

## REVIEW

## Oestrogen deficiency in men: where are we today?

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### Introduction

The precise role of oestrogen in mammals is poorly understood. Actually, the importance of oestrogen for the normal postnatal female physiology is well known, but uncertainty regarding the mechanisms and sites of action in health and disease within a variety of target tissues both in males and females does exist.

Oestrogens exert a wide range of biological effects on a large variety of cell types, for example they regulate cell growth and apoptosis, being major regulators of many physiological functions in the adult, especially those associated with reproduction. Furthermore, oestrogens are involved in the organization and differentiation of the developing organism, notably of the endocrine system, nervous system and peripheral reproductive structures. Remarkably, oestrogen receptors (ERs) were found at very early stages of embryonic development and it seemed obvious that oestrogen insensitivity would be a lethal condition as a consequence of the defective implantation of the blastocyst (1). Indeed, until recently no condition of oestrogen insensitivity had been reported, unlike well-documented inactivating mutations of other members of the steroid/thyroid/retinoid receptor gene superfamily, such as androgen (2), thyroid hormone (3, 4) and glucocorticoid hormone receptors (5). Thus, the general acceptance that oestrogens are essential for life worked until the beginning of this decade, when sporadic cases of congenital oestrogen deficiency in humans were reported (6–11) and a viable line of mutant mice lacking functional ERs was created (12, 13). Since then, the idea of a pivotal role of oestrogen for survival has begun to look thin in the light of these new findings, which undoubtedly raise many questions in turn.

Here we will discuss recent breakthroughs in our understanding of the consequences of congenital oestrogen deficiency, which vary widely within the target tissues, from the skeletal to the reproductive tract. In particular, this review addresses many aspects of congenital oestrogen deficiency in the male, including recent developments in molecular studies and treatment options.

Until now, two different conditions have been identified as possible underlying causes of congenital oestrogen deficiency in humans: (a) oestrogen resistance

caused by disruptive mutations of the ER gene (11); and (b) aromatase deficiency due to mutations of the gene encoding aromatase cytochrome P450 (P450arom) (6–10, 14–17), the enzyme responsible for aromatization of the A ring of androgens to form the A ring characteristic of oestrogens. Whatever the mechanism, all the tissue- and organ-specific physiological responses triggered by oestrogens are lacking.

### ERs

A detailed description of the structure and function of the ERs is beyond the scope of this review and has already been published (18–20). Here, we recall some simple concepts.

It is well known that all hormones require cellular receptors for their expression. In general, ERs are ligand-inducible transcription factors, which regulate the expression of target genes after hormone binding. Along with other members of the nuclear receptor superfamily, ERs consist of a hypervariable N terminus involved in the transactivation function, a highly conserved DNA-binding domain responsible for specific DNA binding and dimerization, and a C-terminal domain involved in ligand binding, nuclear localization and ligand-dependent transactivation function (18). Also the C-terminal domain shows a high conservation of amino acid sequence, most noticeably in regions known to be essential in contacting ligand (21–24). Actually, recent reports have excited this field by identifying a ligand-independent activation of the ER in both normal and neoplastic target cells (25, 26). Briefly, studies in rodents and in a human endometrial carcinoma cell line devoid of ER have indicated that growth factors, such as epidermal growth factor, are able to mimic the effects of oestrogens by coupling the growth factor and the ER signalling pathways. As a result, the two pathways, which can initiate distinct signals to modulate responses in their respective target genes, may also interact to converge in a tissue-specific response when the growth factor signal activates the ER-mediated response in oestrogen targets (13, 25, 26). Furthermore, an increasing body of evidence indicates that ER variants and mutations occur naturally in tissues and cell lines, raising the question whether they may have a role in tissue-specific oestrogen action. Their

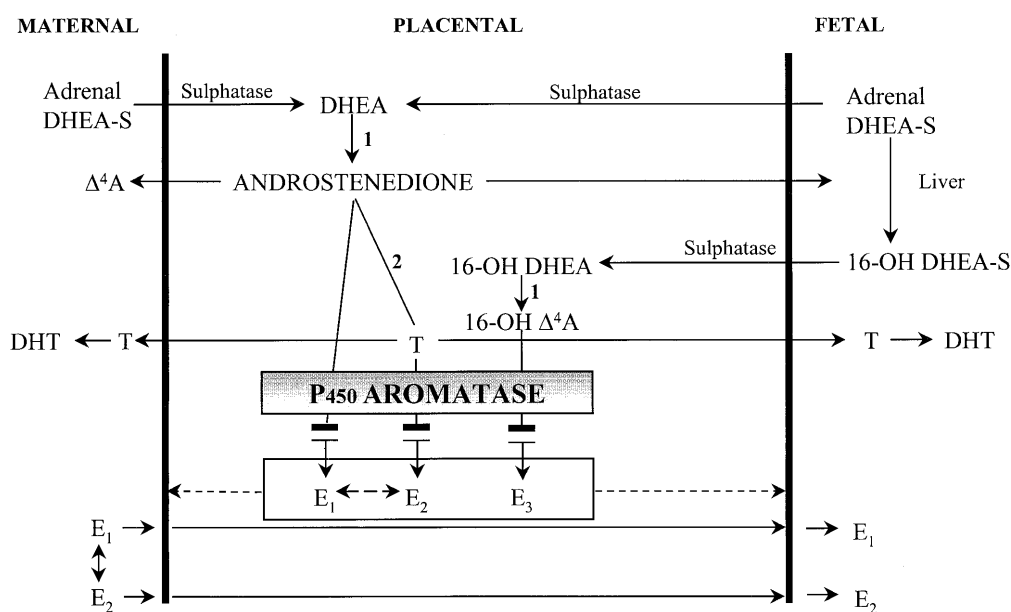
function, however, remains a mystery (for review see reference 20). Going further upstream, researchers have now succeeded in discovering an additional subtype of the ER (27–30), designated ER- $\beta$ , which is highly homologous with the previously cloned ER (ER- $\alpha$ ), thereby complicating the interpretation of earlier data on the distribution and function of ER in target tissues. Interestingly, when compared with the ER- $\alpha$ , ER- $\beta$  shows an overlapping but non-identical tissue distribution. There is no doubt that this finding will open up new perspectives for research in biology and clinical medicine, for example in stirring a debate as to whether different subtypes of receptors mediate distinct biological effects of oestrogens.

### Aromatase

The biosynthesis of  $C_{18}$  oestrogens from  $C_{19}$  androgens is catalysed by an enzyme complex known as oestrogen synthetase or aromatase, located in the endoplasmic reticulum of oestrogen-producing cells and responsible for aromatization of the A ring of androgens to form the phenolic A ring of oestrogens by three consecutive oxidation reactions (for review see reference 31). This enzyme complex comprises two components: (a) P450arom, the product of the CYP19 gene (32, 33), which binds the  $C_{19}$  substrate and catalyses the insertion of oxygen into the molecule, thus leading to the formation of  $C_{18}$  oestrogens; and (b) NADPH-cytochrome P450 reductase, a ubiquitous flavoprotein which conveys reducing equivalents to any form of cytochrome P450 it meets.

P450arom belongs to a large family of cytochrome P450 oxidases with a variable substrate-binding domain and a conserved haem-binding domain. Evidence exists that a single species of P450arom is able to metabolize all three  $C_{19}$  substrates – testosterone, androstenedione, and  $16\alpha$ -hydroxydehydroisoandrosterone – although the major oestrogen produced in each tissue is quite tissue specific, depending on the principal source of the  $C_{19}$  steroid presented to the oestrogen-synthesizing enzyme (31, 34). Thus, in women adipose tissue synthesizes primarily oestrone, since the major source of substrate is androstenedione produced by the adrenal cortex. Again, the human placenta produces a large amount of oestriol, the principal product of placental aromatase activity, as a consequence of the high concentration of  $16\alpha$ -hydroxydehydroisoandrosterone derived by two subsequent enzymatic activities: the fetal  $16\alpha$ -hydroxylation of dehydroepiandrosterone sulphate, whose production from both the fetal and the maternal adrenal gland is particularly increased during gestation, and the subsequent sulphate moiety cleavage by the placental sulphatase (Fig. 1).

In humans, the CYP19 gene is a large gene spreading over 75 kb on chromosome 15 (15q21.1) and consists of at least 16 exons. It is widely expressed in a number of tissues throughout the body, such as ovary (31, 35–38), placenta (31, 32, 38–42), testis (36, 43–54), adipose tissue (31, 55–62), muscle (55), liver (63), various sites in the brain including the hypothalamus (64–68), hair follicles (69), genital skin fibroblasts (70, 71), fetal tissues (31, 60, 72–74),



**Figure 1** Pathway of steroid biosynthesis in the fetoplacental unit in aromatase deficiency of the fetus. DHEA-S: dehydroepiandrosterone sulphate; DHEA: dehydroepiandrosterone;  $\Delta^4$ A: androstenedione;  $16\text{OH}-\Delta^4$ A:  $16\text{-hydroxyandrostenedione}$ ; T: testosterone; DHT: dihydrotestosterone;  $E_1$ : oestrone;  $E_2$ : oestradiol;  $E_3$ : oestriol; 1:  $3\beta$ -hydroxysteroid dehydrogenase; 2:  $17\text{-hydroxysteroid oxidoreductase}$ .

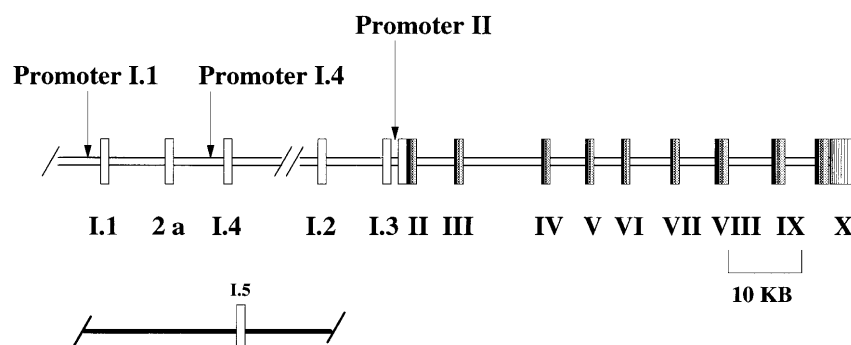
endometriosis (75) and in many human tumours including endometrial cancer (76), breast cancer (77–83), germinomas (84) and adrenocortical tumours (85). Interestingly, in most other vertebrate species aromatase activity is not so widely expressed as in humans and in many higher primates, but rather is limited to the gonads and the brain (31). In rodents there is no evidence of oestrogen biosynthesis either in adipose tissue or in the placenta. The significance of oestrogen biosynthesis in the human placenta and adipose tissue remains poorly understood. Recently, sporadic cases of female pseudohermaphroditism at birth coupled with maternal progressive virilization during the third trimester of gestation as a result of aromatase deficiency have shed new light on this issue (6, 7, 9, 10). Seven well-documented cases of aromatase deficiency have been described so far (6–10, 14–17). Of these, five were reported in females with pseudohermaphroditism (6–10, 14–16) and two in adult males (14, 17). Therefore, evidence is accumulating to suggest a putative role of placental aromatization in protecting the fetus from excessive androgen exposure.

Recent work has led to the claim that tissue-specific regulation of the CYP19 gene expression in the human is controlled in part by mechanisms involving tissue-specific promoters and alternative splicing in a tissue-specific manner (37, 40, 58–62, 71, 73, 86–88), such that in each tissue the expression of P450arom is regulated by binding of specific transactivating factors to defined genomic response elements upstream of the promoter. Briefly, the coding region of human CYP19 gene spans only nine exons beginning with exon 2, while there are a lot of untranslated first exons – corresponding to the 5' termini of the P450arom transcripts – which are spliced into a P450arom transcript in a tissue-specific fashion due to the use of tissue-specific promoters (Fig. 2). For example, expression in the placenta uses a distal promoter (untranslated exon I.1) located more than 40 kb upstream from the translational start codon in exon II, while the expression in the adipose tissue uses a

promoter (untranslated exon I.4) located 20 kb downstream from exon I.1. Because splicing of the untranslated exons occurs at a common 3' splice junction upstream of the start of translation, the primary structure of the protein product of the gene is always the same, notwithstanding the tissue site of expression. Therefore, a unique human P450arom enzyme exists and is encoded by a single-copy gene, while transcripts in different tissues differ only in the 5' termini (for review see reference 87). The comprehension of the mechanisms involved in the transcription of the CYP19 gene in different cell types may be of particular interest in the field of cancer research too, since aromatase activity in adipose tissue seems to be implicated in the development of breast and endometrial cancer (76–83), although the case is far from certain.

### The history of congenital oestrogen deficiency

As mentioned above, it had long been assumed that oestrogen action was critical for survival on the basis of the crucial role that oestrogens play in the earlier phases of development and differentiation, notably at the time of blastocyst implantation in the uterus (1), but this has recently been questioned. The opposite view focuses particularly on the studies by Kenneth Korach and colleagues (12, 13). They used the techniques of homologous recombination to disrupt expression of the mouse ER gene and produced a line of transgenic mice lacking the  $\alpha$ -form of ER (ER- $\alpha$  gene knockout, or ERKO, mice). Surprisingly, both male and female animals were viable with normal gross external phenotypes in contrast to the view that oestrogen action was essential for life. In addition, prenatal male and female reproductive tracts developed to some extent without ER action. In adulthood, female mice homozygous for the ER- $\alpha$  disruption were infertile with hypoplastic uteri, mammary agenesis and cystic haemorrhagic follicles with disrupted gametogenesis due to oogenesis blocked at the secondary follicle stage (12, 13, 89). Although



**Figure 2** Schematic structure of the human CYP19 gene, the gene encoding P450arom. The tissue-specific expression of P450arom is achieved mainly by tissue-specific alternative usage of different promoters. To date, however, the complete scenario remains to be depicted and researchers have been finding new promoters bit by bit (see reference 88).

early studies reported only a decreased fertility in male mice homozygous for the mutant ER- $\alpha$  gene disruption (12, 13), more recent reanalysis of the genotypes revealed that the males that had produced offspring were heterozygous for the mutant ER- $\alpha$  gene (90) and that male ERKO mice had relatively normal accessory sex structures, but were actually infertile with clearly disrupted spermatogenesis, reduced sperm count, motility and viability (89, 91, 92). Furthermore, ER- $\alpha$  gene disruption affected male sexual behaviour, notably the expression of intromissions and ejaculations, with only minor effects on the motivation to mount females (89, 93–95). To date, however, there is little agreement as to what neuronal cell population should be involved in the disruption of male sexual behaviours in ERKO males (93–95).

Taken together, these findings gave insight into the controversial issue of the crucial role of oestrogen for survival. Thus, the prevailing view that the disruption of the ER gene would be lethal began to be inadequate, even if the possibility that oestrogen has any role in the early development cannot be completely ruled out in the light of recent observations showing blastocysts and two-cell embryos to express ER mRNA (96).

In 1994 oestrogen resistance due to a naturally occurring disruptive mutation in the ER- $\alpha$  gene was first detected in a 28-year-old man by Smith *et al.* (11). The patient had a history of continuing linear growth into adulthood, tall stature (204 cm), unfused epiphyses, osteoporosis, delayed bone age and progressive genu valgum. Both his early growth and his pubertal development were normal. He had a full beard and normal male genitalia with testes of normal volume and consistency. The intriguing laboratory tests revealed high serum oestradiol, oestrone, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) concentrations in contrast to normal serum testosterone and growth hormone (GH) levels. Semen analysis showed a normal sperm density ( $25 \times 10^6/\text{ml}$ ) with a decreased viability (18%). Biochemical parameters also revealed insulin resistance and increased bone turnover. These data were consistent with the hypothesis of primary oestrogen resistance. High-dose transdermal ethinyl oestradiol was given without any target tissue response occurring, despite a 10-fold increase in the serum free oestradiol concentration. Analysis of the ER gene of the amplified genomic DNA obtained from lymphocytes of the patient revealed the substitution of thymine for cytosine at codon 157 of exon 2 in both alleles, resulting in a premature stop codon. As a result, the translated protein would be severely truncated, lacking both the DNA-binding and hormone-binding domains. The patient's parents, as well as three of his four sisters, were heterozygous carriers of the mutation. The disease would therefore be inherited as an autosomal recessive trait (11).

Congenital oestrogen deficiency conditions may conceivably arise from aromatase deficiency as well as

being caused by oestrogen resistance and this has indeed been the case.

In 1991, a little before Korach and colleagues created their experimental animal model, Shozu and colleagues had described the first case of female pseudohermaphroditism due to placental aromatase deficiency in a Japanese infant (6). Apart from masculine-appearing external genitalia (i.e. enlarged phallic-like structure, complete fusion of posterior scrotolabial folds which showed rugation as in a scrotum, and a single meatus at the base of the phallic-like structure), the infant, a 3210 g female baby (46,XX) with an Apgar score of 10 at 1 min after birth, was in good health and had no evidence of adrenal dysfunction. Her mother had shown progressive virilization during the third trimester of pregnancy coupled with low urinary excretion of oestrogens for her gestational age and very high serum levels of testosterone, 5 $\alpha$ -dihydrotestosterone and androstenedione. This pattern was confirmed in the cord serum. After delivery both maternal and neonatal serum levels of androgens fell to normal values. Furthermore, maternal virilization gradually disappeared. Data obtained from the determination of aromatase and sulphatase activity of the delivered placenta showed that the former was very low while the latter was normal as compared with values for controls (6). A subsequent study revealed that such a placental aromatase deficiency was the consequence of a point mutation in the consensus splicing donor sequence at the 5' terminus of intron 6 of the aromatase gene as a result of a genetic defect in the fetus (7). Interestingly, given the normal reproductive functions in the mother, the study indicated that the placental aromatase, which protects the mother and the fetus from exposure to adrenal androgens, is expressed only in the parts of fetal origin, shedding new light on this issue (7). Afterwards, DNA sequence analysis of the aromatase gene among the family of the patient revealed that the disease had been inherited as an autosomal recessive trait (7).

Actually, it has been two decades since placental aromatase deficiency was first reported by Mango *et al.* by incubation studies *in vitro* of the delivered placenta in a primigravida with low urinary oestrogens during the third trimester of pregnancy (97). However, neither the mother nor the female infant exhibited evidence of virilization. In addition, the coexistence of aromatase inhibitors was not investigated by the authors. As a result, the case described by Mango and colleagues in 1978 seems to be a clinical entity different from that described by Shozu *et al.* in 1991 (6), which can now be included in the congenital oestrogen deficiency.

Two years following the first report by Shozu and colleagues, Simpson's group described both the clinical features and the molecular basis of congenital aromatase deficiency in an adult 46,XX female (9, 10). Like the patient reported by Shozu and colleagues, at birth she presented with non-adrenal female

pseudohermaphroditism, but no sign of maternal virilization except acne was noted during pregnancy. At the age of 18 years she had sexual infantilism with an enlarged clitoris and no evidence of breast development in addition to primary amenorrhoea, hypergonadotrophic hypogonadism and multicystic ovaries. At 17 months of age normal internal female genital structures had been identified at laparotomy. By direct sequencing of the amplified genomic DNA from the patient, the authors identified two missense point mutations in the aromatase gene in exon 10, at bp 1303 in one allele and at bp 1310 in the other allele. Thus, the patient was found to be a compound heterozygote for these mutations, one of which she had inherited from her mother. The origin of the other mutation could not definitively be demonstrated because neither her father nor other siblings were living. Of interest, oral replacement treatment with ethinyl oestradiol and medroxyprogesterone acetate resulted in a regression of the ovarian cysts and a decrease of serum gonadotrophins together with a pubertal growth spurt of 13 cm, breast development and menarche.

In 1995, Morishima *et al.* (14) reported a novel point mutation in the aromatase gene both in a female with all the cardinal features of aromatase deficiency syndrome as previously described (6, 7, 9, 10) and in her brother, the first male to be identified with congenital aromatase deficiency. Both of them were homozygotes for the mutation, a single base change at bp 1123 in exon 9 of the gene, while the parents were obligate heterozygotes, a finding consistent with autosomal recessive inheritance. Interestingly, the phenotype of the affected adult male sibling (tall stature with continuing linear growth in adulthood, delayed bone age and osteoporosis) was similar to that previously described by Smith *et al.* in a young man with oestrogen resistance due to a disruptive mutation in the ER gene (11).

In 1996, Portrat-Doyen *et al.* described another case of female pseudohermaphroditism caused by a heterozygous mutation (R457X) in the aromatase gene, resulting in a stop codon in exon 10 (15).

Most recently, Mullis and colleagues reported on a female with pseudohermaphroditism due to total aromatase deficiency as a consequence of two new point mutations in the CYP19, resulting in two stop codons: the allele inherited from her mother was a base deletion occurring at P408 (CCC; exon 9), while the allele inherited from her father was a point mutation at the splicing site between exon 3 and intron 3. As a result, the patient was a compound heterozygote for a point mutation/deletion (16).

Finally, in 1997 Carani and colleagues reported the effect of oestrogen treatment in a young man with aromatase deficiency caused by a novel, homozygous inactivating mutation of the aromatase gene, a single base change at bp 1094 in exon 9 (17). Such a therapy resulted in a complete epiphyseal closure after 9 months

coupled with an increase of spinal bone mineral density. In contrast, a previous treatment with testosterone, which was given before the diagnosis had been achieved in the attempt to arrest his persistent linear growth in adulthood, had no effect on skeletal maturation.

## Phenotypes and genotypes

It is therefore likely that life without oestrogen is indeed possible. To date, eight well-documented cases of congenital oestrogen deficiency – five females and three males – have been reported in the medical literature (6–11, 14–17). Table 1 summarizes the clinical features of these subjects. All but one were the consequence of aromatase deficiency (6–10, 14–17), while a unique case was the result of oestrogen resistance caused by an inactivating mutation in the ER- $\alpha$  gene (11).

Although a comprehensive reappraisal of the effects of congenital oestrogen deficiency in the female is beyond the aim of this review, we can affirm that it may not be so easy as it originally seemed to predict the phenotype. There is no question that an excess of androgens during the early development of the embryo leads to a pseudohermaphroditism in the female fetus as well as a transient virilization in the mother (Fig. 1). Again, most of the clinical features accompanying puberty in the female subject, i.e. absent breast development, primary amenorrhoea, multicystic ovaries and further virilization, seem to be obvious. However, revealing how oestrogen deficiency affects the reproductive tract throughout postnatal life and why placentally derived oestrogen is needed to offset fetal androgen only solves part of the puzzle. We still need to know, for example, the precise mechanisms by which oestrogen regulates the pubertal growth spurt and epiphyseal closure, because all the female patients reported so far have been treated with oestrogens since 15 years of age or earlier in childhood.

But one of the more intriguing questions raised by this recent work is undoubtedly the consequence of congenital oestrogen deficiency in the male. As mentioned above, the phenotype of the man affected with oestrogen resistance caused by an inactivating mutation of the ER- $\alpha$  gene (11) appears now to be extremely similar to that described later in two men with aromatase deficiency (14, 17). Interestingly, all these subjects had osteoporosis, tall stature with continuing linear growth into adulthood, unfused epiphyses, bilateral genu valgum and an eunuchoid proportion of the skeleton with a ratio of the upper to the lower segment of 0.84–0.88 (average for men, 0.96). Figure 3 shows the appearance of the patient affected with aromatase deficiency described in a previous report (17). On the other hand, some differences have also been shown.

In particular, the possible development of infertility as well as insulin resistance remains controversial

**Table 1** Clinical features in congenital oestrogen deficiency.

	Female	Male
Fetal life	Mother's transient virilization during second half of pregnancy*	Mother's transient virilization during second half of pregnancy*
External genitalia at birth	Ambiguous genitalia (Prader stage IV–V) with a greatly enlarged phallic-like structure (clitoridomegaly), complete fusion of posterior labioscrotal folds, and a single meatus at the base of the phallic-like structure	Normal male external genitalia
Childhood	Delayed bone age	Unremarkable?
Puberty	Absent breast development; primary amenorrhoea; further enlargement of clitoris; virilization and normal development of axillary and pubic hair; absent growth spurt; delayed bone age	Normal pubertal development; delayed bone age; normal growth spurt?
Adulthood	Severe oestrogen deficiency; virilization; multicystic ovaries; osteoporosis; infertility; unfused epiphyses? (if untreated); tall stature?	Giantism (stature >3 SD) and continuing linear growth in adulthood; unfused epiphyses; eunuchoid skeleton; bilateral genu valgum; osteoporosis; infertility?

\* This feature has not been clearly exhibited in all the cases (10, 17).

(11, 14, 17). Furthermore, as summarized in Table 2, major differences in the lipid profile have recently been reported (17, 98). Whether or not these discrepancies may be the result of the different pathogenesis in the oestrogen deficiency (oestrogen resistance vs aromatase deficiency) remains to be elucidated. The recent work identifying a separate subtype of ER (ER- $\beta$ ) could provide new tools in these issues. An additional argument complicating the debate on the possible link between oestrogen deficiency and male infertility is the increasing evidence of non-genomic actions of oestrogens in reproductive tissues, which are independent of the classic model of steroid hormone receptor–target cell interaction and subsequent transcription of specific genes (for review see reference 99). In this context, the condition of oestrogen resistance with high serum concentration of oestrogens (11) is quite different from that of aromatase deficiency, in which the serum concentration of oestrogens is very low or not detected (14, 17).

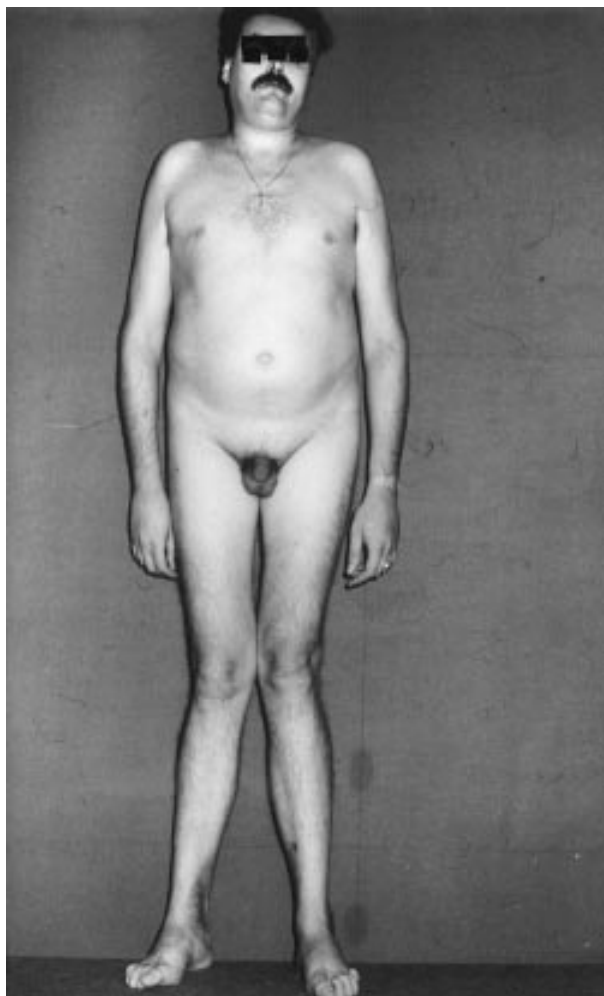
Again, the occurrence of varying phenotypes in the two men with aromatase deficiency described so far is compelling, even though some of the data must be viewed with caution (14, 17). The patient reported by Morishima and colleagues showed macrorchidism with normal testicular consistency, but semen analysis was not carried out (14). In contrast, our patient had small testicles and semen analysis revealed a sperm count of less than  $1 \times 10^6$ /ml with 100% immotile spermatozoa (17). How should we account for such a discrepancy? One might argue that in our patient factor(s) other than aromatase deficiency had arisen during fetal development to impair his reproductive health. According to a still unproved hypothesis, for example, the exposure to

environmental oestrogens *in utero* might affect the multiplication of Sertoli cells during fetal life, thus leading to falling sperm counts in adulthood (100). Interestingly, data obtained from ERKO mice have recently suggested the presence of a distinct oestrogen-responsive gene via a pathway that does not appear to involve nuclear ER- $\alpha$  or ER- $\beta$  and that could mediate also the effects of environmental oestrogens, such as xenoestrogens (101). On the other hand, the recent cloning and characterization of multiple CYP19 loci encoding different P450arom isoforms in pig (102) and goldfish (103) might give new insight. In fact, these isoforms appear to be differentially expressed in tissues such as gonads and the nervous system. What is unknown today is whether multiple CYP19 genes will be isolated from human tissues too. In that case, there should be potentially another level of complexity in the expression of aromatase besides tissue-specific usage of multiple promoters and untranslated first exons, and this may mean another possible explanation for the varying phenotypes of aromatase-deficient subjects.

Here we will summarize our knowledge on the relationship between gene defect(s) and the corresponding affected function of the translated protein.

The homozygous mutation in exon 2 of the ER- $\alpha$  gene reported by Smith and colleagues resulted in a premature stop codon at position 157 (TGA instead of CGA). As a result, the transcript would be a severely truncated protein, expected to be functionally inert (11).

As summarized in Table 3, most of the gene defects in the CYP19 gene described so far were point mutations which led to a loss of function. However, the resulting aromatase activity, as determined by



**Figure 3** A 38-year-old patient with aromatase deficiency (17). He is a well-masculinized man with evident genu valgum and eunuchoid body habitus.

expression studies in COS-1 cells – or in COS-7 cells (7) – showed some minimal differences depending on the involved site and the type of substitution (Table 3). In general, structural changes need not affect function if they are distant from the active site or, in contrast, if they are close to the active site but they can be coupled in such a way that function is conserved.

Interestingly, analysis of aromatase cDNA isolated from a placental cDNA library of the patient described by Harada's group early in the 1990s (6, 7) showed a number of gene defects both in the 3' non-coding region and in the coding region. Of these, only one – the insertion of 87 bp into the junction between exon 6 and intron 6 – was significant as a cause of aromatase deficiency. In fact, the other changes were also found in the placental aromatase cDNA of healthy women as a result of human genetic polymorphisms. In contrast, the insertion fragment between exon 6 and

intron 6 – the consequence of a point mutation (GT was altered to GC) at the 5' terminus of intron 6 leading to a cryptic consensus donor sequence with a downstream shift of the splicing point – resulted in translation of a larger protein with 29 extra amino acids, having only a trace of activity during the expression in COS-7 cells (7). Although both the catalytic and haem-binding regions of aromatase were not interrupted by this highly hydrophobic extra peptide, conformational changes might have arisen out of the insertion position, which is assumed to be the region of bipolar helix structure.

Until now, missense mutations within the critical haem-binding region of the enzyme encoded by exon 10 have been identified in two female patients (9, 15). The former was a compound heterozygote for two different point mutations, one of which encoded tyrosine instead of cysteine 437 (C437Y). Because cysteine 437 is a common residue in all cytochrome P450s and is believed to be the fifth coordinating ligand of the haem iron (104), the transcript of the C437Y allele would be expected to be inactive. And this was indeed the case (9).

In recent years, point mutations were also found in exon 9, resulting in a single base change at position 375 (R375C) in a young female and her brother (14), and at position 365 (R365Q) in a male patient (17). Both of these regions are believed to play an important role in aromatase function (104, 105).

Finally, another case of compound heterozygosity has been recently reported in a female baby, the first well-documented female with no apparent active aromatase activity (16). A point mutation ignoring the splicing site between exon 3 and intron 3, with a stop codon 3 bp downstream, was identified in the allele inherited from her father. Moreover, in the allele inherited from her mother a base pair deletion (C) was found in the Pro (P408;CCC, exon 9), which caused a frameshift resulting in a nonsense codon 111 bp (37 amino acids) downstream in the aromatase gene. As a result, no active transcript was found. As expected, at birth the patient had an extreme degree of masculinization of the external genitalia, with a 2.9 cm long phallic-like structure (Prader V). This case report may be paradigmatic of the putative role of human placental aromatase to offset fetal androgen.

### The role of oestrogens in skeletal maturation and mineralization

Over the last decade remarkable progress has been made in our understanding of the role of oestrogens in growth and skeletal maturation. Until recently, it seemed that many oestrogen actions on bone were indirect. It was also thought that androgens were directly implicated in growth and bone maturation, at least in the male. Really, it had long been assumed that pubertal growth was an androgen-dependent process

**Table 2** Hormonal and biochemical parameters in male congenital oestrogen deficiency.

Hormonal and biochemical parameters	Oestrogen resistance	Aromatase deficiency
In serum		
LH	↑	↑
FSH	↑	↑
Testosterone	N	↑ or N
Oestradiol	↑↑	↓↓ (not detected)
HDL-cholesterol	↓	↓ or N
LDL-cholesterol	↓	↑
VLDL-cholesterol	↓	—
Total cholesterol	↓	↑
Triglycerides	N	↑
Apolipoprotein A-1	↓	—
Pre-β-1 HDL	N	—
Glucose	↑	N
Insulin	↑	↑ or N
Glycosylated haemoglobin (%)	↑	N
Alkaline phosphatase	↑	↑ or N
Bone-specific alkaline phosphatase	↑	—
Osteocalcin	↑	↑ or N
PTH	N	N
PRL	N	N
GH	N	N
IGF-I	↑	N
In urine		
Pyridinoline	↑	↑

↑ = increased values, ↓ = decreased values, N = normal values. PTH = parathyroid hormone; PRL = prolactin.

also in the female, where it would have had to be mediated by adrenal androgen. These concepts may now need to be reconsidered.

Evidence for the presence of ER in bone tissues comes from recent studies (106, 107). Work by Korach *et al.* on ERKO mice has provided preliminary information about bone density by histomorphometry – a method used to study changes at the cellular level – according to which ERKO mice seem to have 10% lower bone density than age-matched wild-type animals (108). Actually, the issue remains somewhat controversial and there is little information on the direct effect of oestrogens on skeletal maturation and mineralization. Some noteworthy advances in basic research have quite recently provided new insights into this field. In fact, contradicting a previous study (108), the subsequent work by Korach's group, by scanning the femurs by peripheral quantitative computerized tomography, has shown that female ERKO mice have higher bone mineral density in the femurs than wild-type mice, while ovariectomy caused a loss of bone mineral density in specific regions of the femur, namely the midshaft region. In contrast, ovariectomy-induced bone loss in wild-type animals occurred in the most distal section of the femur, which is richer in cancellous bone than the midshaft region (109). Then, the authors have suggested that female ERKO mice might use either the ER-β or a non-ER-α signalling pathway to regulate bone density in specific regions of the femur (109). Though important, this recent study only provides the basis for further

investigations and raises some questions and issues of concern. First of all, it highlights that methods for investigating both the site and magnitude of bone mineral density are essential for avoiding possible misunderstanding. Secondly, although the ovariectomized mouse model is now extensively used to evaluate oestrogen deficiency-induced cancellous osteopenia, to relate results from different studies it is strongly recommended that they be limited to mature animals of comparable age, while the two studies by Korach have used 4- and 9-month-old animals respectively (108, 109). So, it would be prudent to wait for confirmatory data.

What's more, the occurrence in humans of natural inactivating mutations in either the ER gene (11) or aromatase gene (6–10, 14–17) has made this field even more exciting. The 14-year-old female with aromatase deficiency reported by Conte *et al.* had an evident delay in bone age and the pubertal growth spurt did not occur until oestrogen replacement therapy was given (10). The authors speculated that she would have had a short final height if she had not been treated with oestrogens. Well, at the age of 14 years the patient was 153.5 cm tall (–1.5 s.d. for chronological age), but her bone age was unexpectedly delayed at 10 years despite persistent androgen excess. On the basis of the phenotype described later in untreated adult men with aromatase deficiency, showing tall stature, unfused epiphyses, continuing linear growth into adulthood and eunuchoid proportions of the skeleton (Table 1, Fig. 3),



**Table 3** Review of known mutations in the human aromatase P450 (CYP19) gene.

Subject	Gene defect(s)	Transcript(s)	Aromatase activity (%)	Reference
Homozygous female newborn	Point mutation in the consensus splicing donor sequence of the gene (GT → GC at the 5' terminus of intron 6), resulting in a downstream shift of the splicing point and the insertion of 87 bp – the first 87 bp of intron 6 – in the coding region, between exon 6 and intron 6	A larger protein with the insertion of an extra peptide of 29 amino acids	0.3	Harada <i>et al.</i> (7, 8)
Compound heterozygous female (aged 18 years)	Two single base changes in exon X: (1) at bp 1303 (C → T) in one allele; (2) at bp 1310 (G → A) in the other allele	(1) Resulting in a cysteine instead of an arginine at position 435 for single base change (R435C); (2) resulting in a tyrosine instead of a cysteine at position 437 (C437Y)	(1) 1.1; (2) 0.0	Ito <i>et al.</i> (9)
Homozygous female (aged 28 years)	Single base change at bp 1123 (C → T) in exon IX	Resulting in a cysteine instead of an arginine at position 375 (R375C)	0.2	Morishima <i>et al.</i> (14)
Homozygous male (aged 24 years)	Single base change at bp 1123 (C → T) in exon IX (R375C)	Resulting in a cysteine instead of an arginine at position 375 (R375C)	0.2	Morishima <i>et al.</i> (14)
Homozygous female newborn	A mutation (R457X) in exon X, resulting in a stop codon	No active transcript, because the mutation ignores the splice site and a stop codon occurs	—	Portrat-Doyen <i>et al.</i> (15)
Homozygous male (aged 38 years)	Single base change at bp 1094 (G → A) in exon IX	Resulting in a glutamine instead of an arginine at position 365 (R365Q)	0.4	Carani <i>et al.</i> (17)
Compound heterozygous female (aged 3 years)	Allele 1: point mutation (G → A) at the splicing point (canonical GT to mutational AT) between exon and intron III; allele 2: a base pair deletion occurring at P408 (CCC, exon IX) in exon IX	(1) No active transcript, because the mutation ignores the splice site and a stop codon 3 bp downstream occurs; (2) resulting in a frameshift that results in a nonsense codon 111 bp (37 amino acids) further down in the CYP19	0	Mullis <i>et al.</i> (16)

we cannot now completely rule out the possibility that untreated females may develop some of these features. Actually, we do not know the natural history of the congenital oestrogen deficiency in females. Furthermore, no information about the occurrence of pubertal growth spurt is available in men, regardless of which molecular mechanism is responsible for oestrogen deficiency. In fact, all of them were first examined in adulthood and the intervals between measurements of height were too long to permit assessment of a pubertal growth spurt (11, 14, 17). Actually, oestrogen-resistant and aromatase-deficient men achieved their genetic potential for height at the normal age of 16–17 years, followed by continuing growth into adulthood, so that one could speculate that the pubertal growth spurt would have had to occur. But this inference remains to be demonstrated.

Interestingly, in most mammals the height velocity does not vary significantly from infancy to adulthood, so that the pubertal growth spurt in humans is a nearly exclusive phenomenon (110). At present, the hormonal network involved in this process of accelerated growth remains poorly understood, even though the recent data on the role of oestrogens have yielded insight into this issue (111–121). There is a general agreement that many hormones – including a number of cytokines, GH, GH-induced local production of insulin-like growth factor-I (IGF-I) and GH-induced increase in circulating levels of IGF-I (122) – are important in the postnatal longitudinal bone growth. Perhaps the debate has in the past paid insufficient attention to the putative role of oestrogens in the process of skeletal maturation. However, in addition to the recent observation relating congenital oestrogen deficiency to characteristic phenotypes, some other peculiar errors of nature might help us to better understand how oestrogen acts on growth and bone maturation (for reviews see references 114, 115, 119–121).

First of all, 46,XY patients with the complete form of androgen insensitivity syndrome (2) provide a model of unopposed oestrogen action, in which the timing and extent of pubertal growth are similar to those observed in normal females (114, 115, 123). In this condition, aromatization of testicular and adrenal androgen to oestrogen coupled with secretion of oestrogen from the intra-abdominal testicles accounts for the increased serum oestrogen levels and feminization at puberty. Mean peak height velocity is strictly similar to that in normal females and lower than in normal boys, while mean adult final height is taller than in normal women and shorter than in normal men, probably as a result of unknown factors responsible at least in part for taller stature in men than in women and believed to be linked on the Y chromosome (113). This natural model suggests that oestrogen alone is sufficient for a normal pubertal skeletal growth. Furthermore, given the normal body proportions of these subjects, there is not a shred of evidence to support

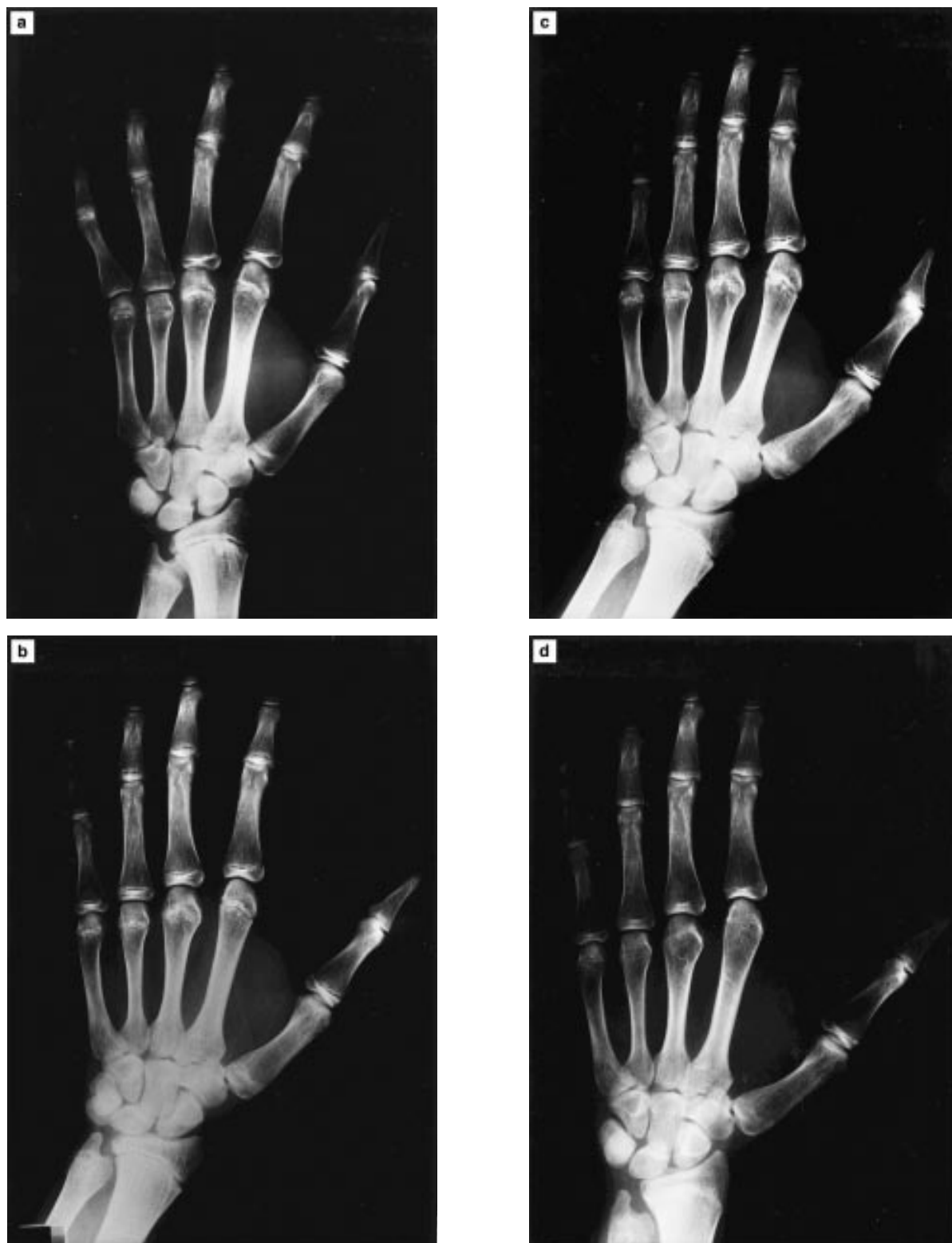
the current view that the eunuchoid body habitus – that is a preferential growth of the arms and legs compared with the spine, resulting in the ratio of the upper to the lower segment smaller than 1 – has to be considered a consequence of an insufficient androgen action.

Another fascinating natural model is the complete deficiency of 17 $\alpha$ -hydroxylase or combined 17 $\alpha$ -hydroxylase/17,20-lyase, in which impaired secretion both of cortisol and of sex steroids occurs together with an overproduction of mineralocorticoids (124). Genetic female patients display sexual infantilism, with primary amenorrhoea and no pubertal development. Genetic males show male pseudohermaphroditism, with a blind vagina, absence of Müllerian structures and female external genitalia. Patients of either sex have an elevated blood pressure and lack of axillary and pubic hair. Of interest, they also show tall stature, eunuchoid body habitus, delayed bone age and osteoporosis, thereby resembling some of the features described in male patients with congenital oestrogen deficiency (11, 14, 17). Such a phenotype, which has classically been related to the impaired secretion of sex steroids, can now conceivably be explained by a deficiency of oestrogen.

With respect to the body proportion, the complete form of androgen insensitivity syndrome, the congenital oestrogen deficiency in men and the complete deficiency of 17 $\alpha$ -hydroxylase/17,20-lyase all reinforce the concept that the eunuchoid skeleton, classically linked to a delay in exposure to sex hormones, is the result of a deficiency of oestrogen rather than of androgen.

In addition, our study on an adult male patient with aromatase deficiency demonstrated that 9 months of treatment with transdermal oestradiol (50  $\mu$ g twice weekly for 6 months, followed by 25  $\mu$ g twice weekly) resulted in a rapid increase in skeletal maturation, with closure of metacarpal and phalangeal epiphyses (Fig. 4c and d). Conversely, 6 months of treatment with high doses of testosterone enanthate, given in an attempt to arrest his persistent linear growth and stimulate epiphyseal closure before the diagnosis had been achieved, had no effect on bone maturation (Fig. 4a and b).

Also, we have to bear in mind that during puberty, along with the skeletal maturation and progressive epiphyseal closure, the mineralization of the growing skeleton rapidly increases, especially in cancellous bone. It is likely that this phenomenon is also under the control of oestrogen. In fact, the density of the lumbar spine measured in a young man with disruptive homozygous mutation of the ER gene and elevated serum testosterone levels (11) was 0.745 g/cm<sup>2</sup> (>2 s.d. below the mean for 15-year-old boys, the patient's bone age). Furthermore, the bone mineral density of the lumbar spine of our male patient with aromatase deficiency increased from 0.93 (normal range for



**Figure 4** X-ray films of the left hand of a man with aromatase deficiency (17) – effects of testosterone and oestradiol treatment. (a) Before testosterone treatment the bone age was 14.8 years (the patient was 31 years old). (b) The bone age after testosterone treatment (testosterone enanthate 250 mg intramuscularly every 10 days for 6 months) was unmodified. (c) Before oestradiol treatment the bone age was still 14.8 years (the patient was 38 years old). (d) bone maturation was achieved after 9 months of treatment with transdermal oestradiol (50  $\mu$ g twice weekly for 6 months, followed by 25  $\mu$ g twice weekly): the metacarpal and phalangeal epiphyses were closed and the bone age was greater than 16 years. (a and d are from reference 17, with permission.)

adolescents in Tanner stage 5, 0.96–1.31) to 1.17 g/cm<sup>2</sup> after 7 months of oestrogen treatment (17).

Finally, Bilezikian *et al.* (125) have recently confirmed our experience on beneficial effects of oestrogen replacement on both the epiphyseal closure and the increase of bone mineral density in a man deficient in aromatase who had previously been described by Morishima *et al.* (14). Interestingly, their data show that the gain in bone mass was evident at different sites as a result of a true net anabolic effect of oestrogens, since it had been continuing after epiphyses closed (125).

In conclusion, oestrogen seems to be the principal sex steroid involved in the final phases of skeletal maturation and mineralization, a phenomenon that takes longer in hypogonadal men and in patients with delayed puberty, who therefore may develop eunuchoid body proportions and osteopenia as a result of insufficient availability of androgen for aromatization to oestrogen (120, 126, 127). Undoubtedly, this is an exciting time for research into metabolic bone diseases.

Paradoxically, as the role of oestrogen on skeletal physiology becomes more certain, the role of androgen is becoming less definite. There is no doubt that androgen alone is ineffective in promoting epiphyseal closure. However, androgen receptors have been demonstrated in human chondrocytes (112) and linear growth can be sustained by androgen alone despite arrested skeletal maturation (11, 14, 17). A possible explanation is that androgen as well as local androgen-induced cytokines may increase chondrocyte proliferation without signalling chondrocyte terminal differentiation (120).

Further evidence for the role of androgen in pubertal skeletal physiology comes from studies carried out in patients with familial male precocious puberty, a rare form of inherited isosexual precocious puberty in which the autonomous Leydig cell activity is due to a constitutively activating mutation of the LH receptor (128) or of the membrane-bound guanine nucleotide-binding protein to which the LH receptor is coupled (129). In the work by Laue and colleagues, both the blockade of androgen action with spironolactone alone and the inhibition of aromatase activity with testosterone alone were unsatisfactory in reverting the skeletal growth to the prepubertal rate in these patients, while a combination of these two drugs was effective in restoring both the growth rate and the rate of bone maturation to normal prepubertal levels (130). On the one hand, this work confirmed that some of the skeletal action of testosterone is mediated via aromatization to oestrogen; on the other hand, it showed that the effect of testosterone on growth is not purely oestrogenic. A putative direct growth-promoting action of androgen has also been suggested by recent studies with non-aromatizable androgens in the treatment of short stature and delayed puberty (131, 132).

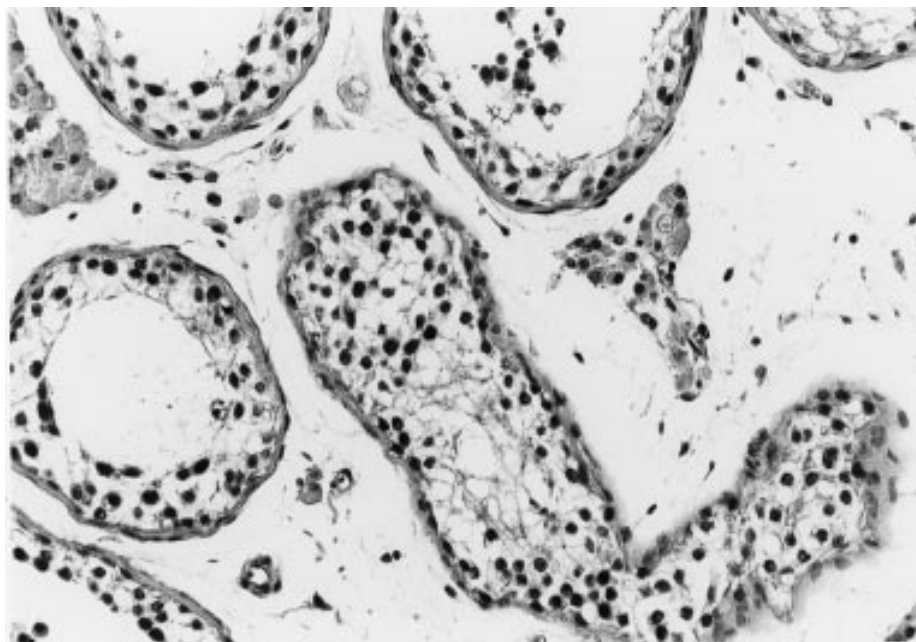
## The role of oestrogen in the control of gonadal function in the male

Congenital oestrogen deficiency offers a natural model also for studying the role of oestrogen in the feedback control of gonadotrophin secretion in men. Both the man with oestrogen resistance and the two men with aromatase deficiency had elevated serum FSH and LH concentrations, despite normal or elevated serum testosterone levels, suggesting that oestrogen has an important part in the regulation of gonadotrophin secretion (11, 14, 17). In this regard, our observations in a male patient with aromatase deficiency provide direct evidence for this hypothesis. In fact, oestrogen treatment caused complete suppression of serum gonadotrophins while androgen treatment had no effect (17). These results did agree with those previously obtained by Finkelstein and colleagues in normal men, in whom a combination of testosterone and an aromatase inhibitor prevented testosterone-induced suppression of gonadotrophins (133). Accordingly, Bagatell *et al.* showed no evidence of any reduction in the gonadotrophin secretion in normal men during the administration of dihydrotestosterone, a non-aromatizable androgen (134).

Another important question is that of the possibility of a causal link between congenital oestrogen deficiency and male infertility. Actually, one must view with concern that supporting evidence for relating oestrogen insensitivity or deficiency to male infertility in humans is lacking in previous reports (11, 14, 17). Semen analysis obtained from the young man with oestrogen resistance (11) revealed a normal sperm density ( $25 \times 10^6$ /ml) coupled with a decreased viability (18%). Each of his testes had a normal volume (20–25 ml). Unlike the patient with aromatase deficiency reported by Morishima *et al.*, who showed macrorchidism with normal testicular consistency (14), our patient had small testicles with severe oligozoospermia (sperm count  $1 \times 10^6$ /ml with 100% immotile spermatozoa). In addition, the occurrence of azoospermia and infertility in one of his brothers who had normal P450arom gene made us unable to assess whether the relationship between oestrogen deficiency and spermatogenic damage was causal (17). Figure 5 shows the histology of the testis in our patient.

Therefore, to date there is still little and controversial evidence for including male infertility in the phenotype of congenital oestrogen deficiency in humans and further studies to assess the possible relationship between congenital oestrogen deficiency and male infertility will clearly be necessary.

However, stirring insight comes from studies in male ERKO mice (89, 91, 92, 135, 136). These animals are infertile and show disrupted spermatogenesis with dilated seminiferous tubules, but one of the possible mechanisms responsible for such a reproductive phenotype has been clarified only recently, when Hess *et al.*



**Figure 5** Histological analysis of the testicular biopsy obtained from an infertile male patient with congenital oestrogen deficiency due to a homozygous inactivating mutation of the P450arom gene (17). The epithelial height is reduced with hypospermatogenesis and germ cell arrest, mainly at the level of the primary spermatocyte. Final magnification  $\times 250$ .

demonstrated that fluid resorption in efferent ducts, which express a large amount of ER in wide-type animals, is greatly impaired in the ERKO mice (92). Perhaps it does not happen at all. As a consequence, the continuing secretion of fluid by Sertoli cells means accumulation not only in the efferent ducts but also in the rete testis and, finally, in the seminiferous tubules, thereby leading to a progressive increase of fluid pressure within the testis. The final step is a disrupted spermatogenesis. In addition, any sperm that can somehow pass into the epididymis may not mature properly, perhaps as a result of excessive dilution of factors which are thought to be important for the epididymal maturation of spermatozoa. Whether their decreased fertilizing ability is only dependent on the abnormal epididymal function still remains a matter of debate. In fact, oestrogens may have other still unknown actions in normal spermatogenesis, given the wide expression of both ER- $\alpha$  and ER- $\beta$  in mammals throughout the male reproductive tract (27, 135, 137, 138).

Of interest, to investigate the consequences of removing the source of oestrogen rather than neutralizing its action, most recently Fisher *et al.* have created mice lacking a functional aromatase enzyme (ArKO mice) by targeted disruption of the CYP19 gene (139). The development of this model would help to clarify the interpretation of the findings that have been reported in ERKO mice and that the recent identification of ER- $\beta$  have made uncertain. The preliminary data of

this work provide further evidence in support of the key role of oestrogens in the negative feedback regulation of gonadotrophins in both males and females. Of particular interest with respect to male phenotype is the finding that, unlike male ERKO mice, male ArKO mice were fertile, even though a decreased fertility became apparent with advancing age. Accordingly, male ArKO mice showed a normal gross morphology of the testicles in contrast to the evident disruption of the seminiferous epithelium reported in ERKO mice. These differences raise the possibility that some actions of oestrogens on the male reproductive tract might be mediated via either the ER- $\beta$  or other putative non-ER- $\alpha$  signalling pathways. Furthermore, though preliminary, this recent work by Fisher and colleagues also suggests that oestrogens might have only minor effects on fertility in the male mouse. However, we still need to know the full range of phenotypic actions of oestrogen in both the male and female reproductive system.

### Metabolic and cardiovascular abnormalities

High levels of fasting blood glucose, an impaired glucose tolerance test and insulin resistance were found in the oestrogen-resistant man in association with clinically evident bilateral axillary acanthosis nigricans (11), while only in one of the two men with aromatase deficiency was an increase in the concentration of fasting serum insulin associated with normal blood

glucose found (14). On the contrary, our patient affected by aromatase deficiency did not show any change in insulin and glucose serum levels (17).

It is difficult to establish whether a causal relationship between oestrogen deficiency and insulin resistance does exist, since the mechanism by which the lack of oestrogen may induce insulin resistance is still unknown. An intriguing hypothesis, however, suggests a possible role of oestrogen in modulating insulin sensitivity of peripheral tissues, a possibility that could in part explain the association between oestrogen deficiency and impaired glucose metabolism (140).

In the male, congenital oestrogen deficiency is associated with an abnormal lipid profile, but data obtained so far have surprisingly led to contrasting results, probably as a consequence of the different pathogenesis (Table 2).

Indeed, in the man with oestrogen resistance the serum levels of apolipoprotein A-I, lipoprotein(a), very-low-density lipoprotein (VLDL), high-density lipoprotein (HDL), low-density lipoprotein (LDL) and total cholesterol were low, while serum levels of triglycerides and pre- $\beta$ -1-HDL were normal (98). The low serum levels of LDL-cholesterol in this patient could be related to the decreased synthesis of its precursor VLDL, given the well-known action of oestrogen in stimulating VLDL triglyceride production (141) and the presence of ERs in the liver (142). The very low concentration of HDL-cholesterol in the oestrogen-resistant man could be the result of the unopposed action of androgen, which is considered the most important determinant of differences between males and females in serum HDL-cholesterol (143–146).

On the contrary, aromatase deficiency is characterized by high levels of triglycerides, LDL-cholesterol, total cholesterol and low levels of HDL-cholesterol (14, 17) (Table 2). The effects of high doses of exogenous testosterone administration on the lipid profile in our patient resulted in a decrease of HDL-cholesterol (17), thereby confirming the role of high doses of exogenous testosterone in decreasing HDL-cholesterol levels (144). In contrast, transdermal oestrogen treatment caused an increase of HDL-cholesterol levels and a decrease of triglycerides, LDL-cholesterol and total cholesterol levels (17). Even though these changes may be the result at least in part of reduced concentrations of serum testosterone, it is now evident that the abnormal lipid profile can be modified by oestrogen treatment in aromatase-deficient men. Taken together, these findings indirectly suggest an important role of oestrogens in modulating lipid profile also in men (17, 144), while it is well known that oestrogen administration in postmenopausal women results in increased plasma HDL-cholesterol (145), thereby restoring the protective role of oestrogen on prevention of cardiovascular disease (147).

However, the differences in lipid profile between oestrogen-resistant and aromatase-deficient men

remain unclear. In theory, it could be possible that the effects of ER- $\alpha$  and ER- $\beta$  actions on lipid metabolism modulation are different and/or in opposite directions. In the oestrogen-resistant man reported by Smith *et al.* (11) the ER- $\alpha$  was blocked but it is possible that some oestrogen functions were sustained by ER- $\beta$  action, while in aromatase-deficient men the non-detected levels of serum oestrogens could not assure any oestrogen activity on lipid metabolism.

The relationship between cardiovascular function and oestrogen deficiency has been investigated only in the young man with oestrogen resistance, who showed an impaired flow-dependent (nitric oxide-mediated) vasodilatation in peripheral arteries in response to an ischaemic stimulus despite a normal rapid vascular responsiveness to nitroglycerine, thereby indicating a preserved response of vascular smooth muscle to nitric oxide (98). These findings have been interpreted as a result of an endothelial dysfunction, perhaps an impaired vascular synthesis/release of nitric oxide (98). On the other hand, the rapid vascular responsiveness to oestradiol appeared to be intact in the oestrogen-resistant man (98). Accordingly, Komesaroff *et al.* have recently shown the existence of a rapid onset, rapid offset, non-genomic action of oestradiol, at plasma concentrations within the physiological range for premenopausal women, on the cutaneous microvasculature in young healthy men, through a mechanism that is specific to the endothelium (148).

Adjunctive information on the cardiovascular system in the oestrogen-resistant man has come from the work by Sudhir *et al.* (144). Using electron-beam computed tomography scanning of the coronary arteries, the authors have shown calcium deposition in the left anterior descending artery, despite no evidence of reduction of artery blood flow on myocardial perfusion scintigraphy performed during an exercise stress test (144). However, it remains to be demonstrated whether the association between premature coronary artery disease and the absence of functional ER- $\alpha$  is causal. Recent data obtained from ovariectomized female ERKO mice using the mouse carotid arterial injury model (149) have given evidence that the classical ER- $\alpha$  is not required to mediate the inhibitory effects of oestradiol on the vasculature injury response, suggesting a role of a novel oestrogen signalling pathway in vascular tissue (150).

## Concluding remarks

Until now, the recent work concerning oestrogen deficiency in men has raised far more questions than it has answered. There is, however, a general agreement that these findings have shaken the foundations of the experimental edifice on which the prevailing view on skeletal maturation has rested. What appears now to be absolutely clear is that the role of oestrogen in the epiphyseal closure and bone mineralization is crucial

not only in women but also in men. Similarly, an increasing body of evidence indicates that oestrogen exerts an essential effect in both sexes for the physiological inhibition of gonadotrophin secretion. Together, these results would not be surprising if we consider the general principle of parsimony operating in evolutionary genetics. And this is precisely what the aromatase gene provides. Nature has once again demonstrated its ability to create enlightening variations of a beautiful theme.

However, there is still a long way to go to unravel all the mysteries of the fascinating condition of congenital oestrogen deficiency in men. But the recent findings discussed in this review may yet indicate the path to success. Furthermore, research in progress could further improve our knowledge.

The history of ER- $\beta$  is only at the beginning (27–30). Moreover, coupled with the previously reported animal model of oestrogen deficiency known as the ERKO mouse, in which the ER- $\alpha$  is deleted by targeted disruption, the recent characterization of ArKO mice by targeted disruption of the CYP19 gene (139) will shed new light. In fact, the phenotype of male ArKO mice is quite different from that of male ERKO mice. Based on the discussion above, this may mean that different ERs (ER- $\alpha$ , ER- $\beta$  and possibly other isoforms) coordinately regulate a number of phenotype actions of oestrogen. Therefore, one might argue that, in the male, the lack of only one isoform of ER could be paradoxically more harmful than the nearly absolute absence of oestrogen. In the mouse, this seems indeed to be the case (91, 92, 139).

In addition, the validation of an ultrasensitive recombinant cell bioassay for determination of oestradiol (151) would have straightforward clinical implications, notably in the management of this rare condition. In particular, with regard to the treatment of aromatase deficiency in the male, although our study has definitively demonstrated that replacement therapy with oestrogen can lead both to the epiphyseal closure and to a relevant increase of bone mineral density (17), it remains to be clarified when to initiate the treatment, at what doses, and for how long. Whether or not lower doses will prove to be effective is being actively investigated in further studies. The long-term goal of therapy should be to use the smallest dose that ensures a physiological bone maturation throughout puberty without interfering with the normal development of secondary sex characteristics, relieves the patient's symptoms, and prevents the early development of osteopenia, a phenomenon that could become one of the major problems in adulthood. We firmly believe that the recent ultrasensitive recombinant cell bioassay for oestradiol (151), which is approximately 100-fold more sensitive than existing assays, will open up new perspectives in the field of oestrogen physiology and subsequently also in the management of congenital oestrogen deficiency.

Finally, as previously suggested (10, 14, 17, 152, 153), data available so far have indicated that congenital deficiency of oestrogen seems not to affect gender identity and sex-related behaviour in humans, while oestrogens play an important role in sexually dimorphic brain functions both in non-primate mammals (154) and in avian species (155). However, the possible role of sex steroids in sexual dimorphism of the human brain is still a matter of debate. A more detailed analysis of psychosexual orientation and sexual activity is now in progress in our patient with aromatase deficiency before and after transdermal oestradiol treatment, and preliminary data by psychometric questionnaires suggest a putative positive role of oestrogens on male sexual behaviour. But further progress in this area awaits the elucidation of the role of oestrogen in the higher central nervous system, including the mechanism(s) controlling the aromatization of androgens by central neuroendocrine tissues (for review see reference 67).

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