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GnRH and GnRH receptors in the pathophysiology of the human female reproductive system

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BACKGROUND: Human reproduction depends on an intact hypothalamic–pituitary–gonadal (HPG) axis. Hypothalamic gonadotrophin-releasing hormone (GnRH) has been recognized, since its identification in 1971, as the central regulator of the production and release of the pituitary gonadotrophins that, in turn, regulate the gonadal functions and the production of sex steroids. The characteristic peculiar development, distribution and episodic activity of GnRH-producing neurons have solicited an interdisciplinary interest on the etiopathogenesis of several reproductive diseases. The more recent identification of a GnRH/GnRH receptor (GnRHR) system in both the human endometrium and ovary has widened the spectrum of action of the peptide and of its analogues beyond its hypothalamic function.

METHODS: An analysis of research and review articles published in international journals until June 2015 has been carried out to comprehensively summarize both the well established and the most recent knowledge on the physiopathology of the GnRH system in the central and peripheral control of female reproductive functions and diseases.

RESULTS: This review focuses on the role of GnRH neurons in the control of the reproductive axis. New knowledge is accumulating on the genetic programme that drives GnRH neuron development to ameliorate the diagnosis and treatment of GnRH deficiency and consequent delayed or absent puberty. Moreover, a better understanding of the mechanisms controlling the episodic release of GnRH during the onset of puberty and the ovulatory cycle has enabled the pharmacological use of GnRH itself or its synthetic analogues (agonists and antagonists) to either stimulate or to block the gonadotrophin secretion and modulate the functions of the reproductive axis in several reproductive diseases and in assisted reproduction technology. Several inputs from other neuronal populations, as well as metabolic, somatic and age-related signals, may greatly affect the functions of the GnRH pulse generator during the female lifespan; their modulation may offer new possible strategies for diagnostic and therapeutic interventions. A GnRH/GnRHR system is also expressed in female reproductive tissues (e.g. endometrium and ovary), both in normal and pathological conditions. The expression of this system in the human endometrium and ovary supports its physiological regulatory role in the processes of trophoblast invasion of the maternal endometrium and embryo implantation as well as of follicular development and corpus luteum functions. The GnRH/GnRHR system that is expressed in diseased tissues of the female reproductive tract (both benign and malignant) is at present considered an effective molecular target for the development of novel therapeutic approaches for these pathologies. GnRH agonists are also considered as a promising therapeutic approach to counteract ovarian failure in young female patients undergoing chemotherapy.

CONCLUSIONS: Increasing knowledge about the regulation of GnRH pulsatile release, as well as the therapeutic use of its analogues, offers interesting new perspectives in the diagnosis, treatment and outcome of female reproductive disorders, including tumoral and iatrogenic diseases.

Key words: endocrinology / female infertility / GnRH AG/ANTAG / gonadotrophin / menstrual cycle

Introduction

Human reproduction, as well as the expression of the different sexual characteristics, depends on an intact hypothalamic–pituitary–gonadal (HPG) axis. The main actors of HPG include the gonadotrophin-releasing hormone (GnRH), the gonadotrophins, LH and FSH, and the gonads. Thus, GnRH, produced by hypothalamic neurons, controls the synthesis and release of pituitary gonadotrophins, which in turn stimulate the production of sex steroids from the gonads. The multi-level organization of this axis guarantees fine-tuned modulation of such fundamental functions.

GnRH release is under the regulatory actions of different neurotransmitter and neuropeptidergic inputs and its characteristic pulsatile secretion drives the main events through female reproductive life, such as the onset of puberty and the regulation of normal ovulatory cycles. Alteration of GnRH pulse pattern is observed both in physiological and pathological conditions.

A GnRH/GnRH receptor (GnRHR) system is also expressed in female reproductive tissues where it is associated with autocrine/paracrine effects, both in physiological and in pathological conditions. In the normal endometrium, this system regulates processes that are crucial for trophoblast local invasion and for embryo implantation. In the ovary, this system is involved in the control of follicular development and corpus luteum function. For gynaecological diseases, GnRH analogues are mainly utilized in those situations in which the main goal of the treatment is the blockade of ovarian estrogen secretion. However, it is now well established that locally expressed GnRHRs are associated with a significant antiproliferative activity (Limonta *et al.*, 2012). This suggests that these receptors might be considered as a molecular target for novel GnRH analogue-based therapeutic strategies for these pathologies. More recently, GnRH agonists, in parallel with chemotherapy, have been proposed as a novel therapeutic approach for the preservation of fertility in young female patients undergoing chemotherapy.

In this review, we will present an overview and new perspectives on the specific role of the GnRH/GnRHR system in female reproductive functions, both in physiological and in pathological conditions. The possible role of the locally expressed GnRH/GnRHR system as an effective molecular target for novel treatment strategies in gynaecological diseases (both benign and malignant) will also be discussed.

Methods

By an analysis of research articles published in English until June 2015, we summarize, in a comprehensive way, what it is actually known about the functions of GnRH in the physiopathology of the reproductive axis and fertility, with particular attention to human females, as well as the novel pleiotropic functions of GnRH receptors at the level of female reproductive tissues and the pharmacological utilization of GnRH analogues in central and peripheral reproductive diseases.

GnRH and GnRH receptors

GnRH

Gonadotrophin-releasing hormone (GnRH) was one of the earliest hypothalamic-releasing hormones to be sequenced and characterized. It is a decapeptide (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) whose structure was discovered in 1971 by the group of the Nobel laureate Andrew V. Schally (Baba *et al.*, 1971; Schally *et al.*, 1971) (Table I); later, its role as the key regulator of the pituitary-gonadal axis became increasingly clear (Conn and Crowley, 1994; Millar, 2005).

In humans, the GnRH gene is located, as a single gene copy, on the short arm of chromosome 8 (8p21-p11.2) and is organized into four exons and three introns (Seeburg *et al.*, 1987; Hayflick *et al.*, 1989). This gene encodes a 92 amino acid prohormone that is cleaved enzymatically and further modified into the secretory granules. The precursor

Table I Amino acid sequences of natural GnRH isoforms.

GnRH (also designed GnRH-I**)**	pGlu^{1}-His^{2}-Trp^{3}-Ser^{4}-Tyr^{5}-Gly^{6}-Leu^{7}-Arg^{8}-Pro^{9}-Gly^{10}-NH_2
GnRH-II	pGlu^{1}-His^{2}-Trp^{3}-Ser^{4}-His^{5}-Gly^{6}-Trp^{7}-Tyr^{8}-Pro^{9}-Gly^{10}- NH_2
GnRH-III	pGlu^{1}-His^{2}-Trp^{3}-Ser^{4}-His^{5}-Asp^{6}-Trp^{7}-Lys^{8}-Pro^{9}-Gly^{10}- NH_2

consists of: (i) a signal peptide (23 amino acids) which directs intracellular packaging and secretion, (ii) the GnRH decapeptide, (iii) a three amino acid (Gly-Lys-Arg) proteolytic processing site, and (iv) a 56 amino acid GnRH-associated protein (GAP) that is secreted with GnRH but whose function is still unclear.

It is now widely recognized that GnRH is synthesized in a small subset of hypothalamic neurons; these neurons secrete the neurohormone in a pulsatile way into the hypophyseal portal blood system through which it is transported to the anterior pituitary gland. By binding to specific receptors (gonadotrophin-releasing hormone receptors, GnRHRs) on pituitary gonadotropes, GnRH stimulates the biosynthesis and the release of the two gonadotrophins (luteinizing hormone, LH; follicle-stimulating hormone, FSH), thus ultimately regulating gametogenesis and gonadal steroidogenesis in both sexes (Conn and Crowley, 1994; Millar, 2005). GnRH pulsatility is critical for both the maintenance of gonadotrophin gene expression and the physiological pattern of gonadotrophin secretion (see below).

GnRH isoforms

It is now well accepted that, in addition to the classical form of GnRH, several other isoforms of the decapeptide exist in vertebrates. In all of these isoforms of the decapeptide, both the N-terminal (Glp-His-Trp-Ser) and C-terminal (Pro-Gly-NH_2) amino acid sequences are conserved. In particular, a second form of GnRH has been identified in most vertebrates, including humans. This form is commonly referred to as 'chicken GnRH-II', or simply GnRH-II, and its structure is uniquely conserved from fish to mammals. In humans, GnRH-II is encoded by a gene located on chromosome 20 and it shows 70% similarity to the classical GnRH at the amino acid level, differing from its structure by three aminoacids: His^{5}, Trp^{7}, Tyr^{8} (Chen *et al.*, 1998; White *et al.*, 1998; Leung *et al.*, 2003) (Table I). GnRH-II is widely distributed in the central nervous system, where it seems to act as a neuromodulator of sexual behaviour (Chen *et al.*, 1998; Millar, 2005). In the monkey hypothalamus, it has been shown that the two peptides are synthesized in two completely distinct cell populations. Specifically, the classical GnRH isoform has a diffuse expression pattern, whereas GnRH-II appears to be concentrated in specific nuclei, such as the supraoptic, paraventricular, suprachiasmatic, as well as in the medial basal hypothalamus (Latimer *et al.*, 2000). GnRH-II is also expressed in different peripheral tissues, including tissues of the female reproductive system, such as the endometrium, ovary and placenta (as well as in tumours derived from these tissues) (Cheon *et al.*, 2001; Millar, 2003; Hong *et al.*, 2008).

Another interesting form of GnRH (referred to as 'GnRH-III') has been isolated from sea lamprey (*Petromyzon marinus*); this decapeptide has 60% homology with GnRH, with four different amino acids: His^{5}, Asp^{6}, Trp^{7}, Lys^{8} (Sower *et al.*, 1993) (Table I). In the lamprey, this peptide has been shown to play crucial roles in the control of the reproductive functions in terms of gametogenesis and steroidogenesis (Deragon and Sower, 1994).

GnRHRs

The GnRHR was first cloned from an immortalized murine gonadotrope-derived cell line (αT3-1) and then from the pituitaries of several species, including humans. The human pituitary GnRHR is a 328-amino acid protein that is encoded by a gene located on chromosome 4 (4q13), composed of three exons and two introns (Fan *et al.*, 1994). The human GnRHR belongs to the family of rhodopsin-like G protein-coupled receptors (GPCR) containing seven transmembrane domains and an extracellular amino-terminal domain (35 amino acids) with two putative glycosylation sites (Stojilkovic *et al.*, 1994; Neill, 2002; Kakar *et al.*, 2004; Millar, 2005). This G protein-coupled receptor is characterized by the presence of a uniquely short carboxy-terminal (1–2 amino acids) cytoplasmic tail, that has been shown to be important for receptor internalization and desensitization (at least for other G protein-coupled receptors); for this reason, the pituitary GnRHR internalizes relatively slowly and it does not undergo rapid desensitization (Kaiser *et al.*, 1997; Hislop *et al.*, 2000).

In pituitary gonadotropes, the intracellular signalling cascade triggered by GnRHR activation has been extensively elucidated. Once activated by GnRH (or by GnRH agonistic ligands), pituitary GnRHRs couple to the $\alpha_{q/11}$ subunit of G protein ($G_{\alpha q/11}$) that, in turn, activates phospholipase Cβ (PLCβ), leading to increased intracellular levels of diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3). As a consequence, DAG will activate the intracellular protein kinase C (PKC) and IP_3 will trigger the release of Ca^{2+} from intracellular stores. Through PKC, the GnRHR activates downstream signalling pathways operating within the mitogen-activated protein kinase (MAPK) cascades, including ERK, JNK, and p38 MAPK (Kraus *et al.*, 2001; McArdle *et al.*, 2002; Cheng and Leung, 2005; Naor, 2009). Phospholipases D and A2 are also sequentially activated by GnRHR ligands, leading to an additional and prolonged activation of PKC (Naor, 1991; Kraus *et al.*, 2001). However, the $G_{s/cAMP}$ pathway is also involved in the initial response of pituitary gonadotropes to GnRH or GnRH analogues (Liu *et al.*, 2002) and these signalling pathways provide the crucial link for the transmission of signals from the receptor located in the cell membrane to the nucleus, leading to gonadotrophin synthesis and secretion (Liu *et al.*, 2002).

To be activated, pituitary GnRHRs require a pulsatile stimulation by GnRH. Based on this observation, native GnRH is administered in a pulsatile delivery pattern in clinical situations in which the ultimate aim is to restore the gonadal functions (Dwyer *et al.*, 2010; Han and Bouloux, 2010); in contrast, chronic stimulation of GnRHRs by natural GnRH, or by GnRH agonists, after the initial and transient increase in gonadotrophin release ('flare effect') event, desensitizes gonadotrope cells, leading to the situation of medical castration (see below).

A receptor specific for GnRH-II (referred to as 'type II' GnRHR) was initially cloned in non-human primates. It belongs to the family of GPCR receptors, and its intracellular tail contains several potential phosphorylation sites, involved in the desensitization and internalization processes (Millar *et al.*, 2001). Later, it was demonstrated that, in humans, the

gene coding for the type II GnRHR reveals a frameshift in coding exon 1 and a premature internal stop codon in the sequence coding its extracellular loop. Based on these observations, it is now well accepted that a functional full-length type II GnRHR protein is not expressed in humans (Neill, 2002; Millar *et al.*, 2004; Neill *et al.*, 2004; Millar, 2005). This conclusion is further supported by experimental observations demonstrating that, in peripheral tissues, such as uterine endometrium and prostate cancer cells, GnRH-II agonists exert antimotility/antiproliferative effects through the activation of the classical form of the GnRHR (Montagnani Marelli *et al.*, 2009; Wu *et al.*, 2015).

In humans, as reported for GnRH-II, also GnRH-III seems to exert its effects through the activation of the classical form of GnRHRs (Montagnani Marelli *et al.*, 2015); however, GnRH-III seems to exert a significant anti-tumour effect on human cancer cells expressing the GnRHR, while causing very low activity on gonadotrophin secretion in mammals (Kovacs *et al.*, 2002).

In this review, the terms 'GnRH' and 'GnRHR' will be used to refer to the classical ('type I') forms of GnRH and GnRHR, respectively.

GnRH analogues

Soon after the discovery of GnRH, its key role in the control of the reproductive functions and the relevance of its possible clinical applications for the treatment of reproductive-related diseases became increasingly clear. However, natural GnRH has a half-life of 2–4 min which can be mainly accounted for by degradation of the glycine-leucine bond between amino acids 6 and 7. For this reason, several GnRH synthetic analogues have been developed and they can be divided into agonists and antagonists.

GnRH agonists

GnRH agonists bind to GnRHRs mimicking the activity of the natural decapeptide. Synthetic GnRH agonists have been designed based on the observation that the native peptide is rapidly degraded in the circulation and this degradation occurs at the Gly residue in position 6; moreover, the COOH terminus of the decapeptide is crucial for the binding to the receptor while its NH_2-terminal end is essential not only for the binding to the receptor but also for receptor activation (Sealfon *et al.*, 1997). Thus, GnRH agonists have been developed by replacing Gly^6 with a D-amino acid, increasing the plasma half-life compared with the native hormone; moreover, some of these compounds present a deleted Gly^{10}-amide with the addition of an ethylamide residue to Pro^9, increasing the affinity for GnRHRs (Karten and Rivier, 1986; Conn and Crowley, 1994; Engel and Schally, 2007). At present, triptorelin, leuprolide, goserelin and buserelin (Table II) represent the GnRH agonist mostly used in clinical trials. High doses and prolonged administration of GnRH agonist, after an initial stimulation of gonadotropes (the so called 'flare effect'), suppresses the activity of the pituitary-gonadal axis, through down-regulation of GnRHRs, and are indicated, for instance, for the treatment of central precocious puberty, endometriosis, and polycystic ovarian disease (see below). These compounds are also widely utilized for the treatment of hormone-dependent tumours (Akaza, 2011; Limonta *et al.*, 2012; Tammela, 2012; Limonta and Manea, 2013; Labrie, 2014).

GnRH antagonists

GnRH antagonists have been designed and developed with the aim to obtain compounds that might block the pituitary-gonadal axis without triggering the undesirable flare effect. These compounds exert their effects by competitively binding to and blocking GnRHRs on pituitary gonadotropes, thus causing a rapid and sustained inhibition of gonadotrophin secretion (Schally, 1999; Herbst, 2003; Coccia *et al.*, 2004; Padula, 2005; Montagnani Marelli *et al.*, 2006). Early antagonists presented a very complex structure, with multiple amino acid substitutions, and they were found not to be suitable for clinical applications because of undesirable side effects due to histamine release, such as oedematogenic effects and other anaphylactic reactions (Schally, 1999; Padula, 2005). GnRH antagonists devoid of undesirable oedematogenic effects were then developed. These compounds contain Ac-D-Nal-D-Cpa-D-Pal in the N-terminal of the peptide and D-Ala in position 10; in addition, they present different amino acid substitutions in positions 5, 6, and 8 (Schally, 1999; Cook and Sheridan, 2000; Herbst, 2003). GnRH antagonist, such as cetrorelix, ganirelix, abarelix and degarelix (Table II) had the highest overall inhibitory activity and receptor binding affinity. Among these compounds, abarelix was shown to possess immediate-onset systemic allergic reactions and for this reason it was withdrawn from the US market. GnRH antagonists have many clinical applications in reproductive medicine and in gynaecology; cetrorelix and ganirelix have

Table II Amino acid sequences of GnRH agonists and antagonists.

GnRH	$pGlu^1$-His^2-Trp^3-Ser^4-Tyr^5-Gly^6-Leu^7-Arg^8-Pro^9-Gly^{10}-NH_2
Agonists	
Buserelin	$pGlu^1$-His^2-Trp^3-Ser^4-Tyr^5-D-Ser(tBu) ^{6}-Leu^7-Arg^8-Pro^9 - NHEt
Goserelin	$pGlu^1$-His^2-Trp^3-Ser^4-Tyr^5-D-Ser(tBu) ^{6}-Leu^7-Arg^8-Pro^9 $AzaGly^{10}$- NH_2
Leuprolide	$pGlu^1$-His^2-Trp^3-Ser^4-Tyr^5-D-Leu^6-Leu^7-Arg^8-Pro^9- NHEt
Triptorelin	$pGlu^1$-His^2-Trp^3-Ser^4-Tyr^5-D-Trp^6-Leu^7-Arg^8-Pro^9-Gly^{10}-NH_2
Antagonists	
Abarelix	Ac-D-Ala^1-D-Cpa^2-D-Ala^3-Ser^4-Tyr^5-D-Asp^6-Leu^7-Lys(iPr)^8-Pro^9-D-Ala^{10}-NH_2
Cetrorelix	Ac-D-Nal^1-D-Cpa^2-D-Pal^3-Ser^4-Tyr^5-D-Cit^6-Leu^7-Arg^8-Pro^9-D-Ala^{10}-NH_2
Degarelix	Ac-D-Nal^1-D-Cpa^2-D-Pal^3-Ser^4-Aph(Hor)^5-D-Aph(Cba)^6-Leu^7-Lys(iPr)^8-Pro^9-D-Ala^{10}-NH_2
Ganirelix	Ac-D-Nal^1-D-Cpa^2-D-Pal^3-Ser^4-Tyr^5-D-hArg(Et)$_2^6$-Leu^7-hArg(Et)$_2^8$-Pro^9-D-Ala^{10}-NH_2

tBu: tert-butyl; Et: ethyl; AzaGly: aza-glycine; Ac: acetyl; Cpa: chlorophenylalanine; Pal: 3-pyridylalanine; Cit: citrulline; Aph: 4-aminophenylalanine; Hor: L-hydroorotyl; Cba: carbamoyl; iPr: isopropyl; Nal: 2-naphtylamine; hARg: homoarginine

been proved to be particularly efficient in the control of ovarian stimulation protocols for prevention of a premature luteinizing hormone surge in assisted reproduction technology (Coccia *et al.*, 2004; Al-Inany *et al.*, 2011; Tan and Bukulmez, 2011) (see below).

Development of GnRH neurons and related diseases

A unique neuronal network of approximately 1500 hypothalamic neurons initiates and maintains reproductive function in mammals (Wray and Hoffman, 1986). It accomplishes this task by coordinating the synthesis and pulsatile secretion of GnRH, from this neural network (Merchenthaler *et al.*, 1984). In humans, GnRH is synthetized and secreted by parvicellular neurons that extend from the preoptic area to the infundibular nucleus of the hypothalamus (Lehman *et al.*, 1986). GnRH neurons project from these nuclei to the organum vasculosum of the lamina terminalis and to the external zone of the median eminence, a highly specialized and plastic structure that allows delivery of the decapeptide GnRH to the anterior pituitary through the hypophyseal portal system.

The origin of GnRH neurons has been mainly studied in small animal models such as mice, but it has been subsequently found to be a conserved process that occurs in many other species, including humans. Thus, in the early embryo, GnRH neurons are found in the nasal region, in the olfactory placode, and migrate into the brain during fetal development. The route of migration is through the cribriform plate, into the olfactory bulb and then caudally to the hypothalamus. As to how these neurons find their way to the final positions in the brain has been the subject of considerable investigation in the past decades, and, although some of the molecular mechanisms have been elucidated, the complete picture is still unknown (Cariboni *et al.*, 2007).

The migration of GnRH neurons is intimately associated with the olfactory system. Specifically, it has been shown that GnRH neurons use the intermingled olfactory and vomeronasal axons extending toward the olfactory bulb as a scaffold along which to migrate. Once they have entered into the forebrain, they follow a transient branch of the vomeronasal/terminal nerve to reach their final positions. In humans, GnRH neurons can be detected in the olfactory placode at embryonic week (EW) 5.5–6; they start to migrate in close association with the terminal nerve and enter the forebrain at EW 6.5, reaching the hypothalamus by EW 9.0. Their migration is considered complete between the 13th and the 16th week of gestation (Quinton *et al.*, 1997). In most species, the distribution pattern of GnRH neurons in the hypothalamic region appears to be already established before birth; however, their complete maturation, with the development of correct synaptic connectivity and functions, may be attained at the time of puberty (Kim *et al.*, 1999).

Developmental defects of GnRH neuron migration can lead to defective or absent GnRH secretion, resulting in a heterogeneous family of genetic disorders, such as central hypogonadotropic hypogonadisms (HH), characterized by reduced or failed sexual maturation and competence. Alterations of olfactory/vomeronasal patterning can also lead to defective GnRH neuron migration with olfactory dysfunction (Cariboni and Maggi, 2006; Forni and Wray, 2015). Thus, HH can occur with a normal sense of smell (normosmic HH or nHH) or in association with anosmia (Kallmann syndrome; KS).

Hypogonadism describes a reduced activity of the gonads, reflected by low levels of circulating sex steroids, that is testosterone in males and estrogen and progesterone in females. The term hypogonadotropic indicates that the hypogonadism found in hypothalamic HH is caused by low levels of the two gonadotrophins LH and FSH related to a deficit of GnRH (Mitchell *et al.*, 2011).

The failure of GnRH production in HH can be due either to the absence of the GnRH neurons inside the hypothalamus or to the inability of the hypothalamus to release GnRH in the correct pulsatile manner or to the lack of gonadotrope cell responsiveness (Sykiotis *et al.*, 2010).

To date at least sixteen different genes affecting the development and function of the GnRH neuron have been implicated in causing HH. However, genetic testing and inheritance prediction are quite problematic since the causative genes show all forms of inheritance and no one gene defect is common to all HH cases. In addition, HH is no longer considered a monogenic disorder, but instead a complex genetic condition, where oligogenic and complex genetic–environmental interactions take place (Mitchell *et al.*, 2011). The genes so far identified (Fig. 1) encode for factors required for GnRH neuron development, differentiation and function. Some of them, such as the fibroblast growth factor receptor 1 (FGFR1) and its ligand fibroblast growth factor 8 (FGF8), heparan sulphate 6-O-sulphotransferase 1 (HS6ST1) and nasal embryonic LH releasing hormone Factor (NELF) are involved in the correct embryonic differentiation of the GnRH-secreting neurons. Other genes, such as ANOS1, semaphorin 3A (SEMA3A), prokineticin 2 (PROK2) and prokineticin receptor 2 (PROKR2), encode for molecular signals that control the correct migration of the GnRH neurons (Cariboni *et al.*, 2011; Hanchate *et al.*, 2012) while others, such as SEMA3E, regulate the survival of GnRH neurons in the hypothalamus (Cariboni *et al.*, 2015). TAC3 and TACR3 genes, encoding for neurokinin B and its receptor NK3, as well as KISS1 and KISS1R genes, encoding for kisspeptin 1 and its receptor, are involved as upstream signals in the activation and control

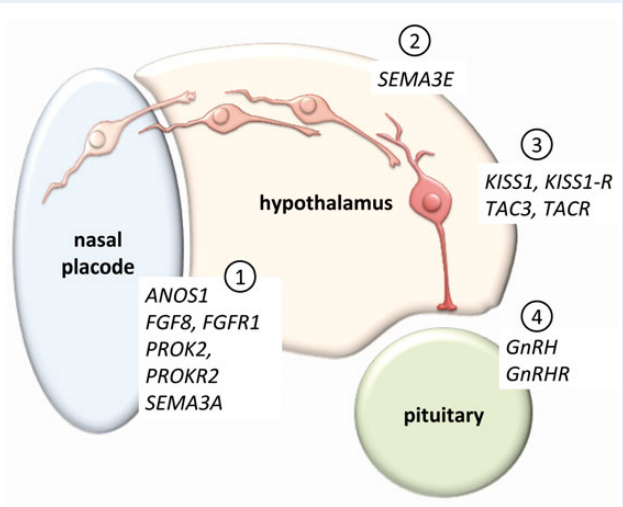

Figure 1 Schematic drawing showing the development of GnRH neurons from their place of origin (nasal placode) to their final destination (hypothalamus). Some of the genes found to affect different phases of this developmental process and found to be mutated in patients with central HH are marked.

of GnRH release. Finally, mutations of the genes for GnRH itself and its receptor (GnRHR) are also causative of HH (Bonomi *et al.*, 2012).

HH is diagnosed at the time of puberty by the absence, complete or partial, of GnRH-mediated release of LH and FSH. A normal anterior pituitary structure and function or the absence of secondary causes have to be considered for a correct diagnostic framework (Buck Louis *et al.*, 2013). The association of low serum gonadotrophins and gonadal steroids with a compromised sense of smell is predictive of KS. In such patients, magnetic resonance imaging (MRI) should be considered to ascertain the hypoplasia or aplasia of the olfactory bulbs and tracts and to exclude hypothalamic or pituitary lesions as causative of HH (Dodé and Hardelin, 2009).

Most cases of HH are sporadic, consistent with the affected individuals being typically infertile, but the familial transmission has also been well described. The condition has a low prevalence, estimated at 1 in 4000 for male nHH cases overall and 1:50 000 for KS. There is a well-reported gender discrepancy between male and female HH cases with approximately 3–5 males diagnosed for each female case. However, this may not be explained by a biological/genetic reason, as the only gene mapping on the X chromosome found so far is ANOS1, which has an incidence of mutation similar to the autosomic genes. Instead, this discrepancy might be due to a phenotypic verification, since girls showing a primary amenorrhoea undergo hormonal therapy and are diagnosed much later in life, or not diagnosed at all, possibly due to the wide spectrum of diseases associated with primary amenorrhoea (such as polycystic ovary syndrome and stress) (Bry-Gauillard *et al.*, 2010; Hu *et al.*, 2012). It is therefore important that both boys with delayed or absent puberty and girls with primary amenorrhoea are referred to genetic counselling for HH. An early and correct diagnosis of HH would enable more timely management of affected newborns (Bouvattier *et al.*, 2012) and improve the response to hormonal therapy (Kousta *et al.*, 1996; Büchter *et al.*, 1998; Bouvattier *et al.*, 2012). Thus, either combined gonadotrophin therapy (human chorionic gonadotrophin [hCG] and human menopausal gonadotrophins [hMG] or recombinant FSH [rFSH]) or pulsatile GnRH therapy, are used to stimulate spermatogenesis or folliculogenesis; a gradual increase of gonadal steroids (testosterone or hCG injections in males; estrogen and progestin in females) are administered to induce and maintain secondary sex characteristics (Meczekalski *et al.*, 2013).

Only about 40% of HH cases have been found to be associated to gene mutations. This is possibly due to the difficulty in performing linkage studies because of the small number of families affected by this disease and to the high proportion of sporadic mutations. However, we expect that the advent of next-generation sequencing combined with the application of in silico prediction tools and animal mouse models will help to unravel the complex genetics of HH.

GnRH neuron function and GnRH secretion

Control of GnRH secretion

GnRH pulsatile secretion

GnRH neurons undergo a fine-tuned modulation to synchronize the pulsatile release of GnRH into the hypothalamic-hypophyseal portal vessels to induce a correct secretion of gonadotrophins (Marshall *et al.*, 1991; Kalra, 1993; Tsutsumi and Webster, 2009). Although a pulsatile release of gonadotrophin seems compulsory for GnRH neuron function, the relevance of such episodic GnRH release is evident considering that continuous infusion of GnRH rapidly suppresses both LH and FSH secretion and that it may be readily reversed with a return to pulsatile stimulation.

Whereas short-term pulsatile treatment with GnRH results in up-regulation of pituitary GnRHRs, a prolonged high-dose treatment induces a loss of the GnRH response due to rapid uncoupling of the GnRH receptor from its intracellular signalling molecules followed by down-regulation of receptor number (Loumaye and Catt, 1982; Cheng *et al.*, 2000; McArdle, 2012); this characteristic is exploited clinically by administration of long-acting GnRH agonist to shut down the reproductive axis. Consequently, one explanation for the pulsatile secretion of GnRH is to avoid the down-regulation of the GnRHR in the pituitary gonadotrope cells.

Most of the data on GnRH pulsatility has been validated in several animal models, from mouse to monkey. In humans, the measurement of GnRH levels in peripheral blood does not accurately reflect hypothalamic secretion and so the evaluation of serum LH levels, detected by the assay of its specific β-subunit, has been used as a marker of GnRH pulse generator activity. However, it is known that the assay of the glycoprotein free α-subunit (FAS) is tightly correlated with fast GnRH pulses, due to its shorter half-life (Landy *et al.*, 1990; Hayes and Crowley, 1998). Therefore, the data reported here on GnRH pulses in humans, when not otherwise specified, will be related to plasma LH or FAS measurement.

GnRH pulses occur approximately every 2 h in the adult male; in females, the pattern of pulsatile GnRH release is more complicated and shows variations during the different reproductive stages, which influence the development of sex functions, and during the ovulatory cycle.

In all species, the GnRH pulse generator is the main regulator of the surge mode of gonadotrophin release because it drives the rapid rise in estradiol (E2) secretion in the late follicular phase that triggers the preovulatory LH surge (Moenter *et al.*, 1991).

The female pattern of gonadotrophin secretion therefore includes both a pulse and a surge phase, which are regulated independently. Moreover, the synthesis of both LH and FSH is regulated by the frequency of GnRH pulses, with FSH favoured by slow pulse frequencies (<1 pulse/2–3 h) and LH favoured by fast pulse frequencies (1 pulse/60–90 min).

Role of ovarian steroids on GnRH secretion

Several central and peripheral signals are involved in the modulation of GnRH neuronal activity and peptide secretion (Hrabovszky and Liposits, 2013); some of these signals are stimulatory (e.g. kisspeptin, norepinephrine and neuropeptide Y), some are inhibitory (e.g. endogenous opioids, interleukin-1, progesterone) and some can be either stimulatory or inhibitory (e.g. E2). In addition, GnRH itself may exert a regulatory role of its own secretion through an ultra-short feedback loop (DePaolo *et al.*, 1987).

In females, the two modes of GnRH and gonadotrophin secretion occur at different times during the ovarian cycle (Fig. 2A). Differential regulatory effects of sex steroids on GnRH secretion and pulsatility are evident in many mammals and in women: progesterone exerts an inhibitory action while E2 can have both stimulatory and inhibitory effects, depending upon the stage of the menstrual cycle. While estrogen receptor beta (ERβ) is expressed in a subset of GnRH neurons, the presence of

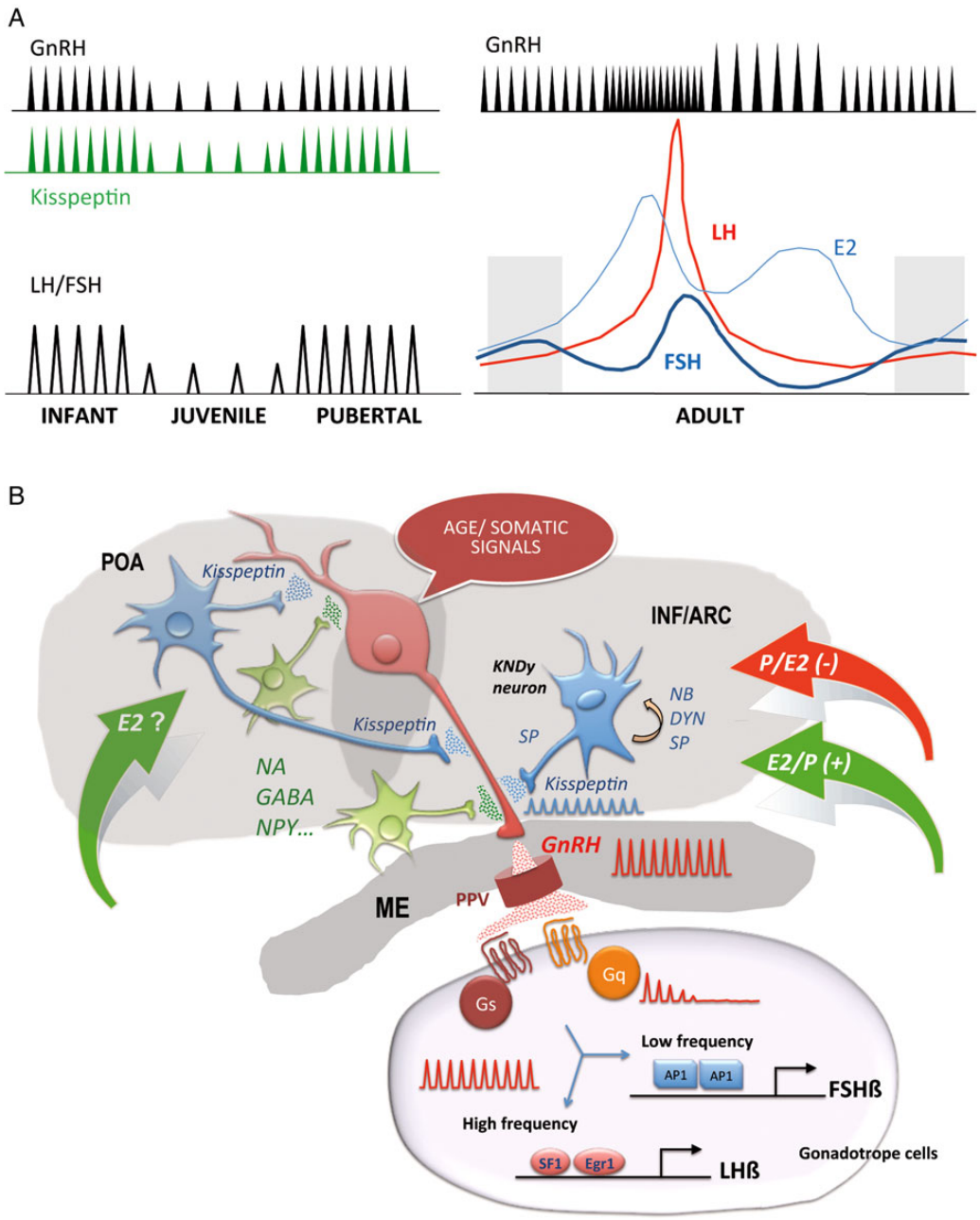

Figure 2 (**A**) Representation of the variations in pulsatile secretion of GnRH during the human female lifespan. (**B**) Summary of the physiological mechanisms possibly involved in the control of GnRH secretion and its action on gonadotrope cells. (POA: preoptic area; INF: infundibular region; ARC: arcuate nucleus; ME: median eminence; NPY: neuropeptide Y; GABA: gamma aminobutirric acids; NA: noradrenaline; NB: neurokinin B; DYN: dynorphin; SP: substance P; E2: estradiol; P: progesterone; SF1: steroidogenic factor 1 transcription factor; Egr1: early growth response 1 transcription factor; AP1: activating protein 1 transcription factor; Gs: Gs alpha subunit; Gq: Gq/11 subunit).

ERα and of progesterone receptors have not been completely clarified (Herbison and Pape, 2001; Hu *et al.*, 2008). It has also been proposed that the actions of ovarian steroids on GnRH pulsatility may be indirect and mediated by peptidergic neurons afferent to GnRH cells (see below).

Both the frequency and the amplitude of the GnRH pulses are influenced by estrogen and progesterone, which drive a diurnal variation in pulse frequency, with a lower frequency at night, and a variation during the menstrual cycle, with a lower frequency and increased amplitude during the luteal phase, mainly mediated by progesterone (Soules *et al.*, 1984; McCartney *et al.*, 2007). During the ovulatory cycle, a 'tonic' pulsatile secretion of GnRH is controlled by the negative feedback actions of ovarian steroids; E2 is responsible for the inhibition of pulse amplitude while progesterone inhibits pulse frequency (Karsch, 1987). At the end of the follicular phase, the rapid elevation of E2 concentrations, produced by maturation of the pre-ovulatory follicle, induces the pre-ovulatory surge phase of GnRH and LH release through a

positive feedback action. E2 may also increase the pituitary sensitivity to GnRH pulses by inducing the expression of GnRH receptors at the gonadotrope level.

Androgens also appear to prevent the normal negative feedback suppression of LH in polycystic ovary syndrome (PCOS) affected women (see below), and the administration of the anti-androgen flutamide may restore the sensitivity of reproductive axis to the negative feedback of E2 and progesterone (Eagleson *et al.*, 2000).

Neuronal control of GnRH secretion

The pulsatile and the surge releases of GnRH also seem to be mainly controlled by different subsets of neurons afferent to GnRH neurons: the neurons located in the infundibular region/arcuate nucleus (INF/ARC) mediate the normal pulsatile phase, whereas the ones located in the anteroventral periventricular nucleus (AVPV) of the hypothalamus in rodents (Smith *et al.*, 2005) or in the preoptic area (POA) in human and non-human primates (Smith *et al.*, 2010; Hrabovszky, 2014), mediate the surge phase.

Recently, two different hypotheses on the neurobiological bases of the hypothalamic GnRH pulse generator have been proposed and have been discussed by Plant and coworkers (Plant, 2015). The first hypothesis proposes that pulsatility in the GnRH neuronal network is intrinsic to the GnRH neuron itself. The second one supports the notion that peptidergic neurons residing in the INF/ARC are responsible for the pulse generation (Piet *et al.*, 2015; Plant, 2015).

The first hypothesis is supported by the original observation that the pulse generator is cell-autonomous (Wetsel *et al.*, 1992) and it has been determined, by *in vivo* and *in vitro* observations, that the activity of each GnRH neuron is spontaneously synchronized (Advis *et al.*, 2003) due to an extensive inter-cellular communication. The second hypothesis is based on the observation that a number of neurotransmitters and neuropeptides may act directly, or as intermediaries of circulating gonadal steroid levels, on GnRH pulse secretion in humans (Hrabovszky and Liposits, 2013). In particular, INF and POA neurons producing kisspeptin are considered the main candidates for the regulation of GnRH pulsatile release (Fig. 2B).

Kisspeptin and neurokinin B

Kisspeptin is a potent GnRH secretagogue and the principal regulator of the secretion of GnRH. Indeed, kisspeptin fibres project to GnRH cell bodies and to GnRH processes; GnRH neurons also express kisspeptin receptors (Piet *et al.*, 2015). The role of kisspeptin neurons in the regulation of GnRH release has been recently and elegantly reviewed by Skorupskaite and coworkers in this journal (Skorupskaite *et al.*, 2014); briefly, the critical roles of kisspeptin in the onset of puberty, the regulation of sex steroid-mediated feedback and the control of fertility in adulthood are well recognized, supporting its role as a GnRH pulse generating peptide (Terasawa *et al.*, 2013). Our knowledge of the kisspeptin-GnRH relationships are mainly from animal models, but restricting our report to humans or nonhuman primates: kisspeptin neurons have been mainly detected in brain POA and INF (Hrabovszky and Liposits, 2013); they provide a direct input to GnRH neurons (Dahl *et al.*, 2009); and they show a sexual dimorphism (Hrabovszky *et al.*, 2010).

The kisspeptin neurons present in POA and INF seems to exert different types of control of GnRH secretion (Lehman *et al.*, 2010). During the follicular phase of the ovulatory cycle, increasing production of ovarian E2 may activate INF kisspeptin neurons, through the binding to both intracellular and membrane E2 receptors, possibly by increasing the expression of progesterone receptors (Mittelman-Smith *et al.*, 2015; Stephens *et al.*, 2015). This overrides the normal negative feedback of E2 (mediated by a suppression of kisspeptin release by INF neurons) with an increase of GnRH pulse frequency and amplitude that will lead to the LH surge, triggering ovulation (Lehman *et al.*, 2010; Watanabe *et al.*, 2014). Although the mechanism leading to E2 positive feedback has been confirmed to involve AVPV kisspeptin neurons in mice and the corresponding POA kisspeptin neurons in non-human primates and ewe (Lehman *et al.*, 2010; Watanabe *et al.*, 2014), the role of the human POA kisspeptin subpopulation in steroid feedback has yet to be determined.

Following ovulation, luteinization of the granulosa cells leads to increased progesterone production which, in the presence of lower levels of E2, down-regulates kisspeptin expression in INF neurons which slows GnRH pulse frequency causing a decrease in LH and a slow progressive increase in FSH production, to induce a new cycle of ovulation (Rance, 2009). The ability of kisspeptin to modulate GnRH pulses has suggested its pharmacological utilization. Kisspeptin analogues have been found to rapidly stimulate GnRH release in healthy and subfertile women; however, upon chronic exposure this is followed by a suppression of the reproductive axis, as observed for GnRH agonists (Prague and Dhillo, 2015).

An increasing number of direct hypothalamic modulators of kisspeptin neurons have been identified, uncovering a complex system of neuroendocrine factors that ensure proper gonadotropic function. Many kisspeptin neurons in the INF co-express other peptides, namely neurokinin B, a tachykinin, and dynorphin, an endogenous opioid peptide (Lehman *et al.*, 2010; Hrabovszky *et al.*, 2012; Navarro, 2012; Skrapits *et al.*, 2015); the acronym KNDy is used to indicate these cells (Cheng *et al.*, 2010). Such peptides (and others, such as substance P) have been proposed to act in a coordinated fashion to shape kisspeptin secretion through paracrine/autocrine actions.

However, although there are unresolved questions concerning the role of KNDy peptides in GnRH pulse generation, there is a consensus that NKB operates upstream of kisspeptin to modulate downstream GnRH pulsatility. Studies conducted in monkeys, have shown that neurokinin B exerts a kisspeptin-mediated stimulatory action on GnRH release (Ramaswamy *et al.*, 2010, 2011), whereas dynorphin is generally recognized as an inhibitor of the release of GnRH (Lehman *et al.*, 2010). In humans, loss of function mutations in either the gene for neurokinin B (TAC3) or in the gene for its receptor (TAC3R) are associated with HH and a delayed or absent puberty phenotype (Topaloglu *et al.*, 2009).

Substance P

Substance P (SP), a member of the tachykinin family, has been found to participate in the timing of puberty onset in mouse, possibly as a player in the putative pulse generator activity of KNDy neurons or by a direct action on GnRH axon terminals (Simavli *et al.*, 2015). In human, SP is expressed in the hypothalamus (Chawla *et al.*, 1997) and GnRH fibres co-localize with SP-positive fibre plexuses (Borsay *et al.*, 2014). Some indirect evidence supports a role of SP in primate central control of fertility; human and monkey studies demonstrate that plasma SP levels vary across the menstrual cycle (Kerdelhué *et al.*, 2006) and the high ratio of children displaying precocious puberty after traumatic brain injury seems to correlate with higher SP levels detected in their brains (Kaulfers *et al.*, 2010). Nevertheless, the determination of the role of SP GnRH release in humans requires further investigation.

RFamide-related peptides, gonadotrophin inhibitory hormone and other factors

The neuronal network that controls GnRH release was further complicated by the isolation, in quail, of a new neuropeptide able to inhibit gonadotrophin synthesis and release, named gonadotrophin inhibitory hormone (GnIH) (Tsutsui *et al.*, 2000). GnIH belongs to a family of peptides, which includes kisspeptin and other neurotransmitters, having an RFamide (Arg-Phe-NH3) motif in their structure. RFamide-related peptides-3 (RFRP-3) has been identified as the human orthologue of avian GnIH; RFRP-3 neurons are located in the dorsomedial region of the hypothalamus and project to GnRH neurons that express its specific GPR147 receptors. Although the relationship between GnIH/RFRP and reproductive function has been characterized in animal models (Tsutsumi *et al.*, 2010), only recently a modulatory effect of the RFRP-3/GPR147 system on a possible regulation of human pubertal development has been suggested (Pietranera *et al.*, 2015).

Moreover, other endogenous factors (e.g. leptin, ghrelin, NPY, orexin, insulin, galanin, oxytocin, proopiomelanocortin, etc.), some of which are co-expressed in human KNDy neurons or upstream of kisspeptin action (Verma *et al.*, 2014; Skrapits *et al.*, 2015), as well as environmental factors (e.g. stress and changes in energy stores) may affect the release of GnRH and the activity of the pulse generator (Hrabovszky and Liposits, 2013) (Fig. 2B).

GnRH action on gonadotrope cells

The differential expression and secretion of gonadotrophins depends on the capacity of gonadotrope cells to decode GnRH signals.

At the pituitary level, GnRH interacts with high affinity G-protein coupled receptors present on the plasma membrane of the gonadotrophin-producing cells. As described above, pituitary GnRH receptors may couple to both Gαq/11 and Gαs subunits and their differential activation may provide the basis for the decodification of frequency and amplitude-dependent signalling by GnRH receptors.

Many studies have defined the mechanisms of down-regulation of the signalling induced by tonic continuous exposure to GnRH, which has a pharmacological, but not physiological, importance (McArdle, 2012). Studies carried out in cell models demonstrate that after continuous GnRH exposure, the Gαs/cAMP pathway shows a rapid initial response that is rapidly deactivated. On the contrary, during the same GnRH regimen, the Gαq/11 pathway remains constantly activated (Liu *et al.*, 2002). With a pulsatile GnRH treatment, a correspondent pulsatile stable response is mediated by the Gαs/cAMP signalling pathway; however, the Gαq/11 pathway shows an initial pulsatile response followed by a rapid decrease of pulse amplitude suggestive of desensitization. Therefore, the Gαs and the Gαq/11 pathways seem to mediate an adaptive and desensitization response, respectively, to pulses of GnRH (Tsutsumi and Webster, 2009).

After receptor activation, the intracellular signal has to drive gonadotrophin expression. According to the different regulation of gonadotrophins by pulsatility, *in vitro* studies have shown that several intracellular signal cascades (e.g. MAPKs; see above) as well as transcription factors have been identified to independently modulate gonadotrope-specific expression of LHβ and FSHβ genes (see (Lim *et al.*, 2009; Tsutsumi and Webster, 2009)). High frequency GnRH pulses induce LHβ expression mainly by the activation of steroidogenic factor 1 (SF1) and early growth response 1 (EGR1) (Fortin *et al.*, 2009) transcription factors; under the same GnRH pulse regimen, the expression of FSHβ is inhibited by the activation of a series of co-repressors. In contrast, in the presence of low frequency GnRH pulses, a decrease in EGR1 levels leads to a prevalent action of co-repressors of the LHβ promoter (Luxardi *et al.*, 2007), whereas a decrease in co-repressors allows the activation of the FSHβ promoter driven by AP-1 transcription factor family members (c-fos, c-jun) (McGillivray *et al.*, 2007) (Fig. 2B).

Dysregulation of pulsatile GnRH release

Several physiological responses and pathological conditions that cause reproductive failure are associated with dysregulated GnRH pulse release. For instance, functional hypothalamic amenorrhoea (e.g. due to a prolonged state of negative energy balance induced by either fasting or exercise) can be partly explained by variable inhibitory alterations of the GnRH pulse-generator (see Liu and Patel, 2013). In obese animal models, insulin receptor signalling to GnRH neurons increases GnRH pulsatile secretion, contributing to reproductive dysfunction (DiVall *et al.*, 2015).

Aging is another factor that influences changes in hypothalamic, pituitary and gonadal function (Hall, 2007; Veldhuis *et al.*, 2009). In women, the overall level of GnRH increases with aging, suggesting that aging alters the pituitary responsiveness to GnRH (Hall, 2007). The depletion of ovarian follicles and the decrease in estrogen levels, with loss of negative feedback, are responsible for this increase in GnRH secretion. However, in younger post-menopausal women, gonadotrophin and GnRH pulses occur approximately every 50–55 min, similarly to a normally cycling woman in the late follicular phase and midcycle surge. However, the frequency of GnRH and gonadotrophin pulses decreases with aging, independently of the ovarian hormonal milieu, possibly due to an impairment of the hypothalamic components of the reproductive axis (Hall, 2007; Hall *et al.*, 2000).

In women, several reproductive disorders, such as hypothalamic amenorrhoea, hyperprolactinemia, PCOS and hypogonadotropic hypogonadism, develop in conditions of disruption of the normal pulsatile GnRH secretion. Regarding PCOS, the observed changes in GnRH pulsatility have been attributed to fetal androgen excess, which may alter the steroid negative and positive feedback regulatory mechanisms (Roland and Moenter, 2014). Recently it has also been proposed that neurokinin B and leptin may play an essential role in the activation of GnRH neurons and in the initiation of an increased LH pulse frequency under a PCOS-like androgen excess and that kisspeptin may coordinate their stimulatory effects on LH release (Yan *et al.*, 2014).

GnRH pulsatility in the onset of puberty

GnRH plays a key role in the onset of puberty. In primates, including humans, the GnRH neuronal network is fully active during the late stages of gestation and early neonatal period; it then becomes quiescent during childhood until its full reactivation at puberty.

Therefore, the activity of the neuroendocrine reproductive axis during development can be distinguished in three phases (on-off-on). The first is characterized by a sustained GnRH pulsatility present during fetal life (16th week of intrauterine life) and between the 4th and 10th weeks of post-natal life (on). This phase has been called mini-puberty since it is accompanied by a significant gonadotrophin and steroid hormone secretion but, in contrast with the true puberty, neither ovulation nor spermatogenesis are present (Plant *et al.*, 2005). Mini-puberty is followed by a phase of relatively stable low GnRH pulsatility and gonadal

quiescence (off) that covers infancy and juvenile development. A sustained GnRH pulsatility is then reactivated (on) at the termination of juvenile development, triggering the puberty phase (Fig. 2A).

Different mechanisms seem to control the changes of GnRH secretion during these three developmental phases. During infancy, the pulse generation centre seems to reside in KNDy ARC neurons and the intermittent release of kisspeptin results in a corresponding pattern of GnRH release into the portal circulation and gonadotrophin secretion. In fact, the observed hypogonadism in infants carrying a mutation of kisspeptin receptor (Semple *et al.*, 2005) is indicative of a central control of the induction of the infant GnRH pulsatile secretion. The high activity of the GnRH pulse generator during infancy is therefore determined by developmental events in the brain (Shinkawa *et al.*, 1983) but modulated by the negative feedback effect exerted by gonadal steroid hormones.

The decrease in GnRH release during late infancy and the juvenile period is due to neurobiological mechanisms (either a decrease in stimulatory signals or an activation of inhibitory signals) impinging on the ARC GnRH pulse generation circuits; it may occur in the absence of gonads and it characterizes the delay in puberty onset present in primates (Grumbach and Kaplan, 1990). Therefore a central 'brake', rather than the endocrine reproductive axis, is the limiting step to puberty onset (Plant, 2015). Nevertheless, during this hypogonadotropic condition, an adult-like hypothalamic content of GnRH is observed and the exposure to pulsatile GnRH or analogues can induce a transient premature pubertal pattern (Plant, 2015). Recent data have shown that juvenile quiescence is characterized by a low pulsatile release of kisspeptin (Guerriero *et al.*, 2012) indicating, once again, its involvement in the control of the GnRH pulse generator mechanism. In the female, the open loop activity of the GnRH pulse generator during juvenile development is only partially suppressed, and the negative feedback signals from the ovary contribute to the prepubertal restraint on gonadotrophin secretion (Pohl *et al.*, 1995); this observation could explain the earlier onset of puberty in females compared with males and the higher frequency of GnRH-dependent precocious puberty in girls (Witchel and Plant, 2014).

Puberty is initiated when high amplitude and high frequency releases of kisspeptin and GnRH are reactivated due to the weakening of the central brake. The reactivation of the GnRH pulse leads to an increase in gonadotrophin release and to gonadal stimulation. The consequent production of ovarian hormones can exert a first negative feedback phase and the delay present between menarche and first ovulation is actually due to the initial rise in E2 at the reactivation of the pulse generator (Rapisarda *et al.*, 1983). Afterwards, a decrease in the sensitivity of steroid negative feedback increases its positive feedback capability and critical levels of E2 can trigger an increase of the pulsatile release of kisspeptin and GnRH which leads to increased LH secretion and to ovulation. Finally, the timing of the reactivation of the GnRH pulse could be due to both internal and external sources (Sisk and Foster, 2004); the existence of an hypothalamic pubertal clock or of brain sensors, which is also able to monitor circulating signals of somatic development, may co-ordinate the reactivation of the GnRH pulse (Plant, 2015).

GnRH and GnRH receptors in female peripheral sexual organs

Besides its well-known endocrine function as the key regulator of the pituitary-gonadal axis, GnRH may directly regulate some extrapituitary reproductive tissues through the activation of locally expressed GnRHRs. The expression of GnRHRs in peripheral tissues was first demonstrated, by radioreceptor assay, in the human placenta in 1981 (Currie *et al.*, 1981). These observations were later confirmed by studies reporting the presence of the mRNA and the cDNA for these receptors in human placental cells (Lin *et al.*, 1995). The GnRH/GnRHR system has also been shown to be expressed in other female reproductive tissues, such as the ovary and the endometrium; this prompted researchers to highlight its possible autocrine/paracrine effects in these tissues, both in physiological and in pathological conditions (Grundker *et al.*, 2002a; Harrison *et al.*, 2004; Cheung and Wong, 2008; Wu *et al.*, 2009; Limonta *et al.*, 2012).

Peripheral versus central GnRHRs

It is now well established that GnRHRs in peripheral reproductive tissues, both female and male, share the same cDNA nucleotide sequence and protein molecular size with the pituitary receptor (Chi *et al.*, 1993; Kakar *et al.*, 1994; Limonta *et al.*, 1999, 2012). However, the pharmacological profile of peripheral receptors appears to be different from that of the receptors at pituitary level. First of all, according to some authors, GnRH agonists bind peripheral GnRHRs at a lower affinity than they bind to pituitary receptors (Iwashita *et al.*, 1986; Limonta *et al.*, 1992; Dondi *et al.*, 1994); on the other hand, high affinity GnRH receptors have been described by others in some peripheral tissues (Imai *et al.*, 1994; Irmer *et al.*, 1995). Moreover, GnRH analogues, which behave as antagonists at the pituitary level (such as cetrorelix), trigger the same biological effects of GnRH agonists in peripheral tissues, particularly in tumours of the reproductive tissues (Kleinman *et al.*, 1994; Yano *et al.*, 1994).

The different pharmacological profile of GnRHRs in different cellular environments is still an open question and has been the matter of intensive debate. First, it has been postulated that differences at the molecular level might exist between pituitary and peripheral GnRHRs. For instance, even if the sequences of the receptor transcripts are identical, the corresponding proteins could undergo alternative post-translational processing, generating proteins with different degrees of glycosylation or phosphorylation (Rama and Rao, 2001). Moreover, Millar and co-authors have proposed the 'ligand-induced selective signalling' theory. This concept proposes that the GnRHR may assume different conformations, according to the type of the cell in which it is expressed. Each GnRH analogue, either agonist or antagonist, can bind to the receptor in a selective way in order to activate specific, and different, intracellular signalling pathways, triggering different biological effects (Millar *et al.*, 2008). In line with these observations, it has been widely reported that GnRHRs in pituitary gonadotropes and in peripheral tissues also differ in terms of intracellular signalling pathway (Neill, 2002; Millar *et al.*, 2008).

As mentioned above, pituitary GnRHRs are associated with a Gαq/11 and with a Gαs subunits. In contrast, activation of GnRHRs in peripheral tissues (more specifically in tumour cells derived from these tissues) has been shown to be coupled to the Gαi pathway, leading to a decrease in intracellular cAMP levels. This effect is then followed by the activation of different intracellular signalling cascades, such as MAPK (p38 MAPK, ERK1/2, and JNK), phosphatidylinositol-3-kinase (PI3K), and phosphotyrosine phosphatase. The three MAPKs have been shown to mediate the antiproliferative/proapoptotic effects of GnRH analogues in several endometrial and ovarian cancer cell lines (Kang *et al.*, 2000; Gunthert *et al.*, 2002; Kim *et al.*, 2004). The PI3K/Akt signalling pathway is also a well-recognized regulator of cell growth/survival. In

ovarian cancer cells, GnRH agonists have been reported to induce apoptosis and to reduce the invasive behaviour of cancer cells by interfering with PI3K/Akt activity (Chen *et al.*, 2007). Finally, a decreased intracellular level of cAMP causes activation of protein tyrosine phosphatase, which interferes with the expression/activity of growth factors (EGF, IGF-I) and their receptors, thus silencing their specific intracellular signalling pathways (Imai *et al.*, 1996; Gunthert *et al.*, 2005; Marelli *et al.*, 2006; Limonta *et al.*, 2012).

These intracellular molecular events mediate the antiproliferative/proapoptotic activity of GnRH analogues in tumour cells, including those of the female reproductive system (Imai *et al.*, 2000; Grundker *et al.*, 2002a, b; Marelli *et al.*, 2006; So *et al.*, 2008; White *et al.*, 2008; Wu *et al.*, 2009; Limonta *et al.*, 2012) (see below).

The GnRH/GnRHR system in the endometrium

The expression of GnRH and GnRHRs in both the epithelium and the stroma of the human endometrium has been widely reported. In particular, the expression of GnRH and GnRHR mRNA in epithelial and stromal endometrial cells reveals a dynamic pattern, being significantly elevated in the secretory phase of the menstrual cycle. Moreover, both GnRH and GnRHR are expressed *in vivo* by the human endometrium throughout the menstrual cycle, with an increase during the luteal phase (Raga *et al.*, 1998). More recently, also the GnRH-II isoform of the decapeptide was shown to be dynamically expressed in endometrial cell lines with high immunoreactivity in the secretory phase (Cheon *et al.*, 2001). In line with these observations, GnRH-II agonists have been demonstrated to promote the motility of human decidual endometrial stromal cells through activation of the matrix metalloproteinases MMP-2 and MMP-5 and of the urokinase-type plasminogen activator (Chou *et al.*, 2003; Wu *et al.*, 2015). Taken together, these observations indicate that locally expressed GnRH peptides (both GnRH and GnRH-II), through the activation of their cognate receptors, may regulate the proteolytic degradation of the extracellular matrix of the endometrial stroma and the motility of decidual endometrial stromal cells, which are crucial processes for trophoblast invasion of the maternal endometrium and for embryo implantation (Wu *et al.*, 2009; Yu *et al.*, 2011).

It is well established that a precisely synchronized cross-talk between the endometrium and the embryo during the so-called 'window of implantation' is necessary for blastocyst implantation and subsequent embryo development. Through the production of several growth factors and cytokines, the endometrium and the embryo induce changes to each other to increase uterine receptivity. As mentioned above, during the luteal phase of the menstrual cycle, the human endometrium expresses high levels of both GnRH and GnRHRs, thus favouring the adhesion of the human blastocyst to the endometrial epithelial surface. In addition, the GnRH/GnRHR system is also expressed in the placenta and, specifically, after embryo-endometrial adhesion, in both cytotrophoblast and syncytiotrophoblast. In particular, the highest expression of both the peptide and its receptors is reached in the first trimester of gestation, the period in which hCG is produced by the placenta. Based on this observation, GnRH and GnRHRs have been proposed to be involved in the regulation of hCG synthesis and secretion (Casan *et al.*, 2000; Yu *et al.*, 2011). Moreover, endometrial GnRH has also been proposed to act in a paracrine way through the activation of placental GnRHRs, thus contributing to the regulation of hCG secretion. Taken together, these observations strongly support the notion that a precisely organized GnRH-based cross-talk between embryo and endometrium is strictly necessary not only for embryo implantation/early development, but also for hCG secretion to maintain progesterone production from the trophoblast during the first trimester of gestation.

These conclusions are supported by data obtained from different clinical trials. Orvieto *et al.* (2008) have investigated the differential effects of GnRH analogues (agonists versus antagonists) during ovarian stimulation on endometrial receptivity in women undergoing *in vitro* fertilization (IVF). These authors reported that women undergoing ovarian stimulation using the GnRH agonist protocol showed significantly higher endometrial thickness and a higher pregnancy rate, suggesting a better endometrial receptivity (Orvieto *et al.*, 2008). In line with these observations, a Cochrane review of clinical trials comparing the GnRH antagonist to the long protocol of GnRH agonist in assisted reproductive technology (ART) cycles pointed out a significantly lower clinical pregnancy rate and ongoing pregnancy/live birth in the antagonist groups compared with the agonist groups (Al-Inany *et al.*, 2006).

It is known that stimulation of the ovaries causes a luteal phase defect, as the corpus luteum is unable to produce sufficient progesterone. Based on the biological evidence described above, GnRH agonists have been indicated as an important luteal phase support in ART cycles. These compounds can exert a positive effect not only by maintaining the corpus luteum by LH secretion, but also by exerting an additional direct effect at the level of both the endometrium and the embryo, through activation of the locally expressed GnRH/GnRHR system.

Tesarik *et al.* (2006) reported that, in women undergoing embryo implantation after ovarian stimulation and intracytoplasmic sperm injection (ICSI), the administration of the GnRH agonist triptorelin in the luteal phase (6 days after ICSI) induced a significant improvement of implantation and live birth rates as compared with placebo. Luteal phase GnRH agonist was also associated with increased serum hCG, estradiol and progesterone levels (Tesarik *et al.*, 2006).

An additional study evaluated the effects of luteal phase administration of GnRH agonist (single dose) on pregnancy, implantation and live birth rates in patients who received a GnRH antagonist-based COH protocol. In this study, patients were subdivided in two groups: one group received progesterone and an additional dose of GnRH agonist on Day 6 after ICSI, while the second group received only progesterone. It was found that the patients in the luteal phase agonist group had significantly higher rates of implantation, clinical pregnancy rates and live birth rates, although the number of embryos transferred and their grade were similar in the two groups (Isik *et al.*, 2009).

In line with these data, Orvieto and coworkers (Orvieto *et al.*, 2015) recently described a case of a normal responder patient with repeated implantation failure who underwent the combination of the ultrashort GnRH agonist/GnRH antagonist COH protocol, followed by endometrial injury, and a natural cycle of frozen-thawed embryo transfer. After conception, the patient received daily progesterone, with an additional single injection of hCG and GnRH agonist, on the day of embryo transfer and 4 days later, respectively. This treatment procedure resulted in clinical pregnancy. The authors discuss that the efficacy of this protocol is mainly based on the ability of hCG to further improve the function of the corpus luteum together with the observation that mid-luteal GnRH agonist treatment is associated with higher pregnancy rates, possibly explained by the direct effect of the peptide on the endometrium and on the corpus luteum (Orvieto *et al.*, 2015).

These observations are in agreement with the results recently reported by a Cochrane review of 94 randomized controlled trials comparing the luteal phase support using progesterone, hCG and/or GnRH agonist supplementation, in ART cycles. The objective of this review was to determine the relative effectiveness and safety of the different treatment protocols utilized for the luteal phase support. The results of this study indicated that GnRH agonist, in addition to progesterone, during the luteal phase is associated with higher rates of live birth/ongoing pregnancy and clinical pregnancy compared with progesterone alone (van der Linden *et al.*, 2015). Similar results have been reported in different systematic reviews and meta-analyses (Oliveira *et al.*, 2010; Kyrou *et al.*, 2011; Aboulghar *et al.*, 2015). Taken together, these data support the notion that a precisely organized GnRH-based cross-talk between embryo and endometrium is strictly necessary not only for embryo implantation/early development, but also for hCG secretion to maintain progesterone production from the trophoblast during the first trimester of gestation.

On the other hand, Martins and coworkers (Martins *et al.*, 2015) could not definitely confirm these clinical observations. In their recent article, these authors report the results of a systematic review and meta-analysis aimed to identify, appraise and summarize not only the effectiveness but also the safety of adding GnRH agonist during the luteal phase for women undergoing ART protocols. The authors conclude that, to date, the evidence of a positive effect of GnRH agonist administration during the luteal phase on ongoing pregnancy is of low quality; moreover, the safety of this treatment is unclear, based on the reported adverse perinatal outcomes and congenital malformations. Additional studies are at present ongoing and their results will help to decide the relevance of adding GnRH agonist in ART/IVF protocols during the luteal phase.

The GnRH/GnRHR system in the ovary

Within the female reproductive system, the GnRH/GnRHR system has also been shown to play a role in ovarian development. The expression of GnRH and GnRHRs was first reported in rat ovarian tissue. Specifically, the expression of GnRHRs in the rat ovary depends on the cell cycle stage, being elevated in the granulosa cells from graafian follicles, in atretic follicles and in developing corpora luteal cells (Bauer-Dantoin and Jameson, 1995). In humans, GnRH and GnRHRs are highly expressed in the granulosa cells of preovulatory follicles and also in the granulosa luteal cells, but not in theca luteal cells (Choi *et al.*, 2006).

In human granulosa cells, GnRH and GnRH-II induce apoptosis, by interfering with the IGF-I/Akt signalling pathway while activating the proteolytic caspase cascade involving the initiator caspase-8 and the effector caspases 3 and 7 (Hong *et al.*, 2012). Granulosa cells, surrounding the growing oocyte, secrete a variety of growth factors that regulate oocyte growth and differentiation (Eppig, 2001). Thus, GnRH-induced apoptosis of granulosa cells may be linked not only to follicle atresia but also to poor oocyte quality; this information may be particularly relevant in triggering ovulation with GnRH and FSH during IVF.

In luteal cells, the GnRH/GnRHR system has been proposed to be involved in the processes of luteinization/luteolysis. Specifically, GnRH induces structural luteolysis in corpora lutea, through the up-regulation of molecules that are involved in the remodelling of the extracellular matrix, such as the matrix metalloproteinase MMP-2 and the membrane type 1-MMP (Cheung *et al.*, 2006; Walters *et al.*, 2008).

In conclusion, in the human ovary the GnRH/GnRHR system is expressed in a temporal- and spatial-specific way and is involved in follicular development and corpus luteum function.

Pharmacology of GnRH and GnRH analogues in human female reproduction and diseases

GnRH analogues for stimulating or to blocking the reproductive axis

The assessment of the physiological role of GnRH and its pulsatile secretion indicates that women with an ovulatory defect resulting from deficient GnRH secretion could be induced to ovulate by a pulsatile GnRH treatment. Actually, patients with hypogonadotropic hypogonadism, either women affected by Kallmann syndrome (i.e. primary GnRH deficiency) or by hypothalamic amenorrhoea (decreased GnRH release in the presence of an intact hypothalamus), generally have the highest response rates to a pulsatile regimen of GnRH administration (Belchetz *et al.*, 1978; Crowley and McArthur, 1980; Leyendecker *et al.*, 1980).

Since 1980, pulsatile GnRH has been utilized to induce ovulation in women affected by diseases due to disordered endogenous GnRH secretion, such as PCOS, late-onset congenital adrenal hyperplasia and hyperandrogenic anovulation, and resistance to clomiphene citrate stimulation (see Scheiber and Liu, 2011). However, whereas pulsatile GnRH is effective in hypothalamic HH, it is not in PCOS. Therefore, the indication of a pulsatile GnRH therapy should be accurately evaluated on the basis on the physiopathology of the infertility.

Efficient absorption of GnRH occurs after intravenous (IV), subcutaneous (SC), intramuscular (IM), nasal, or sublingual administration (Scheiber and Liu, 2011), although the IV route seems to be characterized by better pharmacokinetic data (Keller and Gerber, 1973; Reid *et al.*, 1981). IV administration of pulsatile GnRH induces efficient episodic releases of LH and FSH. On the contrary, SC administration may result in a slower sustained rise in LH and FSH without a normal pulsatile pattern (Liu and Yen, 1984); nevertheless this route can be used for specific needs (Eckert *et al.*, 1996).

The success rate of the treatment depends on the dose and route of administration of GnRH; generally, pulses of approximately 1 μg of the peptide induce, within 4 min, a significant rise in GnRH in the range of concentrations reported in the pituitary portal blood (40–2000 pg/ml) in humans (Filicori *et al.*, 1986). High rates of ovulation and pregnancy have been reported with a 60–90 min of pulse frequency (Filicori *et al.*, 1991; Blacker, 1992).

The suitability of pulsatile GnRH therapy in inducing ovulation led to the consideration of its use for IVF techniques. High doses (10 μg/pulse) of GnRH treatment have been found to induce the development of multiple (2–5) follicles in normal, eumenorrhoeic women (Martin, 1993).

GnRH agonist and GnRH antagonist can be used to stimulate or block, respectively, the pituitary-gonadal axis.

The pulsatile administration of GnRH agonist can be successfully used to induce puberty in HH patients (Raivio *et al.*, 2007). GnRH agonist also provides a useful alternative to hCG treatment for the induction of LH release (Tavaniotou *et al.*, 2002) and ovulation for IVF, although applicable only in an ovarian stimulation protocol with GnRH antagonist,

which leaves the pituitary still responsive to the agonist (Orvieto *et al.*, 2006). The advantages of using GnRH agonist reside in a more physiological LH and FSH surge (with a better oocyte quality) (Humaidan *et al.*, 2005) and in a reduced risk of ovarian hyperstimulation syndrome (OHSS), compared with hCG treatment, because of the rapid induction of luteolysis (Casper, 2015; Mittelman-Smith *et al.*, 2015).

The ability of GnRH antagonist to suppress the endogenous LH surge without an initial stimulatory effect is the main advantage of these compounds over the agonists in assisted reproduction. Because of this, GnRH antagonist treatment can be started after several days of ovarian stimulation and requires less gonadotrophin exposure, thus reducing the incidence of OHSS. The overall shorter total time of treatment and the reduced side effects ameliorate the acceptance of the GnRH antagonist treatment by patients. Whether GnRH antagonist protocols might result in slightly decreased clinical pregnancy rates compared with the GnRH agonist protocols is still a debated issue (Blumenfeld, 2001; Tan and Bukulmez, 2011).

In women undergoing IVF, GnRH agonist may also improve the luteal phase support, induced by hCG, progesterone or estrogen, when used to trigger the final oocyte maturation, to improve pregnancy, implantation and live birth rates (Youssef *et al.*, 2014; van der Linden *et al.*, 2015).

GnRH agonist treatments can be used to treat estrogen-dependent conditions such as central precocious puberty, and can be used in preparation for surgery or in women approaching menopause (Carr *et al.*, 1993; Carel and Lèger, 2008; Carel *et al.*, 2009). Possible side effects of GnRH agonist therapy are related to steroid hormone deficiency and are similar to menopausal symptoms, including, vaginal dryness and hot flushes. In addition, osteoporosis may develop under a prolonged GnRH agonist exposure. Therefore, GnRH agonist treatment should be generally limited to 6 months unless concomitant estrogen replacement therapy is administered.

For more than 30 years, GnRH agonist has been considered an effective treatment for girls (up to age 6) with central precocious puberty. GnRH agonist treatments have a positive effect on adult stature, which may be improved by concomitant exposure to growth hormone (Li *et al.*, 2014). The treatment of early puberty with GnRH agonist is safe and pubertal maturation is achieved usually within 1 year after treatment discontinuation, with no apparent abnormalities in reproductive function, although some concerns about the possible consequences of long-term treatment have been raised. Indeed, the data available so far confirm the reversibility of the hypothalamic-pituitary-ovarian axis suppression and the presence of normal menstrual cycles without altered values for bone or body composition in the majority of female patients after cessation of GnRH agonist treatment (Thornton *et al.*, 2014). Even the observed high prevalence of PCOS (Fuqua, 2013) seems to be linked to the disease itself (Ibáñez *et al.*, 2009) rather than to GnRH agonist therapy. However, further studies are needed to address these possible limitations. It is possible that GnRH antagonists are more appropriate as they do not show the flare effect and decrease gonadotrophin levels in 24–72 h.

GnRH analogues in benign gynaecological diseases

Based on their ability to bind to pituitary GnRHRs and to interfere with the activity of natural GnRH, GnRH analogues (both agonists and antagonists), the key regulators of the so-called 'reproductive hormone cascade' (Millar and Newton, 2013), have found extensive applications for the treatment of different hormone-dependent diseases. However, as mentioned above, GnRHRs are also expressed in peripheral tissues, and specifically in female reproductive tissues, where they have been found to exert autocrine/paracrine effects not only in physiological but also in pathological conditions (Harrison *et al.*, 2004; Cheung and Wong, 2008; Wu *et al.*, 2009; Yu *et al.*, 2011; Limonta *et al.*, 2012). Thus, also locally expressed GnRHRs might be considered effective molecular targets for the treatment of these pathologies.

Endometriosis is a common gynaecological condition, affecting approximately 6–10% of women in their reproductive years, and one of the main causes of infertility in women (Eskenazi and Warner, 1997). It is defined as the dissemination of endometrial tissue (glands and stroma) to ectopic sites, most commonly in the ovaries, fallopian tubes, and other tissues of the pelvic cavity (Hickey *et al.*, 2014). Endometriotic tissues are affected by the same cyclical hormonal influences as normal endometrium, and cyclical bleeding from ectopic endometrial tissue is responsible for the development of the main symptoms, such as inflammation, chronic pelvic pain, dysmenorrhoea, abnormal bleeding, dyspareunia and infertility; urinary or bowel symptoms are less commonly reported symptoms (Nnoaham *et al.*, 2011).

Despite the incidence of endometriosis in women, the pathogenic mechanisms underlying this condition are still largely unknown. Hormonal, genetic and immune factors are believed to be involved in the aetiology of endometriosis (Montgomery *et al.*, 2008; Herington *et al.*, 2011; Rahmioglu *et al.*, 2014). Recently, also some environmental factors (such as bisphenol A or phthalates) have been suggested to be involved in the development of this pathology (Buck Louis *et al.*, 2013).

Endometriosis is usually treated with surgery or with medical therapies. Surgery (laparoscopic surgery) is the first-line therapy, which is aimed at removing endometriotic tissue implants and adhesions, restoring normal anatomy, reducing disease progression and providing symptomatic relief. On the other hand, endometriosis is known to be an estrogen-dependent disease; thus, medical treatments leading to the suppression of the ovarian function represent the mainstay of long-term management for this pathology. These treatments include oral contraceptives, progestins (such as the levonorgestrel-releasing intrauterine system, LNG-IUD, or the oral progestin dienogest), and GnRH analogues (Bizzarri *et al.*, 2014; Brown and Farquhar, 2015). However, it must be underlined that the ovary is not the only source of estrogens to be targeted while treating endometriosis. It is actually well established that the aromatase enzyme is expressed in endometrial tissue and represents the main factor responsible for estrogen synthesis in ectopic endometrium (Meresman *et al.*, 2005). These locally produced estrogens promote the growth and invasion of endometrial lesions and favour the onset of pain and prostaglandin-mediated inflammation (Velasco *et al.*, 2006), and may be responsible for the progression of endometriosis during medical suppression of ovarian function (Bulun *et al.*, 2002). Based on these considerations, aromatase inhibitors (such as letrozole, anastrozole and exemestane) are often utilized for the treatment of endometriosis, usually in association with drugs that lead to the down-regulation of ovarian activity. Novel drugs are also in development to target endometriosis-associated inflammation, angiogenesis, adhesion and tissue invasion (Platteeuw and D'Hooghe, 2014; Zito *et al.*, 2014).

Among these treatment strategies, long-term administration of GnRH agonists (leuprolide, goserelin) represents a second-line therapy (after surgery), based on the effectiveness of these compounds to induce a

hypogonadal state, which deprives the existing disease of estrogen support, and amenorrhoea, which prevents new peritoneal seedlings (Platteeuw and D'Hooghe, 2014; Zito *et al.*, 2014). These synthetic peptides have been consistently proven to be effective in relieving pain in women with endometriosis (Surrey and Hornstein, 2002); moreover, their effects are readily reversible after stopping GnRH agonist administration. Importantly, in women with endometriosis undergoing assisted reproduction, 3 months of treatment with GnRH agonist has been reported to increase pregnancy rates (Brown and Farquhar, 2014). Side effects of GnRH agonist therapy are related to recurrence of pelvic pain and to steroid hormone deficiency, similar to menopausal symptoms, such as hot flashes, sweating, bone loss, vaginal dryness (see above) (Prentice, 2001). These latter side effects may be avoided by 'add-back' regimens, such as low-dose combined estrogen-progestin, progestins alone, bisphosphonate, and selective estrogen receptor (ER) modulators, such as raloxifene (Friedman and Hornstein, 1993; Mukherjee *et al.*, 1996; Surrey, 1999; Palomba *et al.*, 2002).

GnRH antagonists have also been introduced with the aim to reduce estrogen levels and, consequently, to suppress endometriosis-associated pelvic pain (Kupker *et al.*, 2002; Brown and Farquhar, 2014). As previously mentioned, in contrast to GnRH agonist, these compounds exert their effect by competing with endogenous GnRH for pituitary receptors, being devoid of the undesired initial flare effect. GnRH antagonists provide a more rapid suppression of gonadotrophin release from the pituitary gland, thus enabling shorter treatment regimens in ovarian stimulation for assisted reproduction (Huirne and Lambalk, 2001; Finas *et al.*, 2006). In a pilot study, women with endometriosis were treated with cetrorelix (3 mg) weekly for 8 weeks; all patients reported a symptom-free period and regression occurred in more than half of them (Kupker *et al.*, 2002). More recently, small organic molecules have been discovered offering the prospect of an orally active GnRH antagonist (Betz *et al.*, 2008; Pelletier *et al.*, 2008). Phase I and II clinical trials with one of these drugs, elagolix, in women with laparoscopically confirmed endometriosis have demonstrated the safety of this compound and its efficacy in partial and reversible suppression of ovarian estrogen production resulting in improvements in endometriosis-related pain; bone mineral density was found to be minimally but significantly reduced by the treatment (Diamond *et al.*, 2014). Phase III clinical trials are currently underway and elagolix may become a valuable addition to the armamentarium of pharmacological agents to treat endometriosis-related pain (Ezzati and Carr, 2015).

As underlined above, both GnRH and GnRHRs are expressed in human eutopic and ectopic endometrium where they may play a role of autocrine and paracrine regulation in endometriosis (Imai *et al.*, 1994). This suggests that GnRH analogues, in addition to their inhibitory activity on the pituitary-ovarian axis, might also exert a direct action on endometriotic cells. In line with this observation, it has been reported that GnRH agonists significantly reduce cell proliferation in endometriosis, by enhancing the apoptotic index, increasing the expression of the pro-apoptotic proteins BAX and FAS ligand, and decreasing the expression of the anti-apoptotic protein BCL-2 (Bilotas *et al.*, 2007; Khan *et al.*, 2010). Moreover, it is now well established that ectopic endometriosis lesions secrete chemotactic molecules recruiting immune cells to the peritoneal fluid and these, in turn, secrete pro-inflammatory cytokines, further stimulating the proliferation of the lesion (Lebovic *et al.*, 2001). GnRH agonists reduce inflammation and angiogenesis in endometriotic tissues by decreasing the secretion of pro-mitogenic cytokines such as interleukin-1β and the proangiogenic factor VEGF (Khan *et al.*, 2010; Nirgianakis *et al.*, 2013).

Interestingly, also the GnRH-II isoform of the decapeptide has been suggested to be involved in the molecular mechanisms underlying endometriosis development. Expression of GnRH-II was reported to be significantly reduced in eutopic and ectopic endometrium of women with endometriosis, suggesting that endogenous GnRH-II may be endowed with antiproliferative activity (Morimoto *et al.*, 2005). In line with these observations, GnRH-II has been reported to significantly inhibit endometriotic cell proliferation, to induce apoptosis and to decrease VEGF secretion (Huang *et al.*, 2013).

These data provide theoretical and experimental basis for exploring new GnRH peptide-based treatments for endometriosis.

GnRH analogues in gynaecological tumours

The molecular pathogenesis of both endometrial and ovarian cancers remains poorly understood, resulting in a limited cure rate in the treatment of advanced cases. As underlined above, it is now well established that GnRHRs are expressed in peripheral tissues of the female reproductive tract, such as the endometrium and the ovary. The observation that these receptors are also expressed in tumour cells derived from tissues where they are associated with a significant anti-cancer activity suggests that they might represent a molecular target for novel GnRH analogue-based therapeutic strategies for these pathologies (Imai *et al.*, 2000; Grundker *et al.*, 2002b; Grundker and Emons, 2003; Marelli *et al.*, 2006; So *et al.*, 2008; White *et al.*, 2008; Wu *et al.*, 2009; Limonta *et al.*, 2012).

Endometrial cancer (EC) is the most frequent gynaecological malignancy in the world (Burke *et al.*, 2014). It is a malignant neoplasm of the epithelial portion of the endometrium and it is sporadic in almost 90% of the cases (Win *et al.*, 2015). The sporadic endometrial tumourigenesis has two recognized subtypes: estrogen-associated type I carcinoma and estrogen-independent type II carcinoma (Burke *et al.*, 2014; da Silva *et al.*, 2015). Type I carcinoma is the most common type and it is believed to be related to prolonged unopposed estrogen exposure, as occurs in estrogen replacement therapy prescribed to control menopausal symptoms, chronic anovulation (polycystic ovary syndrome), use of tamoxifen (partial ER αagonist in endometrium), obesity (endogenous estrogen production via aromatization in adipose tissues), and ageing (Burke *et al.*, 2014; da Silva *et al.*, 2015). This type of EC, which expresses both estrogen and progesterone (PR) receptors, has a favourable behaviour and good response to hormonal therapies. Type II EC is estrogen-unrelated and is classified as a poorly differentiated cancer. This type of EC has a negative or weakly positivity for expression of ER and PR and usually occurs at an older age than the type I tumour (Burke *et al.*, 2014; da Silva *et al.*, 2015). Most women are diagnosed with early-stage disease and treated surgically (Burke *et al.*, 2014; Jiang *et al.*, 2015; Melamed *et al.*, 2015), while radiation therapy is the most common adjuvant treatment so far considered (Burke *et al.*, 2014). Treatments for women with advanced disease or disease recurrence include chemotherapy, endocrine therapy (Burke *et al.*, 2014), or novel targeted therapeutic strategies (angiogenesis, EGFR, HER2, mTOR inhibitors). Unfortunately, most of patients with advanced disease remain incurable.

Epithelial ovarian cancer (EOC) is the first cause of death among gynaecologic malignancies and the fifth most common cause of

cancer-related death in women (Siegel *et al.*, 2014). The risk for EOC increases with age; more than 80% of all the cases are diagnosed in post-menopausal women (Desai *et al.*, 2014). Most ovarian cancers (80%) are detected in an advanced phase, in which the tumour has already spread to different parts of the body, giving rise to metastases (as reported by Federation Internationale de Gynecologie et d'Obstetrique, Figo, Stage III-IV). Surgery and chemotherapy as well as anti-angiogenic agents (bevacizumab) represent the treatment of choice in cases of advanced stages or early stages with a high risk of recurrence (Desai *et al.*, 2014; Yoshida *et al.*, 2015). However, despite the improved responses to both standard and recently developed therapies, both EC and EOC patients very frequently relapse and succumb to disease progression. A better understanding of the molecular mechanisms underlying the development and progression of these aggressive tumours might help identifying novel molecular targets of intervention to improve the therapeutic options for advanced or relapsed diseases.

The expression and the role of the GnRH/GnRHR system in tumours of the female reproductive tract have been extensively investigated over the past 20–30 years. GnRHRs are widely expressed in both endometrial and ovarian cancer cell lines and in about 80% of the respective primary tumours (Imai *et al.*, 1994; Grundker *et al.*, 2002a). Similarly, the decapeptide GnRH is expressed in cancer cells as well as in the majority of biopsy samples of gynaecological cancers (Grundker *et al.*, 2002a). Activation of locally expressed GnRHRs significantly decreases cell proliferation and metastatic behaviour, indicating that this system behaves as a negative regulator of tumour growth. These observations strongly support the notion that these receptors might represent a molecular target for novel, GnRH-analogue based, therapeutic approaches for gynaecological tumours. Thus, extensive research has been performed to confirm this hypothesis.

GnRH agonists have been widely reported to exert a significant antiproliferative effect on both endometrial and ovarian cancer cell lines as well as on primary cell cultures (Emons *et al.*, 1993; Chatzaki *et al.*, 1996; Shibata *et al.*, 1997). In these cancer cell lines, the GnRH agonist triptorelin inhibits cell proliferation by interfering with the estradiol-induced expression of the c-*fos* protooncogene (Grundker *et al.*, 2004). The proliferation of both endometrial and ovarian cancer cells is also inhibited by GnRH antagonists (Emons *et al.*, 1993; Kleinman *et al.*, 1994), confirming that these compounds behave similarly to the agonists at the level of GnRHRs expressed in tumour cells. These *in vitro* observations were further confirmed in preclinical studies using gynaecological cancer cells xenografted into nude mice (Yano *et al.*, 1994); moreover, positive results in terms of partial remission/disease stabilization were also obtained in women with platinum-resistant ovarian cancer treated with the GnRH antagonist cetrorelix (Verschraegen *et al.*, 2003). More recently, after the discovery of the second form of GnRH, GnRH-II, in several human tissues, including those of the reproductive tract, the possible role of GnRH-II in the control of tumour growth was investigated. It was reported that the native GnRH-II as well as GnRH-II analogues could exert significant antiproliferative effects on human ovarian and endometrial cells, through the activation of the classical form (type I) of the GnRHR (Eicke *et al.*, 2006; Fister *et al.*, 2009). However, despite this consistent *in vitro* and *in vivo* evidence of a direct anti-tumour activity of GnRH analogues, their application as anti-tumour compounds in endometrial and ovarian cancers is limited to the cases in which the main goal of the treatment is the blockade of the gonadal estrogen secretion, such as in tumours where a dependence on estrogens has been demonstrated.

At present, targeted therapeutic strategies are increasingly utilized for the treatment of several types of tumours (Abou-Jawde *et al.*, 2003). GnRH derivative peptides can be used as carriers/targeting moieties for the specific delivery of chemotherapeutic drugs, based on their binding to GnRHRs expressed on cancer cells. The development of these cytotoxic hybrids was based on the higher expression of GnRHRs in tumours compared with the normal corresponding tissues. This development was then achieved by linking a traditional anticancer drug to a GnRH derivative peptide, which acts as a carrier moiety, to specifically target the drug to cancer cells. The major goal of this targeted chemotherapeutic approach is to increase the efficacy and selectivity of cytotoxic drugs, while sparing normal tissues, and decreasing the peripheral toxicity of chemotherapy (Schally and Nagy, 1999; Engel *et al.*, 2012; Limonta and Manea, 2013). These GnRH-based cytotoxic bioconjugates bind to GnRHRs on cancer cells, and are then internalized by endocytosis before being degraded in lysosomes to release the free anticancer drug that can accumulate in the nucleus and exert its cytotoxic effect.

The first examples of these compounds were developed in the laboratory of Andrew Schally in the late 1980s and they consisted of a GnRH analogue to which various chemotherapeutic drugs (D-melphalan, cyclopropane, doxorubicin, methotrexate, etc.) were attached. These compounds were found to be effective in reducing tumour growth *in vitro* and *in vivo* (Bajusz *et al.*, 1989; Janaky *et al.*, 1992). Thus, more potent cytotoxic bioconjugates were developed. At present, the most used cytotoxic hybrid contains the GnRH analogue [D-Lys^6]-GnRH which is covalently coupled to the anthracycline antibiotic doxorubicin (AEZS-108); in this hybrid, the cytotoxic agent connected to glutaric acid through an ester bond is conjugated to the ε-amino group of D-Lys^6 (Bajusz *et al.*, 1989; Nagy *et al.*, 1996; Nagy and Schally, 2005; Schally *et al.*, 2011).

AEZS-108 was shown to bind with high affinity to GnRH receptors on cancer cells, including endometrial and ovarian cancer cells, and on biopsy specimens. After binding and internalization, the GnRH-based cytotoxic analogue induces apoptosis, independent of the multidrug resistance 1 system, suggesting that the bioconjugates may overcome the mechanisms of resistance (Westphalen *et al.*, 2000). This compound was less toxic and more efficacious than doxorubicin in inhibiting the growth of the GnRH receptor-positive human endometrial and ovarian cancer cells xenotransplanted into nude mice (Grundker *et al.*, 2002b; Engel *et al.*, 2005, 2007).

Based on these preliminary *in vitro* and *in vivo* experiments, AEZS-108 was introduced into clinical trials. The first phase I trial was designed to determine the maximum tolerated dose of the compound and its pharmacokinetics (Emons *et al.*, 2010). The study included 17 women with histologically confirmed GnRHR positive gynaecological tumours for which standard curative or palliative strategies were no longer available. This study reported that the maximum tolerated dose of AESZ-108 in the absence of supportive medication was 267 mg/m^2 and recommended this dose as starting dose for further clinical trials (Emons *et al.*, 2010).

In a phase II trial, patients with advanced or recurrent endometrial cancer, expressing the GnRHR, were treated with AESZ-108 for a planned duration of six treatments at the dose suggested by the previous trial. The results obtained demonstrated a complete remission in 5% and

a partial remission in 18% of patients. Stable disease for at least 6 weeks was observed in 44%. The median time to progression was 7 months, and the median overall survival was 15 months. The most frequently adverse effects were neutropenia (12%) and leucopenia (9%) (Engel *et al.*, 2012; Emons *et al.*, 2014).

In another phase II study, the efficacy of AESZ-108 was evaluated in women with taxane-pretreated platinum-resistant GnRHR-positive ovarian cancer. The patients received up to six cycles of the GnRH-based bioconjugate at the dose of 267 mg/m^2. This study reported that 14.3% of patients had a partial remission, and 38% had disease stabilization (Engel *et al.*, 2012; Emons *et al.*, 2014). Taken together, the results of these phase II clinical trials demonstrate that AESZ-108 has significant anti-tumour activity and low toxicity in women with advanced or recurrent GnRHR positive gynaecological cancers.

In conclusion, these promising results suggest that GnRH-based cytotoxic bioconjugates will prove useful in treating GnRHR expressing gynaecological tumours, even in their latest and most aggressive phase. A schematic representation of the central versus peripheral actions of GnRH analogues in gynaecological benign (endometriosis) and malignant (endometrial and ovarian cancer) diseases is shown in Fig. 3.

GnRH analogues for fertility preservation in female patients undergoing chemotherapy

Cancer incidence is increasing in young women, together with a significant increase in the long-term survival of these patients (Siegel *et al.*, 2015). Chemotherapy and radiation therapy are still the main treatments. Chemotherapeutic drugs induce DNA alterations and oxidative damage, causing apoptotic death not only in somatic cells but also in oocytes. Moreover, radiotherapy not only induces ovarian failure, but it also affects the uterus by reducing vascularity and damaging the myometrium, leading to endometrial insufficiency; these effects result in adverse reproduction outcomes. Thus, the development of effective strategies to minimize gonadal damage and preserve ovarian function and fertility (the so called 'oncofertility') has become a major issue not only for oncologists but also for gynaecologists and endocrinologists (Waimey *et al.*, 2013; Blumenfeld *et al.*, 2014; De Vos *et al.*, 2014; Eftekhar *et al.*, 2014; Blumenfeld and Evron, 2015; Mahajan, 2015). So far, various fertility preservation methods have been established, although they still have some limitations.

Oocyte cryopreservation has been developed to preserve fertility in young women (without a partner) (Blumenfeld *et al.*, 2014; Tomasi-Cont

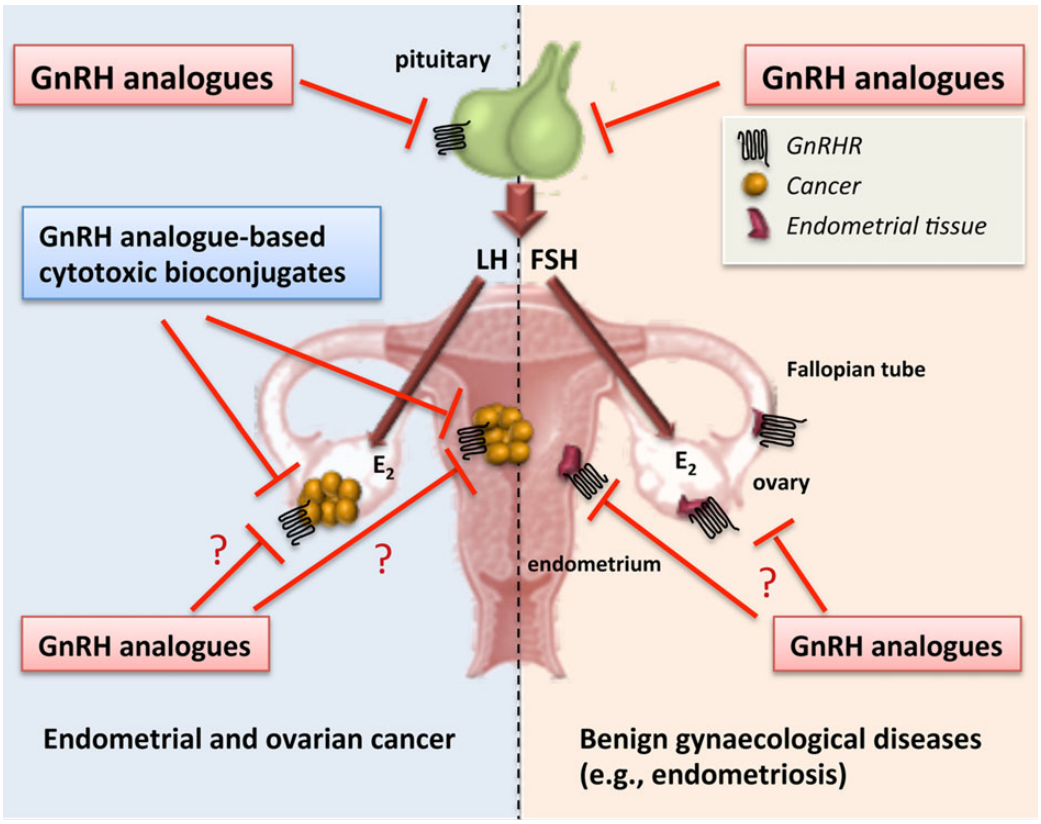

Figure 3 Schematic representation of the central and peripheral actions of GnRH analogues in benign (endometriosis) and malignant (endometrial and ovarian cancer) gynaecological diseases. Central action: classical action of GnRH analogues, agonists and antagonists, at the pituitary level. These compounds cause down-regulation of pituitary GnRHRs, thus blocking the pituitary-ovarian axis and suppressing estrogen secretion. Peripheral action: Based on *in vitro* and preclinical experimental models, it has been suggested that GnRH analogues might exert an additional and direct antiproliferative effect on benign and malignant tissues of the female reproductive tract expressing the GnRHR. Additionally GnRH analogue-based cytotoxic bioconjugates bind to GnRHRs expressed on cancer cells and are internalized to release the cytotoxic drug. The advantage of these treatments is the specific delivery of cytotoxic agents to cancer cells.

et al., 2014). However, this method does not guarantee future fertility and the opportunities for conception largely depend on the number of cryopreserved oocytes.

Another established fertility preservation method is IVF followed by cryopreservation of the embryos; this strategy is usually applied to adult female cancer patients (Linkeviciute et al., 2014; Cardozo et al., 2015). GnRH antagonists are usually utilized to minimize the risk of ovarian stimulation and GnRH agonists (e.g. triptorelin) are then used to trigger ovulation (instead of hCG) (Linkeviciute et al., 2014; Cardozo et al., 2015). This technique requires the postponement of chemotherapy for 10–14 days. Oocyte recovery rate as well as cumulative pregnancy and cumulative live birth rate per transfer are not compromised with this approach (Cardozo et al., 2015). However a major disadvantage occurs in women with estrogen receptor-positive cancers, such as breast cancer. In this case an aromatase inhibitor (e.g. letrozole) should be administered during ovarian stimulation to avoid the possible negative effects of high estrogen levels (Franco et al., 2012; Linkeviciute et al., 2014).

Ovarian tissue cryopreservation is aimed at obtaining ovarian cortical tissue that is rich in primordial follicles; this is done by either laparoscopy or laparotomy. Ovarian tissue is usually dissected into small fragments that are cryopreserved; after completion of chemotherapy, the tissue is transplanted into the pelvis (orthotopic transplant) or outside the pelvis-abdominal wall (heterotopic transplant). The first successful heterotopic technique, in terms of pregnancy, has been recently reported in a patient who had both ovaries removed because of ovarian cancer (Stern et al., 2013). The major advantage of this technique is that it does not delay the start of chemotherapy and avoids the risk of ovarian stimulation; moreover, it is the only form of fertility preservation applicable to prepubertal girls. At present, however, ovarian tissue cryopreservation is still considered as an experimental approach (Tomasi-Cont et al., 2014; Blumenfeld and Evron, 2015).

Based on these results, the pharmacological 'ferto-protective adjuvant therapy' has been introduced with the aim of minimizing the gonadotoxic effects of chemotherapy thus preventing premature ovarian failure (POF). This therapeutic approach is based on the administration of adjuvant therapy, based on a GnRH agonist, during or prior to chemotherapy (Blumenfeld and Evron, 2015; Mahajan, 2015). These compounds, by suppressing the activity of the pituitary-ovarian axis, simulate a prepubertal hormonal condition, and their application is based on the rationale that preventing premature ovarian failure is better than treating it. It must also be taken into consideration that GnRH agonists do not interfere with the efficacy of standard cancer treatments. After the initial 'flare' effect, GnRH agonists exert their classical effect by down-regulating pituitary GnRHRs, resulting in a hypo-estrogenic condition. However, additional mechanisms of action have been suggested to sustain GnRH agonist utilization in female cancer patients. GnRH agonists can: decrease ovarian perfusion secondary to the low estrogen levels; exert a direct inhibitory activity effect based on the expression of GnRHRs (associated with a specific antitumour activity) on these cells; and protect the ovarian germinative stem cells (Blumenfeld and Evron, 2015; Mahajan, 2015).

The primordial and primary follicles are gonadotrophin-independent and lack FSH receptors and antral follicles depend on gonadotrophins and secrete estrogens that, in turn, control FSH secretion. In the normal ovary, an equilibrium state exists in which developing antral follicles act in a paracrine way to keep primordial/primary follicles in a state of dormancy by secreting various growth factors, such as transforming growth factor-β (TGF-β), bone morphogenetic proteins (BMP), and growth differentiation factor-9. Exposure to chemotherapeutic drugs not only kills the developing follicles, resulting in decreased estrogen levels and increased FSH secretion, but it also disturbs this balance, thus accelerating primordial/primary follicles activation which results in the so-called 'burn-out' effect. GnRH agonists determine a decrease in FSH secretion so that the recruitment of additional primordial follicles by developing antral follicles and their burn-out is minimized (Roness et al., 2013; Blumenfeld and Evron, 2015).

Most of the studies so far performed demonstrate that treatment of female cancer patients with GnRH agonists in parallel with chemotherapy is associated with a significant decrease in premature ovarian failure and with preservation of cyclic ovarian function (Blumenfeld and Evron, 2015; Mahajan, 2015). In particular, it has been recently reported that, in gynaecological malignancies (uterine and ovarian cancer), GnRH agonists appear to protect ovarian function and preserve the ability to achieve pregnancy following chemotherapy. In a recent paper, the effects of goserelin were evaluated on ovarian failure, pregnancy outcomes, disease-free and overall survival in premenopausal women undergoing chemotherapy for breast cancer. In this study, 218 patients were evaluated. The administration of goserelin in combination with chemotherapy resulted in a higher percentage of pregnant women (21%) when compared with the chemotherapy alone group (11%); another positive effect of adding goserelin to cytotoxic therapy was the improvement of disease-free and overall survival (Moore et al., 2015). However, contradictory results have also been reported on the benefits of GnRH agonist administration for fertility preservation in young breast cancer patients (Turner et al., 2013; Vitek et al., 2014).

In conclusion, young female cancer patients facing chemotherapy should be counselled about ovarian preservation options, including the co-treatment with GnRH agonists. So far, GnRH agonists have not been approved by the Food and Drug Administration for fertility preservation; however, they may used 'off label'.

Conclusions and future perspectives

The most recent data on the regulation of GnRH release as well as on the therapeutic use of its analogues offer interesting new perspectives in this field.

First of all, the key role played by kisspeptin and KNDy neurons in the control of GnRH release suggests new lines of intervention based on the utilization of kisspeptin, Neurokinin B or dynorphin analogues to modify the activity of the GnRH pulse modulator (Skorupskaite et al., 2014). This approach could lead to a modulation, rather than a complete activation/suppression, of GnRH release, thus reducing the possible GnRH analogue-related side effects (e.g. flare effect, osteopenia).

In deed administration of exogenous kisspeptin has been proposed for the treatment of hypogonadotropic hypogonadism, delayed puberty, hypothalamic amenorrhoea and for the induction of an ovarian stimulation for IVF with a reduced risk of OHSS. Conversely, kisspeptin antagonist therapy could have a therapeutic application for the treatment of post-menopausal women, precocious puberty, PCOS, endometriosis and uterine fibroids (Celik et al., 2015). On the other hand, the efficacy of Neurokinin B administration on gonadotrophin secretion in healthy men and women has not been proven (Jayasena et al., 2014). The

assessed relationship between GnRH release and nutritional status could also have a diagnostic implication in the near future, through the evaluation of serum or tissue levels of metabolic peptide markers (leptin and ghrelin) in patients suffering from infertility and their possible use in substitution of GnRH analogues (Celik *et al.*, 2015).

A GnRH/GnRHR system is expressed in the female reproductive system, both in physiological and pathological conditions. In the endometrium, this system is involved in the embryo implantation/early development processes, as well as in the control of hCG secretion from placental cells to maintain progesterone production during the first trimester of gestation.

GnRH analogues represent a second-line therapy for endometriosis, mainly based on their ability to suppress the pituitary-ovarian axis. However, they have also been shown to exert an additional and direct inhibitory effect on endometriotic cell proliferation and angiogenic properties.

In tumours of the female reproductive system, GnRHRs are associated with a significant antiproliferative effect. At present, these receptors are considered an effective molecular target for novel therapies such as GnRH analogue-based cytotoxic bioconjugates.

Finally, there are ongoing clinical trials investigating the efficacy of GnRH agonists in the preservation of ovarian function in young female chemotherapy-treated cancer patients.

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Authors' roles

R.M. and P.L. wrote the draft of the main part of the manuscript and contributed to determining the idea, the scope, the organization and the final revision of the manuscript; A.M.C. wrote the drafts of the section on Development of GnRH neurons; M.M.M. wrote the drafts of the section on GnRH biochemistry; R.M.M. prepared the drafts of the section on GnRH analogues in tumours; V.A. acquired bibliographic data, edited and prepared the figures; M.M. acquired bibliographic data, edited and prepared tables and figures. All authors thoroughly revised and approved the manuscript before submission.

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Conflict of interest

None declared.

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