Da: "Endocrine Reviews" <em@editorialmanager.com>
Data: 12 luglio 2017 22:44:01 CEST
A: "Manuela Simoni" <manuela.simoni@unimore.it>
Oggetto: Endocrine Reviews / Proposal Decision er.2017-00175
Rispondi a: "Endocrine Reviews" <publications@endocrine.org>

CC: endoreviews@endocrine.org

07/10/2017

Dear Dr. Simoni:

Thank you for your interest in submitting a manuscript to Endocrine Reviews on "Two hormones for one receptor: Evolution, biochemistry and pathophysiology of LH and hCG". We shall be pleased to consider it for publication.
The Editors felt that your outline was somewhat slim and would want assurances that you plan to cover the role of hCG in maintenance of the corpus luteum in early pregnancy, in view of studies indicating that even with an increase in LH support the corpus luteum will last only a few days longer than normal, while hCG results in corpus luteal maintenance for a full 6-8 weeks. Also please include the role of hCG in ovarian hyperstimulation syndrome which does not occur in the absence of hCG administration, as well as a discussion of cross talk of hCG with the thyroid axis.

At your earliest convenience, let us know your planned submission date.

Note that once an author is scheduled to write a manuscript about a particular subject, we hold the topic in reserve until the manuscript is submitted and reviewed. Therefore, it is important for both scheduling and journal content that we receive your manuscript as close as possible to the time we agree upon for your submission.

The journal is flexible on the length of reviews, allowing the author to determine the most appropriate length for the topic being covered. However, as a general guideline, submitted manuscripts should be 15 to 40 double-spaced text pages in length, 6000 to 25,000 words, with about 100 to 300 references and 4 or 5 figures and tables. Exceptions to these parameters require approval by the Editor-in-Chief. The bibliography should be comprehensive for current or recent citations, but doesn't need to include all previous references. However, it is essential to cite early seminal contributions. Appropriate recent reviews should be cited whenever possible.

All manuscripts submitted to Endocrine Reviews undergo peer review by at least three referees. Nearly all undergo a revision and subsequent review by at least one referee. We encourage you to submit the names of four potential referees along with your manuscript. For more information on the review process, see the Purpose, Scope, and General Information section of the Instructions to Authors (http://edrv.endojournals.org/site/author/itoa.xhtml).

Starting in 2017, reviews will be illustrated with figures commissioned from professional illustrators and created in collaboration with authors; these figures will maintain a consistent visual style and will be ideal for use as educational slides.

Thank you for your interest in Endocrine Reviews. The Editors look forward to receiving your manuscript.

Sincerely yours,
Leonard Wartofsky, M.D.
Editor-In-Chief

Endocrine Reviews
publications@endocrine.org

Two hormones for one receptor: evolution, biochemistry, actions and pathophysiology of LH and hCG

Livio Casarini, Daniele Santi, Giulia Brigante, Manuela Simoni

Endocrine Reviews
Endocrine Society

Submitted: February 23, 2018
Accepted: June 08, 2018
First Online: June 13, 2018

Endocrine Reviews; Copyright 2018 DOI: 10.1210/er.2018-00065

Differences between LH and hCG

Two hormones for one receptor: evolution, biochemistry, actions and pathophysiology of LH and hCG

Livio Casarini[1,2], Daniele Santi[1,3], Giulia Brigante[1,3], Manuela Simoni[1,2,3]

1. Department of Biomedical, Metabolic and Neural Sciences, University of Modena and Reggio Emilia, Modena, Italy

2. Center for Genomic Research, University of Modena and Reggio Emilia, Modena, Italy

3. Unit of Endocrinology, Department of Medical Specialties, Azienda Ospedaliero-Universitaria, Modena, Italy

Received 23 February 2018. Accepted 08 June 2018.

Luteinizing hormone (LH) and chorionic gonadotropin (CG) are glycoproteins fundamental for sexual development and reproduction. Since they act on the same receptor (LHCGR), there is a general consensus that LH and hCG are equivalent. However, separate evolution of LHβ and hCGβ subunits occurred in primates, resulting in two molecules sharing ~85% identity and regulating different physiological events. Pituitary, pulsatile LH production results in a ~90 min half-life molecule targeting the gonads, to regulate gametogenesis and androgen synthesis. Trophoblast hCG, the "pregnancy hormone", exists in several isoforms and glycosylation variants with long half-lives (hours), angiogenic potential, and acts on luteinized ovarian cells as a progestational. The different molecular features of LH and hCG lead to hormone-specific LHCGR binding and intracellular signaling cascades. In ovarian cells, LH action is preferentially exerted through kinases, pERK1/2 and pAKT, resulting in irreplaceable proliferative/anti-apoptotic signals and partial agonism on progesterone production *in vitro*. In contrast, hCG displays notable cAMP/PKA-mediated steroidogenic and pro-apoptotic potential, which is masked by estrogen action *in vivo*. *In vitro* data are confirmed by large dataset from assisted reproduction, since the steroidogenic potential of hCG positively impacts on the number of retrieved oocytes, while LH impacts pregnancy rate (*per* oocyte number). Interestingly, Leydig cell *in vitro* exposure to hCG results in qualitatively similar cAMP/PKA and pERK1/2 activation as compared to LH, as well as testosterone. The supposed equivalence of LH and hCG is debunked by such data highlighting their sex-specific functions, thus deeming it an oversight caused by incomplete understanding of clinical data.

LH and hCG regulate specific physiological events. Indeed, recent *in vitro* and *in vivo* data demonstrated that LH and hCG can not be used equivalently for clinical treatments.

Essential points

- In the last decade, the two hormones LH and hCG were considered equivalent since they bind the same receptor, clearly activating the classically known cAMP/PKA steroidogenic pathway.
- Clinical evidences of small or undetectable different outcomes between LH or hCG usage underlined this concept.
- Recent *in vitro* studies demonstrated that intracellular signaling, downstream events and cell fate are specifically mediated by LH and hCG.
- LH activates preferentially ERK1/2- and AKT-dependent proliferative signals, while hCG is mainly progestinic, supporting the physiological roles of the two hormones.
- In the last twenty years, studies comparing the use of commercial LH and hCG preparations in reproductive medicine provided clinical evidence of the differences observed *in vitro*, confirming *in vitro* results.

These data indicate that LH and hCG have unreplaceable roles, overthrewing the old concept that they are equivalent and revisiting the basis on which clinicians decide the application of these hormones.

I. Introduction

Luteinizing hormone (LH) and the primate-specific chorionic gonadotropin (CG) are glycoproteins fundamental for sexual development and reproduction. Both hormones have been considered equivalent for long time, since they bind the same receptor, the LHCGR (1), which is mainly expressed in the gonads, and similarly activate the classical cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) steroidogenic pathway. In clinical practice human CG (hCG) is the hormone of choice when LH activity is needed, e.g. in the treatment of hypogonadotropic hypogonadism (HH) or in assisted reproduction technologies (ART), because it is easily purified in high concentration from urine of pregnant women. By an historical perspective, human LH of pituitary origin was difficult to obtain and lacked of full biological activity since embedding a proteolytic site leading to internally cleaved hormones, thus displaying half of the activity of the intact molecule (2,3). Human recombinant LH became only recently available for clinical use in ART, but there is insufficient experience to draw conclusions about different, specific indications for LH and hCG in clinical practice. As a result, the idea that human LH and CG (hCG) may be used indifferently remains dogmatic. However, since evolution led to the appearance of several copies of the *CGB* gene in primates, the equivalence between the two gonadotropins with "LH activity", interacting with the single receptor, needs a critical reassessment. Physiological considerations suggest that LH is unique and fundamental for gametogenesis regulation, while the evolutionary onset of CG genes might be linked to the requirement of different signals specifically supporting pregnancy in primates. Modern technologies and recent *in vitro* data allow the evaluation of multiple signaling pathways, activated by a number of LHCGR interactors (4,5). A recent report suggests that a complete picture of LH and hCG action might be missing when exclusively investigating the classical cAMP/PKA steroidogenic pathway (6). *In vivo* and *in vitro* studies using rodents or rodent-derived cells provided informative results in understanding the physiology of gonadotropins (7,8). However, since rodents do not have *CGB* genes, they do not produce CG and the murine LH receptor is not identical to the human one (88% of identity between *Rattus norvegicus* Lhr and LHCGR). Some recent evidence suggests that the human LHCGR possesses a specific region capable of distinguishing between LH and hCG (9). These studies raise the question whether hCG and LH really are equivalent and fully interchangeable in humans. In addition, since rodents are still being used to calibrate gonadotropin preparations used in medical practice (10) and the physiology of LH action was mainly obtained from murine models, another question is whether the primate-specific nature of the dual ligand system provided by LH and CG has been completely characterized. Some clinical (11) and *in vitro* (6,12) comparisons of commercial LH and hCG preparations have been performed in the last fifteen years, revealing the existence of several peculiarities but leaving many unanswered questions.

In this article we review current evidence about similar and different functions of LH and hCG.

Two ligands for one receptor. Why?

Ligands and receptors evolved along with their molecular targets, resulting in exclusive, hormone-specific regulation of pathways and physiological functions (13). In primates, the existence of LH and hCG as ligands for the same receptor suggests that a separation of hormone-specific roles occurred, resulting in different, hormone-specific physiological functions. These distinct roles may exist to fulfill different requirements for the regulation of fetal development (in females) and gametogenesis (in both sexes). These reproductive

functions are accompanied by the prevalent – or even exclusive – time- and sex-dependent presence of only one of the two hormones, pointing towards specific physiological targets reasonably regulated by different endocrine signals (6). LH- and hCG-specific actions exerted at the molecular level are underpinned by different molecular structures associated with each hormone (14), which arise from a different set of related genes (14), along with source cell-dependent post-translational modifications (15). Although heretofore considered "equivalent" both hormones divergently evolved in primates and are characterized by differences at the genetic, molecular and physiological level.

Although the specificity of LH and hCG signals is not completely elucidated, it is supported by the different nature of their target cells. In women of fertile age, LH exerts its best-known functions in the ovary, where it mediates proliferative signals in the granulosa cells co-expressing the follicle-stimulating hormone (FSH) receptor (FSHR) and LHCGR, and stimulates androgen synthesis, mainly androstenedione, in theca cells exclusively expressing LHCGR. Moreover, LH induces luteinization of granulosa cells, progesterone synthesis and *corpus luteum* maintenance during the luteal phase of the menstrual cycle. In males, Leydig cells in the testis are targeted by LH, which induces testosterone production, as the major synthesized androgen. LH is therefore essential for reproduction in both sexes. On the other hand, hCG physiological action is only exerted in females as a massive progesterone stimulator in the *corpus luteum* and mediating placental growth during pregnancy, while it is not produced in males. These considerations suggest a sex-specific role of each hormone in humans and other primates. Such roles are quite different (gametogenesis vs. pregnancy) and most likely not fully interchangeable. In fact, clinical experience shows that steroidogenesis and gametogenesis can be supported by hCG administration in both sexes, but is human (primate) pregnancy sustainable by LH in the absence of hCG? In assisted reproduction, hCG is administered to provide LH-like activity based on the androgen-stimulating potential of the molecule. However, is the action of both molecules on gamete maturation identical? Compelling results were provided by a comparison between LH and hCG administered to mouse oocytes cultures at the germinal vesicle stage *in vitro*. A greater maturation rate following hCG treatment was revealed, while LH positively impacted early embryonic development (16). These data, albeit obtained using mouse tissues, suggest that different signals are mediated by each ligand, resulting in different effects on the physiological target. This is but one example of a number of recent *in vitro* and clinical studies providing a novel, unexpected view of LH- and hCG- specific roles. In the following paragraphs we will define such different roles starting from evolutionary considerations.

II. Phylogenies and evolution of LH and hCG

Molecular structures of gonadotropins and their receptors are overall conserved during evolution and share similarities with several other ligands and receptors across the phylogenic tree. This hints at a common ancestral origin of these molecules and the promiscuity of molecular mechanisms involved in endocrine regulation. Gonadotropins are glycoprotein hormones belonging to the superfamily of cystine knot growth factors (CKGF). Members of the CKGF group share an arrangement of six disulfide-linked cysteine residues that achieve a structurally related "knot" conformation, in spite of a relatively low sequence homology (17). Glycoprotein hormones possess a common α and a specific β subunit, assembled to form a *non*-covalently linked heterodimer (18) acting on specific leucine-rich repeat (LRRs), rhodopsin-like G protein-coupled receptors (GPCR). It was estimated that the evolutionary origin of glycoprotein hormones and their receptors occurred at the origin of the metazoans: hence, they organize the regulation of a wide range of endocrine systems as well as reproductive and metabolic functions that differentiated during evolution (19). Such evolutionary issues were inferred by evaluating genetic structure of gonadotropin and their

receptors encoding genes. A number of glycoprotein hormones, i.e. gonadotropins, and receptor variants developed as an endocrine adaptation to specific environmental conditions and physiological changes required to improve reproductive success (fitness) tested by natural selection (20). As an example, fertile window length, menopausal age and high pregnancy success may be the result of species-specific, or even individual-specific, optimizations of reproduction implemented to best balance fitness and selective pressure. In primates, the appearance of choriogonadotropic hormones acting as additional ligands for one receptor suggests that different levels of regulation are required to manage gametogenesis and pregnancy.

Glycoprotein hormones across the phylogenic tree

Glycoprotein hormones and their receptors revealed common structural folds suggesting a common evolutionary origin of ligand-receptor pairs, likely resulting in similar and even promiscuous binding and signaling mechanisms. Binding specificity would be driven by the protein sequence spanning between the 10th-12th cysteine residues of the hormone, consisting of similar sequences and falling within LRR domains of their receptors (21). In insects, the only molecule related to vertebrate glycoprotein hormones is the bursicone hormone, an approximately 40 kDa protein displaying cystine knot heterodimeric structure and pro-apoptotic activity at the epidermal level (22). The analysis of the bursicone hormone-encoding DNA sequence revealed high identity with glycoprotein hormone-like peptides found in silkworm, sea urchin, jellyfish and corals, suggesting their evolutionary proximity (19). Similar homologies were found by comparing the DNA sequences encoding the hormone receptors, demonstrating that the ligand-receptor system underwent co-evolution. Interestingly, co-expression of the *LHCGR* gene is coupled to the canonical cAMP pathways in transgenic *Drosophila melanogaster* like in humans, further supporting conservation of the intracellular machinery necessary for GPCR signaling in invertebrates (23). An evolutionary step towards vertebrates may be provided by a glycoprotein hormone of adenohypophysial origin found in hagfish. This hormone consists of bound α and β subunits and the presence of their mRNAs matches the developmental stages of the gonad (24), likely providing the earliest pituitary-gonadal system in vertebrates. In basal vertebrates, such as the hagfish, unique glycoprotein hormones regulate all the cognate gonadotropin functions found in mammals, by acting on distinct receptors (fshr and lhr) mediating gametogenesis (25). This ancestral form of glycoprotein hormone, homologues of which are conserved in vertebrates and some invertebrates, is known as thyrostimulin and is capable of increasing intracellular cAMP in cell systems expressing lamprey glycoprotein hormone receptor (26). The evolutionary history of more physiologically specialized gonadotropins started about 927 million years ago, when the ancestral gene encoding the thyrostimulin β subunit repeatedly duplicated resulting in an LHβ encoding gene from which the thyroid-stimulating (TSHβ) and the follicle stimulating hormone β subunits (FSHβ) encoding genes subsequently originated (27). Further molecular specializations occurred in primates and equids, where CGβ and CGβ-like LHβ molecules respectively, developed as gonadotropins of pregnancy.

Co-evolution of glycoprotein ligands and their receptors testifies to the specificity of the glycoprotein-mediated signals demonstrated in cartilaginous fishes, providing an ancient representation of the current diversity of reptilian, avian, and mammalian endocrine systems (28). However, endocrine signal promiscuity is retained in some organisms such as zebrafish, where fsh binds both fshr and lhr, while only lh is specific for its own receptor (29). As a consequence of this incomplete functional differentiation, the endocrine control of spermatogenesis in zebrafish relies on fshr expression in both Leydig and Sertoli cells (30). Interestingly, fish gonadotropin receptors may be activated by mammal cognate ligands and *vice versa*, suggesting a limited number of specific binding residues conserved during

evolution, even in the case of hCG binding to fish lhr (31). The sharing of inter-specific binding capability between gonadotropins and their receptors is maintained across fish and mammals as a legacy of common ancestral evolution. Moving from invertebrates to primates, on the other hand, increasing complexity and specificity of glycoprotein hormones and receptors is the requisite of the refined function of the pituitary-gonadal axis: the result is an increasing endocrine specialization with species evolution.

***LHB* gene duplication: the appearance of choriogonadotropin**

LHβ and CGβ subunits are encoded by a cluster of tandem genes, located in humans on chromosome 19q13.32, which embeds a total of eight genes and pseudogenes (32). There are six transcriptionally active *CGB* genes (33) and related promoters (34). The *LHB*/*CGB* gene cluster reaches the highest complexity in *Homo sapiens*, while other primates feature a simpler organization characterized by fewer *CGB* genes. Since most of the 5% difference between humans and chimpanzees is due to genomic insertions and deletions (35), *CGB* genes putatively evolved by repeated duplications of an ancestral *LHB* gene common to all species of the primate lineage, to meet specific physiological requirements (36,37). High crossover activity putatively occurred in the human *LHB*/*CGB* cluster and resulted in sequence inversions generating palindromic genes (38). One of the most accredited theories explains the rise of *CGB* genes along with the increasing glycosylation rate of CGβ molecules as evolutionary adaptations to elevated energy demands for fetal development: the resulting highly specialized regulation of angiogenetic signals and myometrial invasion is necessary to support hemochorial placentation in humans and higher primates (15). Thus, six *CGB* genes are found in humans, four to five in great apes and three to one among *Macaca*, *Callicebus* and *Aotus* (39). The highly conserved *CGB1* and *CGB2* gene sequences in humans and great apes suggest they may have relevance for implantation and placental development in higher primates (40). Other *CGB* genes, namely *CGB*, *CGB5*, *CGB7* and *CGB8*, are recognized to be a source of hCG products during human pregnancy (41). Accordingly, simpler primates (e.g. strepsirrhine) and other mammals have only one *LHB* gene and feature epitheliochorial placentation characterized by the presence of both the uterine epithelium and the maternal vascular endothelium during pregnancy. The theory explaining the rise of *CGB* genes has recently been extended (20). Sex-specific functions, i.e. placentation, folliculogenesis and ovulation in females and spermatogenesis in males, would be driven by the expression of sex-specific genes belonging to the same genomic cluster. In this context, the *LHB* gene is relegated to maintenance of physiological functions common to both males and females, since they may need similar regulation at the intracellular level, while *CGB* genes permitted independent evolution of signals specifically required for placentation in females, as an example of sexual dimorphism. This strategy might have arisen to solve an *intralocus* sexual conflict, which occurs when traits encoded by the same *locus* lead to conflicting fitness outcomes between the two sexes (42). However, the evolution of placentation in primates might be linked to the gene encoding the glycoprotein hormone α subunit (common glycoprotein alpha, *CGA*) (43). This gene has two splice variants, one of which is only found in anthropoid primates with the exonization of an Alu sequence. The additional encoding sequence results in a N-terminal extension improving protein stability. Similarly, *CGB* genes are characterized by an additional DNA sequence compared to *LHB*. Although a certain grade of similarity is shared among genes encoding glycoprotein hormones β subunits, *CGB*s display a peculiar extension of about 90 nucleotides compatible with 30 amino acids at the C-terminal region (44). This carboxyl-terminal extension (CTP) could have originated from the loss of the stop codon occurring together with the duplication of an ancestral *LHB* gene, resulting in inclusion of the 3'-untranslated region within the protein coding sequence, which is absent in all known mammalian *LHB* genes, except equids (Table 1). The human CTP domain contains four potential O-linked glycosylation sites, is

enriched in serine, threonine and proline residues (45) and may be targeted by specific antibodies due to the immunological potential of the glycosylated sites (46).

[TABLE 1]

Classical views of LH and hCG physiology

Although the diversification of endocrine axes and the appearance of CG molecules suggest that different LH receptor-dependent intracellular functions are required to regulate gametogenesis and pregnancy, experimental and clinical observations in women and female primates failed to clearly distinguish the actions of these molecules during gametogenesis (47) and luteal regression (48). Thus, it is a widely accepted opinion that these two molecules are equivalent. However, the evaluation of gonadotropin-specific functions in human physiology points to distinct, cell-specific roles for each molecule. In ovarian granulosa cells, proliferative signals directly delivered by LH are not necessarily exerted through the synthesis of androgens, which instead are the main product of LH action in theca cells. On the other hand, hCG naturally only replaces LH in the luteal phase and beyond, during pregnancy, when steroid production is mainly limited to progesterone.

In humans, gametogenesis progresses thanks to an orchestrated regulation by FSH, LH, growth factors and steroid hormones. In women, the ovarian follicle is the functional unit deputed to oocyte maturation and growth, characterized by a dynamic structure of somatic cells surrounding the gamete (49). Folliculogenesis starts about 20 weeks after conception, when the follicular population comprises 4-7 million oocytes at the resting primordial stage. Pools of these follicles progressively mature by undergoing morphological and molecular changes, and passing through the primary and secondary, gonadotropin-independent stages. Subsequently, from the pubertal onset, monthly recruitment is guided by the expression of FSH receptor (FSHR) and LHCGR at relatively low but progressively increasing levels (50) conferring follicular sensitivity to FSH. This is the proliferative signal for granulosa cells, which start to replicate and synthesize steroid hormones, inducing antrum formation and supporting oocyte maturation. At the early follicular phase, relatively low LH levels are produced, and it is commonly accepted that these are enough to mediate androstenedione synthesis by LHCGR-expressing theca cells (51). On the other hand, relatively high levels of FSHR are exposed at the surface of granulosa cells, which express low levels of LHCGR (52) as well, suggesting a physiological role of the putative interaction described to occur between these two receptors (53) in modulating gonadotropin signals (54). If the coexistence of FSHR and LHCGR has a biological significance, it is exquisitely granulosa cell-specific, since FSHR is absent in theca cells. Most importantly, these data suggest that LH- and FSH-dependent synergetic action in granulosa cells, not provided by each gonadotropin *per se*, is required to properly guide follicular growth. At this stage, the best-known role of theca cells still is to supply androstenedione, as the substrate for estrogen production in granulosa cells, supporting follicular growth and oocyte maturation/metabolism. As pituitary FSH release and follicular FSHR expression decline, LH levels increase along with the progression towards the large antral stage, when follicular growth is gonadotropin and estrogen-dependent. However, both FSH and LH production still co-exist at this stage, with both proliferative and apoptotic signals occurring in the dominant and atretic follicles, respectively. In the late follicular phase, LHCGRs fully replace FSHRs in granulosa cells of the dominant follicle, to induce both ovulation and the changes in metabolic state necessary to luteinization, i.e. massive, exclusively LH-driven progesterone production. Therefore, while both FSHR- and LHCGR-mediated life and death signals are simultaneously present in granulosa cells, only LH-dependent signals of increasing potency are delivered to theca cells over the entire follicular phase up to ovulation. Thereafter, the second half of the menstrual cycle depends on progestational signals transitorily supported by LH in the *corpus luteum*. Only in the case of pregnancy is corpus luteum function maintained by trophoblast hCG, because LH levels

decrease as a consequence of the negative feedback exerted at the hypothalamo-pituitary level by high progesterone concentrations. Therefore, physiologically, the steroidogenic LH activity in the ovary is not naturally taken over by hCG prior to this stage. The progression of pregnancy features steadily increasing progesterone levels, which, during the first 7-10 weeks of gestation are due to the hCG action on the *corpus luteum* (55). It is well known that hCG stimulates the maternal androgen production required for fetal development and pregnancy progression (56). Moreover, it seems that both placental and adrenal androstenedione and testosterone play a role in cervical and myometrium remodeling and parturition (56), while excessively high androgen levels might compromise maternal health (57). Apart from these functions, little is known about the direct action of hCG on androgen synthesis, whereas the role of the gonadotropin is classically associated to progesterone synthesis during pregnancy.

hCG is secreted in high amounts, especially in the first trimester of pregnancy and acts as an essential, potent steroidogenic factor. Other functions have been, however, hypothesized for hCG, e.g. immunosuppressive and angiogenic functions, especially during the early weeks of pregnancy (58), as well as the capability of enhancing steroid-mediated signals by activating cAMP- and extracellular-regulated kinase 1/2 (ERK1/2)-mediated production of the progesterone receptor in endometrial cells (59). The exposure to maternal hCG is crucial for fetal sex steroid production and activation of the hypothalamus-pituitary-gonadal (HPG) axis, which impacts fertility potential in adulthood (60). After birth, maternal estrogens decline in the newborn, leading to the rise of FSH and LH characterizing the neonatal period dubbed mini-puberty. This results in a surge of pituitary gonadotropins of a magnitude only comparable to the levels obtained much later, at puberty (61).

FSH and LH signaling are fundamental for male gametogenesis as well, since these hormones act on Sertoli and Leydig cells, respectively, providing mechanical and endocrine support to sperm production. The hormonal control of spermatogenesis and, especially, its dependence on FSH or LH, are extremely species-specific among mammals (25), although testosterone is in general an essential requirement for the progression of gamete maturation. Testosterone is produced by Leydig cells upon LH stimulation and sustains Sertoli cell function and spermatogenesis progression, albeit being partially converted to 17-β-estradiol by the aromatase enzyme, promoting anti-apoptotic signals, likely together with gonadotropins (62,63). Given the steroidogenic role of Leydig cells, which indeed express LHCGR, but not FSHR, it is understandable that hCG found clinical utility in replacing LH functions. Since the use of LH for treatment of male HH is still limited, even in the era of recombinant gonadotropins, no substantial data are available to differentiate its action from that of hCG in males.

Studies focused on the metabolic fate of gonadotropins indicated that only about the 22% of hCG is excreted in the urine, while the retained hormone is resorbed and degraded mainly in the kidney and, in a lesser extent, liver, and ovary (64). In the kidney, these molecules are metabolized to β core fragments deprived of galactose, sialic acid and CTP fragment, suggesting that these modifications are required for urinary excretion. In fact, sugar moieties play a key role in establishing the circulatory half-life of LH and hCG. Routes and rates of LH/CG distribution and elimination were compared in rats and piglets, revealing that high quantity of radio-labelled porcine LH (pLH) is accumulated in the kidneys within 10 min from injection, while eCG plasma concentration is 80% after 1 h and the hormone is not accumulated in any organ (65). Taking together, these data indicate that LH is eliminated from serum by renal trapping, resulting in rapid removal compared to CG. Other, minor routes for gonadotropin elimination may be found in the liver, through binding of sulfated oligosaccharides to a specific receptor (S4GGnM) expressed in the Kupffer cells (66). Gonadotropins are subsequently processed in the kidney to be excreted with urine as residual, highly similar LHβ and hCGβ core structures, identical to the original pituitary and

trophoblast core molecules (67). This is due to the relatively high stability of the gonadotropin structure, which is nearly identical among LH and CG molecules of humans and other primates (68), suggesting that urinary excretion of highly similar gonadotropin core metabolites might be evolutionarily conserved.

In summary, LH and hCG are involved in the regulation of multiple physiological functions, but their specificity is underrated due to their action through the same receptor and clinical experience derived from the use of readily available hCG only in the treatment of HH and in controlled ovarian stimulation (COS) for ART. In the latter case, gametogenesis may be clinically supported by administration of exogenous FSH and hCG, thereby at least partially replacing LH action. However, clinical data did not provide representative models for understanding LH- and hCG-specific functions *in vivo* so far. For instance, stimulation of multiple oocyte production in the clinical setting of COS is far from replicating the natural oocyte selection, since it results in multifollicular development in a mono-ovulatory species. This effect is due to the pharmacological gonadotropin dosages, which do not necessarily elicit physiological patterns of estrogen production and oocyte selection.

Evolutionary convergence: trophoblast LH and pituitary CGs

The endocrine adaptation to pregnancy results in different, interesting evolutionary strategies exhibited across the phylogenic tree of mammals. The analysis of the *lhb* genomic *locus* of several species suggests that the CTP fragment might be produced by a number of organisms by frameshift of the gene transcription (69). Data suggest that the concept relating the CTP fragment with placentation of primates may be extended to all mammals (70), which share the potential to produce glycoproteins bearing the CTP peptide which likely possesses the key characteristics of hCG. A proof of concept may be provided by the bovine, which produces a pregnancy LHβ variant featuring a CTP fragment. This molecule is produced by decryption of the 3' region of the *lhb* gene, resulting in a glycoprotein hormone which is however poorly O-glycosylated and displays lower half-life compared to hCG, not supporting the evolution of CG molecules in bovines (71).

A placental gonadotropin was described in equids long ago (72,73). This hormone is known as equine choriogonadotropin (eCG), suggesting a similitude to CG molecules of primates. However, both β subunits of equid pituitary LH (eLH) and trophoblast eCG are products of the same *lhb* gene. They differ in source of production and N-linked glycosylation, which is higher for eCG than eLH, while O-linked glycosylation is crucial for both hormones to maintain binding activity (74). Moreover, both eCG and eLH demonstrated binding capability for fshr (75), which results even higher for eLH than eCG (76), suggesting a role in mediating FSH-like signals. In fact, eCG capability of promiscuous fshr activation was described in most mammals (77,78), while, however, could be negligible in horses (79). These gonadotropins consist of glycosylation variants providing a case of evolutionary convergence between equids and primates: different strategies are adopted to support the same physiological process.

Interesting data were provided by studies of primates, which seem to be an evolutionary counterpart to bovines and equids when considering LH and CG. Two decades ago, LH bioactivity in the New World marmoset monkey *Challithrix jacchus* was demonstrated to be produced by a pituitary choriogonadotropin (mCG) sharing about 80% identity with hCG (80). mCG displays multiple activities regulating development, gametogenesis and pregnancy, likely due to a glycosylation pattern similar to human LH and a CTP structure similar to hCG. On the other hand, the marmoset monkey LHCGR lacks the amino acid sequence encoded by exon 10 of the gene, which corresponds to an extracellular portion of the receptor (81). Although the molecular structures of gonadotropin and their receptors will be detailed in the next chapter, the presence of a CG molecule and the lack of exon 10-encoded sequence in *C. jacchus* LHCGR (also known as LHCGR type II) in the

entire New World monkey lineage, provide interesting information about the LH/CG-receptor functioning, which reaches its maximum complexity in primates (82). Indeed, a previous study suggested that the CTP fragment is essential to induce LHCGR type II activation (83). In light of these studies, an interesting finding described an 18-year old patient with Leydig cell hypoplasia characterized by the absence of exon 10-encoded portion of the LHCGR, who was unresponsive to endogenous LH (84). As a consequence, this boy had relatively high serum LH and very low testosterone levels, delayed pubertal development and small testicles, indicating deficit of the LH signal. Surprisingly, testosterone biosynthesis and spermatogenesis were recovered by hCG treatment. hCG also induced cAMP production during functional analysis *in vitro* assessed in exon 10-deficient *LHCGR*-transfected cells, which LH failed to stimulate despite binding to receptor (85). This clinical case confirmed the importance of the amino acid region encoded by the exon 10 of *LHCGR* to discriminate between the two natural ligands, supporting the concept of co-evolution of the ligand-receptor structure as a strategy to regulate gametogenesis and placentation in primates (Figure 1).

[FIGURE 1]

While it might be intuitive to find the structure-function relationship of placental gonadotropins, the role of hCG and hCGβ molecules found in the human pituitary (86) is still unknown. They were detected in the serum of both men (87) and women (88), and would be released in a pulsatile fashion. It was postulated that pituitary hCG molecules might play a role in the regulation of the menstrual cycle (89) and ovarian pathogenesis (90), but further evidence is needed to support this issue.

III. Different sources, molecular structures and biochemical properties

The genetic differences of LH and hCG reflect gonadotropin-specific molecular structures, post-translational modifications and biochemical properties at least partially established in the secretory pathway, involving endoplasmic reticulum and Golgi apparatus of the source cell (91). The secretion of LH by gonadotrope cells of the anterior pituitary is controlled by the gonadotropin-releasing hormone (GnRH) (92), a peptide produced by the hypothalamus, under kisspeptin regulation (93), in a pulsatile fashion and released into the portal bloodstream. GnRH binds its seven-transmembrane receptor (GnRHR) expressed in gonadotrope cells, mainly activating phospholipase C (PLC), ERK1/2-, β-catenin, calmodulin and PKA-dependent signaling (94,95). The preferential LH or FSH synthesis depends on the frequency of GnRH pulses: low frequencies are linked to FSH production, while higher frequencies are synchronized with waves of LH synthesis (96). In women, the preovulatory stage of the menstrual cycle is characterized by a GnRH surge corresponding to the LH increase inducing ovulation. Given the dependence of gonadotropin production on kisspeptin and GnRH, a new model of ovarian physiology was proposed, where follicle maturation and selection, ovulation and luteal phase occur under the strict control of the neuroendocrine system (97). In fact, the relationship between gonadal and pituitary functions was shown by experiments in Lhr knockout (LuRKO) and ovariectomized mice (98). In both these models, high expression of gonadotropin subunit genes occurred, reflecting morphological changes in gonadotrope cells, which display secretory granules larger than in wild-type mice. In contrast, in GnRH-deficient mice expressing low gonadotropin mRNA and protein levels, gonadotrope cells were smaller and featured fewer secretory granules. Interestingly, a variety of LHβ molecules may be produced in humans, even if not completely functionally characterized yet. They differ in glycosylation patterns, resulting in specific molecular weights (99).

The secretion of hCG by trophoblast cells occurs in a *non*-pulsatile, increasing manner, reaching the peak around the first trimester of pregnancy, and is not coupled to GnRH production. A wide variety of hCG isoforms and glycosylation variants are produced

during this period, promoting trophoblast invasion of maternal decidua. Most interestingly, and in contrast to LH, hCG production is not subjected to immediate down-regulation by steroid hormones. It could be speculated that steroidogenic hCG-mediated signals are constantly delivered during pregnancy as a requirement to maintain proper progesterone production, while the different nature of LH is aimed at more transitory events, such as luteinization, which need not be prolonged. A suggestive explanation of the molecular mechanism underlying constant hCG activity may be provided by the discovery of an about 50 kDa-truncated form of LHCGR, specifically expressed by placenta and choriocarcinoma cells, the presence of which is concomitant to the absence of hCG down-regulation (100). The presence of this truncated receptor would be opposed to the appraisal of full-length (about 90 kDa) LHCGR at the term-placenta, which suggests the existence of a feedback mechanism regulating hCG action *via* receptor downregulation. While these findings should be independently confirmed, it is known that hCG production is constant but undergoes qualitative dynamic changes over pregnancy. It has been suggested that the pattern and abundance of hCG molecules is individual-specific, thus, choriogonadotropin acts as a dynamic, autocrine factor which changes qualitatively throughout the first trimester of pregnancy (101). hCG isoforms consist of different polypeptide products selectively transcribed by *CGB* genes (102), while the oligosaccharide structures differentially linked to the hCG backbone determine the glycosylation variants and would depend on the enzymatic *milieu* of trophoblast cells, which differs from that of pituitary gonadotrope cells (103).

The effects of oligosaccharide structures, especially O-linked, are apparent in the serum half-life differences between the two hormones, which is of 90 minutes for LH and 34 hours for hCG (104). In particular, O-linked glycosylation is abundant in the CTP fragment of hCG and could be important to determine hormone-specific physiological roles, providing a strategy for developing chimeric gonadotropins with slow metabolic clearance (105). In gonadotrope cells, gonadotropin glycosylation is modulated by a number of hypothalamic and gonadal endocrine factors, such as estrogens and androgens (106). These steroids may also impact on sialylation and sulfation of the oligosaccharide, further modulating half-life and biopotency, thus extending the qualitative range of signals delivered to the gonads. On the other hand, sulfation of oligosaccharides is critical for hormone half-life and bioactivity. Indeed, this post-translational modification is evolutionarily preserved among glycoprotein α subunits, from teleost fishes to mammals (107). Finally, it is reasonable, albeit speculative, that the pulsatile release of a short half-lived LH would provide a fine-tuned stimulus optimized to regulate life signals mediating follicle growth, while hCG molecules trigger the relatively potent stimulus for sustained and prolonged progesterone synthesis and angiogenesis. In summary, two different gonadotropins, LH and hCG, should be recognized at the receptor level in order to reach a finer level of regulation, not allowed by a unique ligand, mediating specific intracellular signals required to optimize reproduction and development.

Production of alpha and beta subunits

Production of glycoprotein α subunit is a rate-limiting step for gonadotropin heterodimer formation. The α subunit, encoded by the 9.4 Kb *CGA* gene (108), transcripts of which are found in pituitary and placenta, serves for glycoprotein hormone heterodimer assembly occurring in the endoplasmic reticulum of source cell (109). Both hCGα and β subunits are characterized by three loops defined by a cysteine knot and experimental evidence proved the importance of the second loop of the α subunit in dimer formation. These results were obtained using chimeric molecules, where the second loops of hCGα and β subunits were swapped, resulting in β-β homodimers capable of receptor binding and activation of signal transduction, albeit at lower levels than wild-type hCG, and inactive α-α homodimers (110). The knowledge of how the formation of glycoprotein hormone

heterodimers occurs was improved by several attempts to develop the crystal structure of hCG over a five-year period from 1989 to 1994 (111–113). These models revealed interesting features of the molecule, mostly the seat belt-like structure composed of a segment of the β subunit wrapping around the α subunit loop αL2 linked by a disulfide bridge between two cysteine residues at positions 26 and 110 of the polypeptide chain. However, a complete view of the tridimensional dimer structure was strongly hampered by the oligosaccharide structures bound to hCG. Again, experiments using mutant α subunits lacking the disulfide bonds between cysteine residues at positions 7-31 and 59-87 provided a further step in understanding the procedure of gonadotropin heterodimer formation (114). Disruption of these structures did not significantly affect hCG or FSH heterodimer formation, while it negatively impacted LH heterodimerization in transfected Chinese hamster ovary (CHO) cells and in the rat pituitary tumor cell line, GH3. On the other hand, N-linked glycans at position 13 and 30 are required for the efficient hCGβ folding and formation of disulfide bonds between the residues at position 23-72, 93-100, and 26-110 (115). These data suggest that regions recognized by the α subunit for assembly are different in LHβ and hCGβ.

New insights on formation of LH and hCG dimers were provided by recent advancements allowing the evaluation of molecular interactions by means of a bioinformatic approach. This *in silico* analysis revealed that dimer assembly might follow different β subunit-specific modes, depending on the presence or absence of the hydrophobic tail of LHβ and hCGβ, respectively (116). The LHβ subunit is not completely folded when docking with the α subunit occurs. In this case, the α subunit acts as a scaffold using the cystine knot to enhance β-subunit cystine knot formation. The heterodimer is then stabilized by the interaction between loop 2 of the α subunit and the β subunit hydrophobic tail, which form the seat belt-like structure. This LH-specific mode of dimer formation was named “wraparound” and differs from the assembly of the hCGα-β dimer, which was named “threading”, in order to explain the mechanism by which the α subunit passes between a β subunit folded before the docking. An alternative mechanism, similar to the “wraparound” model, also was proposed for hCGα-β dimer formation (117,118). However, these data suggest that different heterodimer assembly may have arisen as a strategy to control the production of the two hormones. In fact, while hCG assembly is efficiently performed, *in vitro* experiments using chimeric gonadotropins revealed that LH assembly and secretion is less vigorous (119). Faster rate of hCG than LH secretion presumably reflects their massively constitutive and GnRH-regulated secretory pathways, respectively. The CTP fragment of hCG plays a role in determining heterodimer formation, since CTP-truncated forms of the chorionic hormone resulted in a 60% decreased efficiency of dimerization. Interestingly, removal of the LHβ C-terminal octapeptide increased the rate of hormone secretion by transfected CHO cells, confirming the relevance of the carboxy-terminal region of LH and hCG for their physiology.

Previous attempts to produce recombinant LHβ and hCGβ in transfected GH3 cells *in vitro* revealed the role of the N-terminal region in the disulfide-linked aggregation of LHβ subunits, whereas hCGβ was exclusively secreted as a monomeric molecule. Further investigation using mutant gonadotropins demonstrated that N-glycosylation on the asparagine at position 13, which LHβ lacks, prevents hCGβ aggregation in culture medium (120). These data indicate that glycosylation in the N-terminal region of the two gonadotropins plays a role in the maintenance of the correct folding and secretion in the absence of the α subunit (Figure 2). Most importantly, this finding may explain the measurable presence of free hCGβ subunits during pregnancy, when high activity of *CGB* genes leads to massive protein production, while free LHβ subunits are relatively rare (121).

[FIGURE 2]

The LHβ subunit

LHB gene transcription is stimulated upon GnRH binding to its receptor, activating Gq/11 proteins and stimulating phospholipase C to mediate inositol 1,4,5-triphosphate and diacylglycerol pathways. These intracellular messengers lead to protein kinase C (PKC) activation, intracellular calcium increase and β-catenin signaling regulating the expression of the *LHB* gene. The latter is under the control of GATA and chicken ovalbumin upstream promoter-transcription factors (COUP-TFI and COUP-TFII) (122,123), whereas the secretion of LH is regulated by the increase in intracellular calcium (124–126). The LHβ monomer was described several decades ago (127). It is an about 22 kDa glycoprotein of 121 amino acids subjected to post-translational modifications, which provide more than fifteen variants of the hormone, with molecular weights spanning from 11 to 24 and featuring specific bioactivity (99). The core fragment is structurally close to that of hCGβ except for the absence of the CTP fragment. Noteworthy, nomenclature of the amino acid position within LHβ and hCGβ subunits originated from these first sequence determinations (127–129), which excluded the 20-amino acid long signal peptide. A new nomenclature including the length of signal peptide was proposed later (130) and is used to indicate amino acid positions in this article.

Glycosylation, sulfonation and sialylation are the main LH modifications, which occur at different rates during the ovarian cycle (131) and with age (132). In particular, the LHβ subunit classically displays one N-glycosylation site at position 50 of the polypeptide chain, while two additional oligosaccharide structures are linked to the α subunit (133). LH heterogeneity consists of a prevalent glycoform carrying three oligosaccharide structures, except during mid-cycle when di-glycosylated LH is the main LH variant produced. Moreover, acidic forms of LH molecules increase with age and are mostly present in elderly women. Since the removal of these oligosaccharide structures by endoglycosidase treatment did not substantially change the LH steroidogenic activity, their functional significance may be related to other aspects of physiology (134). Oligosaccharides may be sulfonated or sialylated, resulting in different half-lives of the glycoprotein. Sulfated oligosaccharides consist of branches terminating in SO4-4GalNAc β 1,4, while sialylated oligosaccharides consist of a number of different structures featuring two or three branches and one to three sialic acid moieties (135). LH molecules with two or three sulfonated N-acetylgalactosamine (SO(3)-GalNAc) residues show shorter half-lives than less sulfonated LH, suggesting their rapid removal by hepatic Kupffer cells, whereas higher-sialylated gonadotropin isoforms have extended half-lives, likely due to the masking of the sulfonated oligosaccharides to S4GGnM binding and sequestration (136,137). However, rather than SO(3), sialic acid may be linked to LH GalNAc residues, likely resulting in extended half-life anyway (138).

hCGβ isoforms and glycosylation variants

In humans, a number of CGβ isoforms and variants are provided by transcription of *CGB* genes and different patterns of glycosylation, respectively. These types of hCG are known as "classical" hCG, hyper- and hypoglycosylated hCG, nicked isoforms and hCG lacking the CTP fragment, core fragments and free β subunits. These molecules represent a palette of multiple hCGβs differentially detectable as urinary products by specific immunoassays (139,140).

Although in-depth functional characterizations of CGβ isoforms *in vitro* are missing, the expression pattern of *CGB* genes might be a determinant of the status of pregnancy or miscarriage. Although the regulation of *CGB* gene transcription is unclear, it is likely under the control of growth factors, cytokines, ligands of the nuclear peroxisome proliferator-activated receptor γ (PPARγ) and steroid hormones, acting through the activation of cAMP-mediated signals (141). In normal pregnancies, all *CGB* transcripts were found, especially *CGB*, *CGB5*, *CGB7* and *CGB8*, which achieve a 1000- to 10000-fold higher expression levels than *CGB1* and *CGB2* genes (142). Ectopic pregnancy is characterized by perturbation of *CGB* expression patterns, in this case featuring a relatively high amount of *CGB1* and *CGB2*

transcripts, while globally reduced *CGB* expression has been associated with miscarriage. Interestingly, relatively high amounts of *CGB1* and *CGB2* gene transcripts were found in the testis of healthy males, suggesting that they may play a still unknown role in male reproduction (142).

Glycosylation is the post-translational modification providing more than a hundred hCGβ variants, depending on the combination of glycans specifically elaborated by the source cell, and attaching two potential N- and four potential O-glycosylation sites in hCGβ (143). Indeed, hCGβ isoforms possessing only one N-glycan are known and only three out of four O-glycosylation sites are typically decorated with carbohydrate (144,145). Moreover, up to fourteen glycoforms of the α subunit wre found, differing by sialylation, oxidation and N-terminal truncation (146). Since hCG as well as LH are administered for infertility treatment as a mixture of different glycoforms resulting from the manufacturing processes and potentially featuring specific biochemical properties and bioactivities, the production of homogeneous hormone samples by chemical synthesis should be of great interest for clinical applications (147). Interestingly, during pregnancy of aneuploid fetuses, the profile of hCG glycoforms is different from that detectable in pregnancy of normal karyotyped fetuses (148). Although fully informative characterizations of these hCG glycoforms *in vitro* are missing, it was suggested that they are related to specific biological activities and functions, mainly angiogenic (149), *in vivo* (150). These conclusions were driven by experiments evaluating cell growth and migration *in vitro* mediated by hyperglycosylated hCG molecules (hCG-H) (151), predominantly produced during the early stages of pregnancy by extravillous cytotrophoblasts or by choriocarcinoma, and consistent with the positive regulation of early trophoblast invasion (152). hCG-H would have lower steroidogenic and higher proliferative potential than the “classical” form of hCG produced later (153). It should therefore be secreted during the very early days of pregnancy as an essential mediator of cell proliferation and maternal tissue invasion by fetal cells. In fact, insufficient hCG-H during the first days of pregnancy might be predictive of miscarriage (154), highlighting the fundamental role of this molecule for placentation and embryo development. Similar conclusions were drawn by analyzing the sera of women undergoing recurrent miscarriage, where anti-trophoblast antibodies inhibited the hCG-H release by the JEG-3 cell line *in vitro* (155). Interestingly, alternative, "hyperbranched" glycoforms linked to both α and β subunits are predominantly produced during aberrant pregnancy and by choriocarcinoma rather than in normal pregnancy, suggesting that the activity of Golgi processing enzymes takes place differently in malignancy (156). Hyperbranching may reflect the presence of free α and β subunits, which dimer formation is impaired by largely tri- and tetra-antennary glycans in contrast to those biantennary associated with classic hCG (157). The angiogenetic potential of hyperglycosylated hCGs is a crucial issue for pregnancy success, since a proper blood flow is required to avoid embryo hypoxia. This role may be exerted *via* activation of cAMP-responsive elements located in the promoter region of vascular endothelial growth factor-encoding genes (158). Interestingly, it has been proposed that angiogenic functions and trophoblast invasion of hCG-H could rely on growth factor-like activities putatively mediated through interaction between the hormone and the transforming growth factor β (TGFβ) receptor II (Tβ-RII), independently from the classical hCG signaling (159) (Table 2). A similar mechanism of action was suggested to explain tumorigenicity of free hCGβ subunits in *BRCA1* gene-defective breast cancer (160). This is a suggestive hypothesis requiring further confirmation before being accepted, since an independent study revealed the opposite findings and suggested that experimental biases, such as TGFβ contaminations in the hCG preparations, may have affected the results (161). While the molecular mechanism underlying the proliferative potential of hCG-H is still unclear, it has been confirmed that these molecules are secreted by several tumor cells, even in the male (162), likely as a glycosylated

product occurring after metabolic reprogramming of tumor cells (163). These data suggest that hCG-H may be a tumor marker and promoter (164).

[TABLE 2]

LH and hCG binding to the human LHCGR: binding differences to *non*-human receptors

Human gonadotropins display binding capability to *non*-human receptors. While indicative of overall structure preservation of these ligands and receptors during evolution, this is also relevant for clinical treatment of human infertility. LH and hCG dosage for clinical purpose is established by evaluating their biological activity against a standard using animal models not expressing the human receptor (165), suggesting some limits to their translation to the human and usage in clinical practice. The first evidence of LH and hCG receptor-binding was provided several decades ago, by *in vitro* studies using rat Leydig cells. These experiments found similar sets of binding sites and binding capacity for both hormones to the rat Lhr, evaluated by equilibrium association constants (Ka; hCG Ka = 7.6×10^{-10} M; LH Ka = 2.5×10^{-10} M) and association rate constants (K_1; hCG $K_1 = 3.4 \times 10^8$ M/min; LH K_1 4.0×10^8 M/min) (12). However, higher binding affinity was suggested for hCG than LH, due to its longer persistence at the receptor level, and higher half-time of bound hormone (hCG = 25.0 h; LH = 9.2 h), thereby resulting in a reduced dissociation rate for hCG. These results suggest that the two hormones interact differently with the receptor, as a consequence of distinct amino acid sequences. Interestingly, it was observed that maximal hCG-mediated cAMP increase occurred at relatively low (<1%) Lhr occupancy in rat Leydig cells, adumbrating the existence of "spare" Lhr (166). This concept arose by evaluating the discrepancy between hCG binding and dose-response curves calculated for cAMP, which appeared to be left-shifted, and it has been recently used to explain the putative, steroidogenic activity exerted by LH during the antral phase of folliculogenesis (167,168). The putative existence of "spare" LHCGRs in the human ovary may be explained by the requirement for sustained androgen synthesis by theca cells, avoiding receptor down-regulation by LH. If so, then hCG must show a different action at the receptor level, during pregnancy, when the hormone is massively produced and constantly stimulates progesterone synthesis, somehow avoiding LHCGR downregulation. Although suggestive, these are largely conjectures, since the existence of "spare" LHCGRs was only suggested *in vitro*. Since hCG is not the natural ligand of rat Lhr, the parallelism between *in vitro* findings using rat Leydig cells and the physiology of the human ovary should be interpreted cautiously (169). On the other hand, LH-like signals may be driven by FSH through FSHR-LHCGR heterodimerization in granulosa cells (170), which should be favored by the about 1:100 ratio between LHCGR and FSHR amounts at the early antral stage (52). Moreover, ligand affinity to rat and human receptor might not be similar, as suggested by hCG and bovine LH, which both exhibit similar affinities for the rat Lhr, but different binding affinities for LHCGR (1000-10000 fold better for hCG than bovine LH) (171). These differences are due to an isoleucine residue falling within the C-terminal end of LRR2 of LHCGR, comparable to a serine residue in LRR2 of the rat Lhr, which would determine the LH-specific binding affinity (172). *In vitro* comparisons of human LH, hCG and some of their *non*-human, rat, equine, bovine, ovine, and porcine counterparts, in the mouse tumor Leydig MLTC1 cell line, revealed molecule-specific control of adenylate cyclase activity, raising the question of whether hCG may be a reliable reference ligand in *non*-human LH receptor-expressing systems (173). These data suggest that Lhr and LHCGR might not be comparable and mediate receptor-specific responses. While the assumption of "spare" ovarian LHCGRs should at least be confirmed by binding experiments using radio-labelled LH and cells expressing the human LHCGR, it is clear that most of our knowledge of LH and hCG binding has been provided by experiments using cell models expressing *non*-human receptors, relying on the intra- and inter-species promiscuity between gonadotropins and their receptors but ignoring their differences. For

instance, conformational changes of LH and hCG occurring upon receptor binding were first demonstrated using rat gonadal cells (174). The importance of specific contact sites, such as the intercysteine loop sequence of both LHβ and hCGβ, was likewise demonstrated, revealing that the amino acid region 58-77 of these subunits is exposed on the surface of the molecule and participates in rat Lhr binding (175), along with Lysine residues at position 22 of hCGβ and 124 of both gonadotropins (176). However, it is worth of noting that the hCGα subunit directly interacts with the LHCGR extracellular domain (177), participating in hormone-receptor binding, and it is not to be excluded that a similar interaction occurs upon LH binding.

Informative results regarding LH and hCG binding affinity for LHCGR were provided by experiments evaluating the displacement of radio-labelled hCG by increasing molar concentrations of the hormones incubated together with membrane lysates of LHCGR-transfected COS-7 cells (85). LH displayed an about 8-fold higher, albeit not significantly different, half-maximal inhibitory concentration *vs.* hCG (IC_{50}; hCG IC_{50} = 1.7 pM; LH IC_{50} = 13.0 pM), demonstrating a quite similar binding affinity for LHCGR between the two gonadotropins. In any case, these experiments should be repeated evaluating the displacement of radio-labeled LH to draw definitive and clear-cut conclusions about the binding features of both molecules. On the other hand, most of the current knowledge depicting the interaction between LHCGR and its ligands was provided by classical experiments using mutated human receptor or chimeric hCG. It is known that hormone β subunits contact specific amino acid residues of LHCGR LRRs β-strands, especially 3 and 6 (178), which play a role in the formation of a “sled-like” tridimensional structure typical of the gonadotropin-receptor extracellular domain, as well as in hormone binding and activity (179). Analysis of the chimeric hCG β-β dimer confirmed the need of this subunit for receptor binding, but, surprisingly, this dimer bound two receptor molecules with three-fold lower affinity than classical hCG and failed to elicit any cAMP response (180). These results demonstrate that the α subunit is involved in LHCGR binding and activity. In fact, experiments using an hCG analog, obtained by fusing the C-terminus of the α subunit and the N-terminus of the β subunit through a CTP fragment, confirmed that both the N- and the C-terminal portions are involved in receptor binding and activation (181), while the seatbelt-like structure of hCG is only minimally involved in LHCGR binding (182). Finally, both the α and β subunits of hCG possess the first three β-hairpin loops, structurally similar to those of the tumor necrosis factor (TNF) and the nerve growth factor (NGF), not involved in receptor binding (183). It is remarkable that the corresponding structures of TNF and NGF were instead crucial for interacting with their respective receptors.

Interesting results were provided by mutagenesis of LHβ and hCGβ, revealing hormone-specific biochemical features embedded in the polypeptide structure of the two molecules. The glutamine residue at position 74 of the β subunit plays a key role in dimer formation. Substitution of this glutamine with a basic amino acid residue, arginine or lysine, resulted in subunit association decreasing to less than 20% compared to wild-type and formation of inactive, mutated LH and hCG dimers which failed to induce progesterone synthesis in the MA-10 cell line. Interestingly, neutral (alanine) or acidic (glutamic acid) residue substitution at position 74 resulted in mildly (50-60%) decreased subunit association and lack of mutant LH binding, while mutant hCG retained full activity (184). While the amino acid residue at position 74 is crucial for both LH and hCG heterodimer formation, structural characteristics intrinsic to the protein chains result in functional differentiation between LH and hCG in spite of similar sequences. Most importantly, these data suggest that the two gonadotropins may bind the receptor differently, but further mutagenesis experiments would be needed to fully clarify this issue. Overall, they seem to interact similarly with the LRRs domain (185), while a second, less known binding site falling within position 285-354 and belonging to the hinge

region of LHCGR may be involved in hormone-specific contacts. Advancements were provided by means of a bioinformatic approach evaluating the LH- and hCG-specific interaction with the hinge region. In this case, an extroflexion consisting of a sulfated tyrosine located at position 331 (sTyr331) would play a key role in discriminating between the two hormones, relying on a specific spatial conformation of the receptor hinge region (Figure 3). The protein segment carrying the sTyr331 features a "U-shaped" structure displaying proximity between the amino acid sequence encoded by the *LHCGR* exon 10 and an adjacent helix. Both LHβ and hCGβ first bind the LRRs domain of the receptor. However, while spatial occupancy provided by hCG binding to LRRs contributes to the LHCGR conformational change by contacting the whole "U" structure, the smaller sized LH needs to interact with the sTyr331 extroflexion to induce proper conformational assembly of the receptor (9). Interestingly, the deletion of the amino acid sequence encoded by exon 10 would result in a modification of the "U" structure, consisting in the shift of the adjacent helix, which spatially replaces the exon 10-encoded sequence, shifting Tyr331 to a special position not permissive for LH accommodation. As a consequence, LH signaling would be impaired (85) while hCG retains its functional properties due to the preservation of the "U" structure binding and conformational change of LHCGR. These data were supported by experiments altering the exon 10-encoded sequence by introducing a double proline mutation at position 303 and 305. In this case, disruption of the "U-shaped" structure while preserving the proper spatial location of the sTyr331 residue negatively impacted on hCG but not LH signaling (9).

[FIGURE 3]

Since LH and hCG display a specific interaction with LHCGR, different conformational changes of the receptor may occur, depending on the hormone. In the HEK293 cell line, co-expression of signaling-deficient LHCGR and binding-deficient LHCGR (186) allowed evaluation of the ligand-induced intermolecular cooperation (53). While treatment by hCG was linked to full cAMP activation, LH failed to induce an intracellular increase in second messenger (187). hCG-induced cAMP increase was the result of binding-deficient receptor activation by signaling-deficient LHCGR capable of hormone binding, which relies on receptor dimer formation and on the ligand-receptor complex undergoing specific conformational changes. This is the so-called LHCGR "trans-activation" and it was suggested occurring under hCG treatment, while LH would be able to induce mainly self (cis)-activation of receptors capable of hormone binding (186). These results and, in general, receptor cis- and trans-activation was independently supported (53,188,189), even if opposite results were also provided (190), questioning the concept of ligand-specific functional rescue between LHCGR molecules (190).

In summary, as a result of common evolution, cross-interaction between ligands (LH and hCG *vs* murine lh, etc.) and receptors (human LHCGR, murine lhr, etc.) from different species may be demonstrated *in vivo* and *in vitro*, together with species-specific patterns of ligands for one receptor, as adaptations to maintain effective biological responses. However, human LH and hCG display their own specific molecular interactions with human and *non*-human receptors, resulting in hormone-dependent modulation of the downstream intracellular signaling and physiology. This will be considered in detail in the next paragraphs.

IV. LH- and hCG-specific intracellular events

LHCGR binding to its ligands triggers a number of subsequent events mediating the activation of multiple signal transduction pathways (1). These events start after hormone interaction with its high-affinity binding site located in the extracellular domain of the receptor, which, however, is not capable of generating intracellular signaling *per se*. The bound receptor undergoes a conformational change impacting the hinge region and subsequently the transmembrane domain. However, contacts between the extracellular

domain and loops (191), especially the second and third extracellular loops, are necessary for proper signaling activation. These secondary, low-affinity contacts established by the hormone compelled to interact with extracellular loops and the hinge region play a key role in signal generation (192). The spatial conformation of the activated LHCGR is linked to different, independently activated signaling cascades, depending on various cell-specific intracellular interactors of the receptor, which mainly consist of G proteins (193) and β-arrestins (194). While the presence of LHCGR at the cell surface is linked to weak basal signals existing as an equilibrium between stimulatory and inhibitory signals, maximal production of high-affinity signal occurs upon hormone binding.

While the intracellular events described above are overall common to all glycoprotein hormone receptors, the existence of ligand-LHCGR specific interactions suggests that qualitatively and quantitatively different patterns of intracellular signaling cascades may be differentially activated by LH and hCG. These features could rely on peculiar LHCGR conformational changes induced by ligands, as well as the presence of other interacting glycoprotein hormone receptors and the intracellular enzymatic *milieu* of target cells. All these factors likely contribute jointly to differentiate LH from hCG physiology.

Classical views and new insights on LHCGR-mediated signaling

Knowledge of LHCGR-mediated intracellular events progressively increased over the past few decades, revealing a complex picture of gonadotropin functions, not explainable exclusively with the old concept of steroidogenesis as the main endpoint of both LH and hCG functions, exerted *via* cAMP/PKA activation and intracellular calcium ion (Ca2+) increase. This classical assumption presumably originated when cAMP and steroid hormones were the main – or even only – molecules analyzable using the first assays available. Modern experimental techniques have revealed the existence of several intracellular LHCGR interactions and multiple signaling cascades, calling for a re-evaluation of LH- and hCG-mediated signals.

It is common knowledge that LH and hCG induce simultaneous increase of the second messenger cAMP and Ca^{2+} through the LHCGR (195). These two events occur relatively early after receptor activation, within less than one minute (196,197), and belong to two separate, G protein-dependent signaling pathways (198). Spatial conformation of the activated receptor leads to G protein stimulatory Gαs subunit dissociation from the βγ dimer, thus activating the adenylyl cyclase membrane enzyme, which, in turn, catalyzes the conversion of ATP into cAMP. This second messenger, before its metabolization to AMP by phosphodiesterase enzymes (PDEs), induces PKA activation and transcription factor cAMP response element-binding protein (CREB) phosphorylation (199). However, relatively high intracellular cAMP concentrations were linked to pro-apoptotic effects in granulosa cells, along with progesterone synthesis and androgen conversion to estrogens (200,201). In the theca cell, phosphorylated CREB (pCREB) binds *CRE* DNA target sequences modulating the transcription of steroidogenic enzyme-encoding genes, such as *STARD1* and *CYP19A1*, and synthesis of androstenedione. Interestingly, phosphorylation of the extracellular-regulated kinase 1/2 (ERK1/2; pERK1/2) occurs as a downstream event to PKA activation in theca cells concomitant to CREB phosphorylation, inhibition of progesterone synthesis and stimulation of androgens synthesis, by differently modulating the transcription of genes encoding steroidogenic enzymes (202). Most importantly, ERK signaling is linked to proliferation and viability in all gonadal steroidogenic cells (203,204), as well as to anti-apoptotic processes (205), revealing the central role of the molecule in regulating GPCR signals, including LH and hCG functions and reproduction. However, the activation of pERK1/2 is linked to several other intracellular processes following gonadotropin stimulation. It is required for the steroidogenic response to LH in certain cell types, such as Leydig cells (206), for receptor mRNA downregulation (203) and for modulating the

activation of GPCR kinases (GRKs) involved in receptor phosphorylation and subsequent internalization by β-arrestins (204). The recruitment of β-arrestins, by itself, is responsible for a second pERK1/2 activation (207) as a likely opposing effect to cAMP pro-apoptotic events mediated by GPCRs (208).

Gonadotropin-induced mobilization of intracellular Ca^{2+} was first investigated in hCG-treated, transfected cells expressing the murine Lhr, and was associated with PLC activation (209). The signaling cascade is triggered by the Gαq protein activating PLC, with subsequent cleavage of phosphatidylinositol 4,5-bisphosphate (PIP_2) to diacyl glycerol (DAG) and inositol trisphosphate (IP_3). IP_3 binds calcium channels located in the endoplasmic reticulum, resulting in Ca^{2+} release in a hormone concentration-dependent manner. Although both the cAMP/PKA- and the PLC/Ca^{2+}-pathways are activated simultaneously, the hCG half-maximal effective dose (EC_{50}) is 20-fold higher for Ca^{2+} mobilization than that needed for cAMP recruitment, and is independent of receptor density, demonstrating that LHCGR carries a dual signaling potential (210). Ca^{2+} binds the calcium-modulated protein calmodulin (CaM) resulting in downstream activation of CaM kinases, which control cholesterol transport into mitochondria and steroidogenesis (211,212). Moreover, Ca^{2+} signaling was associated with proliferative effects *in vitro* (213). Simultaneously to these events, βγ dimer of G protein may lead to PIP_2 phosphorylation to PIP_3 by phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), thus activating protein kinase B (AKT). AKT might also be activated through the epidermal growth factor (EGF) receptor (EGFR) and exerts anti-apoptotic roles as well as inhibition of *CYP19A1* expression (205,214), thus negatively modulating steroidogenesis, at least partially. On the other hand, activation of AKT as well as ERK1/2 signaling is necessary for *STARD1* expression and it is a pathway preserved across mammalian gonads and adrenal glands of mammals to mediate gonadotropin- and adrenocorticotropic hormone (ACTH)-induced steroidogenesis, respectively (215). Taken together, gonadotropin signaling may stem from a balance between opposing steroidogenic and pro-apoptotic *versus* proliferative and anti-apoptotic intracellular events providing the endocrine regulation of reproduction.

An interesting and relatively recent development consists in the dependence of the gonadotropin-mediated signaling on receptor concentration, due to preferential coupling to β-arrestins/ERK1/2- and AKT-pathways at relatively low receptor density as an alternative to the canonical cAMP/PKA-pathway (200,216,217). These data provide a compelling, albeit speculative, regulatory mechanism that may contribute to differentiating gonadotropin signals, depending on physiological requirements. In fact, LHCGR expression is a dynamic event during the menstrual cycle (52) and may be associated with different LH-dependent roles. For instance, during the follicular and luteal phase of the menstrual cycle, the equilibrium between LH-driven proliferative and steroidogenic signals might be differentially regulated through LHCGR coupling, modulating granulosa cell proliferation, luteinization, androgen or progesterone synthesis. At the early antral stages, LHCGR-mediated signals mainly consist of the regulation of proliferative signals delivered to granulosa cells and these may occur as a result of unique patterns of intracellular signaling cascades, plausibly activated by FSHR-LHCGR heterodimers (54,169,218). The LH-dependent androgenic potential of ovarian follicles progressively increases together with LHCGR expression levels in theca cells during the late antral stage, suggesting that receptor density is linked to specific signaling patterns, relying on variable, LHCGR concentration-dependent Gαs protein coupling accompanied by increasing PLC activation (210) and cellular metabolic changes. These data exacerbate the specificity of LH as an irreplaceable ligand of LHCGR during physiological follicular phases.

Agonist-induced desensitization is well-known feature of LHCGR and is characterized by the organization of large receptor aggregates at the cell membrane (219)

preceding their internalization, which, in turn, is the main determinant of downregulation (220) regulated by GRKs, β-arrestins and other modulators (221). Interestingly, experimental evidences support the formation of ligand-dependent LHCGR aggregate structures, suggesting formation of large LHCGR complexes induced by hCG than LH binding (222). LHCGR aggregation may be a determinant for localization of a number of receptors within endosomes, in order to determine trafficking and recycling of these molecules. Persistent cAMP signaling may be induced by the internalized receptor forming complexes with both G proteins and β-arrestins (223), and suggested it is likely modulated via the adaptor protein interacting with pleckstrin homology domain and leucine zipper 1 (APPL1) (224). The molecular mechanism underlying persistent cAMP signaling is of great physiological relevance, since it could play a crucial role in sustaining LH functioning at mid-cycle in the female (218), thus bypassing the potential arrest of steroidogenic signals due to LHCGR downregulation. Whether hCG action is mediated through similar receptor features during pregnancy is unknown. A study evaluating whether LHCGR kinetics of internalization is linked to LH- and hCG-specific treatment by fluorescent microscopy failed to find any difference in human primary granulosa lutein cells (6). However, the existence of hormone-specific LHCGR trafficking cannot be excluded since hCG displays higher potency in β-arrestin 2 recruitment than LH, at least in the transfected HEK293 cell line (225).

The analysis of LHCGR-mediated signaling cascades provides a complex picture of intracellular events occurring upon LH and hCG binding to this receptor, suggesting that hormone-specific signals may occur at different levels and result in a refined, cell-specific modulation of the biological effect.

The steroidogenic pathway

Modulation of the steroidogenic response is of crucial relevance for the preservation of different physiological functions, such as gametogenesis and pregnancy, when estrogens, androgens or progesterone variability occur as major stage- and sex-specific products. Therefore, LH and hCG might be linked to different controls of the steroidogenic pathway regulating different, specific functions. Differences in intracellular signaling may be induced upon LH or hCG binding to LHCGR, likely depending on hormone-specific conformational changes at the receptor level (9). Since these spatial changes impact the intracellular portions of LHCGR, resulting in G protein activation, it is plausible that LH and hCG treatment are linked to activation of hormone-specific patterns of signaling pathways. In fact, mechanistic *in vitro* experiments, performed using bioluminescence resonance energy transfer (BRET) technology and transfected cell models, revealed lower LH- than hCG-dependent levels of Gαq protein activation as well as intracellular Ca^{2+} increase, whereas no differences in Gαs protein subunit recruitment and formation of dimeric or oligomeric complexes was observed (226). These data indicate that the hormone-induced conformation of the receptor impacts activation of LHCGR intracellular interactors and downstream signaling pathways.

Results indicative of different steroidogenic potentials related to LH and hCG were provided by the evaluation of cAMP production in human primary granulosa luteal cells (6), naturally expressing LHCGR. Dose-response experiments showed that hCG is about 5 times more potent than LH in inducing cAMP production, a result exacerbated by the different EC_{50} values of the two hormones (about 100 pM for hCG and 500 pM for LH). This result was later confirmed in transfected COS-7 and HEK293 cells (6,225), in the mouse tumor Leydig MLTC1 cell line (225), as well as in goat granulosa (227) and mouse Leydig primary cells (8), where hCG exhibited higher potency than LH in spite of different EC_{50}s from those observed in human primary granulosa cells, likely indicative of cell-specific LHCGR expression and coupling to intracellular interactors. Interestingly, equipotent, non-saturating concentrations (EC_{50}) of both gonadotropins induced similar *plateau* cAMP levels, reached in about one hour, following however different kinetics (6). In particular, LH-induced cAMP

response is rapid and reaches a plateau after 10 minutes, while hCG treatment requires more time, suggesting the existence of different regulatory mechanisms underlying steroidogenesis mediated by LHCGR bound to each ligand. However, the translation of *in vitro* data into physiology is not immediate, due to the several perturbing factors present *in vivo*, which may lead to biased evaluations and affect data interpretation. For instance, addition of FSH to LH and hCG *in vitro* resulted in a 5-fold increase in potency of hCG in inducing cAMP production, but did not have any effect on LH-specific response in human granulosa cells (228). This finding highlights the relevance of the co-existence of LH and FSH over the follicular phase of the menstrual cycle, which should not alter the FSH-mediated steroidogenic signal, while hCG addition to FSH administered during COS cycles might not lead to similar effects at the molecular level.

cAMP recruitment reflects the downstream pCREB activation and *STARD1* gene expression, which is more sustained upon hCG treatment, in human primary granulosa cells (229), and potentiated by FSH co-treatment (228). The *STARD1* gene encodes the steroidogenic acute regulatory protein (StAR) enzyme modulating cholesterol transport into mitochondria, a rate-limiting step for steroid synthesis. Since these results were corroborated by a greater activity of the *CRE*-reporter gene following hCG rather than LH stimulation, in both MLTC1 and transfected HEK293 cell lines (225), the higher levels of cAMP/PKA-pathway activation obtained by hCG versus LH treatment in different cell models are likely driven by intrinsic characteristics of the ligand-receptor complexes, rather than cell-specific intracellular enzymatic environments. These data, therefore, strengthen the hypothesis that hCG has greater steroidogenic potential than LH, reflecting the role exerted during pregnancy by choriogonadotropins in supporting massive progesterone production. On the other hand, the dual – proliferative and androgen-stimulating – roles exerted by LH in granulosa and theca cells, respectively, might not require induction of the steroidogenic pathway at the levels necessary in pregnancy. It is reasonable that androstenedione synthesis by theca cells is mainly supportive for conversion to estradiol, and its levels are indeed similar over the whole antral phase (230). Therefore, high androgenic potential should not be required to support follicle growth. Interestingly, long-term (48-72 hours) treatment of granulosa cells with LH resulted in higher *CYP19A1* gene expression compared to hCG (229), although no differences were found in the short-term (12-24 hours) (6). The positive action of LH on *CYP19A1* gene expression, which encodes the aromatase enzyme, is consistent with the requirement for suitable estrogen production to support ovarian follicle growth. On the other hand, LH steroidogenic activity should be focused on oocyte growth by converting progesterone to androgens as well as eventually to estrogens, and on transient maintenance of the *corpus luteum*, actions requiring only limited progesterone production as a basal substrate for androgen synthesis. In contrast, the role of hCG in sustaining pregnancy is exerted through massive progesterone production, which could be exacerbated in granulosa cells by high levels of cAMP/PKA-pathway activation, at least *in vitro* (228).

Since the collection of sufficient primary theca cells and the development of stable theca cell lines (231) suitable for *in vitro* experiments are challenging, *in vitro* evaluation of androgen synthesis is often performed using adrenal or Leydig cell lines. In the mouse Leydig tumor-derived MLTC1 cell line, progesterone dose-response curves produced by LH and hCG treatment resulted in lower EC_{50} and higher *plateau* level achieved upon hCG compared to LH treatment, while testosterone dose-response curves are similar and differ only from the LH and hCG EC_{50} values, reflecting the hormone potency inferred by cAMP data (225). In this cell model, progesterone is a precursor of testosterone and its synthesis is strictly connected with StAR activity, the gene expression of which is activated more by hCG than by LH, thereby explaining the higher, hCG-induced *plateau* level of progesterone. Although these data were obtained in Leydig cells, they corroborated hCG function in

pregnancy, suggesting that the steroidogenic role of hCG is focused on the control of efficient, massive progesterone production, which could be useless, or even counterproductive during the follicular phase. Instead, in the Leydig MLTC1 cell line, LH- and hCG-mediated testosterone production turned out to be similar and reflected cAMP data, suggesting that synthesis of the final steroid (testosterone) is regulated by quantitative stimuli and substrate availability, with no need for qualitative control of earlier events.

The scientific literature provides several data comparing the effects of pituitary LHs and chorionic gonadotropins of humans and other mammals (232,233), based on promiscuity of glycoprotein hormones and their receptor systems across the phyla. As a matter of fact, differences in cAMP production induced by human LH and hCG, and mediated by rodent Lhr (8,225), match those found in human granulosa cells (6,228). However, downstream LH- and hCG-induced pCREB, *Stard1* gene expression, as well testosterone synthesis are all highly similar, in mouse primary Leydig cells, *in vitro* (8). This effect may be the result of a balance between stimulatory and inhibitory signals obtained by treatment with human gonadotropins through the cAMP/PKA- and ERK1/2-pathways. Most importantly, these data indicate that only a quantitatively, but not qualitatively different intracellular response occurs in rodent primary Leydig cells. This is likely due to their enzymatic equipment, capable of triggering testosterone synthesis like an “on/off switch”, more than discriminating between steroidogenic and proliferative signals, as occurs in granulosa cells. This concept may find support in observations recently described in a case-report. In a hypogonadic, hypophysectomized man sequentially treated by LH and hCG (234), both gonadotropins showed similar efficacy in inducing testosterone production.

The inconsistency between human granulosa and rodent Leydig cells may be due to differences in amino acid sequences between LHCGR and mouse Lhr: they share only 80% identity at the hinge region (8), which is responsible for discriminating of LH- and hCG-mediated signaling (9), suggesting that the evolutionary divergence between the two species impedes the qualitative discrimination of intracellular signals mediated by human hormones despite effective receptor binding. These data provide relevant insights for the so-called “Van Hell” *in vivo* bioassay (165), currently used for inferring gonadotropin dosages for clinical purpose in humans by only evaluating testosterone-dependent endpoints (235,236). *In vivo* bioassays aim to assess the biological activity, measured in terms of organ weight gain, of pharmacological preparations injected into living rodents. However, these assays might not be appropriate for detecting the full spectrum of gonadotropic bioactivity mediated by LHCGR in human cells.

Taken together, LH and hCG retain different steroidogenic potentials modulated in a cell-specific manner. While a qualitative discriminatory capability between LH- and hCG-mediated signals is displayed by granulosa cells, steroidogenesis, mainly androgen production, in Leydig and theca cells is regulated by quantitative signals. These data highlight the specific roles for each gonadotropin in their target cells, providing experimental evidence that LH and hCG are not equivalent.

Proliferative and pro-apoptotic signals

Proliferative signals control antral follicle growth in the presence of both FSH and LH (169), while hCG physiologically acts on LHCGR-expressing cells dedicated to progesterone synthesis. Pregnancy is also characterized by angiogenic events, consistent with cell proliferation and possibly mediated by hCG-H, growth factors and steroids. It is however plausible that LH and “classical” hCG differentially impact cell proliferation, given the specific molecular properties required to optimize follicle growth and massive progesterone production. Discrimination of qualitatively different LH- and hCG-mediated signaling by LHCGR was demonstrated in human primary granulosa lutein cells by assessing activation of phospho-proteins. Both gonadotropins showed maximum activation of ERK1/2- and AKT-

pathways at a concentration of 100 pM, resulting, however, in a hormone-specific phosphorylation pattern of these kinases (6). LH treatment resulted in higher and more sustained pERK1/2 and pAKT activation compared to hCG (6,229), reflecting the proliferative and anti-apoptotic roles (237,238) exerted by LH during the antral phase of follicular development. Moreover, the range of effective of LH concentrations is widened by presence of FSH combined with LH, but not with hCG, confirming that gonadotropin-specific activities are potentiated upon FSH co-treatment (228). Interestingly, LH and hCG displayed different ratios between the cAMP EC_{50} and the maximally activating concentrations required for pERK1/2 and pAKT activation (about 5:1 for LH and 1:1 for hCG), in granulosa cells. These data suggest that LH has a higher proliferative and anti-apoptotic potential than hCG, in granulosa cells *in vitro*. This is an example of biased signaling, consisting of activation of different intracellular endpoints evoked by varying concentration and ligand.

Expression of proliferative and anti-apoptotic genes, in granulosa cells, reflects hormone-specific signal transduction. The expression of growth-promoting *AREG* gene (6), which encodes epidermal growth factor (EGF)-similar amphiregulin, and of the *CCND2* gene (229), encoding cell cycle regulator, G1/S-specific cyclin-D2, is upregulated more by LH than hCG. In contrast, the high steroidogenic potential of hCG, which relies on efficient cAMP production, is linked to expression of pro-apoptotic genes, in primary granulosa cells *in vitro*, especially in the presence of FSH. Although both LH and hCG elicited increased *TP53* pro-apoptotic gene expression, the procaspase 3-encoding gene *CASP3* was positively modulated upon hCG treatment, while LH fails to obtain similar results, likely due to upregulation of the anti-apoptotic X-linked inhibitor of apoptosis protein-encoding gene *XIAP* (228). This finding should be confirmed in other cell types. However, gene expression data supported a dose-dependent cell viability decrease in human granulosa cells maintained 24-72 hours under hCG stimulation *in vitro*, whereas LH resulted in the opposite effect by counteracting cell death (228,229). Similar conclusions were drawn from a study comparing the long-term effects of LH and hCG in goat granulosa cells *in vitro*. While hCG induced relatively high cAMP intracellular levels and reduced cell viability, LH treatment resulted in marked phosphorylation of ERK1/2 and increased rate of cell proliferation (227).

Whether hCG is capable of mediating pro-apoptotic effects *in vivo*, at least during the follicular phase of the menstrual cycle, should be investigated in-depth, since the molecule was associated with both proliferation and inhibition of cancer cell growth (239,240), and given the proliferative role exerted during placentation. It is plausible that hCG-dependent life signals are cell-specific and sensitive to a particular hormone isoform or glycosylation variant (153). Anyway, proliferative and anti-apoptotic signals should be transmitted through ERK1/2- and AKT-pathways (151,241), which are preferentially activated by LH, rather than hCG, at least in human primary granulosa cells *in vitro*. Interestingly, hCG displayed higher efficiency in recruiting β-arrestin 2 than LH, which has a 13-fold higher EC_{50} (hCG EC_{50} about 10 nM, LH EC_{50} 130 nM) and even acts as a partial agonist, not reaching hCG-dependent *plateau* levels (225). Although these data were obtained in the MLTC1 cell line, they are likely to also be valid in human granulosa cells, since the recruitment of β-arrestins is dependent on GPCR activation by agonists (242). Most importantly, these data reflect the lower efficacy and efficiency of LH compared to hCG in inducing progesterone production, indicating that β-arrestins are involved in steroidogenesis, as demonstrated by decreased progesterone levels upon depletion of β-arrestins by siRNA probes (225). β-arrestins are involved in ERK1/2 phosphorylation and GPCR internalization (208), as well as proliferative signals in granulosa cells (200). It is conceivable that relatively high amounts of LH would be required to down-regulate the LHCGR-mediated steroidogenic and pro-apoptotic signaling (243), in light of the less efficient cAMP production induced by LH as compared to hCG.

These data support the idea that LH has greater proliferative potential than hCG, at least in granulosa cells, reflecting its physiological function as follicle growth regulator (Table 3).

[TABLE 3]

Cross-talk between gonadotropin- and steroid hormone-mediated signaling

The effect of LH and hCG on signal transduction may precisely be revealed by *in vitro* experiments, where perturbations and interactions between signaling pathways activated by different hormones and paracrine factors are missing. For instance, pro-apoptotic effects linked to hCG addition were hardly reproduced in ART cycles *in vivo*, and showed weak effects or even antithetical results (244,245). This concept is also valid for cancer cell growth (239,240), which may be both positively or negatively susceptible to the presence of the hormone, depending on the cell type. Therefore, intracellular effects clearly dissected *in vitro* may be masked *in vivo*.

In the gonads, LH and hCG induce steroid hormone production, activating the same pattern of intracellular signaling cascades. Steroid compounds, such as glucocorticoids, have been associated with proliferation of granulosa cells, as well as protection against cAMP- and p53-induced apoptosis (246). These molecules act through activation of pAKT and pERK1/2 counteracting intracellular death signals. During the follicular phase of the menstrual cycle, gonadotropin functions converge in production of progesterone, which is further converted to androgen in theca cells. Androgen is transferred to granulosa cells, where it is transformed to estrogen, leading to potent proliferative effects and inducing follicle growth *in vivo*. Indeed, estradiol activates the AKT-pathway *in vitro* (247), and estrogen receptor-beta (ERβ) knockout mice are characterized by impaired follicular development (248). It is reasonable that estradiol could counteract pro-apoptotic signals mediated by high cAMP intracellular levels, obtained during treatment by FSH and hCG administration in COS. This concept was confirmed *in vitro* by adding estradiol to hCG or LH treatment of human granulosa cell cultures (229). The effect of steroid addition is predominant on that of either LH or hCG alone, triggering high levels of pAKT activation, preventing hCG-dependent procaspase 3 cleavage and decreased cell viability. This molecular mechanism may explain why hCG addition to FSH in COS for ART is associated with multiple follicular development, reasonably sustained by high estrogen levels and avoiding natural follicular *atresia*.

Progesterone is the main steroid produced during the luteal phase, as well as by human primary granulosa lutein cells *in vitro*, and it is responsible for the protection from apoptosis linked to hCG treatment (249). On the other hand, it is well known that both LH and hCG, as well as progesterone, promote ERK1/2 and AKT phosphorylation. They also induce expression of *AREG* and epiregulin-encoding gene *EREG*, two EGF-like ligands with positive effects on granulosa lutein cell viability *in vivo* and preservation of the *corpus luteum* in primates (250) and rodents (251). The same intracellular pathways lead to the growth of progesterone receptor-expressing cancer cells (252), strengthening the evidence for proliferative and anti-apoptotic effect mediation by steroid hormones through activation of signaling cascades common to gonadotropins. These data support the tumorigenic potential described in hCGβ-overexpressing transgenic mice, where human gonadotropin and high levels of progesterone may co-assist the development of multiple cancers (253,254). However, since extragonadal tumors were totally abolished by ovariectomy in these mice, it is plausible that the tumorigenic potential of hCG is exerted through aberrant ovarian functions, rather than by a direct gonadotropin effect (7). Interestingly, the apoptotic effect seems to be directly dampened by hCG treatment in Leydig cells (255), where signals induced by both LH and hCG converge on a similar balance between cAMP production and ERK1/2 phosphorylation, as well as downstream steroidogenesis (8), suggesting the different nature of LHCGR-mediated signaling within testis and ovary (Figure 4).

[FIGURE 4]

V. Polymorphisms and mutations

Several single-nucleotide polymorphisms (SNPs) and mutations falling within gonadotropins and their receptor genes have been described (20,256). They may modulate or impair hormonal response or receptor function, impacting reproductive function or leading to disease. Generally, spontaneous mutations occur as random events and are silent, not resulting in amino acid changes at the protein level. Some of them however cause changes in the amino acid sequence and are linked to clinical phenotypes. SNPs instead result in mild phenotypes without strong repercussions on reproductive success of individuals. They have a frequency ≥1% in a given population and contribute to the endocrine ethnic background. Most SNPs and mutations impacting LH and hCG signaling are carried by LHCGR (257).

Among the several SNPs and mutations falling within the *LHB*/*CGB* gene cluster, only a few of them have been associated with specific phenotypes. A better understanding of the consequences of LHβ and CGβ mutations is provided by the study of transgenic mice models. The *LHB* gene knockout male mice presented hypogonadism, with low testosterone levels and hypoplastic Leydig cells, yet displayed normal serum FSH levels, while female mice were infertile and featured anovulation and degenerated antral follicles (258). The mouse phenotype partially matches clinical observations in humans, where *LHB* gene mutations lead to a severe, eunuchoid phenotype in males (259). Mouse models may therefore not provide a full comprehension of human LH physiology. Administration of exogenous FSH is enough to sustain follicular development and ovulation in hypophysectomized female mice, while this hormone fails to do the same in Lhr knockout mice (260). These findings support the concept that the presence of gonadotropin receptors, rather than LH, is required to sustain gametogenesis in mice. Transgenic mice overexpressing both the *CGB* and the *CGA* subunits were expected to replicate the human phenotype linked to activating mutations of LHCGR, consisting of asymptomatic women and men developing precocious puberty and testis tumors. Surprisingly, these mice featured the opposite phenotype, with a normal phenotype in males, while precocious puberty, obesity and luteinized ovaries with luteomas and hemorrhagic cysts were present in females (7).

Altogether, while transgenic mice may not be fully representative of human physiology, they provided new insights to comprehend LH/hCG- and LHCGR-specific functions emerging from clinical data involving SNPs and mutations described below.

LHβ and hCGβ polymorphisms

Few LHB and CGB gene polymorphisms were studied in conjunction with their clinical phenotype. Overall, they do not severely impact fertility and most of them lack molecular characterization *in vitro*, not providing clear insights of their suggested effects *in vivo*. One exception is provided by the most common *LHB* gene variant (V-LH), which was discovered in the Finnish population, which displays a double polypeptide change, namely tryptophan to arginine at position 28 and isoleucine to threonine at position 35 of the amino acid chain (261,262), and introduces a glycosylation site (263) hiding the molecule from a specific anti-LH antibody (264). V-LH exhibited reduced serum half-life and bioactivity *in vivo* compared to wild-type LH, as well as decreased receptor binding activity and potency for progesterone production *in vitro* (265), and induced preferential IP_3-related signaling, rather than cAMP/PKA (266). Perturbation of the signaling cascade by V-LH might impact granulosa cell survival and follicle development. In cumulus cells of heterozygous women undergoing ART, high levels of apoptotic markers were found, such as DNA fragmentation index and cleaved caspase-3, and they negatively influenced the success rate of intracytoplasmic sperm injecton (ICSI) procedures (267). However, increased expression of this variant, identified by SNPs within the promoter region in linkage *disequilibrium*, resulting in an about 40% higher activity compared to that of the "normal" *LHB* promoter,

compensates for the weaker hormone bioactivity (268). The frequency of V-LH was lower in obese women affected by polycystic ovary syndrome (PCOS) than in healthy and *non*-obese PCOS women, suggesting that V-LH may provide protection from developing symptomatic PCOS in obese women (269). Nonetheless, this LH variant was associated with infertility in homozygous Japanese women (270), while Baltic, V-LH carrier men affected by idiopathic infertility have higher serum LH levels than healthy men (271). Interestingly, V-LH was found in a 18-year-old man affected by the fertile eunuch syndrome (272). This patient displayed hypogonadism and normal responses to treatment by exogenous GnRH and hCG. Overall, however, the ethnicity-related clinical features displayed by V-LH and the mild phenotypes suggest that it simply represents an example of phenotypic variations due to genetic polymorphisms as the basis of human diversity.

A SNP (rs1056917) in exon 3 of the *LHB* gene was found more frequently in South Indian PCOS women (273). This SNP is characterized by the synonymous amino acid change "T" to "C" at position 294 of the gene sequence. Its contribution to PCOS pathogenesis is thus unexplained. It is conceivable that the polymorphism might impact on the expression or functions of other molecules, since it falls within the palindromic *RUVBL2* gene sequence, encoding for a protein interacting with the activating transcription factor 2 (ATF2/CRE-BP1) (274). Several other SNPs falling within or close to the *LHB* gene sequence were found to be associated with central precocious puberty (275) or infertility (276) in women, but their contribution in defining the endocrine phenotype should be evaluated together with other polymorphisms related to hormones and their receptors. Moreover, these results should be independently confirmed in other populations and supported by functional *in vitro* data, which is still lacking.

Among the *CGB* genes SNPs and mutations previously described in association with miscarriage (41) and mentioned in the chapter "hCGβ isoforms and glycosylation variants", a polymorphism falling within the *CGB5* gene displayed inefficient assembly when co-transfected with the *CGA* gene in the Chinese hamster ovarian (CHO) cell line. This naturally occurring variant is associated with infertility in women and is characterized by a valine to methionine exchange at codon 79 (277). Seventy-one hCGβ variants deriving from *CGB5* and *CGB8* genes might be predictive of recurrent miscarriage in European populations (278), suggesting the relevance of these transcripts in sustaining pregnancy. Moreover, since heterozygous haplotypes calculated for these SNPs are relatively frequent, they may be subjected to balancing selection in Europeans. Overall, associations between *CGB* gene SNPs and miscarriage are relatively rare and molecular mechanisms supporting clinical data are unclarified.

In general, as in the case of SNPs and common variants in other genes, SNPs in *LHB* and *CGB* genes are unlikely to be major determinants of diseases. Rather, they contribute to human phenotypic variation and, together with other SNPs, may be relevant in discrete genomic clusters associated with particular reproductive problems, e.g. PCOS (279).

LHβ and hCGβ mutations

Possibly because of the physiological role played in a specific time-window and in one sex only, inactivating hCG mutations are rare and, presumably, incompatible with successful pregnancy. Indeed, one of the first mutations affecting hCG function was somatic and was found in the α subunit secreted by undifferentiated carcinoma cells of the femoral region (280). This mutation consisted of a substitution of glutamic acid by an alanine at position 56, which changed the hydrophobic profile of the molecule, resulting in misfolding and impairment of dimerization with the β subunit. Similar effects at the molecular level have been associated with a genomic *CGB5* gene mutation leading to a valine to leucine swap at position 76 of the β subunit (281). The mutated hCGβ is only capable of 10% dimeric assembly yet shows increased potency in inducing cAMP production. Nevertheless, enhanced

steroidogenic signaling was not sufficient to replace loss of function due to impaired dimerization, as this mutation was found in a Northern European patient affected by recurrent miscarriage. *CGB8* gene mutations have also been described and found in individuals of the same population (281), albeit without a link to pathogenic phenotypes. A proline to arginine change in position 93 resulted in two-fold reduction in the β subunit secretion without affecting its biological activity, while no effects were associated with an arginine to tryptophan substitution at position 28. Altogether, these data indicate that genomic mutations within the genes encoding for the two major hCG transcripts result in mild consequences that can be tolerated.

While mutations in the *CGB* genes predominantly result in miscarriage, mainly due to misfolding of the β subunit and impaired dimer formation (281), *LHB* gene mutations result in phenotypes featuring hypogonadism, decreased or impaired spermatogenesis, delayed puberty and low testosterone levels in males, and amenorrhea in females. Such symptoms might be associated with infertility in both sexes and may be due to disruption of *LHB* splice sites (259) or in the signal peptides (282). Mutations disrupting the cystine knot motif, such as a glycine to aspartic acid mutation at position 56 may result in defective heterodimer formation, undetectable serum LH levels and hypogonadism (283). A similar phenotype was described in a patient carrying a lysine deletion at position 40 of LHβ, which impaired release of hormone dimers (284). Since mutations falling within the *LHB* gene do not impair receptor function, long-term administration of exogenous hCG to these patients induces virilisation and testicular growth, testosterone synthesis and spermatogenesis, in conjunction with fertility (285). Interestingly, these data strengthen the hypothesis that LH and hCG may be equivalent in inducing proper Leydig cell function and fertility, which mainly relies on testosterone synthesis in males and might be switched on by an on-off molecular mechanism, rather than qualitatively different hormonal signals (8). In this case, it cannot be excluded that signaling cascades are maximally activated by low amounts of occupied receptors, confirming prior findings in rodent' Leydig cells *in vitro* (166). In fact, certain mutants of LHβ may display residual *in vitro* activity sustaining low testosterone synthesis, but enough to support normal spermatogenesis and fertility (286). Studies in siblings carrying the same *LHB* mutations totally disrupting LH signaling, revealed different, sex-specific clinical effects. While normal pubertal maturation may be conserved in women, this is not the case in men (282), suggesting that, in women, the regulation of estrogen production needs to be sustained by FSH acting on granulosa cells, supported by basal androgen production originating from theca cells. Moreover, in the ovary, LH-mediated signals might plausibly be driven by LHCGR-FSHR heterodimers activated by FSH upon the onset of puberty, as well as during early antral follicular stages (169), while Leydig cells are the only LH target and source of proper testosterone levels required to support male secondary sex characteristics. Nevertheless, inactivating mutations of LHβ subunit have been associated with ovulatory disorders (287), suggesting the fundamental relevance of proper LH signaling for the ovarian cycle.

Receptor mutations and polymorphic variants

Although several SNPs were found within the *LHCGR* gene, their contribution in determining clinical phenotypes is mostly weak. An exon 10 SNP, p.N312S (rs2293275), was found in association with spermatogenic damage and is highly prevalent among infertile male patients (288). However, while the number of LHCGR marker SNPs impacting male reproductive function is very low, it is remarkable that the very gene is a genetic hot spot for polycystic ovary syndrome (PCOS) in Han Chinese population (289).

A common polymorphism of LHCGR consists of the addition of two amino acid residues, a leucine (L) and a glutamine (Q), at codons 19–20 within exon 1, originating from a CTCCAG insertion at positions 55–60 of the gene (290). This receptor variant, known as

"ins18LQ," is common among Caucasians but absent in other populations, such as Japanese. Although the double-amino acid residues insertion does not severely affect the LH- and hCG-mediated cAMP production, the polymorphism is linked to a more active signal peptide and to an adverse outcome in breast cancer patients (291).

The *LHCGR* gene mRNA variants comprise highly expressed, primate-specific transcripts including a cryptic exon located between the sixth and seventh exon with unknown function (292). This exon is named "6A" and is responsible for three different mRNA receptor variants differing in length based on the location of stop codons. The resulting mRNA variants, in addition to the "classical" LHCGR, include two truncated forms consisting of exons 1 to 6 and one "6A" of different length, and a full-length transcript with exon "6A" between exons 6 and 7. Mutations falling within this region are causative of aberrant gene transcription leading to Leydig cell hypoplasia type II (293), while SNPs in exon "6A" were associated with testosterone levels in male infertile patients (294). Especially, levels of this hormone were more elevated in "G" homozygous men than in those carrying the "T" allele of the exon "6A" SNP rs68073206. Interestingly, mutations of this cryptic exon were discovered for the first time in 46,XY patients affected by sex development disorders and characterized by female phenotype, a blind-ending vagina and primary amenorrhea (292). Based on current knowledge of human testis development, the presence of testicular structures, along with a phenotype largely resembling testicular feminization (which is due to the lack of androgen action), suggested that the patient was not at all responsive to endogenous LH and maternal hCG. Therefore, it is plausible that isoforms deriving from exon "6A" may be linked to the discrimination between hCG- and LH-mediated signals. Since our knowledge about the control of human testicular development by fetal pituitary LH or maternal hCG is limited and provided by individuals affected by genomic mutations and data from *non*-human mammals, the putative role of exon "6A" in discriminating LH and hCG remains unclear.

Interesting data on LH- and hCG-specific functions were provided by the deletion of the *LHCGR* exon 10-encoded sequence, described in the chapter "Evolutionary convergences: trophoblast LH and pituitary CGs". As discussed above, the mutated receptor is capable of transmitting hCG, but not appropriate LH signals in spite of binding both hormones (85), resulting in male hypogonadism with normal male phenotype (84). The clinical evidence derived from naturally occurring mutations or deletions of LHCGR exons 6A and 10 demonstrate that these sequences of the receptor are essential for LH and hCG action and may be instrumental in discriminating between the two hormones, although the mechanism remains largely unknown.

VI. Pathophysiology of LH and hCG

Specific profiles and levels of LH and hCG may be associated with pathological conditions, such as hypogonadism, cancer and endocrine disorders. These effects may be due to excessive or low hormone activity, altering normal physiology and leading to a wide range of clinical phenotypes. Since gonadotropins modulate cell growth, it is reasonable to assume that their action is linked to proliferation of cancer cells and tumorigenesis. Moreover, extra-gonadal action of LH and hCG may produce clinical effects. For instance, pregnancies of fetuses affected by trisomy 21 are typically characterized by high levels of hCG in maternal serum, in spite of low hCG synthesis capability by the placenta (295). This apparent paradox may be caused by high activity of sialyltransferase-1 and fucosyltransferase-1 enzymes in trisomy-21 trophoblast cells, resulting in highly glycosylated and acidic hCG molecules, displaying reduced activity *in vitro*. hCG synthesis peaks at around 10 weeks in trisomy-21 pregnancies and declines to lower levels than in chromosomally normal pregnancies. In any case, since no association between sialylated hCG isoforms and trisomy-21 pregnancy were found (296,297) while fucosylation was even not investigated, any conclusion should be

interpreted carefully. However, highly glycosylated molecules are more persistent due to increased half-life (295). Moreover, high hCG levels during pregnancy may induce aberrant expression of LHCGR in adrenal glands of the mother, resulting in the increased risk of adrenal hyperplasia and transient Cushing syndrome due to cortisol release in response to the hCG-induced signals (298). Several other clinical effects related to aberrant LH and hCG signaling, i.e. hypogonadism, precocious puberty, PCOS, miscarriage and cancer, are described in this chapter.

Hypogonadism and precocious puberty

Hypogonadism is a pathology characterized by decreased gonadal activity and hormone production, and may be caused by impairment of LH-mediated signaling (299). In males, the disease may arise from testis (primary) or from dysfunction of the hypothalamic-pituitary unit (secondary or central hypogonadism) (300), although several hypogonadal patients remain idiopathic, suggesting a possible polygenic nature (301). The phenotype associated with an impaired HPG axis may vary depending on the severity of the disease. Most severe cases may be linked to rare autosomal recessive conditions, such as inactivating LHCGR mutations, typically interfering with the development of male external genitalia and testicular descent, and resulting in phenotypically female, 46,XY patients with Leydig cell hypoplasia type 1. They are unresponsive to both endogenous LH and exogenous hCG administration and are characterized by primary hypogonadism and sexual differentiation disorder, featuring the absence of Leydig cells, lack of masculinization and pubertal maturation with female-like phenotype and external genitalia. Milder phenotypes could be linked to *LHB* mutations affecting hormone functioning, resulting in infertile individuals with male external genitalia and maldescended testes, micropenis and/or hypospadias. In this case, given the presence of a functional LHCGR, hCG therapy showed efficacy in inducing testosterone production and may restore fertility. The clinical picture is strictly connected to testosterone levels, insufficient to support male sexual development. The total blockade of Leydig cell function is largely attributed to mutations impairing receptor transport to the cell membrane (302) and activation of the G protein-dependent signaling cascades (303), but mutations affecting LH binding have also been described (304). An inactivating, homozygous mutation was found in a 46,XY hypogonadic patient with delayed puberty, normal prepubertal male phenotype and descended testes, displaying a glutamine to arginine substitution at codon 54 of LHβ (305). The resulting β subunit was capable of forming a heterodimer with the α subunit but failed to bind the receptor *in vitro*. This patient was treated with long-term hCG administration, resulting in testicular enlargement, virilisation and spermatogenesis. Similar phenotypes and histories were found in consanguineous, hypogonadic patients bearing the deletion of lysine at position 40 of the LHβ, linked to intracellular retention of the hormone (284). These data allow for the comparison of the effects of mutations impairing LHCGR or LHβ functions in the male. While the first are linked to severe, female-like phenotypes, LHβ mutations are compatible with male phenotype and exogenous hCG-induced spermatogenesis (306). Taken together, these findings suggest that maternal hCG, together with the presence of intact LHCGR, can, at least in part, compensate the absence of fetal pituitary LH and support the development of a male phenotype, although the presence of maldescended testis and micropenis suggest that proper production of functional LH molecules is required to fully support secondary sex characteristics.

Inactivating mutations falling within the *LHB* gene were described in infertile females, where the phenotype displayed normal external genitalia and spontaneous breast and pubic hair development at puberty. Menarche was delayed or even normal, yet these patients were oligo-amenorrhoic and characterized by failure in achieving ovulation and normal LH, estradiol and progesterone levels in the ovulatory or luteal phase. Since women with *LHB* mutations have functional LHCGR, they may successfully be treated with LH or hCG (304),

differently from hypogonadic women with *LHCGR* inactivating mutations, which are unresponsive to gonadotropin treatment.

Precocious puberty is defined as the onset of puberty at a relatively young age and may be determined by several factors, such as hypothalamic or pituitary dysfunctions, McCune-Albright syndrome or sex hormone-secreting tumors. LH signaling is one of the factors regulating the onset of puberty. However, while LHCGR activating mutations, resulting in sustained tonic cAMP production due to an aberrant ratio between the Gαs (stimulating) and Gαi (inhibitory) protein activation (307), were linked to familial, male-limited, precocious puberty, no phenotype is observed in females. No LHβ mutations are known to cause excessive LH activity.

Polycystic ovary syndrome

PCOS is a common endocrine disorder affecting 5-20% of women worldwide and defined as the coexistence of at least two out of three typical features: polycystic ovaries, ovulatory dysfunction and hyperandrogenism (308). Other symptoms may occur in association with the disease, such as type-2 diabetes, metabolic syndrome, adrenal dysfunction, obesity and/or insulin resistance (309). Since PCOS has likely maintained an overall constant prevalence over centuries, albeit being linked to anovulatory subfertility, it has been addressed as an evolutionary paradox (310,311). *Intralocus* sexual conflict was proposed to explain the persistence of genetic *loci* linked to increased reproductive success in males in conjunction with a risk of developing the disease in females (279,312). In fact, PCOS has a polygenic nature and genome-wide association studies (GWAS) found both *FSHR* and *LHCGR* genes to be two of several hot spots for the disease (313,314), suggesting the relevance of gonadotropin signaling in its pathogenesis. However, endocrine disrupting chemicals modulating sex hormone-dependent signals might be associated with the disease (315). Hyperandrogenism is a major determinant of the disease and is indicative of excessive androgens produced by theca cells, exposed to relatively high LH levels. Moreover, androgen and estradiol response to FSH stimulation is higher in PCOS than in healthy women, suggesting that molecular mechanisms regulating paracrine signals between granulosa and theca cells are amplified in individuals carrying the disease (316). As a consequence of high estrogen levels, the feedback mechanism regulating pulsatile gonadotropin production is altered in PCOS women, resulting in low FSH levels, a high LH:FSH ratio and impairment of follicle selection and ovulation (317). In humans, cyclic gonadotropin production recover following treatment with the estrogen receptor antagonist clomiphene citrate, which temporarily restores proper feedback mechanisms at the pituitary level (318). Polycystic ovaries and adrenal disturbances are recapitulated in the phenotype of transgenic, LH-overexpressing female mice, supporting the role played by this hormone in PCOS pathogenesis (319). Interestingly, hCG-overexpressing female mice displayed a slightly different ovarian phenotype, characterized by multiple *corpora lutea* and enhanced estradiol, progesterone and testosterone levels, along with prolactin-linked adenomas (7). Different phenotypes of female mice overexpressing LH and hCG strongly support the view of a different *in vivo* action of these two gonadotropins in rodents.

The polycystic ovary appearance is one of the characteristics which may be used for the diagnosis of PCOS and consists of the recruitment of several follicles reaching the antral stages without completing maturation. Provided that *LHCGR* is of central importance for the understanding of the disease, theca cell androgenic functions and granulosa cell proliferation are modulated by different genes (320), such as *DENND1A*, *INSR* and *RAB5B*, whose functions are involved in modulating proliferative signals through the activation of AKT- and ERK1/2-pathways. The activity of these kinases is increased in the ovary of PCOS women (321) and this picture is compatible with exaggerated LH-, as well as estrogen-dependent stimuli. Interestingly, SNPs falling within the *LHB* gene may be linked to increased PCOS

risk as well. This is the case of the two SNPs inducing a tryptophan to arginine change at position 28 and an isoleucine to threonine change at position 35 of LHβ (322), which are in linkage *disequilibrium* and might contribute to elevated testosterone levels in Brazilian PCOS women. Moreover, it was suggested that the V-LH variant might have been a contributing factor in the development of the disease in a Japanese woman (323), although another study failed to find a similar association in Turkish women (324). This issue should be further investigated and independently confirmed in other populations, it is nonetheless suggestive of altered functioning of proliferative and androgenic LH activity. Most importantly, the glycosylation profile of LH molecules may change depending on steroid hormone levels, influencing gonadotropin activity (325). Although the molecular mechanism underlying the control of biologically active LH isoform production by the pituitary is unknown, age-specific profiles of LH glycosylation were reported among PCOS women. Notably, mainly alkaline LH species were found in adult PCOS patients (326), while basic isoforms featuring high *in vitro* activity were predominant among adolescent girls affected by the disease and positively correlated with androgen levels (327). It is plausible that highly bioactive LH isoforms result in elevated androgen levels and this hypothesis is consistent with the inhibition of steroid synthesis by GnRH antagonists in PCOS women (328).

Given their chronic overexposure to "LH activity", PCOS women require particular attention when ovarian stimulation by exogenous gonadotropins is required in the framework of ART. Coherently with the presence of high estradiol levels and the development of excess antral follicles, which may lead to excessive response to gonadotropin stimulation (316), PCOS women may have an increased risk of developing ovarian hyperstimulation syndrome (OHSS) and multiple pregnancies in ART (329). Thus, ovulation induction with a GnRH agonist has been proposed as a better choice than hCG (330,331), consistent with the high steroidogenic potential of the choriogonadotropin, which may be linked to OHSS. However, the matter is still under debate and the clinical symptomatology spectrum related to PCOS together with ovarian morphology may be a determinant for response to hCG exposure (332), even in pregnancy, when levels of endogenous free hCGβ molecules differ between healthy and PCOS women (333). Interestingly, a clinical study comparing ART performance between Caucasian and Yoruba women from Western Nigeria, triggered by hCG, revealed higher estradiol levels and prevalence of symptoms typically related to polycystic ovarian syndrome among African females, who are more exposed to an increased risk of OHSS and twin pregnancies. This finding suggests that ethnicity could be a determinant for PCOS. The anovulatory condition linked to the disease may lead to maintenance of follicular reserve for a longer time as compared to healthy women and this concept is corroborated by the converging effects of genes regulating the age of menopause and LH levels (334). PCOS could therefore be viewed as an evolutionary strategy to prolong the fertile window at the cost of a decreased number of ovulatory cycles (335). Although suggestive, this hypothesis should be discussed further, since no evidence of evolutionary advantages was experimentally demonstrated and subfertility linked to PCOS may result in significant effects in modern times. Today, especially in Western society, fertility and pregnancy are sought at a relatively late reproductive woman age (20) and ovarian stimulation may be performed in the framework of ART using commercial hormone preparations, a setting quite different from a natural cycle in a non-medicalized society.

Miscarriage

Most miscarriages occur within the first trimester of pregnancy and are mainly due to placental or fetal abnormalities. Embryo aneuploidies are typical causes of miscarriage (336) and other clinical conditions, such as diabetes and obesity may be risk factors for poor pregnancy outcomes (337). These data point out the importance of proper metabolic and endocrine function in regulating embryo implantation and development. The action of LH is

central in follicle growth and uterus preparation, while hCG is required to support pregnancy, and both these aspects impact pregnancy success. A dual function might be assumed for LH action: it is linked to estrogen-mediated proliferative events in the uterine epithelial cells during the follicular phase, resulting in increased endometrial thickness, while it becomes mainly steroidogenic during the luteal phase, when the increase in progesterone levels is associated with the secretory action in the uterus. These changes of steroidogenic potential are accompanied by the 1 to 100-fold increase of *LHCGR* expression, from the early antral stage up to ovulation (52), and are very intense in the *corpus luteum* (338). Antral follicle growth requires proliferative signals preferentially and directly exerted by LH, as demonstrated *in vitro*, and estradiol, which increases *via* the cAMP/PKA-pathway together with receptor number. In fact, estradiol may be used as a marker for successful pregnancy outcome (339). The maintenance of large LHCGR numbers in the *corpus luteum* reasonably predisposes later, massive progesterone synthesis induced by LH and, in case of pregnancy, hCG. Thus, LH and hCG should ensure proper progesterone levels which, in turn, mediate events preparatory to and supportive of pregnancy. Indeed, women undergoing recurrent miscarriage may achieve better pregnancy outcomes through progesterone supplementation (340). These data illustrate the role of both LH and hCG in endometrial preparation and maintenance of *corpus luteum* through adequate steroid production. It is reasonable that endometrial preparation by LH addition in ART cycles may provide adequate stimuli leading to lower pregnancy loss (341), while hCG could not provide similar activity and leads to different effects in pregnancy outcomes (11). As the hormone of pregnancy, hCG exerts its specific functions during the first trimester, resulting in angiogenetic effects and increased progesterone synthesis. These roles are irreplaceable, as demonstrated by reports describing the association between recurrent miscarriage and mutations of the hCGβ-encoding genes, which impair signal transduction and alter the biochemical properties of the molecule (281). Notably, in women undergoing successful pregnancy, serum and urinary levels of the hormone are different, compared to those measured in the case of miscarriage (342). Thus, specific hCG functions and support of embryo development also depends on hormone levels.

Cancer

Since LH and hCG may activate intracellular signaling cascades regulating cell proliferation and anti-apoptotic events, it was suspected that they might retain tumorigenic potential and be involved in cancer formation *via* LHCGR-induced signals (343). As a matter of fact, mouse models revealed that tumorigenesis at gonadal and extra-gonadal sites is related to excessive gonadotropin levels (254). Similar conclusions are suggested by *CGB* gene mutations, leading to aberrant hCG production and linked to gestational trophoblastic neoplasia (344). An interesting case-report provided evidence of hCG-dependent tumorigenesis: a woman under chemotherapy due to metastatic renal cell carcinoma who became pregnant during the treatment break period developed a dramatic growth of the tumor (345). This event was consistent with hCG production and LHCGR expression in cancer cells, while clinical abortion coincided with rapid tumor regression, suggesting that the angiogenic potential of high hCG was proportional to hormone levels. On the other hand, the relationship between cancer cell growth and production of hCGβ and hCG-H molecules would be suspected (346), since they are the major isoforms produced over the first weeks of pregnancy. Their tumorigenic potential may rely on ERK1/2- and AKT-pathways, which is surprising since these pathways are preferentially activated by LH rather than hCG. Overall, hyperglycosylated hCG molecules were suspected to induce proliferative signals required to enhance maternal immune cell modulation, embryo implantation and trophoblast invasion at the early pregnancy stages (347). *In vitro* findings confirmed the proliferative potential of hCGβ and hCG-H (348). These data are consistent with the hypothesis that hCGβ and hCG-H exert different intracellular signaling than the “classical”, dimeric hCG (343), and that their

action should be focused on activation of cAMP/PKA-mediated steroidogenic signals (6,227) fundamental for massive progesterone production during pregnancy. This molecular mechanism could be the basis of a protective effect against breast cancer (349), since relatively high intracellular cAMP levels may trigger the activation of apoptotic processes in certain cell types (350,351), including breast cancer cells (352) and testicular germ cell tumors (162). Moreover, high LHCGR expression levels may result in increased sensitivity to hCG-induced pro-apoptotic signals, suggesting that receptor levels are a prognostic value, at least in some ovarian cancers (353). On the other hand, hCG steroidogenic potential may be transposed *in vivo* as increased production of estrogen metabolites and growth factors displaying pro-angiogenic and proliferative activity (354), thus indirectly inducing variable effects, depending on the enzymatic *milieu* of target cells.

"Overload" of steroidogenic, LHCGR-induced signals might also be provided by excessive LH activity. The lesson of mouse models overexpressing human LH proves that gonadotropin tumorigenic potential is a result of hyperstimulated ovaries, which are induced to produce high estrogen levels (355). Similar results were observed in hCG-overexpressing mice (356), demonstrating that, in rodents, high levels of both human gonadotropins produces comparable steroid-dependent effects *in vivo*, as suggested using mouse Leydig cells *in vitro* (8). Interestingly, gonadectomy of certain mouse strains is linked to Lhr- and estrogen-independent adrenal tumors, which are phenotypically different to those developed in mice overexpressing LH, hCG or Fshr (357,358), suggesting that the adrenal gland may be a target of gonadotropin action (359). These data are suggestive of molecular mechanisms putatively linking adrenal tumors and high LH levels encountered during human menopause. A gonadotropin-responsive adrenocortical adenoma was reported in a menopausal woman, featuring relatively high testosterone levels in response to stimulation of cancer cells by endogenous LH (360). Similar effects may arise in LHCGR-overexpressing adrenal tumors found in some cases involving menopausal and pregnant women (361). Large amounts of receptors would trigger an aberrant activation of the Wnt-pathway by binding endogenous gonadotropins, stimulating differentiation of adrenocortical cells, and similar tumorigenic effects may be due to activating LHCGR mutations in males, providing high basal cAMP and inositol phosphate levels (362). In this case, both excessive testosterone synthesis and proliferative signals may be linked to Leydig cell tumors as well as prostate cancer, and downregulation of the HPG axis by GnRH antagonists may be applied as clinical treatment to counteract aberrant cell growth (363).

In conclusion, causality between gonadotropins and cancer is not sufficiently supported by the scientific literature. Tumorigenesis may rather be linked to the action of steroids, which, in certain cases, are synthesized by tumor cells and induce proliferative events. On the other hand, cancer cells may display aberrant transcription of gonadotropins and their receptor genes, as well as other hormones and receptors (364) such as steroids, GnRH, growth factors, etc. In particular, the presence of a specific receptor expression *per se* may not be indicative of cell function, since the opposite effects may depend on quantitative expression of GPCRs (210). Given the tumor-specific nature of the expression of these factors, the resulting effects are unpredictable. The complexity of tumor cell metabolism provides a unique picture which should be evaluated on a case-by-case basis, and specific treatments entrusted to clinicians' decision.

VII. Clinical applications

Gonadotropins are clinically employed in both sexes when endogenous production is impaired, as in the case of HPG disruption. Indeed, in hypogonadotropic (e.g. secondary or central) hypogonadism, gonadotropin administration represents the most physiological therapeutic approach. The administration of steroids, i.e. estradiol/progesterone in women or

testosterone in men, is preferred in selected cases. However, when the physiological HPG activity is to be restored, and the direct stimulation of the gonads is necessary, gonadotropin administration becomes mandatory. This therapy consists of daily or weekly subcutaneous/intramuscular injections of biological compounds, more expensive than steroids and cumbersome for the patient. Despite these challenges, this is the only therapeutic approach, which can mimic the complex balance of gonadotropin stimulation of the gonad.

Although the most frequent gonadotropin application is in controlled ovarian stimulation (COS) for ART, this approach remains largely empirical and not sustained by strong scientific evidence. Moreover, the business related to this treatment makes the current pharmacological schemes for COS widely industry rather than science-driven. There was no real scientific interest in establishing the best gonadotropin combination in clinical practice during the last few decades. However, hypogonadotropic hypogonadism (HH), characterized by the lack of endogenous gonadotropin secretion, represents the best *in vivo* model to compare different actions of gonadotropins, evaluating their kinetics and efficacy.

In this chapter, we assess the current LH/hCG biological compounds and their clinical applications.

Urinary and recombinant preparations

LH and hCG are both available as registered drugs (Table 4). Their biological activity (i.e. calibration) is evaluated by *in vivo* bioassays, assessing the gonadotropin effects in living rats or mice (236). The "Van Hell" bioassay is the standard method in pharmacopeia to assess gonadotropin bioactivity (165). This method, developed in the 1960s, is based on daily subcutaneous injection for 5 days of a fixed gonadotropin dose in 21-28 day-old immature male rats until the final measurement of seminal vescicles weight gain (165). Both LH and hCG are calibrated using the "Van Hell" method, comparing their bioactivity to an International Standard (365). This method shows two main limitations. First, the animal model used is unable to discriminate between LH and hCG. Second, this bioassay evaluates gonadotropin steroidogenic activity and not the full spectrum of molecular actions (366). Indeed, mouse Leydig cells were recently used *in vitro* to detect biological differences between LH and hCG. In addition to the qualitative differences previously demonstrated in human granulosa cells (6,228,229), LH and hCG resulted in quantitatively different early intracellular actions of cAMP/PKA pathways and steroidogenesis (8). These results suggest that the "Van Hell" method might not evaluate gonadotropin bioactivity correctly, and certainly not fully since it only evaluates testosterone dependent endpoints (8,367). Thus, the calibration of LH and hCG compounds by the "Van Hell" bioassay does not consider the differences in the molecular action described above.

[TABLE 4]

Historically, the first gonadotropin isolation dates back to the third decade of the last century, when two compounds, with FSH and LH activities, were extracted from the urine of pregnant and postmenopausal women and named Prolan A and B, respectively (368). Only in the 1950s were the early gonadotropin compounds produced, first from human pituitaries then from urine when the human menopausal gonadotropin (hMG) was purified from postmenopausal women (369). These first compounds showed a FSH:LH activity ratio of 1:1, ensured by both LH molecules and supplementation of hCG derived from the urine of pregnant women. However, a large amount of impurities was detected, consisting of LH subunits, growth factors, glycoproteins, binding proteins and immunoglobulins (370). All these residues caused high batch-to-batch variability and could influence hMG biological action, reducing efficacy and exposing patients to possible adverse events (370). The urinary purification process was subsequently improved, eliminating all residual extraneous activities, but leading to progressive LH loss. In order to maintain the FSH:LH ratio 1:1, LH activity was replaced by urinary hCG (371). Thus, in highly purified (HP)-hMG compounds,

the impurities percentages dropped to 30%. hCG molecules represent 95% of the remaining protein content (372). Hence, hMG compounds with highest purity were defined as those containing the lowest amount of LH and the highest hCG concentration (372). However, hCG molecules added to hMG are calibrated *in vivo* in rodents against an LH standard. Thus, the amount of hCG in the final compound is the number of molecules capable of producing a biological effect equivalent to that of the LH standard. This process depends largely on half-life and does not consider the molecular differences between LH and hCG. Therefore, the number of hCG molecules in the final preparation could be significantly lower than the number of LH molecules needed to obtain the same biological action with a disequilibrium, in molar terms, between receptor and ligand. This concept must not be understated since the different number of LH/hCG molecules competing with the same number of receptors could be relevant in terms of LHCGR occupancy and activation (169).

Overall, while FSH is easily obtained by urine purification, LH is lost during the chromatography steps, reducing the efficiency of obtaining urine-derived LH preparations. In contrast, u-hCG preparations were readily developed because of the abundance of hCG in the urine of pregnant women (373). Thus, urine-derived biological compounds containing LH alone were not available and until the advent of DNA technologies, only u-hCG compounds have been used to obtain LH activity in clinical practice. Recombinant DNA technologies led to the production of gonadotropins in CHO cell lines, with high rates of safety and consistency. Currently, six recombinant gonadotropins are available: follitropin α, follitropin β, follitropin γ (r-FSH), lutropin α (r-LH), choriogonadotropin α (r-hCG) and chorifollitropin α (374). r-LH and r-hCG possess greater purity as compared to the urine-derived counterparts, and consist of a mixture of isoforms exhibiting a high degree of glycosylation heterogeneity, structurally and biologically comparable to endogenous gonadotropins, with slight differences due to post-translational modifications (366).

Alongside classical gonadotropins, recombinant DNA technology allows for the creation of new chimeric compounds, e.g. long acting FSH (chorifollitropin α) (375). In this case, the FSH β subunit was coupled to the carboxyl-terminal part (CTP) of the hCG β subunit (376,377). Chorifollitropin shows *in vitro* and *in vivo* pharmacological activity comparable to r-FSH, but with longer half-life (about 65 hours) (378). This in an interesting example of how DNA technologies could mix several gonadotropin features in the same compound.

Different clinical effects: ovarian stimulation, luteal support and other aspects

The gonadotropin administration in COS during ART is the current *in vivo* model in which the different clinical effects of biological compounds containing LH and hCG can be evaluated. In this setting, although a truly standardized approach does not exist, FSH administration is always provided to obtain multifollicular growth (379). In addition to this, LH or hCG administration may be added to support FSH action, mimicking the physiological, concerted action of LH and FSH. Indeed, LH physiologically regulates follicular growth, stimulating theca cell production of androgens, which, in turn, serve as a substrate for estradiol production in granulosa cells (380). In the late follicular phase, the estradiol rise acts through a positive feedback causing the LH surge needed for ovulation (381). Finally, LH stimulates progesterone production from the *corpus luteum*, which is then maintained by hCG if pregnancy occurs. To mimic this complex gonadotropin-mediated process during COS, regulatory agencies allow the addition of LH, hCG or hMG to FSH, since they are still considered equivalent in clinical terms. No specific guidelines are available to select which patient may benefit from and should be treated with a specific combination: the selection remains largely empirical, if nor arbitrary.

In contrast, hCG is generally used at the end of the COS phase, to trigger final oocyte maturation. Physiologically, however, it is LH that is responsible for final follicular maturation and follicle rupture with oocyte expulsion from the follicle (382). Despite this

difference between COS protocols and physiology, mainly due to the historical availability of hCG but not of LH, the final ART outcome did not change when u-hCG or r-hCG were compared to LH (382). Thus, no specific evidence supports the use of LH instead of hCG for triggering ovulation. In 1979, the use of a GnRH agonist to induce an endogenous LH surge sufficient to trigger ovulation was proposed (383). This alternative to hCG was not exploited until the use of GnRH antagonists for pituitary down regulation in COS, with a shorter and reversible action compared to the agonists (384,385). Thus, under GnRH antagonists, the pituitary gland remains responsive and a single GnRH agonist bolus is sufficient to displace the antagonist, activate the receptor and induce the endogenous LH release, mimicking the physiological midcycle gonadotropin surge (383). This surge consists of a short ascending limb of about 4 hours and a long descending limb of about 20 hours (386). This pattern is slightly different from the physiological midcycle surge, which lasts 48 hours and shows three consecutive phases (387). Despite GnRH agonist triggering could be more physiological and could reduce the adverse event risk (388,389), neither large retrospective studies (390) nor randomized clinical trials (RCTs) (391,392) found differences between GnRH agonists and hCG for triggering ovulation. Thus, specific evidence favouring one or another ovulation trigger scheme are not available, considering the final ART outcome. In particular, specific hCG actions are obtained with this treatment, such as sustained progesterone production, angiogenesis promotion, blood flow and nutrition to the fetus (149), umbilical cord development and uterine growth synchronization (380). Thus, hCG substitution by LH or GnRH agonist for triggering results in the loss of all these essential hCG activities after fertilization. As a consequence, when GnRH agonist trigger is used, an appropriate luteal support with progesterone and estradiol should be considered (393). While this issue is not fully clarified and needs further investigation, it is established that luteal support with progesterone is used in all cycles. Some authors proposed a modified luteal support when GnRH agonist trigger is chosen, using hCG either in one, 1500 IU single bolus (394) or in repeated 250-500 IU boluses (395), or with LH addition (396). However, a recent meta-analysis showed that the GnRH agonist trigger use, together with a luteal support by LH activity, leads to similar ART outcomes compared to clinical protocols with hCG trigger (388). This analysis showed a notably high heterogeneity in the results, limiting its clinical significance.

COS represents the best *in vivo* example in which gonadotropin combinations are used in pharmacological doses, although the various protocols are neither based on physiology nor on sound scientific evidences. Obviously, LH and hCG are interchangeable in this "unphysiological" context and it is surprising how well ART works in spite of sometimes disparate and "creative" stimulation protocols. It is difficult to draw physiological conclusions about LH and hCG from the supra physiological setting of COS. HH should represent a better model to understand the different efforts *in vivo* of LH and hCG, if they exist.

Clinical experience with LH and hCG: the present and the future

LH activity is needed in clinical practice in the management of both female and male hypogonadism when estrogen and androgen production should be stimulated through the direct action on theca and Leydig cells, respectively. Although both LH and hCG are currently commercially available, historically, only hCG has been used, as it was the only readily available LHCGR ligand. In theory, both LH and hCG might be used now in spite of the complete lack of scientific evidence in favor of one versus the other. This is true in many countries for female hypogonadism, but not in males, in whom only hCG administration is permitted by regulatory agencies. It is widely demonstrated that hCG is efficient at restoring eugonadism in HH men. Indeed, in the case of male hypogonadal hypogonadism, the standard therapeutic approach has been based on hCG administration since the 1950s (397).

In this setting, convincing evidence about the efficacy of hCG administration is available, considering different dosages and schemes, ranging from 1000 IU every other day (398) up to 5000 IU two times weekly (399). However, the reason for hCG administration being preferred to LH in clinical practice resides in the historic availability of commercial preparations, rather than systematic, evidence-based demonstrations. Indeed, no clinical trials have compared LH to hCG in male HH, thus far. Only a single case report is available in the literature, comparing a daily low hCG dose (75 IU) to r-LH (75 IU daily) administration in a man with HH following surgery for a pituitary adenoma (234). In this man, either hCG or r-LH restored eugonadism without exhibiting a significant difference. A careful comparison of the *in vivo* action of LH and hCG in HH men is needed. In 19 healthy men under pituitary suppression by a GnRH antagonist, it was demonstrated that a daily LH dose of 112.5 IU restored testosterone levels to the normal range (400). This trial showed that there were no differences when comparing bolus to pulsatile administration, suggesting that the physiological pulsatile LH secretion pattern is not strictly needed to obtain Leydig cell stimulation (400). These two examples indicate that low doses of LH may be sufficient to stimulate testicular androgen production, to an extent similar to that obtained by apparently much higher doses of hCG (5000 IU/weekly for hCG vs 787.5 IU/weekly for LH), revealing that the dosage of LH necessary to stimulate physiological testosterone production might be much lower than expected. If so, this would clearly indicate a differential action of LH and hCG *in vivo*, in human males. Clinical studies with LH in HH male are urgently needed to explore this.

In women, the main LH clinical application remains COS during ART. Several trials evaluated different ART outcomes using LH instead of hCG to support follicular growth (Figure 5) (11). Despite their large number, these studies show high heterogeneity and low quality, hindering the development of standard protocols based on scientific evidence. Thus, the gonadotropin stimulation during COS, claimed to be “personalized” by ART doctors remains a peculiar example of personalized medicine, in that personalization is not based on selection of the appropriate stimulus according to objective criteria, but rather on the “personal” beliefs of the prescriber. This challenge is further complicated by the increasing number of young infertile couples seeking ART and the corresponding cost of gonadotropins and procedures, which leaves the pharmacological approach widely industry-driven.

In this confusing setting, several clinicians proposed LH activity addition to FSH during COS. This relatively new combination is widely debated in the literature, in particular regarding poor responders and women of advanced age (401–403). Available evidence does not clearly support the hypothesis of increased pregnancy rates using LH combined with FSH in unselected women (404), and the number of published meta-analyses almost outweigh the number of RCTs available on this topic, suggesting the difficulty to design an objective meta-analysis to identify the best COS approach, distinguishing the real benefit of one treatment compared to another (403). Apart from the paucity of adequately powered studies and the extremely high heterogeneity of treatments used, this topic is weakened by the heterogeneity of endpoints evaluated in RCTs (403). Indeed, the vast majority of RCTs, as well as of meta-analyses, focused on the ART clinical outcome. However, when the effectiveness of LH/hCG addition is the topic of investigation, the first endpoint to be evaluated must be the ovarian response, i.e. the first measurable parameters of gonadotropin action. Pregnancy and live birth rates, on the contrary, are final results of ART and are influenced by an increasing number of factors (e.g. sperm contribution, endometrial receptivity and implantation, placenta, etc…). A recent meta-analysis combined the results available in the literature, considering all gonadotropin combinations and all ART outcomes, demonstrating that FSH alone obtains greater oocyte numbers compared to hMG or FSH + LH (11). The LH activity addition is useful to reduce the amount of FSH needed and to improve oocyte quality, but

only when LH is used rather than hCG. This could be a further suggestion that, by reducing the FSH doses used, FSH activity in granulosa cells is shifted from pro-apoptotic to proliferative pathways, like those activated by LH (200,228,229). Only 5 studies directly compared LH to hCG, however, none clearly reported the hCG dosage combined with FSH, thereby preventing any estimation of the real exposure of the ovary to LH/hCG in terms of molar relationship between LH or hCG and the LHCGR.

With the limitation of the pharmacological rather that physiological setting, these results suggests that it is difficult to establish the correct amount of LH to be used in clinical practice, which might be different depending on the clinical setting (HH or COS) and gender. In addition, considering the difficulty of measuring *in vivo* LH bioactivity using approved bioassays, the debate remains fully open. The correct appraisal of the clinical application of LH should probably combine *in vitro* and *in vivo* demonstrations.

[FIGURE 5]

Complications of LH and hCG therapy

In the evaluation of possible clinical applications of LH and hCG, adverse events must also be considered. The most relevant iatrogenic COS adverse event remains OHSS, which is the final result of an exaggerated ovarian response (405). It rarely occurs when ART is applied to unselected women (from 1 to 8%), but its incidence increases when high-risk women are treated (405). This risk is associated with low body mass index, young age, high estradiol serum levels, high interleukin concentrations, high vascular endothelial growth factor (VEGF) and renin-angiotensin system activation, although specific predictive parameters are not available so far (406). Moreover, either the elevated estradiol serum levels obtained at the day of ovulation or the large number of follicles developed during COS could predispose to OHSS (406). These OHSS predisposing conditions could be related to gonadotropin action and should be carefully evaluated to predict the occurrence of adverse ART events.

hCG, rather than LH, is generally used to trigger ovulation. Besides differences at the molecular level, LH and hCG show different circulating half-lives (389). The sustained luteotropic activity induced by hCG could lead to side effects through the release of vasoactive substances (e.g. VEGF) and prostaglandins acting directly on the ovarian follicles. These direct and indirect effects could result in OHSS (389). Triggering ovulation with a GnRH agonist instead of a classical hCG bolus seemed to reduce OHSS risk (407,408). A recent meta-analysis claimed a reduced OHSS rate in GnRH agonist- compared to hCG-triggered cycles, although no statistical significance was reached (388). These considerations confirm the lack of clear knowledge of OHSS pathophysiology. In order to enhance knowledge of underlying pathophysiological mechanisms and to reduce the incidence of this adverse event, the two different clinical OHSS presentations should be considered separately (405). First, early OHSS occurs within 9 days after hCG administration as a final, prolonged hCG effect on already stimulated ovaries. This OHSS clinical picture could probably benefit from the replacement of the hCG trigger by a GnRH agonist. Late OHSS occurs more than 10 days after triggering, representing the ovarian response to the endogenous hCG rise after fertilization (405). This late complication does not benefit from hCG substitution and further research is needed to better understand this condition and its prevention.

Alongside OHSS, other clinical COS complications must also be considered. Among these, the most frequent adverse effect is the cycle cancellation, which occurs in 11.5-17.4% of women of advanced age (409). This event leads to important socio-economic consequences due to the need to repeat COS, further exposing women to OHSS risk and increasing the economic burden. The main reason for cycle cancellation remains an inadequate response to gonadotropin stimulation. This probably results from both the lack of standardization in COS protocols and the high heterogeneity of clinical responses. The only

chance to reduce the cycle cancellation rate is a truly, evidence-based personalization of COS schemes, which requires rigorous clinical studies.

In HH men, the only adverse event that must be considered during hCG/LH treatment is excessive serum testosterone levels, with possible negative effects on red blood cells, liver and prostate gland. However, this effect is the result of androgen action on sensitive tissues rather than a direct consequence of hCG administration. It will be interesting to explore whether HH treatment by LH rather than hCG will be able to reduce the rate of adverse events. Similar conclusions may be applied to pharmacological treatment of cryptorchidism. The pathology is identified by absence of one or both undescended testes to the scrotum and is linked to germ cell loss, infertility and increased testicular cancer risk (410). Descent of testes may be induced by hCG administration or by surgery. Interestingly, treatment of cryptorchidism with hCG was associated with germ cell apoptosis and impaired reproductive function in the adult (411). This effect is consistent with the cAMP/PKA-dependent pro-apoptotic potential of hCG and might plausibly be dampened by administering LH instead of choriogonadotropin. However, the use of LH as a treatment for cryptorchidism has never been reported.

VIII. Beyond reproduction: effects of hCG on thyroid and adrenal glands

Thyroid hormones and TSH are involved in metabolism and development regulation, and increasing evidence highlights a role for these molecules even in reproduction (412), revealing cross-talk between the gonadal and thyroid axes in vertebrates (413). High structural and biochemical similarities between the gonadotropins and TSH (414), as well as their receptors, underlie such interactions. Above all, the binding affinity of LHCGR and TSH receptor (TSHR) for their respective ligands relies on amino acidic residue at positions 171-260 of the extracellular domains. These were considered to lead to chimeric receptors for hormone cross-interaction studies in the past (415). hCG-TSHR chimeras revealed 13-fold cAMP increases in transfected HEK293 cells and the effect was mimicked by high concentrations ($1.0x10^4$-10^5 ng/ml)(416) of hCG acting on wild-type TSHR *in vitro*, demonstrating cross-activation of the canonical cAMP/PKA-pathway (417), along with iodide uptake and tri-iodothyronine secretion in cultured human thyrocytes (418). An *in vitro* comparison between LH and hCG using a CHO cell line permanently expressing the *TSHR* cDNA (CHO-JP09 cells), revealed that LH is more potent than hCG in inducing intracellular cAMP increase and parallel line analysis demonstrated the overall equivalency of 1.0 µM hCG = 0.1 µM LH = $0.1x10^{-3}$ µM TSH (419). Interestingly, a mutant hCG lacking the CTP fragment displayed similar potency to LH, suggesting that the C-terminal peptide might act as a protective factor to prevent hyperthyroidism due to hCG cross-reactivity during pregnancy, when circulating concentrations of this hormone are extremely high. On the other hand, both LH and hCG are able to displace TSH bound to its receptor, thereby revealing an unexpected, about 162-fold greater thyrotropic activity in LH than hCG, equivalent to 44.0 and 0.3 µIU of TSH activity per 1.0 IU of LH and hCG, respectively (420). The fact that, in CHO-JP09 cells, enzymatic digestion of hCG resulted in deglycosylated and/or desialylated molecules with higher potency in activating cAMP compared to classical hCG (421), is suggestive of a plausible evolutionary conservation of the TSH dependence of thyroid function during the early weeks of pregnancy, and similar results were obtained with hCG preparations from patients with trophoblastic disease. These studies supported the concept of thyrotropic action of gonadotropins, especially hCG, which reaches relatively high serum levels during pregnancy or in patients affected by hCG-secreting tumors. It is indeed well known that the rise of hCG levels over the 9th-18th weeks of pregnancy matches the fall of TSH levels and sustains continuous thyroid hormone production (422): this likely depicts the dual function of hCG consisting of negative feedback exerted through thyroid hormones at the pituitary while

supporting thyroid function. These effects are emphasized during twin pregnancies, which features prolonged, higher hCG levels than single pregnancies, as well as more frequently increased free thyroxine and suppressed TSH levels (423). On the other hand, insufficient thyroid stimulation could result in low thyroid hormone levels and may lead to spontaneous abortion (424). Taken together, the metabolic regulation by thyroid hormones might be, in part, dependent on hCG during pregnancy and may be essential to support fetal development.

Since TSHR and thyroid hormone receptors are expressed in endometrial cells, their ligands might be involved in the physiological regulation of this tissue (425), although clear-cut evidence remains to be found. Given the relatively high potency of LH in inducing TSHR-mediated cAMP production *in vitro* (419,420), a role for gonadotropins exerted at the level of the female reproductive system through the TSHR cannot be excluded. In fact, it is noteworthy that LH and TSH release by pituitary seem to be synchronized, at least during the menstrual cycle, largely measurable as a serum concentration peak of both hormones (426). Also, it is known that hypothyroidism before puberty leads to delayed sexual maturity, implying thyroid hormone action on gonadal functions (427). On the other hand, promiscuity of endocrine signals was demonstrated even in simple vertebrates, such as fish (29), suggesting that it is a conserved feature regulating certain physiological functions. It is not surprising that hypo- and hyperthyroidism are associated with menstrual disturbances, anovulation and subfertility in females (428). The cross-talk between thyroid and gonadal functions is anyway far from being thoroughly elucidated. The relatively recent discovery of thyrostimulin (429), a gonadotropin-like molecule acting on the TSHR, and considered the most ancestral glycoprotein hormone sheds new light on ovarian functioning. The production of thyrostimulin by oocytes has been described, suggesting that this molecule, rather than TSH, may act as a paracrine factor in the ovary by binding TSHR expressed in granulosa cells (430), where it would induce proliferative signals simultaneously activating cAMP/PKA-, ERK1/2- and AKT-pathways (431). Although suggestive, these findings should be confirmed by clinical evidence, required to understand the whole picture of the thyroid-gonadal interaction.

Interaction between LH/hCG and the adrenal gland was described in healthy women during the menopausal transition, as an effect linked to increasing serum LH concentration, and during the first trimester of pregnancy, due to hCG production. The adrenal gland intriguingly shares a common developmental origin with gonadal cells (432): it is characterized by different types of cells secreting mainly glucocorticoids, as well as androgens and a small amount of estrogens. During the menopausal transition, the lack of progesterone negative feedback to the pituitary, occurring together with arrest of follicular maturation, results in increasing levels of LH molecules acting on LHCGR expressed in adrenal cortical cells (433). The adrenal gland response consists of the synthesis of pregnenolone metabolites obtained *via* the Δ5-steroidogenic pathway (434), mainly dehydroepiandrosterone and androstenediol (433), as well as cortisol (435). Most importantly, chronically elevated LH levels are suspected to be linked to an increased risk of adrenal tumors (435), even though further studies are required to elucidate the impact of LH on adrenal function. As a matter of fact, long-term elevated LH levels induce Lhr expression in mouse adrenal glands, a first step required for initiating cortical tumors (436), and it is possible that such an effect also occurs in humans (437). hCG action in the adrenal gland was mentioned in the "Pathophysiology of LH and hCG" chapter. In certain cases, increased hCG was suspected to be linked to adrenal LHCGR expression during pregnancy, resulting in high cortisol levels and transitory Cushing syndrome (298). However, hCG-stimulated synthesis of adrenal steroids is marginal in healthy women (438) and transient Cushing syndrome is a relatively rare event.

IX. Conclusions

The evolution of different glycoprotein hormones and their receptors accompanied the separation of distinct endocrine axes optimizing endocrine and metabolic functions, which developed independently in vertebrates. Inter-species ligand-hormone binding is possible, depending on the phylogenetic distance of the species, suggesting evolutionary conservation of key amino acid residues fundamental for the preservation of biochemical properties of the proteins. Intra-species interactions between endocrine axes are also possible, due to the common developmental origin of the hormone target cells, or as a means of optimizing certain metabolic functions. However, the complexity of the physiological mechanisms mediating placentation of certain mammals, such as equids and primates, required a more profound regulatory level, making the role of gonadotropins of pituitary and chorionic origin distinguishable. It is plausible that this complexity achieved its maximal expression in humans, where the largest number of genes encoding for LHβ/CGβ molecules likely evolved from a single ancestral one. Although these hormones may exist in more than one isoform and glycosylation variant, each displaying specific biochemical properties, their physiological roles may be separated according to specific functions required for gamete maturation and pregnancy support (Table 5).

[TABLE 5]

LH production is maintained in both males and females during the fertile age, when their functions depend on the specific enzymatic *milieu* of target cells. In women, the known androgenic role of LH is fulfilled by an overall flattened production of androstenedione by theca cells, as the major steroid serving as a substrate to be converted to the proliferative factor, estradiol, by granulosa cells under FSH stimulation. However, both progestational and proliferative LH-dependent effects have been shown to be relevant specifically in granulosa cells by *in vitro* experiments, revealing previously unknown regulatory molecular mechanisms suggesting that the aforementioned metabolic functions are fundamental for proper oocyte maturation. These roles are exerted through a fine-tuned modulation of the balance between the steroidogenic cAMP/PKA-pathway on the one hand, and proliferative/anti-apoptotic signals mediated through ERK1/2- and AKT-pathway activation on the other, thus exhibiting the capability of biased signaling mediated under synergistic modulation exerted by FSH. Hence, female folliculogenesis is assisted by two LH- and one FSH-target cells and characterized by both LHCGR and FSHR expression as a key factor for sustaining reproductive functions. This dual regulatory system might be involved in dampening signaling defects, resulting in overall mild pathological phenotypes, not necessarily leading to failed pubertal development or complete infertility, as in the case of PCOS. LH androgenizing function is maintained in the male counterpart of theca cells, the Leydig cell, where the steroidogenic pathway is driven towards testosterone synthesis as a major product required for male sexual development and reproduction. In this case, the lack of LH function leads to individuals with impaired sexual development, exacerbated by LHCGR inactivating mutations. Defective hCG signaling is naturally linked to pregnancy defects, mainly miscarriage, due to inappropriate progesterone synthesis, while excessive hCG signals may increase the risk of maternal, transitory Cushing syndrome or adrenal tumors. Trophoblast hCG may appear in women when the gonadal steroidogenic machinery is set to produce exclusively progesterone by luteinized ovarian cells, thus not requiring any discrimination between proliferative and steroidogenic signals. This was demonstrated by hCG treatment of granulosa cells *in vitro*, where hCG was not able to reproduce LH-specific proliferative signals but exhibited a relatively high steroidogenic potential. Even more so, LH and hCG treatment of mouse Leydig cells failed to reveal any qualitative differences, revealing the sex-specific nature of LH and hCG signaling. These data underline the significance of LH and hCG application to fertility treatments, especially in females, where

they are classically used as equivalents. This is an oversight caused by misinterpretation of clinical data provided by studies performed in the ART context, where LH- and hCG-specific effects are masked by those following the massive estrogen production induced to achieve multi-follicular development, not normally occurring in humans. The clinical comparison of LH and hCG in HH is needed to clarify many outstanding questions.

Acknowledgements
This work was supported by a grant to the Department of Biomedical, Metabolic and Neural Sciences (University of Modena and Reggio Emilia (Italy) from the Italian Ministry of University and Research in the Departments of Excellence Programme. We thank Eric A. Zizzi, B.Sc., for language editing.

Corresponding author: Prof. Manuela Simoni, Unit of Endocrinology, Dept. Biomedical, Metabolic and Neural Sciences, University of Modena and Reggio Emilia, via P. Giardini 1355, 41126 Modena, Italy. Email: manuela.simoni@unimore.it; phone: +390593961815.

Reprint requests: Prof. Manuela Simoni, Unit of Endocrinology, Dept. Biomedical, Metabolic and Neural Sciences, University of Modena and Reggio Emilia, via P. Giardini 1355, 41126 Modena, Italy. Email: manuela.simoni@unimore.it; phone: +390593961815.

Disclosure summary:
The authors have nothing to disclose

References

1. Ascoli M, Fanelli F, Segaloff DL. The lutropin/choriogonadotropin receptor, a 2002 perspective. *Endocr Rev.* 2002;23(2):141–174.
2. Stockell Hartree A, Shownkeen RC. Studies of human pituitary LH containing internally cleaved beta subunit. *J Mol Endocrinol.* 1991;6(1):101–109.
3. Ward DN, Glenn SD, Nahm HS, Wen T. Characterization of cleavage products in selected human lutropin preparations. A protease-sensitive site in human lutropin beta subunit. *Int J Pept Protein Res.* 1986;27(1):70–78.
4. Lohse MJ, Benovic JL, Codina J, Caron MG, Lefkowitz RJ. beta-Arrestin: a protein that regulates beta-adrenergic receptor function. *Science.* 1990;248(4962):1547–1550.
5. Galet C, Ascoli M. Arrestin-3 is essential for the activation of Fyn by the luteinizing hormone receptor (LHR) in MA-10 cells☆. *Cell Signal.* 2008;20(10):1822–1829.
6. Casarini L, Lispi M, Longobardi S, Milosa F, La Marca A, Tagliasacchi D, Pignatti E, Simoni M. LH and hCG Action on the Same Receptor Results in Quantitatively and Qualitatively Different Intracellular Signalling. *PLoS One.* 2012;7(10):e46682.
7. Rulli SB, Kuorelahti A, Karaer Ö, Pelliniemi LJ, Poutanen M, Huhtaniemi I. Reproductive Disturbances, Pituitary Lactotrope Adenomas, and Mammary Gland Tumors in Transgenic Female Mice Producing High Levels of Human Chorionic Gonadotropin. *Endocrinology.* 2002;143(10):4084–4095.
8. Riccetti L, De Pascali F, Gilioli L, Potì F, Giva LB, Marino M, Tagliavini S, Trenti T, Fanelli F, Mezzullo M, Pagotto U, Simoni M, Casarini L. Human LH and hCG stimulate differently the early signalling pathways but result in equal testosterone synthesis in mouse Leydig cells in vitro. *Reprod Biol Endocrinol.* 2017;15(1):2.
9. Grzesik P, Kreuchwig A, Rutz C, Furkert J, Wiesner B, Schuelein R, Kleinau G, Gromoll J, Krause G. Differences in Signal Activation by LH and hCG are Mediated by the LH/CG Receptor's Extracellular Hinge Region. *Front Endocrinol (Lausanne).* 2015;6:140.

10. Robyn C, Diczfalusy E. Bioassay of antigonadotrophic sera. 2. Assay of the human chorionic gonadotrophin (HCG) and luteinising hormone (LH) neutralising potencies. *Acta Endocrinol (Copenh).* 1968;59(2):261–276.
11. Santi D, Casarini L, Alviggi C, Simoni M. Efficacy of Follicle-Stimulating Hormone (FSH) Alone, FSH + Luteinizing Hormone, Human Menopausal Gonadotropin or FSH + Human Chorionic Gonadotropin on Assisted Reproductive Technology Outcomes in the "Personalized" Medicine Era: A Meta-analysis. *Front Endocrinol (Lausanne).* 2017;8:114.
12. Huhtaniemi IT, Catt KJ. Differential Binding Affinities of Rat Testis Luteinizing Hormone (LH) Receptors for Human Chorionic Gonadotropin, Human LH, and Ovine LH. *Endocrinology.* 1981;108(5):1931–1938.
13. Schöneberg T, Hofreiter M, Schulz A, Römpler H. Learning from the past: evolution of GPCR functions. *Trends Pharmacol Sci.* 2007;28(3):117–121.
14. Policastro PF, Daniels-McQueen S, Carle G, Boime I. A map of the hCG beta-LH beta gene cluster. *J Biol Chem.* 1986;261(13):5907–5916.
15. Cole LA. hCG and hyperglycosylated hCG in the establishment and evolution of hemochorial placentation. *J Reprod Immunol.* 2009;82(2):112–118.
16. Dinopoulou V, Drakakis P, Kefala S, Kiapekou E, Bletsa R, Anagnostou E, Kallianidis K, Loutradis D. Effect of recombinant-LH and hCG in the absence of FSH on in vitro maturation (IVM) fertilization and early embryonic development of mouse germinal vesicle (GV)-stage oocytes. *Reprod Biol.* 2016;16(2):138–146.
17. Sun PD, Davies DR. The Cystine-Knot Growth-Factor Superfamily. *Annu Rev Biophys Biomol Struct.* 1995;24(1):269–292.
18. Bousfield GR, Dias JA. Synthesis and secretion of gonadotropins including structure-function correlates. *Rev Endocr Metab Disord.* 2011;12(4):289–302.
19. Roch GJ, Sherwood NM. Glycoprotein Hormones and Their Receptors Emerged at the Origin of Metazoans. *Genome Biol Evol.* 2014;6(6):1466–1479.
20. Casarini L, Santi D, Marino M. Impact of gene polymorphisms of gonadotropins and their receptors on human reproductive success. *Reproduction.* 2015;150(6):R175–R184.
21. Moyle WR, Campbell RK, Myers R V., Bernard MP, Han Y, Wang X. Co-evolution of ligand-receptor pairs. *Nature.* 1994;368(6468):251–255.
22. Dewey EM, McNabb SL, Ewer J, Kuo GR, Takanishi CL, Truman JW, Honegger H-W. Identification of the Gene Encoding Bursicon, an Insect Neuropeptide Responsible for Cuticle Sclerotization and Wing Spreading. *Curr Biol.* 2004;14(13):1208–1213.
23. Graves J, Markman S, Alegranti Y, Gechtler J, Johnson RI, Cagan R, Ben-Menahem D. The LH/CG receptor activates canonical signaling pathway when expressed in Drosophila. *Mol Cell Endocrinol.* 2015;413:145–156.
24. Uchida K, Moriyama S, Chiba H, Shimotani T, Honda K, Miki M, Takahashi A, Sower SA, Nozaki M. Evolutionary origin of a functional gonadotropin in the pituitary of the most primitive vertebrate, hagfish. *Proc Natl Acad Sci U S A.* 2010;107(36):15832–15837.
25. Huhtaniemi I. A short evolutionary history of FSH-stimulated spermatogenesis. *Hormones (Athens).* 2015;14(4):468–478.
26. Sower SA, Decatur WA, Hausken KN, Marquis TJ, Barton SL, Gargan J, Freamat M, Wilmot M, Hollander L, Hall JA, Nozaki M, Shpilman M, Levavi-Sivan B. Emergence of an Ancestral Glycoprotein Hormone in the Pituitary of the Sea Lamprey, a Basal Vertebrate. *Endocrinology.* 2015;156(8):3026–3037.
27. Li MD, Ford JJ. A comprehensive evolutionary analysis based on nucleotide and amino acid sequences of the alpha- and beta-subunits of glycoprotein hormone gene family. *J Endocrinol.* 1998;156(3):529–542.

28. Buechi HB, Bridgham JT. Evolution of specificity in cartilaginous fish glycoprotein hormones and receptors. *Gen Comp Endocrinol.* 2017;246:309–320.
29. Bogerd J, Granneman JCM, Schulz RW, Vischer HF. Fish FSH receptors bind LH: how to make the human FSH receptor to be more fishy? *Gen Comp Endocrinol.* 2005;142(1–2):34–43.
30. García-López A, de Jonge H, Nóbrega RH, de Waal PP, van Dijk W, Hemrika W, Taranger GL, Bogerd J, Schulz RW. Studies in zebrafish reveal unusual cellular expression patterns of gonadotropin receptor messenger ribonucleic acids in the testis and unexpected functional differentiation of the gonadotropins. *Endocrinology.* 2010;151(5):2349–2360.
31. Aizen J, Kowalsman N, Kobayashi M, Hollander L, Sohn YC, Yoshizaki G, Niv MY, Levavi-Sivan B. Experimental and computational study of inter- and intra- species specificity of gonadotropins for various gonadotropin receptors. *Mol Cell Endocrinol.* 2012;364(1–2):89–100.
32. Julier C, Weil D, Couillin P, Côté JC, Nguyen VC, Foubert C, Boué A, Thirion JP, Kaplan JC, Junien C. The beta chorionic gonadotropin-beta luteinizing gene cluster maps to human chromosome 19. *Hum Genet.* 1984;67(2):174–177.
33. Bo M, Boime I. Identification of the transcriptionally active genes of the chorionic gonadotropin beta gene cluster in vivo. *J Biol Chem.* 1992;267(5):3179–84.
34. Hollenberg AN, Pestell RG, Albanese C, Boers ME, Jameson JL. Multiple promoter elements in the human chorionic gonadotropin beta subunit genes distinguish their expression from the luteinizing hormone beta gene. *Mol Cell Endocrinol.* 1994;106(1–2):111–119.
35. Britten RJ. Divergence between samples of chimpanzee and human DNA sequences is 5%, counting indels. *Proc Natl Acad Sci U S A.* 2002;99(21):13633–13635.
36. Nagirnaja L, Rull K, Uusküla L, Hallast P, Grigorova M, Laan M. Genomics and genetics of gonadotropin beta-subunit genes: Unique FSHB and duplicated LHB/CGB loci. *Mol Cell Endocrinol.* 2010;329(1–2):4–16.
37. Hallast P, Saarela J, Palotie A, Laan M. High divergence in primate-specific duplicated regions: human and chimpanzee chorionic gonadotropin beta genes. *BMC Evol Biol.* 2008;8(1):195.
38. Hallast P, Nagirnaja L, Margus T, Laan M. Segmental duplications and gene conversion: Human luteinizing hormone/chorionic gonadotropin gene cluster. *Genome Res.* 2005;15(11):1535–1546.
39. Maston GA, Ruvolo M. Chorionic gonadotropin has a recent origin within primates and an evolutionary history of selection. *Mol Biol Evol.* 2002;19(3):320–335.
40. Hallast P, Rull K, Laan M. The evolution and genomic landscape of CGB1 and CGB2 genes. *Mol Cell Endocrinol.* 2007;260–262:2–11.
41. Rull K, Laan M. Expression of β-subunit of HCG genes during normal and failed pregnancy. *Hum Reprod.* 2005;20(12):3360–3368.
42. Pennell TM, Morrow EH. Two sexes, one genome: the evolutionary dynamics of intralocus sexual conflict. *Ecol Evol.* 2013;3(6):1819–1834.
43. Chen H, Chen L, Wu Y, Shen H, Yang G, Deng C. The exonization and functionalization of an Alu-J element in the protein coding region of glycoprotein hormone alpha gene represent a novel mechanism to the evolution of hemochorial placentation in primates. *Mol Biol Evol.* 2017;34(12):3216–3231.
44. Fiddes JC, Goodman HM. The cDNA for the beta-subunit of human chorionic gonadotropin suggests evolution of a gene by readthrough into the 3'-untranslated region. *Nature.* 1980;286(5774):684–687.
45. Bousfield GR, Butnev VY, Butnev VY. Identification of twelve O-glycosylation sites in equine chorionic gonadotropin beta and equine luteinizing hormone ss by solid-phase Edman degradation. *Biol Reprod.* 2001;64(1):136–147.

46. Bidart JM, Bellet DH, Alberici GF, Van Besien F, Bohuon C. The immune response to a synthetic peptide analogous to the 109-145 beta hCG carboxyl-terminus is directed against two major and two minor regions. *Mol Immunol.* 1987;24(4):339–345.
47. Mak SMJ, Wong WY, Chung HS, Chung PW, Kong GWS, Li TC, Cheung LP. Effect of mid-follicular phase recombinant LH versus urinary HCG supplementation in poor ovarian responders undergoing IVF – a prospective double-blinded randomized study. *Reprod Biomed Online.* 2017;34(3):258–266.
48. Zeleznik AJ. In vivo responses of the primate corpus luteum to luteinizing hormone and chorionic gonadotropin. *Proc Natl Acad Sci U S A.* 1998;95(18):11002–11007.
49. Wallace WHB, Kelsey TW. Human ovarian reserve from conception to the menopause. Vitzthum VJ, ed. *PLoS One.* 2010;5(1):e8772.
50. Yung Y, Aviel-Ronen S, Maman E, Rubinstein N, Avivi C, Orvieto R, Hourvitz A. Localization of luteinizing hormone receptor protein in the human ovary. *Mol Hum Reprod.* 2014;20(9):844–849.
51. Chappel SC, Howles C. Reevaluation of the roles of luteinizing hormone and follicle-stimulating hormone in the ovulatory process. *Hum Reprod.* 1991;6(9):1206–1212.
52. Jeppesen JV, Kristensen SG, Nielsen ME, Humaidan P, Dal Canto M, Fadini R, Schmidt KT, Ernst E, Yding Andersen C. LH-receptor gene expression in human granulosa and cumulus cells from antral and preovulatory follicles. *J Clin Endocrinol Metab.* 2012;97(8):E1524-E1531.
53. Ji I, Lee C, Song Y, Conn PM, Ji TH. *Cis* - and *Trans* -Activation of Hormone Receptors: the LH Receptor. *Mol Endocrinol.* 2002;16(6):1299–1308.
54. Jonas KC, Chen S, Virta M, Mora J, Franks S, Huhtaniemi I, Hanyaloglu AC. Temporal reprogramming of calcium signalling via crosstalk of gonadotrophin receptors that associate as functionally asymmetric heteromers. *Sci Rep.* 2018;8(1):2239.
55. Tal R, Taylor HS, Burney RO, Mooney SB, Giudice LC. Endocrinology of Pregnancy. In: De Groot LJ, Chrousos G, Dungan K, Feingold KR, Grossman A, Hershman JM, Koch C, Korbonits M, McLachlan R, New M, Purnell J, Rebar R, Singer F, Vinik A, eds. Endotext. South Dartmouth (MA): MDText.com, Inc.; 2000.
56. Makieva S, Saunders PTK, Norman JE. Androgens in pregnancy: roles in parturition. *Hum Reprod Update.* 2014;20(4):542–559.
57. Chen T, Surcel H-M, Lundin E, Kaasila M, Lakso H-A, Schock H, Kaaks R, Koskela P, Grankvist K, Hallmans G, Pukkala E, Zeleniuch-Jacquotte A, Toniolo P, Lehtinen M, Lukanova A. Circulating Sex Steroids during Pregnancy and Maternal Risk of Non-epithelial Ovarian Cancer. *Cancer Epidemiol Biomarkers Prev.* 2011;20(2):324–336.
58. Cole LA. hCG physiology. *Placenta.* 2013;34(12):1257.
59. Tapia-Pizarro A, Archiles S, Argandoña F, Valencia C, Zavaleta K, Cecilia Johnson M, González-Ramos R, Devoto L. hCG activates Epac-Erk1/2 signaling regulating Progesterone Receptor expression and function in human endometrial stromal cells. *Mol Hum Reprod.* 2017;23(6):393–405.
60. Pitteloud N, Dwyer A. Hormonal control of spermatogenesis in men: Therapeutic aspects in hypogonadotropic hypogonadism. *Ann Endocrinol (Paris).* 2014;75(2):98–100.
61. Rey RA. Mini-puberty and true puberty: Differences in testicular function. *Ann Endocrinol (Paris).* 2014;75(2):58–63.
62. Pentikäinen V, Erkkilä K, Suomalainen L, Parvinen M, Dunkel L. Estradiol acts as a germ cell survival factor in the human testis in vitro. *J Clin Endocrinol Metab.* 2000;85(5):2057–2067.
63. Pareek TK, Joshi AR, Sanyal A, Dighe RR. Insights into male germ cell apoptosis due to depletion of gonadotropins caused by GnRH antagonists. *Apoptosis.* 2007;12(6):1085–1100.

64. Nisula BC, Blithe DL, Akar A, Lefort G, Wehmann RE. Metabolic fate of human choriogonadotropin. *J Steroid Biochem.* 1989;33(4B):733–737.
65. Klett D, Bernard S, Lecompte F, Leroux H, Magallon T, Locatelli A, Lepape A, Combarnous Y. Fast renal trapping of porcine luteinizing hormone (pLH) shown by 123I-scintigraphic imaging in rats explains its short circulatory half-life. *Reprod Biol Endocrinol.* 2003;1(1):64.
66. Fiete D, Srivastava V, Hindsgaul O, Baenziger JU. A hepatic reticuloendothelial cell receptor specific for SO4-4GalNAc beta 1,4GlcNAc beta 1,2Man alpha that mediates rapid clearance of lutropin. *Cell.* 1991;67(6):1103–1110.
67. Birken S, Chen Y, Gawinowicz MA, Agosto GM, Canfield RE, Hartree AS. Structure and significance of human luteinizing hormone-beta core fragment purified from human pituitary extracts. *Endocrinology.* 1993;133(3):985–989.
68. Birken S, Gawinowicz MA, Maydelman Y, Milgrom Y. Metabolism of gonadotropins: comparisons of the primary structures of the human pituitary and urinary LH beta cores and the chimpanzee CG beta core demonstrate universality of core production. *J Endocrinol.* 2001;171(1):131–141.
69. Ben-Menahem D, Grotjan HE. Strategies for construction of luteinizing hormone beta subunit analogs with carboxyl terminal extensions in non-primate, non-equid mammalian species. *Mol Cell Endocrinol.* 2007;260–262:205–211.
70. Bousfield GR, Liu WK, Sugino H, Ward DN. Structural studies on equine glycoprotein hormones. Amino acid sequence of equine lutropin beta-subunit. *J Biol Chem.* 1987;262(18):8610–8620.
71. Gabay R, Rozen S, Samokovlisky A, Amor Y, Rosenfeld R, Kohen F, Amsterdam A, Berger P, Ben-Menahem D. The role of the 3' region of mammalian gonadotropin β subunit gene in the luteinizing hormone to chorionic gonadotropin evolution. *Mol Cell Endocrinol.* 2014;382(2):781–790.
72. Allen WR, Moor RM. The origin of the equine endometrial cups. I. Production of PMSG by fetal trophoblast cells. *J Reprod Fertil.* 1972;29(2):313–316.
73. Sherman GB, Wolfe MW, Farmerie TA, Clay CM, Threadgill DS, Sharp DC, Nilson JH. A single gene encodes the beta-subunits of equine luteinizing hormone and chorionic gonadotropin. *Mol Endocrinol.* 1992;6(6):951–959.
74. Bousfield GR, Butnev VY, Butnev VY. Identification of twelve O-glycosylation sites in equine chorionic gonadotropin beta and equine luteinizing hormone ss by solid-phase Edman degradation. *Biol Reprod.* 2001;64(1):136–147.
75. Butnev VY, Gotschall RR, Baker VL, Moore WT, Bousfield GR. Negative influence of O-linked oligosaccharides of high molecular weight equine chorionic gonadotropin on its luteinizing hormone and follicle-stimulating hormone receptor-binding activities. *Endocrinology.* 1996;137(6):2530–2542.
76. Butney VY, Gotschall RR, Butnev VY, Baker VL, Moore WT, Bousfield GR. Hormone-specific inhibitory influence of alpha-subunit Asn56 oligosaccharide on in vitro subunit association and follicle-stimulating hormone receptor binding of equine gonadotropins. *Biol Reprod.* 1998;58(2):458–469.
77. Guillou F, Combarnous Y. Purification of equine gonadotropins and comparative study of their acid-dissociation and receptor-binding specificity. *Biochim Biophys Acta* 1983;755(2):229–36.
78. Stewart F, Allen WR. The binding of FSH, LH and PMSG to equine gonadal tissues. *J Reprod Fertil Suppl.* 1979;(27):431–440.
79. Stewart F, Allen WR. Biological functions and receptor binding activities of equine chorionic gonadotrophins. *J Reprod Fertil.* 1981;62(2):527–536.

80. Simula AP, Amato F, Faast R, Lopata A, Berka J, Norman RJ. Luteinizing Hormone/Chorionic Gonadotropin Bioactivity in the Common Marmoset (Callithrixjacchus) is Due to a Chorionic Gonadotropin Molecule with a Structure Intermediate between Human Chorionic Gonadotropin and Human Luteinizing Hormone. *Biol Reprod.* 1995;53(2):380–389.
81. Gromoll J, Wistuba J, Terwort N, Godmann M, Müller T, Simoni M. A new subclass of the luteinizing hormone/chorionic gonadotropin receptor lacking exon 10 messenger RNA in the New World monkey (Platyrrhini) lineage. *Biol Reprod.* 2003;69(1):75–80.
82. Troppmann B, Kleinau G, Krause G, Gromoll J. Structural and functional plasticity of the luteinizing hormone/choriogonadotrophin receptor. *Hum Reprod Update.* 2013;19(5):583–602.
83. Müller T, Gromoll J, Simula AP, Norman R, Sandhowe-Klaverkamp R, Simoni M. The carboxyterminal peptide of chorionic gonadotropin facilitates activation of the marmoset LH receptor. *Exp Clin Endocrinol Diabetes.* 2004;112(10):574–579.
84. Gromoll J, Eiholzer U, Nieschlag E, Simoni M. Male Hypogonadism Caused by Homozygous Deletion of Exon 10 of the Luteinizing Hormone (LH) Receptor: Differential Action of Human Chorionic Gonadotropin and LH. *J Clin Endocrinol Metab.* 2000;85(6):2281–2286.
85. Müller T, Gromoll J, Simoni M. Absence of exon 10 of the human luteinizing hormone (LH) receptor impairs LH, but not human chorionic gonadotropin action. *J Clin Endocrinol Metab.* 2003;88(5):2242–2249.
86. Hoermann R, Spoettl G, Moncayo R, Mann K. Evidence for the presence of human chorionic gonadotropin (hCG) and free beta-subunit of hCG in the human pituitary. *J Clin Endocrinol Metab.* 1990;71(1):179–186.
87. Odell WD, Griffin J. Pulsatile Secretion of Human Chorionic Gonadotropin in Normal Adults. *N Engl J Med.* 1987;317(27):1688–1691.
88. Odell WD, Griffin J. Pulsatile Secretion of Chorionic Gonadotropin during the Normal Menstrual Cycle*. *J Clin Endocrinol Metab.* 1989;69(3):528–532.
89. Cole LA, Gutierrez JM. Production of human chorionic gonadotropin during the normal menstrual cycle. *J Reprod Med.* 2009;54(4):245–250.
90. Merhi Z, Pollack SE. Pituitary origin of persistently elevated human chorionic gonadotropin in a patient with gonadal failure. *Fertil Steril.* 2013;99(1):293–296.
91. Bielinska M, Rzymkiewicz D, Boime I. Human luteinizing hormone and chorionic gonadotropin are targeted to a regulated secretory pathway in GH3 cells. *Mol Endocrinol.* 1994;8(7):919–928.
92. Kochman K, Kerdelhué B, Zor U, Jutisz M. Studies of enzymatic degradation of luteinizing hormone-releasing hormone by different tissues. *FEBS Lett.* 1975;50(2):190–194.
93. Skorupskaite K, George JT, Anderson RA. The kisspeptin-GnRH pathway in human reproductive health and disease. *Hum Reprod Update.* 2014;20(4):485–500.
94. Gardner S, Maudsley S, Millar RP, Pawson AJ. Nuclear Stabilization of β-Catenin and Inactivation of Glycogen Synthase Kinase-3β by Gonadotropin-Releasing Hormone: Targeting Wnt Signaling in the Pituitary Gonadotrope. *Mol Endocrinol.* 2007;21(12):3028–3038.
95. Thompson IR, Kaiser UB. GnRH pulse frequency-dependent differential regulation of LH and FSH gene expression. *Mol Cell Endocrinol.* 2014;385(1–2):28–35.
96. Jayes FC, Britt JH, Esbenshade KL. Role of gonadotropin-releasing hormone pulse frequency in differential regulation of gonadotropins in the gilt. *Biol Reprod.* 1997;56(4):1012–1019.
97. Lunenfeld B, Bühler K. The neuro control of the ovarain cycle – a hypothesis. *Gynecol Endocrinol.* 2017:1–5.

98. Abel MH, Widen A, Wang X, Huhtaniemi I, Pakarinen P, Kumar TR, Christian HC. Pituitary gonadotrophic hormone synthesis, secretion, subunit gene expression and cell structure in normal and follicle-stimulating hormone β knockout, follicle-stimulating hormone receptor knockout, luteinising hormone receptor knockout, hypogonadal and ovariectomised female mice. *J Neuroendocrinol.* 2014;26(11):785–795.
99. Stanton PG, Pozvek G, Burgon PG, Robertson DM, Hearn MT. Isolation and characterization of human LH isoforms. *J Endocrinol.* 1993;138(3):529–543.
100. Merz WE. The primate placenta and human chorionic gonadotropin. *Exp Clin Endocrinol.* 1994;102(3):222–234.
101. Evans J, Salamonsen LA, Menkhorst E, Dimitriadis E. Dynamic changes in hyperglycosylated human chorionic gonadotrophin throughout the first trimester of pregnancy and its role in early placentation. *Hum Reprod.* 2015;30(5):1029–1038.
102. Cole LA. hCG, five independent molecules. *Clin Chim Acta.* 2012;413(1–2):48–65.
103. Cole LA. hCG, the wonder of today's science. *Reprod Biol Endocrinol.* 2012;10(1):24.
104. Mann K, Lamerz R, Hellmann T, Kümper HJ, Staehler G, Karl HJ. Use of human chorionic gonadotropin and alpha-fetoprotein radioimmunoassays: specificity and apparent half-life determination after delivery and in patients with germ cell tumors. *Oncodev Biol Med.* 1980;1(4–5):301–312.
105. Furuhashi M, Shikone T, Fares FA, Sugahara T, Hsueh AJ, Boime I. Fusing the carboxy-terminal peptide of the chorionic gonadotropin (CG) beta-subunit to the common alpha-subunit: retention of O-linked glycosylation and enhanced in vivo bioactivity of chimeric human CG. *Mol Endocrinol.* 1995;9(1):54–63.
106. Ulloa-Aguirre A, Maldonado A, Damián-Matsumura P, Timossi C. Endocrine regulation of gonadotropin glycosylation. *Arch Med Res.* 32(6):520–532.
107. Manzella SM, Dharmesh SM, Beranek MC, Swanson P, Baenziger JU. Evolutionary conservation of the sulfated oligosaccharides on vertebrate glycoprotein hormones that control circulatory half-life. *J Biol Chem.* 1995;270(37):21665–21671.
108. Fiddes JC, Goodman HM. Isolation, cloning and sequence analysis of the cDNA for the alpha-subunit of human chorionic gonadotropin. *Nature.* 1979;281(5730):351–356.
109. Xing Y, Myers R V., Cao D, Lin W, Jiang M, Bernard MP, Moyle WR. Glycoprotein Hormone Assembly in the Endoplasmic Reticulum. *J Biol Chem.* 2004;279(34):35426–35436.
110. Moyle WR, Myers R V, Wang Y, Han Y, Lin W, Kelley GL, Ehrlich PH, Rao SN, Bernard MP. Functional homodimeric glycoprotein hormones: implications for hormone action and evolution. *Chem Biol.* 1998;5(5):241–254.
111. Lustbader JW, Birken S, Pileggi NF, Kolks MA, Pollak S, Cuff ME, Yang W, Hendrickson WA, Canfield RE. Crystallization and characterization of human chorionic gonadotropin in chemically deglycosylated and enzymatically desialylated states. *Biochemistry.* 1989;28(24):9239–9243.
112. Lapthorn AJ, Harris DC, Littlejohn A, Lustbader JW, Canfield RE, Machin KJ, Morgan FJ, Isaacs NW. Crystal structure of human chorionic gonadotropin. *Nature.* 1994;369(6480):455–461.
113. Wu H, Lustbader JW, Liu Y, Canfield RE, Hendrickson WA. Structure of human chorionic gonadotropin at 2.6 A resolution from MAD analysis of the selenomethionyl protein. *Structure.* 1994;2(6):545–558.
114. Furuhashi M, Suzuki S, Suganuma N. Disulfide bonds 7-31 and 59-87 of the alpha-subunit play a different role in assembly of human chorionic gonadotropin and lutropin. *Endocrinology.* 1996;137(10):4196–4200.

115. Feng W, Matzuk MM, Mountjoy K, Bedows E, Ruddon RW, Boime I. The asparagine-linked oligosaccharides of the human chorionic gonadotropin beta subunit facilitate correct disulfide bond pairing. *J Biol Chem.* 1995;270(20):11851–11859.
116. Bernard MP, Lin W, Kholodovych V, Moyle WR. Human lutropin (hLH) and choriogonadotropin (CG) are assembled by different pathways: a model of hLH assembly. *J Biol Chem.* 2014;289(20):14360–14369.
117. Darling RJ, Wilken JA, Miller-Lindholm AK, Urlacher TM, Ruddon RW, Sherman SA, Bedows E. Functional Contributions of Noncysteine Residues within the Cystine Knots of Human Chorionic Gonadotropin Subunits. *J Biol Chem.* 2001;276(14):10692–10699.
118. Ruddon RW, Sherman SA, Bedows E. Protein folding in the endoplasmic reticulum: Lessons from the human chorionic gonadotropin β subunit. *Protein Sci.* 1996;5(8):1443–1452.
119. Muyan M, Furuhashi M, Sugahara T, Boime I. The carboxy-terminal region of the beta-subunits of luteinizing hormone and chorionic gonadotropin differentially influence secretion and assembly of the heterodimers. *Mol Endocrinol.* 1996;10(12):1678–1687.
120. Suzuki S, Furuhashi M, Suganuma N. Additional N-glycosylation at Asn(13) rescues the human LHbeta-subunit from disulfide-linked aggregation. *Mol Cell Endocrinol.* 2000;160(1–2):157–163.
121. Corless CL, Matzuk MM, Ramabhadran T V, Krichevsky A, Boime I. Gonadotropin beta subunits determine the rate of assembly and the oligosaccharide processing of hormone dimer in transfected cells. *J Cell Biol.* 1987;104(5):1173–1181.
122. Lo A, Zheng W, Gong Y, Crochet JR, Halvorson LM. GATA transcription factors regulate LH gene expression. *J Mol Endocrinol.* 2011;47(1):45–58.
123. Zheng W, Horton CD, Kim J, Halvorson LM. The orphan nuclear receptors COUP-TFI and COUP-TFII regulate expression of the gonadotropin LHβ gene. *Mol Cell Endocrinol.* 2010;330(1–2):59–71.
124. Millar RP. GnRHs and GnRH receptors. *Anim Reprod Sci.* 2005;88(1–2):5–28.
125. Caunt CJ, Finch AR, Sedgley KR, Oakley L, Luttrell LM, McArdle CA. Arrestin-mediated ERK activation by gonadotropin-releasing hormone receptors: receptor-specific activation mechanisms and compartmentalization. *J Biol Chem.* 2006;281(5):2701–2710.
126. Salisbury TB, Binder AK, Grammer JC, Nilson JH. Maximal Activity of the *Luteinizing Hormone* β-Subunit Gene Requires β-Catenin. *Mol Endocrinol.* 2007;21(4):963–971.
127. Sairam MR, Li CH. Human pituitary lutropin. Isolation, properties, and the complete amino acid sequence of the beta-subunit. *Biochim Biophys Acta.* 1975;412(1):70–81.
128. Morgan FJ, Birken S, Canfield RE. The amino acid sequence of human chorionic gonadotropin. The alpha subunit and beta subunit. *J Biol Chem.* 1975;250(13):5247–5258.
129. Talmadge K, Boorstein WR, Fiddes JC. The human genome contains seven genes for the beta-subunit of chorionic gonadotropin but only one gene for the beta-subunit of luteinizing hormone. *DNA.* 1983;2(4):281–289.
130. den Dunnen JT, Antonarakis SE. Mutation nomenclature extensions and suggestions to describe complex mutations: a discussion. *Hum Mutat.* 2000;15(1):7–12.
131. Wide L, Eriksson K. Dynamic changes in glycosylation and glycan composition of serum FSH and LH during natural ovarian stimulation. *Ups J Med Sci.* 2013;118(3):153–164.
132. Wide L. Median charge and charge heterogeneity of human pituitary FSH, LH and TSH. II. Relationship to sex and age. *Acta Endocrinol (Copenh).* 1985;109(2):190–197.
133. Esteves SC, Alviggi C. The Role of LH in Controlled Ovarian Stimulation. In: Ghumman S, ed. Principles and Practice of Controlled Ovarian Stimulation in ART. New Delhi: Springer India; 2015:171–196.

134. Cole LA, Metsch LA, Grotjan HE. Significant steroidogenic activity of luteinizing hormone is maintained after enzymatic removal of oligosaccharides. *Mol Endocrinol.* 1987;1(9):621–627.
135. Green ED, Baenziger JU. Asparagine-linked oligosaccharides on lutropin, follitropin, and thyrotropin. I. Structural elucidation of the sulfated and sialylated oligosaccharides on bovine, ovine, and human pituitary glycoprotein hormones. *J Biol Chem.* 1988;263(1):25–35.
136. Wide L, Eriksson K, Sluss PM, Hall JE. Serum Half-Life of Pituitary Gonadotropins Is Decreased by Sulfonation and Increased by Sialylation in Women. *J Clin Endocrinol Metab.* 2009;94(3):958–964.
137. Burgon PG, Stanton PG, Robertson DM. In vivo bioactivities and clearance patterns of highly purified human luteinizing hormone isoforms. *Endocrinology.* 1996;137(11):4827–4836.
138. Weisshaar G, Hiyama J, Renwick AG, Nimtz M. NMR investigations of the N-linked oligosaccharides at individual glycosylation sites of human lutropin. *Eur J Biochem.* 1991;195(1):257–268.
139. Cole LA. Immunoassay of human chorionic gonadotropin, its free subunits, and metabolites. *Clin Chem.* 1997;43(12):2233–2243.
140. Birken S, Kovalevskaya G, O'Connor J. Metabolism of hCG and hLH to multiple urinary forms. *Mol Cell Endocrinol.* 1996;125(1–2):121–131.
141. Handschuh K, Guibourdenche J, Cocquebert M, Tsatsaris V, Vidaud M, Evain-Brion D, Fournier T. Expression and regulation by PPARgamma of hCG alpha- and beta-subunits: comparison between villous and invasive extravillous trophoblastic cells. *Placenta.* 2009;30(12):1016–1022.
142. Rull K, Hallast P, Uuskula L, Jackson J, Punab M, Salumets A, Campbell RK, Laan M. Fine-scale quantification of HCG beta gene transcription in human trophoblastic and non-malignant non-trophoblastic tissues. *Mol Hum Reprod.* 2008;14(1):23–31.
143. Zhu H, Qiu C, Ruth AC, Keire DA, Ye H. A LC-MS All-in-One Workflow for Site-Specific Location, Identification and Quantification of N-/O- Glycosylation in Human Chorionic Gonadotropin Drug Products. *AAPS J.* 2017;19(3):846–855.
144. Liu C, Bowers LD. Mass Spectrometric Characterization of the β-Subunit of Human Chorionic Gonadotropin. *J Mass Spectrom.* 1997;32(1):33–42.
145. Valmu L, Alfthan H, Hotakainen K, Birken S, Stenman U-H. Site-specific glycan analysis of human chorionic gonadotropin beta-subunit from malignancies and pregnancy by liquid chromatography--electrospray mass spectrometry. *Glycobiology.* 2006;16(12):1207–1218.
146. Toll H, Berger P, Hofmann A, Hildebrandt A, Oberacher H, Lenhof HP, Huber CG. Glycosylation patterns of human chorionic gonadotropin revealed by liquid chromatography-mass spectrometry and bioinformatics. *Electrophoresis.* 2006;27(13):2734–2746.
147. Fernández-Tejada A, Vadola PA, Danishefsky SJ. Chemical Synthesis of the β-Subunit of Human Luteinizing (hLH) and Chorionic Gonadotropin (hCG) Glycoprotein Hormones. *J Am Chem Soc.* 2014;136(23):8450–8458.
148. Brun S, Leguy MC, Bruneel A, Fournier T, Anselem O, Guibourdenche J. hCG in screening for aneuploidy: A possible role for its glycoforms? *Placenta.* 2014;35(6):425–427.
149. Berndt S, Perrier d'Hauterive S, Blacher S, Péqueux C, Lorquet S, Munaut C, Applanat M, Hervé MA, Lamandé N, Corvol P, van den Brûle F, Frankenne F, Poutanen M, Huhtaniemi I, Geenen V, Noël A, Foidart J-M. Angiogenic activity of human chorionic gonadotropin through LH receptor activation on endothelial and epithelial cells of the endometrium. *FASEB J.* 2006;20(14):2630–2632.
150. Fournier T, Guibourdenche J, Evain-Brion D. Review: hCGs: different sources of production, different glycoforms and functions. *Placenta.* 2015;36 Suppl 1:S60-5.

151. Prast J, Saleh L, Husslein H, Sonderegger S, Helmer H, Knöfler M. Human chorionic gonadotropin stimulates trophoblast invasion through extracellularly regulated kinase and AKT signaling. *Endocrinology.* 2008;149(3):979–987.
152. Guibourdenche J, Handschuh K, Tsatsaris V, Gerbaud P, Leguy MC, Muller F, Evain Brion D, Fournier T. Hyperglycosylated hCG Is a Marker of Early Human Trophoblast Invasion. *J Clin Endocrinol Metab.* 2010;95(10):E240–E244.
153. Cole LA. Hyperglycosylated hCG. *Placenta.* 2007;28(10):977–986.
154. Kovalevskaya G, Birken S, Kakuma T, Ozaki N, Sauer M, Lindheim S, Cohen M, Kelly A, Schlatterer J, O'Connor JF. Differential expression of human chorionic gonadotropin (hCG) glycosylation isoforms in failing and continuing pregnancies: preliminary characterization of the hyperglycosylated hCG epitope. *J Endocrinol.* 2002;172(3):497–506.
155. von Schönfeldt V, Rogenhofer N, Ruf K, Thaler CJ, Jeschke U. Sera of patients with recurrent miscarriages containing anti-trophoblast antibodies (ATAB) reduce hCG and progesterone production in trophoblast cells in vitro. *J Reprod Immunol.* 2016;117:52–56.
156. Elliott MM, Kardana A, Lustbader JW, Cole LA. Carbohydrate and peptide structure of the alpha- and beta-subunits of human chorionic gonadotropin from normal and aberrant pregnancy and choriocarcinoma. *Endocrine.* 1997;7(1):15–32.
157. Blithe DL. N-linked oligosaccharides on free alpha interfere with its ability to combine with human chorionic gonadotropin-beta subunit. *J Biol Chem.* 1990;265(35):21951–21956.
158. Brouillet S, Hoffmann P, Chauvet S, Salomon A, Chamboredon S, Sergent F, Benharouga M, Feige JJ, Alfaidy N. Revisiting the role of hCG: new regulation of the angiogenic factor EG-VEGF and its receptors. *Cell Mol Life Sci.* 2012;69(9):1537–1550.
159. Berndt S, Blacher S, Munaut C, Detilleux J, Perrier d'Hauterive S, Huhtaniemi I, Evain-Brion D, Noël A, Fournier T, Foidart J-M. Hyperglycosylated human chorionic gonadotropin stimulates angiogenesis through TGF-β receptor activation. *FASEB J.* 2013;27(4):1309–1321.
160. Sengodan SK, Nadhan R, Nair RS, Hemalatha SK, Somasundaram V, Sushama RR, Rajan A, Latha NR, Varghese GR, Thankappan R k, Kumar JM, Chil A, Anilkumar T V, Srinivas P. BRCA1 regulation on β-hCG: a mechanism for tumorigenicity in BRCA1 defective breast cancer. *Oncogenesis.* 2017;6(9):e376.
161. Koistinen H, Hautala L, Koli K, Stenman U-H. Absence of TGF-β Receptor Activation by Highly Purified hCG Preparations. *Mol Endocrinol.* 2015;29(12):1787–1791.
162. Lempiäinen A, Sankila A, Hotakainen K, Haglund C, Blomqvist C, Stenman U-H. Expression of human chorionic gonadotropin in testicular germ cell tumors. *Urol Oncol Semin Orig Investig.* 2014;32(5):727–734.
163. Varki A, Kannagi R, Toole B, Stanley P. Glycosylation Changes in Cancer.; In: Varki A, Cummings RD, Esko JD, Stanley P, Hart GW, Aebi M, Darvill AG, Kinoshita T, Packer NH, Prestegard JH, Schnaar RL, Seeberger PH, eds. Essentials of Glycobiology. 3rd edition. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 2015-2017.
164. Cole LA, Butler SA. Hyperglycosylated human chorionic gonadotropin and human chorionic gonadotropin free beta-subunit: tumor markers and tumor promoters. *J Reprod Med.* 2008;53(7):499–512.
165. Van Hell H, Matthijsen R, Overbeek GA. Effects of human menopausal gonadotrophin preparations in different bioassay methods. *Acta Endocrinol (Copenh).* 1964;47:409–418.
166. Catt KJ, Dufau ML. Spare gonadotrophin receptors in rat testis. *Nat New Biol.* 1973;244(137):219–221.

167. Younis JS. Commentary: Efficacy of Follicle-Stimulating Hormone (FSH) Alone, FSH + Luteinizing Hormone, Human Menopausal Gonadotropin or FSH + Human Chorionic Gonadotropin on Assisted Reproductive Technology Outcomes in the "Personalized" Medicine Era: A Meta-analysis. *Front Endocrinol (Lausanne).* 2017;8:264.
168. Chappel SC, Howles C. Reevaluation of the roles of luteinizing hormone and follicle-stimulating hormone in the ovulatory process. *Hum Reprod.* 1991;6(9):1206–1212.
169. Casarini L, Santi D, Simoni M, Potì F. "Spare" Luteinizing Hormone Receptors: Facts and Fiction. *Trends Endocrinol Metab.* 2018;29(4):208–217.
170. Mazurkiewicz JE, Herrick-Davis K, Barroso M, Ulloa-Aguirre A, Lindau-Shepard B, Thomas RM, Dias JA. Single-Molecule Analyses of Fully Functional Fluorescent Protein-Tagged Follitropin Receptor Reveal Homodimerization and Specific Heterodimerization with Lutropin Receptor. *Biol Reprod.* 2015;92(4):100.
171. Cosowsky L, Lin W, Han Y, Bernard MP, Campbell RK, Moyle WR. Influence of subunit interactions on lutropin specificity. Implications for studies of glycoprotein hormone function. *J Biol Chem.* 1997;272(6):3309–3314.
172. Galet C, Ascoli M. The differential binding affinities of the luteinizing hormone (LH)/choriogonadotropin receptor for LH and choriogonadotropin are dictated by different extracellular domain residues. *Mol Endocrinol.* 2005;19(5):1263–1276.
173. Nguyen T-MD, Filliatreau L, Klett D, Combarnous Y. Comparative effects of sub-stimulating concentrations of non-human versus human Luteinizing Hormones (LH) or chorionic gonadotropins (CG) on adenylate cyclase activation by forskolin in MLTC cells. *Gen Comp Endocrinol.* 2018. 261:23-30.
174. Moyle WR, Pressey A, Dean-Emig D, Anderson DM, Demeter M, Lustbader J, Ehrlich P. Detection of conformational changes in human chorionic gonadotropin upon binding to rat gonadal receptors. *J Biol Chem.* 1987;262(35):16920–16926.
175. Keutmann HT, Charlesworth MC, Mason KA, Ostrea T, Johnson L, Ryan RJ. A receptor-binding region in human choriogonadotropin/lutropin beta subunit. *Proc Natl Acad Sci U S A.* 1987;84(7):2038–2042.
176. Xia H, Huang J, Chen TM, Puett D. Lysine residues 2 and 104 of the human chorionic gonadotrophin beta subunit influence receptor binding. *J Mol Endocrinol.* 1993;10(3):337–343.
177. Hong Soh, Ji I, Ji TH. The α-Subunit of Human Choriogonadotropin Interacts with the Exodomain of the Luteinizing Hormone/Choriogonadotropin Receptor. *Endocrinology.* 1999;140(6):2486–2493.
178. Vischer HF, Granneman JCM, Noordam MJ, Mosselman S, Bogerd J. Ligand selectivity of gonadotropin receptors. Role of the beta-strands of extracellular leucine-rich repeats 3 and 6 of the human luteinizing hormone receptor. *J Biol Chem.* 2003;278(18):15505–15513.
179. Smits G, Campillo M, Govaerts C, Janssens V, Richter C, Vassart G, Pardo L, Costagliola S. Glycoprotein hormone receptors: determinants in leucine-rich repeats responsible for ligand specificity. *EMBO J.* 2003;22(11):2692–2703.
180. Roy S, Setlur S, Gadkari RA, Krishnamurthy HN, Dighe RR. Translational fusion of two beta-subunits of human chorionic gonadotropin results in production of a novel antagonist of the hormone. *Endocrinology.* 2007;148(8):3977–3986.
181. Narayan P, Gray J, Puett D. A biologically active single chain human chorionic gonadotropin analog with altered receptor binding properties. *Endocrinology.* 2000;141(1):67–71.
182. Bernard MP, Lin W, Cao D, Myers R V, Xing Y, Moyle WR. Only a portion of the small seatbelt loop in human choriogonadotropin appears capable of contacting the lutropin receptor. *J Biol Chem.* 2004;279(43):44438–44441.

183. Shao K, Purohit S, Bahl OP. Effect of modification of all loop regions in the alpha- and beta-subunits of human choriogonadotropin on its signal transduction activity. *Mol Cell Endocrinol.* 1996;122(2):173–182.
184. Hu SB, Johnson L, Roche PC, Keutmann HT. A functional determinant in human luteinizing hormone and chorionic gonadotropin: differential effect of mutations about beta-GLN-54. *Endocrinology.* 1997;138(4):1627–1633.
185. Jiang X, Dias JA, He X. Structural biology of glycoprotein hormones and their receptors: insights to signaling. *Mol Cell Endocrinol.* 2014;382(1):424–451.
186. Rivero-Müller A, Chou Y-Y, Ji I, Lajic S, Hanyaloglu AC, Jonas K, Rahman N, Ji TH, Huhtaniemi I. Rescue of defective G protein-coupled receptor function in vivo by intermolecular cooperation. *Proc Natl Acad Sci U S A.* 2010;107(5):2319–2324.
187. Grzesik P, Teichmann A, Furkert J, Rutz C, Wiesner B, Kleinau G, Schülein R, Gromoll J, Krause G. Differences between lutropin-mediated and choriogonadotropin-mediated receptor activation. *FEBS J.* 2014;281(5):1479–1492.
188. Lee C, Ji I, Ryu K, Song Y, Conn PM, Ji TH. Two defective heterozygous luteinizing hormone receptors can rescue hormone action. *J Biol Chem.* 2002;277(18):15795–15800.
189. Jeoung M, Lee C, Ji I, Ji TH. Trans-activation, cis-activation and signal selection of gonadotropin receptors. *Mol Cell Endocrinol.* 2007;260–262:137–143.
190. Zhang M, Guan R, Segaloff DL. Revisiting and questioning functional rescue between dimerized LH receptor mutants. *Mol Endocrinol.* 2012;26(4):655–668.
191. Alvarez CA, Narayan P, Huang J, Puett D. Characterization of a Region of the Lutropin Receptor Extracellular Domain Near Transmembrane Helix 1 That Is Important in Ligand-Mediated Signaling. *Endocrinology.* 1999;140(4):1775–1782.
192. Ji TH, Ryu KS, Gilchrist R, Ji I. Interaction, signal generation, signal divergence, and signal transduction of LH/CG and the receptor. *Recent Prog Horm Res.* 1997;52:431–453; discussion 454.
193. Herrlich A, Kühn B, Grosse R, Schmid A, Schultz G, Gudermann T. Involvement of Gs and Gi proteins in dual coupling of the luteinizing hormone receptor to adenylyl cyclase and phospholipase C. *J Biol Chem.* 1996;271(28):16764–16772.
194. Nakamura K, Lazari M de FM, Li S, Korgaonkar C, Ascoli M. Role of the Rate of Internalization of the Agonist-Receptor Complex on the Agonist-Induced Down-Regulation of the Lutropin/ Choriogonadotropin Receptor. *Mol Endocrinol.* 1999;13(8):1295–1304.
195. Gudermann T, Nichols C, Levy FO, Birnbaumer M, Birnbaumer L. Ca2+ mobilization by the LH receptor expressed in Xenopus oocytes independent of 3',5'-cyclic adenosine monophosphate formation: evidence for parallel activation of two signaling pathways. *Mol Endocrinol.* 1992;6(2):272–278.
196. Trehan A, Rotgers E, Coffey ET, Huhtaniemi I, Rivero-Müller A. CANDLES, an assay for monitoring GPCR induced cAMP generation in cell cultures. *Cell Commun Signal.* 2014;12(1):70.
197. Ayoub MA, Landomiel F, Gallay N, Jégot G, Poupon A, Crépieux P, Reiter E. Assessing Gonadotropin Receptor Function by Resonance Energy Transfer-Based Assays. *Front Endocrinol (Lausanne).* 2015;6:130.
198. Herrlich A, Kühn B, Grosse R, Schmid A, Schultz G, Gudermann T. Involvement of Gs and Gi proteins in dual coupling of the luteinizing hormone receptor to adenylyl cyclase and phospholipase C. *J Biol Chem.* 1996;271(28):16764–16772.
199. Montminy MR, Bilezikjian LM. Binding of a nuclear protein to the cyclic-AMP response element of the somatostatin gene. *Nature.* 1987;328(6126):175–178.
200. Casarini L, Reiter E, Simoni M. β-arrestins regulate gonadotropin receptor-mediated cell proliferation and apoptosis by controlling different FSHR or LHCGR intracellular signaling in the hGL5 cell line. *Mol Cell Endocrinol.* 2016;437:11–21.

201. Zwain IH, Amato P. cAMP-induced apoptosis in granulosa cells is associated with up-regulation of P53 and bax and down-regulation of clusterin. *Endocr Res.* 27(1–2):233–249.
202. Tajima K, Yoshii K, Fukuda S, Orisaka M, Miyamoto K, Amsterdam A, Kotsuji F. Luteinizing Hormone-Induced Extracellular-Signal Regulated Kinase Activation Differently Modulates Progesterone and Androstenedione Production in Bovine Theca Cells. *Endocrinology.* 2005;146(7):2903–2910.
203. Menon B, Franzo-Romain M, Damanpour S, Menon KMJ. Luteinizing Hormone Receptor mRNA Down-Regulation Is Mediated through ERK-Dependent Induction of RNA Binding Protein. *Mol Endocrinol.* 2011;25(2):282–290.
204. Ren X-R, Reiter E, Ahn S, Kim J, Chen W, Lefkowitz RJ. Different G protein-coupled receptor kinases govern G protein and -arrestin-mediated signaling of V2 vasopressin receptor. *Proc Natl Acad Sci U S A.* 2005;102(5):1448–1453.
205. Tai P, Shiraishi K, Ascoli M. Activation of the Lutropin/Choriogonadotropin Receptor Inhibits Apoptosis of Immature Leydig Cells in Primary Culture. *Endocrinology.* 2009;150(8):3766–3773.
206. Martinat N, Crépieux P, Reiter E, Guillou F. Extracellular signal-regulated kinases (ERK) 1, 2 are required for luteinizing hormone (LH)-induced steroidogenesis in primary Leydig cells and control steroidogenic acute regulatory (StAR) expression. *Reprod Nutr Dev.* 45(1):101–108.
207. Tohgo A, Choy EW, Gesty-Palmer D, Pierce KL, Laporte S, Oakley RH, Caron MG, Lefkowitz RJ, Luttrell LM. The Stability of the G Protein-coupled Receptor-β-Arrestin Interaction Determines the Mechanism and Functional Consequence of ERK Activation. *J Biol Chem.* 2003;278(8):6258–6267.
208. Revankar CM, Vines CM, Cimino DF, Prossnitz ER. Arrestins Block G Protein-coupled Receptor-mediated Apoptosis. *J Biol Chem.* 2004;279(23):24578–24584.
209. Gudermann T, Birnbaumer M, Birnbaumer L. Evidence for dual coupling of the murine luteinizing hormone receptor to adenylyl cyclase and phosphoinositide breakdown and Ca2+ mobilization. Studies with the cloned murine luteinizing hormone receptor expressed in L cells. *J Biol Chem.* 1992;267(7):4479–4488.
210. Zhu X, Gilbert S, Birnbaumer M, Birnbaumer L. Dual signaling potential is common among Gs-coupled receptors and dependent on receptor density. *Mol Pharmacol.* 1994;46(3):460–469.
211. Manna PR, Pakarinen P, El-Hefnawy T, Huhtaniemi IT. Functional Assessment of the Calcium Messenger System in Cultured Mouse Leydig Tumor Cells: Regulation of Human Chorionic Gonadotropin-Induced Expression of the Steroidogenic Acute Regulatory Protein. *Endocrinology.* 1999;140(4):1739–1751.
212. Pezzi V, Clark BJ, Ando S, Stocco DM, Rainey WE. Role of calmodulin-dependent protein kinase II in the acute stimulation of aldosterone production. *J Steroid Biochem Mol Biol.* 1996;58(4):417–424.
213. Mertens-Walker I, Bolitho C, Baxter RC, Marsh DJ. Gonadotropin-induced ovarian cancer cell migration and proliferation require extracellular signal-regulated kinase 1/2 activation regulated by calcium and protein kinase C. *Endocr Relat Cancer.* 2010;17(2):335–349.
214. Andric N, Ascoli M. Mutations of the lutropin/choriogonadotropin receptor that do not activate the phosphoinositide cascade allow hCG to induce aromatase expression in immature rat granulosa cells. *Mol Cell Endocrinol.* 2008;285(1–2):62–72.
215. Light A, Hammes SR. Membrane receptor cross talk in steroidogenesis: Recent insights and clinical implications. *Steroids.* 2013;78(6):633–638.

216. Tranchant T, Durand G, Gauthier C, Crépieux P, Ulloa-Aguirre A, Royère D, Reiter E. Preferential β-arrestin signalling at low receptor density revealed by functional characterization of the human FSH receptor A189 V mutation☆. *Mol Cell Endocrinol.* 2011;331(1):109–118.
217. Donadeu FX, Ascoli M. The Differential Effects of the Gonadotropin Receptors on Aromatase Expression in Primary Cultures of Immature Rat Granulosa Cells Are Highly Dependent on the Density of Receptors Expressed and the Activation of the Inositol Phosphate Cascade. *Endocrinology.* 2005;146(9):3907–3916.
218. Lyga S, Volpe S, Werthmann RC, Götz K, Sungkaworn T, Lohse MJ, Calebiro D. Persistent cAMP Signaling by Internalized LH Receptors in Ovarian Follicles. *Endocrinology.* 2016;157(4):1613–21.
219. Hunzicker-Dunn M, Barisas G, Song J, Roess DA. Membrane organization of luteinizing hormone receptors differs between actively signaling and desensitized receptors. *J Biol Chem.* 2003;278(44):42744–42749.
220. Nakamura K, Lazari MF, Li S, Korgaonkar C, Ascoli M. Role of the rate of internalization of the agonist-receptor complex on the agonist-induced down-regulation of the lutropin/choriogonadotropin receptor. *Mol Endocrinol.* 1999;13(8):1295–1304.
221. Hirakawa T, Galet C, Kishi M, Ascoli M. GIPC binds to the human lutropin receptor (hLHR) through an unusual PDZ domain binding motif, and it regulates the sorting of the internalized human choriogonadotropin and the density of cell surface hLHR. *J Biol Chem.* 2003;278(49):49348–49357.
222. Roess DA, Horvat RD, Munnelly H, Barisas BG. Luteinizing Hormone Receptors Are Self-Associated in the Plasma Membrane. *Endocrinology.* 2000;141(12):4518–4523.
223. Thomsen ARB, Plouffe B, Cahill TJ, Shukla AK, Tarrasch JT, Dosey AM, Kahsai AW, Strachan RT, Pani B, Mahoney JP, Huang L, Breton B, Heydenreich FM, Sunahara RK, Skiniotis G, Bouvier M, Lefkowitz RJ. GPCR-G Protein-β-Arrestin Super-Complex Mediates Sustained G Protein Signaling. *Cell.* 2016;166(4):907–919.
224. Sposini S, Jean-Alphonse FG, Ayoub MA, Oqua A, West C, Lavery S, Brosens JJ, Reiter E, Hanyaloglu AC. Integration of GPCR Signaling and Sorting from Very Early Endosomes via Opposing APPL1 Mechanisms. *Cell Rep.* 2017;21(10):2855–2867.
225. Riccetti L, Yvinec R, Klett D, Gallay N, Combarnous Y, Reiter E, Simoni M, Casarini L, Ayoub MA. Human Luteinizing Hormone and Chorionic Gonadotropin Display Biased Agonism at the LH and LH/CG Receptors. *Sci Rep.* 2017;7(1):940.
226. Jonas KC, Fanelli F, Huhtaniemi IT, Hanyaloglu AC. Single molecule analysis of functionally asymmetric G protein-coupled receptor (GPCR) oligomers reveals diverse spatial and structural assemblies. *J Biol Chem.* 2015;290(7):3875–3892.
227. Gupta C, Chapekar T, Chhabra Y, Singh P, Sinha S, Luthra K. Differential response to sustained stimulation by hCG & LH on goat ovarian granulosa cells. *Indian J Med Res.* 2012;135:331–340.
228. Casarini L, Riccetti L, De Pascali F, Nicoli A, Tagliavini S, Trenti T, La Sala GB, Simoni M. Follicle-stimulating hormone potentiates the steroidogenic activity of chorionic gonadotropin and the anti-apoptotic activity of luteinizing hormone in human granulosa-lutein cells in vitro. *Mol Cell Endocrinol.* 2016;422:103–114.
229. Casarini L, Riccetti L, De Pascali F, Gilioli L, Marino M, Vecchi E, Morini D, Nicoli A, La Sala GB, Simoni M. Estrogen modulates specific life and death signals induced by LH and hCG in human primary granulosa cells in vitro. *Int J Mol Sci.* 2017;18(5). pii: E926.
230. Kristensen SG, Mamsen LS, Jeppesen J V., Bøtkjær JA, Pors SE, Borgbo T, Ernst E, Macklon KT, Andersen CY. Hallmarks of Human Small Antral Follicle Development: Implications for Regulation of Ovarian Steroidogenesis and Selection of the Dominant Follicle. *Front Endocrinol (Lausanne).* 2018;8:376.

231. Wickenheisser JK, Nelson-DeGrave VL, McAllister JM. Human ovarian theca cells in culture. *Trends Endocrinol Metab.* 2006;17(2):65–71.
232. Saint-Dizier M, Foulon-Gauze F, Lecompte F, Combarnous Y, Chopineau M. The additional N-glycosylation site of the equine LH/CG receptor is not responsible for the limited cyclic AMP pathway activation by equine chorionic gonadotropin relative to luteinizing hormone. *Reprod Biol.* 2011;11(2):157–164.
233. Klett D, Meslin P, Relav L, Nguyen TMD, Mariot J, Jégot G, Cahoreau C, Combarnous Y. Low reversibility of intracellular cAMP accumulation in mouse Leydig tumor cells (MLTC-1) stimulated by human Luteinizing Hormone (hLH) and Chorionic Gonadotropin (hCG). *Mol Cell Endocrinol.* 2016;434:144–153.
234. Santi D, Spaggiari G, Casarini L, Fanelli F, Mezzullo M, Pagotto U, Granata ARM, Carani C, Simoni M. Central hypogonadism due to a giant, "silent" FSH-secreting, atypical pituitary adenoma: effects of adenoma dissection and short-term Leydig cell stimulation by luteinizing hormone (LH) and human chorionic gonadotropin (hCG). *Aging Male.* 2017;20(2):96–101.
235. Tyrey L. Human chorionic gonadotropin: properties and assay methods. *Semin Oncol.* 1995;22(2):121–129.
236. Christin-Maitre S, Vasseur C, Fauser B, Bouchard P. Bioassays of gonadotropins. *Methods.* 2000;21(1):51–57.
237. Qiu M, Quan F, Han C, Wu B, Liu J, Yang Z, Su F, Zhang Y. Effects of granulosa cells on steroidogenesis, proliferation and apoptosis of stromal cells and theca cells derived from the goat ovary. *J Steroid Biochem Mol Biol.* 2013;138:325–333.
238. Brown C, LaRocca J, Pietruska J, Ota M, Anderson L, Duncan Smith S, Weston P, Rasoulpour T, Hixon ML. Subfertility Caused by Altered Follicular Development and Oocyte Growth in Female Mice Lacking PKBalpha/Akt11. *Biol Reprod.* 2010;82(2):246–256.
239. Rao C V. Protective Effects of Human Chorionic Gonadotropin Against Breast Cancer: How Can We Use This Information to Prevent/Treat the Disease? *Reprod Sci.* 2017;24(8):1102–1110.
240. Pennanen P, Peltonen S, Kallionpää RA, Peltonen J. The effect of estradiol, testosterone, and human chorionic gonadotropin on the proliferation of Schwann cells with NF1 +/− or NF1 −/− genotype derived from human cutaneous neurofibromas. *Mol Cell Biochem.* 2017. doi:10.1007/s11010-017-3227-2.
241. Kayampilly PP, Menon KMJ. AMPK Activation by Dihydrotestosterone Reduces FSH-Stimulated Cell Proliferation in Rat Granulosa Cells by Inhibiting ERK Signaling Pathway. *Endocrinology.* 2012;153(6):2831–2838.
242. Lohse MJ, Hoffmann C. Arrestin Interactions with G Protein-Coupled Receptors. *Handbook of experimental pharmacology*. 2014;219:15-56.
243. Bradbury FA, Menon KMJ. Evidence That Constitutively Active Luteinizing Hormone/Human Chorionic Gonadotropin Receptors Are Rapidly Internalized. *Biochemistry.* 1999;38(27):8703–8712.
244. Check JH, Davies E, Brasile D, Choe JK, Amui J. A prospective comparison of in vitro fertilization (IVF) outcome following controlled ovarian hyperstimulation (COH) regimens using follitropin alpha exclusively or with the addition of low dose human chorionic gonadotropin (hCG) and ganirelix. *Clin Exp Obstet Gynecol.* 2009;36(4):217–218.
245. Berkkanoglu M, Isikoglu M, Aydin D, Ozgur K. Clinical effects of ovulation induction with recombinant follicle-stimulating hormone supplemented with recombinant luteinizing hormone or low-dose recombinant human chorionic gonadotropin in the midfollicular phase in microdose cycles in poor responders. *Fertil Steril.* 2007;88(3):665–669.

246. Sasson R, Shinder V, Dantes A, Land A, Amsterdam A. Activation of multiple signal transduction pathways by glucocorticoids: protection of ovarian follicular cells against apoptosis. *Biochem Biophys Res Commun.* 2003;311(4):1047–1056.
247. Li Q, He H, Zhang Y-L, Li X-M, Guo X, Huo R, Bi Y, Li J, Fan H-Y, Sha J. Phosphoinositide 3-Kinase p110δ Mediates Estrogen- and FSH-Stimulated Ovarian Follicle Growth. *Mol Endocrinol.* 2013;27(9):1468–1482.
248. Couse JF, Yates MM, Deroo BJ, Korach KS. Estrogen Receptor-β Is Critical to Granulosa Cell Differentiation and the Ovulatory Response to Gonadotropins. *Endocrinology.* 2005;146(8):3247–3262.
249. Hirata R, Hojo T, Sano M, Hayashi N, Okuda K. Potential role of hCG in apoptosis of human luteinized granulosa cells. *J Reprod Dev.* 2015;61(1):67–73.
250. Puttabyatappa M, Brogan RS, VandeVoort CA, Chaffin CL. EGF-Like Ligands Mediate Progesterone's Anti-Apoptotic Action on Macaque Granulosa Cells1. *Biol Reprod.* 2013;88(1):18.
251. Kariagina A, Xie J, Leipprandt JR, Haslam SZ. Amphiregulin Mediates Estrogen, Progesterone, and EGFR Signaling in the Normal Rat Mammary Gland and in Hormone-Dependent Rat Mammary Cancers. *Horm Cancer.* 2010;1(5):229–244.
252. Salazar M, Lerma-Ortiz A, Hooks GM, Ashley AK, Ashley RL. Progestin-mediated activation of MAPK and AKT in nuclear progesterone receptor negative breast epithelial cells: The role of membrane progesterone receptors. *Gene.* 2016;591(1):6–13.
253. Faraoni EY, Camilletti MA, Abeledo-Machado A, Ratner LD, De Fino F, Huhtaniemi I, Rulli SB, Díaz-Torga G. Sex differences in the development of prolactinoma in mice overexpressing hCGβ: role of TGFβ1. *J Endocrinol.* 2017;232(3):535–546.
254. Huhtaniemi I, Rulli S, Ahtiainen P, Poutanen M. Multiple sites of tumorigenesis in transgenic mice overproducing hCG. *Mol Cell Endocrinol.* 2005;234(1–2):117–126.
255. Yamashita S, Tai P, Charron J, Ko C, Ascoli M. The Leydig Cell MEK/ERK Pathway Is Critical for Maintaining a Functional Population of Adult Leydig Cells and for Fertility. *Mol Endocrinol.* 2011;25(7):1211–1222.
256. Casarini L, Pignatti E, Simoni M. Effects of polymorphisms in gonadotropin and gonadotropin receptor genes on reproductive function. *Rev Endocr Metab Disord.* 2011;12(4):303–321.
257. Casarini L, Huhtaniemi IT, Simoni M, Rivero-Müller A. Gonadotropin Receptors. In: Simoni M, Huhtaniemi IT, eds. *Endocrinology of the Testis and Male Reproduction. Endocrinology.* Springer, Cham; 2017:123–168.
258. Ma X, Dong Y, Matzuk MM, Kumar TR. Targeted disruption of luteinizing hormone beta-subunit leads to hypogonadism, defects in gonadal steroidogenesis, and infertility. *Proc Natl Acad Sci U S A.* 2004;101(49):17294–17299.
259. Lofrano-Porto A, Barra GB, Giacomini LA, Nascimento PP, Latronico AC, Casulari LA, da Rocha Neves F de A. Luteinizing hormone beta mutation and hypogonadism in men and women. *N Engl J Med.* 2007;357(9):897–904.
260. Pakarainen T, Zhang F-P, Nurmi L, Poutanen M, Huhtaniemi I. Knockout of luteinizing hormone receptor abolishes the effects of follicle-stimulating hormone on preovulatory maturation and ovulation of mouse graafian follicles. *Mol Endocrinol.* 2005;19(10):2591–2602.
261. Pettersson K, Ding YQ, Huhtaniemi I. An immunologically anomalous luteinizing hormone variant in a healthy woman. *J Clin Endocrinol Metab.* 1992;74(1):164–171.
262. Nilsson C, Jiang M, Pettersson K, Iitiä A, Mäkelä M, Simonsen H, Easteal S, Herrera RJ, Huhtaniemi I. Determination of a common genetic variant of luteinizing hormone using DNA hybridization and immunoassays. *Clin Endocrinol (Oxf).* 1998;49(3):369–376.

263. Suganuma N, Furui K, Kikkawa F, Tomoda Y, Furuhashi M. Effects of the mutations (Trp8 --> Arg and Ile15 --> Thr) in human luteinizing hormone (LH) beta-subunit on LH bioactivity in vitro and in vivo. *Endocrinology.* 1996;137(3):831–838.
264. Furui K, Suganuma N, Tsukahara S, Asada Y, Kikkawa F, Tanaka M, Ozawa T, Tomoda Y. Identification of two point mutations in the gene coding luteinizing hormone (LH) beta-subunit, associated with immunologically anomalous LH variants. *J Clin Endocrinol Metab.* 1994;78(1):107–113.
265. Liao WX, Goh HH, Roy AC. Functional characterization of a natural variant of luteinizing hormone. *Hum Genet.* 2002;111(2):219–224.
266. Jiang M, Lamminen T, Pakarinen P, Hellman J, Manna P, Herrera RJ, Huhtaniemi I. A novel Ala(-3)Thr mutation in the signal peptide of human luteinizing hormone beta-subunit: potentiation of the inositol phosphate signalling pathway and attenuation of the adenylate cyclase pathway by recombinant variant hormone. *Mol Hum Reprod.* 2002;8(3):201–212.
267. Bosco L, Ruvolo G, Luparello C, Ferrari S, Valerio D, Santi D, Piomboni P, Sarcina E, Lispi M, Roccheri MC. Gene Expression and Apoptosis Levels in Cumulus Cells of Patients with Polymorphisms of FSHR and LHB Undergoing in Vitro Fertilization Program. *Cell Physiol Biochem.* 2017;43(6):2391–2404.
268. Jiang M, Pakarinen P, Zhang FP, El-Hefnawy T, Koskimies P, Pettersson K, Huhtaniemi I. A common polymorphic allele of the human luteinizing hormone beta-subunit gene: additional mutations and differential function of the promoter sequence. *Hum Mol Genet.* 1999;8(11):2037–2046.
269. Tapanainen JS, Koivunen R, Fauser BC, Taylor AE, Clayton RN, Rajkowa M, White D, Franks S, Anttila L, Pettersson KS, Huhtaniemi IT. A new contributing factor to polycystic ovary syndrome: the genetic variant of luteinizing hormone. *J Clin Endocrinol Metab.* 1999;84(5):1711–1715.
270. Liu S, Ogata T, Maruyama T, Yoshimura Y, Ishizuka B. Association of common LH variant with hyperfunctional promoter in a Japanese infertile woman. *Endocr J.* 2005;52(6):781–784.
271. Punab AM, Grigorova M, Punab M, Adler M, Kuura T, Poolamets O, Vihljajev V, Žilaitienė B, Erenpreiss J, Matulevičius V, Laan M. 'Carriers of V-LH among 1593 Baltic men have significantly higher serum LH'. *Andrology.* 2015;3(3):512–519.
272. Shiraishi K, Naito K. Fertile eunuch syndrome with the mutations (Trp8Arg and Ile15Thr) in the beta subunit of luteinizing hormone. *Endocr J.* 2003;50(6):733–737.
273. Dasgupta S, Sirisha PVS, Neelaveni K, Anuradha K, Sudhakar G, Reddy BM. Role of luteinizing hormone β-subunit gene variants among South Indian women with polycystic ovary syndrome. *Gene.* 2012;494(1):51–56.
274. Cho SG, Bhoumik A, Broday L, Ivanov V, Rosenstein B, Ronai Z. TIP49b, a regulator of activating transcription factor 2 response to stress and DNA damage. *Mol Cell Biol.* 2001;21(24):8398–8413.
275. Zhao Y, Chen T, Zhou Y, Li K, Xiao J. An association study between the genetic polymorphisms within GnRHI, LHβ and FSHβ genes and central precocious puberty in Chinese girls. *Neurosci Lett.* 2010;486(3):188–192.
276. Lamminen T, Jiang M, Manna PR, Pakarinen P, Simonsen H, Herrera RJ, Huhtaniemi I. Functional study of a recombinant form of human LHbeta-subunit variant carrying the Gly(102)Ser mutation found in Asian populations. *Mol Hum Reprod.* 2002;8(10):887–892.
277. Miller-Lindholm AK, Bedows E, Bartels CF, Ramey J, Maclin V, Ruddon RW. A naturally occurring genetic variant in the human chorionic gonadotropin-beta gene 5 is assembly inefficient. *Endocrinology.* 1999;140(8):3496–3506.

278. Rull K, Nagirnaja L, Ulander V-M, Kelgo P, Margus T, Kaare M, Aittomäki K, Laan M. Chorionic gonadotropin beta-gene variants are associated with recurrent miscarriage in two European populations. *J Clin Endocrinol Metab.* 2008;93(12):4697–4706.
279. Casarini L, Brigante G. The Polycystic Ovary Syndrome Evolutionary Paradox: a Genome-Wide Association Studies–Based, in silico, Evolutionary Explanation. *J Clin Endocrinol Metab.* 2014;99(11):E2412–E2420.
280. Nishimura R, Shin J, Ji I, Middaugh CR, Kruggel W, Lewis R V, Ji TH. A single amino acid substitution in an ectopic alpha subunit of a human carcinoma choriogonadotropin. *J Biol Chem.* 1986;261(23):10475–10477.
281. Nagirnaja L, Venclovas Č, Rull K, Jonas KC, Peltoketo H, Christiansen OB, Kairys V, Kivi G, Steffensen R, Huhtaniemi IT, Laan M. Structural and functional analysis of rare missense mutations in human chorionic gonadotrophin β-subunit. *Mol Hum Reprod.* 2012;18(8):379–390.
282. Basciani S, Watanabe M, Mariani S, Passeri M, Persichetti A, Fiore D, Scotto d'Abusco A, Caprio M, Lenzi A, Fabbri A, Gnessi L. Hypogonadism in a patient with two novel mutations of the luteinizing hormone β-subunit gene expressed in a compound heterozygous form. *J Clin Endocrinol Metab.* 2012;97(9):3031–3038.
283. Valdes-Socin H, Salvi R, Daly AF, Gaillard RC, Quatresooz P, Tebeu P-M, Pralong FP, Beckers A. Hypogonadism in a patient with a mutation in the luteinizing hormone beta-subunit gene. *N Engl J Med.* 2004;351(25):2619–2625.
284. Potorac I, Rivero-Müller A, Trehan A, Kiełbus M, Jozwiak K, Pralong F, Hafidi A, Thiry A, Ménagé J-J, Huhtaniemi I, Beckers A, Daly AF. A vital region for human glycoprotein hormone trafficking revealed by an LHB mutation. *J Endocrinol.* 2016;231(3):197–207.
285. Valdes-Socin H, Salvi R, Thiry A, Daly AF, Pralong FP, Gaillard R, Beckers A. Testicular effects of isolated luteinizing hormone deficiency and reversal by long-term human chorionic gonadotropin treatment. *J Clin Endocrinol Metab.* 2009;94(1):3–4.
286. Achard C, Courtillot C, Lahuna O, Méduri G, Soufir J-C, Lière P, Bachelot A, Benyounes H, Schumacher M, Kuttenn F, Touraine P, Misrahi M. Normal spermatogenesis in a man with mutant luteinizing hormone. *N Engl J Med.* 2009;361(19):1856–1863.
287. Takahashi K, Karino K, Kanasaki H, Kurioka H, Ozaki T, Yonehara T, Miyazaki K. Influence of missense mutation and silent mutation of LHbeta-subunit gene in Japanese patients with ovulatory disorders. *Eur J Hum Genet.* 2003;11(5):402–408.
288. Simoni M, Tüttelmann F, Michel C, Böckenfeld Y, Nieschlag E, Gromoll J. Polymorphisms of the luteinizing hormone/chorionic gonadotropin receptor gene: association with maldescended testes and male infertility. *Pharmacogenet Genomics.* 2008;18(3):193–200.
289. Chen Z-J, Zhao H, He L, Shi Y, Qin Y, Shi Y, Li Z, You L, Zhao J, Liu J, Liang X, Zhao X, Zhao J, Sun Y, Zhang B, Jiang H, Zhao D, Bian Y, Gao X, Geng L, Li Y, Zhu D, Sun X, Xu J, Hao C, Ren C, Zhang Y, Chen S, Zhang W, Yang A, Yan J, Li Y, Ma J, Zhao Y. Genome-wide association study identifies susceptibility loci for polycystic ovary syndrome on chromosome 2p16.3, 2p21 and 9q33.3. *Nat Genet.* 2011;43(1):55–59.
290. Piersma D, Berns EMJJ, Verhoef-Post M, Uitterlinden AG, Braakman I, Pols HAP, Themmen APN. A Common Polymorphism Renders the Luteinizing Hormone Receptor Protein More Active by Improving Signal Peptide Function and Predicts Adverse Outcome in Breast Cancer Patients. *J Clin Endocrinol Metab.* 2006;91(4):1470–1476.
291. Rodien P, Cetani F, Costagliola S, Tonacchera M, Duprez L, Minegishi T, Govaerts C, Vassart G. Evidences for an Allelic Variant of the Human LC/CG Receptor rather than a Gene Duplication: Functional Comparison of Wild-Type and Variant Receptors. *J Clin Endocrinol Metab.* 1998;83(12):4431–4434.

292. Kossack N, Simoni M, Richter-Unruh A, Themmen APN, Gromoll J. Mutations in a Novel, Cryptic Exon of the Luteinizing Hormone/Chorionic Gonadotropin Receptor Gene Cause Male Pseudohermaphroditism. *PLoS Med.* 2008;5(4):e88.
293. Kossack N, Troppmann B, Richter-Unruh A, Kleinau G, Gromoll J. Aberrant transcription of the LHCGR gene caused by a mutation in exon 6A leads to Leydig cell hypoplasia type II. *Mol Cell Endocrinol.* 2013;366(1):59–67.
294. Liu W, Han B, Zhu W, Cheng T, Fan M, Wu J, Yang Y, Zhu H, Si J, Lyu Q, Chai W, Zhao S, Song H, Kuang Y, Qiao J. Polymorphism in the Alternative Donor Site of the Cryptic Exon of LHCGR: Functional Consequences and Associations with Testosterone Level. *Sci Rep.* 2017;7:45699.
295. Frendo J-L, Guibourdenche J, Pidoux G, Vidaud M, Luton D, Giovangrandi Y, Porquet D, Muller F, Evain-Brion D. Trophoblast production of a weakly bioactive human chorionic gonadotropin in trisomy 21-affected pregnancy. *J Clin Endocrinol Metab.* 2004;89(2):727–732.
296. Sutton JM, Cole LA. Sialic acid–deficient invasive trophoblast antigen(sd-ITA): a new urinary variant for gestational Down syndrome screening. *Prenat Diagn.* 2004;24(3):194–197.
297. Talbot JA, Spencer K, Abushoufa RA. Detection of maternal serum hCG glycoform variants in the second trimester of pregnancies affected by Down syndrome using a lectin immunoassay. *Prenat Diagn.* 2003;23(1):1–5.
298. Plöckinger U, Chrusciel M, Doroszko M, Saeger W, Blankenstein O, Weizsäcker K, Kroiss M, Hauptmann K, Radke C, Pöllinger A, Tiling N, Steinmüller T, Huhtaniemi I, Quinkler M, Bertherat J, Lacroix A, Rahman N. Functional Implications of LH/hCG Receptors in Pregnancy-Induced Cushing Syndrome. *J Endocr Soc.* 2017;1(1):57–71.
299. Latronico AC, Anasti J, Arnhold IJ, Rapaport R, Mendonca BB, Bloise W, Castro M, Tsigos C, Chrousos GP. Brief report: testicular and ovarian resistance to luteinizing hormone caused by inactivating mutations of the luteinizing hormone-receptor gene. *N Engl J Med.* 1996;334(8):507–512.
300. Basaria S. Male hypogonadism. *Lancet.* 2014;383(9924):1250–1263.
301. Topaloğlu AK. Update on the Genetics of Idiopathic Hypogonadotropic Hypogonadism. *J Clin Res Pediatr Endocrinol.* 2017;9(Suppl 2):113-122.
302. Vezzoli V, Duminuco P, Vottero A, Kleinau G, Schülein R, Minari R, Bassi I, Bernasconi S, Persani L, Bonomi M. A new variant in signal peptide of the human luteinizing hormone receptor (*LHCGR*) affects receptor biogenesis causing leydig cell hypoplasia. *Hum Mol Genet.* 2015;24(21):6003–6012.
303. Kremer H, Kraaij R, Toledo SP, Post M, Fridman JB, Hayashida CY, van Reen M, Milgrom E, Ropers HH, Mariman E, Themmen APN, Brunner HG. Male pseudohermaphroditism due to a homozygous missense mutation of the luteinizing hormone receptor gene. *Nat Genet.* 1995;9(2):160–164.
304. Arnhold IJ, Lofrano-Porto A, Latronico AC. Inactivating Mutations of Luteinizing Hormone β-Subunit or Luteinizing Hormone Receptor Cause Oligo-Amenorrhea and Infertility in Women. *Horm Res Paediatr.* 2009;71(2):75–82.
305. Weiss J, Axelrod L, Whitcomb RW, Harris PE, Crowley WF, Jameson JL. Hypogonadism caused by a single amino acid substitution in the beta subunit of luteinizing hormone. *N Engl J Med.* 1992;326(3):179–183.
306. Yang X, Ochin H, Shu L, Liu J, Shen J, Liu J, Lin C, Cui Y. Homozygous nonsense mutation Trp28X in the LHB gene causes male hypogonadism. *J Assist Reprod Genet.* 2018. doi:10.1007/s10815-018-1133-5.

307. Lee C, Ji I, Ji TH. Distinct Mechanisms of cAMP Induction by Constitutively Activating LH Receptor and Wild-Type LH Receptor Activated by hCG. *Endocrine.* 2004;25(2):111–116.
308. Azziz R, Carmina E, Chen Z, Dunaif A, Laven JSE, Legro RS, Lizneva D, Natterson-Horowtiz B, Teede HJ, Yildiz BO. Polycystic ovary syndrome. *Nat Rev Dis Prim.* 2016;2:16057.
309. Rosenfield RL, Ehrmann DA. The Pathogenesis of Polycystic Ovary Syndrome (PCOS): The Hypothesis of PCOS as Functional Ovarian Hyperandrogenism Revisited. *Endocr Rev.* 2016;37(5):467–520.
310. Ünlütürk U, Sezgin E, Yildiz BO. Evolutionary determinants of polycystic ovary syndrome: part 1. *Fertil Steril.* 2016;106(1):33–41.
311. Fessler DMT, Natterson-Horowitz B, Azziz R. Evolutionary determinants of polycystic ovary syndrome: part 2. *Fertil Steril.* 2016;106(1):42–47.
312. Casarini L, Simoni M, Brigante G. Is polycystic ovary syndrome a sexual conflict? A review. *Reprod Biomed Online.* 2016;32(4):350–361.
313. Shi Y, Zhao H, Shi Y, Cao Y, Yang D, Li Z, Zhang B, Liang X, Li T, Chen J, Shen J, Zhao J, You L, Gao X, Zhu D, Zhao X, Yan Y, Qin Y, Li W, Yan J, Wang Q, Zhao J, Geng L, Ma J, Zhao Y, He G, Zhang A, Zou S, Yang A, Liu J, Li W, Li B, Wan C, Qin Y, Shi J, Yang J, Jiang H, Xu J, Qi X, Sun Y, Zhang Y, Hao C, Ju X, Zhao D, Ren C, Li X, Zhang W, Zhang Y, Zhang J, Wu D, Zhang C, He L, Chen Z-J. Genome-wide association study identifies eight new risk loci for polycystic ovary syndrome. *Nat Genet.* 2012;44(9):1020–1025.
314. Chen Z-J, Zhao H, He L, Shi Y, Qin Y, Shi Y, Li Z, You L, Zhao J, Liu J, Liang X, Zhao X, Zhao J, Sun Y, Zhang B, Jiang H, Zhao D, Bian Y, Gao X, Geng L, Li Y, Zhu D, Sun X, Xu J, Hao C, Ren C, Zhang Y, Chen S, Zhang W, Yang A, Yan J, Li Y, Ma J, Zhao Y. Genome-wide association study identifies susceptibility loci for polycystic ovary syndrome on chromosome 2p16.3, 2p21 and 9q33.3. *Nat Genet.* 2011;43(1):55–59.
315. Palioura E, Diamanti-Kandarakis E. Polycystic ovary syndrome (PCOS) and endocrine disrupting chemicals (EDCs). *Rev Endocr Metab Disord.* 2015;16(4):365–371.
316. Wachs DS, Coffler MS, Malcom PJ, Shimasaki S, Chang RJ. Increased Androgen Response to Follicle-Stimulating Hormone Administration in Women with Polycystic Ovary Syndrome. *J Clin Endocrinol Metab.* 2008;93(5):1827–1833.
317. Franks S, Adams J, Mason H, Polson D. Ovulatory disorders in women with polycystic ovary syndrome. *Clin Obstet Gynaecol.* 1985;12(3):605–32.
318. Dickey RP, Holtkamp DE. Development, pharmacology and clinical experience with clomiphene citrate. *Hum Reprod Update.* 1996;2(6):483–506.
319. Kero J, Poutanen M, Zhang F-P, Rahman N, McNicol AM, Nilson JH, Keri RA, Huhtaniemi IT. Elevated luteinizing hormone induces expression of its receptor and promotes steroidogenesis in the adrenal cortex. *J Clin Invest.* 2000;105(5):633–641.
320. McAllister JM, Legro RS, Modi BP, Strauss JF. Functional genomics of PCOS: from GWAS to molecular mechanisms. *Trends Endocrinol Metab.* 2015;26(3):118–124.
321. Nelson-Degrave VL, Wickenheisser JK, Hendricks KL, Asano T, Fujishiro M, Legro RS, Kimball SR, Strauss JF, McAllister JM. Alterations in mitogen-activated protein kinase kinase and extracellular regulated kinase signaling in theca cells contribute to excessive androgen production in polycystic ovary syndrome. *Mol Endocrinol.* 2005;19(2):379–390.
322. Batista MCP, Duarte E de F, Borba MD dos R, Zingler E, Mangussi-Gomes J, dos Santos BTA, de Moraes OL, Hayashida SAY, Baracat EC, da Rocha Neves F de A, Maciel GAR, Bachega TASS, Barra GB, Lofrano-Porto A. Trp28Arg/Ile35Thr LHB gene variants are associated with elevated testosterone levels in women with polycystic ovary syndrome. *Gene.* 2014;550(1):68–73.

323. Kurioka H, Takahashi K, Irikoma M, Okada M, Ozaki T, Ueda T, Miyazaki K. Diagnostic difficulty in polycystic ovary syndrome due to an LH-beta-subunit variant. *Eur J Endocrinol.* 1999;140(3):235–238.
324. Elter K, Erel CT, Cine N, Ozbek U, Hacihanefioglu B, Ertungealp E. Role of the mutations Trp8 => Arg and Ile15 => Thr of the human luteinizing hormone beta-subunit in women with polycystic ovary syndrome. *Fertil Steril.* 1999;71(3):425–430.
325. Lobo RA, Shoupe D, Chang SP, Campeau J. The control of bioactive luteinizing hormone secretion in women with polycystic ovary syndrome. *Am J Obstet Gynecol.* 1984;148(4):423–428.
326. Ding YQ, Huhtaniemi I. Preponderance of basic isoforms of serum luteinizing hormone (LH) is associated with the high bio/immune ratio of LH in healthy women and in women with polycystic ovarian disease. *Hum Reprod.* 1991;6(3):346–350.
327. Ropelato MG, Garcia-Rudaz MC, Castro-Fernandez C, Ulloa-Aguirre A, Escobar ME, Barontini M, Veldhuis JD. A preponderance of basic luteinizing hormone (LH) isoforms accompanies inappropriate hypersecretion of both basal and pulsatile LH in adolescents with polycystic ovarian syndrome. *J Clin Endocrinol Metab.* 1999;84(12):4629–4636.
328. Hayes FJ, Taylor AE, Martin KA, Hall JE. Use of a gonadotropin-releasing hormone antagonist as a physiologic probe in polycystic ovary syndrome: assessment of neuroendocrine and androgen dynamics. *J Clin Endocrinol Metab.* 1998;83(7):2343–2349.
329. Balen AH. Ovulation induction in the management of anovulatory polycystic ovary syndrome. *Mol Cell Endocrinol.* 2013;373(1–2):77–82.
330. Engmann L, Siano L, Schmidt D, Nulsen J, Maier D, Benadiva C. GnRH agonist to induce oocyte maturation during IVF in patients at high risk of OHSS. *Reprod Biomed Online.* 2006;13(5):639–644.
331. Krishna D, Dhoble S, Praneesh G, Rathore S, Upadhaya A, Rao K. Gonadotropin-releasing hormone agonist trigger is a better alternative than human chorionic gonadotropin in PCOS undergoing IVF cycles for an OHSS Free Clinic: A Randomized control trial. *J Hum Reprod Sci.* 2016;9(3):164.
332. Cela V, Obino MER, Alberga Y, Pinelli S, Sergiampietri C, Casarosa E, Simi G, Papini F, Artini PG. Ovarian response to controlled ovarian stimulation in women with different polycystic ovary syndrome phenotypes. *Gynecol Endocrinol.* 2017:1–6.
333. Karsli MF, Gultekin IB, Cakmak B, Yeral MI, Seckin KD, Alt Nboga O, Kucukozkan T. Do we need readjustment of the biochemical parameters in first trimester combined aneuploidy screening test in women with polycystic ovary syndrome? *Prenat Diagn.* 2014;34(11):1073–1076.
334. Saxena R, Bjonnes AC, Georgopoulos NA, Koika V, Panidis D, Welt CK. Gene variants associated with age at menopause are also associated with polycystic ovary syndrome, gonadotrophins and ovarian volume. *Hum Reprod.* 2015;30(7):1697–1703.
335. Corbett S, Morin-Papunen L. The Polycystic Ovary Syndrome and recent human evolution. *Mol Cell Endocrinol.* 2013;373(1–2):39–50.
336. Kajii T, Ferrier A, Niikawa N, Takahara H, Ohama K, Avirachan S. Anatomic and chromosomal anomalies in 639 spontaneous abortuses. *Hum Genet.* 1980;55(1):87–98.
337. Abell S, Nankervis A, Khan K, Teede H. Type 1 and Type 2 Diabetes Preconception and in Pregnancy: Health Impacts, Influence of Obesity and Lifestyle, and Principles of Management. *Semin Reprod Med.* 2016;34(2):110–120.
338. Saner-Amigh K, Mayhew BA, Mantero F, Schiavi F, White PC, Rao C V., Rainey WE. Elevated Expression of Luteinizing Hormone Receptor in Aldosterone-Producing Adenomas. *J Clin Endocrinol Metab.* 2006;91(3):1136–1142.

339. Melnick AP, Pereira N, Murphy EM, Rosenwaks Z, Spandorfer SD. How low is too low? Cycle day 28 estradiol levels and pregnancy outcomes. *Fertil Steril.* 2016;105(4):905–909.e1.
340. Saccone G, Schoen C, Franasiak JM, Scott RT, Berghella V. Supplementation with progestogens in the first trimester of pregnancy to prevent miscarriage in women with unexplained recurrent miscarriage: a systematic review and meta-analysis of randomized, controlled trials. *Fertil Steril.* 2017;107(2):430–438.e3.
341. Chen C-D, Chiang Y-T, Yang P-K, Chen M-J, Chang C-H, Yang Y-S, Chen S-U. Frequency of low serum LH is associated with increased early pregnancy loss in IVF/ICSI cycles. *Reprod Biomed Online.* 2016;33(4):449–457.
342. Lohstroh PN, Overstreet JW, Stewart DR, Nakajima ST, Cragun JR, Boyers SP, Lasley BL. Secretion and excretion of human chorionic gonadotropin during early pregnancy. *Fertil Steril.* 2005;83(4):1000–1011.
343. Iles RK, Delves PJ, Butler SA. Does hCG or hCGβ play a role in cancer cell biology? *Mol Cell Endocrinol.* 2010;329(1–2):62–70.
344. Layman LC, Edwards JL, Osborne WE, Peak DB, Gallup DG, Tho SP, Reindollar RH, Roach DJ, McDonough PG, Lanclos KD. Human chorionic gonadotrophin-beta gene sequences in women with disorders of HCG production. *Mol Hum Reprod.* 1997;3(4):315–20.
345. Mangel L, Bíró K, Battyáni I, Göcze P, Tornóczky T, Kálmán E. A case study on the potential angiogenic effect of human chorionic gonadotropin hormone in rapid progression and spontaneous regression of metastatic renal cell carcinoma during pregnancy and after surgical abortion. *BMC Cancer.* 2015;15(1):1013.
346. Głodek A, Jankowska A. CGB activates ERK and AKT kinases in cancer cells via LHCGR-independent mechanism. *Tumor Biol.* 2014;35(6):5467–5479.
347. Evans J. Hyperglycosylated hCG: a Unique Human Implantation and Invasion Factor. *Am J Reprod Immunol.* 2016;75(3):333–340.
348. Lee C-L, Chiu PCN, Hautala L, Salo T, Yeung WSB, Stenman U-H, Koistinen H. Human chorionic gonadotropin and its free β-subunit stimulate trophoblast invasion independent of LH/hCG receptor. *Mol Cell Endocrinol.* 2013;375(1–2):43–52.
349. Gehring C, Siepmann T, Heidegger H, Jeschke U. The controversial role of human chorionic gonadotropin in the development of breast cancer and other types of tumors. *The Breast.* 2016;26:135–140.
350. Aharoni D, Dantes A, Oren M, Amsterdam A. cAMP-Mediated Signals as Determinants for Apoptosis in Primary Granulosa Cells. *Exp Cell Res.* 1995;218(1):271–282.
351. Amsterdam A, Keren-Tal I, Aharoni D. Cross-talk between cAMP and p53-generated signals in induction of differentiation and apoptosis in steroidogenic granulosa cells. *Steroids.* 1996;61(4):252–256.
352. Kumar N, Gupta S, Dabral S, Singh S, Sehrawat S. Role of exchange protein directly activated by cAMP (EPAC1) in breast cancer cell migration and apoptosis. *Mol Cell Biochem.* 2017;430(1–2):115–125.
353. Lenhard M, Tsvilina A, Schumacher L, Kupka M, Ditsch N, Mayr D, Friese K, Jeschke U. Human chorionic gonadotropin and its relation to grade, stage and patient survival in ovarian cancer. *BMC Cancer.* 2012;12(1):2.
354. Henríquez S, Kohen P, Xu X, Veenstra TD, Muñoz A, Palomino WA, Strauss JF, Devoto L. Estrogen metabolites in human corpus luteum physiology: differential effects on angiogenic activity. *Fertil Steril.* 2016;106(1):230–237.e1.
355. Mohammad HP, Abbud RA, Parlow AF, Lewin JS, Nilson JH. Targeted overexpression of luteinizing hormone causes ovary-dependent functional adenomas restricted to cells of the Pit-1 lineage. *Endocrinology.* 2003;144(10):4626–4636.

356. Ratner LD, Gonzalez B, Ahtiainen P, Di Giorgio NP, Poutanen M, Calandra RS, Huhtaniemi IT, Rulli SB. Short-Term Pharmacological Suppression of the Hyperprolactinemia of Infertile hCG-Overproducing Female Mice Persistently Restores Their Fertility. *Endocrinology.* 2012;153(12):5980–5992.
357. Bernichtein S, Peltoketo H, Huhtaniemi I. Adrenal hyperplasia and tumours in mice in connection with aberrant pituitary–gonadal function. *Mol Cell Endocrinol.* 2009;300(1–2):164–168.
358. Doroszko M, Chrusciel M, Belling K, Vuorenoja S, Dalgaard M, Leffers H, Nielsen HB, Huhtaniemi I, Toppari J, Rahman NA. Novel genes involved in pathophysiology of gonadotropin-dependent adrenal tumors in mice. *Mol Cell Endocrinol.* 2017;444:9–18.
359. Bernichtein S, Alevizaki M, Huhtaniemi I. Is the adrenal cortex a target for gonadotropins? *Trends Endocrinol Metab.* 2008;19(7):231–238.
360. de Lange WE, Pratt JJ, Doorenbos H. A gonadotrophin responsive testosterone producing adrenocortical adenoma and high gonadotrophin levels in an elderly woman. *Clin Endocrinol (Oxf).* 1980;12(1):21–28.
361. Teo AED, Garg S, Haris Shaikh L, Zhou J, Karet Frankl FE, Gurnell M, Happerfield L, Marker A, Bienz M, Azizan EAB, Brown MJ. Pregnancy, Primary Aldosteronism, and Adrenal *CTNNB1* Mutations. *N Engl J Med.* 2015;373(15):1429–1436.
362. Liu G, Duranteau L, Carel J-C, Monroe J, Doyle DA, Shenker A. Leydig-Cell Tumors Caused by an Activating Mutation of the Gene Encoding the Luteinizing Hormone Receptor. *N Engl J Med.* 1999;341(23):1731–1736.
363. Newton CL, Anderson RC, Millar RP. Therapeutic Neuroendocrine Agonist and Antagonist Analogs of Hypothalamic Neuropeptides as Modulators of the Hypothalamic-Pituitary-Gonadal Axis. *Endocr Dev.* 2016;30:106-129.
364. Lacroix A, Bourdeau I, Lampron A, Mazzuco TL, Tremblay J, Hamet P. Aberrant G-protein coupled receptor expression in relation to adrenocortical overfunction. *Clin Endocrinol (Oxf).* 2009;73(1):1–15.
365. Baer G, Loumaye E. Comparison of recombinant human luteinising hormone (r-hLH) and human menopausal gonadotropin (hMG) in assisted reproductive technology. *Curr Med Res Opin.* 2003;19(2):83–88.
366. Riccetti L, Klett D, Ayoub MA, Boulo T, Pignatti E, Tagliavini S, Varani M, Trenti T, Nicoli A, Capodanno F, La Sala GB, Reiter E, Simoni M, Casarini L. Heterogeneous hCG and hMG commercial preparations result in different intracellular signalling but induce a similar long-term progesterone response in vitro. *MHR Basic Sci Reprod Med.* 2017;23(10):685–697.
367. Stocco DM, Wang X, Jo Y, Manna PR. Multiple signaling pathways regulating steroidogenesis and steroidogenic acute regulatory protein expression: more complicated than we thought. *Mol Endocrinol.* 2005;19(11):2647–2659.
368. Evans HM, Simpson ME, Austin PR. The recognition and comparison of Prolan and Prolan-like substances. *J Exp Med.* 1933;58(5):561–568.
369. Johnsen SG, Hamburger C. Studies on urinary gonadotrophins. IV. Qualitative difference between the international reference preparation for human menopausal gonadotrophin and other extracts of urine from postmenopausal women. *Acta Endocrinol (Copenh).* 1959;32:497–508.
370. Giudice E, Crisci C, Eshkol A, Papoian R. Composition of commercial gonadotrophin preparations extracted from human post-menopausal urine: characterization of non-gonadotrophin proteins. *Hum Reprod.* 1994;9(12):2291–2299.
371. Wolfenson C, Groisman J, Couto AS, Hedenfalk M, Cortvrindt RG, Smitz JE, Jespersen S. Batch-to-batch consistency of human-derived gonadotrophin preparations compared with recombinant preparations. *Reprod Biomed Online.* 2005;10(4):442–454.

372. van de Weijer BHM, Mulders JWM, Bos ES, Verhaert PDEM, van den Hooven HW. Compositional analyses of a human menopausal gonadotrophin preparation extracted from urine (menotropin). Identification of some of its major impurities. *Reprod Biomed Online.* 2003;7(5):547–557.
373. Zegers-Hochschild F, Fernández E, Mackenna A, Fabres C, Altieri E, Lopez T. The empty follicle syndrome: a pharmaceutical industry syndrome. *Hum Reprod.* 1995;10(9):2262–2265.
374. Casarini L, Brigante G, Simoni M, Santi D. Clinical Applications of Gonadotropins in the Female: Assisted Reproduction and Beyond. *Prog Mol Biol Transl Sci.* 2016;143:85–119.
375. Perlman S, van den Hazel B, Christiansen J, Gram-Nielsen S, Jeppesen CB, Andersen K V, Halkier T, Okkels S, Schambye HT. Glycosylation of an N-terminal extension prolongs the half-life and increases the in vivo activity of follicle stimulating hormone. *J Clin Endocrinol Metab.* 2003;88(7):3227–3235.
376. Fares FA, Suganuma N, Nishimori K, LaPolt PS, Hsueh AJ, Boime I. Design of a long-acting follitropin agonist by fusing the C-terminal sequence of the chorionic gonadotropin beta subunit to the follitropin beta subunit. *Proc Natl Acad Sci U S A.* 1992;89(10):4304–4308.
377. Klein J, Lobel L, Pollak S, Lustbader B, Ogden RT, Sauer M V, Lustbader JW. Development and characterization of a long-acting recombinant hFSH agonist. *Hum Reprod.* 2003;18(1):50–56.
378. Devroey P, Fauser BC, Platteau P, Beckers NG, Dhont M, Mannaerts BM. Induction of multiple follicular development by a single dose of long-acting recombinant follicle-Stimulating hormone (FSH-CTP, corifollitropin alfa) for controlled ovarian stimulation before in vitro fertilization. *J Clin Endocrinol Metab.* 2004;89(5):2062–2070.
379. Edwards RG, Steptoe PC. Induction of follicular growth, ovulation and luteinization in the human ovary. *J Reprod Fertil Suppl.* 1975;(22):121–163.
380. Cole LA. Biological functions of hCG and hCG-related molecules. *Reprod Biol Endocrinol.* 2010;8(1):102.
381. Wide L, Bakos O. More basic forms of both human follicle-stimulating hormone and luteinizing hormone in serum at midcycle compared with the follicular or luteal phase. *J Clin Endocrinol Metab.* 1993;76(4):885–889.
382. Al-Inany HG, Abou-Setta AM, Aboulghar MA, Mansour RT, Serour GI. HMG versus rFSH for ovulation induction in developing countries: a cost-effectiveness analysis based on the results of a recent meta-analysis. *Reprod Biomed Online.* 2006;12(2):163–169.
383. Nakano R, Mizuno T, Kotsuji F, Katayama K, Wshio M, Tojo S. “Triggering” of ovulation after infusion of synthetic luteinizing hormone releasing factor (LRF). *Acta Obstet Gynecol Scand.* 1973;52(3):269–272.
384. Diedrich K, Diedrich C, Santos E, Zoll C, al-Hasani S, Reissmann T, Krebs D, Klingmüller D. Suppression of the endogenous luteinizing hormone surge by the gonadotrophin-releasing hormone antagonist Cetrorelix during ovarian stimulation. *Hum Reprod.* 1994;9(5):788–791.
385. Albano C, Smitz J, Camus M, Riethmüller-Winzen H, Van Steirteghem A, Devroey P. Comparison of different doses of gonadotropin-releasing hormone antagonist Cetrorelix during controlled ovarian hyperstimulation. *Fertil Steril.* 1997;67(5):917–922.
386. Itskovitz J, Boldes R, Levron J, Erlik Y, Kahana L, Brandes JM. Induction of preovulatory luteinizing hormone surge and prevention of ovarian hyperstimulation syndrome by gonadotropin-releasing hormone agonist. *Fertil Steril.* 1991;56(2):213–220.
387. Hoff JD, Quigley ME, Yen SS. Hormonal dynamics at midcycle: a reevaluation. *J Clin Endocrinol Metab.* 1983;57(4):792–796.

388. Haahr T, Roque M, Esteves SC, Humaidan P. GnRH Agonist Trigger and LH Activity Luteal Phase Support versus hCG Trigger and Conventional Luteal Phase Support in Fresh Embryo Transfer IVF/ICSI Cycles-A Systematic PRISMA Review and Meta-analysis. *Front Endocrinol (Lausanne).* 2017;8:116.
389. Castillo JC, Humaidan P, Bernabéu R. Pharmaceutical options for triggering of final oocyte maturation in ART. *Biomed Res Int.* 2014;2014:580171.
390. Bodri D, Guillén JJ, Galindo A, Mataró D, Pujol A, Coll O. Triggering with human chorionic gonadotropin or a gonadotropin-releasing hormone agonist in gonadotropin-releasing hormone antagonist-treated oocyte donor cycles: findings of a large retrospective cohort study. *Fertil Steril.* 2009;91(2):365–371.
391. Acevedo B, Gomez-Palomares JL, Ricciarelli E, Hernández ER. Triggering ovulation with gonadotropin-releasing hormone agonists does not compromise embryo implantation rates. *Fertil Steril.* 2006;86(6):1682–1687.
392. Melo M, Busso CE, Bellver J, Alama P, Garrido N, Meseguer M, Pellicer A, Remohí J. GnRH agonist versus recombinant HCG in an oocyte donation programme: a randomized, prospective, controlled, assessor-blind study. *Reprod Biomed Online.* 2009;19(4):486–492.
393. Benadiva C, Engmann L. Intensive luteal phase support after GnRH agonist trigger: it does help. *Reprod Biomed Online.* 2012;25(3):329–330.
394. Humaidan P, Polyzos NP, Alsbjerg B, Erb K, Mikkelsen AL, Elbaek HO, Papanikolaou EG, Andersen CY. GnRHa trigger and individualized luteal phase hCG support according to ovarian response to stimulation: two prospective randomized controlled multi-centre studies in IVF patients. *Hum Reprod.* 2013;28(9):2511–2521.
395. Castillo JC, Dolz M, Bienvenido E, Abad L, Casañ EM, Bonilla-Musoles F. Cycles triggered with GnRH agonist: exploring low-dose HCG for luteal support. *Reprod Biomed Online.* 2010;20(2):175–181.
396. Papanikolaou EG, Verpoest W, Fatemi H, Tarlatzis B, Devroey P, Tournaye H. A novel method of luteal supplementation with recombinant luteinizing hormone when a gonadotropin-releasing hormone agonist is used instead of human chorionic gonadotropin for ovulation triggering: a randomized prospective proof of concept study. *Fertil Steril.* 2011;95(3):1174–1177.
397. Santi D, Corona G. Primary and Secondary Hypogonadism. In: Simoni M, Huhtaniemi IT, eds. *Endocrinology of the Testis and Male Reproduction. Endocrinology.* Springer, Cham; 2017:687–747.
398. Barrio R, de Luis D, Alonso M, Lamas A, Moreno JC. Induction of puberty with human chorionic gonadotropin and follicle-stimulating hormone in adolescent males with hypogonadotropic hypogonadism. *Fertil Steril.* 1999;71(2):244–248.
399. Kobori Y, Suzuki K, Iwahata T, Shin T, Sato R, Nishio K, Yagi H, Arai G, Soh S, Okada H. Hormonal therapy (hCG and rhFSH) for infertile men with adult-onset idiopathic hypogonadotropic hypogonadism. *Syst Biol Reprod Med.* 2015;61(2):110–112.
400. Veldhuis JD, Liu PY, Takahashi PY, Weist SM, Wigham JR. Analysis of the impact of intravenous LH pulses versus continuous LH infusion on testosterone secretion during GnRH-receptor blockade. *Am J Physiol Integr Comp Physiol.* 2012;303(10):R994–R1002.
401. Mochtar MH, Van der Veen F, Ziech M, van Wely M, Musters A. Recombinant Luteinizing Hormone (rLH) for controlled ovarian hyperstimulation in assisted reproductive cycles. *Cochrane database Syst Rev.* 2007;(2):CD005070.
402. Mochtar MH, Danhof NA, Ayeleke RO, Van der Veen F, van Wely M. Recombinant luteinizing hormone (rLH) and recombinant follicle stimulating hormone (rFSH) for ovarian stimulation in IVF/ICSI cycles. *Cochrane Database Syst Rev.* 2017;5:CD005070.
403. Legro RS, Kunselman AR. A good meta-analysis is hard to find. *Fertil Steril* 2012;97(5):1048–9.

404. König TE, van der Houwen LEE, Lambalk CB. Recombinant LH supplementation in women of 35 years and older undergoing IVF? *Fertil Steril.* 2012;98(1):e10–e11.
405. Smith V, Osianlis T, Vollenhoven B. Prevention of Ovarian Hyperstimulation Syndrome: A Review. *Obstet Gynecol Int.* 2015;2015:1–10.
406. Nastri CO, Ferriani RA, Rocha IA, Martins WP. Ovarian hyperstimulation syndrome: pathophysiology and prevention. *J Assist Reprod Genet.* 2010;27(2–3):121–128.
407. Youssef MA, Van der Veen F, Al-Inany HG, Mochtar MH, Griesinger G, Nagi Mohesen M, Aboulfoutouh I, van Wely M. Gonadotropin-releasing hormone agonist versus HCG for oocyte triggering in antagonist-assisted reproductive technology. *Cochrane Database Syst Rev.* 2014;(10):CD008046.
408. Ozgur K, Humaidan P, Coetzee K. Segmented ART - The new era in ART? *Reprod Biol.* 2016;16(2):91–103.
409. La Marca A, Sunkara SK. Individualization of controlled ovarian stimulation in IVF using ovarian reserve markers: from theory to practice. *Hum Reprod Update.* 2014;20(1):124–140.
410. Wood HM, Elder JS. Cryptorchidism and Testicular Cancer: Separating Fact From Fiction. *J Urol.* 2009;181(2):452–461.
411. Dunkel L, Taskinen S, Hovatta O, Tilly JL, Wikström S. Germ cell apoptosis after treatment of cryptorchidism with human chorionic gonadotropin is associated with impaired reproductive function in the adult. *J Clin Invest.* 1997;100(9):2341–2346.
412. Colicchia M, Campagnolo L, Baldini E, Ulisse S, Valensise H, Moretti C. Molecular basis of thyrotropin and thyroid hormone action during implantation and early development. *Hum Reprod Update.* 2014;20(6):884–904.
413. Duarte-Guterman P, Navarro-Martín L, Trudeau VL. Mechanisms of crosstalk between endocrine systems: Regulation of sex steroid hormone synthesis and action by thyroid hormones. *Gen Comp Endocrinol.* 2014;203:69–85.
414. Cornell JS, Pierce JG. The subunits of human pituitary thyroid-stimulating hormone. Isolation, properties, and composition. *J Biol Chem.* 1973;248(12):4327–4333.
415. Nagayama Y, Russo D, Chazenbalk GD, Wadsworth HL, Rapoport B. Extracellular domain chimeras of the TSH and LH/CG receptors reveal the mid-region (amino acids 171-260) to play a vital role in high affinity TSH binding. *Biochem Biophys Res Commun.* 1990;173(3):1150–1156.
416. Tomer Y, Huber GK, Davies TF. Human chorionic gonadotropin (hCG) interacts directly with recombinant human TSH receptors. *J Clin Endocrinol Metab.* 1992;74(6):1477–1479.
417. Schubert RL, Narayan P, Puett D. Specificity of cognate ligand-receptor interactions: fusion proteins of human chorionic gonadotropin and the heptahelical receptors for human luteinizing hormone, thyroid-stimulating hormone, and follicle-stimulating hormone. *Endocrinology.* 2003;144(1):129–137.
418. Kraiem Z, Sadeh O, Blithe DL, Nisula BC. Human chorionic gonadotropin stimulates thyroid hormone secretion, iodide uptake, organification, and adenosine 3’,5’-monophosphate formation in cultured human thyrocytes. *J Clin Endocrinol Metab.* 1994;79(2):595–599.
419. Yoshimura M, Hershman JM, Pang XP, Berg L, Pekary AE. Activation of the thyrotropin (TSH) receptor by human chorionic gonadotropin and luteinizing hormone in Chinese hamster ovary cells expressing functional human TSH receptors. *J Clin Endocrinol Metab.* 1993;77(4):1009–1013.
420. Carayon P, Lefort G, Nisula B. Interaction of Human Chorionic Gonadotropin and Human Luteinizing Hormone with Human Thyroid Membranes. *Endocrinology.* 1980;106(6):1907–1916.

421. Yoshimura M, Pekary AE, Pang XP, Berg L, Cole LA, Kardana A, Hershman JM. Effect of peptide nicking in the human chorionic gonadotropin beta-subunit on stimulation of recombinant human thyroid-stimulating hormone receptors. *Eur J Endocrinol.* 1994;130(1):92–96.
422. Harada A, Hershman JM, Reed AW, Braunstein GD, Dignam WJ, Derzko C, Friedman S, Jewelewicz R, Pekary AE. Comparison of thyroid stimulators and thyroid hormone concentrations in the sera of pregnant women. *J Clin Endocrinol Metab.* 1979;48(5):793–797.
423. Grün JP, Meuris S, De Nayer P, Glinoer D. The thyrotrophic role of human chorionic gonadotrophin (hCG) in the early stages of twin (versus single) pregnancies. *Clin Endocrinol (Oxf).* 1997;46(6):719–725.
424. Ross HA, Exalto N, Kloppenborg PW, Benraad TJ. Thyroid hormone binding in early pregnancy and the risk of spontaneous abortion. *Eur J Obstet Gynecol Reprod Biol.* 1989;32(2):129–136.
425. Aghajanova L, Stavreus-Evers A, Lindeberg M, Landgren B-M, Sparre LS, Hovatta O. Thyroid-stimulating hormone receptor and thyroid hormone receptors are involved in human endometrial physiology. *Fertil Steril.* 2011;95(1):230–237.e2.
426. Benvenga S, Di Bari F, Granese R, Antonelli A. Serum Thyrotropin and Phase of the Menstrual Cycle. *Front Endocrinol (Lausanne).* 2017;8:250.
427. Weber G, Vigone MC, Stroppa L, Chiumello G. Thyroid function and puberty. *J Pediatr Endocrinol Metab.* 2003;16 Suppl 2:253–257.
428. Koutras DA. Disturbances of menstruation in thyroid disease. *Ann N Y Acad Sci.* 1997;816:280–284.
429. Nakabayashi K, Matsumi H, Bhalla A, Bae J, Mosselman S, Hsu SY, Hsueh AJW. Thyrostimulin, a heterodimer of two new human glycoprotein hormone subunits, activates the thyroid-stimulating hormone receptor. *J Clin Invest.* 2002;109(11):1445–1452.
430. Sun S-C, Hsu P-J, Wu F-J, Li S-H, Lu C-H, Luo C-W. Thyrostimulin, but Not Thyroid-stimulating Hormone (TSH), Acts as a Paracrine Regulator to Activate the TSH Receptor in Mammalian Ovary. *J Biol Chem.* 2010;285(6):3758–3765.
431. Huang W-L, Li Z, Lin T-Y, Wang S-W, Wu F-J, Luo C-W. Thyrostimulin-TSHR signaling promotes the proliferation of NIH:OVCAR-3 ovarian cancer cells via trans-regulation of the EGFR pathway. *Sci Rep.* 2016;6(1):27471.
432. Val P, Lefrançois-Martinez A-M, Veyssière G, Martinez A. SF-1 a key player in the development and differentiation of steroidogenic tissues. *Nucl Recept.* 2003;1(1):8.
433. Lasley BL, Crawford SL, McConnell DS. Ovarian adrenal interactions during the menopausal transition. *Minerva Ginecol.* 2013;65(6):641–651.
434. Miller WL, Auchus RJ. The molecular biology, biochemistry, and physiology of human steroidogenesis and its disorders. *Endocr Rev.* 2011;32(1):81–151.
435. Rao CV. Human adrenal LH/hCG receptors and what they could mean for adrenal physiology and pathology. *Mol Cell Endocrinol.* 2010;329(1–2):33–36.
436. Vuorenoja S, Rivero-Muller A, Kiiveri S, Bielinska M, Heikinheimo M, Wilson DB, Huhtaniemi IT, Rahman NA. Adrenocortical tumorigenesis, luteinizing hormone receptor and transcription factors GATA-4 and GATA-6. *Mol Cell Endocrinol.* 2007;269(1–2):38–45.
437. Mazzuco TL, Chabre O, Feige J-J, Thomas M. Aberrant Expression of Human Luteinizing Hormone Receptor by Adrenocortical Cells Is Sufficient to Provoke Both Hyperplasia and Cushing's Syndrome Features. *J Clin Endocrinol Metab.* 2006;91(1):196–203.
438. Piltonen T, Koivunen R, Morin-Papunen L, Ruokonen A, Huhtaniemi IT, Tapanainen JS. Ovarian and adrenal steroid production: regulatory role of LH/HCG. *Hum Reprod.* 2002;17(3):620–624.

Figure 1. Genetic, structural and functional relationships between mammal LHβ an CGβ molecules. Mice have a unique Lh molecule encoded by a single *Lhb* gene. Mouse Lhβ acts through its receptor, which displays the exon 10-encoded portion at the hinge region, and regulates both gametogenesis and pregnancy. A CTP fragment appears as a product deriving from the equid *Lhb* gene, which is the source of a placental choriogonadotropin. While pituitary eLH regulates gametogenesis, eCG is specific for pregnancy in equids. However, they act on a common receptor with the exon 10-encoded sequence. The New World monkey *C. jacchus* has a peculiar LH/CG system: both gamete maturation and pregnancy are supported by a unique *CGB* gene encoding transcripts of both pituitary and placental origin and displaying the CTP fragment. Physiological functions are ensured by the LHR type II, lacking the exon 10-encoded portion, which binds the CGβ molecule in replacement of the missing LH. In humans, LH and hCG isoforms are encoded by specific genes belonging to a genetic cluster. However, pituitary LH lacking and placental hCG possessing a CTP appear as major products, specifically regulating gametogenesis and pregnancy, respectively. Both human LH and hCG act trough the same receptor displaying the amino acid sequence encoded by exon 10, which is fundamental for activating hormone-specific intracellular signaling. Deletion of LHCGR exon 10- encoded region leads to a truncated receptor capable of binding both LH and hCG, but resulting in impaired LH signaling and male infertility. Production of CGα, encoded by *CGA* genes, is required for proper dimer formation with β subunits, although CGα were not mentioned in the image.

Figure 2. Differential free LHβ and hCGβ subunit release by somatotrope GH3 cells. Both hormones have an asparagine (N) residue, at position 13 of the amino acid chain (N13), which is glycosylated in the hCG molecule. N13 glycosylation prevents the disulfide-linked aggregation of β subunits and is involved in the maintenance of the correct folding in the absence of the α subunit. This mechanism may underlie the secretion of free hCGβ subunits occurring in pregnancy, when these peptides are produced in greater amounts than the α subunits, while free LHβ aggregates remain in the cytoplasm. |=conserved amino acid residues; :=different amino acid residues sharing similar biochemical properties; .=Biochemically different amino acid residues.

Figure 3. Discrimination of LH- and hCG-mediated signaling by the LHCGR hinge region. Both hormones bind the extracellular domain of the receptor, but differently interact with the "U-shaped" portion of the hinge region. While hCG contacts the exon 10-encoded portion, LH spatial conformation leads to the interaction of the hormone with the extroflecting sTyr331 residue (upper panels). These ligand-receptor interactions result in LHCGR conformational changes associated with hormone-specific intracellular signaling. Exon 10 deletion results in the shift of the sTyr residue impairing the interaction with LH, while a contact point of the "U-shaped" structure of the hinge region with hCG is maintained (lower panels). Thus, exon 10 deletion results in a truncated LHCGR unable to mediate proper LH signaling, albeit retaining both LH and hCG binding capability.

Figure 4. Comparison of LH- and hCG-mediated signaling in the ovary and testis. At the mid-antral follicular phase, granulosa cells express both FSHR and LHCGR capable of forming homo/heterodimers, while theca cells express only LHCGR (upper panel). In granulosa cells, hCG displays a higher steroidogenic potential than LH, exerted *via* relatively high levels of cAMP/PKA-pathway activation, and this feature is potentiated in the presence of FSH. hCG action is exacerbated by massive production of progesterone, which is converted to androstenedione in the theca cell, while testosterone is a minor product in the gonads of the female. Androgens serve as a substrate for the aromatase enzyme, which

converts them to estrogens with high proliferative and anti-apoptotic potential. Intracellular cAMP increase is linked to pro-apoptotic stimuli, exacerbated *in vitro* by the absence of theca cell-derived substrate for estrogen synthesis. LH displays lower potency than hCG, in terms of cAMP/PKA-pathway activation, resulting in relatively low steroidogenic and pro-apoptotic potential. LH signals are preferentially exerted *via* phosphorylation of ERK1/2 and AKT, following the recruitment of G protein and β-arrestins and resulting in proliferative/anti-apoptotic events. LH-specific signals are potentiated in the presence of FSH, which reasonably provides the main steroidogenic stimulus in granulosa cells. Theca cell androgenic potential increases together with the progression of antral follicle growth and amount of LHCGR expression, providing sufficient androstenedione to be converted to estradiol. To date, no *in vitro* studies have compared the action of LH and hCG in theca cells. Since Leydig cells are androgenic and exhibit LHCGR expression, they may be considered the male counterpart to theca cells. hCG is more potent than LH in inducing both the cAMP/PKA- and pERK1/2-pathway activation, but results in a qualitatively similar balance of stimulatory and inhibitory steroidogenic signals, as well as downstream testosterone synthesis.

Figure 5. Overall model illustrating effects of LH *versus* hCG supplementation to FSH on ART outcomes. Scatter plots are obtained by meta-analysis (11) and indicate mean differences for each outcome evaluated. Data were interpolated using polynomial function. 95% confidence intervals are not shown. Adapted under Creative Commons CC BY license from Santi D, Casarini L, Alviggi C, Simoni M. Efficacy of follicle-stimulating hormone (FSH) alone, FSH + leuteininzing hormone, human menopausal gonadotropin or FSH + human chorionic gonadotropin on assisted reproductive technology outcomes in the "personalized" medicine era: a meta-analysis. Front Endocrinol (Lausanne) 2017; 8:114.

Table 1. Evolutionary and genetic differences between LH and hCG.

Endpoint	LH	hCG	Ref.
Presence	In all vertebrates	In primates (CGs)	(39)
Evolutionary convergent molecules	*Callithrix jacchus* pituitary CG	Equid choriogonadotropin (eCG)	(39,73)
Number of genes in *non*-primates	1	0	(39)
Number of genes in prosimian	1	0	(39)
Number of genes in *Macaca*, *Callicebus* and *Aotus*	1	1-3	(39)
Number of genes in *C. jacchus*	0 (one pseudogene)	1	(36,80)
Number of genes in great apes	1	4-5	(39)
Number of genes in *Homo sapiens*	1	6	(36,39)
Number of pseudogenes in *Homo sapiens*	0	2	(32)
Highest human gene sequence identity (92%)	*LHB* (PMID NM_000894.2)	*CGB* (GenBank BC041054.1)	n.a.

hCG is referred as the "classical" choriogonadotropin, except where indicated. n.a=not applicable/not available.

Table 2. Molecular differences between LH and hCG.

Endpoint	LH	hCG	Ref.
β amino acid chain length	121 amino acids	145 amino acids	(99,102)
Molecular weight	~26-32 KDa	~37 KDa	(99,102)
Highest human protein sequence identity (85%)	LHβ (PMID NM_000894.2)	CGβ5 (GenBank: AAI06724.1)	n.a.
LH/hCG-specific amino acid sequences	Absence of CTP	28 amino acid CTP extension	(71)
Number of glycosylations	3 (two N-linked in the α subunit; one N-linked in the β subunit)	8 (two N-linked in the α subunit; two N- and one O-linked in the β subunit)	(133)
Total isoforms and glycosylation variants	1 main molecule source of about thirty-nine isoforms chromatographically separated	At least 9 main molecules ("classical" hCG, hCG-H, nicked hCG, nicked hCG missing CTP, nicked hCG-H, asialo hCG, free hCGβ, nicked hCGβ, β-core molecule) source of an unknown number of variants	(99,102)
Molecular targets	LHCGR	LHCGR; Tβ-RII (criticized)	(1,161,163)

Half-life	25 minutes	15-462 hours	(102)

hCG is referred as the "classical" choriogonadotropin, except where indicated. n.a=not applicable/not available.

Table 3. Effective concentration for cAMP, pERK1/2 and pAKT activation, in primary mouse Leydig and human granulosa cells.

Primary cell type	Molecule	cAMP EC_{50}	pERK1/2 ECmax	pAKT ECmax	cAMP:pERK1/2 ratio	Ref.
human granulosa	LH	530 ± 51 pM	100 pM	100 pM	5.3	(6)
	hCG	107 ± 14 pM	100 pM	NA	1.1	(6)
mouse Leydig	LH	192 ± 54 pM	100 pM	/	1.9	(8)
	hCG	18 ± 10 pM	10 pM	/	1.8	(8)

EC_{50}=50% effective concentration; ECmax=min concentration maximally activating; NA=not activating; /=not assessed

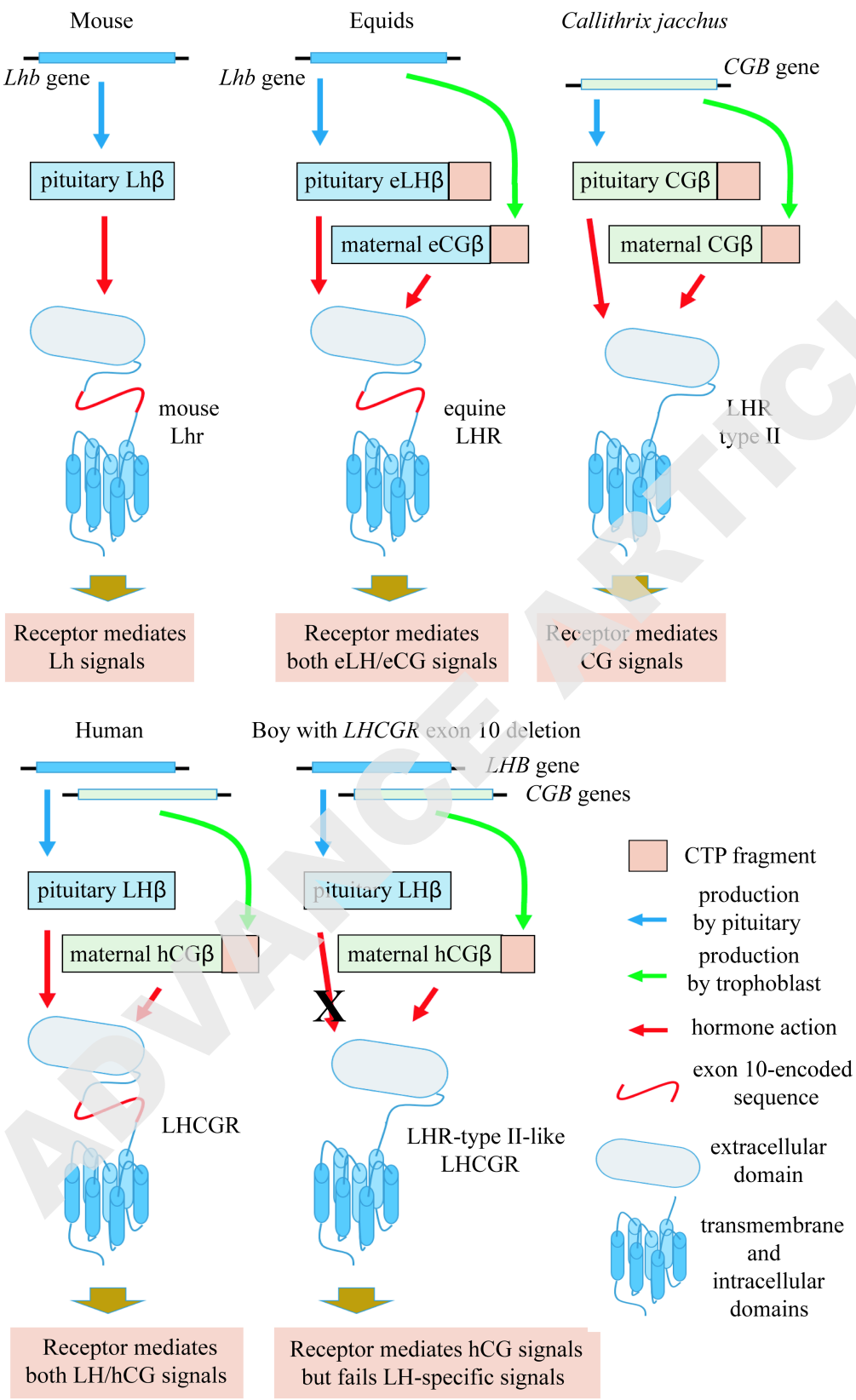
Table 4. Available drugs with LH and/or hCG activity, according to current regulatory agencies

Preparation	Provider	Molecule	Source	Immunoreactivity
Gonasi HP®	IBSA	Choriogonadotropin α	Urinary	hCG
Humegon®	Organon Inc.	Human menopausal gonadotropin	Urinary	FSH + LH + hCG
Luveris®	Merck KGaA	Lutropin α	Recombinant	LH
Menogon®	Ferring Pharmaceuticals	Human menopausal gonadotropin	Urinary	FSH + LH + hCG
Menopur®	Ferring Pharmaceuticals	Human menopausal gonadotropin	Urinary	FSH + LH + hCG
Ovitrelle®	Merck KGaA	Choriogonadotropin α	Recombinant	hCG
Pergogreen®	Serono	Human menopausal gonadotropin	Urinary	FSH + LH + hCG
Pergonal®	Serono	Human menopausal gonadotropin	Urinary	FSH + LH + hCG
Pregnyl®	MSD-Organon	Choriogonadotropin α	Urinary	hCG

Table 5. Physiological differences between LH and hCG.

Endpoint	LH	hCG	Ref.
Non-malignant source cell	Pituitary gonadotrope cells	Trophoblast and placenta; pituitary in very small amount	
Serum concentration in the fetus	~1/400th of the corresponding maternal serum concentrations	~1/400th of the corresponding maternal serum concentrations	
Serum concentration in the prepubertal female	0.0-4.0 IU/l	undetectable-2.3 IU/l	
Serum concentration in the prepubertal male	0.3-0.6 IU/l	undetectable-0.8 IU/l	
Serum concentration in the pubertal female	0.3-31.0 IU/l	undetectable-2.3 IU/l	
Serum concentration in the pubertal male		undetectable-0.8 IU/l	
Serum concentration in *non*-pregnant, fertile-age female	0.0-0.2 mIU/dl (follicular phase); 20.0-105.0 IU/l (mid-cycle peak); 0.4-20 IU/l (luteal phase)	undetectable-2.3 IU/l	
Serum concentration in the fertile-age male	1.8-12.0 IU/l	undetectable-0.8 IU/l	
Serum concetration in *non*-twin pregnancy	n.a.	455.0-142584.0 IU/l (<10 weeks); 3895.0-187852.0 (10-19 weeks); 1542.0-86541.0 (>19 weeks)	
Serum concentration in post-menopausal women	15.0-63.0 IU/l	undetectable-7.3 IU/l	
Physiological functions in the fertile-age female	Androgen synthesis (mainly androstenedione) in theca cells, proliferative and progestinic signals in granulosa cells, support of antral follicle maturation, luteinization, transitory luteal support	Progesterone production by *corpus luteum*, angiogenesis, cytotrophoblast differentiation, maternal immuno-suppressor, support of fetal growth and placentation, inhibition of uterine muscle contraction	(103,257,397)
Physiological functions in the fertile-age male	Androgen synthesis (mainly testosterone) in Leydig cells, support of spermatogenesis	n.a.	(257,397)

hCG is referred as the "classical" choriogonadotropin, except where indicated. n.a=not applicable/not available.



```
hCGβ 1-SKEPLRPRCRPI N ATLAVEK-20
       |:|||||.|.|| | |.|||||
LHβ  1-SREPLRPWCHPI N AILAVEK-20
```

GH3 somatotrope cells