endocrinol 55:41-49

7. **Scholler R, Nahoul K, Castanier M, Rotman J, Salat-Baroux J** 1984 Testicular secretion of conjugated and unconjugated steroids in normal adults and in patients with varicocele. Baseline levels and time-course response to hCG administration. J Steroid Biochem 20:203-215

8. **Baird DT, Horton R, Longcope C, Tait JF** 1969 Steroid dynamics under steady-state conditions. Recent Prog Horm Res 25:611-664

9. **MacDonald PC, Madden JD, Brenner PF, Wilson JD, Siiteri PK** 1979 Origin of estrogen in normal men and in women with testicular feminization. J Clin Endocrinol Metab 49:905-916

10. **Adamski J, Jakob FJ** 2001 A guide to 17beta-hydroxysteroid dehydrogenases. Mol Cell Endocrinol 171:1-4

11. **Bartsch W, Horst HJ, Becker H, Nehse G** 1977 Sex hormone binding globulin binding capacity, testosterone, 5alpha-dihydrotestosterone, oestradiol and prolactin in plasma of patients with prostatic carcinoma under various types of hormonal treatment. Acta Endocrinol (Copenh) 85:650-664

12. **Fiet J, Villette JM, Bertagna C, de Gery A, Hucher M, Husson JM, Raynaud JP** 1987 Plasma hormone levels before and after orchiectomy in prostate cancer patients. Prog Clin Biol Res 243A:33-44

13. **Veldhuis JD, Lizarralde G, Iranmanesh A** 1992 Divergent effects of short term glucocorticoid excess on the gonadotropic and somatotropic axes in normal men. J Clin Endocrinol Metab 74:96-102

14. **Hofman A, Grobbee DE, de Jong PT, van den Ouweland FA** 1991 Determinants of disease and disability in the elderly: the Rotterdam Elderly Study. Eur J Epidemiol 7:403-422

15. **Verjans HL, Cooke BA, de Jong FH, de Jong CM, van der Molen HJ** 1973 Evaluation of a radioimmunoassay for testosterone estimation. J Steroid Biochem 4:665-676

16. **de Jong FH, Hey AH, van der Molen HJ** 1973 Effect of gonadotrophins on the secretion of oestradiol- and testosterone by the rat testis. J Endocrinol 57:277-284

17. **Frolich M, Brand EC, van Hall EV** 1976 Serum levels of unconjugated aetiocholanolone androstenedione, testosterone, dehydroepiandrosterone, aldosterone, progesterone and oestrogens during the normal menstrual cycle. Acta Endocrinol (Copenh) 81:548-562

18. **Van Landeghem AA, Poortman J, Deshpande N, Di Martino L, Tarquini A, Thijssen JH, Schwarz F** 1981 Plasma concentration gradient of steroid hormones across human mammary tumours in vivo. J Steroid Biochem 14:741-747

19. **de Jong FH, van der Molen HJ** 1972 Determination of dehydroepiandrosterone and dehydroepiandrosterone sulphate in human plasma using electron capture detection of 4-androstene-3,6,17-trione after gas-liquid chromatography. J Endocrinol 53:461-474

20. **de Jong FH, Oishi K, Hayes RB, Bogdanowicz JF, Raatgever JW, van der Maas PJ, Yoshida O, Schroeder FH** 1991 Peripheral hormone levels in controls and patients with prostatic cancer or benign prostatic hyperplasia: results from the Dutch-Japanese case-control study. Cancer Res 51:3445-3450

21. **Goderie-Plomp HW, van der KM, de Ronde W, Hofman A, de Jong FH, Pols HA** 2004 Endogenous sex hormones, sex hormone-binding globulin, and the risk of incident vertebral fractures in elderly men and women: the Rotterdam Study. J Clin Endocrinol Metab 89:3261-3269

22. **Hak AE, Witteman JC, de Jong FH, Geerlings MI, Hofman A, Pols HA** 2002 Low levels of endogenous androgens increase the risk of atherosclerosis in elderly men: the Rotterdam study. J Clin Endocrinol Metab 87:3632-3639

23. **Couzinet B, Meduri G, Lecce MG, Young J, Brailly S, Loosfelt H, Milgrom E, Schaison G** 2001 The postmenopausal ovary is not a major androgen-producing gland. J Clin Endocrinol Metab 86:5060-5066

24. **Judd HL, Lucas WE, Yen SS** 1974 Effect of oophorectomy on circulating testosterone and androstenedione levels in patients with endometrial cancer. Am J Obstet Gynecol 118:793-798

25. **Sluijmer AV, Heineman MJ, de Jong FH, Evers JL** 1995 Endocrine activity of the postmenopausal ovary: the effects of pituitary down-regulation and oophorectomy. J Clin Endocrinol Metab 80:2163-2167

26. **Laughlin GA, Barrett-Connor E, Kritz-Silverstein D, von Muhlen D** 2000 Hysterectomy, oophorectomy, and endogenous sex hormone levels in older women: the Rancho Bernardo Study. J Clin Endocrinol Metab 85:645-651

27. **Dowsett M, Cantwell B, Lal A, Jeffcoate SL, Harris AL** 1988 Suppression of postmenopausal ovarian steroidogenesis with the luteinizing hormone-releasing hormone agonist goserelin. J Clin Endocrinol Metab 66:672-677

28. **Rabinovici J, Rothman P, Monroe SE, Nerenberg C, Jaffe RB** 1992 Endocrine effects and pharmacokinetic characteristics of a potent new gonadotropin-releasing hormone antagonist (Ganirelix) with minimal histamine-releasing properties: studies in postmenopausal women. J Clin Endocrinol Metab 75:1220-1225

29. **Longcope C, Hunter R, Franz C** 1980 Steroid secretion by the postmenopausal ovary. Am J Obstet Gynecol 138:564-568

30. **Judd HL, Judd GE, Lucas WE, Yen SS** 1974 Endocrine function of the postmenopausal ovary: concentration of androgens and estrogens in ovarian and peripheral vein blood. J Clin Endocrinol Metab 39:1020-1024

31. **Stege R, Eriksson A, Henriksson P, Carlstrom K** 1987 Orchidectomy or oestrogen treatment in prostatic cancer: effects on serum levels of adrenal androgens and related steroids. Int J Androl 10:581-587

32. **Parker L, Lai M, Wolk F, Lifrak E, Kim S, Epstein L, Hadley D, Miller J** 1984 Orchiectomy does not selectively increase adrenal androgen concentrations. J Clin Endocrinol Metab 59:547-550

33. **Dunn JF, Nisula BC, Rodbard D** 1981 Transport of steroid hormones: binding of 21 endogenous steroids to both testosterone-binding globulin and corticosteroid-binding globulin in human plasma. J Clin Endocrinol Metab 53:58-68

34. **Vermeulen A, Ando S** 1979 Metabolic clearance rate and interconversion of androgens and the influence of the free androgen fraction. J Clin Endocrinol Metab 48:320-326

35. **Bird CE, Masters V, Clark AF** 1984 Dehydroepiandrosterone sulfate: kinetics of metabolism in normal young men and women. Clin Invest Med 7:119-122

36. **Longcope C** 1995 Metabolism of dehydroepiandrosterone. Ann N Y Acad Sci 774:143-148

37. **Laughlin GA, Barrett-Connor E** 2000 Sexual dimorphism in the influence of advanced aging on adrenal hormone levels: the Rancho Bernardo Study. J Clin Endocrinol Metab 85:3561-3568

38. **Kley HK, Deselaers T, Peerenboom H, Kruskemper HL** 1980 Enhanced conversion of androstenedione to estrogens in obese males. J Clin Endocrinol Metab 51:1128-1132

39. **Kley HK, Edelmann P, Kruskemper HL** 1980 Relationship of plasma sex hormones to different parameters of obesity in male subjects. Metabolism 29:1041-1045

40. **Guo SS, Zeller C, Chumlea WC, Siervogel RM** 1999 Aging, body composition, and lifestyle: the Fels Longitudinal Study. Am J Clin Nutr 70:405-411

41. **Schutz Y, Kyle UU, Pichard C** 2002 Fat-free mass index and fat mass index percentiles in Caucasians aged 18-98 y. Int J Obes Relat Metab Disord 26:953-960

42. **Hemsell DL, Grodin JM, Brenner PF, Siiteri PK, MacDonald PC** 1974 Plasma precursors of estrogen. II. Correlation of the extent of conversion of plasma androstenedione to estrone with age. J Clin Endocrinol Metab 38:476-479

43. **Longcope C, Pratt JH, Schneider SH, Fineberg SE** 1978 Aromatization of androgens by muscle and adipose tissue in vivo. J Clin Endocrinol Metab 46:146-152

44. **Matsumine H, Hirato K, Yanaihara T, Tamada T, Yoshida M** 1986 Aromatization by skeletal muscle. J Clin Endocrinol Metab 63:717-720

45. **Bulun SE, Simpson ER** 1994 Competitive reverse transcription-polymerase chain reaction analysis indicates that levels of aromatase cytochrome P450 transcripts in adipose tissue of buttocks, thighs, and abdomen of women increase with advancing age. J Clin Endocrinol Metab 78:428-432

46. **Cleland WH, Mendelson CR, Simpson ER** 1985 Effects of aging and obesity on aromatase activity of human adipose cells. J Clin Endocrinol Metab 60:174-177

47. **Sjoberg B, de la TB, Hedman M, Falkay G, Diczfalusy E** 1979 Circadian variation in systemic hormone levels in healthy men. J Endocrinol Invest 2:131-137

48. **Murono EP, Nankin HR, Lin T, Osterman J** 1982 The aging Leydig cell V. Diurnal rhythms in aged men. Acta Endocrinol (Copenh) 99:619-623

49. **Bremner WJ, Vitiello MV, Prinz PN** 1983 Loss of circadian rhythmicity in blood testosterone levels with aging in normal men. J Clin Endocrinol Metab 56:1278-1281

50. **Luboshitzky R, Shen-Orr Z, Herer P** 2003 Middle-aged men secrete less testosterone at night than young healthy men. J Clin Endocrinol Metab 88:3160-3166

51. **Longcope C, Pratt JH, Schneider S, Fineberg E** 1984 Estrogen and androgen dynamics in liver disease. J Endocrinol Invest 7:629-634

Chapter 6

Association of a CYP 19 (aromatase) polymorphism with plasma (bioavailable) estradiol concentrations in community dwelling men.

Abstract

Mutations in the genes for aromatase and estrogen receptor α (ESR1) in men are associated with increased plasma levels of luteinizing hormone (LH) and testosterone. In this study we investigated whether common polymorphisms in the genes for aromatase (CYP19), ESR1 or ESR2 contribute to interindividual variations in androgen levels in men.

The study is a cross-sectional, single centre study of 341 independently living men aged 40 to 80 years. Testosterone, estradiol, sex hormone binding globulin (SHBG) and LH were measured, and bioavailable fractions of sex steroids were calculated. Genotypes were determined for CYP 19 (T1531C), ESR1 (PvuII and XbaI) and ESR2 (RsaI and AluI). Differences between genotypes for the measured hormones were evaluated using ANOVA and linear regression analyses.

None of the genotypes appeared to be significantly associated with age, BMI, SHBG, LH or androgen levels. Subjects who had the CYP 19 CC genotype (23%) appeared to have 9% lower levels of total and bioavailable estradiol ($p=0.005$ for both comparisons).

We conclude that the CYP 19 CC genotype is associated with 9% lower levels of total and bioavailable estradiol in men. This difference does not appear to be large enough to result in altered LH or androgen levels.

Introduction

Estradiol is a potent inhibitor of gonadotropin release in men. Estrogen administration to men is associated with decreasing levels of both luteinizing hormone (LH) and plasma testosterone (1-3). Increased endogenous estrogen production in men, for instance caused by estrogen producing testicular or adrenal tumours is also characterized by hypogonadotropic hypogonadism (4-6). In men circulating estrogens originate primarily from testicular and adrenal precursor hormones (7;8). Aromatase, the enzyme responsible for conversion of adrenal and testicular androgens to estrogens, is found throughout the body where muscle and adipose tissue are quantitatively the most important tissues for androgen aromatisation (7). Pharmacological reduction of aromatase activity resulting in lower circulating estradiol levels in men is associated with elevated LH and testosterone levels (9;10). Disruptive mutations in the gene for aromatase (CYP 19) are associated with high gonadotrophin and testosterone levels in men (11;12).

To exert its biological effects interaction of estradiol with the estrogen receptors (ER's) is necessary. Two subtypes of this receptor have been described and named ERα and ERβ currently ESR1 (6q25) and ESR2 (14q) respectively. Altered estrogen signalling, resulting from functional differences in ESR1 or ESR2, might be associated with altered levels of LH or testosterone in men. The only man to date described with a disruptive mutation in the ESR1 gene presented with high gonadotrophins and high testosterone levels (13), a pattern similar to that seen in ESR1 knockout mice (14). In mice, ESR2 does not appear to play a prominent role in the feedback of circulating estradiol on gonadotrophin release by the hypothalamus and the pituitary (15). Mutations in the gene for ESR2 in men have not yet been described. All these observations suggest an important effect of circulating estrogens on the activity of the HPG-axis in men. It is well known that polymorphisms in the genes for aromatase and the ERs exist, some of which are functional and associated with quantitative or qualitative alterations in transcript properties. Kristensen et al. (16) investigated a polymorphism located in the untranslated region (3'UTR) of exon 10 of CYP 19. Their data suggested that the T allele was associated with increased mRNA levels and higher aromatase activity probably as a result of a switch from the adipose tissue promoter to the ovarian promoter or increased mRNA stability possibly due to alternative folding of the transcript. For ESR1 the so called PvuII and XbaI polymorphisms have been widely investigated. Functionality of these polymorphisms is assumed based on relationships with clinical phenotypes primarily found in postmenopausal women (17-23) but also in men (24-26). Recently, a polymorphism in ESR2 was shown to be associated with circulating LH levels in men (27). Therefore, we

investigated the relationship between aromatase, ESR1 and ESR2 gene polymorphisms and circulating estrogen, androgen and LH levels in 400 community dwelling men aged 40-80 years. The main focus of our study was to investigate whether these common polymorphisms contribute to interindividual variations in androgen levels.

Subjects and Methods

Subjects

The study is a cross-sectional, single centre study of 400 independently living men aged 40 to 80 years. The study was originally designed to study the relationships between endogenous sex hormones and risk factors or manifestations of chronic diseases. The subjects were recruited by asking female participants of other studies conducted by the department whether they knew a possibly interested male volunteer. Invitation letters were sent to 770 female participants. Eventually 240 men volunteered for participation.

Next, names and addresses of a randomly selected male population aged 40-80 years were drawn from the municipal register of Utrecht, a large sized town in the middle part of the Netherlands. 1230 invitation letters were sent. From this group 390 men volunteered for participation.

From the 630 volunteers we excluded the subjects who did not live independently and subjects who were not physically or mentally able to visit the study center independently (n=16). Of the remaining 614 men 400 men were randomly selected to participate. To obtain equal numbers in each age-decade we sampled 100 men in each decade of age. Data collection took place between March 2001 and April 2002.

All participants gave written informed consent before enrolment and the institutional review board of the Utrecht University Medical Center approved the study.

Height and weight were measured in standing position without shoes. Body mass index (BMI) was calculated as the weight in kilograms divided by the square of the height in meters.

Laboratory measurements

Fasting blood samples were obtained by venapuncture. Cell free serum was immediately stored at –20C. Testosterone was measured after diethyl extraction using an in house radioimmunoassay employing a polyclonal anti-testosterone-antibody (Dr.Pratt AZG 3290). The lower limit of detection of the assay was 0.24 nmol/L and inter-assay variation was 6.0; 5.4 and 8.6% at 2.1; 5.6 and 23 nmol/L respectively. SHBG and LH were measured using an immunometric technique on an IMMULITE 2000 analyzer (Diagnostic Products Corporation,

Los Angeles, California, USA). For SHBG the lower limit of detection was 5 nmol/L and inter-assay variation was 6.1; 4.9 and 6.9% at 11.6; 36 and 93 nmol/L respectively. For LH the lower limit of detection was 0.1 IU/L and inter assay variation was 5.5; 4.1 and 3.9% at 8.5; 21.2 and 43 IU/L respectively. Estradiol was measured after diethylether extraction and Sephadex chromatography using an in house radioimmunoassay employing a polyclonal anti-estradiol antibody. The lower limit of detection was 20 pmol/L and inter-assay variation was 10 and 3.1% at 81 and 660 pmol/L respectively.
Non-SHBG bound testosterone and non-SHBG bound estradiol were calculated using the method described by Sodergard (28).

genotyping

Genomic DNA was isolated from peripheral leucocytes by standard procedures. Genotypes were determined using the Taqman allelic discrimination assay. Primer and probe sequences were optimised using the SNP assay-by-design service of Applied Biosystems (for details see store.appliedbiosystems.com). Reactions were performed on the Taqman Prism 7900HT (384 wells format). The *CYP19* polymorphism is a T to C substitution located 1531 basepairs downstream of the first nucleotide of the transcription initiation site. The PvuII (T>C)and XbaI (A>G) polymorphims in ESR1 are located 397 and 351 bp respectively upstream from the start of exon 2. In the RsaI polymorphism of ESR2, there is a G to A nucleotide exchange at nucleotide 1082 in exon 5. In the AluI polymorphism of this gene there is an exchange of C to T at nucleotide 1730 in the noncoding end of exon 8. We used the genotype data for each of the 2 ESR1 polymorphisms to infer the haplotype alleles present in the population by using the program PHASE, which implements a Bayesian statistical method for reconstructing haplotypes from population genotype data (29). The alleles were defined as haplotypes such as "T-A," representing a thymidine (T) nucleotide at the PvuII polymorphic site and an adenosine (A) nucleotide at the XbaI polymorphic site. For ESR2 no haplotypes were created because of the very low frequency of the A allele of the RsaI polymorphism.

data analysis

One subject was excluded for analysis because of clear hypogonadotropic hypogonadism (LH 0.1 U/l, testosterone 0.24 nmol/l). 58 subjects were excluded for analysis because of missing data on CYP 19, ESR1 or ESR2 genotype. Eventually data from 341 men were analysed. Baseline characteristics of the in-and excluded men were not significantly different. For all parameters mean and SD were calculated. Subjects were grouped according to CYP19, ESR1

or ESR2 genotype. Differences between genotype groups were tested for significance using analysis of variance and were considered significant if the p value was ≤ 0.05.
We used linear regression analyses to study whether there was an independent effect of the CYP19, ESR1, ESR2 genotypes or ESR1 haplotypes on levels of LH, (bioavailable) testosterone and (bioavailable) estradiol. Age, BMI and SHBG were also included in the regression model. Associations were expressed as regression coefficient (β). The linear regression coefficient β indicates the change of the dependent variable for every unit change in the independent variable. All calculations were done using SPSS 11.0 software.

Results

The characteristics of studied men are presented in Tables 1, 2 and 3. Genotype distributions were in Hardy–Weinberg equilibrium. When the subjects were grouped according to CYP19, ESR1, ESR2 genotypes or ESR 1 haplotypes none appeared to be significantly associated with age, BMI, SHBG, LH or androgen levels. ESR1 and ESR2 genotypes or ESR1 haplotypes were not associated with levels of total or bioavailable estradiol. For the CYP 19 gene, subjects homozygous for the C allele appeared to have significantly lower levels of total and bioavailable estradiol (mean difference 8.2 and 6.2 pmol/l respectively, p= 0.02 using analysis of variance; p=0.005 for the CC genotype versus the TC and TT genotypes for both total and bioavailable estradiol; table 4). In tables 5 an 6 the relationships between polymorphisms in CYP 19, ESR 1 and 2 and the levels of total and bioavailable estradiol and total and bioavailable testosterone are presented before and after adjustment for potential confounders. Of the investigated polymorphisms only the 3'UTR CYP 19 polymorphism was an independent determinant of total and bioavailable estradiol levels. The contribution to the explained variance was small (3 and 2 % respectively). In table 6 it is shown that none of the studied polymorphisms appeared to be an independent determinant of total or unbound testosterone levels. None of the investigated polymorphisms was significantly associated with LH levels in our subjects (data not shown). In tables 5 and 6 we also presented the relationships between BMI and the levels of total and bioavailable estradiol and testosterone in our subjects. BMI is positively associated with levels of total and bioavailable estradiol whereas it is negatively associated with levels of total and bioavailable testosterone.

Table 1: Characteristics of 341 men

	Mean ±SD
Age (yrs)	60.3±11.6
BMI (kg/m2)	26.2±3.41
LH (IU/l)	4.68±2.66
Total testosterone (nmol/l)	18.6±5.37
SHBG (nmol/l)	40.4±14.5
Bioavailable testosterone (nmol/l)	10.5±2.69
Estradiol (pmol/l)	91.1±22.8
Bioavailable estradiol (pmol/l)	65.6±17.1

Table 2: allele frequency of investigated polymorhpisms

gene	SNP	genotypes	Frequency (%)
CYP 19	T1531C	TT	90 (26)
		TC	172 (51)
		CC	79 (23)
ESR1	T397C (PvuII)	CC	91 (27)
		CT	163 (48)
		TT	87 (25)
	A351G (XbaI)	AA	140 (41)
		AG	145 (43)
		GG	56 (16)
ESR2	C1730T (AluI)	CC	130 (38)
		CT	171 (50)
		TT	40 (12)
	G1082A (RsaI)	GG	315 (92)
		AG	26 (8)
		AA	0 (0)

Table 3: Frequency of ESR1 (PvuII/XbaI) haplotypes

haplotype	Frequency (%)
TA	337 (49)
CG	257 (38)
CA	88 (13)
TG	0 (0)

Discussion

In this study we investigated whether polymorphisms in the genes for aromatase, ERα or ERβ are associated with plasma estrogen, androgen or LH levels in community dwelling men. We observed that neither the investigated ESR1 polymorphisms nor the ESR2 polymorphisms were associated with total or bioavailable testosterone or estradiol levels in men. These results are in accordance with other studies (24;26;30) in which no relationship was found between circulating hormone levels and ER polymorphisms in men. We did not find a relation between the RsaI polymorhism in ESR2 and LH levels as recently described by Aschim et al. (27) while we have analysed a relatively large number of men and observed similar frequencies of the studied alleles. The mean age of the men in our study was probably higher as compared to the control subjects in the study by Aschim et al. which might explain reduced sensitivity of the hypothalamus-pituitary-gonadal axis to subtle variations in estrogenic signalling in our subjects. However, mean LH and testosterone levels were normal in our study and not much different from the mean levels found in the study by Aschim et al. (27).

Higher aromatase activity is expected to be associated with higher estradiol levels in men, resulting in lower LH and testosterone levels, a pattern also seen in obesity. In the present study, men with the 3'UTR CYP 19 TT and TC genotypes had 9% higher levels of both total and bioavailable estradiol, which is in accordance with the presumed higher enzyme activity as a result of these genotypes (16). A similar association between another CYP 19 polymorphism, a TTTA repeat in intron 4, and estrogen levels in men was described by Gennari et al. (31). Men with a high number of TTTA repeats had significantly higher levels of estradiol; this association might be explained by linkage disequilibrium between the TTTA repeat and the 3'UTR SNP we studied (32). Both in their study and our study no significant relationship was found between these genotypes and circulating testosterone or LH levels despite the fact that estrogens are known for their potent negative effect on gonadotrophin release (7). It might be concluded that changes in estradiol levels brought about by these polymorphisms are too small to alter gonadotropin secretion by pituitary or hypothalamus. However, this conclusion seems to be inconsistent with the observed hormone profiles in obese men. Obesity, which is also associated with increased aromatase activity and increased estrogen levels, is clearly associated with lower levels of both total and bioavailable testosterone levels in men (33-36). Also in our study there is a significant and positive association between BMI and estrogen levels while there is a significantly negative association between BMI and either total or bioavailable testosterone levels (tables 5 and 6).

Table 4: Characteristics of 341 men (mean ±SD) by CYP 19 T1531C genotype

	TT	TC	CC	p
n	90	172	79	
Age (years)	60.1±11.8	60.8±11.5	59.3±11.6	0.64
BMI (kg/m^2)	26.3±3.53	26.1±3.18	26.4±3.75	0.71
LH (IU/l)	4.87±2.44	4.62±2.85	4.62±2.49	0.75
Testosterone (nmol/l)	18.4±5.11	18.6±5.34	18.9±5.73	0.84
SHBG (nmol/l)	40.6±13.8	40.0±14.9	41.2±14.7	0.82
Non-SHBG-testosterone (nmol/l)	10.3±2.70	10.5±2.71	10.5±2.66	0.85
Estradiol (pmol/l)	93.2±21.6	93.0±24.2	84.8±20.2	0.02
Non-SHBG-estradiol (pmol/l)	67.1±16.8	67.0±17.7	60.8±15.6	0.02

p values are obtained using analysis of variance

Table 5: relationships of CYP 19, ESR1 and ESR2 polymorphisms and BMI with total and bioavailable oestradiol levels.

	oestradiol				bioavailable oestradiol			
	unadjusted		adjusted*		unadjusted		adjusted*	
	β±SE	p	β±SE	p	β±SE	p		p
CYP 19 (absence of T allele)	-8.21±1.40	<0.001	-8.96±2.53	<0.001	-6.17±1.05	0.005	-6.26±1.85	0.001
ESR1 (PvuII)	1.65±1.17	0.36	0.90±2.30	0.70	0.92±1.28	0.47	0.83±1.69	0.62
ESR1 (XbaI)	-0.38±1.73	0.82	0.23±2.33	0.92	-0.02±1.30	0.99	0.34±1.70	0.84
ESR2 (AluI)	0.14±1.89	0.94	0.74±1.66	0.66	0.77±1.42	0.59	0.48±1.21	0.69
ESR2 (RsaI)	4.48±4.66	0.34	4.70±4.05	0.25	1.66±3.50	0.64	2.85±2.97	0.34
BMI (kg/m^2)	1.21±0.36	0.001	2.35±0.33	<0.001	1.20±0.27	<0.001	1.67±0.24	<0.001

*Adjusted for age, BMI, testosterone, SHBG, LH, Cyp19, ESR1 (PvuII and XbaI) and ESR2 (AluI and RsaI).

Table 6: relationships of CYP 19, ESR1 and ESR2 polymorphisms and BMI with total and bioavailable testosterone levels.

	testosterone				bioavailable testosterone			
	unadjusted		adjusted*		unadjusted		adjusted*	
	β±SE	p	β±SE	p	β±SE	p		p
CYP 19 (absence of T allele)	0.36±0.69	0.60	-0.05±0.49	0.91	0.06±0.35	0.86	-0.07±0.30	0.82
ESR1 (PvuII)	0.35±0.40	0.39	0.09±0.45	0.84	010±0.20	0.62	0.06±0.28	0.84
ESR1 (XbaI)	-0.27±0.41	0.51	0.01±0.45	0.98	-0.07±0.20	0.74	0.03±0.28	0.91
ESR2 (AluI)	-0.36±0.44	0.42	-0.01±0.32	0.97	-0.06±0.22	0.77	-0.05±0.20	0.81
ESR2 (RsaI)	0.36±1.10	0.75	-0.46±0.78	0.56	-0.08±0.55	0.89	-0.31±0.49	0.52
BMI (kg/m^2)	-0.52±0.08	<0.001	-0.27±0.06	<0.001	-0.21±0.04	<0.001	-0.17±0.04	<0.001

*Adjusted for age, BMI, SHBG, LH, Cyp19, ESR1 (PvuII and XbaI) and ESR2 (AluI and RsaI).

Our results allow a comparison between the effects of CYP 19 3'UTR genotype and BMI on estradiol and testosterone levels in men. From table 5 it can be appreciated that a change in BMI of 1 kg/m^2 is associated with a rise in the level of total estradiol of 2.35 pmol/l. Therefore, to achieve a reduction in the level of total estradiol of 9 pmol/l (the difference between the CYP 19 TT/TC and CC genotypes) a reduction in BMI of 3.8 kg/m^2 is necessary, consistent with a weight loss of 12.3 kg for a man with a body height of 1.80 m. Despite the fact that the absolute difference of estrogen levels between the genotypes is similar to a substantial change in BMI, the CC genotype is not associated with higher androgen or LH levels. Part of this can be explained by the absent effect of aromatase genotype on SHBG levels. Weight loss is not only associated with decreasing estrogen levels but also with increasing SHBG levels. As previously described (37), SHBG and total testosterone are strongly related in our subjects, therefore the rise in testosterone levels associated with weight loss is at least partly mediated by SHBG. When the relationship between BMI and total testosterone is adjusted for the confounding effect of the SHBG level (table 6), a change in BMI of 3.8 kg/m^2 is associated with a significant increase in total testosterone of 1.03 nmol/l. From this comparison between the effects of the CYP 19 genotype and BMI on estrogen and androgen levels in men one might conclude that there is an estrogen and SHBG independent effect of body weight on androgen levels in men and that this effect outweighs the effect of estrogens.

A lower bioavailable testosterone level with a normal LH level suggests reduced gonadal LH sensitivity in obese men. This is supported by the observation that only supraphysiological LH levels were able to restore normal testosterone levels in obese men (38). In massively obese men Isidori et al. found a decreased response to stimulation by HCG (39) and attributed this to the inhibitive effect of leptin on steroidogenesis. In contrast to this, Glass et al. found a normal response to HCG administration in obese men (40). In overtly obese men lower LH levels (41) and lower LH pulse amplitude (42) were found suggesting diminished hypothalamic GnRH drive. Others found normal LH levels and a normal LH response after GnRH administration (43) suggesting at least adequate pituitary functioning.

To summarize, the CC genotype of the studied 3'UTR CYP19 polymorphism is associated with 9% lower estrogen levels in men. Although an effect of altered estrogen levels on LH and testosterone levels could be anticipated, relations between aromatase genotype and androgen levels or LH were not found. This observation argues against an important effect of small fluctuations of circulating estrogen levels, for instance caused by mild weight changes,

on androgen production in men. It appears that subtle alterations in aromatase activity may not be compensated for by increased production of estrogenic precursors in men.

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Reference List

1. **Stewart-Bentley M, Odell W, Horton R** 1974 The feedback control of luteinizing hormone in normal adult men. J Clin Endocrinol Metab 38:545-553

2. **Finkelstein JS, O'Dea LS, Whitcomb RW, Crowley WF, Jr.** 1991 Sex steroid control of gonadotropin secretion in the human male. II. Effects of estradiol administration in normal and gonadotropin-releasing hormone-deficient men. J Clin Endocrinol Metab 73:621-628

3. **Santen RJ** 1975 Is aromatization of testosterone to estradiol required for inhibition of luteinizing hormone secretion in men? J Clin Invest 56:1555-1563

4. **Paja M, Diez S, Lucas T, Ojeda A, Salto L, Estrada J** 1994 Dexamethasone-suppressible feminizing adrenal adenoma. Postgrad Med J 70:584-588

5. **Bulun SE, Noble LS, Takayama K, Michael MD, Agarwal V, Fisher C, Zhao Y, Hinshelwood MM, Ito Y, Simpson ER** 1997 Endocrine disorders associated with inappropriately high aromatase expression. J Steroid Biochem Mol Biol 61:133-139

6. **Veldhuis JD, Sowers JR, Rogol AD, Klein FA, Miller N, Dufau ML** 1985 Pathophysiology of male hypogonadism associated with endogenous hyperestrogenism. Evidence for dual defects in the gonadal axis. N Engl J Med 312:1371-1375

7. **de Ronde W, Pols HA, van Leeuwen JP, de Jong FH** 2003 The importance of oestrogens in males. Clin Endocrinol (Oxf) 58:529-542

8. **de Ronde W, Hofman A, Pols HA, de Jong FH** 2005 A direct approach to the estimation of the origin of oestrogens and androgens in elderly men by comparison with hormone levels in postmenopausal women. Eur J Endocrinol 152:261-268

9. **T'sjoen GG, Giagulli VA, Delva H, Crabbe P, De Bacquer D, Kaufman JM** 2005 Comparative assessment in young and elderly men of the gonadotropin response to aromatase inhibition. J Clin Endocrinol Metab 90:5717-5722

10. **Veldhuis JD, Iranmanesh A** 2005 Short-term aromatase-enzyme blockade unmasks impaired feedback adaptations in luteinizing hormone and testosterone secretion in older men. J Clin Endocrinol Metab 90:211-218

11. **Herrmann BL, Saller B, Janssen OE, Gocke P, Bockisch A, Sperling H, Mann K, Broecker M** 2002 Impact of estrogen replacement therapy in a male with congenital aromatase deficiency caused by a novel mutation in the CYP19 gene. J Clin Endocrinol Metab 87:5476-5484

12. **Morishima A, Grumbach MM, Simpson ER, Fisher C, Qin K** 1995 Aromatase deficiency in male and female siblings caused by a novel mutation and the physiological role of estrogens. J Clin Endocrinol Metab 80:3689-3698

13. **Smith EP, Boyd J, Frank GR, Takahashi H, Cohen RM, Specker B, Williams TC, Lubahn DB, Korach KS** 1994 Estrogen resistance caused by a mutation in the estrogen-receptor gene in a man. N Engl J Med 331:1056-1061

14. **Couse JF, Korach KS** 1999 Estrogen receptor null mice: what have we learned and where will they lead us? Endocr Rev 20:358-417

15. **Couse JF, Yates MM, Walker VR, Korach KS** 2003 Characterization of the hypothalamic-pituitary-gonadal axis in estrogen receptor (ER) Null mice reveals hypergonadism and endocrine sex reversal in females lacking ERalpha but not ERbeta. Mol Endocrinol 17:1039-1053

16. **Kristensen VN, Harada N, Yoshimura N, Haraldsen E, Lonning PE, Erikstein B, Karesen R, Kristensen T, Borresen-Dale AL** 2000 Genetic variants of CYP19 (aromatase) and breast cancer risk. Oncogene 19:1329-1333

17. **Ioannidis JP, Ralston SH, Bennett ST, Brandi ML, Grinberg D, Karassa FB, Langdahl B, van Meurs JB, Mosekilde L, Scollen S, Albagha OM, Bustamante M, Carey AH, Dunning AM, Enjuanes A, van Leeuwen JP, Mavilia C, Masi L, McGuigan FE, Nogues X, Pols HA, Reid DM, Schuit SC, Sherlock RE, Uitterlinden AG** 2004 Differential genetic effects of ESR1 gene polymorphisms on osteoporosis outcomes. JAMA 292:2105-2114

18. **Schuit SC, Oei HH, Witteman JC, Geurts van Kessel CH, van Meurs JB, Nijhuis RL, van Leeuwen JP, de Jong FH, Zillikens MC, Hofman A, Pols HA, Uitterlinden AG** 2004 Estrogen receptor alpha gene polymorphisms and risk of myocardial infarction. JAMA 291:2969-2977

19. **Schuit SC, van Meurs JB, Bergink AP, van der KM, Fang Y, Leusink G, Hofman A, van Leeuwen JP, Uitterlinden AG, Pols HA** 2004 Height in pre- and postmenopausal women is influenced by estrogen receptor alpha gene polymorphisms. J Clin Endocrinol Metab 89:303-309

20. **van Meurs JB, Schuit SC, Weel AE, van der KM, Bergink AP, Arp PP, Colin EM, Fang Y, Hofman A, van Duijn CM, van Leeuwen JP, Pols HA, Uitterlinden AG** 2003 Association of 5' estrogen receptor alpha gene polymorphisms with bone mineral density, vertebral bone area and fracture risk. Hum Mol Genet 12:1745-1754

21. **Weel AE, Uitterlinden AG, Westendorp IC, Burger H, Schuit SC, Hofman A, Helmerhorst TJ, van Leeuwen JP, Pols HA** 1999 Estrogen receptor polymorphism predicts the onset of natural and surgical menopause. J Clin Endocrinol Metab 84:3146-3150

22. **Herrington DM, Howard TD, Hawkins GA, Reboussin DM, Xu J, Zheng SL, Brosnihan KB, Meyers DA, Bleecker ER** 2002 Estrogen-receptor polymorphisms and effects of estrogen replacement on high-density lipoprotein cholesterol in women with coronary disease. N Engl J Med 346:967-974

23. **Shearman AM, Cupples LA, Demissie S, Peter I, Schmid CH, Karas RH, Mendelsohn ME, Housman DE, Levy D** 2003 Association between estrogen receptor alpha gene variation and cardiovascular disease. JAMA 290:2263-2270

24. **Lorentzon M, Lorentzon R, Backstrom T, Nordstrom P** 1999 Estrogen receptor gene polymorphism, but not estradiol levels, is related to bone density in healthy adolescent boys: a cross-sectional and longitudinal study. J Clin Endocrinol Metab 84:4597-4601

25. **McGuigan FE, Murray L, Gallagher A, Davey-Smith G, Neville CE, Van't Hof R, Boreham C, Ralston SH** 2002 Genetic and environmental determinants of peak bone mass in young men and women. J Bone Miner Res 17:1273-1279

26. **Remes T, Vaisanen SB, Mahonen A, Huuskonen J, Kroger H, Jurvelin JS, Penttila IM, Rauramaa R** 2003 Aerobic exercise and bone mineral density in middle-aged finnish men: a controlled randomized trial with reference to androgen receptor, aromatase, and estrogen receptor alpha gene polymorphisms small star, filled. Bone 32:412-420

27. **Aschim EL, Giwercman A, Stahl O, Eberhard J, Cwikiel M, Nordenskjold A, Haugen TB, Grotmol T, Giwercman YL** 2005 The RsaI polymorphism in the estrogen receptor-beta gene is associated with male infertility. J Clin Endocrinol Metab 90:5343-5348

28. **Sodergard R, Backstrom T, Shanbhag V, Carstensen H** 1982 Calculation of free and bound fractions of testosterone and estradiol-17 beta to human plasma proteins at body temperature. J Steroid Biochem 16:801-810

29. **Stephens M, Smith NJ, Donnelly P** 2001 A new statistical method for haplotype reconstruction from population data. Am J Hum Genet 68:978-989

30. **Schuit SC, de Jong FH, Stolk L, Koek WN, van Meurs JB, Schoofs MW, Zillikens MC, Hofman A, van Leeuwen JP, Pols HA, Uitterlinden AG** 2005 Estrogen receptor alpha gene polymorphisms are associated with estradiol levels in postmenopausal women. Eur J Endocrinol 153:327-334

31. **Gennari L, Masi L, Merlotti D, Picariello L, Falchetti A, Tanini A, Mavilia C, Del Monte F, Gonnelli S, Lucani B, Gennari C, Brandi ML** 2004 A polymorphic CYP19 TTTA repeat influences aromatase activity and estrogen levels in elderly men: effects on bone metabolism. J Clin Endocrinol Metab 89:2803-2810

32. **Haiman CA, Stram DO, Pike MC, Kolonel LN, Burtt NP, Altshuler D, Hirschhorn J, Henderson BE** 2003 A comprehensive haplotype analysis of CYP19 and breast cancer risk: the Multiethnic Cohort. Hum Mol Genet 12:2679-2692

33. **Kley HK, Deselaers T, Peerenboom H, Kruskemper HL** 1980 Enhanced conversion of androstenedione to estrogens in obese males. J Clin Endocrinol Metab 51:1128-1132

34. **Kley HK, Edelmann P, Kruskemper HL** 1980 Relationship of plasma sex hormones to different parameters of obesity in male subjects. Metabolism 29:1041-1045

35. **Vermeulen A, Kaufman JM, Giagulli VA** 1996 Influence of some biological indexes on sex hormone-binding globulin and androgen levels in aging or obese males. J Clin Endocrinol Metab 81:1821-1826

36. **Stanik S, Dornfeld LP, Maxwell MH, Viosca SP, Korenman SG** 1981 The effect of weight loss on reproductive hormones in obese men. J Clin Endocrinol Metab 53:828-832

37. **de Ronde W, van der Schouw YT, Muller M, Grobbee DE, Gooren LJ, Pols HA, de Jong FH** 2005 Associations of Sex-Hormone-Binding Globulin (SHBG) with Non-SHBG-Bound Levels of Testosterone and Estradiol in Independently Living Men. J Clin Endocrinol Metab 90:157-162

38. **de Boer H, Verschoor L, Ruinemans-Koerts J, Jansen M** 2005 Letrozole normalizes serum testosterone in severely obese men with hypogonadotropic hypogonadism. Diabetes Obes Metab 7:211-215

39. **Isidori AM, Caprio M, Strollo F, Moretti C, Frajese G, Isidori A, Fabbri A** 1999 Leptin and androgens in male obesity: evidence for leptin contribution to reduced androgen levels. J Clin Endocrinol Metab 84:3673-3680

40. **Glass AR, Swerdloff RS, Bray GA, Dahms WT, Atkinson RL** 1977 Low serum testosterone and sex-hormone-binding-globulin in massively obese men. J Clin Endocrinol Metab 45:1211-1219

41. **Strain GW, Zumoff B, Miller LK, Rosner W, Levit C, Kalin M, Hershcopf RJ, Rosenfeld RS** 1988 Effect of massive weight loss on hypothalamic-pituitary-gonadal function in obese men. J Clin Endocrinol Metab 66:1019-1023

42. **Vermeulen A, Kaufman JM, Deslypere JP, Thomas G** 1993 Attenuated luteinizing hormone (LH) pulse amplitude but normal LH pulse frequency, and its relation to plasma androgens in hypogonadism of obese men. J Clin Endocrinol Metab 76:1140-1146

43. **Castro-Fernandez C, Olivares A, Soderlund D, Lopez-Alvarenga JC, Zambrano E, Veldhuis JD, Ulloa-Aguirre A, Mendez JP** 2000 A preponderance of circulating basic isoforms is associated with decreased plasma half-life and biological to immunological ratio of gonadotropin-releasing hormone-releasable luteinizing hormone in obese men. J Clin Endocrinol Metab 85:4603-4610

Chapter 7

In men peripheral estradiol levels directly reflect the action of estrogens at hypothalamo-pituitary level to inhibit gonadotropin secretion

Abstract

It is well known that estradiol inhibits gonadotropin release in the male by an action at the hypothalamus and pituitary. Reduction of circulating estradiol by 30 to 60% with an aromatase inhibitor results in a powerful stimulation of gonadotropin and testosterone secretion. Due to the tissue specific regulation of aromatase, peripheral estradiol levels may not reflect brain estradiol concentrations.

We evaluated whether local aromatisation of testosterone in the hypothalamus or pituitary is of importance for gonadotropin release and to what extent circulating estrogens affect gonadotropin levels and peripheral testosterone levels.

We suppressed aromatase activity in 10 young healthy men with letrozole 2,5 mg once daily, restored plasma estradiol levels with estradiol patches (first week 100 μg/day, second week 50 μg/day, third week 25 μg/day, fourth week no estradiol patch) and measured plasma testosterone, estradiol, LH, FSH and SHBG levels.

During treatment with letrozole and 100 μg of estradiol per day the mean peripheral estradiol levels were suprafysiological and decreased dose-dependently with the subsequent application of the lower doses. The use of letrozole 2,5 mg alone was associated with a decline in estradiol of 56% compared to baseline. The mean testosterone levels during the study ranged between 179±91 and 955±292 ng/dl (mean±SD). Levels of testosterone, LH and FSH were inversely related to the peripheral estradiol levels and mirrored their fluctuations. Mean SHBG levels did not vary significantly during the course of the study. The relationships between estradiol and testosterone, LH or FSH were non-linear, the HPG axis was extremely responsive to fluctuations when estradiol levels were in the low-normal range. During letrozole use the mean plasma estradiol level needed to restore testosterone, LH and FSH levels to baseline levels was not significantly different from the baseline mean estradiol level.

Local aromatisation of testosterone in the hypothalamo-pituitary compartment is not a prerequisite for expression of the inhibitory action of estrogens on gonadotropin secretion in men. Peripheral estradiol levels directly reflect the inhibitory tone exerted by estrogens on gonadotropin release and are a major determinant of peripheral testosterone, LH and FSH levels.

Introduction

Circulating testosterone levels differ considerably between men (1) and almost 60 % of the interindividual variation in testosterone levels is genetically determined (2). The mechanisms behind this genetic determination remain largely unknown. To identify a testosterone level as abnormal knowledge of these mechanisms is important. One well known determinant of testosterone levels in healthy men is the body mass index (BMI) (3;4). The lower testosterone levels in obese men have been attributed to the lower levels of SHBG associated with higher BMI (5) and to increased conversion of testosterone to estradiol in adipose tissue. Aromatization of testosterone can take place throughout the body, including in the brain (6). It is well known that estradiol inhibits gonadotropin release in the male by an action at the hypothalamus and pituitary (7;8). Aromatase, the enzyme responsible for the conversion of androgens into estrogens has several tissue specific promoters (9). As a consequence the extent of androgen aromatization may vary between tissues in one individual and peripheral estradiol levels might not necessarily reflect estrogen exposure at the level of the pituitary or hypothalamus. Therefore, it remains to be determined whether higher serum estrogen levels in obese men can be directly related to their lower serum testosterone concentrations. Moreover, in men gonadotropin and testosterone secretion are powerfully stimulated by administration of an aromatase inhibitor in the face of only moderate decreases of circulating estrogen concentrations (10;11). This raises the question whether the observed gonadotropin stimulation during aromatase inhibition can be explained by the decrease of circulating estrogen levels alone or rather reflects local blockade of aromatase activity in the hypothalamo-pituitary tissues.

To get a better understanding of the possible role of the local biosynthesis of estradiol in the hypothalamus or pituitary gland in the regulation of the male hypothalamo-pituitary-gonadal axis we monitored serum gonadotropin and testosterone responses to aromatase inhibition and replacement with different doses of estradiol in young men.

Subjects and methods

Subjects

The subjects were 10 apparently healthy male volunteers between the ages of 18 and 40 years. The mean age of the subjects was 29.9±6.47 (range 20-39) years; mean BMI was 24.8±2.82 (range 21.1-29) kg/m^2. Exclusion criteria where: cigarette smoking, pituitary dysfunction, a history of thrombosis, use of any medication and excessive alcohol abuse. Medical history

was uneventful, physical examination was unremarkable and levels of testosterone, estradiol, LH and FSH were within the normal ranges at screening.

Study protocol

This study was approved by the Medical Ethics Committee of the Amsterdam Free University Medical Center and all volunteers gave written informed consent before the start of the study. The study protocol is summarized in figure 1. After baseline blood testing subjects started using letrozole (Novartis AG, Stein, Switzerland) 2,5 mg once daily in the morning for 4 weeks. During weeks 1-3 estradiol was replaced using estradiol patches (Dermestril; Sigma-Tau Ethirama, Utrecht, The Netherlands) with decreasing doses. The dose of the patches was 100, 50 and 25 μg estradiol per day respectively. Patches were applied according to the recommendations of the manufacturer. Patches were replaced on days 4 and 7 of each treatment week or earlier whenever more than 25% of the patch was detached. Blood samples were collected at baseline and at the end of every study week by venepuncture between 8:00 and 11:00 hour a.m. to determine LH, FSH, testosterone, estradiol and SHBG. Blood samples were allowed to clot and following centrifugation serum was stored at -20°C until analysis.

Hormone analysis

Levels of LH, FSH and SHBG were estimated by luminescence based immunoassays using an Immulite 2000 (Diagnostic Products Corporation, Los Angeles, CA). Inter- and intra-assay coefficients of variation were below 4.9, 5.9 and 6.3%, respectively. Testosterone and estradiol were measured using Coat-a-Count radioimmunoassay obtained from the same supplier. Variation coefficients for these assays were below 7.5 and 9.7%.

Statistics

Mean levels for the analyzed hormones and SHBG were calculated grouped by study week. Differences between mean levels were tested for significance using analysis of variance. We combined all measurements under treatment with letrozole and used linear regression analyses to evaluate the relationships between estradiol and LH, FSH or testosterone, using estradiol as the independent variable. To obtain a linear relationship between estradiol and other parameters, all were log transformed. To evaluate whether the relationships between estradiol and LH, FSH or testosterone were different in the samples obtained before and during letrozole use, the baseline levels of estradiol were entered in the previously described

Figure 1: study protocol

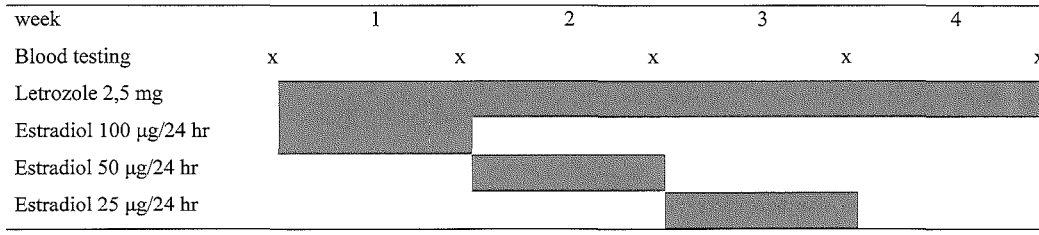


Table 1: mean hormone and SHBG levels in studied subjects (mean±SD)

	baseline	letrozole + estradiol 100	letrozole + estradiol 50	letrozole + estradiol 25	letrozole
Estradiol (pg/ml	28.7±7.81	68.6±38.3	43.7±17.9	26.0±12.3	12.6±7.21
(pmol/l))*	(106±29)	(252±141)	(161±65.7)	(95.4±45.0)	(46.2±26.5)
LH (IU/l)*	4.32±2.11	1.89±0.93	2.91±1.46	4.71±1.98	14.5±6.01
FSH (IU/l)*	4.30±2.18	1.40±0.93	2.10±0.77	4.08±1.83	12.0±6.05
Testosterone (ng/dl	503±97	179±91	339±86	658±196	955±292
(nmol/l))*	(17.4±3.37)	(6.21±3.16)	(11.8±2.98)	(22.8±6.79)	(33.1±10.1)
SHBG (µg/dl	0.72±0.23	0.78±0.26	0.81±0.26	0.76±0.25	0.68±0.21
(nmol/l))	(28.8±9.23)	(31.2±10.6)	(32.5±10.6)	(30.3±10.1)	(27.3±8.37)

*=$p<0.001$ for differences between groups; values in Systeme International units are given in parenthesis

regression equations. The resulting predicted values for LH, FSH and testosterone were compared with baseline levels using the Student's t-test for paired variables.

Results

Hormone and SHBG levels during the study are summarized in table 1. Estradiol levels were lowest during treatment with letrozole alone. The daily use of 2,5 mg letrozole was associated with a decline of the mean serum estradiol concentration of 56%. There was a dose dependent decrease of estradiol levels during estrogen application. During treatment with letrozole and 100 µg of estradiol per day the mean peripheral estradiol level was supraphysiological. During the treatment period, the mean testosterone level ranged between below-normal and high-normal for young males. Levels of LH, FSH and testosterone mirrored the fluctuations in peripheral estradiol levels. The mean SHBG levels did not vary significantly during the course of the study.

The relationships between estradiol and LH, FSH or testosterone were non-linear; the HPG axis appeared to be extremely sensitive to fluctuations when estradiol levels were low. After

logarithmic transformation of the hormone concentrations, linear relationships were obtained between the levels of estradiol and the other hormones during treatment with letrozole and estradiol (open symbols in figures 2-4). The baseline hormone levels, represented by the closed symbols in figures 2-4, visually fitted the line representing the hormone levels obtained under treatment.

In table 2 the actual baseline levels of testosterone, LH and FSH are compared with their respective predicted values. As described, these predicted values were calculated using a regression equation based on the results obtained during treatment with letrozole and various doses of estradiol. None of the differences reached statistical significance.

Discussion

In this study we evaluated whether local aromatisation of testosterone in the hypothalamus or pituitary is of importance for the effect of estradiol on gonadotropin release. When the aromatase inhibitor letrozole was applied to healthy young men estradiol levels declined by 56% which is in accordance with published results (12). Aromatase inhibition was associated with increased serum levels of gonadotropins and testosterone which was expected based on previous reports (8;12), illustrating the inhibitory action of estradiol on gonadotropin release in men. Although it is tempting to ascribe the increased gonadotropin levels to the lower circulating estradiol levels under treatment, such a conclusion is premature since aromatase activity may be different between tissues due to its different tissue specific promoters (9). This could result in higher local estradiol concentrations in the pituitary and/or hypothalamus as compared to the levels in peripheral blood as has been shown to be the case in mammary tumor tissue in postmenopausal women, in whom tissue levels did not differ from those in premenopausal women in spite of much lower peripheral estradiol levels (13;14). Similarly, stimulation of gonadotropin secretion under aromatase inhibition might require inhibition of local aromatase activity in the hypothalamo-pituitary tissues besides the achieved moderate lowering of peripheral estradiol serum concentrations. If local aromatization would play a role in feedback on LH and FSH levels, gonadotropin release under aromatase inhibition would be attenuated by estradiol administration only if the resulting peripheral estradiol levels reached the levels normally present in the brain. By inhibiting aromatase activity while at the same time replacing peripheral estradiol levels the extent of brain aromatization can be evaluated by comparing the estradiol level needed to restore pre-treatment LH and testosterone levels with the baseline serum estradiol concentration in the absence of aromatase blockade. This study shows that during aromatase inhibition in men replacement of the

Figure 2: the relationship between plasma estradiol and LH levels in 10 male subjects. White dots: hormone levels obtained under treatment with letrozole and various doses of estradiol. Black dots: baseline hormone levels. Pearson's coefficient of correlation for the relationship between estradiol and LH during letrozole use (white dots) is 0.87 ($p<0.001$).

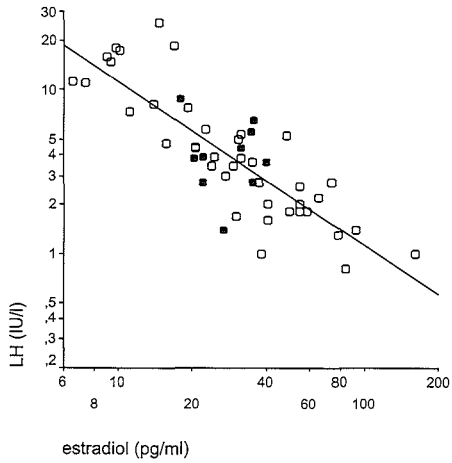
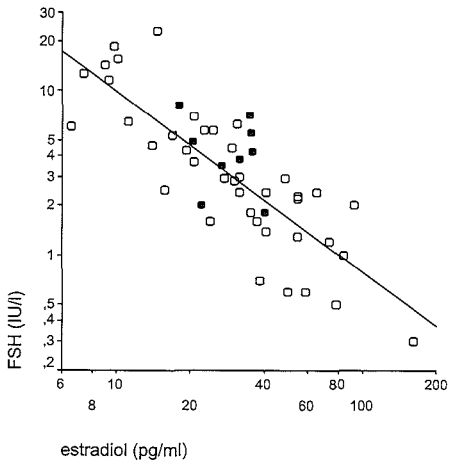


Figure 3: the relationship between plasma estradiol and FSH levels in 10 male subjects. White dots: hormone levels obtained under treatment with letrozole and various doses of estradiol. Black dots: baseline hormone levels. Pearson's coefficient of correlation for the relationship between estradiol and FSH during letrozole use (white dots) is 0.84 ($p<0.001$).



peripheral estradiol concentration to baseline levels is sufficient to normalize gonadotropin and testosterone levels. These results are in accordance with observations in an aromatase deficient man treated with estradiol patches (15). In this man normal LH and testosterone levels were obtained during transdermal estradiol replacement achieving a circulating estradiol level in the physiological range. However, this man showed relatively low levels of LH and testosterone at baseline in the absence of circulating estradiol indicative of an impaired function of the HPG axis in this man. Moreover, FSH levels did not decrease to normal levels after estradiol replacement, probably as a result of low inhibin B levels associated with severe impairment of spermatogenesis.

Our study shows that the male HPG axis is very sensitive to circulating estradiol levels. The relationship between estradiol and testosterone or gonadotropin levels was non linear. Higher estradiol levels have increasingly less effect on circulating LH, FSH and testosterone levels. Varying peripheral estradiol concentrations in the male physiological range resulted in testosterone levels in the low-normal to high-normal range. Although testosterone has an estradiol independent effect on gonadotropin release (16;17), high testosterone levels did not prevent gonadotropins to increase in response to low estradiol levels.

Under normal conditions, only 10 to 20% of circulating estradiol is directly secreted by the testes, the remaining 80% is the product of peripheral aromatization of testosterone or conversion of estrone (18). The adrenal glands also contribute to circulating estradiol levels, through the production of estrone and androstenedione, which can be converted to estradiol and testosterone respectively (6). The production of estradiol depends on the activity of the testes and the adrenal glands and the activity of converting enzymes (19). The estradiol concentration will also be determined by the plasma volume and the metabolic clearance rate. The result is a complex interaction between peripheral levels of estradiol and testosterone. The estradiol concentration is largely dependent on testosterone as a precursor but may also inhibit testosterone production through its effect on gonadotropin release. This explains why in most cross-sectional studies testosterone and estradiol concentrations are positively associated (19). An important difference between the normal physiological situation and the conditions during our study is that here estradiol concentrations were not determined by the serum testosterone concentration. It might be speculated that under normal circumstances the relation between estradiol and testosterone will weaken the overall effect of estradiol on gonadotropin and testosterone levels. If androgen aromatization increases, for instance as a result of weight gain, higher estradiol levels will result in lower testosterone levels thereby decreasing the amount of precursor for estradiol synthesis. Obesity is clearly associated with

Figure 4: the relationship between plasma estradiol and testosterone levels in 10 male subjects. White dots: hormone levels obtained under treatment with letrozole and various doses of estradiol. Black dots: baseline hormone levels. Pearson's coefficient of correlation for the relationship between estradiol and FSH during letrozole use (white dots) is 0.80 ($p<0.001$).

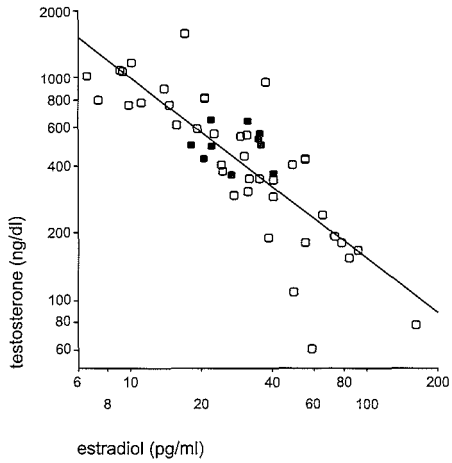


Table 2: actual and predicted values for testosterone, LH and FSH at baseline (mean±SD)

	actual value	predicted value	p for difference
LH (IU/l)	4.32±2.11	4.27±1.25	0.94
FSH (IU/l)	4.30±2.18	3.26±1.05	0.17
Testosterone (ng/dl	503±97	438±105	0.18
(nmol/l))	(17.4±3.37)	(15.2±3.64)	

Values in Systeme International units are given in parenthesis

lower levels of both total and free testosterone (4;20) and the results of the present study tend to support the assumption that this is at least partly mediated by the increased estrogen levels associated with obesity.

This raises an intriguing question: is the male HPG axis primarily driven by circulating testosterone or by estradiol? The results of the present study make a good case for estradiol. However, the increased gonadotropin and testosterone levels in the presence of normal estrogen levels in adult androgen insensitive subjects indicate that there must be a contribution of an androgen receptor mediated effect (21). Nevertheless, it remains to be determined whether the circulating testosterone, when varied within the male physiological range has an estradiol independent, clinically relevant effect on gonadotropin release in men.

In conclusion, circulating estradiol appears to be an important determinant of gonadotropin and plasma testosterone levels in men. The plasma estradiol concentration may help in clinical decision making concerning the cause of low or low-normal testosterone levels in men. However, prerequisites for a correct interpretation is that the used estradiol assay performs adequately in the male physiological range (22) and that the estradiol reference range in men for this assay has been established.

Reference List

1. **Vermeulen A, Verdonck G** 1992 Representativeness of a single point plasma testosterone level for the long term hormonal milieu in men. J Clin Endocrinol Metab 74:939-942

2. **Ring HZ, Lessov CN, Reed T, Marcus R, Holloway L, Swan GE, Carmelli D** 2005 Heritability of plasma sex hormones and hormone binding globulin in adult male twins. J Clin Endocrinol Metab 90:3653-3658

3. **Vermeulen A, Kaufman JM, Deslypere JP, Thomas G** 1993 Attenuated luteinizing hormone (LH) pulse amplitude but normal LH pulse frequency, and its relation to plasma androgens in hypogonadism of obese men. J Clin Endocrinol Metab 76:1140-1146

4. **Zumoff B, Strain GW, Miller LK, Rosner W, Senie R, Seres DS, Rosenfeld RS** 1990 Plasma free and non-sex-hormone-binding-globulin-bound testosterone are decreased in obese men in proportion to their degree of obesity. J Clin Endocrinol Metab 71:929-931

5. **de Ronde W, van der Schouw YT, Muller M, Grobbee DE, Gooren LJ, Pols HA, de Jong FH** 2005 Associations of Sex-Hormone-Binding Globulin (SHBG) with Non-SHBG-Bound Levels of Testosterone and Estradiol in Independently Living Men. J Clin Endocrinol Metab 90:157-162

6. **de Ronde W, Pols HA, van Leeuwen JP, de Jong FH** 2003 The importance of oestrogens in males. Clin Endocrinol (Oxf) 58:529-542

7. **Finkelstein JS, O'Dea LS, Whitcomb RW, Crowley WF, Jr.** 1991 Sex steroid control of gonadotropin secretion in the human male. II. Effects of estradiol administration in normal and gonadotropin-releasing hormone-deficient men. J Clin Endocrinol Metab 73:621-628

8. **Hayes FJ, Seminara SB, Decruz S, Boepple PA, Crowley WF, Jr.** 2000 Aromatase inhibition in the human male reveals a hypothalamic site of estrogen feedback. J Clin Endocrinol Metab 85:3027-3035

9. **Simpson ER, Davis SR** 2001 Minireview: aromatase and the regulation of estrogen biosynthesis--some new perspectives. Endocrinology 142:4589-4594

10. **Veldhuis JD, Iranmanesh A** 2005 Short-term aromatase-enzyme blockade unmasks impaired feedback adaptations in luteinizing hormone and testosterone secretion in older men. J Clin Endocrinol Metab 90:211-218

11. **Leder BZ, Rohrer JL, Rubin SD, Gallo J, Longcope C** 2004 Effects of aromatase inhibition in elderly men with low or borderline-low serum testosterone levels. J Clin Endocrinol Metab 89:1174-1180

12. **T'sjoen GG, Giagulli VA, Delva H, Crabbe P, De Bacquer D, Kaufman JM** 2005 Comparative Assessment in Young and Elderly Men of the Gonadotropin Response to Aromatase Inhibition. J Clin Endocrinol Metab

13. **Van Landeghem AA, Poortman J, Nabuurs M, Thijssen JH** 1985 Endogenous concentration and subcellular distribution of estrogens in normal and malignant human breast tissue. Cancer Res 45:2900-2906

14. **Pasqualini JR, Chetrite G, Blacker C, Feinstein MC, Delalonde L, Talbi M, Maloche C** 1996 Concentrations of estrone, estradiol, and estrone sulfate and evaluation of sulfatase and aromatase activities in pre- and postmenopausal breast cancer patients. J Clin Endocrinol Metab 81:1460-1464

15. **Rochira V, Balestrieri A, Faustini-Fustini M, Borgato S, Beck-Peccoz P, Carani C** 2002 Pituitary function in a man with congenital aromatase deficiency: effect of different doses of transdermal E2 on basal and stimulated pituitary hormones. J Clin Endocrinol Metab 87:2857-2862

16. **Veldhuis JD, Urban RJ, Dufau ML** 1994 Differential responses of biologically active luteinizing hormone secretion in older versus young men to interruption of androgen negative feedback. J Clin Endocrinol Metab 79:1763-1770

17. **Knuth UA, Hano R, Nieschlag E** 1984 Effect of flutamide or cyproterone acetate on pituitary and testicular hormones in normal men. J Clin Endocrinol Metab 59:963-969

18. **Baird DT, Horton R, Longcope C, Tait JF** 1969 Steroid dynamics under steady-state conditions. Recent Prog Horm Res 25:611-664

19. **de Ronde W, Hofman A, Pols HA, de Jong FH** 2005 A direct approach to the estimation of the origin of oestrogens and androgens in elderly men by comparison with hormone levels in postmenopausal women. Eur J Endocrinol 152:261-268

20. **Vermeulen A, Kaufman JM, Giagulli VA** 1996 Influence of some biological indexes on sex hormone-binding globulin and androgen levels in aging or obese males. J Clin Endocrinol Metab 81:1821-1826

21. **Melo KF, Mendonca BB, Billerbeck AE, Costa EM, Inacio M, Silva FA, Leal AM, Latronico AC, Arnhold IJ** 2003 Clinical, hormonal, behavioral, and genetic characteristics of androgen insensitivity syndrome in a Brazilian cohort: five novel mutations in the androgen receptor gene. J Clin Endocrinol Metab 88:3241-3250

22. **Yang DT, Owen WE, Ramsay CS, Xie H, Roberts WL** 2004 Performance characteristics of eight estradiol immunoassays. Am J Clin Pathol 122:332-337

Chapter 8

Endogenous sex hormones, SHBG and the risk of incident vertebral fractures in elderly men and women: the Rotterdam Study

Abstract

In an age-matched case-control study we investigated the association between endogenous sex steroid hormones and incident vertebral fractures in both elderly men and women (aged 67.7 ± 6.8 yrs). Drawn from the Rotterdam Study, participants required radiographs of the lumbar spine at both baseline and follow-up (average time of follow-up: 6.5 years) and frozen blood samples, taken at baseline. 178 men (45 cases) and 454 women (115 cases) were thus selected. Serum estradiol, SHBG, testosterone and insulin were measured, along with BMD at both spine and hip.

Women in the lowest tertile of serum estradiol (≤ 15.5 pmol/l) had a 2.1 times increased risk (95% confidence interval: 1.3–3.5) of incident vertebral fractures, independent of BMD measured at either site. SHBG levels in the lowest two tertiles were associated with a 50% reduction in incident vertebral fracture risk. Women with a combination of both low estradiol and high SHBG had a 7.8 times higher risk of an incident vertebral fracture (95% CI: 2.7 – 22.5, p=0.001), adjusted for age and weight. This increased risk did not change when non-SHBG-bound estradiol was used instead of total estradiol. For men, no clear association could be observed, possibly due to insufficient power. No clear association between testosterone and incident vertebral fractures was observed in either men or women.

Introduction

The physiological fall in estrogen levels after the menopause is well known to be associated with a decreased bone mineral density (BMD) and an increased risk of osteoporotic fractures. Although all postmenopausal women share this drop in estrogen level, they show a varying tendency to suffer from osteoporotic fractures. Differences in residual serum levels of estradiol might in part explain this variation.

Low endogenous estradiol levels are associated with low BMD (1-4). Furthermore, higher estradiol plasma concentrations are associated with a decreased risk of hip fractures (5). Studies on the association between sex steroid hormones and incident vertebral fractures have shown inconsistent results (6-9). These studies were all performed in or before 1985 and therefore could not use recent, more sensitive estradiol assays (10, 11). Recently, the Study of Osteoporotic Fractures has shown that women with an estradiol level below the detection limit of the assay ($\leq$ 18 pmol/l) were at an increased risk of a subsequent fracture at both the vertebrae and hip. This was even more so in women with a simultaneously elevated serum level of sex hormone binding globulin (SHBG) (12). The data suggested a threshold value of estradiol below which the risk suddenly increases.

In men, sex steroid hormones have only been studied in relation to prevalent vertebral fractures and conflicting results were obtained (13-16). The majority of these studies showed no association between prevalent vertebral fractures and estradiol or testosterone. The Rancho Bernardo study (13) however, found an inverse association between estradiol levels and prevalent vertebral fractures in men; such an association was not found in women. To our knowledge no studies on the association between serum sex steroid hormones and incident vertebral fractures have been performed in men.

The aim of this study was to investigate the impact of levels of endogenous sex steroid hormones and SHBG on the incidence of vertebral fractures in both men and women. We performed a nested case-control study within the population–based cohort of the Rotterdam Study.

Materials and methods

Subjects

We designed a nested case-control study amongst the participants of the Rotterdam Study, which is a prospective population-based cohort study of men and women aged 55 years and over. As described elsewhere (17), the study aim is to investigate the occurrence of and risk factors for chronic disabling diseases in an aging population. The total study population

consists of inhabitants of Ommoord, a suburb of Rotterdam, The Netherlands. All 10275 men and women aged 55 year and over were invited to participate in this study between August 1990 and June 1993. From those, 7983 (3105 men) agreed to participate. The Medical Ethics Committee of Erasmus Medical Center, Rotterdam approved the study.

Determinants

At baseline, all participants were extensively interviewed at home on current and past health, potential risk factors and medication. Medication use was scored as baseline usage of more than three different medications per day. Alcohol intake was assessed using a food frequency questionnaire and scored as baseline usage of more than 10 grams of alcohol per day. Current smoking status was scored. At the subsequent research center visit a physical examination was performed. Height and weight were both measured while wearing indoor clothing but without shoes. Non-fasting blood samples were drawn and frozen. Post-load glucose tolerance tests were performed. BMD measurement of both the lumbar spine and femoral neck was performed by dual energy X-ray absorptiometry as described earlier (18) (DXA, Lunar DPX-L densitometer) and lateral radiographs of the spine were taken. There was no correlation between age and time of visit to the research center ($p>0.07$).

Identification of fractures

Both at baseline between 1990 and 1993, and at the second follow-up visit, between 1997-1999, radiographs of the thoraco-lumbar spine were taken following a standard protocol. The distance between source and plate was 120 cm, using a Solarize FV (General Electric CGR, Utrecht, the Netherlands). The follow-up radiographs were available for 3469 individuals (1971 women), who survived until the second follow-up visit and were still able to come to the research center.

All follow-up radiographs were scored morphometrically for the presence of vertebral fractures by the McCloskey/Kanis assessment method as was described previously (19, 20). If a vertebral fracture was present the baseline X-ray was scored as well, to ascertain whether a fracture was incident or prevalent. A fracture was considered incident if no baseline fracture of the vertebra was present and any of the three vertebral heights (anterior, central or posterior) showed a decrease of at least 4.6 mm and 15% on the later spinal film. The detection of fractures was blinded for the baseline steroid hormone status.

Selection of cases and controls

Participants were eligible for the present study if both the baseline and the follow-up spinal X-ray radiographs were available (n=3469). From this group, 176 subjects (5%) had at least one incident vertebral fracture; 162 of those had sufficient baseline blood available. For each case, three age- (within one year) and gender-matched controls were randomly selected from the participants with both X-rays available. Eleven women were excluded because of baseline usage of hormone therapy; no men were on hormone therapy. Thus, 456 women were included (115 cases) and 179 men (45 cases). Of these 633 participants, 588 also had information on BMD available. The average follow-up time was 6.5 ± 0.5 years (range 3.6 - 9.0 years).

Assay methods

Non-fasting blood samples were drawn by veni puncture at the baseline examination in the research center between 8:30 am and 4:00 pm. For the collection of plasma, blood was sampled in 5-ml tubes containing 0.5-ml sodium citrate solution. Platelets were removed by centrifugation and the samples were stored at -80 °C until hormone measurements. The period of storage of frozen serum varied from 7.5 years to 12.5 years. Estradiol, testosterone and SHBG were determined by direct immunoassays; albumin and post-load insulin were also measured. Testosterone was estimated in single measurements by RIA using coated tubes. SHBG was measured in duplicate using double antibody RIA (both: Diagnostic Systems Laboratories, Inc, Webster, TX, USA). The estradiol levels were estimated in duplicate using the ultra-sensitive RIA purchased from the same company. The mean minimum detection limit of this test was 4.8 pmol/l, which enabled us to study the risk of incident vertebral fractures at very low levels of estradiol. Non-detectable estradiol was scored as zero. Inter-assay coefficients of variation, determined on basis of duplicate results of internal quality control serum pools with three different levels of analyte were below 10% for estradiol and SHBG and 12% for testosterone. Intra-assay coefficients of variation were below 10% for both estradiol and SHBG. As measures of biologically active estradiol, the free fraction of estradiol and non-SHBG-bound estradiol were calculated according to the method as described by Södergård et al. (21). Albumin was measured by photometry (Boehringer, Mannheim, Germany). Due to the limited amount of plasma per patient, not all hormone levels could be measured in all subjects. Estradiol levels were available for 632 subjects, SHBG levels for 647 subjects, testosterone for 496 subjects and albumin for 494 subjects. The case-control ratio varied from 1:2.8 to 1:3.1.

A non-fasting glucose tolerance test was used to determine post-load insulin levels. Participants' blood was drawn two hours after they drank a glucose drink containing 75 grams in 200-ml water. Insulin was measured in duplicate by RIA (Medgenix, Brussels, Belgium), intra-assay coefficients of variation was 8.0% and the inter-assay coefficient of variation 13.7%. Participants were classified as diabetics when they reported the use of anti-diabetic medication or when the pre- or post load serum glucose level, measured by the glucose hexokinase method, was equal to or higher than 11.1 mmol/l. Participants already classified as diabetics were excluded from the glucose tolerance test. Therefore, insulin data are only available for the 529 participants with no or undiagnosed insulin resistance or diabetes mellitus.

Data analysis

Differences in baseline characteristics were compared by means of a two-sided t-test for continuous variables and a χ^2-test for categorical variables. One man was excluded from further analysis due to an extremely high estradiol level (>500 pmol/l). All analyses were performed for men and women separately.

An aggregated mean of the various hormone levels was calculated for all controls matched to one case. These aggregated means were subsequently compared to the hormone levels of the cases. Differences between these levels were tested by a paired sample T-test. Because the hormone levels were not normally distributed, the impact of estradiol, non-SHBG-bound estradiol and SHBG on the incidence of vertebral fractures was analyzed in tertile groups using a conditional logistic regression model, to account for the age matching. Adjustment was made for weight. All analyses were subsequently repeated with additional adjustment for femoral neck BMD. To explore the possibility of interaction, the analyses on tertiles of serum estradiol level in association with incident vertebral fractures were repeated in strata of tertiles of SHBG, thus creating 9 groups of women. We used the group of women with a serum estradiol level in the highest tertile and a serum SHBG level in the lowest tertile as the reference. A general linear model was used to analyze the association between BMD and estradiol levels, SHBG levels and non-SHBG-bound estradiol levels with adjustment for age and weight. The p-for-trend was calculated by a linear regression method with the above-mentioned parameters. SPSS 9.0 for Windows was used for all analyses (SPPS Inc. Chicago, Illinois).

Table 1:Baseline characteristics of controls and subjects with incident vertebral fractures

		No vertebral fractures	Vertebral fractures
Women	n	n = 339	n = 115
Age (yr)	454	68.3 (6.9)	68.4 (6.8)
Weight (kg)	453	70.2 (10.3)	67.7 (11.6)*
Lumbar spine BMD (g/cm^2)	419	1.04 (0.18)	0.92 (0.16)**
Femoral neck BMD (g/cm^2)	412	0.82 (0.13)	0.75 (0.13)**
Current smoking (%)	450	14.0	26.3**
Prevalent vertebral fractures (%)	454	9.4	30.0**
Medication use (%)	454	17.1	20.9
Diabetes (%)	405	9.2	7.8
Alcohol use (%)	385	21.5	29.9
Use of walking aid (%)	446	4.8	8.9
Men	n	n = 133	n = 45
Age (yr)	178	66.1 (6.3)	66.3 (6.2)
Weight (kg)	177	80.7 (11.1)	78.7 (10.1)
Lumbar spine BMD (g/cm^2)	169	1.14 (0.19)	1.02 (0.18)**
Femoral neck BMD (g/cm^2)	166	0.87 (0.12)	0.82 (0.11)*
Current smoking (%)	176	25.8	34.1
Prevalent vertebral fractures (%)	178	3.7	20.0**
Medication use (%)	178	11.9	6.8
Diabetes (%)	155	13.7	2.6
Alcohol use (%)	138	58.7	47.1
Use of walking aid (%)	175	2.3	2.3

Values are means with standard deviations or percentages; *: $p < 0.05$, **: $p < 0.005$

Results

Women

Women with an incident vertebral fracture had lower weights and lumbar spine BMD than women without incident vertebral fractures. They smoked more and at baseline had a higher prevalence of vertebral fractures (Table 1). Women with incident vertebral fractures had lower levels of both total and non-SHBG-bound estradiol levels, although not statistically

significant, whereas higher levels of SHBG and lower post load insulin levels were observed (Table 2).

BMD in women was positively correlated with estradiol (Figure 1), albeit that the magnitude of the difference in BMD across the tertiles of estradiol was small. BMD in women was also inversely correlated with incident vertebral fractures (Table 1). Women with a total estradiol level in the lowest tertile (≤15.5 pmol/l) had a two-times increased risk of an incident vertebral fracture as compared to women in the highest tertile (Table 3a). This association appeared to be independent of femoral neck BMD, although BMD was associated with both incident vertebral fractures (Table 1) and estradiol (Figure 1). Results did not change when adjusted for lumbar spine BMD instead of femoral neck BMD. Analyses repeated with either non-SHBG-bound or free estradiol resulted in similar risk estimates (data not shown for free estradiol). Post-load insulin levels were not significantly correlated with BMD (figure 1).

Female participants in the lowest tertile of SHBG levels had a 50% decreased risk for a vertebral fracture (Table 3a). However, when adjustment was made for insulin levels, the association of SHBG with incident vertebral fractures appeared to be non-significant, with odds ratios of 0.67 (0.38 – 1.17) for the first tertile and 0.70 (0.43 – 1.16) for the second tertile with a p-for-trend of 0.25. The association of SHBG with incident vertebral fractures was dependent on BMD (Table 3a). Results for any of the hormones considered did not change when analyses were restricted to first-event cases, excluding the participants with prevalent fractures (data not shown). Furthermore, results remained similar with additional adjustment for current smoking status.

Women with both low estradiol levels (lowest tertile) and high SHBG serum levels (highest tertile) were at an almost 8-fold increased risk of incident vertebral fractures when compared to women with both low levels of SHBG and high levels of total estradiol (OR 7.8, 95% CI: 2.7–22.5, p=0.001; Figure 3). This group comprised of almost 15 percent of the women in our study population (68 out of 463). The other women in the study group, with either a high SHBG level or a low estradiol level, were also at an increased risk compared to this reference group. This risk however was only two-fold increased. Analyses using categories of non-SHGB-bound estradiol instead of total estradiol versus categories of SHBG led to similar results.

Men

Men who suffered an incident vertebral fracture were only different from their controls in lumbar spine and femoral neck BMD and in the prevalence of vertebral fractures (Table 1).

Table 2: Comparison of means of hormonal and humoral factors in controls and subjects with incident vertebral fractures

		No vertebral fractures	Vertebral fractures	Difference (95% CI)
Women	n	n = 339	n = 115	
Estradiol (pmol/l)	454	23.0 (1.5)	20.5 (3.0)	-2.6 (-9.6 / 4.3)
Non-SHBG bound estradiol (pmol/l)	289	14.0 (1.1)	10.5 (1.6)	-3.5 (-7.7 / 0.7)
Testosterone (nmol/l)	359	1.84 (0.06)	1.66 (0.13)	-0.19 (-0.48 / 0.11)
Albumin (nmol/l)	365	43.0 (0.2)	43.3 (0.3)	0.3 (-0.3 / 1.0)
SHBG (nmol/l)	454	53.1 (2.0)	63.9 (5.1)	10.8 (-1.0 / 20.6)
Insulin (pmol/l)	388	66.3 (3.7)	49.0 (2.7)	-17.3 (-28.1 / -6.6)
Men	n	n = 133	n = 45	
Estradiol (pmol/l)	178	49.0 (1.5)	60.0 (12.3)	11.0 (-14.9 / 36.9)
Non-SHBG bound estradiol (pmol/l)	102	34.7 (1.7)	50.0 (17.0)	15.3 (-21.3 / 51.9)
Testosterone (nmol/l)	137	14.4 (0.6)	14.5 (1.7)	0.1 (-3.7 / 3.9)
Albumin (nmol/l)	129	43.1 (0.3)	43.3 (0.6)	0.2 (-1.1 / 1.5)
SHBG (nmol/l)	178	41.2 (1.7)	43.0 (2.4)	1.8 (-4.0 / 7.6)
Insulin (pmol/l)	141	51.4 (3.6)	45.6 (5.4)	-5.8 (-19.9 / 8.3)

Values are case-pooled means with standard error of the mean. Differences are tested with a paired sample T-test, comparing cases and means for pooled matched controls

There was an indication that, compared to the control group, men with incident vertebral fractures less often suffered from diabetes mellitus. Although not statistically significant, men with an incident vertebral fracture appeared to smoke more often. None of the hormone levels were statistically different between cases and controls, although a trend similar to that for women could be observed for SHBG and insulin (Table 2). In men, estradiol levels were associated with BMD, independent of age and weight (Figure 2). Lower non-SHBG-bound estradiol, higher SHBG and lower post-load insulin levels were associated with lower BMD, although not statistically significant. None of the measured hormones was associated with incident vertebral fractures (Tables 2 and 3b).

Due to the limited number of male cases, there was insufficient power to analyze the relative risk of an incident vertebral fracture in men who combined low serum estradiol levels (lowest tertile) with high SHBG serum levels (highest tertile).

Discussion

In this population-based case-control study, in postmenopausal women, low levels of total estradiol, low levels of non-SHBG-bound estradiol and high levels of SHBG were all associated with an increased risk of an incident vertebral fracture, independent of BMD. Women with both an SHBG level in the highest tertile and an estradiol level in the lowest tertile were at a nearly 8-times increased risk of an incident vertebral fracture. In men, no association between sex steroid hormones and incident vertebral fractures could be observed. Women in the lowest tertile of serum estradiol were at a 2.1 time increased risk of an incident vertebral fracture (95% CI: 1.3–3.5). This observation is very similar to the results of a study by Garnero et al. (22), who found an odds ratio of 2.2 (1.2-4.0) for both vertebral and hip fractures, and of a study performed by Cummings et al. (OR 2.5, 1.4–4.2) (12). Ettinger et al. (4) extended the results of Cummings in the same cohort but then for prevalent vertebral fractures (OR 2.5, 1.4-5.0). They were not able, however, to find any influence of oestradiol on prevalent vertebral fractures in their validation set as summarized in Table 4.

It is thought that low estradiol levels influence bone remodeling via an uncoupling of bone resorption and bone formation, favoring osteoclastic bone resorption (23). Lower estradiol levels are indeed associated with lower BMD (1, 4), as confirmed by our study. Therefore, it might be that the association between serum estradiol levels and incident vertebral fractures is in part caused through effects on BMD. Yet, when the analyses were repeated with additional adjustment for femoral neck or lumbar spine BMD, results remained unchanged. This suggests that differences in BMD alone are not a likely explanation for the observed association. Apart from a lower BMD, the uncoupling in bone resorption and bone formation due to estrogen deficiency also results in a deterioration of the bone micro-architecture, resulting in a lower quality of bone. An increase in the depth of the resorption cavity may result in a perforation of the trabecular elements. Thus, given a certain BMD, bone with a better quality of the trabecular structure will be more resistant to fractures (24-26). Estrogen deficiency is also thought to result in an increased apoptosis of the osteocytes (27).

Table 3a: Associations between tertiles of estradiol, SHBG and insulin levels in women and the risk of suffering an incident vertebral fracture

Tertiles		Range	OR adj. for age and weight (95% CI)	p for trend	OR adj. for age, weight and femoral neck BMD (95% CI)	p for trend
Estradiol		pmol/l		n = 451		n = 408
	1	0.0 - 15.5	2.09 (1.26 - 3.46)		2.31 (1.27 – 4.19)	
	2	15.6 - 25.0	1.42 (0.84 - 2.39)	0.01	1.31 (0.71 - 2.43)	0.02
	3	25.1 – 69.5	1 (reference)		1 (reference)	
Non-SHBG bound estradiol		pmol/l		n = 288		n = 255
	1	0.0 – 8.3	2.09 (1.09 - 4.00)		2.39 (1.12 – 5.07)	
	2	8.4 - 15.8	1.41 (0.69 - 2.85)	0.07	1.54 (0.72 – 3.30)	0.08
	3	15.9 – 51.3	1 (reference)		1 (reference)	
SHBG		nmol/l		n = 452		n = 407
	1	0.0 - 42.9	0.51 (0.30 - 0.87)		0.67 (0.37 - 1.20)	
	2	43.0 - 58.5	0.61 (0.39 - 0.99)	0.05	0.63 (0.36 - 1.11)	0.22
	3	58.6 - 167.9	1 (reference)		1 (reference)	
Insulin		pmol/l		n = 381		n = 355
	1	0.0 – 34.9	1.41 (0.85 – 2.36)		1.31 (0.70 – 2.46)	
	2	35.0 – 64.9	1.03 (0.59 – 1.80)	0.31	0.94 (0.49 – 1.81)	0.51
	3	65.0 – 530.0	1 (reference)		1 (reference)	

The capacity of bone to repair micro-damage and to modulate the effects of mechanical strain is believed to be dependent on these osteocytes (28). If estrogen acts as a permissive factor for osteocyte viability, this would explain the fact that the risk of an incident vertebral fracture is increased in women in the lowest tertile of estradiol levels only. In addition, osteocyte death might also play a role in explaining the independence of BMD of the association between estradiol levels and incident vertebral fractures (29).

In our study, increased levels of SHBG are associated with an increased risk of incident vertebral fractures in women. Several other studies have found that an increased level of SHBG is a risk factor for osteoporotic fractures (5, 12, 22, 30; see Table 4). As SHBG is a transport protein that binds estradiol, its impact on fractures is most often regarded as a proxy for the bioavailability of estradiol. Nonetheless, a combination of low estradiol and high SHBG significantly increases the risk to around 8-fold, more than could be expected from the levels for both hormonal factors separately. Interestingly, the SOF study (12) found the same 8-fold elevated risk for a subgroup of women with higher levels of SHBG and lower levels of

Table 3b: Associations between tertiles of estradiol, SHBG and insulin levels in men and the risk of suffering an incident vertebral fracture

	Tertiles	Range	OR adj. for age and weight (95% CI)	p for trend	OR adj. for age, weight and femoral neck BMD (95% CI)	p for trend
Estradiol		pmol/l		n = 177		n = 165
	1	0.0 – 40.5	1.37 (0.61 – 3.06)		1.00 (0.38 – 2.62)	
	2	40.6 – 53.8	1.33 (0.60 – 2.95)	0.71	1.27 (0.51 – 3.13)	0.82
	3	53.9 – 110.1	1 (reference)		1 (reference)	
Non-SHBG bound estradiol		pmol/l		n = 101		n = 93
	1	0.0 – 28.5	1.10 (0.33 – 3.69)		0.92 (0.25 – 3.36)	
	2	28.6 – 38.7	1.66 (0.59 – 4.73)	0.30	2.45 (0.77 – 7.86)	0.17
	3	38.8 – 72.9	1 (reference)		1 (reference)	
SHBG		nmol/l		n = 177		n = 165
	1	9.9 – 34.2	0.75 (0.34 – 1.68)		0.67 (0.26 – 1.73)	
	2	34.3 – 47.7	0.86 (0.40 – 1.84)	0.78	0.90 (0.38 – 2.17)	0.70
	3	47.8 – 109.9	1 (reference)		1 (reference)	
Insulin		pmol/l		n = 129		n = 123
	1	1.0 – 27.9	1.88 (0.75 – 4.73)		1.97 (0.68 – 5.73)	
	2	28.0 – 56.9	1.62 (0.65 – 4.01)	0.39	1.63 (0.58 – 4.60)	0.44
	3	57.0 – 174.0	1 (reference)		1 (reference)	

estradiol with approximately the same boundaries for the estradiol levels (16 vs. 18 pmol/l). Furthermore, our results were essentially the same when analyzing non-SHBG-bound estradiol instead of total estradiol. This suggests that SHBG either has an effect on bone different from the effect through estradiol bioavailability, or is a proxy for another factor that reduces vertebral fractures. Previously, SHBG was also shown to be associated with an increase in the rate of bone loss independent of sex steroid hormone levels, which supports the idea of an additional SHBG action other than through affecting bioavailability of estradiol (31). It has been hypothesized that estrogen-dependent tissues express an SHBG-receptor, albeit so far it has not been identified (32-34). Research in breast cancer patients suggests that SHBG could amplify or dampen the effect of estrogen on target tissue via the receptor (35). If the latter would be the case the combination of low estradiol and high SHBG could have an enhanced effect, resulting in a strong increase of the risk of an incident vertebral fracture. Already in 1988, Von Schoultz hypothesized that SHBG is controlled by other factors than steroid hormones, with IGF-I as one of the stronger candidates (36).

Table 4: Review of the recent literature on hormones and fracture risk

Author, year, reference	Gender	Average age (yr)	Endpoint	N	Hormones	Results, OR (95% CI interval)
Cummings et al. 1998 (12)	♀	73	Inc. VF & inc. HF	402 (138 inc. VF) 476 (133 inc. HF) E_2 data: 274 controls, 89 HF, 96 VF	E_2, SHBG, E_1, T, PTH, Vit D	VF: adj. for age & weight E_2 (< 18 pmol/l): 2.5 (1.4 – 4.2) SHBG (≥ 34.7 nmol/l): 2.3 (1.2 – 4.4) T (≤ 2.4 pmol/l) : 1.4 (0.8-2.4) Similar results for inc. HF
Ettinger et al. 1998 (4)	♀	72	Prev. VF	247 (on average 15% prevalence of VF)	E_2	adj. for age & weight E_2 (< 18 pmol/l): 2.5 (1.4 – 5.0) Validation cohort: no association between E_2 and prevalent VF
Garnero et al. 2000 (22)	♀	64	Inc. osteoporotic F	435 (58 osteoporotic F of which 21 prev. VF)	E_2, SHBG, E_1, T, DHEAS	adj. for age, prev. VF, physical activity E_2 (< 39.6 pmol/l): 2.2 (1.2 – 4.0) SHBG (≥ 71.8 nmol/l): 1.6 (0.9 – 2.9) Additionally adj. for BMD E_2 (< 39.6 pmol/l): 2.1 (1.2 – 3.9)
Chapurlat et al. 2000 (5)	♀	81	Inc. HF	848 (212 inc HF)	E_2, SHBG	adj. for age & weight E_2 (≥ 36 pmol/l): 0.7 (0.5 – 1.1) SHBG (≥ 89.5 nmol/l): 1.61 (0.99 – 2.62)
Barrett-Connor et al. 2000 (13)	♀ & ♂	♀: 72 ♂: 66	Prev. VF	♀: 288 (61 prev. VF) ♂: 352 (28 prev. VF)	$E_{2\ total,\ bio}$, E_1, $T_{total,\ bio}$, DHT, DHEAS, DHEA, androstenedione	adj. for age ♀: $E_{2,\ total}$ (< 12 pmol/l): 0.7 (0.3 – 1.5) ♂: $E_{2,\ total}$ (< 56 pmol/l): 4.2 (1.2 – 14.2) $E_{2,\ bio}$ (< 37 pmol/l): 5.1 (1.2 – 21.5)
Center et al. 2000 (16)	♂	72	Osteoporotic F	437 (24 osteoporotic F)	E_2, SHBG, T, PTH, Vit D, IGF-1	adj. for age & BMD E_2 (- 1 SD): NS SHBG (+1 SD): 1.6 (1.1 - 2.3)
Legrand et al. 2000 (48)	♂	50	Prev. VF	120 (36 prev. VF)	E_2, SHBG, T, bone resorption markers	adj. for age & BMI E_2 (- 1 SD): NS SHBG (+1 SD): 2.0 (1.2 – 3.5)

Abbreviations used: VF = vertebral fractures, HF= hip fractures, F= fractures, inc.=incident, prev.=prevalent, bio=bioavailable,NS=not significant

There is an inverse relationship between IGF-I and SHBG (37), and insulin has been shown to inhibit the hepatic production of SHBG in women (38, 39), whereas simultaneously insulin levels are associated with an increased BMD (40-42). Hence, the risk associated with higher SHBG concentrations could also be due to for instance lower insulin or lower IGF-1 levels (39, 43).

Weight strongly modifies the effect of insulin on incident vertebral fractures as the difference in insulin levels between cases and controls looses its significance when adjusted for weight. Hyperinsulinemia and insulin resistance might have been present in this elderly study population.

Most studies performed in men so far have focussed on the correlation between sex hormones and BMD. Most, but not all, found that bioavailable or total estradiol levels are associated with BMD (2, 3, 15, 44, 45), just as we showed that BMD was associated with total estradiol. Estradiol has also been found to be associated with bone turnover markers (15, 46). These studies did not find a role for SHBG, except for one small case-control study (47). Two recent studies investigated prevalent osteoporotic fractures as endpoint instead of BMD in men (16, 48). Both studies observed that high SHBG levels form a risk factor for osteoporotic fractures in men, independent of estradiol and BMD, with a risk estimate similar to that found in women. No studies with incident vertebral fractures as an endpoint were reported sofar.

We did not observe any association between hormonal factors and the risk for incident vertebral fractures in men, neither for SHBG nor for estradiol or testosterone, although a non-significant difference in the same direction as in women could be observed. As the incidence of vertebral fractures is lower in men than in women, relatively few men could be included in the current study. Our negative findings could, therefore, be a result of low statistical power. Men have higher estradiol serum levels than postmenopausal women. If estradiol indeed acts as a permissive factor for osteocytes, it could well be that the serum levels of estradiol found in men are well above the minimal required level for osteocyte viability. Then, in men, no association between estradiol and incident vertebral fractures would be expected. Also, endogenous sex hormones might be more strongly associated with BMD than with the risk of an incident vertebral fracture in men, as our results suggest as well. During aging, bone loss is partly compensated by concurrent bone formation on the periosteal bone surface; this bone formation is greater in men than in women. Therefore, men appear to compensate their internal bone loss better compared to women, and as a result are more fracture resistant despite a similar areal bone density (49).

Figure 1: Mean lumbar spine BMD per tertile group of hormone level in all women adjusted for age and weight. Error bars show standard errors.

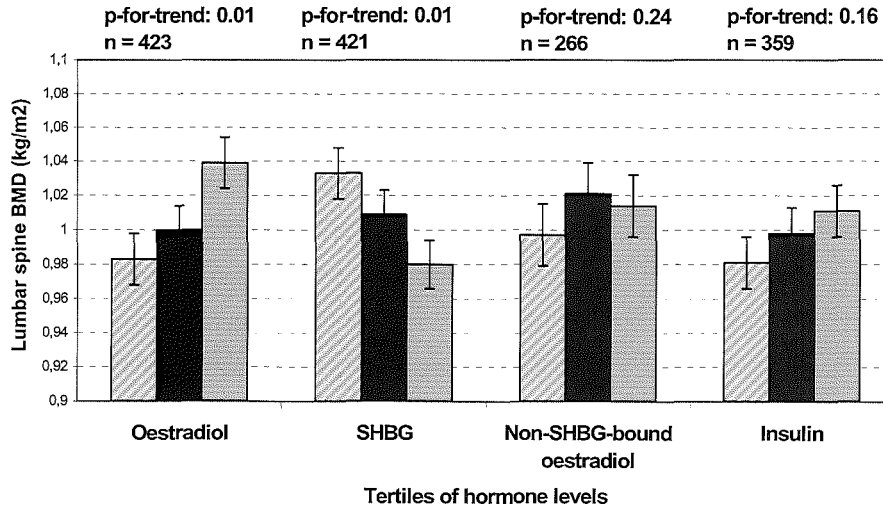
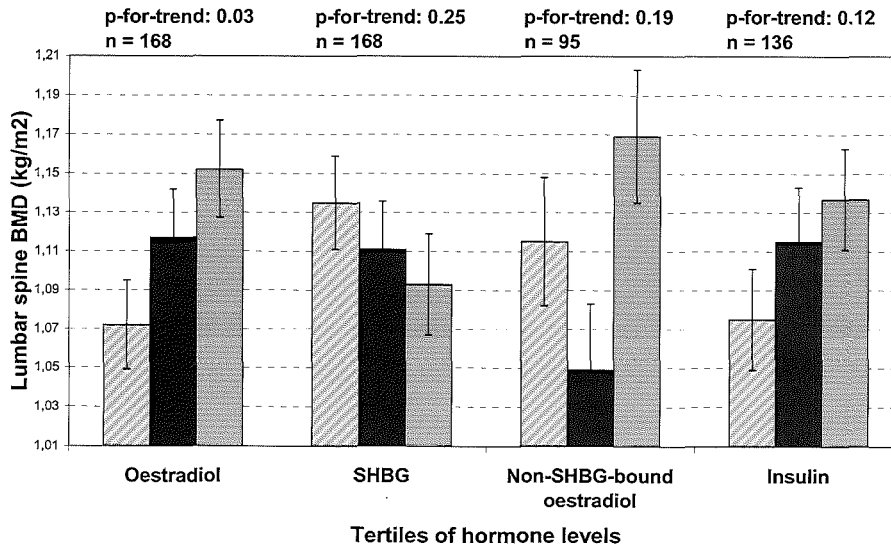


Figure 2: Mean lumbar spine BMD per tertile group of hormone level in all men adjusted for age and weight. Error bars show standard errors.



Our study has several limitations. The subjects were elderly people from Northern European descent; our results may therefore not be generalizable to other populations. The study was performed with a case-control design that might have led to selection bias and information bias. For participants to be included two spinal radiographs were needed. Participants, therefore, had to be mobile enough to be able to visit the study center. This undoubtedly will have led to a health selection bias, whereby participants with higher morbidity were less likely to be included. This bias however applied equally to cases and controls, which makes it unlikely that the validity of the study was affected. However, this health selection bias will affect the generalizability of the study as only mobile elderly people have been studied. Information bias is unlikely to have influenced the results of this prospective case control design: as the study was nested in a population cohort the exposure measure could be determined in blood samples that were drawn at baseline. Furthermore, the outcome validation, spinal fractures, was blinded for the exposure status, i.e. hormone levels. The long interval between blood collection and vertebral fracture assessment, the determination of the hormonal status by a single measurement and the inability to collect the blood at a fixed time of the day can have only diluted the association (50-53).

In conclusion, in postmenopausal women, independent of BMD, both serum estradiol and SHBG are risk factors for incident vertebral fractures. In men, no clear association between sex steroid hormones and incident vertebral fractures could be observed. The subgroup of women with both low estradiol and high SHBG levels was at an almost 8-fold increased risk of incident vertebral fracture.

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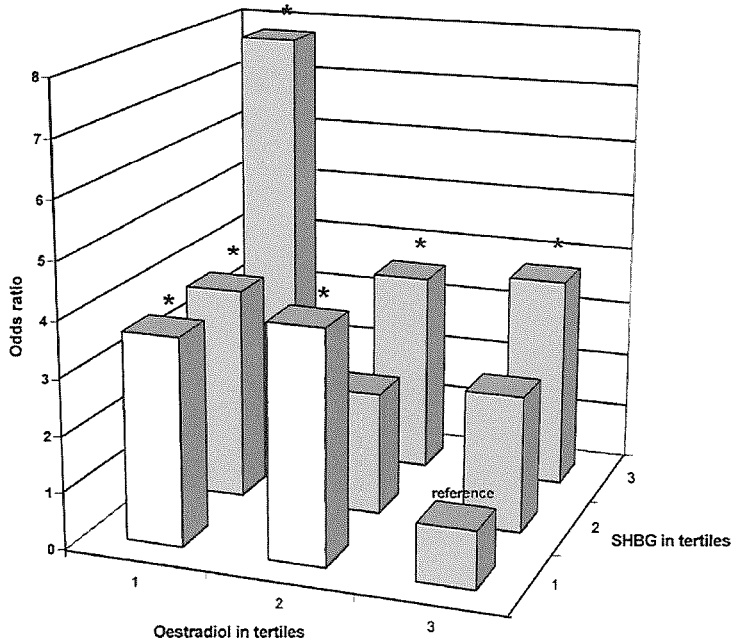
Figure 3: Odds ratios for incident vertebral fractures in tertile groups of estradiol and SHBG in women, adjusted for age and weight.



*: p-value < 0.05

References

1. **Greendale GA, Edelstein S, Barrett-Connor E** 1997 Endogenous sex steroids and bone mineral density in older women and men: the Rancho Bernardo Study. J Bone Miner Res 12:1833-43

2. **Slemenda CW, Longcope C, Zhou L, Hui SL, Peacock M, Johnston CC** 1997 Sex steroids and bone mass in older men. Positive associations with serum estrogens and negative associations with androgens. J Clin Invest 100:1755-9

3. **Khosla S, Melton LJ, 3rd, Atkinson EJ, O'Fallon WM, Klee GG, Riggs BL** 1998 Relationship of serum sex steroid levels and bone turnover markers with bone mineral density in men and women: a key role for bioavailable estrogen. J Clin Endocrinol Metab 83:2266-74

4. **Ettinger B, Pressman A, Sklarin P, Bauer DC, Cauley JA, Cummings SR** 1998 Associations between low levels of serum estradiol, bone density, and fractures among elderly women: the study of osteoporotic fractures. J Clin Endocrinol Metab 83:2239-43

5. **Chapurlat RD, Garnero P, Breart G, Meunier PJ, Delmas PD** 2000 Serum estradiol and sex hormone-binding globulin and the risk of hip fracture in elderly women: the EPIDOS study. J Bone Miner Res 15:1835-41

6. **Riggs BL, Ryan RJ, Wahner HW, Jiang NS, Mattox VR** 1973 Serum concentrations of estrogen, testosterone, and gonadotropins in osteoporotic and nonosteoporotic postmenopausal women. J Clin Endocrinol Metab 36:1097-9

7. **Marshall DH, Crilly RG, Nordin BE** 1977 Plasma androstenedione and oestrone levels in normal and osteoporotic postmenopausal women. Br Med J 2:1177-9

8. **Aloia JF, Cohn SH, Vaswani A, Yeh JK, Yuen K, Ellis K** 1985 Risk factors for postmenopausal osteoporosis. Am J Med 78:95-100

9. **Davidson BJ, Riggs BL, Wahner HW, Judd HL** 1983 Endogenous cortisol and sex steroids in patients with osteoporotic spinal fractures. Obstet Gynecol 61:275-8

10. **Riggs BL, Khosla S, Atkinson EJ, Dunstan CR, Melton LJ 3rd** 2003 Evidence that type I osteoporosis results from enhanced responsiveness of bone to estrogen deficiency. Osteoporos Int. 2003 Jul 18 [Epub ahead of print].

11. **Cummings SR, Duong T, Kenyon E, Cauley JA, Whitehead M, Krueger KA** 2002 Serum estradiol level and risk of breast cancer during treatment with raloxifene. JAMA 287(2):216-20

12. **Cummings SR, Browner WS, Bauer D, Stone K, Ensrud K, Jamal S, Ettinger B** 1998 Endogenous hormones and the risk of hip and vertebral fractures among older women. Study of Osteoporotic Fractures Research Group. N Engl J Med 339:733-8

13. **Barrett-Connor E, Mueller JE, von Muhlen DG, Laughlin GA, Schneider DL, Sartoris DJ** 2000 Low levels of estradiol are associated with vertebral fractures in older men, but not women: the Rancho Bernardo Study. J Clin Endocrinol Metab 85:219-23

14. **Francis RM, Peacock M, Marshall DH, Horsman A, Aaron JE** 1989 Spinal osteoporosis in men. Bone Miner 5:347-57

15. **Resch H, Pietschmann P, Woloszczuk W, Krexner E, Bernecker P, Willvonseder R** 1992 Bone mass and biochemical parameters of bone metabolism in men with spinal osteoporosis. Eur J Clin Invest 22:542-5

16. **Center JR, Nguyen TV, Sambrook PN, Eisman JA** 2000 Hormonal and biochemical parameters and osteoporotic fractures in elderly men. J Bone Miner Res 15:1405-11

17. **Hofman A, Grobbee DE, de Jong PTVM, van den Ouweland FA** 1991 Determinants of disease and disability in the elderly: the Rotterdam Elderly Study. Eur J Epidemiol 7:403-22

18. **Burger H, van Daele PL, Algra D, van den Ouweland FA, Grobbee DE, Hofman A, van Kuijk C, Schutte HE, Birkenhager JC, Pols HAP** 1994 The association between age and bone mineral density in men and women aged 55 years and over: the Rotterdam Study. Bone Miner 25:1-13

19. **Van der Klift M, De Laet CE, McCloskey EV, Hofman A, Pols HAP** 2002 The incidence of vertebral fractures in men and women: the Rotterdam Study. J Bone Miner Res 17:1051-6

20. **McCloskey EV, Spector TD, Eyres KS, Fern ED, O'Rourke N, Vasikaran S, Kanis JA** 1993 The assessment of vertebral deformity: a method for use in population studies and clinical trials. Osteoporos Int 3:138-47

21. **Sodergard R, Backstrom T, Shanbhag V, Carstensen H** 1982 Calculation of free and bound fractions of testosterone and estradiol- 17 beta to human plasma proteins at body temperature. J Steroid Biochem 16:801-10

22. **Garnero P, Sornay-Rendu E, Claustrat B, Delmas PD** 2000 Biochemical markers of bone turnover, endogenous hormones and the risk of fractures in postmenopausal women: the OFELY study. J Bone Miner Res 15:1526-36

23. **Riggs BL, Khosla S, Melton LJ, 3rd** 1998 A unitary model for involutional osteoporosis: estrogen deficiency causes both type I and type II osteoporosis in postmenopausal women and contributes to bone loss in aging men. J Bone Miner Res 13:763-73

24. **Riggs BL, Khosla S, Melton LJ, 3rd** 2002 Sex Steroids and the Construction and Conservation of the Adult Skeleton. Endocr Rev 23:279-302

25. **Manolagas SC** 2000 Birth and death of bone cells: basic regulatory mechanisms and implications for the pathogenesis and treatment of osteoporosis. Endocr Rev 21:115-37

26. **Veenland JF, Link TM, Konermann W, Meier N, Grashuis JL, Gelsema ES** 1997 Unraveling the role of structure and density in determining vertebral bone strength. Calcif Tissue Int 61:474-9

27. **Tomkinson A, Gevers EF, Wit JM, Reeve J, Noble BS** 1998 The role of estrogen in the control of rat osteocyte apoptosis. J Bone Miner Res 13:1243-50

28. **Noble BS, Reeve J** 2000 Osteocyte function, osteocyte death and bone fracture resistance. Mol Cell Endocrinol 159:7-13

29. **Tomkinson A, Reeve J, Shaw RW, Noble BS** 1997 The death of osteocytes via apoptosis accompanies estrogen withdrawal in human bone. J Clin Endocrinol Metab 82:3128-35

30. **van Hemert AM, Birkenhager JC, De Jong FH, Vandenbroucke JP, Valkenburg HA** 1989 Sex hormone binding globulin in postmenopausal women: a predictor of osteoporosis superior to endogenous oestrogens. Clin Endocrinol (Oxf) 31:499-509

31. **Stone K, Bauer DC, Black DM, Sklarin P, Ensrud KE, Cummings SR** 1998 Hormonal predictors of bone loss in elderly women: a prospective study. The Study of Osteoporotic Fractures Research Group. J Bone Miner Res 13:1167-74

32. **Porto CS, Musto NA, Bardin CW, Gunsalus GL** 1992 Binding of an extracellular steroid binding globulin to membranes and soluble receptors from human breast cancer cells (MCF-7 cells). Endocrinology 130:2931-6

33. **Strel'chyonok OA, Avvakumov GV, Survilo LI** 1984 A recognition system for sex hormone binding protein-estradiol complex in human decidual endometrium plasma membranes. Biochim Biophys Acta 802:459-66

34. **Hryb DJ, Khan MS, Rosner W** 1985 Testosterone-estradiol binding globulin binds to human prostatic cell membranes. Biochem Biophys Res Comm 128:432-40

35. **Fortunati N** 1999 Sex hormone-binding globulin: not only a transport protein. What news is around the corner? J Endocrinol Invest 22:223-34

36. **von Schoultz B, Carlstrom K** 1989 On the regulation of sex-hormone-binding globulin--a challenge of an old dogma and outlines of an alternative mechanism. J Steroid Biochem 32:327-34

37. **Pfeilschifter J, Scheidt-Nave C, Leidig-Bruckner G, Woitge HW, Blum WF, Wuster C, Haack D, Ziegler R** 1996 Relationship between circulating insulin-like growth factor components and sex hormones in a population-based sample of 50- to 80-year-old men and women. J Clin Endocrinol Metab 81:2534-40

38. **Pasquali R, Vicennati V, Bertazzo D, Casimirri F, Pascal G, Tortelli O, Labate AM** 1997 Determinants of sex hormone-binding globulin blood concentrations in premenopausal and postmenopausal women with different estrogen status. Virgilio-Menopause-Health Group. Metabolism 46:5-9

39. **Lecomte P, Lecureuil N, Lecureuil M, Lemonnier Y, Mariotte N, Valat C, Garrigue MA** 1998 Sex differences in the control of sex-hormone-binding globulin in the elderly: role of insulin-like growth factor-I and insulin. Eur J Endocrinol 139:178-83

40. **Reid IR, Evans MC, Cooper GJ, Ames RW, Stapleton J** 1993 Circulating insulin levels are related to bone density in normal postmenopausal women. Am J Physiol 265:E655-9

41. **Stolk RP, Van Daele PL, Pols HAP, Burger H, Hofman A, Birkenhager JC, Lamberts SWJ, Grobbee DE** 1996 Hyperinsulinemia and bone mineral density in an elderly population: The Rotterdam Study. Bone 18:545-9

42. **Thomas T, Burguera B, Melton LJ, 3rd, Atkinson EJ, O'Fallon WM, Riggs BL, Khosla S** 2001 Role of serum leptin, insulin, and estrogen levels as potential mediators of the relationship between fat mass and bone mineral density in men versus women. Bone 29:114-20

43. **Janssen JAMJL, Burger H, Stolk RP, Grobbee DE, de Jong FH, Lamberts SWJ, Pols HAP** 1998 Gender-specific relationship between serum free and total IGF-I and bone mineral density in elderly men and women. Eur J Endocrinol 138:627-32

44. **van den Beld AW, de Jong FH, Grobbee DE, Pols HAP, Lamberts SWJ** 2000 Measures of bioavailable serum testosterone and estradiol and their relationships with muscle strength, bone density, and body composition in elderly men. J Clin Endocrinol Metab 85:3276-82

45. **Amin S, Zhang Y, Sawin CT, Evans SR, Hannan MT, Kiel DP, Wilson PW, Felson DT** 2000 Association of hypogonadism and estradiol levels with bone mineral density in elderly men from the Framingham study. Ann Intern Med 133:951-63

46. **Szulc P, Munoz F, Claustrat B, Garnero P, Marchand F, Duboeuf F, Delmas PD** 2001 Bioavailable estradiol may be an important determinant of osteoporosis in men: the MINOS study. J Clin Endocrinol Metab 86:192-9

47. **Gillberg P, Johansson AG, Ljunghall S** 1999 Decreased estradiol levels and free androgen index and elevated sex hormone-binding globulin levels in male idiopathic osteoporosis. Calcif Tissue Int 64:209-13

48. **Legrand E, Hedde C, Gallois Y, Degasne I, Boux de Casson F, Mathieu E, Basle MF, Chappard D, Audran M** 2001 Osteoporosis in men: a potential role for the sex hormone binding globulin. Bone 29:90-5

49. **Seeman E** 2002 Pathogenesis of bone fragility in women and men. Lancet 359:1841-50

50. **Cauley JA, Gutai JP, Kuller LH, Powell JG** 1991 Reliability and interrelations among serum sex hormones in postmenopausal women. Am J Epidemiol 133:50-7

51. **Toniolo P, Koenig KL, Pasternack BS, Banerjee S, Rosenberg C, Shore RE, Strax P, Levitz M** 1994 Reliability of measurements of total, protein-bound, and unbound estradiol in serum. Cancer Epidemiol Biomarkers Prev 3:47-50

52. **Potischman N, Falk RT, Laiming VA, Siiteri PK, Hoover RN** 1994 Reproducibility of laboratory assays for steroid hormones and sex hormone-binding globulin. Cancer Res 54:5363-7

53. **Phillips GB, Yano K, Stemmermann GN** 1988 Serum sex hormone levels and myocardial infarction in the Honolulu Heart Program. Pitfalls in prospective studies on sex hormones. J Clin Epidemiol 41:1151-6

Chapter 9

General discussion

Diagnosing hypogonadism

The regulation of the plasma testosterone concentration in men can be described as a classical endocrinological feedback system. Hypothalamic Gonadotropin Releasing Hormone (GnRH) stimulates the release of Luteinizing Hormone (LH) by the pituitary and eventually the secretion of testosterone by the testicular Leydig cells. Testosterone, secreted into the blood and carried back to the brain attenuates GnRH and gonadotropin release thereby completing the feedback loop and keeping the plasma testosterone level within close limits. Although appealing in its simplicity this model raises one critical question; which factors determine the plasma testosterone concentration in men; in other words what controls the "androstat"? This information is crucial when interpreting the plasma testosterone concentrations of men with symptoms reminiscent of androgen deficiency. The signs and symptoms typically associated with testosterone deficiency, decreased bone mass, lean body mass and bodily hair, loss of libido, decreased testicular volume and erectile function, were shown to be rather non-specific (1;2). Therefore, a diagnosis of hypogonadism relies heavily on biochemical criteria. When testosterone levels are extremely low there is general consensus that testosterone replacement therapy is necessary. However, there is a considerable proportion of men with nonspecific symptoms who present with mildly decreased or low-normal plasma testosterone concentrations. This situation is mostly encountered in older men since ageing in men is associated with declining circulating testosterone levels and is associated with signs and symptoms reminiscent of androgen deficiency (2). There is much debate whether or not this so called "late onset hypogonadism", "(partial) androgen deficiency of the aging male", "male climacteric" or "andropause" should be treated with androgen replacement (3). The small-scaled randomized controlled trials executed up to now have not shown unequivocal evidence for the benefits of this therapy neither have they relieved concerns about possible harm.

As described in chapter **1** it has become evident that estradiol, aromatized from testosterone is responsible for some of the effects originally attributed to testosterone. Estradiol has an important role in gaining and maintaining bone mass, closing of the epiphyses and the feedback on gonadotropin release by the pituitary. Since estradiol may be a determinant of the circulating testosterone concentrations but may also be involved in the development of the clinical syndrome associated with androgen deficiency, evaluation of estradiol levels in men seems appropriate. However, the interpretation of estradiol levels in men is probably even more difficult than the interpretation of testosterone concentrations. Only little is known about the determinants of estradiol, its interaction with testosterone and the minimal tissue level

needed to prevent symptoms of estrogen deficiency. To increase our understanding of the determinants of androgen and estrogen concentrations in men the following questions were addressed.

-Sex Hormone Binding Globulin (SHBG) is an important testosterone and estradiol binding protein in human plasma. SHBG prevents bound hormone from diffusing out of the blood stream to interact with the intracellular hormone receptors. When the non-SHBG-bound fraction of testosterone is considered bioactive, an increase in SHBG concentrations will cause an increased secretion of GnRH by the hypothalamus and LH secretion by the pituitary. Low levels of non-SHBG-T will subsequently lead to increased LH and testosterone production via a decreased negative feedback signal on the hypothalamus and pituitary. Via this mechanism SHBG may influence the testosterone production rate. However, SHBG will also prevent bound hormone from entering the hepatocytes thereby decreasing its metabolic clearance rate. As a result SHBG may be a determinant of the plasma levels of testosterone and estradiol. How are levels of SHBG related to total and bioavailable estradiol and testosterone levels in men?

-If the SHBG concentration is associated with the plasma levels of testosterone and estradiol, what is the relative impact of age related changes in SHBG levels on plasma (bioavailable) testosterone levels in men?

-The dialysis and ammonium sulphate precipitation techniques for the measurement of free or bioavailable testosterone are often recommended but rarely used because they are expensive, time consuming and labor intensive. In both clinical practice and scientific reports calculated estimates of the free or bioavailable testosterone fraction are mostly used. Is there much difference between the results of the algorithms to calculated bioavailable and free fractions of testosterone?

- The origin of estrogens in men is not easily assessed. Estrone and estradiol are secreted by adrenal glands and testes (4;5). A substantial portion of circulating estrogens however is derived from conversion of adrenal and testicular androgens in fat and muscle tissue by the aromatase enzyme (6;7). Finally, the 17β- hydroxysteroid dehydrogenases, found throughout the body, can convert estradiol to estrone and testosterone to androstenedione and vice versa (8). What is the relative contribution of testes and adrenal glands to the circulating pool of estrogens in men?

- Estradiol is a potent inhibitor of gonadotropin release by hypothalamus and pituitary in men. Disruptive mutations in the genes for aromatase (CYP 19) and estrogen receptor α (ESR1) are associated with high gonadotropin and testosterone levels in men (9-11). Therefore, what is

the relationship between aromatase, ESR1 and ESR2 gene polymorphisms and circulating estrogen, androgen and LH levels in men?
- Aromatase, the enzyme responsible for the conversion of androgens into estrogens has several tissue specific promoters (12). As a consequence the extent of androgen aromatization may vary between tissues in one individual and plasma estradiol levels might not always reflect estrogen exposure at the pituitary or hypothalamus. Is local aromatization of testosterone in the hypothalamus or pituitary important for the effect of estradiol on gonadotropin release?
- In adult aromatase deficient men, estrogen deficiency is associated with very low bone mineral density (BMD) (13-15). Are estradiol levels associated with low BMD or fractures in men?

SHBG and its association with plasma testosterone and estradiol levels in men

In chapter **2** the association between levels of SHBG and total testosterone in men is described. It is evident that SHBG is an important determinant of total testosterone levels; higher SHBG levels are associated with higher levels of total testosterone and vice versa. This association can be explained by the effect of SHBG on production or clearance rates of testosterone. Testosterone is tightly bound to SHBG. Therefore, during passage through the liver SHBG will prevent bound hormone to leave the blood stream and enter the hepatocytes (16) . This subject has been extensively investigated (6;17;18) using the constant hormone infusion technique. As expected, hormones with the highest binding affinity for SHBG showed the lowest metabolic clearance rates (MCR) (6;16).

A strong positive correlation between the bioavailable testosterone fraction (non-SHBG-T(%)) and the MCR of testosterone (MCR_T) ($r=0.83$, $p<0.001$) was observed in men and women (17). Using linear regression analysis and the data published by Vermeulen et al. (17) the MCR_T in a particular person can be estimated from the bioavailable testosterone fraction (table 1; equation 1). In this equation the constants k_1 and k_2 reflect all factors that can influence MCR_T aside from SHBG such as smoking status (19) and weight (20) but not age (20). In the small scale studies performed by Vermeulen et al. (17) the variance of these parameters appeared to be small which is reflected in the high correlation between MCR_T and non-SHBG-T (%). The production rate of testosterone PR_T is equal to the product of the MCR_T and the plasma concentration of testosterone (PC_T) (table 1; equation 2). When in this equation MCR_T is substituted by non-SHBG-T (%) it can be deducted that the PR_T is relative

to the product of non-SHBG-T (%) and the PC_T which equals the bioavailable testosterone concentration. This results in some rules of thumb for the clinical endocrinologist to be used in the interpretation of testosterone levels in men; the ratio between the PR_T and the MCR_T can be directly estimated from the ratio between non-SHBG-T (nmol/l) and non-SHBG-T (%)(table 2).

In the studied men in chapter **2** no association was found between SHBG and bioavailable testostosterone (nmol/l) which suggests that the production rate of testosterone in these men was not associated with SHBG levels. As a result, the observed positive association between SHBG and total testosterone has to be completely attributed to the effect of SHBG on testosterone clearance. This hypothesis is supported by experiments in rats. Castrated male rats were substituted with implants releasing testosterone at a constant rate. Infusion of SHBG resulted in increased plasma concentrations of testosterone but hardly affected bioavailable testosterone levels (21).
However, in several studies in women no positive association was found between levels of SHBG and plasma levels of testosterone despite a wide range of SHBG levels (22;23). Although Kalish et al. (24) found a weak but positive association between SHBG and testosterone in postmenopausal women (unadjusted for other variables) they also found a strong negative association between SHBG and bioavailable testosterone. The negative relationship between SHBG levels and bioavailable estradiol levels found in chapter 2 also argues against an important effect of SHBG on hormone clearance. If we assume that SHBG has an effect on the metabolic clearance rate of testosterone this should also hold true for estradiol although to a lesser extent due to the lower binding affinity of SHBG for estradiol (25). For the reasons described above one would not expect a negative relationship between SHBG and bioavailable estradiol. The absent association between levels of SHBG and total testosterone in women indicates that the strong positive association between these parameters in men may be driven by effects of SHBG on the production rate of testosterone. Clarifying these points is important for several reasons. If we assume that the association between total testosterone and SHBG is primarily driven by the effect of SHBG on the production rate of testosterone, higher SHBG levels will contribute to lower bioavailable testosterone levels in men in whom the HPG axis is partially dysfunctional. Older men with high SHBG levels will be more at risk for developing biochemical hypogonadism. The age associated increase in SHBG levels may thus contribute to the age associated decline in bioavailable testosterone levels.

Table 1: estimation of MCR_T and PR_T

$\mathbf{MCR_T = k_1 * non\text{-}SHBG\text{-}T(\%) + k_2}$	equation 1 according to Vermeulen et al.1979 ($k_1 = 10.9$ and $k_2 = 248$)
$\mathbf{PR_T = MCR_T * PC_T}$	equation 2
Combining equation 1 and 2	
$\mathbf{PR_T = (k_1 * non\text{-}SHBG\text{-}T(\%) + k_2) * PC_T}$	

PC_T=plasma concentration of testosterone in nmol/l, PR_T = production rate of testosterone in nmol/day; MCR_T =metabolic clearance rate of testosterone in L/day; k_1 and k_2 = constant; non-SHBG-T(%) = fraction of bioavailable T

Table 2: Pearsons coefficients of correlation for the associations between the estimated metabolic clearance rates and production rates of testosterone with levels of total and bioavailable testosterone in men.

	Estimated MCR_T	Estimated PR_T
SHBG	-0.95^{***}	0.13^{*}
Total testosterone	-0.41^{***}	0.87^{***}
Bioavialable testosterone (%)	$\mathbf{1.0}^{***}$	0.07
Bioavailable testosterone (nmol/l)	0.32^{***}	$\mathbf{0.96}^{***}$

Therefore we studied the relationship between SHBG and total testosterone in two groups of males in whom the functionality of the HPG axis might be questioned; male infants and older men (chapter 3). In male infants mean SHBG levels were much higher as compared to adult men. In the first weeks after birth total testosterone levels may reach adult levels but there is a strong decline with increasing age, resulting in prepubertal levels between the age of 3 to 6 months. SHBG was strongly associated with total testosterone levels in these infants which may suggest that during the first months of life the HPG axis is functional and sensitive to androgen feedback. This is supported by the observation that castration of neonatal monkeys results in elevated LH levels comparable to those found in castrated adults (26). Bioavailable testosterone levels were low compared to adult men. The absent relationship between bioavailable testosterone and SHBG suggests that these high SHBG levels are not the cause of the low bioavailable testosterone levels. A lower and age related setpoint for the HPG axis during the first months of life might thus be postulated.

In adult men the positive association between SHBG and total testosterone is maintained up to the oldest age group. This shows that the increased SHBG levels in older men are not the cause of the lower bioavailable testosterone levels. In fact, the increasing mean SHBG level

with age and the positive association between SHBG and testosterone should result in an increase in total testosterone levels with increasing age. However, the mean total testosterone levels decreases. This means that SHBG partially prevents the age related decline of total testosterone levels.

A limitation of the study is that mean testosterone levels, even in the oldest men, were relatively high compared to the mean levels for bioavailable and total testosterone levels reported in the literature (27). The high mean testosterone levels may indicate that the HPG-axis in these men is functioning rather well and can be expected to respond adequately to varying SHBG levels. On the other hand, this study shows that even in men with a well functioning HPG axis, aging is associated with declining bioavailable testosterone levels independent of SHBG levels.

Estimation of free and bioavailable testosterone

When free or bioavailable testosterone are supposed to be the bioactive fractions of testosterone, measurement of this fraction is preferred. However, accurate measurement of free or bioavailable fractions is complicated, expensive and labor intensive and therefore not used for routine estimation of free or bioavailable testosterone levels in the Netherlands. Calculation of bioavailable or free testosterone is generally regarded as an accurate method to estimate free and bioavailable testosterone levels. For both scientific purposes and for clinical practice calculating bioavailable or free testosterone is therefore well accepted. The two most widely used equations for calculating bioavailable or free testosterone are those described by Vermeulen et al. (28) and Sodergard et al. (25). These algorithms rely on the law of mass action which states that the free and bioavailable fractions of testosterone can be calculated once the concentrations of testosterone, SHBG and albumin are known in combination with the binding constants for the binding of testosterone to the respective binding proteins. The concentrations of testosterone, SHBG and albumin can be measured with standard techniques whereas the binding constants have been obtained experimentally. These algorithms were validated by comparing calculated estimates with measured values using a gold standard technique. The principal differences between the two algorithms are the values for the association constants. Recently three papers were published in which three other algorithms for estimating the concentration of free or bioavailable testosterone were proposed based on measured levels in large numbers of samples using a gold standard technique (29-31). Knowing that all algorithms were based on or validated by gold standard techniques the results obtained should not be largely different.

However, in chapter **4** we showed that two algorithms to calculate bioavailable testosterone, based on measured concentrations using the ammoniumsulfate technique, gave very different results (29;30). Obtained levels were relatively low when compared to traditional estimates of bioavailable testosterone and were significantly associated with SHBG levels. The results of one of the algorithms were highly correlated with total testosterone levels which makes it questionable whether calculated bioavailable or free testosterone levels obtained using this algorithm have any additional value above the total testosterone measurements. This algorithm has been placed on the internet (www.him-link.com) for distant users but its use should be discouraged based on the above mentioned limitations. The ammoniumsulfate precipitation technique, the gold standard technique for the measurement of bioavailable testosterone, relies on the complete precipitation of SHBG and bound hormone by adding ammoniumsulfate without precipitating albumin bound hormone. The amount of ammoniumsulfate to be added to the patient material appears to be very critical (32). A slight change in ammoniumsulfate concentration may lead to incomplete precipitation of SHBG or precipitation of albumin bound testosterone resulting in aberrantly high or low estimates of bioavailable testosterone.

In chapter **4** we also evaluated the results of an algorithm to calculated free testosterone based on measurements in almost 4000 men using a ultrafiltration method (31). The results correlated very strongly with total testosterone levels. Moreover there was a significant correlation between results obtained and SHBG levels. A strong association between free or bioavailable testosterone levels and SHBG levels not only raises questions regarding the reliability of the estimates but also introduces fundamental problems when interpreting these estimates. Confounding of these results by SHBG can not be solved by establishing testosterone and SHBG assay specific reference ranges.

The results of the Vermeulen and Sodergard algorithms were fairly concordant and were not or only mildly associated with SHBG levels. As expected on the basis of the association constants used, the Sodergard algorithm gave higher results as compared to the Vermeulen algorithm. The absent relationship between estimates of free or bioavailable testosterone obtained using the Sodergard and Vermeulen algorithms and SHBG levels add to their credibility and make them applicable for clinical practice as long as method specific reference ranges are obtained.

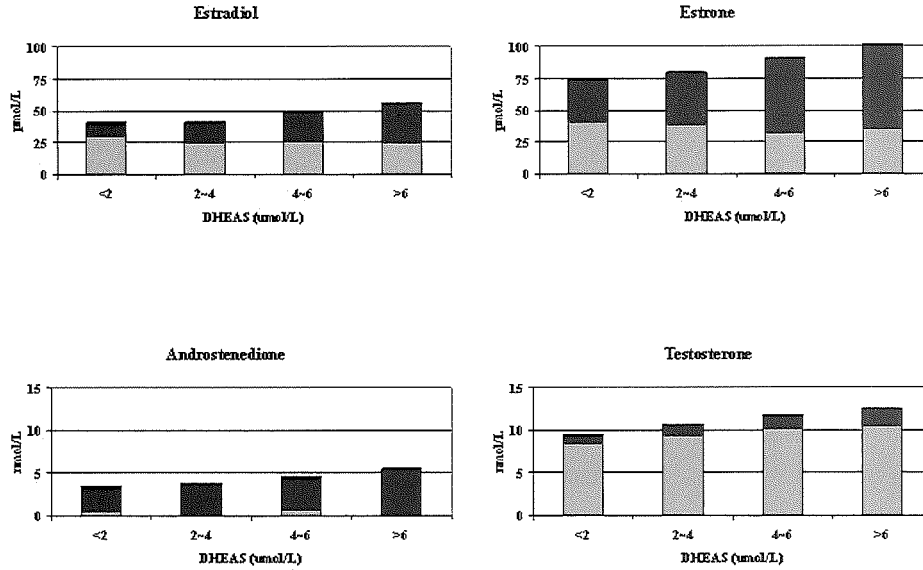
This study shows that one should be very cautious when applying published algorithms to one's data. It should be noted that even gold standard methods to estimate free or bioavailable testosterone may be seriously flawed. Users of these algorithms should never compare their

results with reference ranges or treatment thresholds published in the literature and should always obtain method and laboratory specific reference ranges. With every method, either an algorithm or a measurement, there should be no association between SHBG and estimates of free or bioavailable testosterone levels in healthy men and there should be no strong association between estimates of free or bioavailable testosterone and total testosterone.
As described in chapters **1** and **2** circulating estradiol is also partially bound to SHBG and albumin. We did not compare different techniques or algorithms to estimate free or bioavailable estradiol levels. However, because free and bioavailable fractions of estradiol and testosterone are obtained using the same techniques similar concerns as described in chapter **4** may relate to estimates of free and bioavailable estradiol.

The origin of estrogens in males

As described in chapter **1** some of the clinical effects formerly attributed to testosterone actually represent effects of estradiol. This became particularly evident in men with aromatase deficiency. Aromatase is the enzyme responsible for conversion of androgens into estrogens. Men with estrogen deficiency caused by a mutation in the CYP 19 gene suffer from low BMD and unfused epifyses and have high gonadotropin and testosterone levels (10;11;14;15). Direct precursors of estrogen synthesis are testosterone and androstenedione, produced by the testes and adrenal glands respectively. In chapter **5** we estimated the origin of estrogens in men by comparing hormone levels with those obtained in postmenopausal women. For this comparison we made several assumptions: the postmenopausal ovary is not endocrinologically active, the testes do not contribute to the circulating DHEAS concentration and the metabolism of hormones is similar in men and women. Since the mean DHEAS level is much higher in men as compared to women we stratified for DHEAS levels. According to our calculations up to 56 % of E2, 66 % of E1 and 17 % of T can have its direct or indirect origin in the adrenal glands of elderly men (figure 1).
The testes contribute only little to peripheral A levels (maximum 14%). These estimates are in agreement with earlier results from experiments using radio labeled hormone infusion techniques (7).

Figure 1: DHEAS stratified calculated relative contributions of the testes (gray) and adrenals (black) to peripheral concentrations of E2, E1, A and T.



A polymorphism in CYP 19 is a determinant of peripheral estradiol levels.

The plasma concentration of estradiol is not only dependent on the concentration of its main precursors but also on the amount and activity of the aromatase enzyme. Aromatase is present in adipose tissue (33;34), muscle (33) and bone (35;36). A well known determinant of estradiol concentrations in men is obesity. Obesity is associated with increased plasma levels of estradiol (37;38) probably as a result of increased androgen aromatisation in adipose tissue. In chapter **6** we present the association between plasma estradiol levels with BMI in men. As expected there was a clear positive association between BMI and plasma estradiol levels. We also investigated whether a polymorphism in the aromatase gene was associated with altered estradiol levels in these men. Kristensen et al. (39) showed that a C-T substitution, located in the untranslated region (UTR) of exon 10 of CYP 19 was associated with increased aromatase mRNA levels. Their data suggested that the T allele was associated with higher aromatase activity probably as a result of a switch from the adipose tissue promoter to the ovarian

promoter or increased mRNA stability due to alternative folding of the transcript (39). We have shown that the T allele, present in 77% of the studied men was associated with a 9% higher mean estradiol level. The studied polymorphism was not associated with levels of LH or testosterone. This is somewhat unexpected, since estradiol is known for its potent suppressive effects on gonadotropin secretion in men. In our subjects there was a negative relationship between BMI and both total and bioavailable testosterone levels. Part of this association is driven by SHBG; SHBG is negatively associated with BMI and positively associated with total testosterone (chapter **1** and **2**). As a result, obese men have lower SHBG and lower total testosterone levels. The lower bioavailable testosterone levels in obese men may also be caused by the increased inhibition of gonadotropin release as a result of the higher plasma concentrations of estradiol. We calculated that a 9% change in the estradiol concentration, associated with a 3.9 kg/m^2 change in BMI is associated with a 1.05 nmol/l change in the total testosterone concentration. However, a 9% difference in estradiol level associated with the presence or absence of the T allele was *not* associated with testosterone levels. Therefore, one might conclude that there is an estrogen and SHBG independent effect of body weight on androgen levels in men and that this effect outweighs the effect of estrogens. Obesity may have estradiol independent effects on gonadtropin secretion or on the responsiveness of the Leydig cells to gonadotropins. Perhaps, the difference in estradiol level brought about by this aromatase polymorhism is to small to have a significant effect on gonadotropin and circulating testosterone levels. Alternatively, it may be hypothesized that extensive aromatisation of androgens takes place in or near the pituitary. In that case circulating estradiol levels may not represent the estrogenic tone exerted in the pituitary and hypothalamus.

Effect of circulating estradiol on male HPG axis activity

To obtain a better insight into the effect of circulating plasma estradiol levels on HPG axis activity in men we studied the effect of varying doses of estradiol on serum concentrations of LH and testosterone in healthy young men (chapter **7**). We lowered estradiol levels in these men using the aromatase inhibitor letrozole. As expected (40) lower estradiol levels were associated with high levels of LH, FSH and testosterone. During aromatase inhibition, estradiol levels were increased by adding transdermal estradiol using patches releasing on average 25, 50 or 100 micrograms per day. Peripheral estradiol levels had very strong effects on LH, FSH and testosterone levels. Varying estradiol levels in the male physiologic range resulted in gonadotropin and testosterone levels ranging from high normal to low normal.

During aromatase inhibition the estradiol level needed to restore LH, FSH and testosterone to baseline levels was not significantly different from the baseline estradiol concentration. From this we conclude that aromatisation of androgens in or near the hypothalamus or pituitary is not a prerequisite for the suppressive effect of estradiol on gonadotropin release. As a result circulating estradiol levels directly reflect the inhibitory tone exerted by estrogens on gonadotropin release and appear to be a major determinant of peripheral testosterone, LH and FSH levels in men.

The plasma estradiol concentration might thus, in addition to the SHBG concentration, add to the interpretation of the plasma testosterone concentration in men. However, there are some difficulties in interpreting the interaction between estradiol and testosterone concentrations. First of all, testosterone is an important precursor of estradiol. Therefore, in most cross-sectional studies there is a positive correlation between testosterone and estradiol levels (chapter **5**). The result is that estradiol is both dependent on testosterone for its synthesis whereas it also restrains testosterone production through its effects on gonadotropin production. Testosterone may also have an estradiol independent effect on gonadotropin release. Although there is strong evidence that testosterone can inhibit gonadotropin release without being aromatised first (41-43) the relative contribution of testosterone to the feedback inhibition of gonadotropin release remains to be determined.

Estradiol levels are associated with bone mineral density BMD in men

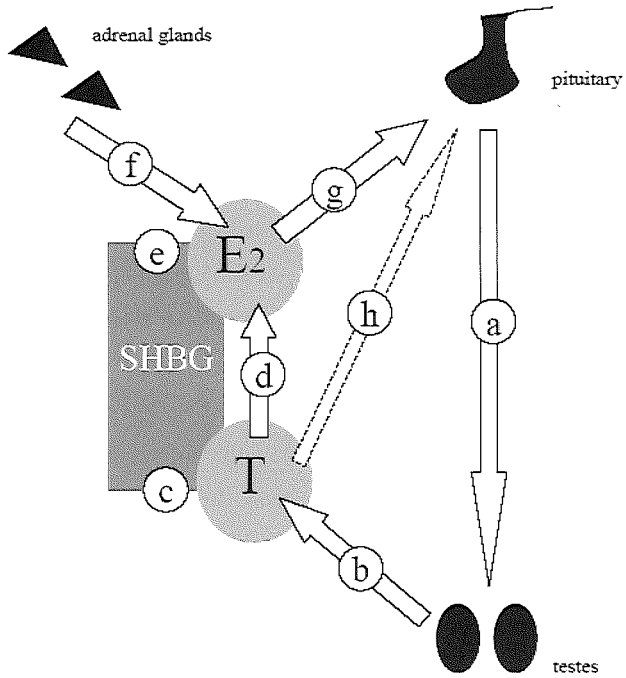
In chapter **8** we evaluated whether peripheral estradiol levels were associated with BMD and vertebral fractures in elderly men and postmenopausal women. Women with low estradiol and high SHBG levels had an increased vertebral fracture risk. In men estradiol levels were significantly associated with BMD which is in line with current evidence as reviewed in chapter **1**. Higher SHBG levels were associated with lower BMD although not significantly. Sex hormones and SHBG were not associated with fracture risk in men.

As described in chapter **5** the mean estradiol level in older men is much higher compared to the mean level in postmenopausal women. Therefore the relationship between plasma estrogen levels and BMD may be less critical in men. Extremely low estrogen levels in men are strongly associated with low BMD but are only encountered in severe hypogonadism or rare genetic disorders such as aromatase deficiency. The presence of testosterone and sufficient amounts of estradiol probably explain the absent relationship between endogenous sex hormones and fracture risk in our study. Khosla et al. proposed a threshold for bioavailable estradiol of 30 pmol/l below which BMD appeared to be strongly and negatively

associated with the plasma bioavailable estradiol concentration in men (44;45). Although a substantial proportion of the men in our study had bioavailable estradiol levels below this threshold, no clear association with BMD or fracture risk was found. Because the number of men in our study was relatively small, the power of the study may have been too small to detect such an association. Moreover, mean total testosterone and total estradiol levels in our studied men were much lower as compared to levels in the men studied by Khosla et al. (45) which may indicate that either the characteristics of the studied men or the steroid hormone assays used were different. Additionally the methods to determine bioavailable hormone levels were different in both studies. Khosla et al used ammoniumsulfate precipitation whereas we have calculated bioavailable levels using the algorithm as described by Sodergard (25). To underline the conclusions reached in chapter **4** the estimates obtained using these two methods appeared to be very different. When the mean levels for total testosterone, total estradiol and SHBG from the Khosla study were entered into the Sodergard algorithm, the estimates for bioavailable testosterone were 3 to 5 times the results presented by Khosla. For bioavailable estradiol results obtained using the Sodergard algorithm were also considerably higher; the bioavailable estradiol fraction in our study was between 70 and 83% and for the older men in the Khosla study between 36 and 51%. Therefore, once again, one should be extremely cautious when interpreting calculated or measured bioavailable hormone levels presented in the literature.

It is questionable whether the plasma estradiol concentration should be added to the work-up of male osteoporosis. Although in most studies in men, estradiol levels are significantly associated with BMD, its impact is relatively small. In most hospitals no detailed reference levels for male estradiol concentrations are present and especially in the lower ranges, standard estradiol assays may not be accurate enough. As described above, there is no clear cut level below which the estradiol level can be considered too low and in most cases, low estradiol levels will be associated with low testosterone levels. Although the relationship between BMD and plasma testosterone concentrations is even weaker than the relationship between BMD and estradiol levels, low testosterone is an established indication for substitution for reasons other than low BMD alone. Knowing that testosterone is an important precursor for estradiol synthesis, substitution of testosterone will also lead to higher circulating estradiol levels. Last but not least, it is unclear in what way low estradiol levels in men should be treated and what the results of this treatment will be. As described in chapter **7** treating eugonadal men with estradiol will result in decreased plasma testosterone levels which, for most men, will be an undesirable side effect of the treatment.

Figure 2: a model for the interaction between testosterone and estradiol in men



Determinants of androgen and estrogen levels in men; conclusions.

The data presented in this thesis allow us to present a model for the interaction between testosterone and estradiol in men (figure 2).

Pituitary derived LH stimulates testosterone production by the testicular Leydig cells (a). Testosterone is secreted in the blood (b) and is partially bound by SHBG (c). SHBG is a determinant of total testosterone and estradiol plasma concentrations in men. The plasma concentration of bioavailable testosterone is not influenced by SHBG binding as a result of decreased testosterone clearance, increased testosterone production or both. Consequently the age related increase of SHBG does not account for the age related decline in non-SHBG-T in healthy adult men and the high SHBG levels in infants are not the cause of the relatively low bioavailable testosterone levels in the first three months of life. Testosterone is aromatized to estradiol (d). The extent of androgen aromatisation is positively associated with BMI and is slightly higher in carriers of the T allele of the studied 3'UTR CYP 19 polymorphism. The

changes in estradiol levels associated with this polymorphism are probably too small to significantly affect gonadotropin secretion.

Estradiol is also bound by SHBG although with lower affinity as compared to testosterone (e). Higher SHBG levels are associated with lower bioavailable estradiol levels. The plasma estradiol concentration is not only dependent on the testosterone concentration but also on the concentrations of androstenedione and estrone secreted by the adrenal glands (f). Circulating estradiol has an important inhibitive effect on LH and FSH secretion and thereby on the plasma testosterone concentration (g). For this effect, local aromatization of androgens near the pituitary or hypothalamus is not a prerequisite. Although not investigated in our studies, testosterone probably has an estradiol independent effect on gonadotropin release by the hypothalamus and/or pituitary (h).

With such a complicated model, which is far from complete, interpreting levels of steroids in men remains challenging. Because of the association between SHBG and total testosterone and estradiol, measuring bioavailable or free fractions is preferred. However, since both measurement and calculation of bioavailable and free testosterone have their limitations, estimates should be interpreted with caution until accurate methods become available for routine measurement of free or bioavailable estradiol and testosterone concentrations.

The results of the studies described in this thesis have generated new intriguing questions and have left other issues unresolved leaving numerous topics for further research. Some of these will be discussed in more detail.

Perspectives for further research

-The male HPG axis setpoint hypothesis

It is generally assumed that the activity of the male HPG axis is tightly regulated. It would be interesting to evaluate whether such a postulated setpoint really exists and whether this setpoint changes under different circumstances. One way of testing this is by partially compromising testicular testosterone production, for instance by administering the enzyme inhibitor ketoconazole (46). One would assume that the decline in testosterone level will be compensated for by an increase in LH production, resulting in normalization of the baseline testosterone levels. If the HPG axis of old men is able to overcome the partial blockade inflicted by ketoconazole administration, this would indicate that the setpoint for bioavailable testosterone is actively reduced with aging which may change the appreciation of lower testosterone levels in older men.

This protocol can be used as a function test for the male HPG axis. If, after ketoconazol administration, testosterone levels return to baseline levels, dysfunction of the HPG axis is highly unlikely. The test may have advantages over current tests such as the GnRH stimulation test or the HCG stimulation test because it tests all components of the male HPG axis.

-The relative influence of testosterone and estradiol on the feedback inhibition of gonadotropin release

The ketoconazole protocol is based on the generally accepted assumption that bioavailable or free testosterone is primarily responsible for the feedback inhibition of gonadotropin secretion in men. However, in chapter **6** we showed that circulating estradiol levels have a very strong effect on LH, FSH and testosterone levels. High testosterone levels could not prevent a rise in LH and FSH when estradiol levels were low. The question arises what the independent effects on LH release are of testosterone and estradiol. In chapter **6** the dependency of estradiol on testosterone levels was artificially bypassed by administering an aromatase inhibitor after which estradiol levels were varied using estradiol patches. In this study testosterone was used as a readout of HPG axis activity. However, the varying testosterone levels may have influenced the gonadotropin response to estradiol manipulation. This could be prevented by repeating the study in gonadectomized men. In such a model the specific effects of estradiol on gonadotropin release can be monitored because the presumed counterregulatory effects of testosterone are absent. The effect of testosterone on LH release, independent of estradiol, will be represented by the difference in the LH responses in agonadal and intact men. Ideally a model will be studied in which circulating testosterone can be manipulated independent of estradiol levels. Although complicated this could be done in men with primary gonadal insufficiency. Estradiol levels can be "clamped" by administering letrozole 2,5 mg once daily in combination with an estradiol patch delivering 25 micrograms per day. In these men testosterone levels can be varied by applying testosterone patches or testosterone gel and the effects on LH levels can be monitored.

-Relative effects of SHBG on clearance and production of testosterone and estradiol in eugonadal men

In chapters **2** and **3** the positive relationship between SHBG and testosterone was described. It is unclear whether this relationship can be explained by the effect of SHBG on testosterone clearance. Ideally the plasma SHBG concentration should be artificially varied and the

response of LH and testosterone measured. To make a definitive discrimination between effects of SHBG on clearance or production rates of testosterone this experiment should be executed in normal subjects and subjects without a functional HPG axis (testosterone treated hypogonadal men). For a correct interpretation of the results, testosterone delivery in the hypogonadal subjects must be stable during the course of the study. Probably this can only be accomplished by using ultra-long acting testosterone depot preparations. SHBG levels can be manipulated by various substances such as thyroxin, oral estrogens, oral androgens or metformin. With all these interventions an effect on clearance or production rates of the studied hormones independent of SHBG can not be excluded. In fact, administration of estrogens to eugonadal men will certainly decrease the production rate of testosterone as described in chapter **6**. Perhaps short term exposure to oral estrogens may suffice to increase SHBG levels for a longer period, allowing the HPG axis to adapt to the altered SHBG level in the absence of exogenous estrogens.

-Evaluation of local hormone processing

The peripheral concentrations of androgens and estrogens can be considered to reflect the mean of the plasma concentrations in different tissues of the body. As described in chapter **1** several enzymes, distributed throughout the body, are capable of converting steroid hormones into more or less active metabolites. The distribution and activity of these enzymes might influence local tissue concentrations. We already alluded to this possibility in chapter **6** in which we investigated whether local aromatization near the pituitary or hypothalamus is a prerequisite for the inhibitory effects of estradiol on gonadotropin release. In this experiment we used two interventions (aromatase inhibition and estrogen replacement) to obtain indirect evidence that the estradiol concentration in the brain is not very much different from the peripheral estradiol concentration. Direct measurement of hormones *in vivo* can be accomplished by using the microdialysis technique (47). Measuring steroid hormones using this technique is hampered by several problems. The hormone concentrations in the tissues are the non-albumin-non-SHBG bound concentrations only representing a limited fraction of the circulating total hormone concentration. Therefore, extremely sensitive assays are needed for proper measurements. The materials of the catheters and tubing have to be chosen carefully to prevent extensive loss of hormones from sticking to the material. Of course, not all tissues can be reached by using this technique and therefore measurements in healthy volunteers will be limited to muscle and fat tissue. However, using this technique we have been able to measure the testosterone level in fat tissue *in vivo* (unpublished data). Further

exploration of this technique is needed to gain more insight in the extent and importance of local steroid hormone modulation.

Reference List

1. **T'Sjoen G, Goemaere S, De Meyere M, Kaufman JM** 2004 Perception of males' aging symptoms, health and well-being in elderly community-dwelling men is not related to circulating androgen levels. Psychoneuroendocrinology 29:201-214

2. **Kaufman JM, Vermeulen A** 2005 The decline of androgen levels in elderly men and its clinical and therapeutic implications. Endocr Rev 26:833-876

3. **Nieschlag E, Swerdloff R, Behre HM, Gooren LJ, Kaufman JM, Legros JJ, Lunenfeld B, Morley JE, Schulman C, Wang C, Weidner W, Wu FC** 2005 Investigation, treatment and monitoring of late-onset hypogonadism in males: ISA, ISSAM, and EAU recommendations. Int J Androl 28:125-127

4. **Saez JM, Morera AM, Dazord A, Bertrand J** 1972 Adrenal and testicular contribution to plasma oestrogens. J Endocrinol 55:41-49

5. **Scholler R, Nahoul K, Castanier M, Rotman J, Salat-Baroux J** 1984 Testicular secretion of conjugated and unconjugated steroids in normal adults and in patients with varicocele. Baseline levels and time-course response to hCG administration. J Steroid Biochem 20:203-215

6. **Baird DT, Horton R, Longcope C, Tait JF** 1969 Steroid dynamics under steady-state conditions. Recent Prog Horm Res 25:611-664

7. **MacDonald PC, Madden JD, Brenner PF, Wilson JD, Siiteri PK** 1979 Origin of estrogen in normal men and in women with testicular feminization. J Clin Endocrinol Metab 49:905-916

8. **Adamski J, Jakob FJ** 2001 A guide to 17beta-hydroxysteroid dehydrogenases. Mol Cell Endocrinol 171:1-4

9. **Smith EP, Boyd J, Frank GR, Takahashi H, Cohen RM, Specker B, Williams TC, Lubahn DB, Korach KS** 1994 Estrogen resistance caused by a mutation in the estrogen-receptor gene in a man. N Engl J Med 331:1056-1061

10. **Herrmann BL, Saller B, Janssen OE, Gocke P, Bockisch A, Sperling H, Mann K, Broecker M** 2002 Impact of estrogen replacement therapy in a male with congenital aromatase deficiency caused by a novel mutation in the CYP19 gene. J Clin Endocrinol Metab 87:5476-5484

11. **Morishima A, Grumbach MM, Simpson ER, Fisher C, Qin K** 1995 Aromatase deficiency in male and female siblings caused by a novel mutation and the physiological role of estrogens. J Clin Endocrinol Metab 80:3689-3698

12. **Simpson ER, Davis SR** 2001 Minireview: aromatase and the regulation of estrogen biosynthesis--some new perspectives. Endocrinology 142:4589-4594

13. **Bilezikian JP, Morishima A, Bell J, Grumbach MM** 1998 Increased bone mass as a result of estrogen therapy in a man with aromatase deficiency. N Engl J Med 339:599-603

14. **Carani C, Qin K, Simoni M, Faustini-Fustini M, Serpente S, Boyd J, Korach KS, Simpson ER** 1997 Effect of testosterone and estradiol in a man with aromatase deficiency. N Engl J Med 337:91-95

15. **Maffei L, Murata Y, Rochira V, Tubert G, Aranda C, Vazquez M, Clyne CD, Davis S, Simpson ER, Carani C** 2004 Dysmetabolic syndrome in a man with a novel mutation of the aromatase gene: effects of testosterone, alendronate, and estradiol treatment. J Clin Endocrinol Metab 89:61-70

16. **Rivarola MA, Singleton RT, Migeon CJ** 1967 Splanchnic extraction and interconversion of testosterone and androstenedione in man. J Clin Invest 46:2095-2100

17. **Vermeulen A, Ando S** 1979 Metabolic clearance rate and interconversion of androgens and the influence of the free androgen fraction. J Clin Endocrinol Metab 48:320-326

18. **Saez JM, Forest MG, Morera AM, Bertrand J** 1972 Metabolic clearance rate and blood production rate of testosterone and dihydrotestosterone in normal subjects, during pregnancy, and in hyperthyroidism. J Clin Invest 51:1226-1234

19. **Longcope C, Johnston CC, Jr.** 1988 Androgen and estrogen dynamics in pre- and postmenopausal women: a comparison between smokers and nonsmokers. J Clin Endocrinol Metab 67:379-383

20. **Longcope C, Baker S** 1993 Androgen and estrogen dynamics: relationships with age, weight, and menopausal status. J Clin Endocrinol Metab 76:601-604

21. **Damassa DA, Gustafson AW** 1988 Effects of chronic infusions of sex steroid-binding protein on the testosterone-mediated inhibition of gonadotropin secretion and maintenance of sex accessory glands in male rats. Endocrinology 123:1885-1892

22. **Sowers M, Derby C, Jannausch ML, Torrens JI, Pasternak R** 2003 Insulin resistance, hemostatic factors, and hormone interactions in pre- and perimenopausal women: SWAN. J Clin Endocrinol Metab 88:4904-4910

23. **Pasquali R, Vicennati V, Bertazzo D, Casimirri F, Pascal G, Tortelli O, Labate AM** 1997 Determinants of sex hormone-binding globulin blood concentrations in premenopausal and postmenopausal women with different estrogen status. Virgilio-Menopause-Health Group. Metabolism 46:5-9

24. **Kalish GM, Barrett-Connor E, Laughlin GA, Gulanski BI** 2003 Association of endogenous sex hormones and insulin resistance among postmenopausal women: results from the Postmenopausal Estrogen/Progestin Intervention Trial. J Clin Endocrinol Metab 88:1646-1652

25. **Sodergard R, Backstrom T, Shanbhag V, Carstensen H** 1982 Calculation of free and bound fractions of testosterone and estradiol-17 beta to human plasma proteins at body temperature. J Steroid Biochem 16:801-810

26. **Plant TM** 1980 The effects of neonatal orchidectomy on the developmental pattern of gonadotropin secretion in the male rhesus monkey (Macaca mulatta). Endocrinology 106:1451-1454

27. **Vermeulen A** 1991 Clinical review 24: Androgens in the aging male. J Clin Endocrinol Metab 73:221-224

28. **Vermeulen A, Verdonck L, Kaufman JM** 1999 A critical evaluation of simple methods for the estimation of free testosterone in serum. J Clin Endocrinol Metab 84:3666-3672

29. **Emadi-Konjin P, Bain J, Bromberg IL** 2003 Evaluation of an algorithm for calculation of serum "bioavailable" testosterone (BAT). Clin Biochem 36:591-596

30. **Morris PD, Malkin CJ, Channer KS, Jones TH** 2004 A mathematical comparison of techniques to predict biologically available testosterone in a cohort of 1072 men. Eur J Endocrinol 151:241-249

31. **Ly LP, Handelsman DJ** 2005 Empirical estimation of free testosterone from testosterone and sex hormone-binding globulin immunoassays. Eur J Endocrinol 152:471-478

32. **Davies R, Collier C, Raymond M, Heaton J, Clark A** 2002 Indirect measurement of bioavailable testosterone with the Bayer Immuno 1 system. Clin Chem 48:388-390

33. **Longcope C, Pratt JH, Schneider SH, Fineberg SE** 1978 Aromatization of androgens by muscle and adipose tissue in vivo. J Clin Endocrinol Metab 46:146-152

34. **Schindler AE, Ebert A, Friedrich E** 1972 Conversion of androstenedione to estrone by human tissue. J Clin Endocrinol Metab 35:627-630

35. **Schweikert HU, Wolf L, Romalo G** 1995 Oestrogen formation from androstenedione in human bone. Clin Endocrinol (Oxf) 43:37-42

36. **Janssen JM, Bland R, Hewison M, Coughtrie MW, Sharp S, Arts J, Pols HA, van Leeuwen JP** 1999 Estradiol formation by human osteoblasts via multiple pathways: relation with osteoblast function. J Cell Biochem 75:528-537

37. **Kley HK, Deselaers T, Peerenboom H, Kruskemper HL** 1980 Enhanced conversion of androstenedione to estrogens in obese males. J Clin Endocrinol Metab 51:1128-1132

38. **Vermeulen A, Kaufman JM, Giagulli VA** 1996 Influence of some biological indexes on sex hormone-binding globulin and androgen levels in aging or obese males. J Clin Endocrinol Metab 81:1821-1826

39. **Kristensen VN, Harada N, Yoshimura N, Haraldsen E, Lonning PE, Erikstein B, Karesen R, Kristensen T, Borresen-Dale AL** 2000 Genetic variants of CYP19 (aromatase) and breast cancer risk. Oncogene 19:1329-1333

40. **T'sjoen GG, Giagulli VA, Delva H, Crabbe P, De Bacquer D, Kaufman JM** 2005 Comparative Assessment in Young and Elderly Men of the Gonadotropin Response to Aromatase Inhibition. J Clin Endocrinol Metab

41. **Faiman C, Winter JS** 1974 The control of gonadotropin secretion in complete testicular feminization. J Clin Endocrinol Metab 39:631-638

42. **Lacroix A, McKenna TJ, Rabinowitz D** 1979 Sex steroid modulation of gonadotropins in normal men and in androgen insensitivity syndrome. J Clin Endocrinol Metab 48:235-240

43. **Veldhuis JD, Urban RJ, Dufau ML** 1994 Differential responses of biologically active luteinizing hormone secretion in older versus young men to interruption of androgen negative feedback. J Clin Endocrinol Metab 79:1763-1770

44. **Khosla S, Melton LJ, III, Atkinson EJ, O'Fallon WM** 2001 Relationship of serum sex steroid levels to longitudinal changes in bone density in young versus elderly men. J Clin Endocrinol Metab 86:3555-3561

45. **Khosla S, Melton LJ, III, Robb RA, Camp JJ, Atkinson EJ, Oberg AL, Rouleau PA, Riggs BL** 2005 Relationship of volumetric BMD and structural parameters at different skeletal sites to sex steroid levels in men. J Bone Miner Res 20:730-740

46. **Nashan D, Knuth UA, Weidinger G, Nieschlag E** 1989 The antimycotic drug terbinafine in contrast to ketoconazole lacks acute effects on the pituitary-testicular function of healthy men: a placebo-controlled double-blind trial. Acta Endocrinol (Copenh) 120:677-681

47. **Lameris TW, van Den Meiracker AH, Boomsma F, Alberts G, de Zeeuw S, Duncker DJ, Verdouw PD, Veld AJ** 1999 Catecholamine handling in the porcine heart: a microdialysis approach. Am J Physiol 277:H1562-H1569

Summary

Testosterone is responsible for the development of the primary and secondary male sex characteristics such as male pattern hair growth, deepening of the voice and increased lean body mass. Testosterone is produced in the testicular Leydig cells as a result of stimulation by pituitary derived luteinizing hormone (LH). On its turn the pituitary LH secretion is regulated by the hypothalamus. Testosterone will feed back onto the pituitary and hypothalamus thereby allowing the hypothalamo-pituitary-testicular (HPG) axis to maintain the plasma testosterone concentration within close limits. The serum testosterone concentration is considered normal when within the reference range as supplied by the laboratory. The lower limit of this reference range represents the 2,5 percentile of a group of apparently healthy young men. However, testosterone levels may vary considerably between individuals. From this it might be concluded that every individual might have a specific setpoint for the regulation of the HPG axis, which means that testosterone levels within an individual may be regulated within much closer limits compared to the reference ranges supplied by the laboratory. Older men may have signs and symptoms reminiscent of testosterone deficiency such as lack of libido, erectile dysfunction and lower bone and lean body mass. In older men the mean testosterone concentration in blood is lower compared to young men. The question is whether the above-mentioned symptoms truly represent testosterone deficiency. For an adequate answer to this question a better understanding of the determinants of the serum testosterone level in men is necessary in order to better differentiate between normal and abnormal levels in a specific individual. In the body testosterone is converted to estradiol. In the past ten years in has become evident that estradiol is responsible for a number of the effects formerly attributed to testosterone. Therefore, measuring estradiol in men seems appropriate. Estradiol levels may vary significantly between men. Also, estradiol and testosterone levels may be interdependent. The aim of the present thesis is to gain more insight into the determinants of the testosterone and estradiol concentrations in men.

In chapter **1** the importance of estradiol in men is described. Estradiol has an important role in aquiring peak bone mass and maintaining bone mass at older age. It is responsible for closure of the epiphyses during puberty and inhibits LH release by the pituitary and thereby testosterone secretion by the testes.
In chapter **2** the importance of sex hormone binding globulin (SHBG) is discussed. Most of the testosterone and estradiol assays only measure total hormone concentrations and cannot discriminate between the bound and unbound fractions of testosterone and estradiol. It is

generally accepted that only the unbound fractions can be considered to be biologically active. Therefore, hormone sensitive tissues, such as the pituitary and hypothalamus will only respond to the unbound hormone concentration. When the SHBG level increases, unbound hormone levels will decrease. As a result the pituitary will increase its LH production and stimulate testosterone production in order to maintain bioavailable hormone concentrations at their predefined levels. SHBG bound hormone is less well cleared from the circulation by the liver. As a result SHBG is a potential determinant of testosterone and estradiol levels in men. The relationships between the serum levels of SHBG, testosterone and estradiol were studied in 400 men and are described in chapter **2**. As expected, higher SHBG levels were associated with higher testosterone and estradiol levels. However, SHBG levels were not associated with bioavailable testosterone levels, whereas there was a negative relationship between SHBG and bioavailable estradiol levels. As a result, SHBG has a potentially negative effect on estrogen action in men.

In chapter **3** these relationships are evaluated in combination with age. The serum SHBG concentration varies considerably throughout life; high levels shortly after birth reaching a plateau in the first year of life which is maintained until puberty and steadily increasing levels after adolescence. Therefore we evaluated the relationship between SHBG and testosterone in a group of male infants and in adult men with ages between 40 and 80 years. In infants mean SHBG levels were high. No relationship was found between the levels of SHBG and bioavailable testosterone. The level of bioavailable testosterone at this age is low but we concluded that the high serum level of SHBG is not the cause of this. In the adult men there was an age related decrease in the level of bioavailable testosterone accompanied by an increase in the level of SHBG. Our analyses showed that the age related change in SHBG is not the cause of the age related decline of bioavailable testosterone levels. In fact, we did not find a significant association between levels of SHBG and bioavailable testosterone at any age and from this we conclude that age related variations in the level of SHBG are not the cause of age associated fluctuations in the level of bioavailable testosterone.

From this study it appeared that in men the concentration of SHBG is a determinant of the total testosterone concentration but not of the bioavailable testosterone concentration. Since the latter is considered bioactive it seems evident to prefer the bioavailable hormone concentration over the total level for the evaluation of a patient's hormone status. However, measuring bioavailable hormone concentrations is a technically demanding and expensive procedure whereas measuring levels of SHBG and total testosterone can be automated and is

relatively cheap. For this reason several labs have independently measured bioavailable testosterone, SHBG and total testosterone in a large number of samples and created an algorithm in order to predict bioavailable testosterone levels on basis of the levels of SHBG and total testosterone in the same sample.
In chapter **4** the results of five algorithms were compared using the SHBG and total testosterone concentrations measured in 399 adult men. From this comparison it appeared that the different algorithms resulted in divergent results even if the algorithms were based on similar measuring techniques. We conclude that one should be very cautious when interpreting calculated and even measured bioavailable testosterone levels.

As described in chapter **1** not only testosterone but also estradiol is of importance for men. In chapter **5** the origin of circulating estrogens in men is investigated. Estradiol is produced by conversion of testosterone via aromatase or by conversion of estrone via the enzyme 17β-hydroxysteroid dehydrogenase. These conversions can take place throughout the body although primarily in muscle and adipose tissue. There are two potential sources for estradiol or its precursors in men; the testes and the adrenal glands. To estimate the relative contribution of the two glands to the pool of circulating estradiol we compared the levels of testosterone, androstenedione, estrone and estradiol of postmenopausal women and elderly men. We assumed that the postmenopausal ovary did not significantly contribute to the levels of the four mentioned hormones and adjusted for the different dehydroepiandrosterone sulphate (DHEAS) levels between men and women. The difference in the estrogen levels between men and women must be the result of steroid secretion from the testes. We estimated that the testes contribute for more than 83% to the serum testosterone concentration in men and between 44 and 72% to the estradiol concentration. The latter is dependent on the activity of the adrenal glands represented by the level of DHEAS. We concluded that both the testes and the adrenal glands contribute substantially to the serum estradiol concentration in elderly men.

For CYP 19, the aromatase gene, several polymorphisms were described. In chapter **6** we investigated the relationship between a polymorphism in the 3' UTR of CYP 19 and found that men who carried the CC genotype, had a 9% lower mean estradiol level as compared to carriers of the T allele. As described in chapter **1** estradiol can inhibit gonadotropin secretion and we assumed that lower estradiol levels might be associated with higher LH and testosterone levels. However, we did not find significant differences in levels of LH or

testosterone between carriers of the various genotypes. Perhaps the difference in mean estradiol level between the groups was too small to significantly affect HPG axis activity.

Aromatase has several tissue specific promoters which makes it plausible to assume that local estradiol levels may vary between tissues within one individual.
One might assume that the tissue concentration of estradiol near the pituitary and hypothalamus may be different from the plasma concentration of estradiol. As a result the serum level of estradiol may not represent the estrogenic effect on gonadotropin secretion. To evaluate whether peripheral estradiol levels give a reliable estimate of the central estradiol concentration near the pituitary and hypothalamus we suppressed aromatase activity in 10 healthy volunteers by administering the aromatase inhibitor letrozole (chapter **7**). As a result the central estradiol concentration became dependent on the estradiol supply from the bloodstream. During aromatase inhibition estradiol levels decreased and levels of LH, FSH and testosterone increased. Subsequently we varied serum estradiol levels by applying estradiol patches and monitored their effect on levels of gonadotropins and testosterone. It appeared that restoring the estradiol concentration to baseline levels sufficed to normalize peripheral levels of testosterone and LH. We concluded that aromatization of testosterone near the hypothalamus and pituitary is not a prerequisite for the action of estradiol on gonadtropin release in men.

As described in chapter **1** estradiol is important for bone maintenance in adult men. In cross sectional studies lower estradiol levels are associated with lower bone mineral density (BMD). A low BMD is associated with an increased fracture risk. In chapter **8** we evaluated whether elderly men and women who developed a vertebral fracture during a mean follow up of 6.5 years differed in their hormone levels compared to subjects without a fracture. In both men and women no significant differences were seen in the mean estradiol level between persons with and without a fracture at the start of the investigation. However, women with the lowest estradiol levels had a significantly higher risk of developing a fracture. This was not found in men. For this difference two explanations can be postulated. First, the group of men was smaller compared to the women which decreased statistical power. Secondly, it is assumed that for the effect on bone a minimal amount of estradiol is necessary to maintain bone mass. As described in chapter **5** older men have higher estradiol levels compared to postmenopausal women. Therefore the relationship between plasma estrogen levels and BMD may be less critical in men.

With our combined results a model can be created for the interaction between estradiol and testosterone in men. LH, produced by the pituitary, stimulates the production of testosterone by the testes. Testosterone is secreted into the blood and partially bound to SHBG. In men SHBG is a determinant of total testosterone but not of bioavailable testosterone. Age related changes in SHBG do not contribute to age related changes in bioavailable testosterone. Testosterone is aromatized to estradiol. The extent of androgen aromatization is associated with the Body Mass Index and with a 3'UTR CYP 19 polymorphism. Estradiol is also partially bound by SHBG. In men the level of SHBG is negatively associated with the level of bioavailable estradiol. The serum estradiol concentration is largely determined by the concentrations of precursor hormones such as testosterone, mainly produced by the testes and androstenedione mainly produced by the adrenal glands. Estradiol has an important inhibitive effect on gonadotropin and testosterone secretion in men. The result is a complicated model, which still causes difficulties in the interpretation of testosterone and estradiol levels in men. Things are even more complicated because estimates of bioavailable testosterone are highly unreliable. Adding an estradiol measurement to the work up of men with suspected hypogonadism in most instances will currently not lead to a more accurate diagnosis. Therefore additional research needs to be performed. Suggestions for these investigations are provided in Chapter 9.

Samenvatting

Testosteron (het mannelijke geslachtshormoon) is verantwoordelijk voor de ontwikkeling van typisch mannelijk kenmerken als baardgroei, verzwaring van de stem en toename van de spiermassa. Testosteron wordt gevormd in de zaadballen (testes). De testes zullen alleen testosteron produceren wanneer zij worden gestimuleerd door een door het hersenaanhangsel (de hypofyse) geproduceerd hormoon, het luteiniserend hormoon (LH). De hypofyse wordt op zijn beurt weer gereguleerd door een deel van de hersenen, de hypothalamus. De hypofyse en hypothalamus kunnen de concentratie van testosteron in het bloed meten en zullen de stimulatie van de testes verminderen wanneer de testosteronconcentratie te hoog dreigt te worden. De hypofyse en hypothalamus treden als het ware op als de thermostaat van de testosteronproductie. De testosteronconcentratie in het bloed wordt normaal genoemd wanneer deze valt binnen de door het laboratorium vastgestelde referentiewaarden. De ondergrens van deze referentiewaarden wordt zodanig gesteld dat 97,5% van een groep ogenschijnlijk gezonde jonge mannen een testosteronspiegel boven deze grens heeft. Er blijkt echter een grote variatie in testosteronconcentratie te zijn tussen mannen onderling. Hieruit zou kunnen worden afgeleid dat iedere man een individueel setpoint heeft waarop de Hypothalamus-Hypofyse-Testes-as is afgesteld, een setpoint dat veel nauwere grenzen kent dan omschreven in de zogenaamde referentiewaarden.

Oudere mannen hebben vaak klachten die passen bij testosterongebrek; verminderde zin in seks en een verminderde bot- en spiermassa. Ook is de testosteronspiegel bij oudere mannen gemiddeld lager dan bij jonge mannen. Het is dan ook de vraag in hoeverre de verouderingsverschijnselen hun oorzaak vinden in de verlaagde testosteronspiegel. Deze vraag laat zich moeilijk beantwoorden zolang niet duidelijk is welke testosteronwaarde als normaal voor de persoon in kwestie kan worden aangehouden.

Testosteron wordt in het lichaam omgezet tot het hormoon oestradiol. De afgelopen jaren is duidelijk geworden dat dit oestradiol verantwoordelijk is voor een belangrijk deel van de effecten die voorheen werden toegeschreven aan testosteron. Het ligt dus voor de hand naast testosteron ook de oestradiolspiegel van mannen te beoordelen. Ook de oestradiolspiegel kan tussen mannen behoorlijk verschillen. Bovendien kunnen testosteron en oestradiol elkaars spiegels beïnvloeden.

Het doel van het onderzoek beschreven in dit proefschrift is helderheid te krijgen over de determinanten van de testosteron en oestradiolconcentraties in het bloed van mannen. Met behulp van deze informatie kan hopelijk beter worden ingeschat of in individuele gevallen de testosteron- en oestradiolspiegels als normaal of abnormaal moeten worden gekwalificeerd.

In hoofdstuk **1** wordt uitgebreid beschreven wat het belang is van oestradiol, het hormoon dat traditioneel als "vrouwelijk" wordt gezien, bij de man. Oestradiol heeft een belangrijke rol bij de opbouw en het behoud van de hoeveelheid kalk in het bot; de botmineraaldichtheid. Ook is oestradiol verantwoordelijk voor het sluiten van de groeischijven tijdens de puberteit. Oestradiol is in staat de afgifte van LH door de hypofyse te remmen en daarmee de productie van testosteron door de testes.

In hoofdstuk **2** wordt ingegaan op het belang van Sex Hormoon Bindend Globuline (SHBG), een eiwit dat in het bloed aanwezig is en zowel testosteron als oestradiol aan zich kan binden. Bij de gangbare bloedbepaling wordt de totale hoeveelheid testosteron of oestradiol in het bloed gemeten en kan geen onderscheid gemaakt worden tussen het hormoon dat wel en het hormoon dat niet aan SHBG gebonden is. De heersende gedachte is dat hormoon dat aan dit SHBG gebonden is niet werkzaam is. Dit betekent dat de hypofyse en hypothalamus, maar ook de rest van het lichaam, alleen zullen reageren op de aanwezigheid van het ongebonden hormoon. Als de SHBG spiegel hoog is zal de hypofyse de indruk kunnen krijgen dat er weinig hormoon beschikbaar is, reageren met een verhoging van de LH productie en daarmee de testosteron aanmaak stimuleren. Aan SHBG gebonden hormoon wordt minder snel door de lever uit het bloed verwijderd. Via beide mechanismen kan SHBG dus de spiegels van testosteron en oestradiol beïnvloeden. De relatie tussen de spiegels van SHBG, testosteron en oestradiol werd bestudeerd in 400 mannen en beschreven in hoofdstuk **2**. Het blijkt dat hogere SHBG spiegels inderdaad samengaan met hogere spiegels van zowel testosteron als oestradiol. Als echter gekeken wordt naar de concentraties van het ongebonden (niet aan SHBG gebonden) hormoon, dan lijkt SHBG geen effect te hebben op de concentratie van ongebonden testosteron maar gaan hogere SHBG bloedspiegels wel gepaard met lagere spiegels van ongebonden oestradiol. SHBG heeft dus een potentieel nadelig effect op de werking van oestradiol.

In hoofdstuk **3** worden deze relaties bekeken in relatie tot leeftijd. De SHBG concentratie kan gedurende het leven behoorlijk variëren; hoge waardes kort na de geboorte, een plateau tot aan de puberteit, een daling van de gemiddelde concentratie tijdens de puberteit en een gestage stijging na de adolescentie. Om die reden werd de relatie tussen de SHBG en testosteronconcentraties beoordeeld bij pasgeborenen en mannen met een leeftijd tussen de 40 en 80 jaar. Bij pasgeborenen worden hoge SHBG spiegels gemeten maar er is geen relatie tussen de hoogte van het SHBG en de concentratie van ongebonden testosteron. De concentratie van ongebonden testosteron op deze leeftijd is laag maar wij concluderen dat de hoge SHBG concentratie hier niet de oorzaak van is. Vanaf de adolescentie neemt de

gemiddelde ongebonden testosteronconcentratie af terwijl de gemiddelde SHBG spiegel stijgt. Het ligt voor de hand te veronderstellen dat de leeftijdsgebonden stijging van het SHBG gehalte de leeftijdsgebonden daling van het ongebonden testosterongehalte veroorzaakt. Onze analyses laten echter zien dat er op geen enkele leeftijd een relevant verband bestaat tussen de concentraties van SHBG en die van het ongebonden testosteron en wij trekken daaruit de conclusie dat leeftijdsgebonden variaties van de SHBG spiegel bij pasgeborenen en bij volwassen mannen niet de oorzaak zijn van leeftijdsgebonden schommelingen van de ongebonden testosteronconcentratie.

Uit het voorgaande is gebleken dat bij mannen de concentratie van SHBG wel een determinant is van de totale testosteronconcentratie maar niet van de ongebonden testosteronconcentratie. Aangezien de laatste biologisch actief is ligt het voor de hand bij voorkeur de ongebonden hormoonconcentratie te betrekken bij de evaluatie van de hormoonstatus van een patiënt. Probleem is dat het meten van de ongebonden hormoonconcentratie lastig en kostbaar is terwijl het meten van de concentraties van totaal testosteron, totaal oestradiol en SHBG vrij eenvoudig is. Om die reden heeft een aantal laboratoria onafhankelijk van elkaar bij een grote groep mannen het SHBG en de totale en ongebonden testosteron concentratie gemeten waarna een algoritme werd gecreëerd waarmee met behulp van alleen de SHBG en totale testosteronconcentratie de ongebonden testosteronconcentratie bij andere personen kon worden voorspeld.
In hoofdstuk **4** worden de uitkomsten van 5 van deze algoritmes met elkaar vergeleken met behulp van testosteron en SHBG concentraties van 399 mannen. Uit deze vergelijking bleek ons dat verschillende algoritmes tot uitlopende resultaten kunnen leiden, zelfs wanneer de algoritmen waren gebaseerd op een zelfde bepalingsmethode voor ongebonden testosteron. De oorzaken van deze uiteenlopende uitkomsten zijn waarschijnlijk gelegen in de onnauwkeurigheid van de bepalingsmethoden voor ongebonden testosteron. Wij concludeerden dat men zeer terughoudend moet zijn met de interpretatie van berekende en zelfs gemeten concentraties van ongebonden testosteron.

Zoals beschreven in hoofdstuk **1** is behalve testosteron ook oestradiol van belang voor de man. In hoofdstuk **5** stelden wij ons de vraag wat precies de herkomst van oestradiol bij de man is. Oestradiol wordt gevormd door omzetting van testosteron via het enzym aromatase of door omzetting van oestron via het enzym 17β-hydroxysteroid dehydrogenase. Deze omzettingsreacties vinden voor een belangrijk deel plaats in spier- en vetweefsel. Er zijn twee

potentiële bronnen voor (de voorlopers van) oestradiol; de zaadballen en de bijnieren. In beide klieren zijn de enzymen aanwezig die noodzakelijk zijn voor de testosteron en oestradiolvorming. Om de relatieve bijdrage van de twee klieren te schatten werden de hormoonconcentraties bij oudere mannen en vrouwen vergeleken. Onze aanname was dat bij de vrouwen, allen na de menopause, alleen de bijnieren bijdragen aan de bloedspiegels van oestradiol en testosteron. Bij de mannen dragen zowel de bijnieren als de zaadballen bij. De verschillen in de bloedspiegels tussen mannen en vrouwen moeten dan worden toegeschreven aan de aan- of afwezigheid van zaadballen. Na correctie voor verschillen in bijnieractiviteit tussen mannen en vrouwen bleek dat bij mannen de zaadballen voor meer dan 83% bijdragen aan de testosteronconcentratie in het bloed en tussen de 44 en 72% bijdragen aan de oestradiolconcentratie in het bloed. Dat laatste is afhankelijk van de activiteit van de bijnieren gemeten aan de spiegel van een ander bijnierhormoon; dehydroepiandrosteron sulfaat (DHEAS). Zowel de bijnieren als de zaadballen dragen dus in belangrijke mate aan de oestradiolconcentratie in het bloed van mannen.

Het stukje erfelijk materiaal dat verantwoordelijk is voor het enzym aromatase blijkt tussen mensen heel gering te kunnen verschillen. Het is voorstelbaar dat kleine verschillen in het erfelijk materiaal effect hebben op de werkzaamheid van het enzym en dus op de efficiëntie waarmee het enzym testosteron omzet in oestradiol. In hoofdstuk **6** vonden wij dat ongeveer een kwart van de onderzochte mannen, allen met een bepaalde variant van het erfelijk materiaal inderdaad een iets lagere gemiddelde serumconcentratie van oestradiol had. Zoals beschreven in hoofdstuk **1** kan oestradiol bij de man de afgifte van het hypofysehormoon LH remmen. Op basis daarvan kan worden verwacht dat een lagere bloedspiegel van oestradiol gepaard gaat met een hogere concentratie van LH en daarmee een hogere concentratie van testosteron. Echter, bij de onderzochte mannen werd geen verschil gevonden in testosteron of LH concentraties. Mogelijk was het verschil in oestradiolconcentratie tussen de groepen te klein om een relevant effect te hebben op de hypothalamus of hypofyse.
Een ander interessant aspect van dit enzym is dat de activiteit ervan in één persoon kan verschillen tussen verschillende weefsels. Het enzym is ook aangetoond in de hersenen. Het is dus voorstelbaar dat de oestradiolconcentratie in de hersenen anders is dan in het bloed omdat de omzetting van testosteron naar oestradiol in de hersenen groter of kleiner is dan in het spier- en vetweefsel. Een gevolg hiervan zou kunnen zijn dat het effect van oestradiol op de hypofyse en hypothalamus niet goed is af te lezen uit de oestradiolconcentratie in het bloed. Om dit te beoordelen werd bij tien gezonde mannen het enzym geremd door toediening van

het geneesmiddel letrozol (hoofdstuk **7**) waardoor de oestradiolconcentratie in de hersenen afhankelijk werd gemaakt van de aanvoer van oestradiol uit het bloed. Door het gebruik van letrozol daalde de oestradiolspiegel in het bloed en werd een stijging gezien van LH en testosteron als gevolg van de verminderde remming van de LH afgifte door de hypofyse. Tijdens de remming van het aromatase werd de reactie van LH en testosteron op verschillende bloedspiegels van oestradiol onderzocht na het toedienen van verschillende doses oestradiol door middel van huidpleisters. Uit dit experiment bleek dat bij het bereiken van de normale oestradiolconcentratie in het bloed ook de concentratie van LH en testosteron normaliseerden. Wij concludeerden hieruit dat er geen groot verschil bestaat tussen de oestradiolconcentraties in de hersenen en in het bloed en dat de concentratie van oestradiol in het bloed dus een goede maat is voor het effect van oestradiol op de hypofyse en hypothalamus.

Zoals beschreven in hoofdstuk **1** is oestradiol ook bij mannen zeer belangrijk voor behoud van de integriteit van het bot. Uit onderzoek is gebleken dat mannen met gemiddeld een lagere oestradiolconcentratie in het bloed doorgaans ook een lagere botmineraaldichtheid hebben. Een lage botmineraaldichtheid is een belangrijke risicofactor voor het krijgen van fracturen. In hoofdstuk **8** werd onderzocht of personen ouder dan 55 jaar die over een periode van gemiddeld 6.5 jaar een wervelfractuur hadden doorgemaakt op hormonaal gebied verschilden van mensen die geen fractuur hadden doorgemaakt. Zowel bij mannen als bij vrouwen werden geen opvallende hormonale verschillen gezien tussen de personen met en zonder fractuur. Wel bleek dat vrouwen die de laagste bloedconcentraties van oestradiol hadden een hoger risico hadden op een fractuur. Bij mannen werd dit niet gevonden. Hiervoor zijn een aantal verklaringen denkbaar. Ten eerste was de groep onderzochte mannen kleiner dan de groep vrouwen hetgeen de kans op het vinden van een verschil verkleint. Ten tweede bestaat het vermoeden dat een bepaalde minimale hoeveelheid oestradiol noodzakelijk is voor behoud van de botminaraaldichtheid. Aangezien mannen, zoals ook beschreven in hoofdstuk **5** gemiddeld een veel hogere oestradiolconcentratie in het bloed hebben dan vrouwen zullen zij minder snel in de gevarenzone belanden.

Met de bovenbeschreven bevindingen kan een model worden gecreëerd voor de beschrijving van de interactie tussen oestradiol en testosteron in mannen. LH, afgegeven door de hypofyse, stimuleert de afgifte van testosteron door de testes. Dit testosteron wordt afgegeven aan de bloedbaan en is deels gebonden aan SHBG. Bij mannen blijkt SHBG een determinant van de totale maar niet van de ongebonden testosteronconcentratie. Leeftijdsgebonden variaties in de

SHBG concentratie blijken niet bij te dragen aan leeftijdsgebonden variaties van de ongebonden testosteronconcentratie. Testosteron wordt omgezet tot oestradiol door het enzym aromatase. De mate van omzetting wordt beïnvloed door de hoeveelheid vetweefsel en door variaties in het enzym. Ook oestradiol wordt deels gebonden aan SHBG. Hogere SHBG concentraties gaan samen met een gemiddeld lagere ongebonden oestradiolconcentratie in het bloed. De concentratie van oestradiol wordt ook bepaald door de concentraties van voorloperhormonen als testosteron, voornamelijk geproduceerd door de testes, en androsteendion en oestron, voornamelijk afkomstig uit de bijnieren. Oestradiol heeft een belangrijk remmend effect op de afgifte van LH door het hersenaanhangsel en daarmee op de concentratie van testosteron in het bloed.
Het resultaat is een gecompliceerd model waarmee de interpretatie van testosteron en oestradiolconcentraties bij de man nog steeds buitengewoon lastig blijft. Dit wordt nog extra gecompliceerd door de bevinding dat het blijkbaar niet goed mogelijk is de concentratie van ongebonden testosteron betrouwbaar te schatten. Het toevoegen van een oestradiolmeting aan de diagnostiek bij mannen met een vermoeden op testosterongebrek draagt in de meeste gevallen waarschijnlijk weinig bij aan een betere diagnose. Aanvullend onderzoek is dus nodig. Aanbevelingen voor aanvullend onderzoek worden gedaan in hoofdstuk **9**.

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Curriculum vitae

De schrijver van dit proefschrift werd geboren op 23 december 1969 te Delft. In 1988 behaalde hij het VWO diploma aan het Christelijk Lyceum te Delft. In dat zelfde jaar begon hij aan de studie geneeskunde aan de Erasmus Universiteit te Rotterdam. Na een muzikale onderbreking van 1 jaar werd in 1994 het doctoraal- en in 1996 het artsexamen afgelegd (cum laude). Van 1996 tot 2002 volgde hij de opleiding tot internist in het Erasmus Medisch Centrum (opleiders prof.dr. M.A.D.H. Schalekamp en prof.dr. H.A.P. Pols) en aansluitend het aandachtsgebied endocrinologie (opleider prof.dr. A.J. van der Lely). Vanaf juli 2003 is hij als staflid verbonden aan de afdeling endocrinologie van het Vrije Universiteit Medisch Centrum te Amsterdam. Gedurende de opleiding tot internist werd een aanvang gemaakt met het in dit proefschrift beschreven onderzoek (promotoren prof.dr. F.H. de Jong en prof.dr. H.A.P. Pols).

De auteur van dit proefschrift is getrouwd met Nannette Huizenga, heeft twee kinderen, Michiel en Iris en woont met veel plezier te Heemstede.