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Variants in the Kisspeptin-GnRH Pathway Modulate the Hormonal Profile and Reproductive Outcomes

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Kisspeptin has been identified as a key regulatory protein in the release of gonadotropin-releasing hormone (GnRH), which subsequently increases gonadotropin secretion during puberty to establish reproductive function and regulate the hypothalamic–pituitary–gonadal axis. The effects of variants in the *KISS1*, *KISS1R,* and *GNRHR* genes and their possible association with assisted reproduction outcomes remain to be elucidated. In this study, we used next-generation sequencing to investigate the associations of the genetic diversity at the candidate loci for *KISS1*, *KISS1R,* and *GNRHR* with the hormonal profiles and reproductive outcomes in 86 women who underwent *in vitro* fertilization treatments. Variants in the *KISS1* and *KISS1R* genes were associated with luteinizing hormone (rs35431622:T>C), anti-Mullerian hormone (rs71745629delT), follicle-stimulating hormone (rs73507529:C>A), and estradiol (rs73507527:G>A, rs350130:A>G, and rs73507529:C>A) levels, as well as with reproductive outcomes such as the number of oocytes retrieved (s35431622:T>C), metaphasis II oocytes (rs35431622:T>C), and embryos (rs1132506:G>C). Additionally, variants in the *GNRHR* UTR3′ (rs1038426:C>A, rs12508464:A>C, rs13150734:C>A, rs17635850:A>G, rs35683646:G>A, rs35610027:C>G, rs35845954:T>C, rs17635749:C>T, and rs7666201:C>T) were associated with low prolactin levels. A conjoint analysis of clinical, hormonal, and genetic variables using a generalized linear model identified two variants of the *KISS1* gene (rs71745629delT and rs1132506:G>C) that were significantly associated with hormonal variations and reproductive outcomes. The findings suggest that variants in *KISS1, KISS1R*, and *GNRHR* genes can modulate hormone levels and reproductive outcomes.

Keywords: kisspeptin, *in vitro* fertilization, single nucleotide variation, *KISS1*, *KISS1R, GNRHR*

Introduction

Proper function of the hypothalamic–pituitary–gonadal (HPG) axis is vital for normal development of sexual organs to achieve reproductive competence at puberty (Knobil, 1974; Knobil *et al.*, 1980). Gonadotropin-releasing hormone (GnRH) is one of the main components of the HPG axis. GnRH neurons are thought to form the final common pathway for central regulation of fertility by receiving input from phenotypically diverse neurons of several brain regions and then projecting to the median eminence, where their peptide hormone is released to reach the adenohypophysis.

Fertility in mammals is initiated at puberty by the pulsatile secretion of GnRH that stimulates the release of the gonadotrophic hormones, luteinizing hormone (LH), and follicle-stimulating hormone (FSH), through the action of GnRH on the anterior pituitary (Belchetz *et al.*, 1978). The action of LH and FSH on the gonads also stimulates the production of sex steroids, gametogenesis, and sexual maturation, and provides hormonal feedback loops to regulate the release of GnRH, LH, and FSH (Simoni and Nieschlag, 1995).

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The effects of GnRH are mediated by a G protein receptor expressed in the plasma membrane of gonadotropes. The GnRH receptor (GnRHR) is the product of the *GNRHR* gene that is mapped at 4q21.2 and composed of three exons encoding a 328-amino-acid protein (NM_000406.2) and a 249-amino-acid protein (NM_001012763.1) (Noel and Kaiser, 2011). Variants in *GNRHR* gene can lead to a wide phenotypic spectrum that varies from pubertal delay to completely deficient GnRH-induced gonadotropin secretion, known as isolated hypogonadotropic hypogonadism (IHH) (Beneduzzi *et al.*, 2014).

Although adequate pulsatile secretion of GnRH is mandatory for acquiring and maintaining reproductive competency, a major advance in the understanding of the neuroendocrine mechanisms controlling GnRH secretion came from the identification of kisspeptins (KP) and their receptor KISS1R, and the subsequent elucidation of their physiological roles in controlling reproduction (Kotani *et al.*, 2001; Muir *et al.*, 2001; Ohtaki *et al.*, 2001; Messager *et al.*, 2005; Smith *et al.*, 2011). Kisspeptins act on GnRH neurons to increase the secretion of gonadotropins during puberty; this interaction regulates the HPG axis in both animal models and humans to promote reproductive function and control (de Roux *et al.*, 2003; Seminara *et al.*, 2003).

The *KISS1* gene, mapped at 1q32, is composed of three exons, of which the first two are noncoding. This gene encodes a 138-amino-acid peptide (NM_002256.3) precursor of kisspeptin that is proteolytically processed into a 54-amino-acid protein and can be further cleaved to 14, 13, and 10 amino acid peptides, widely referred to as ''kisspeptins'' (West *et al.*, 1998; Trevisan *et al.*, 2018). Kisspeptins act through the binding and subsequent activation of the G protein-coupled receptor KISS1R, which is encoded by the *KISS1R* gene mapped at 19p13.3 and composed of five exons (Kotani *et al.*, 2001; Muir *et al.*, 2001; Ohtaki *et al.*, 2001).

The distribution of kisspeptin neurons within the hypothalamus varies among species; however, kisspeptin neurons have been observed to be more abundant in the arcuate nucleus/infundibular region and preoptic region of rhesus monkeys, sheep, and human brains (Franceschini *et al.*, 2006; Rometo *et al.*, 2007). The close association between kisspeptin fibers and GnRH neuron cell bodies in the preoptic area of adult female rats and mice has been proposed to modulate the preovulatory GnRH/LH surge in females (Pielecka-Fortuna *et al.*, 2008).

Kisspeptin plays an important role in the central control of the HPG axis (Navarro *et al.*, 2004); KISS1 neurons in the hypothalamus mediate the negative feedback of estradiol (E2) in the arcuate nucleus area/infundibular region and the positive feedback in the preoptic region (Franceschini *et al.*, 2006; Pielecka-Fortuna *et al.*, 2008; Sébert *et al.*, 2010). Moreover, kisspeptin directly promotes the secretion of LH and growth hormone in the pituitary gland (Gutiérrez-Pascual *et al.*, 2007; Richard *et al.*, 2009). Additionally, *KISS1* and *KISS1R* are expressed in the ovaries where they regulate ovarian progesterone (PGR) synthesis (Peng *et al.*, 2013). Kisspeptin also participates in follicle development and ovulation (Zhai *et al.*, 2017). Thus, kisspeptin is highly involved in female reproduction and endocrinology.

De Roux *et al.* (2003) and Seminara *et al.* (2003) were the first authors to highlight the main roles of the KISS1/KISS1R system in controlling key aspects of the reproductive function. These studies reported deletions and inactivating variants in the *KISS1R* gene of patients suffering from familial or sporadic forms of IHH, a rare condition characterized by defective gonadotropin secretion and infertility of central origin (de Roux *et al.*, 2003; Seminara *et al.*, 2003). Variants in the *KISS1* gene have also been reported in patients with IHH (Topaloglu *et al.*, 2012). Moreover, activating variants in *KISS1* and *KISS1R* genes were also observed in cases of central precocious puberty (Silveira *et al.*, 2010; Teles *et al.*, 2011). *GNRHR*, *KISS1*, and *KISS1R* were also considered as candidate genes for genetic screening of IHH (Boehm *et al.*, 2015).

The understanding of the effects of variants in the *KISS1, KISS1R*, and *GNRHR* genes, and the association with IHH and precocious or delayed puberty are well characterized in the literature. However, the possible associations among variants in these genes and the resulting assisted reproductive outcomes remain to be elucidated. Inspired by these findings, we aimed to identify variants in *KISS1*, *KISS1R*, and *GNRHR* genes of infertile women using next-generation sequencing (NGS) to test for associations among genetic variants at these candidate loci and the hormonal profiles and in vitro fertilization (IVF) outcomes.

Materials and Methods

Patients

A cross-sectional study comprising 86 normoovulatory women (mean age, 32.2 ± 3.5 years old) who underwent IVF treatment at the Human Reproduction and Genetics Center of the Faculdade de Medicina do ABC, Santo André, Brazil was performed (Supplementary Data S1). Infertility was determined to be due to either a male factor (56/86) or a tube-peritoneal factor (30/86).

The inclusion criteria were as follows: ≤38 years old, FSH serum level ≤10.0 mIU/mL, thyroid-stimulating hormone (TSH) serum level of 0.5–4.0 mIU/mL, prolactin (PRL) serum level ≤35.0 ng/mL, body mass index of 18.5–30 kg/m^2, ovulatory cycle of 25–35 days, the presence of both ovaries without morphological abnormalities, and no evidence of endocrine disease. The exclusion criteria were endometriosis, polycystic ovarian syndrome (PCOS), previous history of ovarian surgery or chemo/radiotherapy, and women whose partners had undergone invasive procedures for sperm recovery.

We included in this study only women with male factor or tube-peritoneal factor of infertility since these factors usually does not affect hormonal profile, ovarian reserve, follicle maturation, and the response to controlled ovarian stimulation. Therefore, we would be able to better understand the effects of variants in the *KISS1*, *KISS1R*, and *GNRHR* genes on the hormonal variation and reproductive function. Although the association between endometriosis and infertility is well established, the exact mechanisms of its pathogenesis in causing infertility are varied and several hypotheses have been proposed such as pelvic, ovarian, and uterine factors. Besides, some studies suggested that women with endometriosis may experience decreased ovarian reserve.

Considering PCOS, this disorder is characterized by reproductive, endocrine, and metabolic disturbances. Although the etiopathogenesis of PCOS remains unclear, the

hypothalamic–pituitary–gonadal axis has been proposed to be involved, with observed disturbances in gonadotropin secretion, increased LH levels and perturbed LH and FSH ratios. So, the etiopathogenesis of endometriosis and PCOS itself could interfere in the reproductive competence, and also in the assisted reproductive treatment outcomes.

The investigation into the cause of infertility followed the guidelines of the American College of Obstetricians and Gynecologists (ACOG) and the American Society for Reproductive Medicine (ASRM) (ASRM, 2015; ACOG, 2019) and included a comprehensive medical history, physical examination, hormonal and biochemical profile, testing for sexually transmitted infections, imaging examinations (transvaginal ultrasonography, hysterosalpingography, and hysteroscopy), and semen analysis. Tubal integrity was analyzed by hysterosalpingography and/or laparoscopy, and tubeperitoneal factors were considered as anatomic tubal abnormalities such as tubal obstruction, functional changes caused by pelvic inflammatory disease, and previous tubal surgery.

According to the World Health Organization criteria (WHO, 2010), infertility was considered as male factor infertility when: (1) the initial semen concentration analysis was less than 15 million sperm/mL or 5 million/mL of rapid progressive spermatozoa after sperm processing; (2) less than 40% motile spermatozoa considering both fast progressive and nonprogressive sperm; or (3) asthenospermia with less than 32% if only the rapid progressive sperm.

Clinical data and blood samples were collected only after explaining the objectives of the study and obtaining signed informed consent. The study was approved by the local Research Ethics Committee (CAAE1228914.4.0000.0082).

Collection of samples

We collected 15 mL of peripheral blood from peripheral venipunctures in a tube containing clot-separator gel and a tube containing ethylenediaminetetraacetic acid. After collection, the tubes for the biochemical dosages were centrifuged (1000 rpm for 10 min), and the plasma was aliquoted into microtubes and frozen at −80°C for further determination of TSH, FSH, anti-Mullerian hormone (AMH), LH, PGR, PRL, and E2 concentrations. The tube for DNA extraction was stored at 8°C until extraction.

Hormonal measurements

TSH, FSH, LH, AMH, and E2 were measured at the follicular phase on the second or third day of the menstrual cycle, whereas PGR and PRL were measured at the luteal phase between the 18th and 21st day of the menstrual cycle. Hormonal measurements were performed during the menstrual cycle immediately before controlled ovarian hyperstimulation (COS).

TSH, PGR, PRL, FSH, LH, and E2 were measured using enzyme-linked fluorescent immunoassay (BioMerieux®, Hazelwood, MI). AMH was measured using an AMH Gen II enzyme-linked immunosorbent assay (Beckman Coulter®, Inc., Brea, CA). Information regarding the assays is provided in Supplementary Table S1.

Antral follicle count

Before the start of COS, two-dimensional transvaginal ultrasounds were performed to determine the antral follicle count (AFC) of each ovary using a 7 MHz vaginal transducer (Philips®, Netherlands). Follicles possessing a mean diameter between 2 and 10 mm were counted (Broekmans *et al.*, 2010).

In vitro *fertilization/intracytoplasmatic sperm injection treatment*

COS was performed using fixed doses of recombinant FSH (rFSH) (Puregon®) for 8–14 days starting on the third day of the menstrual cycle (Barbosa *et al.*, 2014). Doses of 100/150 IU of rFSH were indicated on the first treatment for patients with a minimum of four preantral follicles in each ovary. All other cases received 200 IU rFSH. When the largest follicle reached 14 mm, a GnRH antagonist (Orgalutran®) was administered and maintained for follicle growth and monitored by transvaginal ultrasound. When follicles reached 17–20 mm in size, recombinant human chorionic gonadotropin (hCG-Choriomon®) was administered (5000 IU). Oocytes were retrieved after 34–36 h. Luteal phase support began on the day of oocyte retrieval and included 600 mg/day administration of vaginal PGR.

We harvested the cleavage-stage embryos on the third day after fertilization or the blastocysts (on fifth or sixth day after fertilization) for transfer or freezing. The embryos were evaluated according to Istanbul consensus (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology *et al.*, 2011), and preimplantation genetic tests were not performed in any case. Patients who developed ovarian hyperstimulation syndrome (OHSS) had their embryos frozen and transferred in a subsequent cycle. A maximum of two embryos were transferred on the third or fifth day after fertilization, as recommended by ASRM (ASRM *et al.*, 2017). Pregnancy was confirmed by serum βhCG on day 12 after embryo transfer.

NGS

Genomic DNA was extracted from lymphocytes using the salting-out method (Lahiri and Numberger, 1991). The samples were quantified using a Qubit 2.0 (Invitrogen®), and the DNA was diluted to 25 ng/μL. Sequencing of *KISS1*, *KISS1R,* and *GNRHR* was performed using a TrueSeq Custom Amplicon® (Illumina Inc., San Diego, CA), including the exons, UTRs, and flanking regions of these genes. The library preparation followed the TrueSeq Custom Amplicon® protocol, and genes were sequenced using an Illumina Miseq® instrument (Illumina Inc.).

The threshold for coverage depth considered was 10 reads. The allelic balance ratios between variant and reference allele for heterozygous loci were between 0.3 and 0.7, for homozygous reference loci these values were 0–0.1, and for homozygous variant loci these values were 0.9–1.0 ratio. An 80% variant call rate was considered. The included variants exhibited a minor allele frequency of >1%.

The sequencing results were obtained as fastq files from BaseSpace® (Illumina Inc.). The pipeline followed the Burrows-Wheeler Aligner (BWA MEM v.0.1.15) (Li and Durbin, 2009) for alignment to the human reference genome GRCh37 (hg19). Picard v.2.17.4 was used for SAM-to-

BAM file conversion. GATK v.3.7. (Van der Auwera *et al.*, 2013) was used for quality checks, realignment, recalibration, and variant calling, and Atlas2 v.1.4.3 (Challis *et al.*, 2012) was used for multiallelic variant splits. Annovar (Wang *et al.*, 2010) was used to annotate public data, and variants were compared using ABraOM, a web-based public database of Brazilian genomic variants (Naslavsky *et al.*, 2017).

Statistical analyses

Statistical analyses were performed using RStudio version 1.1.383 (R Core Team, 2018). Data normality was verified using the Shapiro–Wilk test. Categorical variables were described in absolute and relative frequencies, and quantitative variables were presented as medians with 95% confidence intervals (CI). The estimation of Hardy–Weinberg equilibrium was evaluated by chi-squared test.

The association of the variants with clinical characteristics, hormonal levels, and reproductive outcomes (AFC, number of oocytes retrieved, metaphasis II oocytes [MII], and embryos) were evaluated using the Mann–Whitney or Kruskal–Wallis tests. For variables that were subjected to Kruskal–Wallis tests, the Dunn test was used to evaluate the differences among the three groups. The association between the variants and pregnancy rate was evaluated using a chi-squared test. Generalized linear models were used to verify the contribution of each variant in the studied genes to the variance observed on the hormonal levels and to reproductive outcomes.

Genotype imputation was performed using multiple correspondence analyses in the absence of patient variant data due the adopted cutoff for variant call. The dominant model was adopted for genetic variant analysis. A log-linear model was used for hormonal profile analysis. The analysis of reproductive outcomes was performed by Poisson regression, with the exception of pregnancy rate analysis, which was performed by binary logistic regression. The variables were selected using a stepwise method. Statistical significance was considered when $p < 0.05$.

Results

The clinical variables, hormonal profiles, and IVF outcomes of the studied participants are described in Table 1.

NGS

The NGS of the *KISS1*, *KISS1R*, and *GNRHR* genes resulted in 41 variants, including six variants in *KISS1R*, 10 in *KISS1,* and 25 in *GNRHR* (Supplementary Data S2). Among these, seven variants were in the exonic region, seven were in the intronic region, 12 were in UTR5′, and 15 were in UTR3′. A total of 34 variants were in Hardy–Weinberg equilibrium.

Two new variants were found in the *GNRH* gene, and these included a C>T substitution in the chromosomal position chr4:68604929 (NM_000406:c.*1269G>A, NM_001012763:c.*1378G>A) in the UTR 3′ region of the gene with allelic counting of C/T:127,123 reads and a T insertion into the chromosomal position chr4:68621343 (NM_000406:c.-1291_-1290insA, NM_001012763:c.-1291_-1290insA) in the UTR 5′ region of the gene with allelic counting of -/T:114,134 reads.

Table 1. Clinical Characteristics, Hormonal Profile, and Reproduction Outcomes of the Women Studied

Variables	
Age (years)	32.0 [31–33.6][a]
BMI (kg/m^2)	23.9 [23.0–24.5][a]
Menarche (years)	12.0 [12.0–13.0][a]
Menstrual cycle interval (days)	28.0 [28.0–29.0][a]
Infertility duration (years)	3.0 [3.0–4.0][a]
Infertility cause *n* (%)	
Male factor	56 (65.1)
Tube-peritoneal	30 (34.9)
TSH (mIU/mL)	1.8 [1.4–2.1][a]
FSH (mIU/mL)	5.9 [5.5–6.5][a]
LH (mIU/mL)	2.6 [2.3–2.7][a]
Estradiol (pg/mL)	44.3 [40.8–48.3][a]
AMH (ng/mL)	4.1 [3.8–4.5][a]
PRL (ng/mL)	15.4 [12.4–17.0][a]
PGR (ng/mL)	8.2 [4.3–10.5][a]
AFC	10.0 [9.0–10.0][a]
Protocol of rFSH *n* (%)	
100/150 IU	64 (74.4)
200 IU	22 (25.6)
Oocytes retrieved	5.0 [4.0–6.0][a]
MII	5.0 [4.0–6.0][a]
Embryos	3.0 [2.0–4.0][a]
Pregnancy rate *n* (%)	26 (36.1)

[a]Median and 95% confidence interval.

AFC, antral follicle count; AMH, anti-Mullerian hormone; BMI, body mass index; FSH, follicle-stimulating hormone; LH, luteinizing hormone; rFSH, recombinant FSH; TSH, thyroid-stimulating hormone; MII, metaphase II oocyte; PGR, progesterone; PRL, prolactin.

The allelic frequencies and variant characteristics are listed in Supplementary Table S2. Figure 1 shows the –log10(*p*-value) of the association tests (Mann–Whitney, Kruskal–Wallis, or chi-squared test) among each variant in the *KISS1, KISS1R,* and *GNRHR* genes along with the hormonal profiles (Fig. 1A) and reproductive outcomes (Fig. 1B). In Figure 1, the *p*-values <0.05 from associations tests are indicated by oversize bars on the plot, and the bars that do not exceed the red circle are $p > 0.05$. Associations among variants in the *KISS1*, *KISS1R,* and *GNRHR* genes with hormonal profiles (blue graphics) and reproductive outcomes (pink graphics) according to genetic model are shown in Figure 2.

Hormonal profile

Considering the variants in the *KISS1* gene, low LH levels were observed in women carrying the heterozygous genotype of *KISS1*/rs35431622:T>C compared with women carrying the wild-type homozygous genotype (TT) ($p = 0.042$, Fig. 2). The variant genotype (CC) was not found in any of the women included in this study. Furthermore, a thymine deletion in the terminal exon of the *KISS1* gene (NC_000001.10:g.204159612delT, NM_002256.3: c.417delA, dbSNP rs71745629) resulted in a stop-loss that was associated with increased AMH levels for

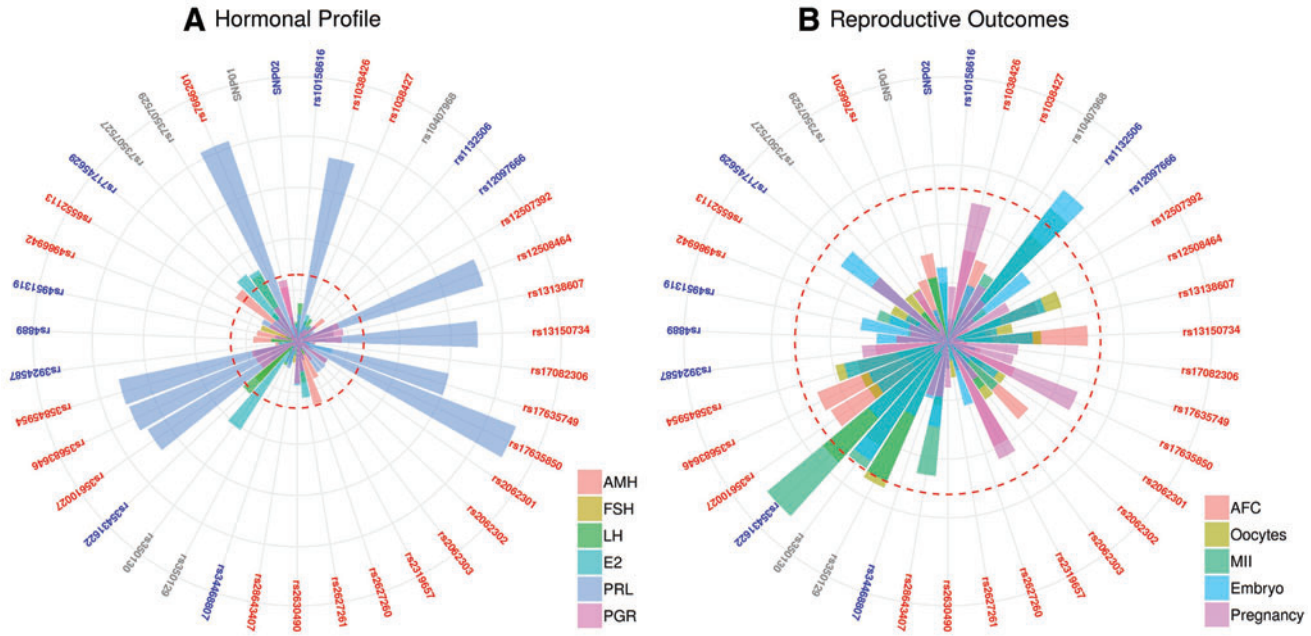


FIG. 1. Spider plot indicating the overall association among variants in the *KISS1, KISS1R*, and *GNRHR* genes and **(A)** hormonal profiles and **(B)** reproductive outcomes. The graphics indicate the –log10 of the *p*-values for the association among the variables. The *red* dashed cycle represents log10 (0.05). The variables that crossed the dashed *red circle* exhibited a significant association with the variant of the gene ($p < 0.05$). The colors in the dbSNP code identify variants in the genes; *GNRHR* is in *red*, *KISS1* is in *blue*, and *KISS1R* is in *gray*. SNP01 (variant in *KISS1R* at chr19:919890T>C); and SNP02 (variant in *KISS1* at chr1:204159549G>C) at reference genome (hg19). The rs35432622 was associated with LH levels, the number of oocytes, and MII. The rs71745629 was associated with AMH levels. Three variants (rs73507529, rs73507527, and 350130) were associated with E2 levels, and rs73507529 was associated with FSH levels. Nine variants (rs1038426:C>A, rs12508464:A>C, rs13150734:C>A, rs17635850:A>G, rs35683646:G>A, rs35610027:C>G, rs35845954:T>C, rs17635749:C>T, and rs7666201:C>T) were associated with PRL levels. The variant rs1132506 was associated with the number of embryos. AFC, antral follicle count; AMH, anti-Mullerian hormone; E2, estradiol; Embryo, the cleavage-stage embryos or blastocysts that were transferred or frozen; FSH, follicle-stimulating hormone; LH, luteinizing hormone; MII, metaphase II oocyte; Oocytes, oocytes recovered by ovarian puncture; PGR, progesterone; PRL, prolactin. Color images are available online.

either a homozygous or heterozygous deletion ($p = 0.033$, Fig. 2).

Within the intron of *KISS1R*, the variant rs73507529:C>A was associated with higher FSH levels in heterozygous women compared with those carrier of the wild-type genotype (CC) ($p = 0.032$, Fig. 2). This variant was also associated with lower E2 levels in heterozygous women ($p = 0.026$, Fig. 2). The variant genotype (AA) was not found in any of the women included in this study. There are two other variants that were associated with E2 levels. The variant *KISS1R*/rs73507527:G>A was associated with lower E2 levels in the heterozygous genotype (GA) compared with the wild-type genotype (GG) ($p = 0.023$, Fig. 2). The variant genotype (AA) was not found in any of the women included in this study. The variant *KISS1R*/rs350130:A>G was associated with lower E2 levels in the AG + GG genotypes ($p = 0.009$, Fig. 2) compared with the AA genotype.

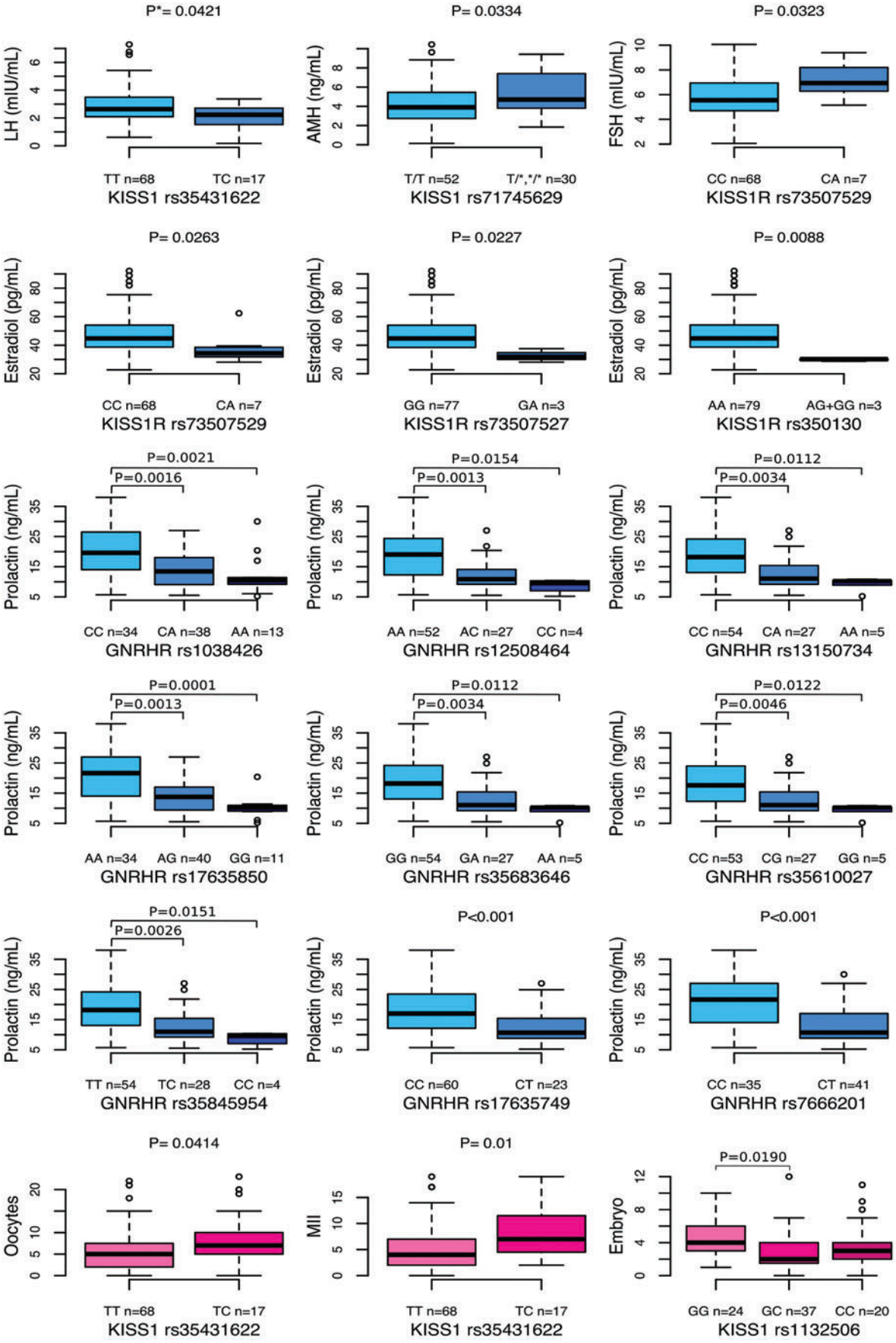
With regard to the *GNRHR* gene, nine variants in the UTR3′ region (rs1038426:C>A, rs12508464:A>C, rs13150734:C>A, rs17635850:A>G, rs35683646:G>A, rs35610027:C>G, rs35845954:T>C, rs17635749:C>T, and rs7666201:C>T) were associated with low PRL levels in the presence of a variant allele ($p < 0.05$, Fig. 2).

Reproductive outcomes

We also tested for associations among genetic variants at *KISS1*, *KISS1R*, and *GNRHR* genes with IVF outcomes such as AFC, number of oocytes retrieved, MII, embryos, and pregnancy rate.

The median number of oocytes retrieved was 5.0 (95% CI, 4.0–6.0), MII was 5.0 (95% CI, 4.0–6.0), and embryos was 3.0 (95% CI, 2.0–4.0). Fourteen (15.9%) out of 86 women had no embryo to transfer. Among these, 12 women demonstrated a poor response to COS, where five had the cycle canceled, four did not have MII, and in three cases the embryos did not develop. The embryos of one patient did not develop properly despite a satisfactory response to COS. Another patient developed OHSS with 26 follicles visualized at ultrasound; however, she later presented with empty follicle syndrome. Pregnancy occurred in 36.1% (26/72) of the women who transferred embryos.

Two variants in *KISS1* were associated with reproductive outcomes (Figs. 1 and 2). *KISS1*/rs35431622:T>C was associated with an increased number of oocytes retrieved and MII in the heterozygous genotype compared with those carrying the wild-type homozygous genotype ($p = 0.041$ and $p = 0.010$, respectively). Additionally, women carrying



the GG genotype of *KISS1*/rs1132506:G>C presented with more embryos compared with that CT ($p=0.019$). There were no genetic variants associated with AFC.

Conjoint analysis of variables

Considering the clinical, hormonal, and genetic variables, we performed a conjoint analysis using generalized linear models to estimate the contribution of each variable to the studied phenotype. The data are shown in Supplementary Table S3.

Two variants in the *KISS1* gene were associated with hormonal profiles and reproductive outcomes. The presence of the variant NC_000001.10:g.204159612delT (rs71745629) contributed to a 76.0% increase in the AMH levels and to a 35.0% and 70.3% decrease in PGR and PRL levels, respectively. Regarding the reproductive outcomes, this variant contributed to a 52.6% and 58.5% decrease in the number of oocytes and embryos, respectively. Furthermore, the presence of the variant *KISS1*/rs1132506:G>C contributed to a 65.0% decrease in AMH levels and to a 19.5% and 44.2% increase in E2 and PRL levels, respectively. Additionally, this variant contributed to a 53.0% and 68.3% decrease in the number of MII and embryos, respectively.

Discussion

Kisspeptins have recently been identified as vital upstream regulators that integrate both central and peripheral signals with GnRH release (Trevisan *et al.*, 2018). It appears that kisspeptin/KISS1R signaling within the GnRH neuronal network is important for pubertal activation and reproduction (Seminara *et al.*, 2003; Trevisan *et al.*, 2018). To the best of our knowledge, this is the first study to evaluate the variants of *KISS1*, *KISS1*R, and *GNRHR* genes in women who have undergone assisted reproductive treatment.

In the present study, the *KISS1*/rs35431622:T>C variant was associated with lower LH levels and an increased number of oocytes and MII. The *KISS1*/rs35431622:T>C is a missense variant that leads to a substitution of glutamine to arginine and no functional analysis of this variant has been reported, however, it can potentially impact the protein structure and function. Alteration in kisspeptin secretion might result in a considerable commotion of the gonadotropic axis (Kaya *et al.*, 2019). Lapatto *et al.* (2007) observed a significant reduction in the concentration of basal FSH and a relatively moderate reduction in basal LH level in *Gpr54/Kiss1r* and *Kiss1* knockout mice

Physiologically low LH levels may lead to inadequate oocyte maturation; however, excessive ovarian stimulation with LH was adversely associated with effects on preovulatory development, including premature luteinization (Balasch and Fábregues, 2006). Recent studies indicate elevated kisspeptin levels in patients with PCOS as well as a higher LH/KP ratio (de Assis Rodrigues *et al.*, 2019; Varikasuvu *et al.*, 2019). The alterations in kisspeptin levels in PCOS are associated with hormonal (hyperandrogenism) and metabolic alterations (insulin resistance and oligo-anovulation) and can alter follicle maturation and reduce oocyte quality. These results reinforce the role of *KISS1* in promoting the secretion of LH and regulating follicular function (Hu *et al.*, 2018a).

Regarding the critical role that *KISS1* and its receptor play in the regulation of fertility and the limited amount of information available regarding variants in the *KISS1* gene, Vaziri *et al.* (2017) analyzed the *KISS1*/rs35431622:T>C variant in women with idiopathic infertility and in fertile controls. The results revealed no differences in the genotype and allele frequencies between these groups; however, the association between the genotype and reproductive outcomes was not evaluated.

KISS1 may exert its action directly on various types of ovarian cells in an autocrine/paracrine manner. Additionally, emerging evidence has indicated that the ovarian *KISS1/KISS1R* system plays an essential role in regulating follicular development, oocyte maturation, hormone secretion, and ovulation (Hu *et al.*, 2018a). *KISS1* and *KISS1R* are expressed in the granulosa and cumulus cells of preantral and antral follicles (Cejudo Roman *et al.*, 2012) that secrete AMH into the follicular fluid and bloodstream until they reach a state that allow them to be receptive to exogenous FSH (García-Ortega *et al.*, 2014).

AMH also inhibits the sensitivity of the antral follicles to FSH during cycle recruitment and the aromatase activity that reduces estrogen biosynthesis. Thus, AMH plays an important role in folliculogenesis by acting on the modulation of follicular recruitment in the granulosa cells, where it regulates the number of growing follicles and their selection for ovulation (Peluso *et al.*, 2014). In this sense, AMH is considered a good ovarian biomarker, and there is a world-wide trend to identify AMH as the best and most unique ovarian biomarker due to its ability to predict ovarian stimulation response during assisted reproduction treatments (Peluso *et al.*, 2014; Di Paola *et al.*, 2018). It should be noted that the response to COS is also influenced by patient age and other markers of ovarian reserve (La Marca and Sunkara, 2014).

A recent study indicated that ovarian *Kiss1* can upregulate the serum levels of AMH in rats (Fernandois *et al.*, 2016). In agreement with this, Hu *et al.*, (2018b) observed that the expression levels of *KISS1* were highly correlated with the serum levels of AMH in women. In the present study, the thymine deletion in the terminal exon of the *KISS1* gene (rs71745629:delT) was associated with higher AMH serum levels and lower number of oocytes and embryos. These contrasting findings reinforce the need for a set of biomarkers, as opposed to a single biomarker, to assess reproductive potential.

FIG. 2. Associations among variants in the *KISS1*, *KISS1R,* and *GNRHR* genes with hormonal profiles and reproductive outcomes in women who underwent assisted reproduction treatment according to the genetic model. *Blue graphics* represent the hormonal profile results. *Pink graphics* represent the reproduction outcomes. Mann–Whitney test was used to compare the two groups, and Dunn test was used to compare three groups. *KISS1/*rs71745629delT. Color images are available online.

E2 levels can influence COS results, including the number of follicles and mature oocytes (de Mattos *et al.*, 2014). We observed that *KISS1R* gene variants (rs73507527:G>A, rs73507529:C>A, and rs350130:A>G) were associated with low E2 levels. The *KISS1R*/rs73507529:C>A variant was also associated with higher FSH levels, and considering generalized linear models, this variant contributed to a 39.65% decrease in AFC. Kisspeptin neurons in the arcuate and anteroventral periventricular regions are E2-sensitive GnRH afferents that are postulated to mediate E2-negative and positive feedback (Oakley *et al.*, 2009; Lehman *et al.*, 2010). E2 extends the action of FSH on granulosa cells to promote their proliferation and increase their expression of FSH receptors, and this, in turn, influences follicle growth, maturation, and ovulation.

In the present study, a variant in *KISS1* (rs35431622:T>C) was also associated with a higher number of retrieved oocytes in women carrying the heterozygous genotype. The same result was observed for MII in the rs35431622:T>C variant. Additionally, the *KISS1*/rs1132506:G>C variant was associated with the number of MII and embryos, reinforcing the role of kisspeptin in follicle development and maturation. This variant also contributes to higher levels of E2 and PRL and lower levels of AMH. These hormonal changes are consistent with the lower number of MII and embryos observed in this study.

For the *GNRHR* gene, nine variants at the UTR3′ region (rs1038426:C>A, rs12508464:A>C, rs13150734:C>A, rs17635850:A>G, rs35683646:G>A, rs35610027:C>G, rs35845954:T>C, rs17635749:C>T, and rs7666201:C>T) were associated with lower PRL levels in the presence of a minor allele, despite being within the normal range. PRL is essential for PGR biosynthesis and luteal cell hypertrophy during pregnancy. Numerous peptides are responsible for the control of PRL, and this includes GnRH, which acts directly on the lactotrophs (Freeman *et al.*, 2000).

GnRH is also secreted by the hypothalamic region and stimulates the hypophysis. Furthermore, there is a close proximity between lactotrophs and gonadotrophs, and GnRH pulse modifications could result in changes in PRL secretion. In humans, higher levels of PRL inhibit granulosa cell luteinization and steroidogenesis. However, PRL receptor knockout mice lack luteal function, and this leads to sterility due to decreased ovulation rate, aberrant folliculogenesis, and implantation failure (Freeman *et al.*, 2000).

Chen *et al.* (2017) investigated the relationship between variants in the *GNRHR* and outcomes in patients with polycystic ovary syndrome undergoing IVF treatment. The authors observed that the rs12644822:G>A, rs3756159:G>A, and rs13138607:G>A variants in the *GNRHR* gene were associated with high AFC, LH, LH/FSH, and testosterone levels and with higher pregnancy rates in these women. The *GNRHR*/rs13138607:G>A variant is frequently found in the general population (Gnomad: 0.46 and ABraOm: 0.54), and similarly, we observed that the minor allele frequency was 0.49, while 35.7% of the women were carriers of the heterozygous genotype and 30.9% were carriers of the variant homozygous genotype. Considering generalized linear models, this variant contributed to a 37.11% decrease in FSH and to a 440.19% increase in AMH levels.

Variants can occur in any region of the genome. In the gene coding regions (exons), the variants can lead to amino acid substitution in the protein and possibly to changes in protein conformation, polarity, and phosphorylation and even cause other functional consequences such as nonprotein formation (Chorley *et al.*, 2008). Otherwise the variants in the noncoding regions of the gene (intron), including the promoter regions or in upstream or downstream regions may affect gene transcription (Chorley *et al.*, 2008), RNA splicing, or gene translation (Ponomarenko *et al.*, 2002; Sadee *et al.*, 2011).

In particular, the UTRs of vast majority of genes have been demonstrated to act as important regulatory elements that exert a strong impact on the posttranscriptional regulation of gene expression where they function together with the complex of different RNA-interacting factors to affect mRNA stability, export to the cytoplasm, subcellular localization, and translation efficiency to influence the total amount of synthesized protein (Moore, 2005; Matoulkova *et al.*, 2012). Therefore, any variant or defect in these regions may strongly affect gene expression and associated cellular viability, growth, and development (Matoulkova *et al.*, 2012). In this context, it is plausible that the variants found at the UTR3′ region of the *GNRHR* gene may lead to posttranscriptional modifications that could influence PRL levels.

To provide better interpretation of these results, it is necessary to consider additional aspects. The data are derived from only one center, and the number of participants studied was relatively small. Further functional analysis of the variants found in *KISS1*, *KISS1R*, and *GNRHR* genes may allow researchers to elucidate their effect on hormonal variations and reproductive function. Despite these limitations, the group of normoovulatory women studied was homogeneous, and the selection criteria adopted potentially diminished the inclusion of patients whose characteristics could interfere with the hormonal and reproductive outcomes.

Conclusion

In summary, these findings suggest that variants in these genes could modulate hormone levels and reproductive outcomes. Therefore, the associations presented in this study are a starting point for further studies to allow for a greater understanding of the role of the Kisspeptin-GnRH system and their variants in human reproduction.

Authors' Contributions

C.M.T., C.P.B. and B.B. conceived the project. B.B. supervised the project. C.M.T. and C.P. were responsible for data acquisition. C.M.T., C.P., R.P., F.M., and H.H. were responsible for processing the samples. C.M.T., M.S.N., J.W., and M.Z. analyzed the NGS data. C.M.T., F.M., F.M.F., H.N., E.M., and B.B. performed the statistical analysis of the data and the presentation of the results. C.M.T., D.M.C., K.A.C., C.P.B., and B.B. interpreted data. All authors contributed to the identification and critical evaluation of the relevant literature and drafting the article, as well as to approve the final version of the article.

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No competing financial interests exist.

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Supplementary Material

Supplementary Data S1
Supplementary Data S2
Supplementary Table S1
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Supplementary Table S3

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