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Review

Kisspeptin/GPR54 System: What Do We Know About Its Role in Human Reproduction?

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Key Words
Kisspeptin • Human Reproduction • Infertility • KISS1 gene • GPR54 gene • Dosage

Abstract
Kisspeptin is involved in the control of human reproduction bridging the gap between the sex steroid levels and feedback mechanisms that control the gonadotropin releasing hormone (GnRH) secretion; however, studies considering this peptide and infertility are limited. We conducted a review and critical assessment of available evidence considering kisspeptin structure, physiology, function in puberty and reproduction, its role in assisted reproduction treatments, kisspeptin dosage and the impact on *KISS1* and *GPR54* genes. Literature searches were conducted in PubMed using keywords related to: (i) kisspeptin or receptors, kisspeptin-1 (ii) reproduction or infertility or fertility (iii) gene and (iv) dosage or measurement or quantification or serum level, in human. Kisspeptin is a product of *KISS1* gene that binds to a G-protein-coupled receptor (GPR54/KISS1R) stimulating the release of GnRH by hypothalamic neurons, leading to secretion of pituitary gonadotropins (LH and FSH) and sexual steroids, which in turn will act in the gonads to produce the gametes. Kisspeptin is being recognized as a crucial regulator of the onset of puberty, the regulation of sex hormone mediated secretion of gonadotropins, and the control of fertility. Inactivating and activating mutations in both *KISS1* or *GPR54* genes were associated with hypogonadotropic hypogonadism and precocious puberty. Despite this, studies considering kisspeptin and infertility are scarce. The understanding of the role of kisspeptin may lead to its use as a biomarker in infertility treatments and use in controlled ovarian hyperstimulation.

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Introduction

Lee *et al.* [1] isolated the kisspeptin from melanoma cell lines and considered it as a 54-amino acid protein from a metastasis suppressor gene, officially named *KISS1* (gene ID: 3814), located at 1q32. In 1999, a G protein coupled receptor (GPR54) was discovered as a member of galanin receptor family, although, this receptor did not show specific binding with galanin [2]. In 2001, independent groups announced that kisspeptin is a natural ligand of GPR54, as a consequence it is referred as kisspeptin receptor (KISS1R) [3–5].

Northern blot and *in situ* hybridization analyses revealed that *GPR54* is expressed in brain regions (pons, midbrain, thalamus, hypothalamus, hippocampus, amygdala, cortex, frontal cortex, and striatum) as well as peripheral regions (liver, pancreas and intestine). *GPR54* is also highly expressed in placenta, pituitary gland, pancreas, gonadotrophs, testicles, ovaries, and spinal cord, suggesting a role in the regulation of endocrine function [2, 3, 5–7].

First evidence related the KISS1/GPR54 system with reproductive control came from two distinct studies that noted mutations in *GPR54* (*KISS1R*) (located at 19p13.3, gene ID: 9291) were associated with hypogonadotropic hypogonadism in humans, characterized by deficient luteinizing hormone (LH) and follicle stimulating hormone (FSH) secretion; leading to delay of reproduction function and infertility [8, 9]. Similar effects were observed in transgenic mice that did not express *Kiss1*/*Gpr54* and showed absence of sexual maturity, with hypo development of gonads, hypogonadotropic hypogonadism, and infertility [9].

Numerous studies have reported the involvement of kisspeptin(s) in the regulation of the hypothalamic–pituitary–gonadal (HPG) axis. The kisspeptin system is conserved among vertebrate species, except for the avian species; however, the general physiological functions of kisspeptin in vertebrates is still unclear. Here, we aimed to highlight aspects of kisspeptin related to the human reproduction system, focusing on human assisted reproduction.

Methods

Literature searches were conducted in Pubmed using the following keywords: (i) kisspeptin or receptors, kisspeptin-1, (ii) reproduction or infertility or fertility, (iii) gene and (iv) dosage or measurement or quantification or serum level. We found 460 articles related to reproduction and 69 articles related to dosage/measurement. We focused on articles related to kisspeptin role in human reproduction. We excluded reviews, comments and articles which main subject was animal reproduction, cancer, cell culture, embryos/fetal development, social environment, behavior and stress. Animal models were used to corroborate with kisspeptin action in humans. A total of 107 articles were analyzed.

Kisspeptin/GPR54 Structure

The first transcript of *KISS1* has 145 amino acids and is cleaved proteolytically delivering the most common form with 54 amino acids; other forms are: 14, 13 and 10-amino acid peptides. The post-translational proteolysis occur at two dibasic residues in pre-pro-kisspeptin (145 amino acid) at positions 66-67 and 123-124 [10] (Fig. 1).

The products from *KISS1* are widely referred to "kisspeptins" as the peptides that possess the highly conserved 10 amino acid RF-amide C terminus core sequence (kp-54, kp-14, kp-13 and kp-10) [3, 5, 11, 12]. The last two amino acid are arginine and phenylalanine that receive an amine group transferred from glycine at position 122 to residue 121 which is the C-terminal end of mature peptide amino acid [10] (Fig. 1).

The decapeptide kp-10 has the minimal length to completely stimulate the GPR54 and consequently increases phosphatidylinositol turnover. For this reason, it is considered the main peptide [9, 13]. GPR54 is a 398-amino acid protein of the Gq class of G proteins coupled to phospholipase C. GPR54 has an extracellular N-terminal domain that is followed

by seven transmembrane helices and ends with a C-terminal cytoplasmic domain of about 70 residues [9, 13]. Bianco and Kaiser [14] predicted the GPR54 structure in cellular membrane considering the amino acids sequence that result in a three intracellular and three extracellular loops, and seven transmembrane helices (Fig. 1).

This intracytoplasmic C-terminal region binds to the catalytic and regulatory subunits of phosphatase 2A and can form complexes with protein partners involved in receptor signaling [13, 15]. As consequence, GPR54 signal increases intracellular Ca^{2+} levels, activate calcium-

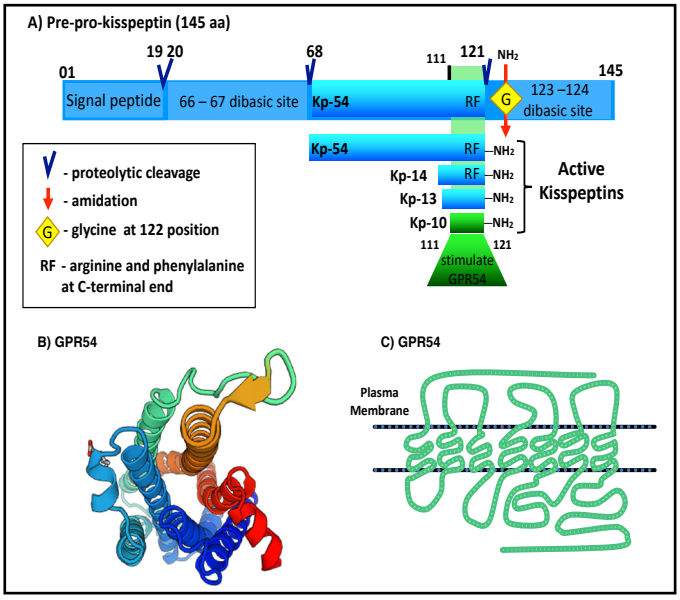

Fig. 1. Kisspeptin and GPR54 protein structure from human. (A) Pre-pro-kisspeptin and path of proteolytic cleavage, resulting in active kisspeptins (kp-54, kp-14, kp13 and kp-10). Kp-10 has the minimal-length for completely stimulate the GPR54 (B) GPR54 structure from SWISS-MODEL (Q969F8 - KISSR_HUMAN)[106] (C) GPR54 structure illustrated in plasma membrane with seven transmembrane helices, an extracellular N-terminal domain and ends with C-terminal cytoplasmic domain.

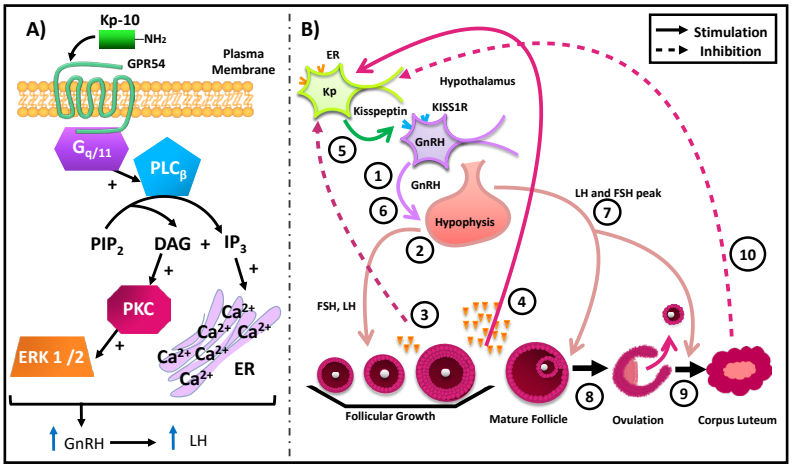

Fig. 2. Kisspeptin/GPR54 cellular signaling and physiologic role in menstrual cycle. A) GPR54 and intracellular signaling. The GPR54 is coupled to $G_{q/11}$ protein, when kisspeptin stimulates its receptor, the $G_{q/11}$ activates phospholipase C (PLC_{β}) that hydrolyzes phosphatidylionositol 4,5-bisphosphate (PIP_2) producing second messengers: inositol 1,4,5-trisphospate (IP_3) and diacylglycerol (DAG). IP_3 stimulates the endoplasmic reticulum (ER) to mobilizes calcium (Ca^{2+}). Increasing intracellular Ca^{2+} levels activate calcium-dependent signaling pathways in GnRH neurons. DAG activates calcium-dependent protein kinase C (PKC) that activates extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2). B) The kisspeptin role in LH pulse. GnRH stimulates the hypophysis (1) that releases FSH and LH (2), which stimulates the follicular growth and begin to produce estradiol (3). The estradiol suppresses hypothalamic–pituitary–gonadal axis (3). In late follicular phase when main follicle achieves around 12mm estradiol level increase and stimulates the kisspeptin (Kp) neurons which have an estradiol receptor (ER) releasing the kisspeptin (4). Kp neurons stimulate the GnRH neurons (5) that induces the LH release (7), ovulation (8) and corpus luteum development (9). Corpus luteum produces progesterone, estradiol and inhibin A that inhibit the hypothalamic–pituitary–gonadal axis (10).

dependent signaling pathways and activates mitogen-activated protein kinases (MAPK) p38, extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2) in GnRH neurons [3] (Fig. 2). In mice, kisspeptins have been reported to act on gonadotropin-releasing hormone (GnRH) neurons directly, by acting on some intrinsic ion channels producing a strong persistent depolarization [16, 17].

Kisspeptins not only increase the GnRH secretion, but also increases *GnRH* expression, as demonstrated in neuronal cell lines (GT1-7 and GN11 cell) [18]. In mouse the *Gnrh* gene was observed as a kisspeptin response element between −3446 bp and −2806 bp that participate in *Gnrh* expression regulation. In addition, GT1-7 cells treated with kisspeptin significantly increased total histone 3 acetylation (H3Ac). It demonstrated an histone-dependent transcription activation and chromatin conformation mediated by kisspeptin [19].

Function in puberty and reproductive system

The KISS1/GPR54 system is essential to signal for increased gonadotrophin secretion during puberty and to establish mammalian reproductive function and regulation of the HPG axis [20, 21]. *KISS1* expression is negligible in prepubertal ovaries and abruptly increases at the time of preovulatory surge of gonadotropins [22]. As consequence, kisspeptin administration in immature rodents and primates was able to induce precocious activation of the gonadotropic axis and precocious pubertal development [23–25]. Additionally, kisspeptin levels are higher in girls with central precocious puberty [26, 27]. In gonadal juvenile male monkeys, however, the continued kisspeptin administration decreases the LH levels, consequently leading to the assumption that kisspeptin secretion is pulsatile, and the continuous stimulation may induce receptor desensitization [28, 29].

Mutations in *KISS1/GPR54* genes result in a great diversity of phenotypes from partial sexual development to severe hypogonadism. This demonstrated that kisspeptin is essential in all phases of development. In fetal development GnRH secretion is required for testicular descent and penile growth. Mutations in *GPR54* results in micropenis and/or cryptorchidism. In neonatal life and puberty kisspeptins stimulate GnRH secretion, gonadotropin maintenance levels and loss-of-function mutations leads to an absence of pubertal development [30]. Mutations in *KISS1/GPR54* could cause infertility in men and women, therefore hormone replacement (usually GnRH) repaired the reproductive function [31, 32].

Human spermatozoa expresses *KISS1*, kisspeptin was located at post-acrosomal region of the sperm head and GPR54 was mainly found in the equatorial segment of the sperm head and around the neck, both demonstrated by immunodetection and the presence of GPR54 was confirmed by western blot [33]. Different status of fertility showed different levels of serum kisspeptin. Fertile men showed significantly higher kisspeptin serum levels compared to infertile men with diverse problems in spermatogenesis [34]. Kisspeptin levels were higher in four men with hypogonadism than in fertile controls. After GnRH treatment the serum kisspeptin decreased suggesting that elevated kisspeptin levels were a consequence of lower negative feedback of gonadal steroids in hypogonadism [35].

Kisspeptin exerts an important stimulatory role in the genesis of LH preovulatory peak [36–39], which is responsible for ovulation deflagration in females. In the nervous system, kisspeptin neurons were connected to GnRH neurons and experiments showed that central or peripheral administration of kisspeptin exerts a potent stimulatory effect on gonadotrophins secretion [40]. It was noted that low doses of intracerebroventricular kisspeptin markedly increased LH and FSH secretion [41, 42]. This occurs through the activation of GnRH neurons, which express *GPR54* [43–45]. The same results were observed in sheep [46], monkeys [24, 28] and humans [47, 48].

In mice, kisspeptin regulates the prolactin release through the inhibition of hypothalamic dopaminergic neurons, whereas this mechanism depends on estradiol in female rodents

[49]. These results confirm the important role played by kisspeptin in physiological regulation of HPG axis.

Sex steroid sensitivity of kisspeptin expressing neurons appears to be conserved among mammals [37, 50] suggesting that this sensitivity is a general feature of all kisspeptin systems among vertebrates. It also indicates that KISS1 neurons mediate sex steroid feedback effects in placental mammals; these neurons receive sex steroid signals from the gonads and modulate the activity of GnRH neurons (Fig. 2) [16, 17]. Neurons that directly receive sex steroids and control the GnRH release (mediators of sex steroid feedback) have been researched because GnRH neuron lacks estrogen receptor alpha [51], which is essential for sex steroid control of reproduction [52]. At the same time, positive or negative steroid feedback regulation can be changed rather easily by the composition of co-expressing transcription factors [53].

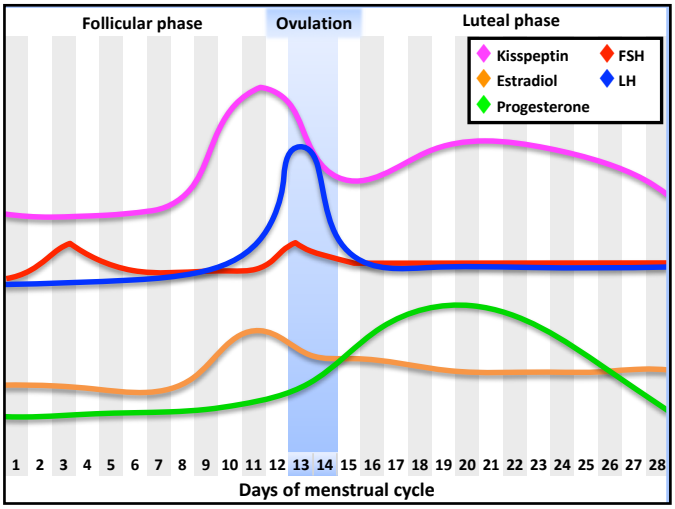

Fig. 3. Kisspeptin levels during menstrual cycle.

Even though kisspeptin was used for *in vitro* fertilization (IVF) treatments, such knowledge is not yet established in the menstrual cycle. Kisspeptin levels increase from early follicular to preovulatory phase and from preovulatory to luteal phase [54, 55], which along with previous data about menstrual cycle guided the Fig. 3 construction. The relation among kisspeptin and follicular growth was tested and the peak was found on the 11th day when the dominant follicle was approximately 1.2 cm. These data suggest a new role for kisspeptin dosage as a potential predictor of follicle development and ovulation [55].

In addition, kisspeptin is associated with follicular maintenance in ovarian tissues. Mural granulosa cells and cumulus cells were collected at oocyte retrieval from oocyte donors in IVF treatment. These cells expressed *KISS1* and *GPR54* observed by mRNA levels and immunofluorescence that showed kisspeptin presence in cytoplasm and cell nuclei and GPR54 in cytoplasm and cell membrane. These results were also confirmed by western blot. *KISS1* mRNA was more expressed in cumulus cells than in mural granulosa cells [56].

Moreover, mutant mice with *Gpr54* haploinsufficiency developed premature ovarian failure phenotype. These mice did not show differences in preantral follicle development (3 weeks), that was estimated by total number and morphologic features, between wild type (WT) and *Gpr54*+/- mice (before puberty). However, in later postnatal ages *Gpr54*+/- mice showed a progressive deterioration of ovarian architecture. By 32 weeks of age, follicle loss became firmly established in *Gpr54*+/- mice ovaries showing a reduced number of both antral and preantral follicles [22].

Taking into account the participation of kisspeptins in folliculogenesis pharmacokinetic studies was developed. Kp-54 and kp-10 have had the similar activities when added to cell culture. However, kp-54 had a longer onset and more duration of action than kp-10 in the same molar concentration in rodents [57]. Pharmacokinetic studies of kisspeptin in humans did not find consensus about the administration route (intravenous bolus, intravenous infusion and subcutaneous bolus) and which kisspeptin type should be used (kp54 or kp-10)[57].

Kp-10, when administrated as intravenous bolus or subcutaneous bolus, had a detection peak at 15 min in both cases; although, degradation was different, undetectable by 30 min and declined to baseline over 45-120 min (half-life approximately 20 min), respectively.

Kp-10 administrated as intravenous infusion showed a half-life approximately 4 min after infusion stopped [58]. Administration of kp-54 as an intravenous bolus in women or men has a peak at 40 min and 30 min, respectively. In women it disappeared after 3h and in men it has a half-life 28 min [47, 58]. Kisspeptin subcutaneous bolus has peak at 15min and disappeared after 3.5 h [59]. *In vitro*, kp-54 added to human plasma showed half-life 55 s [60].

Also, kisspeptin administration showed different responses across the menstrual cycle. In the preovulatory phase, the LH pulses increased immediately after an administration of single bolus at 0.24 nmol/kg of kp-10. In luteal phase a discrete increase of FSH and estradiol also was observed. Although in early follicular phase there were a variety of responses (only half women increase LH levels) [61]. The same was observed in administration of a subcutaneous bolus of kp-54 (at 0, 0.2, 0.4, 0.8, 1.6, 3.2, and 6.4 nmol/kg concentrations) in women with regular menstrual cycle, that leaded to potent elevation of LH release specially in preovulatory phase [59]. In healthy men, intravenous bolus injection of kp-10 (at doses: 0.3, 1.0, 3.0, or 10 nmol/kg) stimulated FSH, LH and testosterone release, specially LH [47, 58]. However, kp-10 and kp-54 intravenous infusion did not achieve the same stimulation of gonadotropins that GnRH produces [62].

In addition, purified native forms of kisspeptins in various vertebrate species are less used and attention is needed to interpreting physiological experiments with kp-10 as kisspeptin ligands since there may exist other slightly different physiological functions of the natural peptides [12].

Human reproduction treatments

Infertility is a public health issue that affects 15–20% of couples at reproductive age [63]. Among the causes of infertility, the main are advanced female age, tubal obstruction and polycystic ovarian syndrome (PCOS). *KISS1* and *GPR54* are expressed in human uterus (endometrium), ovary and oviduct at both mRNA and protein levels [64]. However, the peripherical kisspeptin/GPR54 system function is not clear yet.

Mentioned above, kisspeptin is essential to ovary maintenance and there is evidence that women in distinct reproductive status had different responses to kisspeptin. Intravenous administration of kp-10 in women increased LH in follicular phase (6.3±1.2 to 9.4±1.3 IU/L), postmenopausal (35.3±2.8 to 44.7±3.4 IU/L) and combined oral contraceptive pill (2.2±0.9 to 3.7±1.4 IU/L) [65]. Therefore, it is necessary to conducted more studies about kisspeptin levels and signaling during female reproductive life whereas women are becoming pregnant older. Also, PCOS was associated with higher levels of *KISS1* expression and there is a positive correlation of kisspeptin and LH levels in these patients, suggesting that kisspeptin contributes to hypersecretion of LH which participates in the pathophysiology of this syndrome [66, 67].

The use of *in vitro* fertilization (IVF) allows treatment of some aspects of infertility. In developed countries, it is estimated that 2-3% of all births are results of IVF procedures [68]. IVF is a complex process involving several steps, including puncture of oocyte containing follicles following controlled ovarian hyperstimulation (COH) with recombinant FSH (rFSH), oocyte fertilization, embryo development, embryo transfer to the uterus, and implantation. All of these steps are crucial to the IVF procedure success. However, the first important step is the ovarian response to controlled ovarian hyperstimulation, whose objective is obtain a satisfactory number of mature oocytes; thus, allowing the selection of the most viable embryo for transfer.

The standard protocol in young normoovulatory women can result both in satisfactory or unsatisfactory responses, such as hyper response, poor respondse, and Ovarian Hyper Stimulation Syndrome (OHSS), which requires the rFSH dose adjustment. OHSS is an IVF

complication characterized by multiple ovarian follicles (≥20 follicles) together with possible clinical symptoms, such as ascites, hematological changes (hemoconcentration), pleural effusion, liver and/or coagulation abnormalities, besides ≥4000 IU of serum estradiol, according to the classification proposed by Golan *et al.* [69]. Severe ovarian hyperstimulation is observed in approximately 0.6% of COH [70].

Considering this variability of ovarian response to exogenous gonadotrophins administration in IVF treatments, it is necessary to search ways to prevent the adverse effects. Abbara *et al.* [71] conducted a phase 2, multidose, open-label, randomized clinical trial of 60 women at high risk of OHSS using kp-54 to trigger oocyte maturation and prevent the OHSS. They noted oocyte maturation and embryo formation in 95% and 90%, respectively, of women who received kisspeptin. The highest pregnancy rate was founded in 9.6 nmol/kg kp-54. Also, the OHSS was observed only in 7% (four woman), however did not have moderate, severe, or critical OHSS.

Previously the same group tested a single subcutaneous injection of kp-54 to induce LH surge and egg maturation with different concentrations. They observed that oocyte retrieval was difficult in the 1.6 nmol/kg and 3.2 nmol/kg groups; therefore, they used 6.4 nmol/kg and 12.8 nmol/kg doses to induce the oocyte maturation. This approach resulted in 92% of fertilized oocytes and 58% of high-quality embryos. Kisspeptins were considered safe and well tolerated by patients [72].

Furthermore, patients with high risk of OHSS (AMH ≥5.6 ng/mL or total antral follicle count ≥23, age 18–34 years) were stimulated using daily recombinant FSH injection (112,5 IU of recombinant FSH) and a new protocol was tested comparing single or double subcutaneous injection of kisspeptin-54 (9.6 nmol/kg). The injections were administrated when at least three ovarian follicles ≥18 mm diameter at 36 h prior to oocyte retrieval and double subcutaneous injection was administrated at 36 h prior to oocyte retrieval and 10 h after first injection. The number of patients achieving ≥60% oocyte yield was 45.2% and 71.0% in single and double injection, respectively. In addition pregnancy rate was bigger in double injection group (23.0% x 39.0%) [73]. The data suggest that kisspeptin could be an alternate oocyte trigger preventing OHSS and resulting in a satisfactory pregnancy rate, especially for women with OHSS risk.

Kisspeptin on implantation and pregnancy

When kisspeptin was discovered it was called metastin, which was considered a metastasis suppressor [1], due to its capacity to inhibit cell invasion, altering cell motility and/or adhesion [5]. These properties were essential in embryo implantation as it is a critical step for reproduction success. The nidation is based on trophoblast invasion of the uterine extracellular matrix. It is known that kisspeptin is a factor that together with other proinflammatory cytokines, particularly TNFα, constrain trophoblast invasion and may play a role in the time of delivery, modulating the trophoblast apoptosis [74].

Immunohistochemical and Real Time Polymerase Chain Reaction (RT-PCR) analyses showed presence of *KISS1* and *GPR54* expression in the human ovary, the oviduct, and the uterus (luminal and glandular epithelial cells from endometrium), suggesting a kisspeptin role in the regulation of epithelial functions [64]. The expression of mRNA signatures of trophoblast cells isolated from first trimester (high invasiveness) and term (no/low invasiveness) placenta showed that *KISS1* and *GPR54* were highly expressed in first trimester trophoblasts compared to term gestation. Kp-10 increased intracellular Ca^{2+} levels in isolated first trimester trophoblasts emphasizing Kp-10 as a paracrine/endocrine regulator in fine-tuning trophoblast invasion generated by the trophoblast itself [75]. Corroborating these results, the same was found in canine pregnant uterus and trophoblast cells [76].

Unmated but cycling mice females did not respond to kp-54 injection suggesting that *Gpr54* is weakly expressed in nonpregnant uterus [77]. *Kiss1*-/- female mice were unable to achieve pregnancy due to implantation failure, independently of gonadotropins replacement

and estradiol that restores ovulation and fertilization. The implantation of embryos transferred from *Kiss1-/-* mothers to wild ones demonstrated that is maternal defect [78].

The injection of kp-54 in pregnant mice triggers the signal of MAPK p38 and ERK1/2 phosphorylation on the day of embryo implantation (D4 after of pregnancy), emphasizing that Kiss1/Gpr54 prepare the stromal cells for decidualization and reinforces the kisspeptin role as a paracrine signaling [77].

In women, there is evidence that kisspeptin levels increase each trimester during pregnancy [79] and it could reach a 200-fold higher level in the third trimester when compared with nonpregnant women [80]. Kisspeptin levels below 1630 pmol/L during the first trimester were considered a miscarriage biomarker, since women who suffer miscarriages show kisspeptin levels 60% lower than unaffected pregnancies [81].

In addition, low levels of kisspeptin in the second trimester (16 – 20 weeks) were associated with intrauterine growth restriction [82], while low levels in early and late pregnancy were associated with preeclampsia [82, 83]. These alterations should be considered in fetus development since intrauterine environment, preterm, epigenetic mechanisms and parental programing could participate of diseases development in later life [84], for example, intrauterine environmental factors are linked to the development of cardiovascular disease in later life [85] and alterations in maternal or fetal metabolism are associated with birth weight [86].

In intracytoplasmic sperm injection (ICSI) treatments, kisspeptin levels on the day of human chorionic gonadotropin (hCG) administration were correlated with pregnancy rate. Kisspeptin was higher in the pregnant group with fetal heart beat than the nonpregnant group. However, this result should be interpreted with cation, since the pregnant group also presented better embryo quality [87].

Dosage

Kisspeptin dosage is currently measured by diverse analytical methods without standard values and limited data, which leads to an absence of consensus (Table 1). Even though its dosage was done in different health conditions and measured looking for biomarker in women submitted to IVF [88] and patients with cancer [89, 90]; also was used to investigate fertility status [91]; polycystic ovary syndrome [92]; hypertensive diseases in pregnant women [93] and metabolism such as kisspeptin impact in insulin release and obesity [94–96].

Among analytical detection methods monoclonal assays were not capable of detecting kisspeptin inactive forms, but polyclonal assays could reduce this bias; however, some part of the studies did not specify which molecule was dosed. On the other hand, mass spectrometry seems to overcome many limitations, since it identifies the intact isoforms and their metabolites [57].

Ramachandran *et al.* [97] evaluated different anticoagulants (lithium heparin with 2000 U trasylol, citrate, EDTA, serum clot activator, and serum separator tubes) as a pre-analytical factor that could affect radioimmunoassay (RIA) measurements of kisspeptin serum levels. They observed that an EDTA solution preserve the sample better than traysol, citrate and serum separator. Kisspeptin immunoreactivity concentrations did not significantly change even after repeated freezing. However, this study was conducted using only pregnant women who have a significant increase of kisspeptin levels.

Non-invasive methods were tested considering that kisspeptin is a small oligopeptide and can pass through the glomerular filtration membrane, being able to be test in urine and saliva samples [55]. In nonpregnant women, there is evidence that serum and urine samples are equivalent. Saliva samples differ considerably from serum and urine samples and cannot be considered as an alternative sample [55].

Kisspeptin-10 added to human plasma showed a half-life of 55 seconds *in vitro*, revealing

Table 1. Dosage of kisspeptin by different methodologies and clinical conditions. # Kisspeptin results were converted to ng/mL for comparison purposes. * median and percentile (25-75) were showed. BMI: body mass index; POS: Polycystic Ovary Syndrome; CCR: Colorectal Cancer; SST: Serum Separator Tube; PHI: Pregnancy-Induced Hypertension

Condition	Authors	Study brief description	Methodology	Condition and results (mean ± SD)#			
Kisspeptin collected considering different phases of menstrual cycle	Latif & Rafique [54]	Serum kisspeptin during the menstrual cycle in 30 women (18 – 25 years old).	ELISA kit. Blood collected between 8-10 am, clotted sample, centrifuged within 30 min and frozen at -80°C.	Follicular phase 343.99 ±37.02	Pre-ovulatory phase 615.36 ±23.21	Luteal Phase 944.01 ±48.00	
	Rafique & Latif [96]	Serum kisspeptin levels in 14 women with BMI 18.5-24.99 and between 18 and 25 years old.	ELISA kit. Blood collected between 8-10 am, clotted sample, centrifuged within 30min and frozen at -80°C.	Follicular phase 338.38 ±26.70	Pre-ovulatory phase 584.02 ±32.04	Luteal Phase 922.40 ±64.47	
Kisspeptin collect in follicular phase of menstrual cycle	Zhai et al. [55]	Kisspeptin collected in 15 women submitted to IVF due male infertility between 23 and 35 years old.	KISS ELISA kit - all fractions (BlueGene, Shanghai, China). Collection in the morning, centrifuged, serum was frozen at -80°C collects in specific days.	3rd day 0.23 ±0.03	11th day 0.51 ±0.12	14th day 0.40 ±0.16	
	Mumtraz et al. [88]	Kisspeptin dosage in 124 women (32.2±4.8 years old) submitted to IVF associated with pregnancy results.	KISS-1-ELISA kit (Shanghai, China). Serum collection on second day of menstrual cycle before begin the IVF treatment.	Pre-clinic abortion 0.22 ±0.03	Clinical pregnancy 0.30 ±0.01	Male infertility 0.40 ±0.06	Female infertility 0.26 ±0.02
	Bacopoulou et al. *[91]	Kisspeptin levels in adolescents (12 - 20 years old) with anorexia nervosa (AN) and amenorrhea.	Kp-54 ELISA kit (Bioscience, Netherlands). Serum collect in follicular phase (3-5 day) and randomly in amenorrhea, let 20 min row temperature, centrifuge 20 min at 3000 g, stored -80°C.	General dosage* 0.23 (0.10 - 0.71)	Typical AN* 0.21 (0.11 - 0.60)	Non-typical AN* 0.44 (0.17 - 0.64)	
Kisspeptin collect in women	Chen et al. [27]	Plasma kisspeptin levels in adolescent (n =19) and women (n=23) with PSO and healthy teenagers (n = 20).	ELISA kit kp-54 (Phoenix Pharmaceuticals Inc., USA). Plasma collect at 9 am, sample extraction before dosage.	Health teenagers Undetectable	POS teenagers	PSO women	
Kisspeptin collect in adults	Andreozzi et al. [94]	Plasma kisspeptin levels in adults (216) (47±13 years old), 137 men.	EIA kit Kp-10 (Phoenix Pharmaceuticals Inc. USA). Plasma kisspeptin.	1st tercile 0.33 ±0.07	2nd tercile 0.62 ±0.14	3rd tercile 1.80 ±0.71	
Kisspeptin collected in patients with cancer	Curtis et al. [89]	Kisspeptin in men with prostatic carcinoma and controls with other kinds of cancer.	RIA kit all kisspeptins No information about collect	Patients Undetectable	Controls		
	Canbay et al. [90]	Kisspeptin levels in 88 patients with CCR (57.9 ± 11.75 years old) and 59 healthy controls (57.1 ± 7.44 years old).	ELISA kit kp-54 (Phoenix Pharmaceuticals Inc., USA). Blood collect in EDTA (1mg/ml) tube, centrifuge at 1600g for 25min at 4°C, storage at -80°C and kisspeptin extraction before dosage.	CCR group 86.20 ±20.50	Control group 49.00 ±12.70		
	Ramachandran et al. [97]	Preanalytical factors affecting RIA measurement of kisspeptin in four pregnant (27 ± 8.83 weeks women).	RIA kit kp-54. Samples processed in different tubes (lithium heparin with 2000 U/tube of trasylol, citrate, EDTA and SST and times (0 and 4h).	EDTA 0h 35.73 ±12.89	SST 0h 8.79 ±3.51	EDTA 4h 20.50 ±7.61	SST 4h Undetectable
	Horikoshi et al. [79]	Kisspeptin concentrations in men (12), women (10) and pregnant women in 1st (11=G1); 2nd (16=G2) and 3rd trimester (12=G3).	In house ELISA ki. It used refrigerated tube with EDTA (1mg/mL) collected between 9-12 am. Sample centrifuged 1000g x 25min at 4 °C stored at -80 °C and diluted to dosage.	Men and women Undetectable	1st trimester 1.60 ±0.45	2nd trimester 5.98 ±0.72	3rd trimester 12.49 ±2.14
	Nijher et al. [93]	Kisspeptin concentrations in pregnant woman (78), women with PIH (19) or pre-eclampsia (8).	RIA kit KP-54. Blood samples were collected into lithium-heparin tubes containing 5000 kallikrein inhibitor units of aprotinin (0.2 ml Trasylol), immediately centrifuged and plasma stored at –20°C.	PIH 3.51 ±0.39	Pre-eclampsia 4.58 ±0.46	Pregnant 3.75 ±0.20	
Kisspeptin collected in pregnant women	Jayasena et al. [81]	Kisspeptin serum levels in 981 pregnant women (5.9 to 22.1 weeks) during the antenatal booking visit.	In house RIA kit all dosage all kisspeptin fractions in plasma.	Abortion after collection < 7 days 0.25 ±0.15	7 - 21days 0.92 ±0.10	>21days 1.24 ±0.93	Health pregnancy 2.93 ±1.36
	Jayasena et al. [80]	Kisspeptin serum and urinary levels in 49 pregnant women (34± 0.6 weeks) and 50 healthy non-pregnant women.	In house RIA kit all kisspeptins. Blood collected in lithium heparin tubes (plasma) and urine collected from all subjects in sterile containers. Samples were conserved with 5000 kallikrein inhibitor units of aprotinin, stored at -20°C and kisspeptin extraction before dosage.	Plasma, 3rd Trimester 17.95 ±1.13	Plasma, non-pregnant 0.08 ±0.02	Urine, 3rd Trimester 0,39 ±0.08	Urine, non-pregnant 0.10 ±0.02
	Anne Armstrong et al. [82]*	Kisspeptin levels in pregnant women (317) (16-20 weeks) and women with pre-eclampsia (57) or growth restriction (118).	In house ELISA kp-10. Antibody from Phoenix Pharmaceuticals Inc, USA. Samples stored at -70°C.	Pre-eclampsia* 1.11 (0.60 – 1.9)	Growth restriction* 1.16 (0.44 – 3.9)	Normal gestation* 1.19 (0.49 –2.30)	
	Logie et al. [95]	Kisspeptin levels in obese pregnant women.	ELISA kit kp-10 (Phoenix Pharmaceuticals Inc., Germany.) Blood collected in EDTA tube between 8 and 9 am, centrifuged 1500 g, 10 min at 4°C, plasma dosage.	Geral 16w 1.36 ±0.81	Geral 28w 2.23 ±1.70	Geral 36w 3.18 ±2.77	

a fast degradation rate that requires immediate blood processing after sampling [57, 97]. Chan *et al.* [60] added kisspeptin-10 in human plasma, incubated at 37°C and measure in different times by mass spectroscopy. Kisspeptin was undetectable in samples incubated for more than 60 min. Similar result was observed after kisspeptin-10 intravenous or subcutaneous bolus administration in men and women. In healthy men was noticed a peak of kisspeptin 10 min after injection and undetectable levels after 50 min the same was observed that intravenous kp-10 or kp-54 in healthy women during the follicular phase [58].

Because these data suggest that kisspeptin has a short half-life *in vivo* and *in vitro*, some studies used aprotinin as an inhibitor to kallikrein (a kind of protease) [80, 93]. Other technique used is sample centrifugation at 4˚C immediately after blood collection and plasma or serum at storage -80˚C [79, 97].

Another parameter that should be considered is the circadian control of female reproduction. Some studies describe morning collection; however any study considered the kisspeptin dosage in different moments in the same volunteer. All these aspects show that is necessary a standardization in all steps (collect, pre-analytic and analytic process).

Genetic Diversity of *KISS1* and *GPR54*

Inactivating and activating mutations in *KISS1* and *GPR54* genes are associated with infertility due to isolated hypogonadotropic hypogonadism (IHH) and central precocious puberty (CPP) (Table 2, Fig. 4) [57, 98, 99]. A man with IHH due to a homozygous insertion of a cytosine after nucleotide position 1001 (1001_1002insC) resulted in elongation of *GPR54* protein from 398 to 441 amino acids. After treatment with pulsatile GnRH therapy for 2 years, he had a healthy son following an IVF procedure [31].

GPR54 mutations were showed in a Saudi-Arabian family carrying homozygous single nucleotide variant 443T>C in exon 3, which resulted in a substitution a serine by leucine at position 148 of protein (L148S). Four homozygous male members received treatment and resulted in testicular maturation, spermatogenesis, ejaculation, and subsequent fertility [32]. In the same family, pulsatile GnRH treatment followed by exogenous gonadotropin therapy in an affected woman resulted in fertility restoration. She had two healthy kids after two unsuccessful pregnanices [32].

A homozygous mutation in exon 2 of *GPR54* 305T>C results in a proline substitution for leucine at 102 position of GPR54 (NCBI reference sequence NM_032551.4(*KISS1R*): c.305T>C). This mutation leads to IHH in two families from Arab-Muslim and Syria with

Table 2. Variants of *KISS1* and *GPR54* in different populations related with hypogonadotropic hypogonadism and central precocious puberty. ***** Variants described as results articles and confirmed at ClinVar. ** Study developed in France, but there was not information about family ethnicity. ◊ Variants did not found at ClinVar. [#]Clinvar cited this variant as NM_002256.3(*KISS1*):c.339C>G (p.Asn113Lys). [†] Clinvar cited this variant as NM_002256.3(*KISS1*):c.220C>T (p.Pro74Ser). nIHH - normosmic isolated hypogonadotropic hypogonadism was defined as an absence of spontaneous pubertal maturation, inappropriately normal or low gonadotropin levels together with prepubertal or low testosterone or estradiol levels for age, absence of other pituitary hormonal deficiencies, no evidence of a hypothalamic–pituitary anatomical lesion on imaging and olfactory bulbs were normal [29, 101]. KS - Kallmann's syndrome defined as an idiopathic hypogonadotropic hypogonadism with anosmia [100]. CDP - constitutional delay of puberty was defined by lack of breast development by the age of 13 years in girls and lack of testicular enlargement in boys by the age of 14 years [101]. CPP - central precocious puberty was defined as the development of secondary sexual characteristics before the age of 8 years in girls and 9 years in boys which results from premature activation of the hypothalamic–pituitary–gonadal axis, and gonadotropin independent precocious puberty [105]

Study	Studied population	Main finds*	Ethnic
Seminara et al. [9]	Family of 19 Saudi Arabian (6 with nIHH) 63 nIHH 20 KS 180 controls	Six members of family with nIHH carrying *GPR54* mutation c.443T>C (p. Leu148Ser) in homozygosis. Among unrelated patients a man with nIHH has a *GPR54* mutation in heterozygous c.991C>T (p. Arg331Ter) and 1195T>A (p. Ter399Arg). Among controls (80 North American + 50 Middle Eastern + 50 black from North America people) these variants were not found.	Saudi Arabian Black American
de Roux et al. [8]	10 patients from a family whose parents were first cousin and had 8 children 50 controls	Five siblings with nIHH were homozygous to *GPR54* mutation 155-bp deletion at splice acceptor site intron 4/exon 5 and their parents and one health sibling were heterozygous. One non-affected sibling and 50 controls did not have this mutation.	Study developed in France**
Lanfranco et al. [31]	45 hypogonadal male 50 healthy men as controls	Man with nIHH from consanguineous parents showed a mutation, 1001_1002insC ◊homozygous in *GPR54* (protein elongation from 398 to 441). No other pathogenic variants were found.	German
Tenenbaum-Rakover et al. [100]	11 patients from two consanguineous families with cases of nIHH	Seven patients with nIHH showed homozygous in *GPR54* mutation c.305T>C (p. Leu102Pro) Other people analyzed showed the c.305T>C heterozygous mutation or the normal sequence.	Syria and Israel
Teles et al. [101]	69 patients with nIHH a 30 with CDP 120 controls with normal pubertal development	Two male siblings with nIHH had a mutation in *GPR54* in homozygous 2_-4delGCAinsACCGGCT◊. Their mother was heterozygous. Man with nIHH had heterozygous *GPR54* mutation at c.754G>C (p.Glu252Gln) ◊, it did not demonstrate functional impairment, but it was not found in controls.	Brazilian
Miraoui et al. [99]	199 KS 187 nIHH 155 controls with normal reproductive function	Man with KS heterozygous to *IL17RD* c.2204C>T (p.Ala735Val) and *GPR54* c.581C>A (p. Ala194Asp). Woman heterozygous to *FGFR1* c.682T>G (p.Trp228Asp) and *GPR54* c.565G>A (p.Ala189Thr) with nIHH. Controls did not have these variants.	European-descendent
Brioude et al. [98]	9 patients from two families	In first family, three siblings with hypogonadism had *GPR54* c.937T>C (p.Tyr313His) homozygous mutation with heterozygous parents and non-affected siblings without the variant. The second family has a man with hypogonadism carrying *GPR54* c.305T>C (p.Leu102Pro) and c.1195T>A (p. Ter399Arg) both in heterozygous, his mother was heterozygous to c.305T>C.	Portuguese French Caucasian
Topaloglu et al. [102]	16 patients of a consanguineous family 100 healthy controls	Four siblings with nIHH were homozygous to *KISS1* c.345C>G (p.Asn115Lys)*.Their parents were heterozygous and non-affected siblings were heterozygous or wide type. This mutation was not found in controls.	Kurdish
Teles et al. [105]	Case Report of an adopted girl	A girl with CPP had *GPR54* mutation at c.1157G>C (p. Arg386Pro) in heterozygous.	Brazilian
Silveira et al. [29]	83 CPP 61 nIHH 200 controls with normal puberal development	A boy with CPP was heterozygous to *KISS1* c.369C>T (p.Pro74Ser)†. Two girls with CPP were homozygous to *KISS1* c.417C>G (p.His90Asp) ◊. Both mutations were absent in 400 control alleles and no mutation was identified in the coding region of *KISS1*.	Brazilian

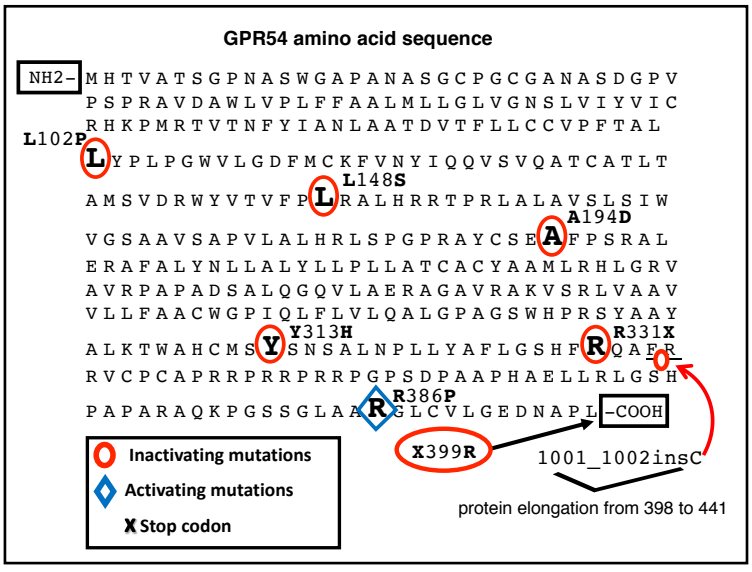

Fig. 4. Alignment of mutations in GPR54. Hypogonadotropic hypogonadism as consequence of inactivating mutations (circle) and central precocious puberty caused by activating mutations (rhombus). An insertion was represented by a red arrow and a black arrow represented a substitution of a stop codon at residue 399 to an arginine (1195T>A p.X399R).

consanguineous history. Features observed among affected people were: low LH and FSH levels in both sex, primary amenorrhea in women and men with puberty delay (some of them had micropenis and bilateral cryptorchidism). All health relatives tested showed the T305C heterozygous mutation or normal sequence [100].

A homozygous *GPR54* gene mutation was identified in two Brazilian siblings with normosmic isolated hypogonadotropic hypogonadism (nIHH). This mutation is characterized by deletion of three nucleotides at position -2 to -4 and insertion of seven nucleotides (IVS2 -2_-4delGCAinsACCGGCT) within the 3' splice acceptor site of intron 2. Computational analysis suggested this variant leads to a truncated receptor. Both boys had micropenis and cryptorchidism at birth and exhibited prepubertal testosterone levels associated with low basal gonadotropin. Their mother carried the same mutation in heterozygous form and had normal pubertal development [101].

Mutations in *KISS1* gene also cause IHH. In a large consanguineous family, a change from cytosine to guanine 345C>G (NCBI reference sequence NM_002256.3:c.345C→G) resulted in a substitution of asparagine by lysine at residue 115 (N115K) in kisspeptin. This region is highly functionally conserved across species such as human, macaque, sheep, mouse, frog and zebrafish. The functional study revealed GPR54 had significantly reduced sensitivity to the mutant kisspeptin protein which was unable to achieve maximal inositol phosphate response [102]. In this family, four women with IHH were all homozygous for the mutation. Their unaffected relatives were wild type homozygous or heterozygous; therefore, this phenotype was considered transmitted as an autosomal recessive trait [102].

These studies showed the role of inactivating mutations in *GPR54* in development of IHH. Animal models reproduce these results as demonstrated in mice with *Gpr54* null mutation that were infertile, have low FSH and LH levels in male and female models [103].

Activating mutations in *KISS1/GPR54* were observed in cases of central precocious puberty (CPP). A mutation in exon 3 of *KISS1* gene caused CPP in a boy, the variant changed a cytosine to thymine at 369 (369C>T) resulting in substitution of proline by serine at position 74 of primary kisspeptin (P74S). Ovarian cell cultures showed that mutated cells (P74S) incubated in 50% human serum had less kisspeptin degradation than wild type, suggesting this mutation increased the time of kisspeptin activity [29].

A *GPR54* mutation 1157C>G in exon 5 showed an autosomal dominant trait. It replaced a proline by arginine at codon 386 (R386P) in the carboxy-terminal tail of the receptor and was identified in an adopted girl with CCP. The functional analysis of R386P in COS-7 reveled wild and mutated type cells internalized the GPR54 upon stimulation, but the most part was recycled back to the cell membrane. There were not differences between wild and

mutated types in affinity of kisspeptin, or levels of receptor expression. R386P did not affect the GPR54 trafficking. Nevertheless, this mutation prolongs kisspeptin response decreasing GPR54 degradation, consequently increasing the number of receptors recycled back to the plasma membrane that could justify the phenotype associated with this mutation [14, 104, 105].

Even though there are many variants related to hypogonadism and precocious puberty, there is still no evidence of variants associated with assisted reproduction outcomes.

Conclusion

Kisspeptins are essential to puberty development and participate in many events in woman reproduction such as LH peak, implantation, and pregnancy. There is evidence that kisspeptin could induce the oocyte maturation during the controlled ovarian hyperstimulation; however, there is not a consensus about route of administration. Also, there is a lack of evidence about *KISS1* and *GPR54* variants influence in assisted reproduction treatments. Kisspeptin dosage data are limited and the results are not clear, due to different methods and lack of knowledge of pre-analytical methods. The role of kisspeptin in reproduction function is incontestable; nevertheless, more experimental studies and clinical trials are needed to explore the role of kisspeptin in assisted reproduction treatments.

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Disclosure Statement

The authors have no conflicts of interest to disclose.

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