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ESTROGENS AND MALE REPRODUCTION

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ABSTRACT

In males, estrogens exert pleiotropic effects by acting on several tissue and organs, including the male reproductive system. The action of estrogens is manifest from prenatal life during which the exposure to estrogen excess might influence the development of some structures of the male reproductive tract. Male fertility is under the control of estrogens, especially in rodents. The loss of function of estrogen receptor alpha and/or of the aromatase enzyme leads to infertility in mice. In men, estrogens are able to exert their actions at several levels through the reproductive tract and on several different reproductive cells. However, the regulation of human male reproduction is more complex and the role of estrogens is less clear compared to mice. During fetal and perinatal life, estrogen acts on the central nervous system by modulating the development of some areas within the brain that are committed to controlling male sexual behavior in terms of setting gender identity, sexual orientation development and the evolution of normal adult male sexual behavior. This organizational, central effect of estrogens is of particular significance in other species (especially rodents and rams), being probably less important in men where psychosocial factors become more determining. Other relevant, non-reproductive physiological events depend on estrogen in men and they involve bone maturation and mineralization as well as metabolic functions. In this chapter we provide an update of estrogen's role in male reproductive function by reviewing the physiological actions of estrogen on male reproduction and the pathophysiology related to estrogen deficiency and estrogen excess. Phenotypes associated with estrogen deficiency and excess in rodents and in man have shed new light on the mechanisms involved in male reproduction, challenging the perception of the predominant importance of androgens in men. It is now clear that the imbalance between estrogen and androgen in men might affect male reproductive function even in presence of normal circulating androgens. Some uncertainties still remain, especially regarding the impact of abnormal serum estrogen levels on male health, particularly due to the fact that estrogen is not routinely measured in men in clinical practice. Advancements in methods to precisely measure estrogens in men, together with a reduction of their costs, should provide better evidence on this issue and inform clinical practice. New basic and clinical research is required to improve our knowledge on the role of estrogen in male reproductive function and men's health in general.

INTRODUCTION

From an historical perspective, estrogens were identified about 85 years ago and estradiol was identified in 1940, reviewed in (1). The first evidence of estrogen production in the male was provided in 1934 by Zondek (2), who documented that male stallions excrete high levels of estrogens in the urine and hypothesized that estrogen production in the male occurs via the intratesticular conversion of androgens into estrogens (1-3).

In men, the conversion of androgens into estrogens was first demonstrated a few years later, when an increase in urinary estrogens after the administration of exogenous testosterone was recorded in normal men (4) (Figure 1).

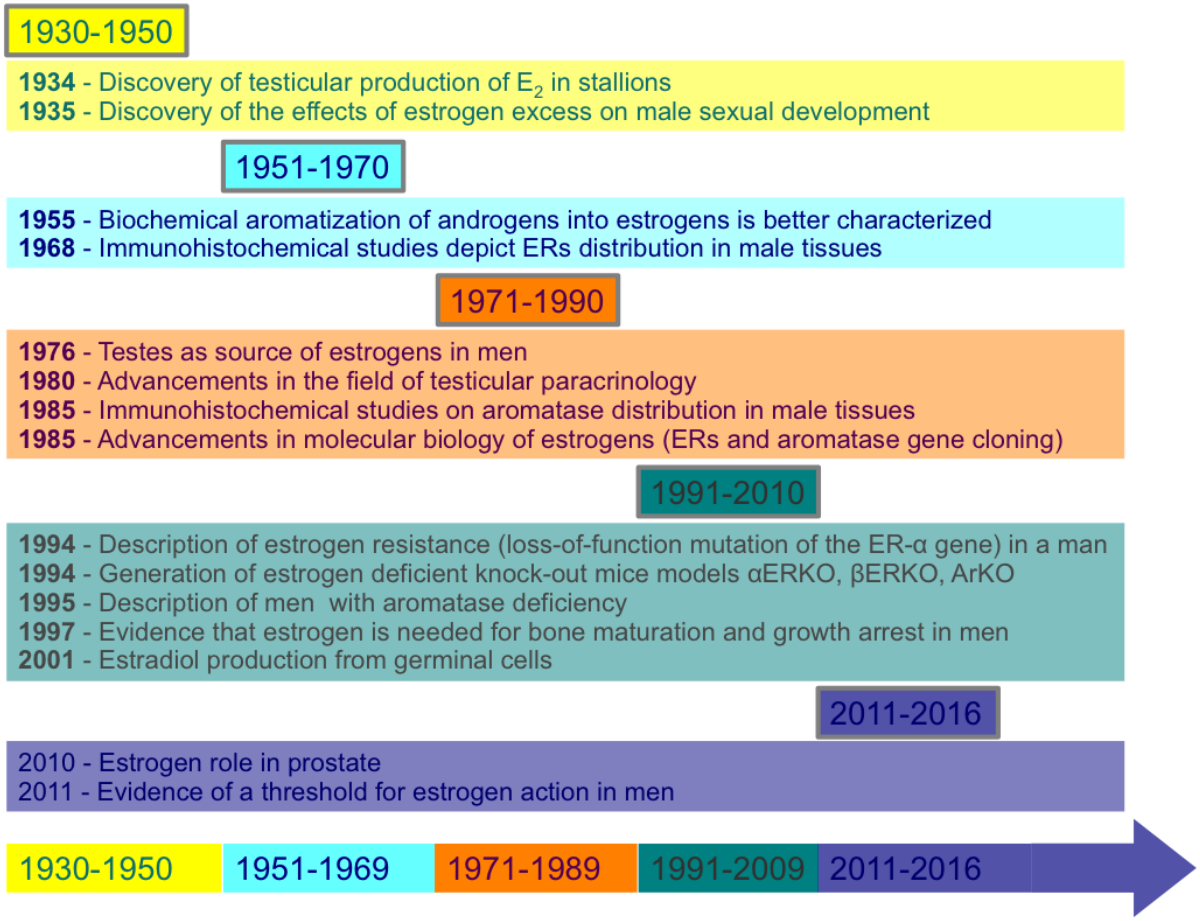

Figure 1. Milestones in the advancement of research in the area of estrogens in men.
[*E_2: 17β-estradiol; ER: estrogen receptor; ERKO: Estrogen Receptor Knock out; ARKO: Aromatase Knock out*]

A more detailed demonstration of estrogen production in the human male was provided several years later in 1979 by MacDonald *et al*. who showed that the aromatization of androgens to estrogens can occur also peripherally in several tissues other than in the testes (5).

Prior to the demonstration of estrogen production in males, the effects of estrogen excess on the development of male reproductive organs had been evident since the 1930s (6). Thus, the concept that male tissues are responsive to estrogens was not new, but it was thought that only a great amount of estrogens was able to induce such changes in males. Notwithstanding the great amount

of data accumulated in the last eighty years, research in the field of estrogen excess and its role on male reproductive system is still ongoing (7) (Figure 1).

The pioneering studies of Zondek and MacDonald opened the way for an appreciation of the physiological roles of estrogens in the male beyond their effects during embryogenesis. Several studies, year after year, provided further data on estrogen's role in men (8-11), since the first pilot studies on estrogens and male reproductive function (12,13).

The progressive development of immunohistochemical studies and the subsequent progress in the field of molecular biology highlighted the actions of estrogens in the male [for further details see (3)] and opened the way for the creation of estrogen null mice (14) (Figure 1).

The detailed characterization of estrogen receptors' structure and function (14,15) together with the discovery and the characterization of genes involved in estrogens synthesis (16) disclosed the biomolecular mechanisms involved in estrogen function and dysfunction pathways. It is now clear that estrogen effects in the male are not confined to reproductive organs but are pleiotropic.

In addition, the development of male transgenic mice lacking functional estrogen receptors or the aromatase enzyme (responsible for estrogen biosynthesis) further contribute to advancements in this field (14,15). Finally, the discovery of mutations in both the human estrogen receptor alpha (17) and aromatase (18,19) genes contributed to an understanding of estrogen's role in human male physiology (10,11,20) (Figure 1).

Nowadays, notwithstanding this long history of studies, reviewed in (21-23), the role of estrogens in the physiology of the male reproductive tract is still not fully understood. The presence of estrogens in the human testis is well documented (24,25), and there is clear evidence that estrogens exert a wide range of biological effects in men and not only in women (9,11,26).

PHYSIOLOGY

Estrogen Biosynthesis in Males

The term estrogen refers to any substance, natural or synthetic, able to interact with the estrogen receptor (ER) (27,28). 17β-estradiol (estradiol) is the prevalent endogenous estrogen form in mammals, although many of its metabolites could be detected with several degrees of estrogenic activity (29). In humans, the three major endogenous estrogens are estrone (E1), estradiol (E2), and estriol (E3) (28) (Figure 2). In males, estrogens mainly derive from circulating androgens. The key step in estrogen biosynthesis is the aromatization of the C19 androgens, testosterone and androstenedione, to form estradiol and estrone, respectively (27). This step is under the control of the aromatase enzyme (27,30) (Figure 2).

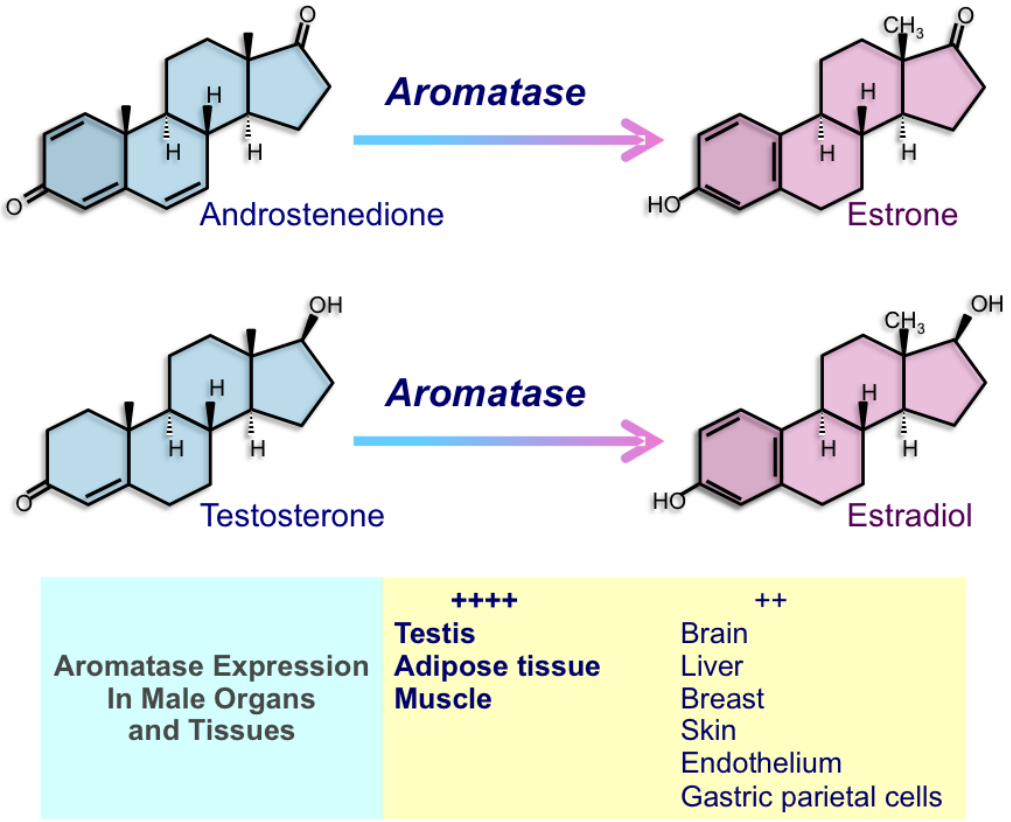

Figure 2. Biochemical pathway of testosterone conversion into estrogen.

However, a wide number of other endogenous products belongs to the category of estrogenic compounds, such as 27-hydroxycholesterol, dehydroepiandrosterone (DHEA), 7-oxo-DHEA, 7α-hydroxy-DHEA, 16α-hydroxy-DHEA, 7β-hydroxyepiandrosterone, Δ4-androstenedione, Δ5-androstenediol, 3α-androstanediol (3α-Adiol), 3β-androstanediol (3β-Adiol), 2-hydroxyestradiol, 2-hydroxyestrone, 4-hydroxyestradiol, and 4-hydroxyestrone and 16α-hydroxyestrone (28). In particular, dihydrotestosterone (DHT), an androgenic metabolite of testosterone that is synthesized by the enzyme alpha reductase, can be metabolised into 3 β-Adiol, an intermediate metabolite with estrogenic activity (27,31). All these molecules differ in terms of ER affinity (28). Various exogenous substances also show estrogenic activity, such as bisphenol A, metalloestrogens, phytoestrogens (e.g., coumestrol, daidzein, genistein, miroestrol) and mycoestrogens (e.g., zeranol) (32). These exogenous estrogens can influence human physiology via environmental exposure or ingestion.

The aromatase enzyme is a P450 mono-oxygenase enzyme complex (16) present in the smooth endoplasmic reticulum, which acts through three consecutive hydroxylation reactions, with the final reaction being the aromatization of the A ring of androgens (16,29) (Figure 2). This enzymatic complex is composed of a ubiquitous and non-specific NADPH-cytochrome P450 reductase, together with the regulated form of cytochrome P450 aromatase (16,24). The latter is highly specific for androgens (33,34). The conversion of androgens into estrogens takes place mainly in the testes, adipose tissue and muscle tissue, even though other male tissues are also involved to a lesser extent (16,29,30) (Figure 2).

The P450 aromatase enzyme is encoded by the *CYP19A1* gene: a gene of 123 kb of length, which consists of at least 16 exons and is located on the long arm of chromosome 15 in the q21.2 region in humans (8,16,29) (Figure 3). This gene belongs to the cytochrome P450 superfamily, similar to other enzymes involved in steroidogenesis (27).

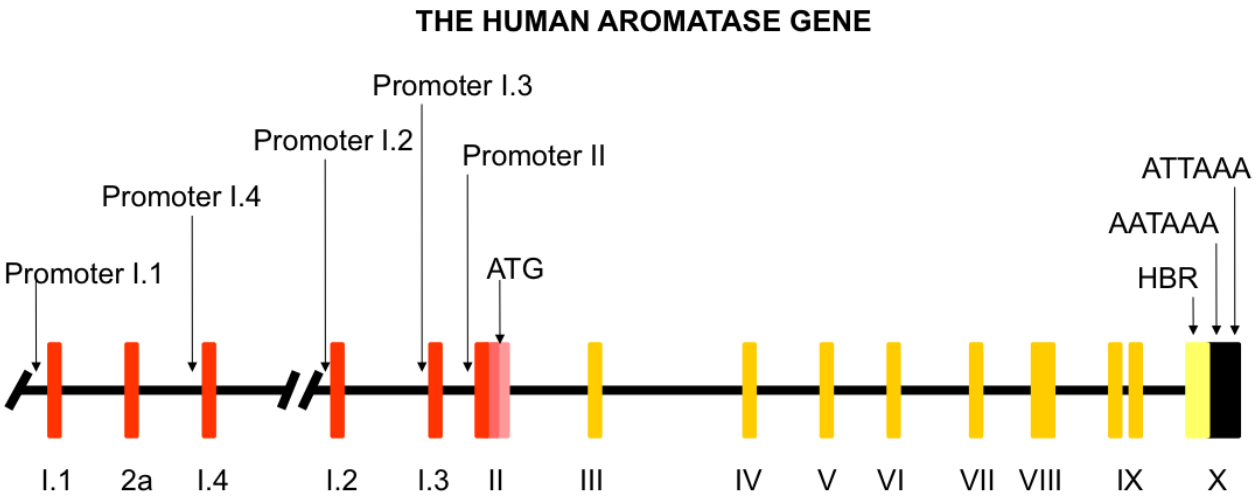

Figure 3. Schematic representation of the human aromatase (*CYP19*) gene.
[*Red bars: first exons associated with upstream alternative, tissue-specific promoters; yellow bars: coding exons; black bar: heme-binding region*]

Circulating estrogens are mainly reversibly bound to sex hormone binding globulin (SHBG), a β-globulin, and, to a lesser degree, to albumin (35). The amount of circulating free estradiol depends on several factors, of which the concentrations of albumin and SHBG are the most important (35). Serum free estradiol may be calculated by a complicated formula using total estradiol, SHBG, and albumin levels or may be measured by means of equilibrium dialysis or centrifugal ultrafiltration methodology; both, however, are too time consuming and expensive to be employed in routine clinical practice (35). When calculating free estradiol, the reliability of the value of total serum estradiol should be considered, since assays commonly used for estradiol in clinical laboratories have poor accuracy when measuring the low serum estrogen characteristic in males (36-38).

Estrogen Actions in Males

Estrogen action is mediated by interaction with specific nuclear estrogen receptors (ERs), which are ligand-inducible transcription factors regulating the expression of target genes after hormone binding (9,29,39). Two subtypes of ERs have been described: estrogen receptor α (ERα) and the more recently discovered estrogen receptor β (ERβ) (29,39). These two ER subtypes show different ligand specificity and transcriptional activity, and mediate the classical, direct, ligand-dependent pathway involving estrogen response elements in the promoters of targets genes and protein-protein interactions with several transcription factors (39). These two different ERs have different transcriptional activity. In particular, ERβ shows a weaker transcriptional activity compared to ERα (39). This difference is due to the presence of different ERβ isoforms, which can modulate estrogen signalling using different pathways and lead to different impacts on the regulation of target genes (39). In addition, it should be remarked that the co-expression of both ERα and ERβ in the same cell determines a complex cross-talk finally resulting in the antagonistic effect exerted

by ERβ on ERα-dependent transcription (39). Thus, the presence/absence of ER subtypes together with their cross-talk determines a cell's ability to respond to different ligands as well as the regulation of transcription of different target genes (39).

ERα in humans is encoded by the *ESR1* gene located on the long arm of chromosome 6, while the *ESR2* gene encodes ERβ and is located on band q22-24 of human chromosome 14 (39). The two ER proteins have a high degree of homology at the amino acid level (39) (Figure 4).

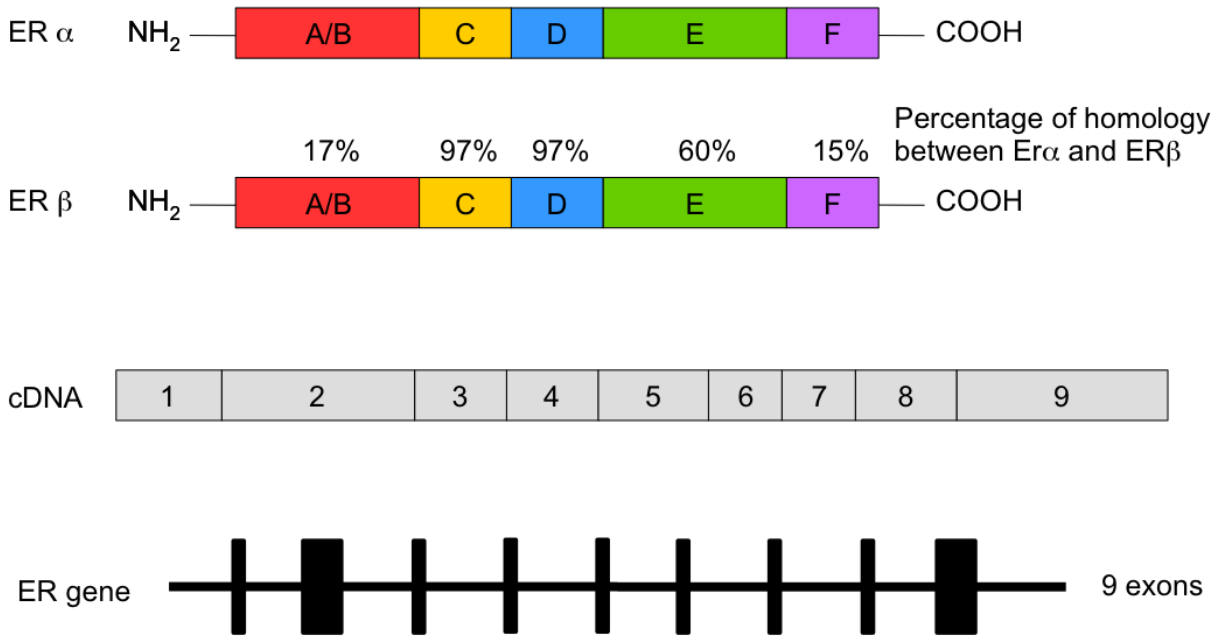

Figure 4. Estrogen receptor gene structure showing the 9 exons (lower panel), cDNA domains (indicating exons), and protein structures of both ERα and ERβ (upper panels: coloured boxes denote the different functional domains of the protein)

ERs are nuclear receptors in which structurally and functionally distinct domains are recognized. Estrogens bind the COOH-terminal multifunctional ligand-binding domain (LBD), whereas the DNA-binding domain recognizes and binds DNA (39). The NH2-terminal domain, the most variable domain, is involved in the transcriptional activation (39). This domain recruits a range of coregulatory protein complexes to the DNA-bound receptor (39). The two ER forms share a high degree of sequence homology except in their NH2-terminal domains. This specificity accounts for different transcriptional effects on different target genes (39). The ER genomic pathway begins with the binding of estrogen to ER (39). This interaction induces conformational changes in the ER, allowing receptor dimerization and subsequent nuclear translocation prior to binding to estrogen response elements or to other regulatory sites within target genes (39). Thereafter, the availability of several coregulatory proteins influences the transcriptional response to estrogen (39).

While it is clear that estrogens regulate transcription via nuclear interaction with their receptors, a non-genomic action of estrogens has been also demonstrated, suggesting a different molecular mechanism involved in some estrogen actions (29,39-41). *In vitro* studies show a very short latency time between the administration of estrogens and the appearance of its biological effects. These actions seem to be mediated by a cell-surface G protein-coupled receptor, known as GPR30, that does not act through a transcriptional mechanism (29,40,41). Rapid effects of

estrogens result from the actions of specific receptors localized most often to the plasma membrane; in particular it seems that a monomeric portion of the ERα is translocated from the nucleus to the plasma membrane (40,41).

Recently, immunohistochemical analysis of murine tissues reported the presence of GPR30 in the male reproductive tract, including testes, epididymis, vas deferens, seminal vesicles and prostate (42). Furthermore, a rapid response to estradiol suggests that non-genomic estrogen actions are involved also in human spermatozoa (43,44). The different intracellular pathways of estrogen action are summarized in Table 1.

Estrogen Actions	Receptors	Mechanism/Pathway	Final effect	Features
Genomic (nuclear actions)	ERα	Transcriptional: nuclear interaction with estrogen-responsive elements	Modulation of estrogen target gene expression	Slow effects (minutes or hours)
	ERβ	Transcriptional: nuclear interaction with estrogen-responsive elements	Modulation of estrogen target gene expression	Slow effects (minutes or hours)
Non Genomic (cell membranes actions)	Estrogen receptors on cells membrane (GPR30)	Cells membrane changes	Changes in ionic transport through cell surface	Rapid effects (seconds)

Table 1. Characteristics of estrogen actions and related biomolecular pathways.
[*Erα: estrogen receptor alpha; ERβ: estrogen receptor beta*].

Aromatase enzyme and ERs are widely expressed in the male reproductive tract both in animals and humans (45,46), implying that estrogen biosynthesis occurs at this site and that both locally produced and circulating estrogens may interact with ERs in an intracrine/paracrine and/or endocrine fashion (29). Today, it is clear that not only testicular somatic cells, but also germ cells constitute a source of estrogens in human (24,47). Thus, the concept of a key role for estrogen in the male reproductive tract is strongly supported by the ability of the male reproductive structures to produce and respond to estrogens (21,45). In men, the aromatase enzyme and ERs are expressed in several tissues including those involved in male reproduction. The distribution and expression of aromatase and ERs described below concerns the male reproductive organs.

Aromatase and ERs in the Male Reproductive Tract

The distribution of ERs and aromatase in both the developing and adult male reproductive tract of rodents and humans is summarized below.

Distribution of ERs and aromatase in fetal rodents

Aromatase and ERs are found at a very early stage of development in the rodent testis, thus suggesting a role for estrogens in influencing testicular development (3,21,48-50).

Leydig cells in fetal rodent testis express ERα before androgen receptor. Moreover, ERα is abundant in the developing efferent ductules, which are the first male reproductive structures to

express ERs during fetal development (51-53). Furthermore, the epididymis also expresses ERα in the fetal rodent. By contrast, it is unclear whether ERα is present within the seminiferous tubules of the fetal testis since conflicting results have been reported in literature (21,24,50).

ERβ is found early in fetal testis, particularly in gonocytes, Sertoli and Leydig cells, with the gonocytes showing the highest expression between 10-16 days post coitum (54). This suggests a role for estrogens in their maturation. In addition, ERβ is expressed by rat Wolffian ducts, the structures from which the efferent ductules and epididymis arise (21,50). ERα is widely expressed in efferent ductules from fetal life to adulthood, implying a crucial role in male reproduction that has been well documented in adult rodents (22,45,53). On the other hand, ERβ is mainly expressed during fetal life, suggesting a major role in the development of male reproductive structures until birth (21).

Aromatase is expressed in both Leydig and Sertoli cells in the fetal rodent testis, but not in gonocytes and immature structures of the seminal tract. ER and aromatase distribution in the fetal testes is summarized in Table 2. The presence of both aromatase and ERs in the developing fetal testis implies a possible involvement of estrogens in the process of differentiation and maturation of developing rodent testis just starting from an early stage of embryogenesis, with ER β possibly playing a greater role than ER α (46,48,49).

	ERα	ERβ	Aromatase
Leydig cells	++	++	+
Sertoli cells	-	++	++
Gonocytes	-	+++	-
Efferent Ducts	+	+	-

Table 2. ERs and aromatase distribution in the rodent fetal testis and efferent ducts. The proposed distribution is based on information from various studies including immunohistochemical and mRNA expression studies.
[*ERα : estrogen receptor alpha; ERβ: estrogen receptor beta*].

Distribution of ERs and aromatase in adult rodent reproductive tract

ERα is expressed (both in terms of mRNA and protein) in the Leydig cells of both adult rats and mice (55) but not in Sertoli cells, and is mainly expressed in the proximal (*rete testis*, efferent ductules, proximal epididymis), rather than in the distal (*corpus* and *cauda* of the epididymis, *vas deferens*) reproductive ducts (21). Furthermore, ERα has been immunolocalized in ciliated and non-ciliated cell nuclei of the epididymal epithelium (52,56). This peculiar distribution explains several important estrogen actions in the proximal ducts, especially within the efferent ductules that are small and convoluted tubules connecting the *rete testis* (an anastomosing network of intricate and tenuous tubules located in the hilum of the testis) to the epididymis (53). In the efferent ductules, estrogens promote fluid reabsorption (45,53,57). Finally, the full-length form of ERα has been detected in purified rat germ cells, using a specific antibody directed against the C-terminal region of the protein (58) (Table 3).

ERβ is expressed (both in terms of mRNA and protein) in Leydig and Sertoli cells in adult rodents (21,50,53) and in monkey germ cells (59); furthermore, it is expressed also in epithelial and peritubular cells of efferent ducts (52,56). The presence of ERβ in rodent germ cells has been the subject of some debate. In addition, there are some controversies about ERβ localization since immunohistochemical studies show discrepancies possibly due to methodological differences(60). Immunolocalization of ERβ in differentiated germ cells of adult rodents has been revealed in various studies (54,61). Conversely, no ERβ immunoreactivity was found in rodent germ cells in other studies (62), while mRNA expression seems to decline from fetal life to adulthood in the rat (60). Nevertheless, ERβ does seem to be involved in the regulation of gonocyte multiplication, which is under the influence of growth factors and estradiol (15), suggesting a functional role for ERβ at least in immature male germ cells.

	ERα	ERβ	GPR30	Aromatase
Leydig cells	+	+ / -	+	+++
Sertoli cells	-	+	+	+
Germ cells	+	++	+	++++
Spermatogonia	+	+	+	+
Pachytene Spermatocytes	+	+	+	+
Round Spermatids	+	+	+	++

Spermatozoa	+	+	?	+
Efferent ductules	++++	+	?	+

Table 3. ERs and aromatase distribution in the adult rodent testis and efferent ducts. The proposed distribution is based on information from various studies including immunohistochemical and mRNA expression studies.
[*ER α: estrogen receptor alpha; ERβ: estrogen receptor beta; GPR30: G protein-coupled receptor*].

GPR30 is widely expressed (both in terms of mRNA and protein) in rodent testis (63). In particular, this receptor is expressed in rat Leydig cells (64) and Sertoli cells (65,66), in the spermatogonia GC-1 cell line (67), in rat pachytene spermatocytes (58), and in round spermatids (68).

Rodent Leydig cells show higher aromatase expression than Sertoli cells (69). Aromatase is also expressed at high levels in germ cells throughout all stages of maturation, with its expression increasing as germ cells mature into spermatids. Aromatase mRNA expression and enzyme activity are present in both rat and mouse germ cells from the pachytene spermatocyte stage, and during their subsequent maturation into round spermatids (50,53,69) (Table 3). Carreau *et al.* demonstrated that aromatase activity in germ cells was more than 50% of that of the whole testis (24). This intensive activity suggests that germ cells may be a major source of estrogen in adult rodents (50,53,69) (Table 3). Specifically, when fully developed spermatids are released from the epithelium, aromatase is present in the residual body (the remains of the spermatid cytoplasm that is removed during spermiation) and is subsequently phagocytosed by the Sertoli cell. Aromatase activity also remains detectable in the cytoplasmic droplet attached to the flagellum when sperm passes through the epididymis, suggesting that mature spermatozoa are able to synthesize their own estrogen as they pass through the efferent ducts (24,70). The ability to synthesize estrogen gradually decreases as the droplet slowly moves to the end of the tail during epididymal transit until it is finally lost. The demonstration of aromatase in sperm is important as it suggests that the sperm itself could control the levels of estrogen present in the luminal fluid, and might directly modulate some functions such as the reabsorption of fluid from the efferent ductules (53).

Distribution of ERs and aromatase in the human male reproductive system

ERs are present in human testis and reproductive tract (24,53,71). In the male fetus both ERβ and aromatase are expressed in Sertoli, Leydig and germ cells from 13 to 24 weeks, whereas ERα expression is absent (72). Furthermore, ERβ immunoreactivity in the epididymis suggests a putative role for locally produced estrogens, the actions of which are likely mediated by ERβ in this site. This supports the importance of estrogens for the prenatal development and function of male reproductive structures, which is well documented in literature (72).

Aromatase and ERβ, but not ERα, continue to be expressed (both in terms of mRNA and protein) during the prepubertal period in men, but their function during infancy remains unclear, especially if the very low levels of both circulating and locally produced sex steroids in this period of life is taken into account (73).

In adult men, ERα is expressed only in Leydig cells, while ERβ has been documented in both Leydig and Sertoli cells and in the efferent ducts (62) (Table 4). The presence of ERs in the human epididymis is still a matter of debate (22), even though ERα has been detected in the nuclei of epithelial cells of the caput of the epididymis (74). Both ERs (ERα and β) have been identified in isolated immature germ cells (24). Furthermore, they were localized in mature spermatozoa (75) and in ejaculated spermatozoa (76). Luconi *et al.* first described an estrogen receptor-related

protein in the sperm membrane (43,44). This protein is able to bind steroid hormones and may act through a calcium-calmodulin dependent pathway, accounting for a well-documented rapid non-genomic action (43,44). Subsequently, the expression (both in terms of mRNA and protein) of both ERs in human ejaculated spermatozoa (76,77) reinforced the concept that estrogens are able to modulate the spermatogenic process from its onset within the testes through to the final process of sperm maturation after ejaculation (3,24,76,77). The ERα and ERβ localize to different regions in human sperm, with ERα present in the compact zone in the equatorial segment of the upper post-acrosomal region of the sperm head, and ERβ in the mid-piece, at the site of the mitochondria (50). This confirms that each type of receptor probably has a distinct role in sperm physiology and in the process of fertilization (78).

	ERα	**ERβ**	**GPR30**	**Aromatase**
Leydig cells	+	+ / -	+	+
Sertoli cells	-	+	+	+
Germ cells				
Spermatogonia	-	+	+	ND
Pachytene Spermatocytes	+	+	-	+
Round Spermatids	+	+	-	+
Spermatozoa	+	++	-	+
Efferent ductules	+	+	?	+

Table 4. ERs and Aromatase distribution in the human testis and efferent ductules. The proposed distribution is based on information from various studies including immunohistochemical and mRNA expression studies..

[*ERα: estrogen receptor alpha; ERβ: estrogen receptor beta; GPR30: G protein-coupled receptor*].

Of particular interest is the demonstration of differential expression in the human testis of wild type ERβ (ERβ1) and of a novel human variant form of ERβ, the latter arising from alternate splicing (known as ERβcx, or ERβ2), (79,80). ERβ2 expression seems to be associated with prevalent, negative inhibition of ER action by inhibiting ERα–induced transactivation (81); it is highest in spermatogonia and Sertoli cells in adult men, suggesting that these cells may be "protected" from estrogen action (79,80). Wild type ERβ1 was mostly present in pachytene spermatocytes and round spermatids, which have been proposed to be more estrogen sensitive (21), yet ERβ1 was low in less mature germ cells (79). In addition, the discovery of several splice variants of ERβ (including ERβ4) in human testicular cells suggests a specific and more complex estrogen action on spermatogenesis (80).

Recently, the cellular distribution of non-genomic GPR30 estrogen receptor in human testicular biopsies was examined (82). Immunohistochemical analysis of testicular sections identified the GPR30 receptor in the cytoplasm of Leydig cells, Sertoli cells and spermatogonia (82). This pattern of localization was further demonstrated by the analysis of GPR30 expression (both in terms of mRNA and protein) in isolated germ cells and in Sertoli cell culture (82). This peculiar distribution suggests that GPR30 may be involved in germ cell differentiation (82). Furthermore, the presence of GPR30 in human spermatozoa has been confirmed at both the mRNA and protein level, with this receptor being localized in the sperm mid-piece (83). The co-expression of the two classic ERs and of the GPR30 receptor in the same area within the spermatozoa (mid-piece and acrosome region) suggests a complex cross-talk among all these receptors.

Aromatase expression in the human testis is present in both somatic and germ cells (46). Specifically, it is expressed in Leydig and Sertoli cells (84,85), in immature germ cells, from pachytene spermatocytes through elongated spermatids (50,84), and ejaculated sperm cells (86). Locally produced estrogens in sperm are proposed to exert a protective action on sperm DNA by preventing sperm DNA damage (87), thus accounting for estrogen's potential role as a survival factor during sperm transit through the seminal vesicles (88). Unlike rodents, aromatase expression in human gametes persists during the transit through the genital tracts, since P450 aromatase has been demonstrated in human ejaculated spermatozoa at three different functional levels: mRNA expression, protein production and activity (76). Therefore, as in rodents, human sperm are considered a potential site of estrogen biosynthesis (3,76,84,85,87). The presence of functional aromatase in human spermatozoa allows the conversion of androgens into estrogens as they transit the reproductive tract, providing free estrogens in the seminal fluid able to act on the cells of the reproductive ducts. Thus, human spermatozoa should be considered a mobile endocrine unit (46,47,89).

In summary, the testes are able to synthesize and respond to estrogens throughout their development (46). The localization of ERα, ERβ and aromatase suggests that estrogen action is likely to be important for testicular and efferent ductule function. Differences among various polymorphisms of ER genes may account for different responses to estrogens in term of sperm count and sperm quality (90,91). The role of estrogens in the male reproductive system is clearer in rodents (see below), and the mapping of ERs and aromatase distribution in the human male reproductive system has led to the suggestion that estrogen plays a role in human male reproduction (3,46,48). As a consequence, a new field of research has evolved, aimed at improving our knowledge on estrogen action on male reproduction, and the molecular mechanisms involved in both animal models and in men.

ROLE OF ESTROGENS IN MALE REPRODUCTION

Estrogens in Animal Male Reproduction: effects of estrogen deficiency

Estrogen-deficient knockout mice are useful models to investigate estrogen action in rodents (15,21). At present, four different lines of estrogen receptor-deficient knockout mice have been generated: 1) ERα knockout (α-ERKO) mice with disrupted ERα gene (92-94); 2) ERβ knockout (β-ERKO) mice, with an inactivated Erβ (95), 3) double ERα and ERβ knockout (αβ-ERKO) mice with non-functioning ERα and ERβ (15), and 4) GPR30 knockout mice (96-98). The αERKO, βERKO and αβERKO mice provide very helpful information on the loss of ER function, leading to estrogen resistance. The knockout of the aromatase gene in aromatase knockout (ArKO) mice is an experimental model useful for investigating the congenital lack of both circulating and locally produced estradiol (15,21,99,100). Estrogen-resistant mice (αERKO, βERKO, and αβERKO) have high levels of circulating estrogens with the non-genomic pathway still likely functional. Aromatase-deficient mice have no circulating estradiol however estrogen receptors could be activated by other estrogenic compounds produced outside the aromatase pathway (e.g. 3β-Adiol) or introduced by diet (e.g. phytoestrogens) (21). Furthermore, in 2009, Sinkevicius et al. created transgenic mice with a G525L point mutation in the ligand-binding domain of ERα (ENERKI mice) (101). This allows differentiation of ligand-dependent vs ligand-independent ER actions since these two different pathways could lead to different actions *in vivo*. The study of fertility of the ENERKI mouse shows that the efferent ductule fluid reabsorption is regulated by ligand-independent actions of ERα, whereas germ cell production and/or viability requires ligand-dependent ERα actions (101).

Recently, the creation of the knockout mice lacking GPR30 estrogen receptor (96,98) allowed an investigation of the reproductive phenotype of mice lacking a functional GPR30, with the results suggesting a minor role of this receptor in male fertility. GPR30 knockout mice did not show abnormalities of endocrine organs, alterations of spermatogenesis and mating behavior, or decreased fertility (97,102). A detailed study of spermatogenesis in this mouse model is, however, still lacking.

Studies on transgenic mice lacking ERs or the aromatase enzyme demonstrate that the lack of estrogen action is compatible with life (20,103). Congenital estrogen deficiency in mice leads to an impairment of male reproductive function ranging from normal fertility with a fully male phenotype in βERKO mice, to complete infertility in both αERKO and αβERKO mice. An intermediate pattern exists for the ArKO mice in which spermatogenesis is normal in young mice, but progressively worsens during aging (15,21,53,57,92-95,99,104). Characteristics of male mouse models are summarized in Table 5.

αERKO	**βERKO**	**αβERKO**	**ArKO**
Infertility	Fully fertile	Similar to α ERKO mice	Normal fertility in young mice, infertility with advancing age
Normal FSH Elevated LH Elevated testosterone Elevated estradiol	--	--	Normal FSH Elevated LH Elevated testosterone Undetectable estradiol
Germ cell loss and dilated seminiferous tubules	Normal testicular histology	Testicular histology similar to α ERKO mice	Histology of the testis is disrupted with advancing age
Impairment of sexual behavior	Normal sexual behavior	Complete suppression of sexual behavior	Impairment of sexual behavior

Table 5. Reproductive phenotype of male mouse models of estrogen deficiency. The *G protein-coupled receptor* (GPR30) knockout mice have normal reproductive phenotype.
[*ERKO: estrogen receptor knockout mice; α: estrogen receptor alpha; β: estrogen receptor beta; ArKO: Aromatase knockout mice*].

Male αERKO mice are infertile as the seminiferous epithelium is atrophic and degenerated, and seminiferous tubules and rete testis are dilated (53,57,94), even though the development of male reproductive tract is largely unaffected (15,92,94,105). The disruption of spermatogenesis is progressive as the testicular histology is normal at postnatal day 10, but starts to degenerate at twenty-thirty days of age (57,94). From 40 to 60 days, tubules are markedly dilated with a corresponding significant increase in testicular volume, while the seminiferous epithelium becomes atrophic (15,57,94). A severe impairment in tubule fluid absorption at efferent ducts level is the cause of infertility in αERKO male mice, and this defect is partially mimicked also by the administration of an anti-estrogen drug in wild-type mice (52,53,57). In the male genital tract, the highest concentration of ERα is in the efferent ducts (57) and the estrogen-dependent fluid reabsorption at this site probably results from estrogen interaction with the ERα that seems to regulate the expression of the Na(+)/H(+) exchanger-3 (NHE3) (52,57). This mechanism appears to be the consequence of the ligand-independent ERα activation (101). In fact, the disruption of ERα, or the use of anti-estrogens, results in a decreased expression of NHE3 mRNA, as well as in

a decrease of other proteins involved in water reabsorption, such as aquaporin I (106,107). The lack of fluid reabsorption in the efferent ductules of αERKO male mice and the consequent dilatation induces a retroactive progressive swelling of the seminiferous tubules (22,45,53,57,94,108). The seminiferous tubule damage results from the increased fluid pressure and severely impaired spermatogenesis coupled with testicular atrophy as seen at the age of 150 days of age (15,45,53,57). When germ cells from αERKO mice are transplanted in wild type mice, they show normal development (109). The αERKO mouse is also characterized by a reduced number, motility, and fertilizing capacity of the sperm levels (Table 5). In addition, αERKO male mice show increased serum luteinizing hormone (LH) and testosterone as well as Leydig cells hyperplasia, together with normal serum follicle-stimulating hormone (FSH) levels (Table 5) (15).

The production of both ArKO (104) and βERKO (95) mice added further insights in this field, supporting the idea that estrogen actions on the male reproductive tract in mice are more complex than previously suggested on the basis of the αERKO mice (15). In fact, unlike αERKO mice, male ArKO mice are initially fully fertile (104), but fertility decreases with advancing age (Table 5) (21,99,100). Furthermore, βERKO mice are fully fertile and apparently reproductively normal in adulthood (Table 5) (95).

The mechanism involved in the development of infertility in ArKO male mice therefore differs from that of the αERKO mice (21). Transgenic mice models suggest that ligand-independent ERα signaling is essential for concentrating epididymal sperm via regulation of efferent ductule fluid reabsorption, while ligand-dependent ERα is involved in germ cell production and/or viability (101). Thus, the lack of estrogen action at the level of the seminiferous epithelium rather than a problem due to impaired fluid reabsorption probably explains infertility in ArKO male mice (21,43). Accordingly, estradiol seems to be necessary for round spermatid survival and estrogen deficiency seems to promote apoptosis before differentiation into elongated spermatid (21,76,88).

Studies of αβERKO mice showed a male phenotype very close to that of αERKO mice characterized by infertility and dilated seminiferous tubules (15,21). On the contrary, βERKO male mice were fully fertile (95). These findings lead to the hypothesis that estrogen activity in the male reproductive tract depends on both the type of estrogen receptor involved, and the site of action through the male reproductive tract. Interestingly, results from mice lacking functional ERs or aromatase point to an important role for estrogen in the maintenance of mating behavior in male mice. For this reason, infertility in αERKO, αβERKO and ArKO mice is at least in part due to the reduction of various components of mating behavior from an early age (Table 5) (15,21).

The function of the hypothalamo-pituitary-testicular axis is impaired in both αERKO and ArKO male mice, leading to elevated serum LH levels in the presence of normal values of FSH, while, as expected, testosterone is augmented and estrogens are higher than normal or undetectable in αERKO and ArKO mice, respectively (21). Thus, negative effects on male reproduction are the direct result of estrogen deprivation in the reproductive structures or of indirect changes in the regulation of sex steroid secretion.

Taken together, all these studies support the concept that a functional ERα, but not ERβ and GPR30, is needed for the development and maintenance of a normal fertility in male mice (14,15,45,48,52,53,57,94,95).

Estrogens in Human Male Reproduction: effects of estrogen deficiency

The demonstration of wide expression of the aromatase enzyme, ERα and ERβ throughout the male reproductive system and within human sperm underlines the role of estrogens in human male reproductive function (3,24,48,89,110). Accordingly, estrogens seem to modulate sperm maturation (43,110), since spermatozoa express ERα and ERβ, and are responsive to estrogens throughout their journey from the testes to the urethra.

Data from human subjects with congenital estrogen deficiency have provided conflicting and confusing results. Only one man with estrogen resistance has been identified; this patient exhibited a mutation in ERα, rendering it unable to respond to estrogen, thus he could be considered to be a human equivalent of the αERKO mouse. However, this man had normal testicular volumes and normal sperm count but with slightly reduced motility (17) (Table 6). This is different from that observed in αERKO mice (15,21,45,57,94) since there was no clinical evidence of obstruction in the man with estrogen resistance, different to that observed in the rodent model (17). However, no data on the histology of the testis and efferent ductules is available from this estrogen-resistant man (17).

The other human model of estrogen deficiency is congenital aromatase deficiency (111). At present, twelve men with aromatase deficiency have been described (Table 6 and Table 7) (18,112-118). For most of them the genetic diagnosis (119,120) and/or the clinical description (19,121-123), as well as the following clinical studies (124-131) were performed by our research group. These patients showed a variable degree of impaired spermatogenesis (3,10,119,120,132). The hormonal pattern of the patients affected by aromatase deficiency is summarized in Table 6 (3,48,111). Testicular size in aromatase-deficient men is normal except for three cases having a smaller testes volume (Table 6). Of the five patients with available semen analysis, three had normal sperm count (114,115,120,123) and the remaining two had oligospermia (19,20,113) from moderate (113) to severe (19) (Table 6). In four of the five patients, however, moderate to severe asthenospermia without teratospermia was reported (19,20,113-115,123) (Table 6). No data on sperm count was available in the other three men with aromatase deficiency (18,112,119) and in case reports (119,120), as well as in the unique aromatase-deficient boy described so far (117,118). Moreover, a variable degree of germ cell arrest, ranging from complete depletion of germ cells to arrest at the stage of primary spermatocytes, was described in three aromatase-deficient men who underwent biopsy of the testes (19,20,121,122) (Table 6).

Furthermore, a history of cryptorchidism was present in three of the nine patients (33,3%) being bilateral in one case (121) and unilateral in the remaining two (114,115,123). These data suggest a possible role of estrogen in testis descent, although this was not seen in the transgenic mouse models. The small number of cases of cryptorchidism among men with aromatase deficiency does not allow any conclusion concerning a possible relationship between estrogen deficiency and the occurrence of abnormalities in testis development and descent.

It should be remarked, however, that a clear cause-effect relationship between infertility and aromatase deficiency was not demonstrated in all these patients (3,111). For this reason, the different degree of fertility impairment found in men with congenital estrogen deficiency does not allow us to establish with certainty whether sperm abnormalities are a consequence of the lack of estrogen action, or are an epiphenomena. Again, this spermatogenetic pattern is different from that observed in ArKO mice (15,21,45,99,100,104).

	Estrogen resistance	Aromatase deficiency

Total subjects	1	12
Subjects diagnosed during adulthood	1	11
Age (mean + DS)	28 years	29.0 ± 4.8 years
REPRODUCTIVE HORMONES		
LH	High	Normal to high
FSH	High	High
Testosterone	Normal	Normal to high
Estradiol	High	Undetectable
EXTERNAL GENITALIA		
Size testis	Normal	Small to normal
Cryptorchidism	Absent	3 cases
SEMEN ANALYSIS		
Sperm count	Normozoospermia	Oligo to normozoospermia
Viability	Asthenozoospermia	Asthenozoospermia
Testis biopsy	Not performed	Depletion or germ cell arrest at primary spematocyte level

Table 6. Reproductive phenotypes of men with congenital estrogen deficiency.
[*LH: luteinizing hormone; FSH: follicle-stimulating hormone*]

The frequency of sperm abnormalities in these patients together with the results from rodent studies suggests a possible role for estrogen in human spermatogenesis, however this requires further elucidation (3,11,25). Our knowledge on estrogen's role in human male reproduction remains far from complete. The data available in the literature suggests that the action exerted by estrogens on male reproductive organs is more complex than that seen in mice and that estrogen alone does not directly control spermatogenesis to the same extent than in rodents.

In addition to human models of congenital estrogen deficiency, other experimental settings have provided information on the role of estrogens on human male fertility.

Studies on the association between ER polymorphisms and infertility in men showed that two polymorphisms of ERα (*XbaI* and *PvuII*) are associated with azoospermia, severe oligospermia and impaired sperm motility (133-135) and the multiallele (TA)n polymorphism with male infertility (91). The *RsaI* polymorphism of the ERβ has been associated with male infertility in one study (90), but not confirmed in another study (135). The ERβ (*RsaI*) polymorphism *AluI* was also associated with sperm motility, while no association with motility was found for the *RsaI* polymorphism (135). Thus, the association of polymorphisms of estrogen-related genes with both sperm concentration and motility, but not with sperm morphology, further supports a putative role of estrogen in controlling sperm production and quality (136).

Furthermore, the investigation of ERs in the nuclear matrix of human spermatozoa showed a reduction of ER levels in the nucleus of idiopathic infertile men compared to normospermic fertile men (137).

Interventional research studies show that the administration of aromatase inhibitors to infertile men with documented impaired testosterone-to-estradiol ratio may result in an improvement of their fertility rate, but further evidence is needed to verify their efficacy and safety (see paragraph below 'Anti-estrogen treatment in men' for further details) (138,139). These results suggest that such modulation of estrogen metabolism will influence sex hormone balance and the HPT axis while dissecting out direct effects of estrogen on spermatogenesis in vivo is extremely challenging.

It seems that exposure to increasing estradiol concentrations might influence glucose metabolism in spermatozoa and that the increase of aromatase activity and estradiol enhances glucose metabolism in capacitated, but not in non-capacitated sperm (77).

It seems probable that most of estrogen actions operating in mice, such as regulation of sperm motility, sperm capacitation, acrosome reaction, and sperm metabolism also occur in men, but the contribution of estrogens to these processes is quantitatively less important in humans. It seems likely that most of these processes in humans are also regulated by other factors in a complex cross-talk system involving also estrogens. This could also explain why high amounts of estrogens or the exposure to an excess of environmental estrogens (or to xenoestrogens with high estrogenic potency) could negatively impact on male fertility. For these reasons, it is apparently difficult to reconcile existing data about effects of both estrogen deficiency and excess on male reproductive function (12,26,140-142).

Regulation of Gonadotropin Feedback

The regulation of gonadotropin feedback is an important and well-documented action of estrogen in males. While testosterone has been classically considered the key hormone for the control of gonadotropin feedback in the male (Figure 5), a role for estrogens was recently clarified in studies performed in normal and GnRH-deficient men.

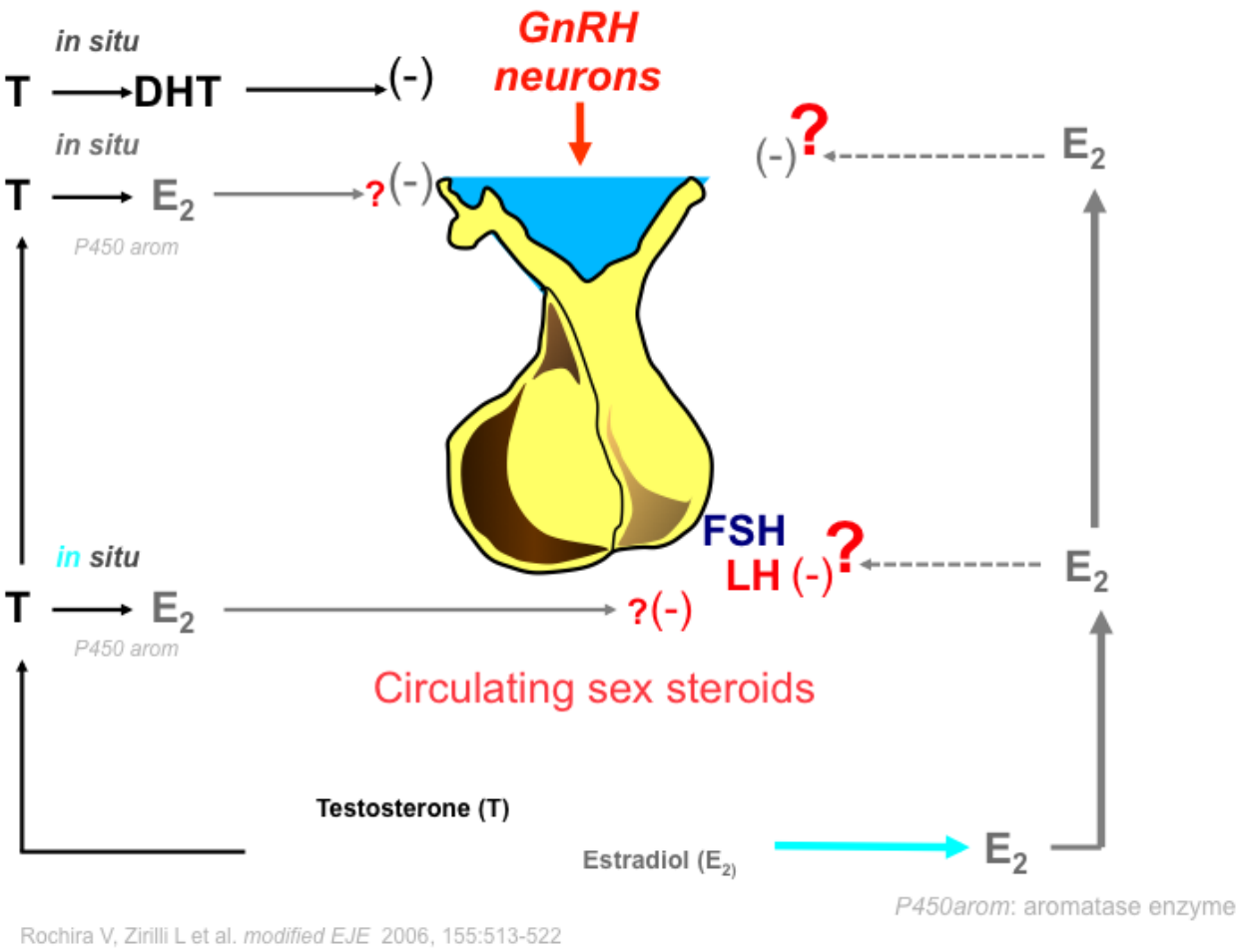

Figure 5. Previous knowledge concerning sex steroids in the control of gonadotropin secretion: testosterone and DHT are the main sex steroids with inhibitory effects on gonadotropin secretion.

The role of estrogen was considered of minor importance and mainly related to locally produced estrogens.

[*T: testosterone; DHT: dihydrotestosterone; E_2: estradiol; GnRH: gonadotropin releasing hormone; LH: luteinizing hormone; FSH: follicle-stimulating hormone*].

The response of the hypothalamic-pituitary gonadal axis to androgens is confirmed by the administration of dihydrotestosterone (DHT), which is able to partially decrease LH and FSH with a concomitant reduction in serum testosterone and estradiol (143) (Figure 5). However, the discovery of men with congenital estrogen deficiency has provided further evidence for a relationship between estrogens and gonadotropin secretion also in men (20). In fact, serum gonadotropins are high in all adult patients with aromatase deficiency, notwithstanding normal to increased serum testosterone levels (111), thus implying that estrogens are also important for the regulation of circulating gonadotropins levels in men.

The effects of estrogens on gonadotropin secretion have been investigated in GnRH-deficient men whose gonadotropin secretion was normalized by pulsatile GnRH administration. Moreover, in order to determine the precise role of sex steroids on the hypothalamo-pituitary-testicular axis, several studies characterized by the administration of testosterone, testosterone plus testolactone (an aromatase inhibitor), or estradiol have been performed (144,145). Testosterone alone induced a significant decrease in mean basal LH and FSH levels as well as of LH pulse amplitude, demonstrating a direct suppressive effect on the pituitary of testosterone and its metabolites. In general, mean LH levels and LH pulse frequency are suppressed to a greater extent in normal control subjects under testosterone administration, suggesting the involvement of a hypothalamic site of action of testosterone (or its metabolites) in suppressing GnRH secretion. In order to discriminate the impact of testosterone from its aromatized products, both groups of subjects were treated with testosterone plus testolactone. The addition of this aromatase inhibitor completely inhibited the testosterone effect on gonadotropin secretion both in normal and GnRH-deficient men, thus leading to a significant increase in mean LH levels in both groups. The latter was greater in normal men who received testolactone alone than in normal men who received testosterone plus testolactone, thus confirming a direct effect of androgens on gonadotropin secretion in normal men. On the basis of the results of these studies, it is clear that the aromatization of testosterone to estradiol is, at least in part, required for normal gonadotropin feedback at the pituitary level (145). In fact, when the same experimental model was applied using estradiol administration instead of testolactone, mean LH and FSH levels as well as LH pulse amplitude decreased significantly during the treatment (144). These studies have demonstrated an important direct inhibitory effect of estradiol on gonadotropin secretion in both GnRH-deficient and normal men (144,145) and support the concept that, at least in part, the inhibitory effect on gonadotropin secretion is mediated by the conversion of testosterone to estradiol. Accordingly, the administration of the aromatase inhibitor letrozole to healthy adult males is able to suppress aromatase activity and serum estradiol levels leading to an increase of gonadotropins (146). Only the restoration of normal circulating estrogens, by means of transdermal estrogen administration, normalized gonadotropin secretion in this setting (146). In contrast, it seems that the 5α-reduction of testosterone to DHT does not play a very important role in pituitary secretion of gonadotropins (147); DHT, in fact, slightly decreases LH and FSH only after long-term administration (143).

All these studies suggest possible estrogen action at the level of hypothalamus. In order to clarify the role of estrogen on the feedback regulation of gonadotropin secretion at hypothalamic level, Hayes *et al*. (148) conducted a study involving men affected by idiopathic hypogonadotropic hypogonadism (IHH), whose gonadotropin secretion was normalized by long-term pulsatile GnRH

therapy, followed by treatment with the aromatase inhibitor anastrozole. They observed that the inhibition of estradiol synthesis led to an increase in mean gonadotropin levels that was greater in normal subjects than in IIH men, suggesting a hypothalamic involvement. The rise in mean LH levels in normal subjects related to the anastrozole effect was the result of an increase in LH pulse frequency and amplitude. The authors concluded that estrogen acts at the hypothalamic level by decreasing GnRH pulse frequency and pituitary responsiveness to GnRH (148). Subsequently, the same group (149) demonstrated that the administration of estradiol in normal subjects, whose endogenous testosterone and estradiol synthesis was inhibited through the use of ketoconazole, reduced mean LH levels by lowering LH pulse frequency, but not amplitude. These authors went on to report that the sex steroid component to FSH negative feedback was not androgenic but rather was mediated by estradiol effects on the frequency of GnRH stimulation (149)(150).

For many years another important unresolved issue has been the relative role of circulating vs. locally produced estrogens in the control of gonadotropin secretion. Now we know that the effects of circulating estrogen are more relevant than that of locally produced, at both the hypothalamic and pituitary level (146) (Figure 6). Accordingly, the administration of both the aromatase inhibitor letrozole and estradiol at different dosages showed that serum testosterone and gonadotropins were inversely related to circulating estradiol, depending on the dose of exogenous estradiol (146). The plasma estradiol required to obtain the same levels of gonadotropins were not different compared to that at baseline, suggesting that aromatase inhibition and the blockade of locally produced estrogens are less important than previously thought (146). In the same year, our group reached the same conclusions using a different approach. In men with aromatase deficiency, we demonstrated that circulating rather than locally produced estrogens are the main inhibitors of LH secretion (128). This implies that the role of locally produced estradiol on gonadotropin feedback at hypothalamic and pituitary levels is relatively modest *in vivo* (Figure 6).

Data available in the literature demonstrate that (i) circulating estrogens are involved in gonadotropin suppression both at pituitary (146) and hypothalamic level (128,149), and (ii) estrogen effects on hypothalamus are independent from central aromatization, but requires adequate amounts of circulating estrogens in normal healthy men (146), in men with IHH (149,150), and in men with aromatase deficiency (128).

The effects of estrogen on gonadotropin secretion at the pituitary level operate from early- to mid-puberty (151,152) into old age in men (153). The administration of an aromatase inhibitor (anastrozole 1 mg daily for 10 weeks) to young men aged 15-22 years (151) resulted in a 50% decrease in serum estradiol concentrations, an increase in testosterone concentrations and an increase in both LH and FSH values during the study protocol. These hormonal parameters were restored after the discontinuation of anastrozole treatment (151). In addition, the administration of letrozole increased serum LH levels, LH pulse frequency and amplitude and the response of LH to GnRH administration in boys during early and mid-pubertal phases, confirming that estrogens act at the pituitary level during early phases of puberty (152). The same mechanism continues to operate during adulthood and early senescence (154), as shown in fifteen eugonadal men, aged 65 years treated with 2 mg anastrozole for 9 weeks, in which serum FSH and LH levels increased significantly, in spite of an increase in serum testosterone levels (153). Similar results were replicated by using letrozole in older men (139). For these reasons, the use of aromatase inhibitors as blockers of the negative feedback on gonadotropin has been tested as a possible strategy useful for the treatment of late-onset male hypogonadism (155). The rationale was that increasing endogenous serum testosterone through the inhibition of the rate of conversion of testosterone into estradiol led to the consequent LH and FSH increase (155). After the first encouraging results

(156-158), this kind of treatment seems to be not effective, especially on large-scale clinical trials and for long periods of time (154,155).

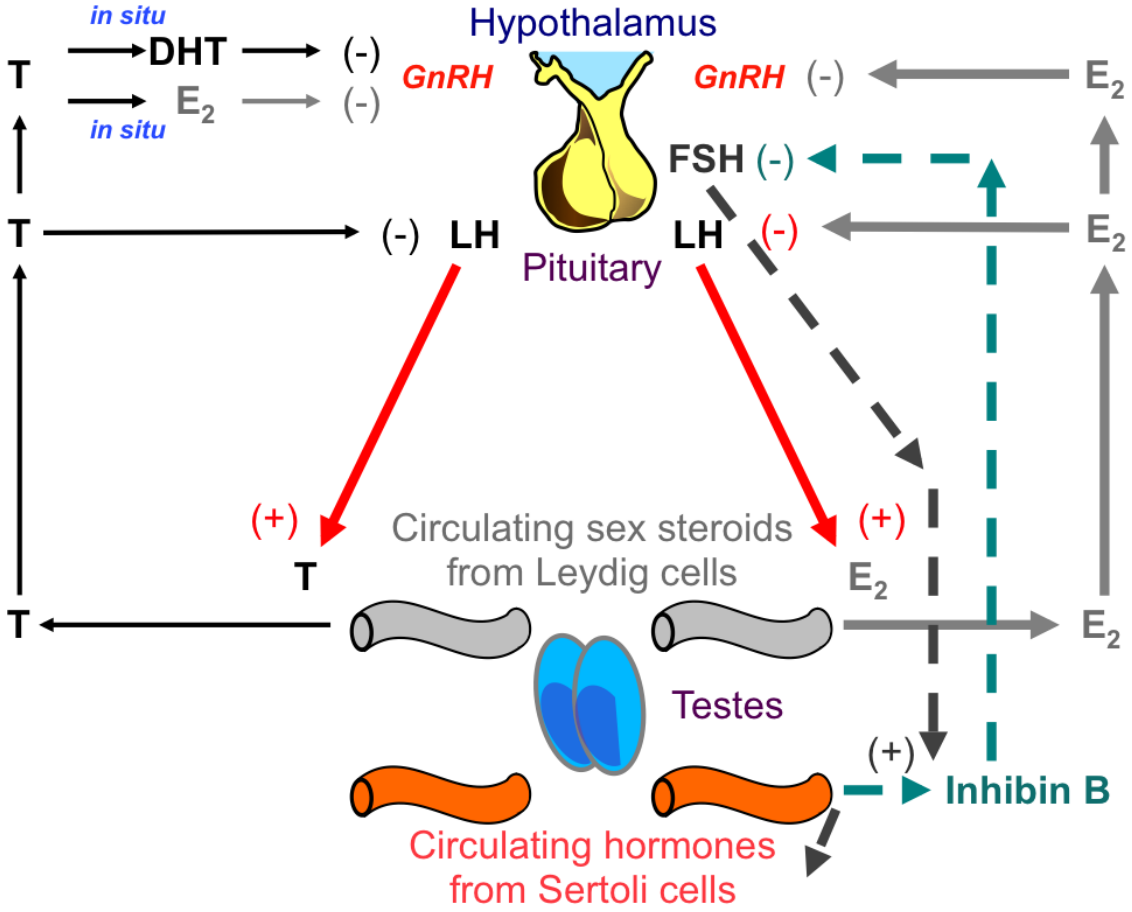

Figure 6. Sex steroid control of gonadotropin secretion after recent advances: estrogens, but not androgens, are the main regulator of gonadotropins and the action of circulating estradiol prevails with respect to that of locally produced estradiol.
[*T: testosterone; DHT: dihydrotestosterone; E_2: estradiol; GnRH: gonadotropin releasing hormone; LH: luteinizing hormone; FSH: follicle-stimulating hormone*]

Previous data suggest that estradiol may modulate GnRH receptor number and function at hypothalamic-pituitary level (159), since ERs were detected in GnRH secreting neurons (160). Moreover, both genomic and non-genomic estrogen actions seems to be involved in the regulation of the gonadotropin feedback in males (160,161), although the precise mechanism remains unclear (162). Nevertheless, it is now well established that androgens need to be converted to estrogens in order to ensure the integrity of the gonadotropin feedback mechanism in men, testosterone itself having a lesser role than previously thought (Figure 6), and circulating estrogen, rather than locally produced estrogen, having a major role at the hypothalamic pituitary level (128,146,150).

In a complementary way, our knowledge on the role of estrogens in gonadotropin feedback has been enhanced through studies of men with congenital estrogen deficiency. The description of a man lacking a functional ERα (17) revealed a remarkable hormonal pattern consisting of normal serum testosterone, high estradiol and estrone levels, but increased serum FSH and LH concentrations (Table 6). Other important information about estrogen's role in the human male

came from the discovery of naturally occurring mutations in the aromatase gene. To date, of the twelve different cases of human male aromatase deficiency that have been described, all were discovered to be aromatase-deficient as adults, except one who was diagnosed as a child (Table 6). Eight of the adult patients with aromatase deficiency had increased basal FSH concentrations (111,119), except for three of them having normal FSH (119). The subject diagnosed during childhood had normal FSH in infancy (117) and high to normal FSH levels at puberty (118). LH was normal except for one subject with elevated (18,112) and two subjects with high to normal LH levels (125,132) (Table 6). Serum testosterone concentrations were generally normal or high-to-normal except for the first case described with elevated serum levels (18,112), and two other aromatase-deficient men with testosterone slightly above the normal range (113,114). Conversely, another man with aromatase deficiency presented with low to normal serum testosterone levels (121,127). In all twelve patients estradiol concentrations were undetectable (111,119) (Table 6). The detection of increased gonadotropin levels despite normal-to-increased serum testosterone levels, in these men, further highlights the key role for estrogen in regulating circulating gonadotropins in men (126,128), In normal men with pharmacologically induced sex steroid deprivation, estradiol but not testosterone, was able to restore normal FSH serum levels (150). Due to the concomitant impairment of the patient's spermatogenesis, complete normalization of serum FSH was not achieved in all aromatase-deficient men during estradiol treatment, even in the presence of physiological levels of circulating estradiol (111), only supraphysiological levels of estrogens were able to normalize FSH (19,111,125,126).

A detailed study of the effects of different doses of transdermal estradiol on pituitary function in two men with congenital aromatase deficiency demonstrated that estrogens might control not only basal secretion of gonadotropins but also their responsiveness to GnRH administration (113,126,128). In these studies, estrogen administration to three male patients with aromatase deficiency caused a decrease in both basal and GnRH-stimulated LH, FSH and α-subunit secretion with a dose-dependent response to GnRH administration (113,126,128). In 2006, Rochira *et al*. (128), demonstrated that estrogen’s effects on LH secretion are exerted both at pituitary and hypothalamic level, as shown by the decrease of basal and GnRH-stimulated secretion of LH and the LH pulse amplitude, and the reduction of the frequency of LH pulses respectively, during estrogen treatment to normalize estradiol serum levels in two aromatase-deficient men. Moreover these data provide evidence that the action of negative feedback exerted by circulating estrogens is more relevant than that of locally produced at the hypothalamic level (128). As previously explained, these data confirm data from healthy men.

Notwithstanding recent advances in the study of estrogen’s role in males, some difficulties remain when data from men with congenital estrogen deficiency are interpreted, particularly if phenotype heterogeneity is considered (132). No abnormalities were found in either gonadotropin secretion or in testis position and size in the patient with congenital aromatase deficiency diagnosed in childhood (117), unlike female newborns (163). For these reasons, the role of estrogens in the hypothalamo-pituitary-testicular axis should become relevant in a later stage of life than infancy in men. Furthermore, the smaller than expected increase in FSH levels (given the prevailing serum testosterone levels and impaired spermatogenesis) in two estrogen-deficient men (128), suggests a possible role of estrogens in priming and maturation of hypothalamus-pituitary-gonadal axis in men (126,127). Thus, the control of gonadotropin feedback exerted by sex steroids during early infancy and childhood remains a matter of debate in the human male.

Estrogens and Prostate

Androgens regulate prostate gland growth and differentiation, particularly during its development. Estrogens also act on prostate growth and differentiation through both ERα, and ERβ (164,165). In rodents, the prostate is sensitive to estrogen exposure during development (166).

Studies on animals have helped to better understand estrogen's role in prostate function. Studies in mice overexpressing aromatase (AROM+) demonstrated that prostate lobes are significantly reduced as a consequence of estrogen excess (167). On the other hand, aromatase-deficient mice presented a hyperplastic prostate gland probably due to the excess of circulating androgens (168) and consistent with hyperplasia of the epithelial, interstitial and luminal compartments (166). Furthermore, McPherson *et al*., using tissue recombination and an ERβ-specific agonist, demonstrated that ERβ activation results in an anti-proliferative response not influenced by systemic androgen levels, or activation of ERα (168). Moreover, studies on ArKO mice demonstrated that the administration of an ERβ-specific agonist reverted the existing hyperplastic epithelial pathology (168).

In terms of prostate carcinogenesis, it is generally assumed that androgenic hormones play a major role in tumor development, since the prostate gland is an androgen-dependent tissue, as is prostate cancer (169). However, considering the fact that testosterone can be converted to estradiol, and that ERs are present in the prostate epithelium (170), theoretically estrogen might also be involved in the induction of prostate cancer. In fact, Bosland *et al*. found that combined treatment of rats with estradiol and testosterone lead to an increased incidence of prostate cancer from 35-40% with androgen alone to 90-100% (171). The estrogen pathways involved at the molecular level in the process of prostate carcinogenesis are very complex (165). Several studies demonstrate that both ERα and β are involved in the transduction of estrogen signaling in prostate cancer such as cell proliferation pathways (165). Furthermore, ERβ seems mainly involved in pro-apoptotic pathways (e.g. FOXO3 and p-53), while ERα is involved in chronic inflammation, and the two ERs seem to act differently on oncogenes playing suppressive (ERβ) and oncogenic (ERα) roles (165).

In humans, selective inhibitors of aromatase are helpful for the evaluation of estrogen *in vivo* effects on prostate. Recently, the combined therapy with transdermal testosterone and the aromatase inhibitor anastrozole in older men with low or low-to-normal serum testosterone (< 350 ng/dL) prevented the increase of prostate volume, but not that of prostate-specific antigen seen in patients treated with testosterone alone (172). This is in line with the above-mentioned experimental results suggesting an active role of estrogens in prostate cell proliferation in prostate carcinogenesis. Conversely, prostate was normal in aromatase-deficient men and did not change in volume during estrogen replacement therapy (Carani & Rochira; data not published data). In the future, if estrogen's role in the prostate is further elucidated, new treatment strategies will be available for benign prostate hypertrophy and cancer, especially in men with concomitant hypogonadism (173).

Estrogens and Male Sexual Behavior

Sex steroids act on several aspects of male sexual behavior (174). Sex steroids, mainly testosterone, modulate adult male sexual behavior in mammals (175). In men, sexual behavior is more complex than in other species since it results also from cognitive processes, cultural environment and an individual system of beliefs (174,176). Thus, sexual behavior does not depend only on hormonal and genetic prerequisites in men (174,176). Traditionally, it was thought that only

testosterone, the male hormone, is responsible for the control of male sexual behavior (177). In the last two decades, the possibility that estrogens may be involved in the control of male sexual behavior has received more attention, and an impact of estradiol on male sexuality has become evident (158).

Testosterone is mainly involved in the control of sexual desire and sexual drive and in the facilitation and maintenance of a normal sexual genital response (174). Erections, especially nocturnal erections, are also under the control of androgens (178,179). The role of estrogen on male sexual behavior has been poorly investigated and knowledge derives mainly from studies performed on animals or from rare models of human estrogen deficiency. The increasing interest on the treatment of transsexuals (180) and on the cross actions of male and female hormones on both sexual behavior (181) and other physiological functions (182) probably have contributed to a better focus on this area of research. In recent years, however, several *in vivo* experimental settings have addressed this issue. As a result, nowadays all studies on steroid sex hormones action on male sexual behavior tend to investigate androgens and estrogens separately (158,183-186). Furthermore, steroid sex hormones may influence both gender-identity and sexual orientation (187,188), even though in humans this action is mitigated by the strong influence of psychosocial factors.

Estrogens and Gender Identity and Sexual Orientation

Testosterone aromatization to estradiol in the brain was traditionally considered the key step in the development of a male brain and in determining sexual dimorphism of the central nervous system in non-primate mammals (189-191). According to Dörner's hypothesis (192), prenatal and perinatal brain exposure to estrogens may be responsible for the establishment of a male brain (188,193), an event occurring only in the male, but not female, brain. Accordingly, ovaries release a very small amount of estrogen, soon inactivated in rodents (3,193), while the testes produce a greater amount of androgen that is converted into estrogen. Thus, circulating estrogens are paradoxically greater in males than in females during fetal life (188) and this accounts for the sexual dimorphism of hypothalamic structures in rodents and other species like sheep (193,194).

The same mechanism seems to be also involved in the differentiation in hypothalamic structures between men and women (192,195). Prenatal hormonal exposure is classically considered to be involved in determining sexual orientation, on the basis of some differences in hypothalamic structures between heterosexual and homosexual men (191,195). This hypothesis is supported by the concept that brain sexual differentiation during fetal life occurs in parallel with the peak of testosterone secretion from the testis and the consequent increase in serum estradiol (188,189,191,193). Accordingly, the intrinsic pattern of mammalian brain development is female, and estrogen is required for the development of a male brain (188,191,192), thus emphasizing the role of locally produced estrogen (193). Permanent changes in the organization of different neural circuits, fundamental for sex-specific regulation of reproductive and sexual behavior, probably also occurs under the effects of estrogen (188,190,191,193,196). Considering all the above mentioned aspects, the lack of estrogen action on the developing brain in males should be considered strictly related to the direction of future development of sexual orientation, and of dimorphism of hypothalamic structures (188,189,191,193,195). Most of the data supporting this evidence, however, came from studies performed in rodents or other species, but not in humans (188,190,191,193).

The role of hypothalamic aromatase activity and expression in partner preference has been elegantly confirmed in rams (197). In this study, the choice of sexual partner was associated with

both the volume of the ovine sexually dimorphic nucleus and different patterns of aromatase expression (197). This provides the first demonstration that differences in aromatase expression within the brain are related to partner choice and to the determination of adult sexual behavior (193,194,197). However, in humans, a clear cause-effect relationship between prenatal exposure to sex steroids (especially estrogens) and the differences in volume of some dimorphic brain areas (e.g. sexually dimorphic nucleus of the preoptic area and the intermediate nucleus) has not been demonstrated (188).

Aromatase-deficient men represent an interesting model to investigate the role of estradiol on human male sexual development and behavior from fetal life through adulthood (3,48,111). All men with aromatase deficiency had male gender-identity and heterosexual orientation (3,18-20,111,113,119-121,123,124,127) (Table 7). The fact that congenital aromatase deficiency does not affect psychosexual orientation and gender-identity in humans suggests that estrogens do not mediate the organizational effects on male sexuality induced by early exposure to androgens. Differently from animals, psychological and social factors are the most relevant determinants of gender role behavior in men, with hormones probably having a minor role compared to other species (3,111,176,194).

In conclusion, aromatase plays a key role in controlling male reproductive behavior especially in animals (rodents and rams), by modulating organizational effects on the developing brain during fetal life (196,198); the latter are mediated by estrogen production within the brain and exposure to circulating estrogens. However, differences among species could explain the essential role of aromatization in rodents, rams, and monkeys (194,198,199) and its poor or minor effect in humans (3,111,124,127) and other primates, respectively (199).

Subjects	Authors	Sexual function	Gender identity	Psychosexual orientation
Estrogen Resistance (Age:28 years)	Smith *et al.* 1994(17)	Libido: normal. Morning erections: normal. Nocturnal emissions: normal. Ejaculations: normal.	Male	Heterosexual
Aromatase Deficiency (Age 24 years)	Morishima *et al.* 1995(18); Bilezikian *et al.* 1998(112)	Libido: modest. Morning erections: normal. Nocturnal emissions: normal. Ejaculations: normal.	Male	Heterosexual
Aromatase Deficiency* (Age 38 years)	Carani *et al.* 1997(19); Carani *et al.* 1999(124)	Libido: normal. Morning erections: normal. Ejaculations: normal.	Male	Heterosexual
Aromatase Deficiency* (Age 28 years)	Maffei *et al.* 2004(121); Carani *et al.* 2005(127)	Morning erections: normal. Libido and sexual activity have not been investigated according to the religious thinking of the patient.	Male	Heterosexual
Aromatase Deficiency (Age 27 years)	Herrmann *et al.* 2002(113); Herrmann *et al.* 2005(116)	Libido: normal. Morning erections: normal. Ejaculations: normal.	Male	Heterosexual
Aromatase	Maffei *et al.*	Libido: normal.	Male	Heterosexual

Deficiency (Age 25 years)	2007(121); Zirilli *et al.* 2009(131)	Morning erections: normal. Ejaculations: normal.		
Aromatase Deficiency (Age 27 years)	Lanfranco *et al.* 2008(123)	Libido: normal. Morning erections: normal. Ejaculations: mild praecox ejaculation.	Male	Heterosexual
Aromatase Deficiency (Age 27 years)	Baykan *et al.* 2013(120)	No sexual dysfunction reported before and during treatment	Not reported	Not reported
Aromatase Deficiency 3 men (26-44 years)	Pignatti *et al.* 2013(119)	No sexual dysfunction reported before and during treatment	Not reported	Not reported

Table 7. Sexual behavior in men with congenital estrogen deficiency.
** Only these two patients underwent an extensive, well-designed study of sexual behavior in terms of psychosexual issues (gender identity and sexual orientation) and sexual function (desire and erectile function), while for other patients the information was obtained by patients' interview and medical history.*

Estrogens and Sexual Behavior

In adult men sexual behavior is partially dependent on testosterone, the main hormone involved in male sexuality (174,177,178). Accordingly, testosterone deficiency frequently causes loss of libido and erectile dysfunction (174,178,200). These are restored by testosterone replacement therapy, which is effective in increasing sexual interest and improving sexual function (174,175,200,201). Other hormones, however, are involved in the control of male sexual behavior, including estrogens (202,203).

In experimental animal models, the knockout of estrogen pathways or a pharmacologically induced estrogen deficiency results in severe impairment of sexual behavior (3,15,21). Accordingly, ArKO mice (204), αβERKO male mice (205) and αERKO mice (15,92) all exhibit a significant reduction in mounting frequency and prolonged latency to mount when compared with wild-type animals (15,21). On the contrary, βERKO mice did not show abnormalities of sexual behavior (15,95). These findings suggest that androgen receptor activation alone is not sufficient for fully normal sexual behavior in rodents and that a normal functioning ERα together with adequate levels of circulating or locally produced estrogen are required for mounting behavior in male mice (3,48).

Less is known about the role of estrogens in sexual behavior in men since the relative importance of testosterone and its metabolite estradiol on male sexual behavior is still not known. In the past five years, only a few studies have investigated the direct effect of estrogen on male sexual behavior (206,207), indirect evidence being available only from rare cases of men with congenital estrogen deficiency (3,48,111,119,120,208) (Table 7). A detailed sexual investigation of aromatase-deficient men documented an increase in all the parameters of sexual activity during estrogen treatment (124,127), with the best outcome in terms of sexual behavior obtained only when a concomitant normalization of both serum testosterone and estradiol was reached (127). These results support the concept that both sex steroids are required for normal sexual behavior in men. Outside the context of congenital lack of estrogens, it is difficult to reach conclusive information on the role of estrogen on male sexual behavior because of the inadequacy of studies and the conflicting results reported in the literature.

Recently, a very elegant study provided evidence-based information on the relative role of testosterone and estradiol on male sexual function in men (158). In this study, a considerable number of healthy men (n. 400) underwent gonadotropin suppression by the administration of a GnRH analogue (goserelin acetate), resulting in testosterone and estradiol suppression (158). In order to investigate the placebo effect and the effects of testosterone and estradiol, subjects were assigned to receive i) placebo, ii) testosterone treatment at different dosages, and iii) testosterone at different dosages plus the aromatase inhibitor anastrozole (158). In the testosterone group, serum testosterone and estradiol varied from physiological levels to low levels according to the different doses of exogenous testosterone in each group and the estradiol to testosterone ratio remaining substantially unchanged in all groups (158). This pharmacologic scheme allowed testing the effects of lowering both serum testosterone and estradiol in a similar way on several physiological functions; the result was a decline of both sexual desire and erectile function in parallel with the decrease of both sex steroids (158). In the testosterone plus anastrozole group, the decline of serum testosterone paralleled that obtained in the testosterone group, while serum estradiol was quite suppressed and changed to a lesser degree in each testosterone dose group, thus fluctuating across very low values (158). In this group, both sexual desire and erectile function were severely affected in patients with low serum levels of estradiol despite normal serum testosterone in patients taking the higher doses of testosterone (158). Conversely, goserelin treatment resulted in the maximum reduction of both sexual desire and erectile function in the placebo group (158). These results confirm observations in aromatase-deficient men (111,124,127) and suggest that estrogen deficiency is largely responsible for the impairment in sexual function occurring when serum testosterone is suppressed in hypogonadal men (158). A possible role of estrogen on male sexual function is also provided by further studies showing that testosterone therapy is more effective on libido when the treatment produces serum estradiol levels greater than 5 ng/dL (183) and that this serum estradiol is directly related to sexual function in men (184). In particular, serum estradiol is associated with sexual activity and desire, but not with erectile function (209). In addition, exogenous estradiol improves sexual desire in men with low testosterone and prostate cancer (210).

Notwithstanding these studies, the role of estrogen in male sexual behavior remains controversial (202) since several studies reached opposite conclusions. In particular, Sartorius *et al.* found that DHT was effective in maintaining male sexual function in healthy, older men, despite its suppressive effect on both testosterone and estradiol, suggesting that male sexual function can be ensured without aromatization (185). Furthermore, other cross-sectional studies failed to demonstrate a clear association between serum estradiol and male sexual function (186,211).

To add further complexity, estrogen action on erectile function seems to be biphasic, since estrogen deficiency may affect the ability to achieve an erection, yet estrogen excess and an increased estradiol to testosterone ratio is associated with an impaired erectile function. Recently, low serum testosterone and high serum estradiol was reported to be associated with erectile dysfunction in Chinese men with sexual dysfunction (212) and erectile dysfunction was more severe in hypogonadal men having concomitant high serum levels of estradiol (213). Even in an experimental rabbit model of the metabolic syndrome, a model used to study erectile function, erectile dysfunction is associated with high serum estradiol rather than with than low testosterone (214).

A possible explanation for these results is that a serum estradiol in the normal male range is required for a fully normal male sexual function in addition to testosterone, while both estrogen deficiency and estrogen excess have a negative impact on male sexual activity (127,184,186).

Finally, estrogen receptors and the aromatase enzyme have been identified in the penile tissue of a large number of species, including humans (215-217) suggesting direct estrogenic activity within the penis. At present, knowledge on estrogen action within the penis derives from the observation that: i) male offspring exposure to estrogen-like endocrine disruptors in utero induces micropenis and hypospadia (140), and that ii) penile development and function is estrogen-dependent in animals (218).

OTHER NON-REPRODUCTIVE PHYSIOLOGICAL ESTROGEN ACTIONS IN MEN

Estrogens and Metabolism

The role of estrogen on glucose and insulin metabolism in men is difficult to establish since it remains a challenge to differentiate androgen from estrogen actions in vivo. In estrogen-deficient men, both insulin resistance and fasting glucose are increased and improve during estrogen treatment (121,129,132), confirming data from mice models (15). Thus, severe estrogen to testosterone ratio imbalance (increased androgens and decreased estrogens) seems to favor the development of insulin resistance in men (121,122,129), not only in estrogen-deficient men (219).

Furthermore, congenital estrogen deficiency is associated with an altered lipid profile (20,132), mainly characterized by higher total cholesterol and triglycerides serum levels, higher low-density lipoprotein (LDL) cholesterol and very low high-density lipoprotein (HDL) cholesterol (10,111). In these patients, estradiol treatment induces a moderate increase of HDL-cholesterol together with a small reduction of triglycerides, total cholesterol, and LDL cholesterol (19,111,113,121), resembling the effects of estrogen on lipid metabolism exerted in females (9).

In hypogonadal men, estrogen deficiency, but not testosterone deficiency is responsible for vasomotor symptoms (i.e. hot flushes), thus reinforcing the concept that in men with hypogonadism several clinical manifestations are due to relative estrogen deficiency rather than to testosterone deficiency *per se* (220).

At present, the impact of these slight metabolic alterations on male reproductive function is unknown.

Estrogens and Bone

There is increasing evidence suggesting that circulating estrogens plays a key role in bone health in men, as in women (221). The relative contribution of androgen versus estrogens in the regulation of the male skeleton, however, is complex and relatively unclear (222). Some estrogen actions on male bone, such as bone maturation and the acceleration of growth arrest, are now well defined (221,223). The important role of estrogen in bone metabolism in men has been characterized in the last 15 years by means of the description of rare case reports of estrogen-deficient men (111,123) and by several epidemiological studies (224,225). Estrogen replacement therapy led to skeletal maturation and improvement of bone mineral density in all aromatase-deficient men described so far (10,111,119) in a dose-dependent way (125) while testosterone treatment did not (19,20,130). During puberty and late adolescence, epiphyseal closure, growth arrest, the achievement of peak bone mass, and final bone maturation are mainly under the control of estrogens and all these processes do not progress in the case of severe estrogen deficiency (19,20,111,221). The eunuchoid body proportions of the skeleton typical of hypogonadal men are the effect of estrogen deficiency during late adolescence and of the disproportional growth between long bones and the appendicular skeleton (10,221). During adulthood, both normal

circulating estradiol and testosterone are required for maintaining bone mineral density in aromatase-deficient men (112,130,131) as well as in the general male population (221,222,224,225). Estrogen action on bone seems to be possible only when circulating estradiol is above a threshold between approximately 15 and 25 pg/mL (221,224). This mechanism has been suggested both for growth arrest and bone maturation (123) and for bone mineral density (BMD) (224) and suggest that circulating estrogens above this threshold are required for optimal skeletal maturation and mineralization in men (208,221). Relative estrogen deficiency, rather than testosterone deficiency, is responsible for bone loss in hypogonadal men as clearly demonstrated when using different doses of exogenous testosterone alone or in combination with a potent aromatase inhibitor in men treated with GnRH analogues (226). In this study, BMD decreased and indices of bone resorption increased only in the group of men treated with both testosterone and anastrozole independently from the dose of exogenous testosterone administered to men with pharmacologically-induced hypogonadism (226). Furthermore, this study confirms that serum estradiol below 10 pg/mL is particularly harmful for bone health (226).

EFFECTS OF ESTROGEN EXCESS

Effects of Exposure to Excess Estrogens in Animals

In order to evaluate the effect of estrogen excess on the reproductive tract, several studies have been performed in various animal species treated with diethylstilbestrol (DES), a synthetic, potent estrogenic compound (227). The period between 13 to 24 weeks of human fetal life corresponds with the highest susceptibility of male reproductive organs to endocrine disruptors (3,46,48,227). Many studies in rodents suggest that the inappropriate exposure to estrogen in utero and/or during the neonatal period impairs the hypothalamic-pituitary-gonadal axis, testicular descent, efferent ductule function and testicular function (21,26,140,142). The latter effect is a direct consequence of the exposure to estrogen excess, of the indirect effect of perturbations in circulating hormones, and of the ability of the efferent ductules to reabsorb fluid. It seems that ERβ may mediate the process through which excess estrogens produce negative effects on male reproduction (21,26,50). The effects of estrogen excess during the neonatal period can induce irreversible alterations of the testis that become manifest in adulthood, consisting of permanent changes in both testis function and spermatogenesis (21,26).

Aromatase Over-Expression in Rodents

The transgenic model of mice overexpressing the aromatase enzyme (AROM+) exhibits highly elevated serum estradiol concentrations together with a decrease of serum testosterone levels due to gonadotropin suppression (167,228). The phenotypic abnormalities of AROM+ males are similar to those developed by mice that are perinatally exposed to estrogens. The most frequent abnormalities include: undescended testes, testicular interstitial cell hyperplasia, hypoandrogenism, and growth inhibition of accessory sex glands (167). The impairment of spermatogenesis observed in AROM+ may be due to multiple factors, including cryptorchidism, abnormal Leydig cell function, testosterone deficiency or hyperestrogenemia (167). Thus, estrogens are thought to inhibit Leydig cell development, growth and function, resulting in the final suppression of androgen production (21). Furthermore, the observation of numerous degenerating germ cells and the absence of spermatids within the seminiferous tubules of AROM+ mice suggest that germ cell development arrests at the pachytene spermatocyte stage (21). However, a possible role of cryptorchidism *per se* on germ cell arrest cannot be excluded since cryptorchidism is known to induce germ cell arrest in rodents (229). Interestingly, the spermatogenic arrest occurred at a

stage where P450arom expression is generally high. The spermatogenic arrest found in the AROM+ mice could be explained, at least in part, by the suppression of FSH action (167,228). In fact, the reduced serum FSH levels associated with normal LH levels provide further evidence of the inhibiting actions of estrogens on FSH secretion in in AROM+ males (167,228).

Effects of Exposure to Excess estrogens in Humans

The observation that the clinical use of DES by pregnant women to prevent miscarriage is associated with a dramatic increase in the incidence of genital malformations in their sons represents the first evidence in humans on the potential for estrogen excess to provoke urogenital malformations (230). The most frequent structural and functional abnormalities include epididymal cysts, meatal stenosis, hypospadias, cryptorchidism and microphallus (230-232). The frequency of abnormalities is dependent on the timing of estrogen exposure; in fact, men who were exposed to DES before the 11th week of gestation (i.e. the time of Műllerian ducts formation) had a two-fold higher rate of abnormalities than those who were exposed later (230,232). These data support the hypothesis that the asynchrony between formation and regression of embryonic reproductive structures is probably strongly influenced by estrogen exposure.

Various reports demonstrated that semen quality of men exposed to DES in utero is significantly worse than in unexposed controls (233), even though sperm concentrations of most of these patients was average, with normal fertility (13). The implications for human spermatogenesis in terms of exposure to environmental estrogens remain less clear. The risk of testicular cancer among men exposed to DES in utero has been a controversial issue and several meta-analyses showed a doubling of testicular cancer risk, together with increased incidences of cryptorchidism, hypospadias, and impaired spermatogenesis (234). However, more direct evidence will be necessary in order to fully understand this issue and particularly to identify the exact estrogenic mechanism of action (234). It is clear that exogenous estrogens could interfere with the development of genital structures if administered during early organogenesis (232). The main effect is an impairment of gonadotropin secretion and the imbalance of estrogen to androgen ratio, which may account for impaired androgen receptor stimulation or inhibition according to the dose, the cell type and the timing of exposure (230,232). Furthermore, it seems that an excess of environmental estrogens could be a possible cause of impaired fertility in humans (140,141,232) since environmental estrogens are associated with an increased risk of subfertility in several studies (235). Although controversial, a proposed progressive decline in sperm count has been reported in some Western countries during the past 50 years, and has been suggested to involve negative effects of environmental contaminants, especially xenoestrogens, on male reproductive function (12,140,230,235).

Aromatase over-expression in humans

Aromatase over-expression causes an increased conversion of androgens to estrogens with a consequent excess of estrogen. Excess estrogen in boys causes gynecomastia, a premature growth spurt, early fusion of epiphyses, and decreased adult height (236). Increased extraglandular aromatization was firstly reported in an adopted boy with prepubertal gynecomastia in 1977 (237). Four families were then described, in which several members had estrogen excess (manifested as gynecomastia in boys and men and premature thelarche in girls) due to increased extraglandular aromatization (238-240), and one case with a gain-of-function mutation of the aromatase gene (236). The latter seemed to be an autosomal dominant inherited disease

(236,239). In adult men, elevated serum estradiol levels induce mild hypogonadotropic hypogonadism due to enhanced negative feedback on pituitary gonadotropins exerted by estrogens (236,239). This inhibitory effect of estrogen on reproductive function appears to be milder in males with aromatase excess syndrome than in patients receiving exogenous estrogens or having estrogen-secreting tumors, probably because serum estradiol and/or estrone levels are lower in the former (239). External genitalia in adult men with aromatase excess syndrome are characterized by normal penile and testicular size (236,239). This clinical reproductive phenotype has been observed also in other patients with aromatase excess syndrome due to gain-of-function mutations of the aromatase enzyme (241). Even though spermatogenesis and sexual behavior were not specifically studied, the adult men described were fertile and reported normal libido (236,239) and sperm count was normal in other studies (241). In these patients, treatment with an aromatase inhibitor reduces estrogen levels and normalized testosterone, LH and FSH serum levels (236), confirming a crucial role of estrogen in the suppression of both gonadotropins in men.

Klinefelter's Syndrome has been classically considered a feminizing syndrome on the basis of signs (gynecomastia) and the observation of circulating estradiol higher than normal (242,243). In the literature, however, the data concerning hyperestrogenism in Klinefelter patients are not solid since they come from single case reports or studies using old assays for the measurement of serum estradiol. Data from mouse models of Klinefelter's are not conclusive about the real increase of circulating estrogens and aromatase expression and activity (244). Infertility in these patients is mainly due to the genetic abnormalities rather than to the hormonal status (245). However, preliminary results from a recent meta-analysis does not confirm that serum estradiol is higher in Klinefelter's patients compared with non-Klinefelter's men, with only the estradiol to testosterone ratio being elevated in Klinefelter's (246).

CLINICAL IMPLICATIONS OF ESTROGENS IN MALES

Diagnostic Aspects: Significance of Serum Estradiol in Men

In adult men, the normal range of serum estradiol is 18-40 pg/mL (66-147 pmol/L). Approximately 50 μg of estradiol are produced daily: about 5-10 μg in the testis (10 to 20%) and the remaining 40-45 μg (80 to 90%) in peripheral tissues (adipose tissue, muscle, breast, brain liver and bone) in which the aromatase enzyme is expressed (3,208).

In some circumstances, the clinical work-up for the evaluation of male infertility may involve the serum estradiol assay when clinical aspects suggestive for aromatase deficiency, coupled with normal to high testosterone and gonadotropins levels and/or history of cryptorchidism are present (Table 6) (247). However, it has to be noted that the accuracy of most of the major commercially available kits for the detection of serum estradiol is poor, especially for low serum levels of estradiol typical of the male range (1,225). Therefore, the assay of serum estradiol is recommended only if the method used has a very high sensitivity and specificity (e.g. 3rd generation RIA) (225). The gold standard test is the gas chromatography/tandem mass spectrometry (35-38,248). A good result in terms of sensitivity could also be obtained by ultrasensitive recombinant cell bioassay, being approximately 100-fold more sensitive than previous estradiol assays with a sensitivity of <0.02 pg/ml estradiol equivalents (249), which is adequate for serum estradiol levels within the normal male range. The limitations of these two methods are their cost and the fact they are time-consuming procedures, which limit their routine use in clinical practice. In recent years however, gas chromatography-tandem mass spectrometry, especially liquid chromatography-tandem mass spectrometry, are more frequently used in

laboratories for clinical routine evaluations of sex steroids, including estrogens (1,36-38,248), thus increasing the availability of precise and accurate sex steroids measurement in a clinical setting (248).

Estrogens and Male Infertility: Clinical and Therapeutic Implications

Estrogens are involved in male fertility and could represent an important factor involved in the pathogenesis of infertility as well as a possible pathway to explore new therapies for human male infertility.

Estrogen Treatment

At present there is no indication to prescribe estrogen compounds to men, except for the treatment of rare diseases such as congenital estrogen deficiency (111,208,247) or in the management of transgender patients. The increasing evidence of the existence of several testosterone actions that are mainly mediated by estrogens theoretically support the concept that tailoring estrogens in the treatment of hypogonadal men may improve the outcome in terms of benefits for patients (156,157). However, at present, there is no evidence on the effectiveness and safety of such therapeutic strategy. In the future, advances in the field of routine clinical measurement of very low amounts of circulating estrogens (1,36-38,248) will open new frontiers for testing the effect of estrogen compound or of SERMs alone or combined to androgens in men with documented mild estrogen deficiency.

Estrogen treatment of aromatase-deficient men

The clinical features common to all aromatase-deficient men are: tall stature, delayed bone maturation, osteopenia/osteoporosis, eunuchoid skeleton, bone pain, and progressive *genu valgum* (10,111,221). Estrogen replacement treatment, at the daily dose of 0.22 to 0.35 μg/kg of transdermal estradiol in adult men, should be started as soon as the diagnosis of estrogen deficiency has been reached (111,247). When the diagnosis is available at birth, or is achieved during infancy, low dosages of exogenous estradiol should be administered at the beginning of puberty (0.8 to 0.12 μg/kg daily) (111,118). The main target of estrogen replacement therapy in these patients is the skeleton in order to promote epiphyseal closure, bone maturation and mineralization and the completion of these physiological processes on time. Accordingly, high doses of estrogen in adult men with aromatase deficiency might be used to lead a rapid completion of skeletal maturation within 6-9 months in adults with epiphyseal cartilages still open, through rapid bone elongation and an increase in height followed by quick epiphyseal closure and growth arrest (111,125,247). Once epiphyseal closure has been achieved, estrogen replacement treatment should be continued lifelong. The main goal is to prevent bone loss and to reduce the risk of cardiovascular disease. In this case, the dose of estradiol should be reduced to ensure serum estradiol within the normal range for adult men (111,247). Moreover, estrogen treatment in aromatase deficient men is effective in normalizing or improving other aspects such as gonadotropin secretion, glucose metabolism, insulin sensitivity, liver function, and circulating lipids (121,122,128-130). When estrogen treatment is started at puberty, the effects of estrogen treatment on spermatogenesis are unknown, but the administration of estrogens in a more physiological way could theoretically be associated with normal spermatogenesis in adulthood. Conversely in adult patients, impaired spermatogenesis is irreversible even when estradiol treatment is administered (111). Other aspects related to estrogen deficiency cannot be modified by estrogen treatment when the treatment is started during adulthood (e.g. eunuchoid body proportions, *genu valgum*, failure in reaching the bone peak mass, normal body weight restoration) (111,122).

Finally, the real impact of estrogen treatment on sexual behavior in adult aromatase-deficient men remains to be determined (111).

Anti-estrogen treatment in men

As estrogens act on gonadotropic feedback inhibition (128,146,149), they could be a good target in the clinical management of male infertility. The rationale is to employ anti-estrogen drugs in order to modulate gonadotropin feedback by blocking the inhibitory effect exerted by estrogen on gonadotropins and to increase both LH and FSH. This will result in increased testosterone and FSH with potential benefits on spermatogenesis (250). However, the real effectiveness of this approach in treating male infertility remains to be established, since conflicting results are available (3,110,138,139,251-254) and this kind of treatment remains empirical and 'off label' (250,251). Thus, the real efficacy of anti-estrogens is far from being elucidated and whether the increase of sperm density induced by anti-estrogens is actually related to a real improvement of both sperm fertility and pregnancy rates is a matter of debate (3,110) (Table 8).

Since the 1960s, anti-estrogen agents have been used as an empirical treatment of male infertility (251,255) based on their modulation of the hypothalamic-pituitary testicular axis. The main classes of drug that have been tested are aromatase inhibitors. They are the most potent blockers of the estrogen-mediated negative feedback on gonadotropins and excites LH and FSH secretion aiming to stimulate spermatogenesis (256). However, no clear evidence of direct effects of anti-estrogens on spermatogenesis exists (250,256), but LH and FSH serum levels generally increase during aromatase inhibitor administration in infertile men (257).

Clomiphene at a dosage of 25-50 mg daily for 3-12 months, or tamoxifen at dosage of 20-30 mg daily for 3-6 months, represent the most frequently used anti-estrogen agents for the treatment of male infertility (258) (Table 8); on the contrary the new generation of selective estrogen receptor modulators does not result in significant changes in male fertility (259) (Table 8).

Treatment	Dose (mg/daily)	Duration (months)	Effects on semen analysis
Anti-estrogens			
Clomiphene	25-50	3-12	Semen volume: No effect or ↑ Total sperm number: No effect or ↑ Sperm concentration: No effect or ↑ Sperm motility: No effect or ↑ Sperm morphology: No effect or ↑
Tamoxifen	20-30	3-6	Semen volume: No effect Total sperm number: No effect Sperm concentration: No effect or ↑ Sperm motility: No effect Sperm morphology: No effect
Tamoxifen and Testosterone undecanoate	20 120 orally	6	Semen volume: No effect Total sperm number: ↑ Sperm concentration: No effect Sperm motility: ↑ Sperm morphology: ↑

Aromatase inhibitors			
Testolactone	2000	8	No effect
Testolactone or Anastrozole	100-200	6	Semen volume: ↑ Total sperm number: ↑ Sperm concentration: ↑ Sperm motility: ↑ Sperm morphology: ↑
Letrozole	2,5	6	Semen volume: No effect Total sperm number: ↑ Sperm concentration: ↑ Sperm motility: ↑ Sperm morphology: No effect

Table 8. Dosages and time duration of oral anti-estrogen and aromatase inhibitors used in male infertility and their different effects on semen analysis in clinical trials reported in literature.

Clomiphene (25-50 mg/day) has been recently studied in a cohort of 86 men with hypogonadism for six months (260). This treatment represented an effective and apparently safe alternative to testosterone supplementation in hypogonadic men wishing to preserve their fertility (260). Furthermore, Ghanem *et al*. have recently found that combined treatment with clomiphene (25 mg/day) and an antioxidant drug (vitamin E) increased the pregnancy rate and improved sperm count and progressive motility in men with idiopathic oligoasthenozoospermia (261). In men with secondary hypogonadism treated with testosterone, enclomiphene (the transisomer of clomiphene) was able to prevent gonadotropin suppression and the related oligospermia compared to placebo (262) (Table 8).

Tamoxifen (20 mg/day) has been also used in combination with oral testosterone undecanoate (120 mg/day) in men affected by idiopathic oligozoospermia. This combined treatment was effective in improving not only the sperm parameters (total sperm number, sperm morphology and motility), but also the pregnancy rate (263). Recently, Moein et al. studied thirty-two azoospermic infertile men with proven non-obstructive azoospermia, administrating Tamoxifen for 3 months (264). Tamoxifen treatment led to the recovery of spermatozoa in the ejaculates of six patients (264). These studies showed that treatment of patients with non-obstructive azoospermia with anti-estrogenic drugs like tamoxifen can improve the results of sperm recovery in testis samples and also increase the chance of pregnancy by microinjection. Also other non-controlled trials suggest improvements in sperm quality or sperm concentration (265,266), however, no well-performed clinical trial has confirmed these results (250) (Table 8). A recent meta-analysis including a very small number of studies supports the empirical use of the estrogen antagonists clomiphene and tamoxifen at the dose of 50 mg and 20 to 30 mg daily based on the finding of the detection of a doubling rate of pregnancy outcome among men with idiopathic infertility (267). The uncertain role of these therapies on male fertility may be related to the fact that idiopathic oligozoospermia constitutes a group of heterogeneous disorders of which only a subgroup might respond to anti-estrogen therapy. However, studies have failed to identify the characteristics of this subgroup and thus physicians cannot distinguish potential responders and non-responders (250).

Few data are available on the effect of aromatase inhibitors in male infertility (Table 8). An old study failed to demonstrate the efficacy of testolactone in the treatment of idiopathic oligozoospermic infertility (257). However, when aromatase inhibitors (testolactone or anastrazole)

were administered in a selected group of infertile men with abnormal baseline testosterone-to-estradiol ratio, an improvement of fertility rate was generally obtained (138). In particular, letrozole treatment improved semen parameters and estradiol to testosterone imbalance in patients with low testosterone and increased estradiol to testosterone ratio (254). In 2011, Saylam *et al*. treated 27 infertile, hypogonadotropic men with 2.5 mg daily of letrozole for six months, finding an improvement of both testosterone serum levels and semen parameters after treatment (139). Thus, it seems that letrozole may facilitate some improvement in infertile men with azoospermia by improving the number of sperm in the ejaculate (139). Accordingly, a further study on the effects of letrozole on sperm parameters showed that letrozole but not placebo was effective in increasing sperm count and improving sperm motility after 6 months of treatment in a small group of 46 patients (22 on letrozole; 24 on placebo) who were azoospermic or cryptozoospermic at baseline (268) (Table 8).

Data concerning the safety of anti-estrogens for treatment of male infertility are scant, especially as far as long-term treatment is concerned (154,155,250,269). Safety data regarding the use of clomiphene and tamoxifen for male infertility is limited, but information on their safety might be indirectly derived from small groups of men with breast cancer (270). Conversely, more data are available on aromatase inhibitors (154,155). Six months of therapy with letrozole seems to not affect psychometric tests, glucose tolerance, serum circulating lipids, markers of bone turnover, and body composition, including BMD, in obese, hypogonadal men(271). In this study, however, moderate aromatase inhibition resulted in serum estradiol still within the normal male range and all the outcomes were obtained after a short period of treatment (271). In the literature, opposite results are available and suggest possible undesired effects of aromatase inhibitors, especially on metabolism and bone. Evidence exists that high-dose aromatase inhibition might lead to several side effects, especially when patients are treated for more than 12 months with an aromatase inhibitor (154,155). Both very short-term and short-term treatment with aromatase inhibitors had deleterious metabolic effects: one study demonstrated a prompt worsening of both insulin sensitivity and lipid profile in young and older men after 28 days of treatment with letrozole (272), while anastrozole reduced insulin sensitivity in healthy men after 6 weeks of treatment (229). In the case of longer treatments (with outcomes obtained after 1 year) vertebral deformities (273) and decreased BMD (139,156,274) were found in young and older men, respectively. In addition, treatment with aromatase inhibitors lowered HDL-cholesterol in peripubertal boys (275) and in both adult and older men (154,272) while data on total and LDL-cholesterol are conflicting(229,272,276). Data available from a very small subset of male patients operated on for male breast cancer and treated with anti-estrogens (most of them with tamoxifen) provides data on long-term effects and major adverse events (270). The authors concluded that side effects and major adverse events did not differ between men and women taking anti-estrogens and that cerbrovascular or coronary events, thromboembolic events (deep venous thrombosis), depression, muscle cramps, and hot flashes might occur also in men during anti-estrogens treatment, hot flashes being the most frequent (270). These data, however, should be regarded with caution due to the small sample size, the lack of a control group, and the difficulties in proving a cause-effect relationship between major adverse events and the use of anti-estrogens in men.

As a result, it should be remarked that none of the drugs belonging to the category of anti-estrogens (i.e. clomiphene, tamoxifen, aromatase inhibitors) is approved for the indication of the treatment of male infertility by regulatory drug agencies (e.g. FDA, EMEA, TPD and TGA Regulations) nor is recommended by guidelines provided by Scientific Societies (e.g NICE, ASA,

EAA etc) for use in idiopathic infertility. At present, all the data available on anti-estrogens in male infertility comes from the use of these drugs for research purposes outside the context of clinical practice. In addition, none of the studies are of adequate design, strength and power. For all these reasons, their clinical use remains anecdotal and is off label (154,155).

In conclusion anti-estrogens, alone or in combination with testosterone, may represent a potential therapy for idiopathic oligozoospermia, however this remains an empirical off-label treatment (250,267). The data set does not yet provide sufficient evidence for these applications, but there is suggestive evidence that encourages further study (267). Further well-designed studies on adequate sample size (and homogeneous groups of men with infertility) are needed to detect their true efficacy in improving the pregnancy rate, or to identify the features of the responders.

CONCLUSIONS

Sex steroids account for sexual dimorphism because they are responsible for the establishment of primary and secondary sexual characteristics, which are under the control of androgens and estrogens in male and female, respectively. Advances in the understanding of the role of estrogens in animal and human models suggest a role for this sex steroid in the reproductive function of both sexes. The fact that both estrogen excess and estrogen deficiency influence male sexual development, testis function, the hypothalamic-pituitary-testis axis, spermatogenesis and ultimately male fertility, highlight the biological importance of estrogen action in males. Thus, estrogens, not only androgens, are responsible for some crucial physiological functions in men like fertility, reproduction and bone health. In particular, the balance of serum estradiol to testosterone ratio is likely crucial for maintaining all these functions, thus suggesting that the homeostatic equilibrium between estrogens and androgens is important for the correct functioning of several physiological systems in men (10,20,29,103,129,138,254). From an evolutionary perspective, this relevance of estrogen actions in males provides an example of the parsimony operating in biological events that are crucial for the evolution of the human species such as growth and reproduction (Figure 7).

This chapter has addressed the reproductive effects of estrogens in males but there are emerging roles for estrogens in non-reproductive tissues. In particular, while testosterone has traditionally been considered the sex hormone involved in bone maturation and growth arrest in men the key role of estrogens on growth has recently been revealed.

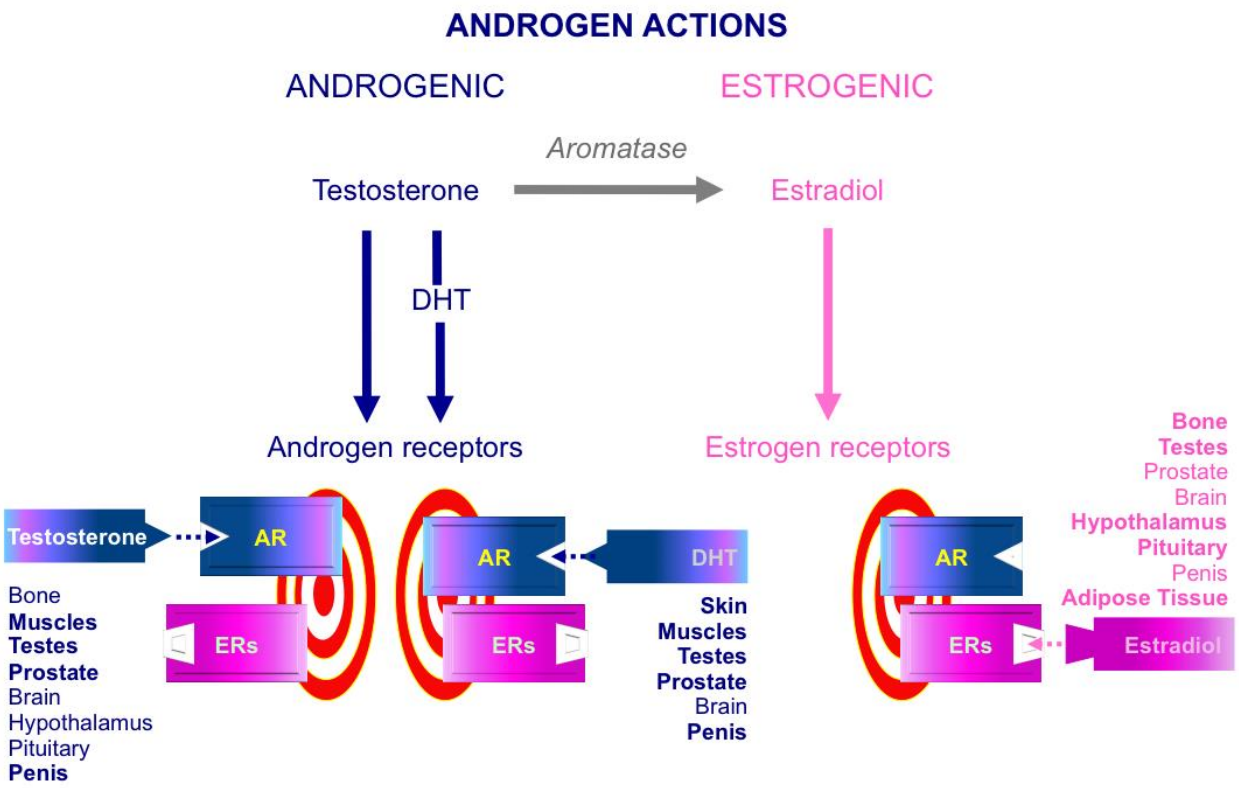

Figure 7. Direct and indirect (estrogen mediated) testosterone actions.
[DHT: dihydrotestosterone, AR: androgen receptor; Ers: estrogen receptors]

A major area of uncertainty is the possible role of estrogen in boys before puberty. It is known that low levels of circulating estradiol are detected in infancy when using ultrasensitive assays, but their significance is not known (208).

Several lines of evidence support the view that estrogens are required for, and in part mediate, androgen actions on several tissues and organs in men (Figure 7). The progresse made in the last twenty years in this field have clarified the importance of estrogen in men but leaves some issues still unsolved. In particular, estrogen actions on bone and on gonadotropin secretion are now well characterized and part of the estrogen action on spermatogenesis is known, but further evidence is needed to clarify several aspects still under debate.

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