OVARIAN STIMULATION, ENDOCRINE RESPONSES AND IMPACT FACTORS AFFECTING THE OUTCOME OF IVF TREATMENT

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PUBLISHED IN: Frontiers in Endocrinology

frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88976-104-3
DOI 10.3389/978-2-88976-104-3

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OVARIAN STIMULATION, ENDOCRINE RESPONSES AND IMPACT FACTORS AFFECTING THE OUTCOME OF IVF TREATMENT

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Citation: Fatemi, H. M., Lawrenz, B., eds. (2022). Ovarian Stimulation, Endocrine Responses and Impact Factors Affecting the Outcome of IVF Treatment. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88976-104-3

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frontiers | Frontiers in Endocrinology

EDITORIAL
published: 14 April 2022
doi: 10.3389/fendo.2022.857089

Editorial: Ovarian Stimulation, Endocrine Responses and Impact Factors Affecting the Outcome of IVF Treatment

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Keywords: ovarian stimulation, assisted reproductive techniques, final oocyte maturation, endocrine evaluation, Oocyte yield

Editorial on the Research Topic

Ovarian Stimulation, Endocrine Responses and Impact Factors Affecting the Outcome of IVF Treatment

OPEN ACCESS

Edited and reviewed by:
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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *18 January 2022*
Accepted: *22 March 2022*
Published: *14 April 2022*

Citation:
Lawrenz B and Fatemi H (2022) Editorial: Ovarian Stimulation, Endocrine Responses and Impact Factors Affecting the Outcome of IVF Treatment. Front. Endocrinol. 13:857089. doi: 10.3389/fendo.2022.857089

INTRODUCTION

Assisted reproductive treatments are a tool to overcome infertility and are used worldwide to treat patients, suffering from this condition. Pregnancy chances have increased over the last decades due to improvement of the techniques for ovarian stimulation, although, there is still a lack of treatment individualization according to the patients' characteristics.

THE MAIN POINTS OF INDIVIDUAL CONTRIBUTIONS

This series includes 11 papers, all original research articles, which are referred to in the order, which would apply for basic assessment, evaluation and treatment of the infertile couple.

Primary assessment of the female partner involves basic characteristics like recording of age, body mass index (BMI) and cycle history. Usually with this information, patients with a polycystic ovarian syndrome (PCOS) are easy to identify and, as Insulin resistance (IR) is a common metabolic problem in these patients (1), this metabolic condition can be confirmed or exclude by performing an oral glucose tolerance test (OGTT). However, also lean patients with regular cycles might have IR (2), impacting the outcome of an ART treatment. In their prospective, observational study, Wang et al. subjected lean, non-PCOS patients to an OGTT and compared the stimulation outcomes between patient with and without IR. In their study,

IR was associated with a slower response to the ovarian stimulation, a poorer oocyte maturation and a decreased proportion of freezable embryos, as compared to non-IR women. Further on, lean patients with IR had a higher prevalence of subclinical hypothyroidism.

Prolactin (PRL) is one of the hormones evaluated usually in the primary assessment, as elevated PRL levels can cause cycle irregularities and anovulation (3). However, in cases with mildly elevated PRL levels, the question remains whether a treatment is indicated to lower the PRL levels prior to initiating an IVF cycle. Zhang et al. evaluated retrospectively the influence of basal PRL levels on the pregnancy outcomes in patients, undergoing ovarian stimulation in a long GnRH-agonist protocol due to tubal or male factor infertility. Based on their findings, the authors concluded that in patients with a basal PRL level within the range of 0–50 ng/mL, higher PRL levels were associated with higher numbers of oocytes, mature oocytes, zygotes, and embryos. Also, cumulative clinical pregnancy rate (CPR) and live birth rate (LBR) increased with increasing PRL levels. These data point to the fact, that for patients with an asymptomatic, mild hyperprolactinemia, planning to undergo an IVF/ICSI treatment, the PRL serum level may be not suppressed to an extremely low level, provided that organic lesions were excluded.

Success rates can be severely impacted by uterine abnormalities, with adenomyosis being one of them (4). It is well known, that pretreatment with GnRH agonists can reduce the size of the adenomyotic areal and therefore reduce the negative impact of the adenomyosis on the IVF outcome. However, the benefit of a long term pretreatment with GnRH agonists, prior to an ovarian stimulation for IVF/ICSI in a long GnRH agonist protocol is discussed controversially and the data of Chen et al. do not support GnRH agonist pretreatment in this setting.

The ovarian reserve parameters Anti-Muellerian-Hormone (AMH) and Antral Follicle Count (AFC) are together with patients' characteristics like age, BMI and the outcome of a possible previously performed ovarian stimulation treatment, the basis for deciding on the gonadotropin dosage (5). The most commonly used protocols are the GnRH (Gonadotropin-Releasing-Hormone)- agonist and GnRH-antagonist protocol. In 2020, a guideline was published by the ESHRE group on ovarian stimulation to summarize the available data (6). Huang et al. evaluated in a retrospective study, whether AMH remains a reliable predictor for the outcome not only in commonly used ovarian stimulation protocols, but also in progestin-primed ovarian stimulation protocols. According to their analysis, AMH correlates well also in this kind of protocol, independent of the dose of medroxyprogesterone acetate, used to prevent ovulation.

The number of retrieved/mature oocytes is crucial for the success (7) and treatment of poor responder patients (8) and remains a challenge for the reproductive medicine specialist. In a retrospective approach, Orvieto et al. analyzed the treatment of patients, who have had – in a conventional stimulation protocol - a previous poor response and who were treated with a combined Stop GnRH-antagonist protocol subsequently. With this approach, a significantly higher numbers of oocytes were retrieved, as well as higher numbers of embryos transferred, as compared to their previous IVF attempt.

During ovarian stimulation, serum FSH reflects the *in vivo* serum FSH levels to which the ovaries are exposed (9). In the search of tools for early individualization of the stimulation protocol, serum delta FSH levels between D6 of gonadotrophin use and basal serum FSH as well as between D6 of gonadotrophin use and D1 of gonadotrophin use have been investigated by Hu et al., in order to predict ovarian response.

Choosing the "correct" timing for the administration of the medication for final oocyte maturation (so called "trigger") is crucial for the retrieval of mature oocytes. The size of the follicles and the measurement of estradiol (E2) are the parameters commonly used to determine the optimal time point. However, due to the multifollicular growth of follicles of varying size, serum E2 levels are supraphysiological and therefore might render E2-measurement unreliable as a determinant of oocyte maturity. To add a diagnostic tool for this decision process, the paper of Lawrenz et al. evaluated the role of Inhibin A, which is only released from a follicle size of 12mm and beyond, as a parameter of oocyte maturity.

Human Choriongonadotropin (hCG) was long considered to be the "gold standard" for final oocyte maturation, however in high responder patients or in oocyte donation patients, the administration of GnRH (Gonadotropin-Releasing-Hormone) – agonist is meanwhile standard to avoid ovarian hyperstimulation syndrome (OHSS) (9). In seldom cases, administration of GnRH-agonist fails to be effective and no oocytes will be retrieved, which leads to disappointment and possibly loss in trust. The study of Cozzolino et al. evaluated the reliability of a urinary LH-self test 12 hours after administration of the GnRH-agonist trigger in oocyte donation patients. As a positive urinary LH test after GnRH-agonist trigger proved to be a reliable tool for retrieving mature oocytes, it could be used in the monitoring process to detect errors in the administration and/or inadequate responses to the trigger and therefore improve the outcome. Due to their different mode of action, the administration of various kinds of "trigger" medications (hCG, GnRH-agonist and kisspeptin) result in altered endocrine profiles. These profiles have been investigated by Abbara et al., revealing distinct differences, which should be taken into account when individualizing treatment protocols according to patients' characteristics.

In a secondary analysis from previously published data, Benmachiche et al. evaluated the correlation of preovulatory LH levels in a GnRH-antagonist protocol and the use of a GnRH-agonist for final oocyte maturation, and the cycle outcome in cycles with fresh embryo transfer and the use of a modified luteal phase support. According to their data, low pre-ovulatory LH levels might reduce the chance for a pregnancy, but further studies are warranted.

Progesterone measurement and the impact of the progesterone levels in the luteal phase on the ART outcome is a "hot-topic" and discussed controversially. Besides progesterone, also 17-OH progesterone (17-OH P4) is produced by the corpus luteum (CL) and 17-OH P4 levels are not influenced by luteal phase support. In order to evaluate whether 17-OH P4 would give a better parameter for the monitoring of the luteal phase,

Thomsen et al. evaluated prospectively the correlation with the ART outcome.

SYNTHESIS AND CONCLUSION

This "Research Topic" includes papers which evaluate the impact/meaningfulness of hormonal parameters "deviating from the well-trodden path" of commonly used hormones and may open new insights into diagnostic and treatment options.

AUTHOR CONTRIBUTIONS

BL: writing the editorial HF: reviewing the editorial. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
published: 29 November 2018
doi: 10.3389/fendo.2018.00690

Mid-Luteal 17-OH Progesterone Levels in 614 Women Undergoing IVF-Treatment and Fresh Embryo Transfer—Daytime Variation and Impact on Live Birth Rates

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OPEN ACCESS

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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *27 June 2018*
Accepted: *02 November 2018*
Published: *29 November 2018*

Citation:
Thomsen LH, Humaidan P, Erb K, Overgaard M, Andersen CY and Kesmodel US (2018) Mid-Luteal 17-OH Progesterone Levels in 614 Women Undergoing IVF-Treatment and Fresh Embryo Transfer—Daytime Variation and Impact on Live Birth Rates. Front. Endocrinol. 9:690. doi: 10.3389/fendo.2018.00690

Introduction: Corpus luteum (CL) produces progesterone (P_4) and 17-OH progesterone (17-OH P_4) during the luteal phase. Contrary to P_4, 17-OH P_4 is not supplied as part of the luteal phase support following IVF-treatment. Therefore, measuring endogenous serum 17-OH P_4 levels may more accurately reflect the CL function compared to monitoring serum P_4 concentrations.

Objective: To explore the correlation between mid-luteal serum 17-OH P_4 levels and live birth rates and to explore the possible daytime variations in mid-luteal serum 17-OH P_4.

Design: Prospective cohort study.

Patients: 614 women undergoing IVF-treatment and fresh embryo transfer.

Intervention: All patients had serum 17-OH P_4 measured 7 days after oocyte pick-up (OPU+7). Furthermore, on OPU+7, seven patients underwent repeated blood sampling during daytime to clarify the endogenous daytime secretory pattern of 17-OH P_4.

Outcome measure: Live birth rate.

Secondary outcome measure: Daytime variation in serum 17-OH P_4 levels.

Results: The highest chance of a live birth was seen with mid-luteal 17-OH P_4 between 6.0 and 14.0 nmol/l. The chance of a live birth was reduced below (RD −10%, $p = 0.07$), but also above the optimal range for 17-OH P_4 (RD −12%, $p = 0.04$). Patients with diminished CL-function (17-OH P_4 <6 nmol/l) displayed clinically stable 17-OH P_4 values, whereas patients with 17-OH P_4 levels >6 nmol/l showed random 17-OH P_4 fluctuations during daytime.

Conclusion: The association between 17-OH P_4 and reproductive outcomes is non-linear, and the negative effect of excessive CL-secretion seems to be just as strong as the negative effect of a reduced CL-function during the peri-implantation period.

Keywords: 17-OH progesterone, progesterone, IVF, live birth, daytime variation

INTRODUCTION

Following ovulation, the human corpus luteum (CL) produces progesterone (P_4) and 17-OH progesterone (17-OH P_4) upon stimulation with luteinizing hormone (LH) or human chorionic gonadotropin (hCG). Progesterone governs the secretory transformation of the endometrium prior to implantation and an adequate luteal P_4 level is crucial for the establishment and maintenance of early pregnancy (1).

During IVF and fresh embryo transfer, the luteal function is disrupted and the success of the treatment is critically dependent on exogenous luteal phase support (2–5). For decades, this exogenous P_4 support has been administered as a standard dose in IVF patients in the firm belief that "one dose fits all." A widely held view has been that the absolute luteal P_4 level does not affect the chance of pregnancy, as long as a minimum P_4 concentration was reached by means of the administration of exogenous luteal phase support (6, 7). However, recent studies have suggested that both very low and very high luteal P_4 levels affect the reproductive outcome negatively (8–12). Thus, in a study by Yovich et al., 529 artificial frozen-thawed cycles with single blastocyst transfer were evaluated (8). The authors reported that the optimal pregnancy and live birth rate was achieved when mid-luteal serum P_4 was in the range of 70–99 nmol/l. Below, but also above this range, the clinical pregnancy rate was significantly reduced from 64 to 44%. Following this, several other papers also reported a lower, as well as, higher luteal P_4 threshold in artificial frozen-thawed embryo transfer cycles (10–12). In IVF cycles with fresh embryo transfer, the mid-luteal P_4 requirement is significantly increased compared to both the natural and the frozen embryo transfer cycle as demonstrated by a work by Humaidan et al. (2). Very recently, our group described the optimal P_4 levels during the early and mid-luteal phase of IVF cycles with fresh embryo transfer (9). In a cohort of 602 patients, we observed that reproductive outcomes seemed consistently decreased below, but most distinctly above a defined optimal P4 range.

Taken together, it seems that both too high and too low luteal P_4 concentrations result in reduced pregnancy rates in both fresh and frozen embryo transfer cycles. The findings of a higher and lower P_4 threshold seem plausible from a biological point of view: A very high P_4 level during the early luteal phase may advance the endometrium leading to asynchrony between embryo development and endometrial receptivity, whereas a very low P_4 level fails to support a sufficient secretory transformation in time for implantation. Both scenarios hamper the chance of a live birth.

The CL produces not only P_4, but also 17-OH P_4 during its lifespan (13). When measuring serum P_4 following fresh embryo transfer with the use of exogenous P_4 luteal support, the serum P_4 value is a combination of the exogenously supplied P_4 and the endogenous luteal P_4 production. As 17-OH P_4 is not supplied as part of the luteal phase support, the serum 17-OH P_4 level may reflect more accurately the true CL function compared to the measurement of total P_4.

The aim of this study was to explore the possible correlation between mid-luteal serum 17-OH P_4 levels and the reproductive outcome in terms of live birth rates following IVF treatment and fresh embryo transfer. Furthermore, if serum 17-OH P_4 should serve as an index for CL function, it is evident that the accuracy of a single measurement is important. Therefore, a second aim of the present study was to explore the daytime variations in serum 17-OH P_4 which might affect the clinical interpretation of the measurement.

MATERIALS AND METHODS

Study Design

Prospective cohort study.

Patient Population

The present cohort of patients has previously been described in papers by our group (9, 14). Briefly, this study included 614 patients undergoing IVF treatment at four public Danish fertility centers—The Fertility Clinic Skive Region Hospital, The Fertility Clinic Horsens Region Hospital, The Fertility Clinic Herlev Hospital and The Fertility Clinic Odense University Hospital—between May 2014 and June 2017. The patient cohort was unselected, representing normal everyday patients treated in the clinics. All participating patients were under the age of 41 and with a body mass index (BMI) <35 kg/m^2 as required by Danish national guidelines for public fertility treatment[1] Treatment choices regarding type of protocol (GnRH agonist or GnRH antagonist) and trigger type (hCG or GnRH agonist) were made on an individual basis by the attending clinician.

Written and oral information was given to 1,482 patients of whom 609 (41%) declined to participate mainly due to the extra visit needed at the clinic for mid-luteal blood sampling 7 days after oocyte retrieval (OPU+7). The final study cohort included 614 patients with embryo transfer and relevant study samples taken (**Figure 1**).

Clinical information regarding primary diagnosis, age, BMI, smoking habits, antral follicle count and basal FSH and LH levels were obtained prior to treatment by the clinical staff. Serum TSH and prolactin levels were within normal range in all patients prior to treatment start. All patients participated once, only. No patients were lost to follow-up.

Ovarian Stimulation

Patients treated in the long GnRH-agonist protocol were down-regulated using daily SC injections of a GnRH antagonist (Suprefact®, Sanofi, Denmark or Gonapeptyl®, Ferring Pharmaceuticals, Denmark) starting in the mid-luteal phase of the preceding cycle and continuing until the day before ovulation induction. Ovarian stimulation was initiated after 12–14 days of down-regulation in case of an endometrial thickness <4 mm. Final follicle maturation was induced with hCG 5,000–10,000 IU (Pregnyl®, MSD, Denmark or Ovitrelle, Merck Biopharma, Denmark) when two or more leading follicles reached a diameter of ≥ 17 mm.

[1]Danish Fertility Society. *Guideline 2016*: Overweight, Obesity and Fertility Treatment.

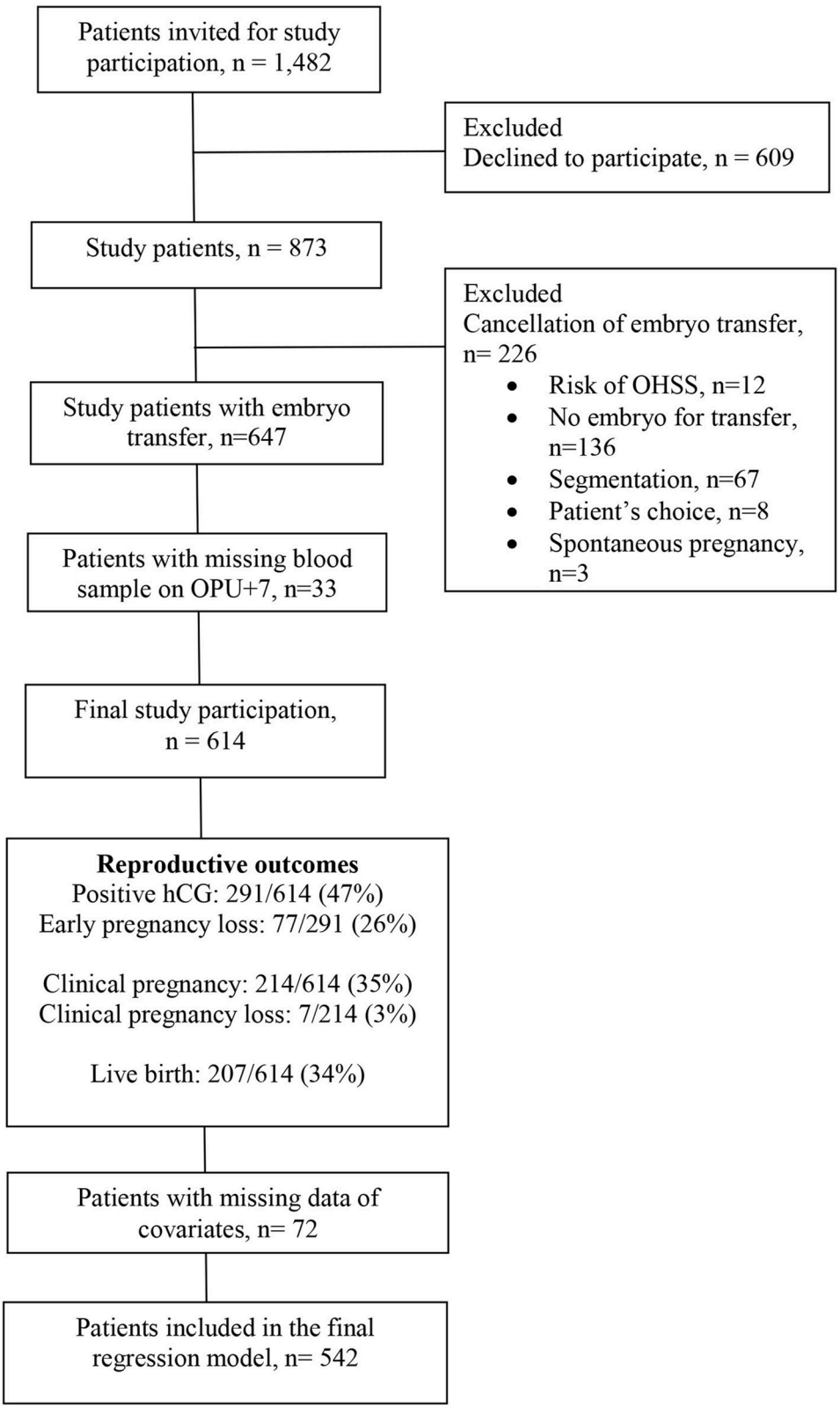


FIGURE 1 | Flowchart of study participation.

If the GnRH antagonist protocol was used, ovarian stimulation commenced on day 2 or 3 of the cycle after a vaginal ultrasound examination. Daily GnRH antagonist co-treatment was started from cycle day 6 and continued up until the day of ovulation induction. When at least two follicles reached a size of ≥17 mm, final oocyte maturation was induced with SC Buserelin 0.5 mg (Suprefact®, Sanofi, Denmark) or hCG 5,000–10,000 IU (Pregnyl®, MSD, Denmark or Ovitrelle, Merck Biopharma, Denmark).

Ovarian stimulation was performed with either hMG (Menopur®, Ferring Pharmaceuticals, Denmark), r-FSH (Gonal-f®, Merck Biopharma, Denmark), or rFSH/LH (Pergoveris, Merck Biopharma, Denmark) alone or in combination with corifollitropin-alfa (Elonva, MSD, Denmark). The initial gonadotropin dosage was determined individually based on previous response to ovarian stimulation, as well as, patient age, body mass index, antral follicle count, and basal levels of follicle stimulating hormone (FSH). Dose adjustments were performed according to ovarian response monitored by transvaginal ultrasound during treatment. Oocyte pick-up (OPU) was carried out 36 h after trigger administration. *In vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI) was performed according to normal clinical practice. A maximum of two embryos were transferred on either days 2, 3, or 5 following oocyte retrieval.

Trained embryologists on site evaluated the quality of all available embryos. All original embryo scores from the four clinics were subsequently evaluated by two independent leading embryologists and allocated a final score from 1 to 3 (1 being a top-quality embryo, 2 being an intermediate embryo, 3 being a low-quality embryo). In case of incongruence, a second evaluation was performed to reach final agreement.

Briefly, a top-quality embryo on day 2 and 3 was described as having four and eight cells, respectively, equally sized blastomeres, <10% fragmentation and no multinucleate cells in accordance with the consensus scoring system for cleavage-stage embryos described by the Alpha Scientist group (15). In case of severe fragmentation (>25%), cell-size not stage-specific or evidence of multinucleation the cleavage embryo was classified as low-quality. The remaining cleavage embryos were classified as intermediate.

A top-quality blastocyst had a day 5 score better than 3BB according to the Gardner standard based on grade of expansion, trophectoderm, and inner-cell mass quality (16). A low-quality blastocyst had a day 5 score <3BB. The remaining blastocysts (3BB, 4BB, 5BB) were described as intermediate.

Luteal Phase Support

All patients received the same vaginal luteal phase support in a standard regimen using 300 mg micronized P_4 daily (Lutinus®, Ferring Pharmaceuticals). Intramuscular P_4 for luteal support was not used in any of the participating patients. A small fraction of patients ($n = 41$) had one bolus of GnRH agonist (Gonapeptyl 0.1 mg) on OPU+7 based on an individual clinical assessment. In patients receiving Gonapeptyl®, 30/41 were treated in the long GnRH agonist protocol and 11/41 in the GnRH antagonist protocol. Patients receiving a bolus of GnRH as luteal phase support were distributed equally across the different 17-OH P4 groups ($p = 0.35$).

In case of a GnRH-agonist trigger, a bolus of hCG on the day of oocyte retrieval (1,500 IU) was given to all patients. Based on the individual ovarian response to stimulation, some patients received an additional bolus of HCG on OPU+5 according to a protocol previously described by Humaidan et al. (17). Vaginal P_4 administration continued until the day of pregnancy testing (hCG trigger) or until 7 completed weeks of gestation (GnRHa trigger).

Blood Sampling

All 614 patients had blood samples performed 7 days after oocyte pick-up (OPU+7) for hormone measurements and 14 days after oocyte pick-up (OPU+14) for pregnancy testing.

On OPU+7, seven patients agreed to have a series of blood samples performed during daytime to assess the possible variation in serum 17-OH P_4 levels over time. These seven women were admitted to the fertility unit at Skive Region Hospital early in the morning and stayed at the clinic for the subsequent 12 h. The starting time for blood sampling was between 6 and 8 a.m. for all patients. Participants were allowed normal daily life activities during the study period. An intravenous cannula was inserted into a vein in the antecubital fossa and blood samples (4 ml) were drawn every 60 min for 12 h ($n = 7$) and for two of these hours every 15 min ($n = 6$ because of difficult venous access in one patient).

After coagulation at room temperature, all blood samples were centrifuged, and serum was isolated and divided into three separate aliquots to allow for analyses at different laboratories. Individual serum samples were stored at −80°C until analysis. Blood samples from the total cohort ($n = 614$) were analyzed for 17-OH P_4 and P_4, whereas the series of blood samples in the small cohort ($n = 7$) were analyzed for 17-OH P_4 and LH.

Hormone Assays

Serum 17-OH progesterone concentrations were measured using liquid chromatography-tandem mass spectrometry (LC-MS/MS) at the Department of Biochemistry, Aarhus University Hospital, Denmark. The assay allowed quantification of 17-OH P_4 in the range 0.37–78.7 nmol/l without dilution of samples. The accuracy was ± 0.32 nmol/l at 17-OH P_4 concentrations of 1.3, ± 0.90 nmol/l at 17-OH P_4 concentrations of 6.4 and ± 6.6 nmol/l at 17-OH P_4 concentrations of 47.0 nmol/l.

Serum P_4 and serum β-hCG concentrations were measured at the Department of Biochemistry, Odense University Hospital, Denmark using commercial automated electro chemiluminescent immunoassays (Immulite® 2000XPi, Siemens Healthcare, Denmark and Architect® i2000SR, Abbott Diagnostics, USA) routinely used for analysis. Serum LH concentrations were measured at the Department of Biochemistry, Viborg Region Hospital, Denmark, using commercial automated electro chemiluminescent immunoassays (Cobas® Modular analytics E170, Roche Diagnostics, Switzerland). The detection limit for P_4 was 0.6 nmol/l, and the in-house inter- and intra-assay coefficients of variation

were 4.4 and 1.6%, respectively. The detection limit for hCG was 1.2 IU/l and the in-house inter- and intra-assay coefficients of variation were 3.4 and 1.7%, respectively. The detection limit for LH was 0.1 IU/l and the in-house inter- and intra-assay coefficients of variation were 3.8 and 1.8%, respectively.

Exposure

Patients were divided into four 17-OH P_4 groups based on raw data of pregnancy outcomes: 17-OH P_4 <6, 6–14, 14.1–30, and >30 nmol/l (**Supplementary Figure 1**). The lower threshold of 6 nmol/l corresponds to the mid-luteal 17-OH P_4 level of the natural cycle (18).

In sensitivity analyses, estimates were also calculated based on 25/50/75 percentiles, as well as, 10/50/90 percentiles (**Supplementary Figure 1**).

Outcome Variables

Serum β-hCG concentration was determined on OPU+14 and was considered positive if β-hCG >10 IU/l. In case of a β-hCG level between 10 and 45 IU/l, a control β-hCG was performed after 48 h. Clinical pregnancy was defined as the presence of a live fetus within an intra-uterine gestational sac at ultrasound examination in gestational weeks 7–8. Early pregnancy loss was defined as (1) patients with an insufficient β-hCG value at the day of pregnancy testing (10–45 IU/l) and decreasing β-hCG values toward null in subsequent hCG-controls (2) patients with a positive hCG but no intra- or extrauterine sac visualized on transvaginal ultrasound in gestational weeks 7–8, and (3) patients with a fetus without visible heartbeat at UL in gestational weeks 7–8. Clinical pregnancy loss was defined as the loss of a viable intrauterine pregnancy up to and including gestational weeks 20+0. Live birth was defined as the delivery of a live infant after gestational weeks 20+0. For description of gestational age, clinical gestational dating was applied using the day of oocyte retrieval as gestational weeks 2+0.

Confounding Factors

The confounding factors included in the regression model were chosen a priori based on a Directed Acyclic Graph (DAG) (**Supplementary Figure 2**). DAGs are visual representations of causal paths between exposure and outcome (19, 20). Drawing and analysis of a DAG can help to identify confounding factors that obscure the real effect of the exposure on the outcome. Based on a structured analysis of the DAG, it is possible to identify a minimum, however sufficient set of covariates to adjust for in the statistical analysis, which will cover all confounding elements. The web application DAGitty was used to draw and analyze the DAGs used in this paper.

Statistical Methods

Data are presented as mean and standard deviation for continuous parametric variables, percentages for categorical variables and median and range for continuous, non-parametric variables. Differences in categorical variables between 17-OH P_4 groups were assessed with Fishers exact test or Pearson's chi-square test when appropriate. Differences in continuous parametric data between the four 17-OH P_4-groups were assessed using one-way analysis of variance (ANOVA) followed by a *post-hoc* pairwise comparison in case of a statistical difference between groups. Normality was checked by QQ-plots, and the assumption of variance homogeneity was tested by Bartlett's test. Kruskal-Wallis test was used in case of non-parametric continuous data.

A multiple logistic regression model was used to assess the association between mid-luteal 17-OH P_4 levels and the hCG test result (positive/negative), clinical pregnancy (yes/no) and early pregnancy loss (yes/no), and live birth (yes/no). The model included the independent variables maternal age (continuous, ln-transformed), maternal BMI (continuous, ln-transformed), smoking (yes/no), final follicle count on the day of trigger (continuous, ln-transformed), late follicular phase P_4 level [dichotomous (>4.77 or ≤4.77 nmol/l)] and day of transfer [dichotomous (cleavage-stage or blastocyst)] for estimates of positive hCG rate, clinical pregnancy rate, and live birth rate. For estimates of early pregnancy loss adjustment was made for maternal age (continuous, ln-transformed), maternal BMI (continuous, ln-transformed), smoking (dichotomous), final follicle count (continuous, ln-transformed), day of transfer [dichotomous (cleavage-stage or blastocyst)] and peak estradiol level on the day of trigger (continuous, ln-transformed). The cut-off for late follicular phase progesterone (>4.77 ng/ml equivalent to >1.5 ng/ml) was chosen based on the results of earlier studies (21, 22).

In case of missing data of covariates, patients were omitted from the final regression analysis ($n = 72$). A $p < 0.05$ was considered statistically significant. All statistical analyses were performed using STATA version 13.

Ethics

The study was conducted according to the declaration of Helsinki for Medical Research and approved by the local Ethics Committee of Central Denmark Region (M-2012-423-12). All patients gave their written and oral consent prior to study participation. ClinicalTrial.gov registration number NCT02129998.

RESULTS

Demographic Data

The population consisted of 614 women undergoing IVF/ICSI treatment followed by fresh embryo transfer on either days 2, 3, or 5. Demographic data are shown in **Table 1**. Overall, patients had a mean age of 32.5 ± 4.6 years and a mean BMI of 25.1± 4.2 kg/m^2. Maternal age, basal LH, basal FSH and smoking did not differ between 17-OH P_4 groups. Paternal age and BMI showed no significant differences between 17-OH P_4 groups (data not shown). Maternal BMI was significantly higher in the low 17-OH P_4 group (17-OH P_4 <6 nmol/l) compared pairwise to any of the other 17-OH P_4 groups (all pairwise $p < 0.001$). Antral follicle count and the distribution of women with PCOS differed significantly

TABLE 1 | Baseline characteristics of study patients in different 17-OH P_4 groups.

17-OH P_4 (nmol/l)	*N*	All	<6	6–14	14.1–30	>30	*p*
Number of patients, *n*	614		183	134	132	165	
Maternal age, years	614	32.5 ± 4.6	33.0 ± 4.9	32.2 ± 4.6	32.7 ± 4.5	32.0 ± 4.2	0.195
Maternal BMI, kg/m^2	614	25.1 ± 4.2	26.3 ± 4.0	24.2 ± 4.1	24.7 ± 4.4	24.5 ± 4.1	<0.001
Maternal smoking,%	614	8	10	8	4	9	0.197
Basal FSH, IU	573*	6.2 (0.1–22.0)	6.1 (0.3–22.0)	6.7 (0.1–17.5)	6.4 (0.3–15.5)	6.0 (1.1–14.7)	0.544
Basal LH, IU	554**	5.4 (0.1–40.0)	5.2 (0.2–40.0)	5.2 (0.1–19.0)	5.4 (0.1–17.0)	5.6 (0.4–24.0)	0.792
Antral follicle count, *n*	614	13 (2–50)	12 (2–33)	14 (3–40)	12 (2–38)	13 (4–50)	0.004
Primary diagnosis,	614						
Unexplained, %		25	21	22	30	29	0.200***
Tubal, %		9	7	8	11	11	0.426
PCO/PCOS, %		11	11	13	5	14	0.028
Endometriosis, %		6.5	7	7	7	5	0.781
Male, %		38	39	45	37	30	0.074
Single/female partner, %		10	14	5	9	10	0.095
Other, %		1	1	0	1	1	0.895

Baseline characteristics are presented as mean ± SD for continuous parametric data and as median (range) for continuous non-parametric data. Categorical data is presented as percentages (%). Data on basal FSH levels were missing in 41 patients (6.7%). Patients with missing data on basal FSH levels were equally distributed across 17-OH P_4 groups ($p = 0.49$). ** Data on basal LH levels were missing in 60 patients (9.8%). Patients with missing data on basal LH levels were equally distributed across 17-OH P_4 groups ($p = 0.43$). ***p-value describes the comparison between the chosen primary diagnosis category and the combined group of all other primary diagnosis categories. SI conversion factor for 17-OH P_4: nmol/l = 3.03 * ng/ml.*

between groups, albeit with no apparent clinically relevant differences.

Cycle Characteristics

A total of 63% of patients were treated in a GnRH antagonist protocol, whereas a long GnRH agonist protocol was used in 37% of patients. Final oocyte maturation was achieved using hCG trigger in 58% of patients and using GnRH agonist trigger in 42% of patients. In total, 64% of patients had a top-quality embryo for transfer (**Table 2**).

The low 17-OH P_4 group (<6 nmol/l) and the high 17-OH P_4 group (>30 nmol/l) both had a significantly higher final follicle count ($p = 0.01$) and a significantly higher number of oocytes retrieved ($p = 0.01$) compared to the two remaining 17-OH P_4 groups. Furthermore, total FSH dose, duration of stimulation and the luteal phase support regime differed between groups (**Table 2**).

Single embryo transfer was applied in 81% and double embryo transfer in 19% of patients. There was no significant difference between the number of embryos transferred across 17-OH P_4 groups ($p = 0.66$) A cleavage-stage embryo transfer was performed on day 2 or 3 in 72% of patients, whereas 28% had a blastocyst transfer on day 5. The study blastocyst transfer rate is in line with the present blastocyst transfer rate for all public IVF clinics in Denmark [2]. The percentages of patients with blastocyst transfer were comparable across the four 17-OH P_4 groups ($p = 0.42$). Likewise, the mean embryo score was similar in different 17-OH P_4 groups for both SET ($p = 0.09$) and DET transfers ($p = 0.92$).

[2]Danish Fertility Society. Annual Report 2017.

Mid-Luteal 17-OH P_4 Levels

The median 17-OH P_4 concentration measured on OPU+7 was 13.2 nmol/l, range 0.5–129.0 nmol/l. The median P_4 concentration was 113 nmol/l, range 16.3–1685.0 nmol/l. There was a significant, positive association between P_4 levels and 17-OH P_4 levels, $p < 0.001$ (**Figure 2A**). Thus, an increase of 100 nmol/l in serum P_4 levels corresponded to an increase in serum 17-OH P_4 levels of 9.5 nmol/l, 95%CI [9.0;9.9]. This ratio between serum 17-OH P_4 and serum P_4 of ∼10% was constant with increasing levels of P_4, $p = 0.67$ (**Figure 2B**). However, a large inter-individual difference in the secretion pattern of P_4 and 17-OH P_4 was noticed. To illustrate this, the 19 patients in the cohort with serum P_4 values between 400–450 nmol/l are marked in red in **Figure 2A**. Despite comparable levels of P_4 in these patients, the range of 17-OH P_4 varied from levels as low as 4.4 nmol/l up to 114 nmol/l.

Patients were equally distributed in the four chosen 17-OH P_4 groups. Out of the total cohort of 614 patients, 30% ($n = 183$) had 17-OH P_4 levels <6 nmol/l, 22% ($n = 134$) had 17-OH P_4 levels between 6 and 14 nmol/l, 21% ($n = 132$) had 17-OH P_4 levels between 14.1 and 30 nmol/l and finally, 27% ($n = 165$) had 17-OH P_4 levels >30 nmol/l. The 72 patients (12%) who were omitted from the final regression analysis due to missing values of covariates, were equally distributed across the four 17-OH P_4 groups ($p = 0.94$).

Reproductive Outcomes

The overall rate for positive hCG per transfer was 47% (291/614), the clinical pregnancy rate per transfer was 35% (214/614) and the overall live birth rate per transfer was

TABLE 2 | Descriptive data of controlled ovarian stimulation, oocytes, embryo transfer, and luteal phase support.

17-OH P_4, nmol/l	All	<6	6-14	14.1-30	>30	*p*
Number of patients (*n*)	614	183	134	132	165	
Protocol						
Antagonist (%)	63	51	58	61	81	<0.001
Long GnRH agonist (%)	37	49	42	39	19	
Total FSH dose (IU)	2,250 (500–7,350)	2,450 (900–6,750)	2,063 (788–7350)	2,475 (900–5025)	1,950 (500–7,350)	<0.001
Stim duration (days)	10.4 ± 2.0	10.4 ± 1.9	10.6 ± 2.1	10.6 ± 2.1	9.9 ± 1.9	0.006
Final follicle count >12 mm on trigger day	10 (1–29)	10 (1–29)	9 (1–22)	9 (3–20)	10 (1–26)	0.005
Mode of triggering for final oocyte maturation						
hCG (%)	58	67	72	61	35	<0.001
GnRH agonist (%)	42	33	28	39	65	
Number of oocytes retrieved (n)	8 (1–28)	9 (1–23)	8 (1–26)	8 (1–24)	9 (2–28)	0.014
Number of fertilized oocytes (n)	8 (1–27)	8 (1–23)	8 (1–23)	7 (1–24)	8 (2–27)	0.033
Single embryo transfer (%)	81	79	79	83	83	0.661
Double embryo transfer (%)	19	21	21	17	17	
At least one top quality embryo for transfer (%)	64	66	59	70	64	0.160
Mean embryo score,						
SET	1.4 ± 0.6	1.4 ± 0.6	1.5 ± 0.7	1.3 ± 0.5	1.5 ± 0.7	0.090
DET	1.7 ± 0.6	1.7 ± 0.6	1.7 ± 0.7	1.6 ± 0.7	1.6 ± 0.6	0.915
Day of transfer,						
Cleavage-stage embryo transfer (%)	72	72	72	77	68	0.418
Blastocyst transfer (%)	28	28	28	23	32	
Luteal phase support						
Vaginal progesterone only (%)	52	61	66	53	31	<0.001*
+ 1 bolus of hCG (%)	17	28	19	9	9	<0.001
+ 2 boluses of hCG (%)	25	4	8	30	56	<0.001
Vaginal P + Gonapeptyl (%)	6	7	7	8	4	0.353

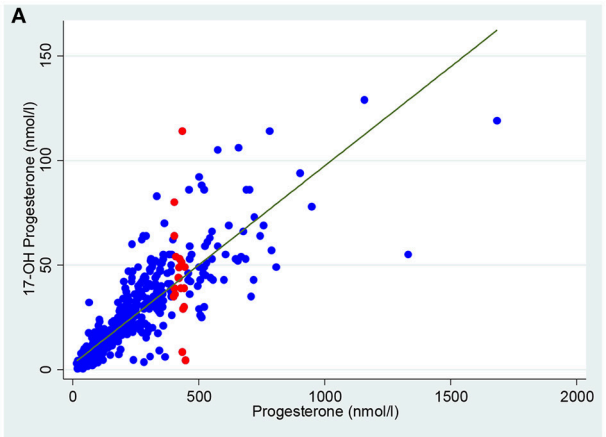
*Descriptive data is presented as mean ± SD for continuous parametric data and as median (range) for continuous non-parametric data. Categorical data is presented as percentages (%).*p-value describes the comparison between the chosen luteal phase support category and the combined group of all other luteal phase support categories. SI conversion factor for 17-OH P_4: nmol/l = 3.03 * ng/ml.*

34% (207/614). The early pregnancy loss rate was 26% (77/291), and the clinical pregnancy loss was 3% (7/214). The minimum and maximum levels of 17-OH P_4 in patients with a live birth were 0.65 nmol/l and 114 nmol/l, respectively.

When evaluating the association between mid-luteal 17-OH P_4 and reproductive outcomes, the optimal serum level of 17-OH P_4 was between 6 and 14 nmol/l. Below but also above this level, the OR for positive hCG, clinical pregnancy and live birth showed a non-linear pattern indicating a negative impact on the reproductive outcomes (**Figure 3**). Thus, OR for live birth in the low 17-OH P_4 group was 0.61, 95% CI [0.36;1.01], $p = 0.06$. Likewise, above the optimal 17-OH P_4 range the OR for live birth was significantly decreased: OR 0.59, 95%CI [0.35;0.98], $p = 0.04$. As seen from **Figure 3**, the association between 17-OH P_4 and reproductive outcomes displays a non-linear pattern and the negative impact of a high mid-luteal 17-OH P_4 level seems to be just as strong as the negative impact of low 17-OH P_4 in the peri-implantation period.

In sensitivity analyses, adding trigger type, or protocol type to the statistical model did not change estimates significantly. Furthermore, when using 25/50/75 percentiles or 10/50/90 percentiles to define four 17-OH P_4 groups, the same non-linear pattern for reproductive outcomes was found as seen with the a priori chosen 17-OH P_4 groups presented above, however, with smaller differences between groups (**Supplementary Figure 1**).

For a reference person (30 years old, BMI 25 kg/m^2, 8 follicles on the day of trigger, late follicular phase P_4 ≤4.77 nmol/l, non-smoker) the chance of a live birth following blastocyst transfer was 53%, 95% CI [42;64%] if mid-luteal 17-OH P_4 was within the optimal range (6–14 nmol/l). With mid-luteal 17-OH P_4 levels above the optimal range, the chance of a live birth decreased significantly to 41%, 95% CI [31;52%], thus an absolute risk difference of −12 percentage points, 95% CI [−22%;−0.01%], $p = 0.04$. With mid-luteal 17-OH P_4 levels below the optimal level, the chance of a live birth was 43%, 95%CI [33;53%], thus an absolute risk difference of −10 percentage points, 95% CI [−21;0.1%], $p = 0.07$.



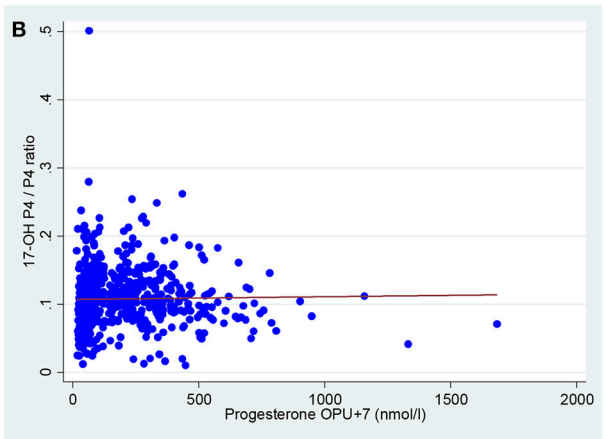


FIGURE 2 | Correlation between mid-luteal serum 17-OH P4 and serum P4 in 614 women undergoing IVF treatment. **(A)** A significant positive correlation was found between serum P_4 and serum 17-OH P_4, $p < 0.001$.Red dots depict the 19 patients in the cohort with P_4 400–450 nmol/l. **(B)** The ratio between serum 17-OH P_4 and serum P_4 was constant at ∼10% throughout the P_4 range, $p = 0.67$. The linear regression line is marked in red.

No significant correlation between mid-luteal 17-OH P_4 levels and early pregnancy loss was found (**Table 3**).

Daytime Variations in Serum 17-OH P_4 Levels

Figure 4 shows the individual daytime variations in mid-luteal serum 17-OH P_4 concentration in seven women undergoing IVF treatment. Three of these women (#4, #5, and #6) had very low endogenous 17-OH P_4 production with median concentrations during daytime between 1.9 and 3.8 nmol/l compared to 13.2 nmol/l for the total study cohort. It is seen from **Figure 4** that in patients with diminished luteal phase 17-OH P_4 production (<6 nmol/l), serum concentrations of 17-OH P_4 displayed a constant pattern though out daytime without any significant fluctuations. In contrast, in patients with 17-OH P_4 levels above 6 nmol/l, sudden fluctuations in 17-OH P_4 occurred randomly in different patients without any obvious common pattern. In patient #1, 17-OH P_4 increased 12.8 nmol/l in just 15 min (12.45–13.00 p.m.). This rise in concentration corresponds to an increase of 53% compared to the median level for the day, and this rise occurred even though LH levels were below the detection limit throughout the study period (LH data not shown).

DISCUSSION

This prospective study, including 614 women undergoing IVF and fresh embryo transfer, aimed at investigating whether the mid-luteal serum 17-OH P_4 concentration–used as an index of corpus luteum (CL) function–affects the reproductive outcome. The results suggest that positive hCG rates, clinical pregnancy rates, and live birth rates are reduced outside the defined optimal range for 17-OH P_4 (6–14 nmol/l). Furthermore, for the first time in IVF patients, we monitored the variation in mid-luteal serum 17-OH P_4 levels showing that patients with diminished CL function displayed a constant hormone pattern without any significant daytime fluctuations in serum 17-OH P_4 concentrations.

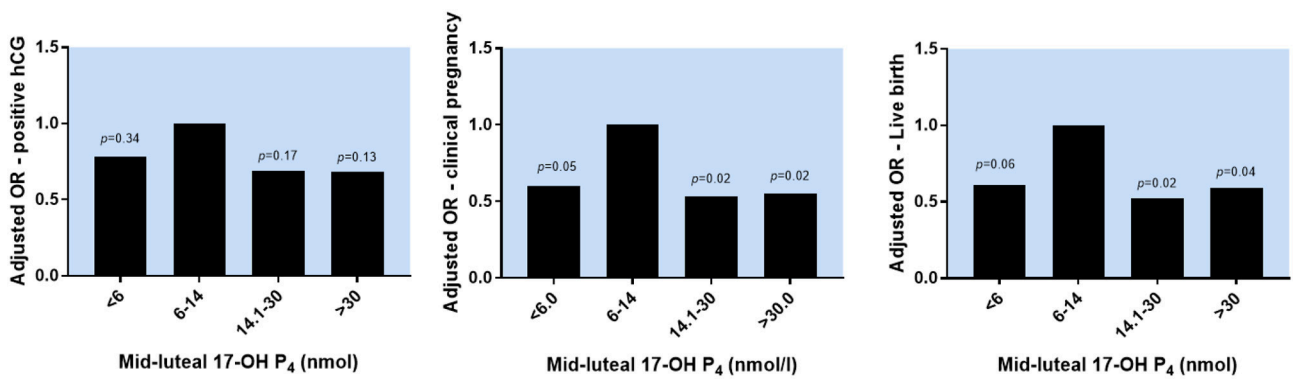


FIGURE 3 | The association between mid-luteal serum 17-OH P_4 levels and reproductive outcomes. OR for positive hCG, clinical pregnancy and live birth in different 17-OH P4 groups adjusted for maternal age, maternal BMI, day of embryo transfer, late follicular P4 levels, smoking and final number of follicles. *P*-values refer to the pairwise comparison between each 17-OH P4 category and the reference group (6–14 nmol/l).

TABLE 3 | Reproductive outcome in different luteal 17-OH P_4 groups.

Cohort for mid-luteal 17-OH P_4 monitoring															
	OR for positive hCG				OR for clinical pregnancy				OR for live birth				OR for early pregnancy loss			
	N	Crude OR [95% CI]	*N*	Final adjusted OR [95% CI]	*N*	Crude OR [95% CI]	*N*	Final adjusted OR [95% CI]	*N*	Crude OR [95% CI]	*N*	Final adjusted OR [95% CI]	*N*	Crude OR [95% CI]	*N*	Final adjusted OR [95% CI]
	291/614	614*		542**	214/614	614*		542*	207/614	614*		542**	77/291	614*		535***
17-OH P_4 < 6 nmol/l	88/183	0.82 [0.53;1.29]	161	0.78 [0.47;1.30]	65/183	0.74 [0.47;1.18]	161	0.60 [0.36;1.01]	62/183	0.74 [0.46;1.17]	161	0.61 [0.36;1.01]	23/88	1.23 [0.61;2.50]	160	1.82 [0.81;4.02]
17-OH P_4 6–14 nmol/l	71/134	1.00	120	1.00	57/134	1.00	120	1.00	55/134	1.00	120	1.00	14/71	1.00	119	1.00
17-OH P_4 14.1–30 nmol/l	58/132	0.70 [0.43;1.13]	115	0.69 [0.40;1.17]	41/132	0.61 [0.37;1.61]	115	0.53 [0.30;0.92]	39/132	0.60 [0.36;1.01]	115	0.52 [0.30;0.91]	17/58	1.18 [0.55;2.54]	111	1.60 [0.69;3.72]
17-OH P_4 >30 nmol/l	74/165	0.72 [0.46;1.14]	146	0.68 [0.41;1.13]	51/165	0.60 [0.37;0.98]	146	0.55 [0.33;0.92]	51/165	0.64 [0.40;1.04]	146	0.59 [0.35;0.98]	23/74	1.32 [0.65;2.69]	145	1.49 [0.67;3.32]

**In the crude OR estimates, all 614 patient with embryo transfer were included. **Due to missing data on the covariate late follicular P_4 level in 72 patients, the final adjusted regression model included 542 patients. Patients with missing data were equally distributed across 17-OH P_4 groups ($p = 0.94$). ***Due to missing data on the covariate peak follicular E_2 level in 79 patients, the final adjusted regression model for early pregnancy loss included 535 patients. Patients with missing data were equally distributed across 17-OH P_4 groups ($p = 0.67$). SI conversion factor for 17-OH P_4: nmol/l = 3.03 * ng/ml. CI, confidence interval.*

FIGURE 4 | Individual daytime variation in mid-luteal serum 17-OH P_4 levels in women undergoing IVF treatment and fresh embryo transfer. **(A)** Daytime variations in mid-luteal serum 17-OH P_4 in four women treated in the long GnRH agonist protocol and trigged for final oocyte maturation with hCG. All patients received vaginal P_4 for luteal phase support. Dotted lines depict 6, 14, and 30 nmol/l, respectively. **(B)** Daytime variations in mid-luteal serum 17-OH P_4 in three women treated in the GnRH antagonist protocol and trigged for final oocyte maturation with a bolus of GnRH agonist. All patents received vaginal P_4 for luteal phase support in combination with one (patient#5 and #6) or two (patient #7) boluses of hCG on the day of oocyte pick-up and 5 days later, respectively. Dotted lines depict 6 and 14 nmol/l.

The CL produces 17-OH P_4, as well as, P_4 during the luteal phase (13). However, the secretion pattern of the two steroids differs. Coinciding with the LH peak, an initial distinct 17-OH P_4 peak occurs reflecting the initial luteinisation and growth of the theca lutein cells—the luteal cell-line capable of 17-OH P_4 synthesis (13). After 2–4 days of decline, the 17-OH P_4 levels increase again—now in parallel with P_4 reaching a second peak during the mid-luteal phase, followed by a decrease toward the end of the luteal phase. In the natural cycle, the mid-luteal ratio of 17-OH P_4/P_4 is reported to be 10–20% (13, 23–26). We found a similar ratio of ∼10% in our cohort and this ratio did not change significantly with increasing levels of P_4. The large inter-individual differences seen in the secretion pattern of P_4 and 17-OH P_4 underline that the CL function is highly individual, and that comparable values of P_4 in individual patients may correspond to very diverse levels of 17-OH P_4. Furthermore, three out of seven randomly chosen patients who participated in the daytime monitoring displayed severely reduced endogenous 17-OH P_4 levels throughout the day (median levels 1.9–3.8 nmol/l). These concentrations are even lower than seen during the mid-luteal phase of the natural

cycle (∼6 nmol/l) (18). Additionally, the very low serum 17-OH P_4 levels were accompanied by low serum P_4 levels (36–55 nmol/l). The exogenous vaginal P_4 supplementation induces a serum P_4 level of ∼30–40 nmol/l, thus underlining that the abovementioned three patients had a severely diminished endogenous P_4 secretion. This occurred although two of the patients had 17 and 19 follicles, respectively, on the day of trigger. Thus, the CL function is individual, and a large number of CLs do not necessarily warrant a high steroid output in the mid-luteal phase. Furthermore, it seems that a severely decreased mid-luteal CL function is not a rare finding following IVF treatment despite a sufficient trigger regimen and luteal phase support.

The biological effect of 17-OH P_4 is not well-described. Whereas, P_4 has a fundamental impact on the decidualization process (27), the maternal immunological adaption in early pregnancy (28) and the dampening of uterine contractions at the time of implantation (29), the endogenous 17-OH P_4 has only very weak progestogen effects (30). The binding affinity of 17-OH P_4 to both P_4 receptors (PR-A and PR-B) is only 1% of that of P_4. Furthermore, upon binding, the capacity of 17-OH P_4 to activate subsequent gene expression is very low and only ∼0.12% of that of P_4 (30). Thus, even though P_4 and 17-OH P_4 are structurally similar and are secreted in parallel from the CL, they seem to work in different ways. In serum, P_4 is tightly bound to cortisol-binding protein (18%) and loosely bound to albumin (80%) whereas only 2% of P_4 is unbound (free) (24, 31). The free form of P_4 is available for diffusion out of capillaries, into cells where it exerts its function (32). A fraction of the secreted P_4 and 17-OH P_4 from the CL is transported directly to the uterus through a counter-current exchange mechanism from the utero-ovarian veins into the utero-ovarian arteries driven by a large concentration gradient (33, 34). This mechanism may function to secure a high biological steroid concentration from the site of production (the ovaries) directly to the target organ (the endometrium) (34, 35). Another fraction of secreted P_4 and 17-OH P_4 from the CL enters circulation directly via the ovarian veins, which terminate in the inferior vena cava (right) and the renal vein on the left (36). The binding affinity of 17-OH P_4 to cortisol-binding protein (CBP) is much greater than that of P_4 and close to that of cortisol (24). It can be speculated that 17-OH P_4 acts by displacing P_4 and cortisol from CBP, thereby increasing the free active hormone concentration locally in the ovarian veins. Thus, this mechanism will ensure a high, free P_4 concentration facilitating the counter-current transport from the venous to the arterial vascular bed and hence, an increased direct transport of P_4 to the endometrium.

It should be emphasized that natural, endogenous 17-OH P_4 differs chemically and biologically from the synthetic progestin 17-OH P_4 caproate (17-OHPC). The latter is a synthetic progestogen (compound with progesterone-like action) and is not produced endogenously (37). The 17-OHPC binds more avidly to the P_4 receptor than natural 17-OH P_4, eliciting a sustained and robust progestogen effect on the endometrium (30). Thus, 17-OHPC can be used as luteal phase support (IM administration) whereas monotherapy with natural 17-OH P_4– with a very weak direct progestogen effect—probably would be inefficient in terms of rescuing the luteal phase following IVF treatment.

From a clinical viewpoint, 17-OH P_4 may be used as a direct biomarker for luteal phase function, as 17-OH P_4 is not supplied as part of the luteal P_4 supplementation regimen. Thus, the measured 17-OH P_4 reflects the endogenous production predominantly from the CL, as only a minor fraction (∼0.5 nmol/l) of circulating mid-luteal 17-OH P_4 originates from the adrenal glands (23, 26).

Our findings of a non-linear association between 17-OH P_4 levels and the reproductive outcomes is in line with other studies examining luteal phase steroid profiles. Following frozen-thawed embryo transfer, work by Yovich et al. (8) and Alsbjerg et al. (10) both showed a diminished chance of ongoing pregnancy if serum P_4 was above or below a defined optimal P_4 range. Similarly, in a previous paper using the present patient cohort, we found a consistently non-linear pattern describing the association between early and mid-luteal P4 levels and reproductive outcomes. Thus, suggesting that both low, as well as, high luteal P4 levels reduce the chance of a positive pregnancy outcome following fresh embryo transfer (9). In that study, P_4 monitoring was performed during the early luteal phase (2–3 days following OPU) or in the mid-luteal phase (OPU+5). The same pattern emerged in this study, measuring serum 17-OH P_4 on OPU+7.

Taken together, the non-linear pattern between luteal steroid levels and reproductive outcomes seems to apply both to P_4 and 17-OH P_4, to different days in the luteal phase (2, 3, 5, or 7 days after OPU) and to both the fresh and frozen embryo transfer cycle (8–11). Furthermore, the above-mentioned studies all found a consistency in the absolute risk reduction (14–20 percentage points) below or above the defined P_4 which is in line with our present results.

In this study, daytime monitoring of 17-OH P_4 showed that patients with a diminished luteal phase function (17-OH P_4 <6 nmol/l) displayed a constant 17-OH P_4 pattern throughout daytime without any significant fluctuations in serum levels. Thus, measurement of luteal 17-OH P_4 concentrations will accurately detect patients with low endogenous 17-OH P_4 levels and, thus, a decreased corpus luteum function. In patients with higher 17-OH P_4 concentrations, fluctuations in serum 17-OH P_4 concentrations occur in a random fashion without any obvious common pattern between patients.

The possible clinical effect of serum fluctuations is demonstrated in patient #7 (**Figure 4**). When measuring 17-OH P_4 levels at 8.00 a.m., the patient would be classified in the optimal 17-OH P_4 range between 6 and 14 nmol/l. However, if measurements were performed at 12.00 p.m., she would be categorized in the 14.1–30 nmol/l group. Similarly, patient #2 shift between 17-OH P_4 group 14.1–30 and >30 nmol/l depending on the time of measurements. Thus, the figure demonstrates that when 17-OH P_4 monitoring is done 7 days after OPU, there is a risk of misclassification of patients if serum 17-OH P_4 >6 nmol/l. The finding, that the magnitude of the 17-OH P_4 fluctuations depends on the 17-OH P_4 concentration, is in total agreement with the P_4 daytime variation on OPU+7 previously shown by our group (14).

We monitored 17-OH P_4 on OPU+7 to explore whether the non-linear association between progestogen levels and reproductive outcome was still present at the time of implantation compared to earlier luteal measurements (2, 3, or 5 days following OPU) (9). Based on the present results, this seems to be the case. Performing the luteal monitoring early in the luteal phase, allows for an intervention based on the results. Thus, better reproductive outcomes may be obtained by additional exogenous luteal P_4 support to the low P_4 or 17-OH P_4 group and by segmentation followed by subsequent embryo transfer in a frozen/thawed cycle in case of a high P_4 or 17-OH P_4 level. In a clinical setting monitoring of P_4 or 17-OH P_4 on OPU+7 is disadvantageous as the clinical consequence of a "too low" or "too high" progestogen level is limited. Thus, at this time of cycle, the embryo is already transferred and the effect of administering additional exogenous P_4 during peri-implantation may be reduced compared to administration earlier in the luteal phase (38). Furthermore, in some IVF patients, peak levels of P4 and 17-OH P4 are seen already on day 5–6 and following this P4 and 17-OH P4 start to decrease. It could be hypothesized that some of the patients with low 17-OH P4 measured on day 7, had sufficient levels of 17-OH P4 earlier in the luteal phase and therefore are classified as "false low" on day 7. These patients could theoretically belong to a group with better pregnancy chance compared to patients with consistently low 17-OH P4 levels throughout the luteal phase. This misclassification of some of the patients could potentially affect the OR in the low 17-OH P4 group and underestimate the effect of low 17-OH P4 on the chance of pregnancy.

Whether the 17-OH P_4 monitoring offers a clinical advantage compared to the more traditional P_4 monitoring may be questioned. The 17-OH P_4 does not seem to display a more stable luteal daytime pattern in patients with sufficient CL function compared to P_4 (14). Furthermore, whereas the analytical performance of P_4 immunoassays is generally high, the immunoassays available for the quantification of 17-OH P_4 suffer from important analytical limitations (39, 40). The specificity of 17-OH P_4 measured by immunoassays is critically limited due to reduced reproducibility and cross reactivity with particularly P_4 (40). To account for this, 17-OH P_4 quantification must be performed using LC-MS/MS to obtain sufficient accuracy. This requires more manual work for the medical laboratory technician compared to a P_4 quantification using standard immunoassays and this more than triples the expense per sample. Finally, as demonstrated by **Figure 2A**, some patients display a low mid-luteal 17-OH P_4 level even though the concomitant measured P_4 level seems sufficient. This phenomenon may reflect an isolated defect in the function of the luteinized theca cells but a sufficient P_4 output from the luteinized granulosa cells (13) and may lead to a misclassification of the patient.

The key strengths of the present study include its prospective design, the large cohort of patients and the systematic approach to the handling of confounding factors by use of Directed Acyclic graphs minimizing the risk of collider stratification (41). Furthermore, all patients received the same type and dose of vaginal P_4 supplementation in the luteal phase ensuring a basis for comparison between patients. Furthermore, the participants included in the study were unselected broadening the generalizability of the findings.

In conclusion, this study shows for the first time that the chance of a live birth is reduced by ∼10 percentage points below, but also above the defined optimal range for 17-OH P_4 measured on OPU+7. This finding supports the emerging evidence that the absolute concentrations of luteal P_4 seem to affect the reproductive outcomes following IVF treatment. Based on the present study, luteal monitoring of 17-OH P_4 levels alone does not seem to offer a better insight into the CL function compared to the monitoring of total P_4 levels.

AUTHOR CONTRIBUTIONS

LT, PH, CA, and KE designed the study. LT drafted the manuscript and UK, CA, KE, MO and PH all contributed to the interpretation of data and critically reviewed the manuscript. All co-authors participated in the conduction of the study and approved the final manuscript.

FUNDING

An unrestricted grant from Ferring Pharmaceuticals, Denmark, supported this study. Furthermore, grants from The Health Research Fund of Central Denmark Region, The Research Foundation of the Hospital of Central Jutland, The Research Foundation of Dagmar Marshall, The Research Foundation of A. P. Møller, The Research Foundation of Aase & Ejnar Danielsen, The Research Foundation of Dir. Jacob Madsen & Hustru Olga Madsen, The Research Foundation of Fam. Hede Nielsen and The Danish Medical Research Grant supported conducting this study. The providers of funding were neither involved in the conduction of the study nor in the writing of the scientific report.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2018.00690/full#supplementary-material

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Conflict of Interest Statement: LT received an unrestricted research grant from Ferring Pharmaceuticals to support this work. PH received unrestricted research grants from MSD, Merck, and Ferring Pharmaceuticals, as well as, honoraria for lectures from MSD, Merck, and Gedeon Richter outside of this work. USK received honoraria for lectures from MSD and Ferring Pharmaceuticals outside of this work. CA received unrestricted research grants from MSD, IBSA, and Ferring Pharmaceuticals, as well as, honoraria for lectures from MSD and IBSA outside of this work.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
published: 28 May 2019
doi: 10.3389/fendo.2019.00325

Anti-müllerian Hormone for the Prediction of Ovarian Response in Progestin-Primed Ovarian Stimulation Protocol for IVF

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Background: The ability of anti-Müllerian hormone (AMH) to predict ovarian response has been studied extensively in gonadotropin-releasing hormone agonist and antagonist treatments, but no information is available regarding its value in progestin-primed ovarian stimulation (PPOS) protocol.

Methods: This retrospective data analysis included 523 patients without polycystic ovary syndrome who underwent their first *in vitro* fertilization/intracytoplasmic sperm injection cycle with PPOS protocol at our center between Jan. 2015 and Jul. 2018. Serum AMH measurements were acquired within 12 months prior to ovarian stimulation using the automated Access AMH assay.

Results: AMH exhibited a significantly positive correlation with the number of retrieved oocytes ($r = 0.744$, $P < 0.001$). For the prediction of poor (<4 oocytes) and high (>15 oocytes) response, AMH had an area under the receiver operating characteristic curve (AUC) of 0.861 and 0.773, corresponding with an optimal cutoff point of 1.26 and 4.34 ng/mL, respectively. When stratified according to the dose of medroxyprogesterone acetate (MPA) (4 mg vs. 10 mg per day), AMH retained its similarly high predictive value for poor (AUC = 0.829 and 0.886, respectively) and high response (AUC = 0.770 and 0.814, respectively) in both groups. Amongst the 314 women who received their first frozen embryo transfer (FET) following PPOS protocol, no significant differences were observed on the rates of biochemical pregnancy, clinical pregnancy, implantation, early miscarriage, multiple pregnancy and ectopic pregnancy (all $P > 0.05$) across AMH quartiles (≤1.43, 1.44-2.55, 2.56–4.35, >4.35 ng/mL). In a multivariable logistic regression model, age was suggested to be the only independent risk factor for clinical pregnancy ($P = 0.011$).

Conclusions: Our data demonstrated that AMH is an adequate predictor of both high and poor ovarian response in PPOS protocol regardless of MPA dose, but it does not associate with pregnancy outcomes in the first FET cycles in a freeze-all strategy.

Keywords: Anti-Müllerian hormone, ovarian response, progestin-primed ovarian stimulation, pregnancy, freeze-all strategy

OPEN ACCESS

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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *21 January 2019*
Accepted: *07 May 2019*
Published: *28 May 2019*

Citation:
Huang J, Lin J, Gao H, Wang Y, Zhu X, Lu X, Wang B, Fan X, Cai R and Kuang Y (2019) Anti-müllerian Hormone for the Prediction of Ovarian Response in Progestin-Primed Ovarian Stimulation Protocol for IVF. Front. Endocrinol. 10:325. doi: 10.3389/fendo.2019.00325

INTRODUCTION

The optimization and individualization of controlled ovarian stimulation (COS) for *in vitro* fertilization (IVF) depends on utilizing patient characteristics and biomarkers to accurately predict ovarian response and tailor intended treatment. The characteristics, such as age, body mass index (BMI), menstrual cycle length, and results from previous IVF cycles are generally considered by clinicians for selection of ovarian stimulation strategies (1). In addition, several different markers of ovarian reserve, which usually refers to the number of available primordial follicles as well as the oocyte quality, have been proposed as predictors of ovarian response with varying degrees of success (2, 3). Of these, biochemical measures, such as basal follicle-stimulating hormone (FSH), estradiol (E_2) and inhibin concentrations, fluctuate substantially during the menstrual cycle and hence their use has been limited (4, 5). Ovarian imaging, particularly antral follicle count (AFC), is largely affected by sonographers' intra- and inter-observer reproducibility and its sensitivity may differ from the resolution of transvaginal ultrasonography equipment (2, 6).

Anti-Müllerian hormone (AMH), a dimeric glycoprotein and a member of the transforming growth factor-β (TGF-β) family, has recently been demonstrated to be a promising surrogate marker of functional ovarian follicle reserve (5, 7). Produced by granulosa cells of preantral and small antral follicles, it acts as a follicular gatekeeper inhibiting initial follicle recruitment and FSH-dependent growth and selection (5). Unlike other ovarian reserve biomarkers, AMH has shown its superiority for good intra- and inter-cycle stability and good measurement repeatability (4, 5). Previous studies have extensively investigated the value of AMH in predicting both high and poor response in either gonadotropin-releasing hormone (GnRH) agonist or GnRH antagonist protocols (8–15), and the efficiency of an AMH-tailored stimulation regimen (1, 16, 17). However, so far no consensus on cutoff points of AMH has been achieved for ovarian response prediction as different COS protocols are inconsistent in the endocrine profile, early follicle recruitment and synchronization of follicular development, consequently resulting in a difference in the amount of oocytes retrieved (18). Besides, the method of AMH measurements in different clinical settings should be taken into consideration. For instance, the AMH concentrations detected by the Diagnostic System Laboratories assay have been reported to be 30% lower than those measured by the Gen I immunoassay (19). In addition, ethnicity has been associated with altered levels of AMH, with Chinese, Black African, Hispanic and South Asian women reported as having lower AMH than Caucasian women (20). Therefore, the predictive models based on AMH cannot be extrapolated directly from one ethnic population to another.

Recently, we reported a new COS protocol named progestin-primed ovarian stimulation (PPOS), in which medroxyprogesterone acetate (MPA), adjuvant to human menopausal gonadotropin (hMG), is used from the early follicular phase as an effective oral alternative to GnRH analogs for the prevention of premature luteinizing hormone (LH) surges during COS (21, 22). Based on the freeze-all policy, the PPOS protocol yields similar amount of oocytes and pregnancy outcomes compared with conventional short protocol in normal ovulatory women undergoing IVF/intracytoplasmic sperm injection (ICSI) (21). Subsequent studies have also proven its efficacy in women with poor ovarian response (23), polycystic ovarian syndrome (PCOS) (24) and advanced ovarian endometriosis (25), and demonstrated its safety in IVF newborns regarding neonatal outcome and congenital malformations (26). However, unlike the direct action on pituitary GnRH receptor in GnRH agonist and antagonist treatment, this new protocol was initially proposed for the consideration that administration of exogenous progestin (P) could inhibit GnRH/LH surge via the P receptor in the hypothalamus and block the E_2-induced positive feedback effects (22, 27). Therefore, differences have been noted between PPOS protocol and other conventional regimens, including the total gonadotropin dose and endocrine changes during COS (21, 23, 24).

The question therefore remains whether AMH can predict ovarian response to PPOS protocol at a level of accuracy comparable to that of GnRH agonist and antagonist treatment. Moreover, given the dearth of evidence concerning the predictive role of AMH among Chinese women undergoing IVF (11, 12, 14), and the controversy on whether AMH has any correlation with IVF outcomes (15, 28–32), the current study attempted to establish the predictive value of AMH in ovarian response and assess the relationship between serum AMH and pregnancy outcomes in IVF using PPOS protocol.

MATERIALS AND METHODS

Study Population and Design

The present work was a retrospective analysis of a cohort study performed at the Department of Assisted Reproduction of Shanghai Ninth People's Hospital affiliated with Shanghai Jiao Tong University School of Medicine. Our study protocol was approved by the hospital's Ethics Committee (Institutional Review Board) (No: 2014–31). We selected patients with measured AMH levels within the previous 12 months before the COS started from January 2015 to July 2018. This time interval has been proven with reliable consistency regarding the predictive value of AMH (33). The inclusion was limited to patients with a regular cycle who underwent their first IVF/ICSI cycle with PPOS protocol regardless of age. Patients were excluded from the study if they met one of the following criteria: (1) diagnosis of PCOS in accordance with the modified Rotterdam diagnostic criteria (34); (2) documented history of ovarian surgery (i.e., laparoscopic ovarian drilling, ovarian endometrioma stripping ,and unilateral oophorectomy); (3) use of hormonal contraceptives for pretreatment before the study cycle; (4) core data missing in the medical records (e.g., without endometrial thickness on the day of embryo transfer).

Endocrine Assays and AFC Measurement

Basal serum concentrations of FSH, LH, E_2, and P were analyzed on menstrual cycle day 3 (MC3) before the start of stimulation using chemiluminescence (Abbott Biologicals B.V., the Netherlands). The analytical sensitivity was as follows: FSH,

0.06 IU/L; LH, 0.09 IU/L; E_2, 10 pg/mL; and P, 0.1 ng/mL. We determined serum levels of AMH with the automated Access AMH assay (Beckman Coulter, Inc., USA). The assay's detection range was between 0.08-24 ng/mL with the detection limit of 0.02 ng/mL. Coefficients of variation were 1.5% (intra-assay) and 3.9% (inter-assay) for low (0.87 ng/mL), 1.4% (intra-assay) and 3.0% (inter-assay) for medium (4.45 ng/mL), and 1.7% (intra-assay), and 3.5% (inter-assay) for high (13.70 ng/mL) AMH levels. The AFC was detailed as the combined number of follicles with diameters between 2 and 10 mm in both ovaries as measured by transvaginal ultrasound scan on MC3.

Ovarian Stimulation Protocol

A description of the PPOS protocol has been presented in detail in our previous publications (21, 35). Briefly, patients were administered with hMG (150 or 225 IU/d; Anhui Fengyuan Pharmaceutical Co., China) and MPA (4 or 10 mg/d; Shanghai Xinyi Pharmaceutical Co., China) from MC3 onward. The initiating dose was 150 IU/d for patients with high AFC (>20) and those with elevated basal FSH (>7 IU/L), while 225 IU/d was used for all other patients. Follicular monitoring, along with measurement of serum FSH, LH, E_2, and P concentrations, were initiated on MC7-8 and performed every 2–4 days. The dose of hMG was adjusted depending on the growing follicles and E_2 level during the stimulation. When the leading follicle reached 18 mm in diameter, the final stage of oocyte maturation was cotriggered using triptorelin (0.1 mg; Decapeptyl, Ferring Pharmaceuticals, Germany) and human chorionic gonadotropin (hCG) (1,000 IU; Lizhu Pharmaceutical Trading Co., China). Transvaginal ultrasound-guided oocyte retrieval was undertaken 34–36 h after trigger. All follicles with diameters over 10 mm were aspirated.

The aspirated oocytes were fertilized *in vitro* by either conventional insemination or ICSI according to semen parameters. The freeze-all strategy was performed for all IVF/ICSI cycles. According to the criteria described by Cummins et al. (36), only embryos classified as top-quality (grade I and II) were cryopreserved via vitrification on day 3 after oocyte retrieval, whereas embryos graded as quality III and IV were subjected to extended culture and observation up to the blastocyst stage. The Gardner and Schoolcraft grade system (37) was then applied to select blastocysts with good morphological grades (grade ≥3BC) for vitrification on day 5 or 6.

Endometrium Preparation and Frozen Embryo Transfer

Endometrial preparation and frozen embryo transfer (FET) were performed as previously described (21). In short, FET was conducted in a natural cycle for patients with regular menstrual cycles, while patients with irregular menstrual cycles were treated with letrozole and, if necessary, in combination with hMG to stimulate monofollicular growth. Hormone replacement therapy (HRT) was recommended for patients with thin endometrium during either natural cycles or stimulated cycles. Up to two embryos per patient were transferred in each FET cycle. The transfer of day 3 or day 5–6 embryos was scheduled according to the timing of ovulation during the natural and mild stimulation cycle and the timing of P administration during HRT. Once a pregnancy was achieved, the luteal support was continued to 10 weeks of gestation.

Outcome Measures

The primary outcome measure was to determine whether serum levels of AMH have any correlation with the number of oocytes retrieved. Furthermore, we aimed to evaluate the ability of AMH to successfully predict high and poor response, and investigate whether the predictive ability differed according to the MPA dose (4 or 10 mg/d) applied in PPOS protocol. The threshold for high response was set at >15 oocytes retrieved (8, 12, 13), while a poor response was defined as <4 retrieved oocytes or cycle cancellation in accordance with the Bologna criteria (9, 12, 13, 38). A normal response was therefore defined as 4–15 oocytes retrieved. The total cumulative dose of hMG and duration of stimulation were recorded, as well as the number of >10 mm and >14 mm follicles on trigger day, number of oocytes retrieved, number of metaphase II oocytes, number of fertilized oocytes, number of two pronuclei (2PN) oocytes and number of embryos available.

In the second part of our study, we attempted to assess the association between AMH concentration and pregnancy outcomes among patients who received their first FET before May 2018. Clinical pregnancy was defined as the presence of a gestational sac regardless of the presence or absence of fetal heart activity, as measured by ultrasound examination 7 weeks after FET. The implantation rate was defined as the number of gestational sacs divided by the number of embryos transferred. The early miscarriage rate was defined as the percentage of patients with spontaneous pregnancy termination prior to the gestational age of 12 weeks.

Statistical Analysis

Statistical analysis was performed using the SPSS (version 20.0; SPSS Inc., USA), MedCalc (version 15.0; MedCalc Software bvba, Belgium) and STATA (version 12.0; StataCorp LLC, USA). Because none of the continuous data studied showed normal distribution under both Kolmogorov-Smirnova and Shapiro-Wilk test, they were presented as median with interquartile range, while categorical data was presented as frequencies with percentages. Between-group statistical differences were assessed by Kruskal-Wallis test and Chi-square test or Fisher's exact test for continuous and categorical variables, respectively. The correlation between baseline variables and the number of oocytes retrieved was evaluated using Spearman's rank correlation coefficients (r). Variables correlated were further included in a multivariate linear regression model to identify the independent determinants related to the number of retrieved oocytes. Receiver operating characteristic (ROC) curves were generated for each of the selected parameters to determine their ability to predict high or poor ovarian response. To assess differences in the predictive ability of AMH in the context of utilized MPA dose (4 mg/d vs. 10 mg/d) in PPOS protocol, the areas under the curves (AUCs) were compared using the method described by DeLong et al. (39). Optimal cutoff points were determined by the combination of specificity and sensitivity closest to the optimal.

For the comparison of pregnancy outcomes, patients were grouped based on AMH quartiles: ≤25th (≤1.43 ng/mL), 25–50th (1.44–2.55 ng/mL), 50–75th (2.56–4.35 ng/mL), >75th (>4.35 ng/mL). A multivariable logistic regression was further performed to investigate the effect of potential risk factors on clinical pregnancy. Potential risk factors, including age, BMI, AMH, MPA dose during COS, number of embryos transferred, endometrial preparation and endometrial thickness on FET day, were introduced into the regression equation by the forward stepwise (likelihood ratio) method. Unadjusted and adjusted odds ratios (ORs) and 95% confidence intervals (CIs) were calculated by the regression models. The −2 log likelihood was used to determine the significance of the models, and the Nagelkerke's R^2 was used to evaluate and explain uncertainty. All *P*-values were based on two-sided tests and $P < 0.05$ was considered to be statistically significant.

RESULTS

Baseline Characteristics, Stimulation Characteristics and Outcomes According to Ovarian Response

Overall, 523 women who underwent the PPOS treatment protocol for their first IVF/ICSI cycle were included in the study. High ovarian response was observed in 77 (14.7%) women, while 182 (34.8%) were categorized as poor responders. The patients' baseline characteristics according to the level of ovarian response were shown in **Table 1**. The three groups differed significantly in age, basal FSH, basal E_2, AMH and AFC (all $P < 0.01$). However, no significant differences were found when BMI, subfertility type, duration and causes, basal LH and basal P were analyzed.

Table 2 demonstrates the stimulation characteristics and outcomes per started cycle. The proportions of patients in different ovarian response categories who received the hMG + MPA (4 mg/d) protocol statistically significantly decreased across the three groups ($P < 0.001$), from 64 (83.1%) in the high response group to 85 (46.7%) in the poor response group. The opposite trend was observed for the hMG + MPA (10 mg/d) protocol applied. The poor-responding patients had received a significantly lower dose of gonadotropin compared with the normal-responding and high-responding patients ($P < 0.001$), while the duration of stimulation was similar among groups ($P = 0.117$). There was a significant between-group difference for the cycle stimulation outcomes, including the number of >10 mm and >14 mm follicles on trigger day, number of oocytes retrieved, number of metaphase II oocytes, number of fertilized oocytes, number of 2PN oocytes as well as number of embryos available (all $P < 0.001$).

Predictive Ability of AMH for Ovarian Response

The level of AMH exhibited a strong positive correlation with the number of oocytes retrieved according to Spearman's rank correlation analysis ($r = 0.744$, $P < 0.001$) (**Supplementary Figure 1A**). A significant but weaker correlation was also shown between AFC, basal FSH, age, basal E_2 and oocyte yield ($r = 0.740$, $P < 0.001$; $r = -0.552$, $P < 0.001$; $r = -0.394$, $P < 0.001$; and $r = -0.122$, $P = 0.005$, respectively), while no significant correlation was observed with regard to BMI ($r = -0.025$, $P = 0.565$) (**Supplementary Figures 1B–F**). After construction of a multivariate linear regression model, the largest influencing independent factor for the number of retrieved oocytes was AFC, followed by AMH, age, MPA dose and basal FSH in order of decreasing importance (**Table 3**). No significant association was observed between total hMG dose and oocyte yield ($P = 0.806$).

The predictive abilities of AMH, AFC, age and basal FSH for ovarian response were further analyzed by ROC curves (**Figure 1**). AMH showed a high accuracy for the prediction of both poor and high response with an AUC of 0.861 (95% CI: 0.825–0.892) and 0.773 (95% CI: 0.725–0.817), respectively. The AMH cutoff value for poor response prediction was 1.26 ng/mL with a sensitivity of 72.0% and a specificity of 86.4%, while the threshold of 4.34 ng/mL was shown to predict high response with a sensitivity of 67.5% and a specificity of 75.8%. The AUC values of AFC were comparable to those of AMH for prediction of poor and high response (AUC = 0.843 [95% CI: 0.806–0.876] and 0.797 [95% CI: 0.751–0.839]; $P_{AMHvs.AFC} = 0.374$ and 0.420, respectively). Basal FSH and age, however, performed significantly worse than AMH. The AUC values of basal FSH for poor and high response were 0.773 (95% CI: 0.731–0.811; $P_{AMHvs.FSH} = 0.001$) and 0.673 (95% CI: 0.621–0.723; $P_{AMHvs.FSH} = 0.021$), and those of age were 0.656 (95% CI: 0.609–0.700; $P_{AMHvs.FSH} < 0.001$) and 0.659 (95% CI: 0.606–0.710; $P_{AMHvs.age} < 0.001$), respectively.

To investigate whether the predictive ability of AMH was affected by the MPA dose applied in PPOS treatment, ROC curves were constructed for poor and high response accordingly (**Figure 2**). The curves revealed that the AUC values of AMH were comparable between hMG + MPA (4 mg/d) and hMG + MPA (10 mg/d) protocol: 0.829 (95% CI: 0.778–0.880) vs. 0.886 (95% CI: 0.834–0.981) for poor response, $P = 0.125$; and 0.770 (95% CI: 0.704-0.835) vs. 0.814 (95% CI: 0.709–0.919) for high response, $P = 0.485$.

Pregnancy Outcomes According to AMH Quartiles

A total of 314 women (60.0%) undergoing FET were stratified according to the 25, 50, and 75th percentiles of the serum AMH concentration. Due to the significant difference in number of embryos available for transfer across AMH quartiles ($P < 0.001$) (**Table 4**), only the first FET cycles were included for analysis. No significant differences, however, were observed among the AMH quartiles for all the analyzed pregnancy parameters, including biochemical pregnancy rate ($P = 0.084$), clinical pregnancy rate ($P = 0.158$), implantation rate ($P = 0.144$), early miscarriage rate ($P = 0.346$), multiple pregnancy rate ($P = 0.132$) and ectopic pregnancy rate ($P = 0.278$), as detailed in **Table 4**.

Unadjusted and adjusted ORs and 95% CIs of the potential risk factors for clinical pregnancy are shown in **Table 5**. Age and number of embryos transferred were significantly related to clinical pregnancy in unadjusted analysis ($P = 0.010$ and $P =$

TABLE 1 | Baseline characteristics according to the type of ovarian response.

	High response (oocytes >15)	Normal response (4≤ oocytes ≥15)	Poor response (oocytes <4)	*P*-value
No. of patients	77	264	182	
Demographics				
Age (years)	30.0 (28.0–32.5)	33.0 (30.0–37.0)	36.5 (32.0–40.0)	<0.001
BMI (kg/m^2)	21.6 (19.9–24.0)	21.1 (19.5–23.0)	21.5 (19.5–23.4)	0.353
Fertility characteristics				
Primary subfertility, *n* (%)	33 (42.9)	125 (47.3)	84 (46.2)	0.785
Secondary subfertility, *n* (%)	44 (57.1)	139 (52.7)	98 (53.8)	
Duration of subfertility (years)	2 (1–4)	2 (1–4)	2 (1–4)	0.965
Cause of subfertility, *n* (%)				
Tubal factor	45 (58.0)	136 (51.5)	92 (50.5)	0.375
Male factor	8 (10.4)	27 (10.2)	15 (8.2)	
Endometriosis	0 (0.0)	17 (6.4)	14 (7.7)	
Unexplained	0 (0.0)	2 (0.8)	3 (1.6)	
Mixed/other	24 (31.2)	82 (31.1)	58 (31.9)	
Endocrinological profile				
Basal FSH (IU/L)	5.16 (4.39–5.78)	5.05 (5.80–6.52)	7.70 (6.04–10.36)	<0.001
Basal LH (IU/L)	3.37 (2.79–4.79)	3.19 (2.34–3.96)	3.18 (2.28–4.26)	0.486
Basal E_2 (pg/mL)	31.0 (23.0–39.0)	34.5 (27.0–44.0)	37.5 (26.0–49.0)	0.009
Basal P (ng/mL)	0.3 (0.2–0.4)	0.3 (0.2–0.4)	0.3 (0.2–0.4)	0.460
AMH (ng/mL)	5.38 (3.48–7.38)	2.79 (1.67–4.34)	0.76 (0.42–1.50)	<0.001
Antral follicle count	16 (12–20)	9 (7–13)	5 (3–7)	<0.001

Data are presented as median (interquartile range) for all continuous variables.

TABLE 2 | Stimulation characteristics and outcomes according to the type of ovarian response.

	High response (*n* = 77)	Normal response (*n* = 264)	Poor response (*n* = 182)	*P*-value
Stimulation characteristics				
hMG + MPA (4mg/d), *n* (%)	64 (83.1)	202 (76.5)	85 (46.7)	<0.001
hMG + MPA (10mg/d), *n* (%)	13 (16.9)	62 (23.5)	97 (53.3)	
Total dose of hMG (IU)	2025 (1800–2250)	2025 (1800–2250)	1575 (1125–1950)	<0.001
Duration of stimulation (days)	9 (9–10)	9 (8–10)	8 (7–9)	0.117
Stimulation outcomes				
No. of >10 mm follicles on trigger day	21.0 (17.0–26.5)	11.0 (8.0–14.0)	3.0 (2.0–5.0)	<0.001
No. of >14 mm follicles on trigger day	15.0 (11.0–20.0)	7.0 (5.0–10.0)	2.0 (1.0–4.0)	<0.001
No. of oocytes retrieved	19.0 (17.0–22.5)	9.0 (6.0–12.0)	2.0 (1.0–3.0)	<0.001
No. of metaphase II oocytes	17.0 (14.0–20.0)	8.0 (6.0–10.0)	2.0 (1.0–3.0)	<0.001
No. of fertilized oocytes	14.0 (12.0–18.0)	6.5 (5.0–9.0)	2.0 (1.0–3.0)	<0.001
No. of 2PN oocytes	12.0 (9.5–14.0)	5.0 (4.0–7.0)	1.0 (1.0–2.0)	<0.001
No. of embryos available	8.0 (3.5–9.0)	3.0 (2.0–5.0)	1.0 (0.0–2.0)	<0.001

Data are presented as median (interquartile range) for all continuous variables.

0.042, respectively). In adjusted analysis, the only independent variable was found to be age ($P = 0.011$). Women ≥41 years had a significantly lower incidence of clinical pregnancy than women <30 years (OR = 0.27, 95% CI: 0.10–0.80).

DISCUSSION

The results of the study provided evidence for the first time that AMH as a single test is adequately predictive of both high and poor ovarian response in patients undergoing PPOS protocol for IVF. This predictive ability is unaltered by the different dose of MPA applied in PPOS treatment. Furthermore, our study found no significant association between AMH level and pregnancy outcomes in the first FET cycles in a freeze-all strategy.

The findings from the current study are in line with previous researches on the high predictive value of AMH for ovarian response using either GnRH agonist or antagonist protocols (8–15). Our data revealed that both AMH and AFC are better

TABLE 3 | Multiple linear regression analysis of possible determinants for number of oocytes retrieved.

Independent variables	Unstandardized coefficients		Standardized coefficients		*P*-value
	β (95% CI)	Std. Error	β	*t*	
(Constant)	7.528 (3.840 to –11.216)	1.877	-	4.010	<0.001
AFC	0.465 (0.364 to –0.566)	0.051	0.370	9.041	<0.001
AMH (ng/mL)	0.926 (0.723 to –1.130)	0.104	0.346	8.924	<0.001
Age (years)	−0.142 (−0.219 to −0.064)	0.040	−0.110	−3.582	<0.001
MPA dose (10 vs. 4 mg)	−1.210 (−2.129 to −0.290)	0.468	−0.082	−2.585	0.010
Basal FSH (IU/L)	−0.147 (−0.281 to −0.012)	0.068	−0.076	−2.143	0.033
Total hMG dose (IU)	0 (−0.001 to −0.001)	0	0.008	0.246	0.806

The model ($R = 0.763$, $R^2 = 0.583$, adjusted $R^2 = 0.578$, $P < 0.001$). The values of the standardized coefficients reflect the independent contributions of each predictor to dependent variables.

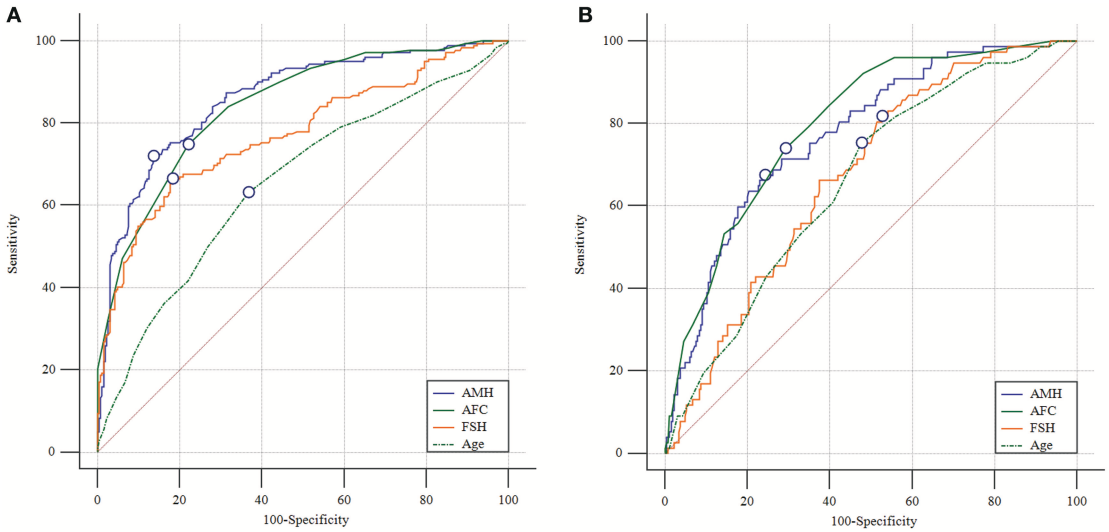

FIGURE 1 | Receiver operating characteristic curves for AMH, AFC, basal FSH and age for ovarian response prediction. **(A)** Prediction of poor (<4 oocytes) response. **(B)** Prediction of high (>15 oocytes) response. The marked point is in correspondence with Youden index.

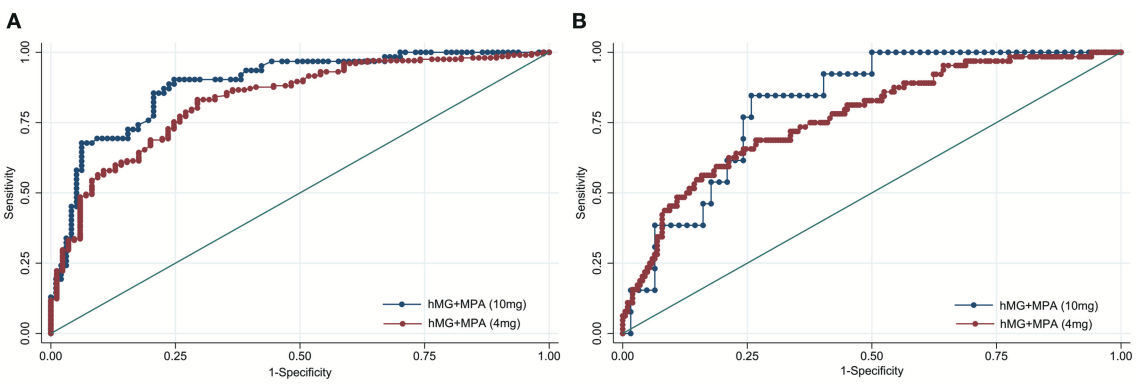

FIGURE 2 | Receiver operating characteristic curves for AMH for ovarian response prediction according to the MPA dose. **(A)** Prediction of poor (<4 oocytes) response. **(B)** Prediction of high (>15 oocytes) response. The diagonal line is the reference line of no discrimination (area under the curve = 0.5).

predictors of ovarian response during COS compared with other traditional measures (i.e., age and basal FSH level). These two markers of ovarian reserve exhibit comparable predictive value for ovarian response in PPOS protocol, in accordance with previous studies indicating that early-follicular phase AFC and AMH have similar correlations to the number of oocytes

TABLE 4 | Pregnancy outcomes of the first FET cycle according to the AMH level.

	AMH quartiles (ng/mL)				*P*-value
	≤1.43	1.44–2.55	2.56–4.35	>4.35	
No. of patients	79	79	78	78	
No. of viable embryos per patient	2 (1–3)	3 (2–4)	4 (3–6)	4 (3–8)	<0.001
AMH (ng/mL)	0.79 (0.46–1.04)	2.01 (1.68–2.23)	3.28 (2.92–3.83)	6.31 (5.19–7.72)	<0.001
Demographics					
Age (years)	37.0 (33.0–40.0)	34.0 (30.0–38.0)	33.0 (29.0–37.0)	31.0 (28.0–34.3)	<0.001
BMI (kg/m^2)	21.0 (19.5–23.4)	20.8 (19.5–23.1)	21.0 (19.4–23.0)	21.9 (20.1–23.8)	0.634
FET characteristics					
Total No. of transferred embryos	122	142	145	143	
Single embryo transfer, *n* (%)	36 (45.6)	16 (20.3)	11 (14.1)	13 (16.7)	<0.001
Double embryo transfer, *n* (%)	43 (54.4)	63 (79.7)	67 (85.9)	65 (83.3)	
Endometrial preparation					
Natural cycle, *n* (%)	17 (21.5)	15 (19.0)	17 (21.8)	8 (10.3)	0.016
HRT, *n* (%)	24 (30.4)	16 (20.3)	9 (11.5)	13 (16.7)	
Mild stimulation, *n* (%)	38 (48.1)	48 (60.8)	52 (66.7)	57 (73.1)	
Endometrial thickness (mm)	9.8 (8.5–11.3)	9.5 (8.3–11.3)	10.1 (8.4–11.2)	10.6 (9.5–12.5)	0.096
Pregnancy outcome, *n/N* (%)					
Biochemical pregnancy rate	30/79 (38.0)	40/79 (56.0)	45/78 (57.7)	35/78 (44.9)	0.084
Clinical pregnancy rate	27/79 (34.2)	39/79 (49.4)	38/78 (48.7)	31/78 (39.7)	0.158
Implantation rate	34/122 (27.9)	48/142 (33.8)	54/145 (37.2)	37/143 (25.9)	0.144
Early miscarriage rate	5/27 (18.5)	5/39 (12.8)	2/38 (5.3)	5/31 (16.1)	0.346
Multiple pregnancy rate	7/27 (25.9)	7/39 (17.9)	15/38 (39.5)	6/31 (19.4)	0.132
Ectopic pregnancy rate	0/27 (0.0)	2/39 (5.1)	0/38 (0.0)	2/31 (6.5)	0.278

Data are presented as median (interquartile range) for all continuous variables.

retrieved (3, 6, 40). Direct comparisons of AFC and AMH in ovarian response prediction have generally shown no significant difference, while a few studies demonstrated that AMH or AFC had stronger predictive value than the other (6). Since each method has its own advantages and drawbacks (2, 6), a combination of both could potentially be used to assess the ovarian reserve comprehensively, although AMH has been found to be a better predictor of oocyte yield in patients with discordant AFC and AMH measurements (41).

PPOS protocol is established based on the inhibitory effects of P on pulsatile GnRH and pituitary LH and FSH discharges, as well as its prevention of E_2-induced positive feedback effects (21, 27). The current study found that MPA 4 mg/d was preferentially used in patients with high response, while MPA 10 mg/d was applied more frequently for poor responders at our center. This is mainly based on the hypothesis that a higher dose of MPA could lead to a deeper pituitary suppression and prevent spontaneous ovulatory LH surge more effectively, especially for women of advanced age, diminished ovarian reserve, and elevated basal LH levels (42). However, a recent prospective randomized controlled trial (RCT) has demonstrated comparable endocrinological characteristics and clinical outcome of PPOS protocol using different doses of MPA (35). The ROC analysis in our study also revealed that the predictive values of AMH for both high and poor response remain constant irrespective of MPA dose, further strengthening that the administration of 4 mg of MPA daily is sufficient for a desirable outcome in women undergoing IVF/ICSI treatment (35).

The cutoff level of AMH should be interpreted with caution and assessed by the evaluation of eventual benefits vs. the possible misclassification of patients. A threshold of 4.34 ng/mL is set for high response in PPOS protocol, which implies an elevated risk of ovarian hyperstimulation syndrome (OHSS) for patients above this level and a need for more intense monitoring of ovarian stimulation. However, coupled with dual trigger (GnRH agonist and a low dose of hCG) for final oocyte maturation and the application of a freeze-all strategy for viable embryos, PPOS protocol allows for nearly complete avoidance of the incidence of OHSS (21, 43). Regarding poor response, the AMH cutoff value is 1.26 ng/mL with a sensitivity of 72.0% and a specificity of 86.4%. Patients with AMH below this threshold should be informed in advance of their relatively low opportunity of achieving pregnancy due to a significantly higher rate of no available embryos (36.9 vs. 7.3%, P <0.001). Nevertheless, it should not be used in isolation as the criterion for withholding fertility treatment (30, 31). Through repeated COS cycles, it is rational to assume an increased cumulative pregnancy rate since the developmental potential of embryos showed no difference between AMH below and above 1.26 ng/mL, as indicated by the similar clinical pregnancy rate following their first FET cycle (39.1 vs. 44.1%, $P = 0.463$).

TABLE 5 | Crude and adjusted odds ratios of confounding factors for clinical pregnancy in the first FET cycle.

Variables	Crude OR (95% CI)	*P*-value	Adjusted OR (95% CI)	*P*-value
Age (years)		0.010		0.011
<30	Reference		Reference	
30–34	1.46 (0.80–2.66)		1.51 (0.82–2.78)	
35–37	1.66 (0.81–3.40)		1.72 (0.84–3.53)	
38–40	1.06 (0.50–2.23)		1.04 (0.49–2.21)	
≥41	0.26 (0.09–0.74)		0.27 (0.10–0.80)	
BMI (kg/m^2)		0.569		–
<18.5	0.82 (0.39–1.72)		-	
18.5–24.9	Reference		-	
≥25	0.70 (0.35–1.42)		-	
AMH (ng/mL)		0.161		-
≤1.43	0.55 (0.29–1.04)		-	
1.44–2.55	1.03 (0.55–1.92)			
2.56–4.35	Reference		-	
>4.35	0.69 (0.37–1.31)		-	
MPA dose during COS (mg)		0.939		-
4	Reference		-	
10	1.02 (0.62–1.67)		-	
No. of embryos transferred		0.042		-
1	Reference		-	
2	1.75 (1.02–3.02)		-	
Endometrial preparation		0.478		-
Natural cycle	Reference		-	
HRT	0.85 (0.42–1.75)		-	
Mild stimulation	0.71 (0.39–1.28)		-	
Endometrial thickness (mm)		0.514		-
<8	0.71 (0.35–1.45)		-	
8–11	Reference		-	
>11	1.10 (0.67–1.80)		-	

Adjusted ORs were adjusted for all covariates in the table using a binary logistic regression model through forward stepwise method. The −2 log likelihood = 405.58, and the Nagelkerke $R^2 = 0.069$.

Accurate prediction of ovarian response is of paramount importance in individualized gonadotropin dose selection (1). Previous cohort studies have shown that AMH-tailored stimulation strategies resulted in a decreased incidence of high and poor response, increased pregnancy and live birth rates, as well as a reduction in costs (16, 17). These findings, however, are challenged by two recent RCTs to some extent (44, 45). In the single-center study by Allegra et al. (44), no significant differences were observed in the clinical pregnancy rate or the number of embryos cryopreserved per patient between FSH starting dose selection based on a nomogram (age, day 3 FSH and AMH) and an age-based strategy, despite a significant increase in the proportion of patients with optimal ovarian response. Another multicenter RCT of 1329 women further demonstrated that individualized FSH dosing based on serum AMH and body weight was non-inferior for ongoing pregnancy and implantation rates as well as the risk of moderate to severe OHSS (45). In our study, patients of poor response require significantly lower dose of gonadotropin than those of normal and high response, in contrast with the higher gonadotrophin dose needed for maximal stimulation in poor responders undergoing long GnRH agonist protocol (10, 12). One potential explanation may be the mechanism that the inhibitory action of P on the GnRH/LH surge is mediated by the classical P nuclear receptor of the hypothalamus rather than pituitary GnRH receptor, and by blockade of the activation and transmission of the E_2-induced signal (27). Therefore, unlike the pituitary desensitization in long agonist protocol, PPOS protocol exhibited an indirect, mild and slow suppression of LH secretion through continuous administration of MPA (21), leading to a lower dose of gonadotropin for stimulating growth and development of fewer follicles. This finding would lay a foundation for future design of prospective well powered studies on the efficacy and safety of different dosing regimens in PPOS protocol determined by an individual's AMH level.

Given that serum AMH concentration correlates strongly with oocyte yield, it is plausible that AMH might also be associated with qualitative outcomes of ovarian stimulation. Several large-scale retrospective analyses have shown a positive association between AMH and implantation, pregnancy and

live birth rates after assisted reproduction (15, 29), with the confirmation from a prospective cohort study even after adjusting for age and oocyte yield (28). However, others have found no such association (30, 31). Due to the conflicting results of accumulating data, a meta-analysis of 19 studies has been carried out recently which suggested that AMH has a weak correlation with implantation and clinical pregnancy but its predictive accuracy is limited (32). To date, this is the first study to demonstrate no significant differences in pregnancy outcomes in the first FET cycles across AMH quartiles in a freeze-all strategy. Instead, age serves as the only risk factor for clinical pregnancy, which is easy to understand since increased age is well-characterized by a reduction in both oocyte quantity and quality and accompanied with a decline in female fecundity (46). Thus, AMH may be less promising in predicting pregnancy chances of women undergoing IVF, although further prospective studies are still awaited.

A major weakness of the current study stems from its retrospective and non-randomized design, although the ascertainment and recall bias were minimized because all the data were gathered and documented in the computerized database. Also, there was no attempt to compare the ability of AMH in ovarian response prediction between PPOS and other conventional COS protocols. Since PPOS protocol is the prior and mainstream COS regimen at our center, and previous studies have extensively investigated the predictive role of AMH in GnRH agonist and GnRH antagonist treatment (11, 12, 14), we therefore decided not to make a direct comparison in this study. Finally, analysis of the association between AMH and pregnancy outcomes was limited in the first FET cycle, without assessing the rates of cumulative clinical pregnancy and live births. Considering that no trial has been published regarding the predictive value of AMH for pregnancy in the freeze-all policy and that FET equals or even surpasses fresh embryo transfer on clinical outcomes following IVF (47), it is essential and vital for further research in this field.

CONCLUSION

Our study demonstrates that AMH is an adequate predictor of both high and poor ovarian response in PPOS protocol, independent of the dose of MPA. However, AMH does not correlate with pregnancy outcomes in the first FET cycles in a freeze-all strategy. Therefore, to render infertility counseling and care more tailored to the patient, AMH level should be determined before embarking on PPOS treatment. Further studies are urgently needed to investigate the efficiency, safety and cost-effectiveness of individualized gonadotropin dosing based on the AMH level prior to IVF.

ETHICS STATEMENT

The present work was a retrospective analysis of a cohort study performed at the Department of Assisted Reproduction of Shanghai Ninth People's Hospital affiliated with Shanghai Jiao Tong University School of Medicine. Our study protocol was approved by the hospital's Ethics Committee (Institutional Review Board) (No: 2014-31).

AUTHOR CONTRIBUTIONS

JH, RC, and YK contributed to the conception and design of the study. JL, HG, YW, XZ, and XL were responsible for data collection and checking. JH performed the data analysis, interpretation and manuscript drafting. BW and XF were involved in data visualization. RC and YK supervised the project administration. All authors read and approved the final manuscript.

FUNDING

This study was funded by the National Key Research and Development Program of China (SQ2018YFC100163) and National Natural Science Foundation of China (81571397, 81771533).

ACKNOWLEDGMENTS

The authors would like to express sincere gratitude to Dr. Hengye Huang, Shanghai Jiao Tong University School of Public Health, for her support in data management and statistical analysis.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2019.00325/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 18 September 2019
doi: 10.3389/fendo.2019.00639

Low LH Level on the Day of GnRH Agonist Trigger Is Associated With Reduced Ongoing Pregnancy and Live Birth Rates and Increased Early Miscarriage Rates Following IVF/ICSI Treatment and Fresh Embryo Transfer

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OPEN ACCESS

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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *28 May 2019*
Accepted: *03 September 2019*
Published: *18 September 2019*

Citation:
Benmachiche A, Benbouhedja S, Zoghmar A and Humaidan P (2019) Low LH Level on the Day of GnRH Agonist Trigger Is Associated With Reduced Ongoing Pregnancy and Live Birth Rates and Increased Early Miscarriage Rates Following IVF/ICSI Treatment and Fresh Embryo Transfer. Front. Endocrinol. 10:639. doi: 10.3389/fendo.2019.00639

Objective: To examine the correlation between serum luteinizing hormone (LH) levels on the day of GnRH agonist (GnRH-a) trigger and reproductive outcomes following *in vitro* fertilization/intracytoplasmic sperm injection (IVF/ICSI) treatment and fresh embryo transfer, and to identify a pre-trigger serum LH threshold which would be compatible with the most optimal cycle outcome.

Design: This study is based on data from a previously published randomized controlled trial conducted from 2014 to 2016.

Patients: A total of 322 participants were enrolled.

Setting: Private IVF center. Intervention(s): GnRH-antagonist-based IVF cycles triggered with GnRH-a. For the purpose of the study, patients were stratified according to preovulatory LH quartiles (Q1-Q4). Main Outcome Measure(s): Ongoing pregnancy rates (OP), live birth rates (LB) and early pregnancy loss (EPL) rates.

Results: The results of the present study showed increasing OP as well as LB rates and decreasing EPL rates with increasing pre-trigger serum LH levels (P for trend < 0.06, 0.07, and 0.02), respectively. The absolute difference between the highest LH(Q4) and the lowest LH (Q1) group was 13.4%, 12.1%, and 12% in OP, LB, and EPL rates, respectively. In multivariate regression analysis, a pre-trigger serum LH level of 1.60 mIU/ml was identified as a threshold below which reproductive outcomes decreased. The ROC curve values were statistically significant for OP, LB, and EPL; the AUC (95% CI) = [0.57 (0.50–0.63) $P < 0.04$; 0.57 (0.50–0.63) $P < 0.05$, and 0.60 (0.51–0.70) $P < 0.04$], respectively. A significant positive correlation was found on the day of GnRH-a trigger between serum LH, the number of follicles, serum P4, and serum E2, $p < 0.03$; $P < 0.03$; and $P < 0.001$, respectively.

Conclusion: Low serum LH levels on the day of GnRH-a trigger is associated with reduced ongoing pregnancy and live birth rates and increased early miscarriage rates. Our findings suggest a lower threshold of serum LH values on the day of GnRH-a trigger necessary to optimize reproductive outcomes in fresh embryo transfer cycles.

Clinical Trial Registration: www.ClinicalTrials.gov, Number: 02053779

Keywords: luteinizing hormone, GnRH agonist trigger, live birth, early miscarriage, fresh embryo transfer

INTRODUCTION

Luteinizing hormone (LH) is essential for normal folliculogenesis and oocyte maturation in the natural ovulatory menstrual cycle (1). As early as at a follicle size of 6–8 mm, granulosa cell LH receptors are expressed, although at a low level, explaining the importance of LH from the early stage of follicular growth (2). Concomitantly, the pulsatile secretion of LH increases in frequency during the cycle and the mean LH level increases gradually from approximately 4.8 to 8 mIU/ml (3–5). Beyond the upper limit of the above-mentioned range, a surge of endogenous gonadotropins (FSH and LH) induces ovulation (4). Conversely, in stimulated IVF cycles, the use of GnRH antagonist during the late follicular phase in order to prevent the occurrence of a premature LH surge results in LH levels significantly lower as compared to the natural cycle, preventing the occurrence of premature LH surges (6–8). Accordingly, when GnRH agonist (GnRH-a) is used for final oocyte maturation, low LH levels will be present after the initiation of the GnRH antagonist co-treatment (9–11), raising concerns that LH levels may be too low for optimal cycle outcomes particularly when FSH only is used for ovarian stimulation. Further, several studies have shown that the surge of gonadotropins induced by a bolus of GnRH-a is short and low, respectively, in terms of duration and amplitude (12–17), and that has a negative effect on the early luteal phase gonadotropin and steroids profile (18, 19). Others recently, explored the possible impact of the LH level on the day of ovulation trigger when GnRH-a was used for final oocyte maturation. Indeed, it was found that low LH levels on the day of GnRH-a trigger were associated with a low mature oocyte yield (20, 21). However, their impact on the probability of pregnancy is still unknown. The primary objective of the present study was to examine the relationship between serum LH levels on the day of ovulation trigger and the reproductive outcomes in patients triggered with a bolus of GnRH-a followed by a modified luteal phase support (LPS) and fresh embryo transfer. The secondary objective was to identify a pre-trigger serum LH threshold, if appropriate, which would be compatible with the most optimal cycle outcome.

MATERIALS AND METHODS

Study Design

A secondary data analysis evaluating the relationship between serum LH levels on the day of GnRH-a trigger and the reproductive outcomes. Data were obtained from a randomized controlled trial exploring the impact of mid-luteal GnRH agonist administration on reproductive outcomes in GnRH-a triggered cycles (NTC: 02053779) (22).

Patients

This study included 322 infertile women who underwent ovarian stimulation, GnRH antagonist co-treatment, GnRH-a trigger and *in vitro* fertilization /intracytoplasmic sperm injection (IVF/ICSI) treatment followed by fresh embryo transfer, using a modified luteal phase support (23–25) at the IVF center Ibn Rochd, Constantine, Algeria, between February 2014 and January 2016.

Blood Samples and Hormone Assays

Serum LH concentrations were measured at the laboratory of the center, Ibn rochd, Constantine, Algeria on the day of ovulation induction for all participants early in the morning. Sera were analyzed immediately using a Vidas kit (BioMerieux, France). All measurements were performed according to the manufacturer's instructions. The detection limit for the VIDAS LH (LH) assay is 0.1 mIU/ml. The Intra and inter assay coefficients of variation were 2.7 and 3.7%, respectively.

Study Protocol

The reproductive outcomes as well as luteal phase gonadotropin and steroid profiles of this study have previously been published (22). In brief, hormonal stimulation was performed with GnRH antagonist co-treatment, using recombinant FSH (Puregon., MSD; Gonal F., Merck Serono) for ovarian stimulation. No LH activity was added. Once the leading follicle had reached a size of 13 mm, co-treatment with a GnRH antagonist (Cetrotide. 0.25 mg; Merck Serono) or (Orgalutran. 0.25 mg; MSD) was initiated and continued up until and including the day of induction of ovulation. Ovulation induction was performed with a single bolus of 0.2 mg triptorelin, s.c. (Decapeptyl. 0.1 mg, Ipsen, France) as soon as $\geq$3 follicles were $\geq$17 mm in diameter, followed by oocyte pick up (OPU) 36 h later. Retrieved oocytes were fertilized by either IVF or ICSI depending on sperm quality.

Embryo Transfer and Luteal Phase Support

In alignment with our local embryo transfer policy, one to three embryos were transferred on day 2 or 3 after OPU. A good quality embryo is defined as follows: the number of cells on day 2 is 4 cells and 7–9 cells by day 3, <20% of fragmentation, and regular sized cells.

For luteal phase support, in addition to a bolus of hCG 1,500 IU, IM (Pregnyl.; MSD) given 1 h after OPU, all patients received

micronized P (600 mg/day) vaginally (Utrogestan.; Laboratoires Besins-Iscovesco, Paris, France) and estradiol (4 mg/day) orally (Progynova. 2 mg; Schering, Madrid, Spain), beginning on the day after oocyte retrieval and continuing until either a fetal heartbeat was detected by ultrasound examination 5 weeks after OPU or a negative pregnancy test. As part of the study set-up, participants were randomized into two groups, of which the study group received a bolus of Triptorelin 0.1 mg (Decapeptyl. 0.1 mg) 6 days after OPU for additional luteal phase support (22).

Statistical Analysis

All statistical analyses were performed using the Statistical Package for Social Sciences (SPSS) version 20.0 (SPSS Inc., USA). Descriptive data are presented as mean ± standard deviation or median and range for continuous variables as appropriate, and percentages for categorical variables. Normality was examined by use of the Shapiro–Wilk test. Spearman rank correlation and Mann–Whitney tests were applied when indicated. Non-parametric ANOVA (Kruskal-Wallis test) was used across the four LH quartiles followed by a *post-hoc pairwise* comparison in case of a statistical difference between groups where appropriate. Percentages or rates were compared by use of Pearson chi-square, and Mantel–Haenszel test was computed for trend analysis. The receiver operating characteristic (ROC) curve was defined for serum LH on day of trigger and the area under the curve (AUC) was calculated. Multivariate logistic regression was used to estimate the odds ratio (OR) for the association between LH value on the day of trigger adjusted for all potential confounders and ongoing pregnancy (OP), live birth (LB), and early pregnancy loss (EPL). The LH level on the day of trigger was assessed as quartiles rather than continuous. Variables were included in the logistic regression model if they demonstrated a $P < 0.03$ for the association with outcome in the unadjusted analyses. The model for OP and LB included variables: serum estradiol (E2) levels and serum prolactin levels on day 2, total dose of GnRH antagonist, serum E2, serum progesterone (P4), number of follicles > 11 mm, and serum LH levels on the day of trigger (the first quartile was taken as the reference category), serum LH levels and serum P4 on OPU+7, number of embryos obtained, number of transferred embryos, embryo quality (good vs. bad), and GnRH-a dose on OPU+6 (yes/no). The model for EPL included the following variables: BMI, serum LH levels on the day of trigger (the first quartile was taken as the reference category), serum LH levels and serum FSH levels on OPU+7, the day of embryo transfer (2 or 3), and the GnRH-a dose on OPU+6 (yes/no). All statistical tests were two sided. $P < 0.05$ was considered statistically significant.

RESULTS

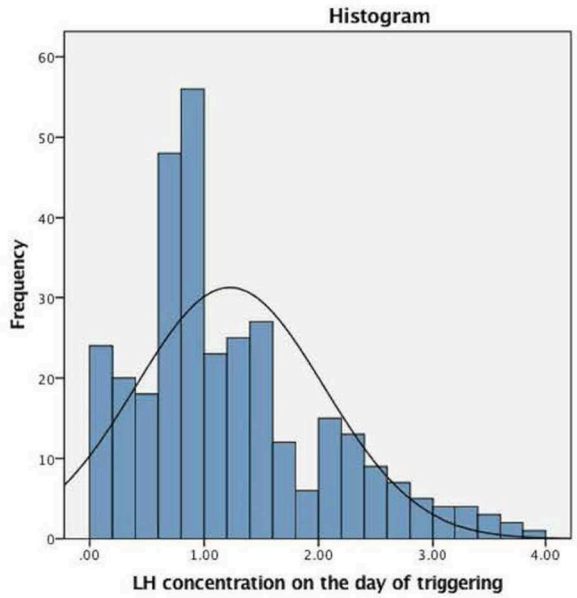
The present study evaluated a total of 322 IVF cycles. Of note, data on preovulatory LH levels were missing in six participants of the original cohort (328 IVF cycles), and hence were dropped from the current analysis. For the purpose of the study, patients were divided into four distinct groups according to their quartile serum LH levels on the day of GnRH-a trigger: [Q1: < 0.68, Q2: 0.68–0.98, Q3: 0.99–1.60, and Q4: > 1.60 mIU/ml] (**Figure 1**).

Demographic data, stimulation, follicles, oocytes, and embryos.

Baseline characteristics, and stimulation outcomes according to quartiles of serum LH levels on the day of trigger are presented in **Table 1**. The four groups (Q1, Q2, Q3, Q4) were comparable as regards age, BMI, days of stimulation, total dose of r-FSH, total dose of GnRH antagonist, P4 on day of trigger, number of embryos, and number of transferred embryos. However, significant differences were seen between the highest quartile (Q4) and the lowest quartile (Q1) as regards number of follicles >11 mm and E2 on day of trigger, $P < 0.04$ and $P < 0.001$ respectively.

Reproductive Outcomes

The relationship between pre-trigger LH and reproductive outcomes is shown in **Figure 2**. On one hand, a trend toward increasing OP rates across the lowest to highest quartile of serum LH levels on the day of GnRH-a trigger was seen as the OP rate increased from 28.9% in the Q1 to 42.3% in the Q4 (P for trend < 0.06). Likewise, a trend toward increased LB rates across the lowest to highest quartile of serum LH levels on the day of GnRH-a trigger was seen as the LB rate increased from 28.9% in the Q1 to 41% in the Q4 (P for trend < 0.07). In contrast, a trend toward decreased EPL rates across the lowest quartile (Q1) to the highest quartile (Q4) of serum LH concentration on the day of GnRH-a trigger was seen as the EPL rate decreased from 13.2% in the lowest quartile (Q1) to 1.2 % in the highest quartile (Q4) (P for trend < 0.02). The absolute difference between the highest and the lowest LH groups was 13.4%, 12.1%, and 12% in OP, LB, and EPL rates respectively. The ROC curve values, for OP, LB, and EPL, are shown in **Figure S1**; the AUC were 0.57, $P < 0.04$; 95% CI (0.50–0.63), 0.57 $P < 0.05$; 95% CI (0.50–0.63) and 0.60, $P < 0.04$; 95% CI (0.51–0.70) respectively. The ROC for EPL outcome has been performed by reversing the dataset labels giving the individuals who got EPL a label of "0" and those who didn't a label of "1". The difference between these areas and the reference line (area 0.5) was statistically significant for the serum LH measurement (**Figure S1**). **Table 2** summarizes the results of a multivariate regression analysis of the OP rates, LB rates and EPL rates. The results show that in addition to the availability of good embryos for transfer, serum LH level is the most valuable independent predictor of the reproductive outcome. **Figure 3** depicts the OR (95% CI) for OP rates, LB rates, and EPL rates according to the quartiles of serum LH (**Figures 3A–C**), respectively. After adjustment for relevant confounders, OP significantly increased in women with the highest quartile (LH > 1.60 mIU/ml) compared to the lowest quartile Q1 (LH < 0.68 mIU/ml; reference category), OR = 2.80, 95% CI (1.32- 5.95), $p < 0.007$. **Figure 3A**, LB significantly increased in women with the highest quartile (LH > 1.60 mIU/ml) compared to the lowest quartile Q1 (LH < 0.68 mIU/ml; reference category), OR = 2.56, 95% CI (1.21–5.40), $p < 0.01$. **Figure 3B**), and EPL significantly decreased in patients with the highest quartile (LH > 1.60 mIU/ml) compared to the lowest quartile Q1 (LH < 0.68 mIU/ml; reference category), OR = 0.09, 95 % CI (0.01–0.75), $p < 0.02$. **Figure 3C**.



Descriptive statistics		
Mean	1.2244	
Std. Error of Mean	.04581	
Median	.9800	
Std. Deviation	.82210	
Skewness	.965	
Std. Error of Skewness	.136	
Kurtosis	.462	
Std. Error of Kurtosis	.271	
Minimum	.10	
Maximum	3.91	
Percentiles	25	.680
	50	.980
	75	1.600

N: Valid= 322, Missing= 6

FIGURE 1 | Descriptive analysis of the serum values of LH (mIU/ml) on the day of GnRH-a trigger.

TABLE 1 | Baseline characteristics, and stimulation outcome based on LH levels on the day of GnRH-a trigger.

Parameter	LH Quartile 1 (<0.68)	LH Quartile 2 (0.68–0.98)	LH Quartile 3 (0.99–1.60)	LH Quartile 4 (> 1.60)	*P*-value
Number	83	83	78	78	
Age (years)	31 (23–39)	32 (23–39)	31 (26–39)	31.50 (21–39)	0.99
Body mass index (kg/m2)	27 (18.6–45.8)	27.6 (19.1–40)	28.1 (19.8–37.8)	27 (18.4–43.4)	0.26
Basal LH (mIU/ml)	4.1 (1.3–19.9)	4.8 (1.2–28.2)	4.2 (1.7–17.4)	5.1 (1.9–15)	0.30
No. of days of stimulation	9 (7–14)	9 (6–13)	9 (7–12)	9 (6–15)	0.85
Total dose of r-FSH (IU)	1800 (1400–2700)	1800 (1200–2925)	1800 (1200–2700)	180 (1125–3375)	0.26
Total dose of antagonist (mg)	1 (0.75–1.50)	1 (0.75–1.25)	1 (0.50–1.50)	1 (0.50–1.50)	0.29
No. of follicles on day of trigger	10 a (5–26)	12 (4–30)	15 (4–30)	15 b (4–24)	0.04
E2 on day of trigger (pg/ml)	1611 a (350–6298)	1953 (304–4300)	1916 (426–3000)	2229 b (536–3000)	0.002
P4 on day of trigger (ng/ml)	0.85 (0.36–2.56)	0.98 (0.38–2.63)	0.94 (0.27–2.86)	0.96 (0.38–4.65)	0.08
No. of oocytes retrieved	7 (3–23)	9 (2–30)	8 (3–25)	7 (1–22)	0.45
No. of embryos	5 (1–17)	5 (1–14)	5 (1–16)	4 (1–14)	0.32
No. of embryos transferred	2 (1–3)	2 (1–3)	2 (1–3)	2 (1–3)	0.53

Values are presented as median (range), Groups were compared using Kruskal-Wallis test.
b>a: Statistically significant difference in the post-hoc analysis over the groups Q4 vs. Q1.
Two-sided P < 0.05 were considered significant.

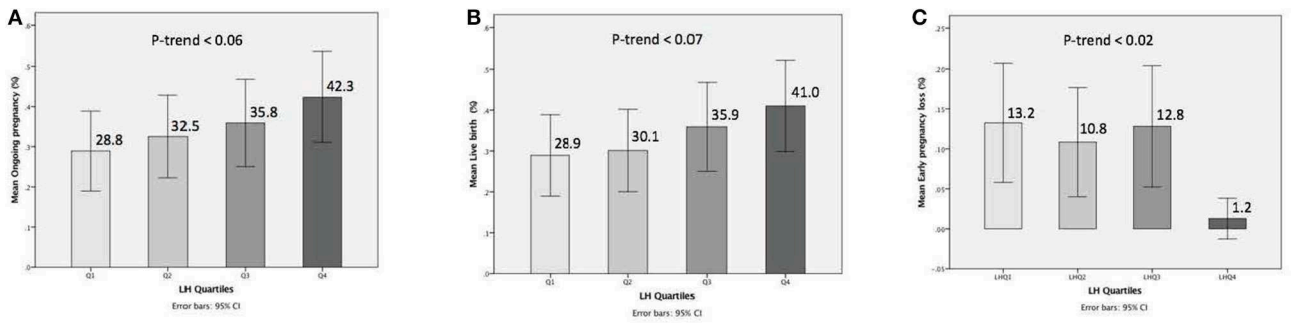


FIGURE 2 | Bar charts represent the ongoing pregnancy **(A)**, the live birth **(B)**, and the early pregnancy loss **(C)** outcomes for LH concentrations when stratified into quartiles. Trend analyzed using Mantel Haenszel test. A trend for increase of ongoing pregnancy, a trend for increase of live birth, and trend for decrease of early pregnancy loss observed with progressively higher concentrations of serum LH (p-trend < 0.06) < (p-trend < 0.07), and (p-trend < 0.02), respectively **(A-C)**. Data are expressed as ongoing pregnancy rates (95% CI) **(A,B)**, and early pregnancy loss rates (95% CI) **(C)** for each quartile of the serum LH levels.

TABLE 2 | Multivariate regression analysis of factors related to the cycle outcome.

Variable	Odds ratio (95 % CI)	*P*-value
Ongoing pregnancy		
Serum LH day of trigger (Q4 vs. Q1)	2.80 (1.32- 5.95)	0.007
Embryo quality (Good vs. Bad)	3.82 (1.68–8.65)	0.001
Embryos (*n*)	1.16 (1.04–1.29)	0.006
Embryos transferred (*n*)	1.59 (1.01–2.50)	0.04
Live birth		
Serum LH day of trigger (Q4 vs. Q1)	2.56 (1.21- 5.40)	0.01
Embryo quality (Good vs. Bad)	3.60 (1.60–8.12)	0.002
Embryos (*n*)	1.16 (1.05–1.30)	0.005
Follicles day of trigger (*n*)	0.92 (0.86–0.99)	0.03
Early pregnancy loss		
BMI (Kg/m2)	0.91 (0.83–1.00)	0.06
Serum LH day of trigger (Q4 vs. Q1)	0.09 (0.01- 0.75)	0.02

Serum LH was compared between the first quartile (<0.68 mIU/ml; reference category). and the rest of quartiles (2–4).

DISCUSSION

To our best knowledge, this is the first study to investigate the association between the LH level on the day of GnRH-a trigger and reproductive outcomes in a large cohort of GnRH antagonist co-treated IVF/ICSI treatment cycles. The results of the present study showed increasing OP as well as LB rates and decreasing EPL rates with progressively higher pre-trigger LH levels (P for trend < 0.06; 0.07; 0.02), respectively (**Figure 2**). After correction for the effect of main confounders, a multivariate regression analysis suggested a serum LH level of 1.60 mIU/ml on the day of GnRH-a trigger as the most appropriate threshold to predict reproductive outcomes (**Table 2**). Thus, patients with LH> 1.60 mIU/ml exhibited significantly better reproductive outcomes than those with LH < 1.60 mIU/ml (**Figure 3**). The ROC curve values, though statistically significant for OP, LB, and EPL, did not allow for accurate prediction; the AUC (95% CI) = [0.57 (0.50–0.63) $P < 0.04$; 0.57 (0.50–0.63) $P < 0.05$, and 0.60 (0.51–0.70) $P < 0.04$], respectively (**Figure S1**). In line with previous reports after human chorionic gonadotropin (hCG) trigger (11, 26–29), the current study using GnRH-a trigger supports the concept that a late follicular phase LH threshold exists below which adverse effects on the reproductive outcomes will occur. Importantly, others previously failed to find any association between LH levels and reproductive outcomes in hCG triggered IVF (30–33). However, studies on the optimal preovulatory LH level in GnRH-a triggered cycles are scarce. Indeed, only two studies showed that low LH level yields a lower number of mature oocytes (20, 21). In contrast, the relationship between pre-trigger LH levels and reproductive outcomes has not been reported before. The area under the curve of LH elicited by a bolus of GnRH-a is significantly less than compared to both the natural cycle and hCG trigger (12–17). Hence, it might be anticipated that low LH levels on the day of GnRH-a trigger might have an even higher impact on assisted reproductive outcomes as compared to hCG trigger. It should be noted that in IVF cycles triggered with hCG, varying cut-off values of LH on the day of trigger have been proposed ranging from 0.5 to 1.2 mIU/ml (11, 26–29), and the majority of them were arbitrarily chosen, and hence not conclusive. In the present study, the threshold of 1.60 mIU/ml suggested by the multivariate regression seems to be slightly higher than the above-mentioned thresholds, assuming that GnRH-a triggered cycles would require a higher LH level to compensate for the inadequacy of the LH activity surge compared to natural as well as hCG triggered cycles. Recently, accumulating evidence has been provided that many potential factors such as GnRH, inhibin, oestradiol, gonadotrophin surging-attenuating factor (GnSAF), and antimüllerian hormone (AMH) may be implicated in the control of circulating LH levels during the follicular phase (34, 35). However, none of these substances fully explain why the LH levels vary from individual to individual. Besides, in antagonist IVF co-treated cycles the circulating LH levels may decrease during the late follicular phase due to the negative feedback of ovarian hormones from multiple follicular developments or after suppressive effect from GnRH antagonist (36). The underlying mechanism by which low pre-trigger LH levels seem to reduce the pregnancy rates has not been fully elucidated. In fact, whether the observed effect of low LH exposure is exerted on the oocyte and/or on the endometrium is not clear. As mentioned, previous studies reported a negative

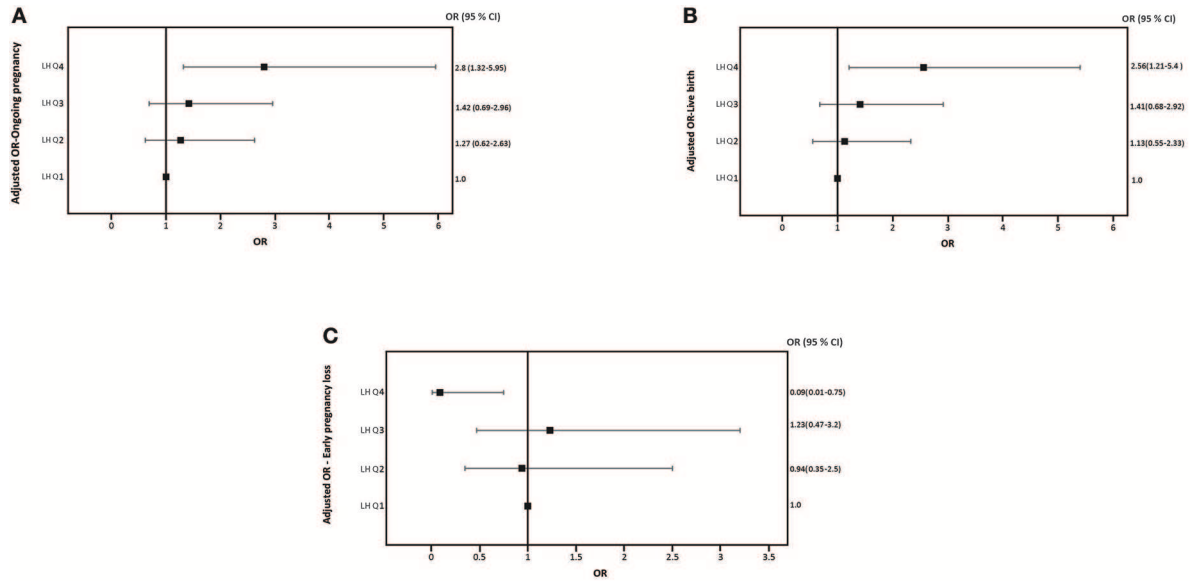


FIGURE 3 | Adjusted Odds ratio (95% CI) for ongoing pregnancy rates **(A)**, adjusted Odds ratio (95% CI) for live birth rates **(B)**, and adjusted Odds ratio (95% CI) for early pregnancy loss **(C)** across quartiles serum LH levels the day of GnRH-a trigger.

impact of low LH levels on the day of GnRH-a trigger as regards mature oocyte yield. Thus, the study by Meyer el al. (20), showed that a low LH level (LH< 0.5 mIU/ml) on the day of GnRH-a trigger leads to a poor oocyte retrieval. Another recent study (21), reported that patients with a suboptimal hormone response to GnRH-a trigger, as defined by a serum LH< 15 mIU/ml on the morning after GnRH-a administration, had significantly lower LH levels on the day of trigger (1.93 ± 4.65 mIU/ml vs. 2.26 ± 2.25 mIU/ml; $P < 0.001$), and significantly lower mature oocytes retrieved (4.10 ± 5.85 vs. 8.29 ± 6.94; $P < 0.001$) compared to those with adequate response (post-trigger LH>15 mIU/ml). In contrast, our data failed to find any significant impact of LH levels on the number of mature oocytes which is in agreement with the results reported by Andersen et al. (37) in hCG triggered IVF cycles showing a significant positive association between the late-follicular-phase LH levels and P4 levels, but not the number of oocytes retrieved. Hence, this discrepancy suggests that the impact of low LH levels (LH< 1.60 mIU/ml) may be more relevant to endometrial receptivity rather than to oocyte and/or embryo development. Moreover, our findings are in accordance with a prior study (28) showing that patients with LH levels < 0.5 mIU/ml before the day of hCG trigger in GnRH antagonist cycles exhibited an impairment of their endometrial receptivity since they had decreased implantation rates and LB rates as compared to patients with LH levels > 0.5 mIU/ml, despite significantly higher number of oocytes retrieved and embryos obtained in the group of patients with low LH levels. Interestingly, the same report found that the addition of LH activity in the form of low- dose hCG before ovulation induction significantly enhanced reproductive outcomes in low LH patients. The aforementioned notion is also consistent with a multicenter study (36), which included 333 IVF patients receiving six different doses of the GnRH antagonist, Ganirelix. Administration of the GnRH antagonist started on day 6 of stimulation. In the two highest dose groups, i.e., 1 mg and 2 mg per day, serum LH levels were suppressed well-below 1IU/l on the day of hCG trigger, 0.6 and 0.4 IU/l, respectively. Importantly, despite the fact that the number of retrieved oocytes and the number of good quality embryos were similar to those seen in lower GnRH antagonist dosing groups, implantation rates were significantly lower and early miscarriage rates significantly higher in the 1 mg and 2 mg per day, groups, with no ongoing pregnancies in the 2 mg per day group. Collectively, the above-mentioned effects could be ascribed to lack of up-regulation of endometrial LH receptors. Importantly, endometrial stromal cell apoptosis seems to be reduced by the administration of low dose of hCG (38, 39). Thus, the addition of LH activity in subgroups with markedly suppressed pre-trigger LH levels may have a positive effect on the regulation of the endometrium and hence, implantation (40–43). More studies, including gene-expression analyses, are required in the future to decrypt potential mechanisms involved in the interaction between circulating LH on the day of ovulation induction and the endometrium, particularly when GnRH-a is used for final oocyte maturation.

In the current study, we found that the LH level on the day of trigger is positively correlated with the number of follicles > 11 mm, E2 levels, and P4 levels ($r = 0.11$, $P < 0.03$, $r = 0.19$, $P < 0.001$, $r = 0.12$, $P < 0.03$, respectively) using Spearman rank correlation (data not shown). Thus, our data concur with previous findings, demonstrating the tight correlation between LH and follicular growth (37, 44). To date, several early studies demonstrated contradicting effects of elevated P4 on the day of hCG trigger and reproductive outcomes (45–49). The results of the present study are consistent with the fact that preovulatory P4 levels do not seem to affect reproductive outcomes. Further, the highest OP rate was found in the group of patients who had the highest late-follicular-phase P4 concentrations (i.e., P4

>1.5 ng/ml) 87.5% (98/112) and thus, developed many follicles which is supported recently by Andersen et al. (37). Our results are also in line with two recent reports showing that the possible negative impact of an elevated P4 on the day of hCG trigger seems to be more pronounced in women with low follicle numbers (50, 51). Importantly, the current published data on P4 elevation and IVF outcomes predominantly derive from hCG triggered cycles (52), whereas, there is still a paucity of information addressing this issue in GnRH-a triggered cycles (53). We recognize the limitations of the present study, including the sample size, which prevents statistical detection of further clinically significant differences, the fact that data derive from a *post-hoc* analysis, and the fact that possible circadian variations in LH and progesterone were not taken into account. Moreover, the findings of the current study can not be extrapolated to single fresh blastocyst stage transfer, which is the current mode of modern practice. Finally, the LH assays currently used do not always accurately reflect the LH bioactivity (54).

CONCLUSION

This is the first study to assess the impact of low late follicular phase LH levels on reproductive outcomes in GnRH-a triggered IVF cycles. A significant positive correlation was found on the day of ovulation trigger between serum LH quartiles and the number of follicles > 11 mm. Low serum LH levels on the day of GnRH-a trigger is associated with a reduction in reproductive outcomes. Future studies in a larger cohort of patients are needed to corroborate our findings.

DATA AVAILABILITY

The raw data supporting the conclusion of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

This secondary data analysis is based on a previously published randomized controlled trial which was conducted according to the declaration of Helsinki for Medical Research and approved by the Ethics Committee of the University hospital Centre Ibn Badis, Constantine, Algeria. All patients provided written and oral informed consent to participate in the study.

AUTHOR CONTRIBUTIONS

AB and PH designed the study, drafted, and edited the manuscript. AB performed data collection, handling of data, and statistical analysis. SB and AZ involved in patient's treatment and review of manuscript. All co-authors accepted the final draft.

FUNDING

This study was entirely funded by the center for Reproductive Medicine Ibn-Rochd, Constantine, Algeria.

ACKNOWLEDGMENTS

The technical assistance by Amel Boularak is gratefully acknowledged. Statistical analyses were performed by Tarik El Ghoulem.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2019.00639/full#supplementary-material

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Conflict of Interest Statement: PH received unrestricted research grants from MSD, Merck, and Ferring Pharmaceuticals, as well as honoraria for lectures from MSD, Merck, and Gedeon Richter outside of this work.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 21 April 2020
doi: 10.3389/fendo.2020.00221

Self-Detection of the LH Surge in Urine After GnRH Agonist Trigger in IVF—How to Minimize Failure to Retrieve Oocytes

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Research question: Urine LH testing may be useful to confirm an LH surge after the GnRH agonist (GnRHa) trigger prior to oocyte retrieval in IVF.

Design: A prospective cohort study, including oocyte donors undergoing ovarian stimulation, treated with a GnRHa trigger for final oocyte maturation. Urine LH testing was performed at home, 12 h after the GnRHa trigger. In the case of a negative result, serum LH and progesterone measurements were done that same day. Donors with no serum LH peak after trigger were re-scheduled using a dual trigger, with GnRHa and hCG.

Results: Three hundred and fifty nine oocyte donors were included in the analysis. Three hundred and fifty six donors had positive urine LH tests, followed by oocyte retrieval. In one case, the LH test was positive, however, no oocytes were retrieved (false positive 1/356). Three LH tests were negative in urine: in one of these three cases, LH was tested again in blood, confirming an LH rise, consistent with an optimal response to the GnRHa trigger; in the other two cases, serum LH was <15 mUI/mL, after which the oocyte retrieval was re-scheduled for 36 h after an being re-triggered, resulting in the retrieval of 19 and 22 MII oocytes, respectively. Considering the cost analysis, it would be a significantly cost-saving strategy, as blood testing would have costed 14,840€ vs. only 185.5€ in urine LH kits.

Conclusions: Urinary testing of the LH surge after GnRHa trigger is easy, safe, reliable, and convenient. In addition, LH urine testing allows identifying donors and patients who could benefit from a rescue hCG trigger after an unsuccessful GnRHa trigger.

Keywords: LH surge, GnRH agonist, urinary test, trigger, oocyte retrieval

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *16 January 2020*
Accepted: *27 March 2020*
Published: *21 April 2020*

Citation:
Cozzolino M, Matey S, Alvarez A, Toribio M, López V, Perona M, Henzenn E, Piró M, Humaidan P and Garcia-Velasco JA (2020) Self-Detection of the LH Surge in Urine After GnRH Agonist Trigger in IVF—How to Minimize Failure to Retrieve Oocytes. Front. Endocrinol. 11:221. doi: 10.3389/fendo.2020.00221

INTRODUCTION

In clinical practice, the total number of oocytes retrieved usually differs from the number of mature follicles observed on ultrasound as not all growing follicles will generate mature oocytes. Thus, it is not uncommon in IVF not to retrieve the same number of oocytes as mature follicles observed on ultrasound.

However, only in a few cases, there is a complete failure to retrieve oocytes after hCG as well as the GnRHa trigger (1, 2). In the case of a GnRHa trigger, failure to retrieve mature oocytes should not always be classified as an Empty Follicle Syndrome (EFS) (3), as the pathophysiology is likely to be different. Based on the available literature, some authors do not support the existence of the EFS, considering the "syndrome" due to errors in the administration of the trigger. Moreover, EFS is defined as a sporadic event in patients with adequate ovarian stimulation and final oocyte maturation trigger, correctly administered (4). Failure to retrieve mature oocytes or cases with a high immaturity rate (2, 5) have been described in 1% of IVF oocyte retrievals (2, 6–8). Even though failure to retrieve oocytes after GnRH agonist triggering is relatively uncommon, there is a need to prevent the occurrence –a frustrating experience for both patients and physicians.

After the final oocyte maturation trigger with a bolus of GnRHa, the subsequent LH surge induced should determine the correct response of the patient to the subcutaneous GnRHa injection. Even more, it could allow the identification of patients in whom pituitary dysfunction might induce an insufficient endogenous LH surge, not enough to maturation of 70% of the oocytes. As a result of increasing worldwide use of GnRHa trigger for final oocyte maturation, primarily to avoid OHSS (9–11), the measurement of serum LH post-trigger has been suggested to prevent EFS. Unfortunately, a consensus on optimal serum LH threshold levels after the trigger has not yet been reached (12–14). Kummer et al. (8) detected low LH values as <15 mIU/ml 10 h after trigger in seven cases of EFS among 508 patients triggered with GnRHa. However, others used different thresholds (6). Interestingly, Shapiro et al. (15) demonstrated a dramatic reduction in the numbers and maturity rates of oocytes when serum LH values 12 h after the trigger was lower than 12 mUI/ml. After the GnRHa trigger, the subsequent LH peak lasting for ∼28–32 h can be measured not only in serum but also in urine, using the same type of urine strips, that are currently used to detect the LH surge during the natural cycle (16).

In our oocyte donation program, the GnRHa trigger is routinely used, and during the years a few donor cycles resulted in an insufficient number of MII oocytes retrieved, even after a reassuring final ultrasound examination with good hormonal profile at the time of trigger. These cases are exceptional and unpredictable. Therefore, detecting the LH surge could be important to avoid failed oocyte retrieval in the event of error in the trigger administration, or an insufficient pituitary response to the GnRHa trigger. This prompted many clinics to recommend donors to have a serum measurement in the fertility clinic 12 h after the GnRHa trigger. However, urine LH measurement performed at home should predict the correct response to the GnRHa trigger, and if needed, the administration of a rescue bolus of hCG (re-trigger) and re-scheduling could avoid EFS or a low follicle mature oocyte ratio (17, 18).

The urinary LH test was firstly used in the context of intrauterine insemination (IUI), as 22% of patients exhibited a premature LH rise (19). In cycles where clomiphene citrate was used, up to 30% premature LH surges were reported, and up to 42% in rFSH cycles (20). Urinary LH testing helped to time the insemination in IUI cycles, thus, when it was performed between 18 and 53 h after a positive urine LH test, reasonable results in terms of live birth rate were obtained (21).

The present study aimed to explore whether the routine use of urine LH testing in oocyte donors 12 h after GnRHa trigger for final oocyte maturation would correctly detect an insufficient LH surge. Moreover, we have tried to identify patients that required a rescue hCG bolus to avoid failed oocyte retrieval.

MATERIALS AND METHODS

We performed a prospective cohort study, enrolling a total of 371 oocyte donors from May 2017 until November 2018. The study was approved by our Institutional Review Board (1701-MAD-006-JG) in compliance with the provision of the Spanish law on Assisted Reproductive Technologies (14/2006).

Oocyte Donors

Oocyte donors were healthy women aged 18–35 years with regular menstrual cycles, no hereditary or chromosomal diseases, who had normal karyotype, body mass index (BMI) of 18–29 kg/m^2, no more than two previous miscarriages, no gynecological or medical disorders, and a negative screening result for sexually transmitted diseases (19). Inclusion in the oocyte donor program also required the donor to have at least six antral follicles per ovary at the beginning of the cycle, and at least 8 follicles larger than 14 mm on the day of ovulation induction. Donor who had polycystic ovary syndrome based on the Rotterdam criteria (20) or sonographically visible endometriosis were excluded. Finally, donors were required to have a smartphone with WhatsApp (WhatsApp Ireland Limited, Dublin, Ireland) available to enter the trial.

Ovarian Stimulation

For planning purposes, an oral contraceptive pill (Microgynon®, levonorgestrel 0.15 mg/ethinylestradiol 0.03 mg, Bayer Hispania, Spain) was administered between 12 and 16 days prior to ovarian stimulation, starting on day 1 or 2 of the menses of the previous cycle. Following a 5-day wash-out period after the last pill, donors started the ovarian stimulation protocol. Donors received daily doses of 150/225 IU of recFSH (Gonal-f®; Merck Serono, Spain) depending on body mass index (BMI) and antral follicle count. A daily dose of 0.25 mg GnRH antagonist (Cetrotide®; Merck Serono, Spain) was introduced when one follicle reached a mean diameter of 13 mm. A single dose of 0.2 mg GnRHa (Decapeptyl®; Ipsen Pharma, Spain) was administered to trigger final oocyte maturation when at least three follicles reached a mean diameter of 17–18 mm. The triggering bolus of GnRHa was self-administered by the donor. To avoid failure to comply prior to treatment all donors received training in a practical nurse-led workshop related to drug preparation and administration; moreover, donors received instructions as to how to download a video available on the clinic's website with a step-by-step drug instruction. As part of the general practice of the clinic, oocyte pick-up was performed under sedation 36 h after GnRHa trigger.

Urine LH Testing

The LH test was performed at home in urine, deriving from the first micturition in the morning, 12 h after the GnRHa trigger. Donors were instructed how to self-administer the GnRHa trigger bolus 36 h prior to oocyte retrieval and to perform the urine LH test the following morning during the first micturition. All donors received the LH urine test from the clinic (LH test, Lab Ruedafarma, Spain). With the instructions, donors were also provided with an emergency phone number for further instructions if they encountered any problems.

The lower sensitivity of the urine LH test mentioned is 25 mIU/mL. To perform the test, donors had to collect the urine in a plastic container and then remove the strip from the protective wrapper. The strip was immersed in the urine sample for 5 s, after which it was removed and placed on a clean, dry, non-absorbent surface. Importantly, donors had previously been thoroughly instructed on the possible outcomes of the test; specifically, in case of a positive result, resulting in two purple lines on the strip. The results were available within 40 s to 10 min.

Pictures of LH Tests

Donors were instructed to send a cell phone picture of the LH test to the nurse via WhatsApp (WhatsApp Ireland Limited, Dublin, Ireland) application for smartphone as soon as the test was performed. In the case of a positive LH test, donors underwent oocyte retrieval the following morning as scheduled. In case of a negative result, the donor was asked to visit the clinic the same day to have a serum LH and progesterone measurement. To evaluate the validity of the test, the false positive rate was calculated by false positive/(true negative + false positive), and the false-negative by false negative/ (true positive +false negative).

Serum LH Analyses in LH Negative Urine Tested Patients

Serum LH levels were analyzed in patients with a negative urine test only, using an automated electro-chemiluminescent immunoassay system (Roche Cobas e411 analyzer). The sensitivity for LH was 0.1 mIU/ml. Donors with no serum LH surge defined as LH <15 mIU/ml (12) at 12 h after injection and with more than 19 ovarian follicles >11 mm were given in the clinic by a nurse a dual trigger consisting of a bolus of GnRHa (0.2 ml Decapeptyl) and hCG, 1000 IU (Ovitrelle, Merck Serono) in order to minimize the risk of OHSS development; oocyte retrieval was subsequently performed 36 h after re-trigger. In donors with <19 follicles >11 mm, hCG 6500 UI (Ovitrelle, Merck Serono) was administered. Patients with a negative urine test, regardless of their serum LH levels, continued with GnRH antagonist the day of the serum LH testing until the new trigger was administered.

Cost Analysis

The cost analysis was performed from a patient perspective and was limited to costs from LH testing to oocyte retrieval. Direct non-medical costs (travel costs) data were based on a previous review (22). The theoretical cost if every patient had come in for serum LH testing, including indirect costs such a travel time and income loss was compared against the cost of a home urine LH kit. For the calculations, we considered serum LH cost 30€ and the urine LH kit cost 0,5€.

RESULTS

Three hundred and seventy one donors were enrolled in the study, and 12 donors were excluded as they did not perform urine LH testing as instructed, leaving 359 donors included in the analysis (**Table 1**). Three donors did not forward the picture to confirm the test results, in four donors the result was unreliable due to the lack of a control line to confirm the correct execution of the LH urine test, and five donors forgot to perform the urine test (**Figure 1**).

Finally, a total of 359 oocyte donors and 359 oocyte retrievals were included in the present analysis. 356 donors (99.16 %) had a positive urine LH test prior to oocyte retrieval. In one donor, the urine LH test was positive (1/356, 0.28 % false-negative rate), however, no oocytes were retrieved. Three urine LH tests were negative. One was false-negative in fact LH was tested in blood the same morning, confirming an LH rise corresponding to 18.6 mUI/mL consistent with an adequate response to the GnRHa trigger bolus. In the other two cases, after the negative urine LH test, serum LH testing in the clinic revealed a serum LH level <15 mUI/mL, resulting in re-scheduling with an hCG trigger, oocyte retrieval after 36 h and the retrieval of 19 and 22 MII oocytes, respectively. **Table 2** summarizes the general characteristics of the four oocyte donors who experienced either false positive or false negative urine LH testing. The false-positive rate was 33% (1/3) and the false-negative rate was 0.28% (1/356), with a sensitivity of 99.7% (355/356), and specificity of 66.7% (2/3) (**Table 3**). Regarding the cost analysis, and considering the cost of the urine LH kit (0,5€ per sample) and the cost of serum LH testing (30€ per sample) in addition to an estimate of the cost of visiting the clinic (10€ round trip), it would be a significantly cost-saving strategy, as blood testing would have costed 14,840€ vs. only 185,5€ in urine LH kits.

TABLE 1 | General demographics of the oocyte donor population included in the study.

Patients	359
Age (years)	25.9 ± 1.4
BMI (kg/m^2)	23.1 ± 1.2
Antral follicular count	18.3 ± 5.1
Duration of stimulation (days)	12.1 ± 1.4
Total gonadotrophin dose (IU)	1,861 ± 689
Estradiol (pg/mL)	3,105 ± 664
Number of oocytes retrieved	19.2 ± 4.9
Number of MII oocyte	13.7 ± 2.6

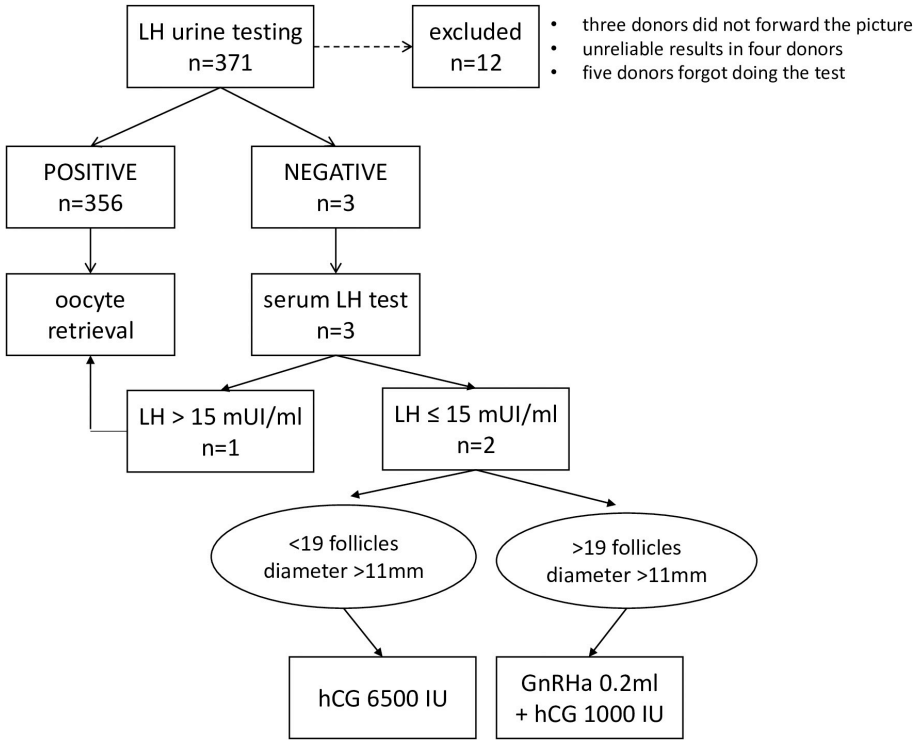

FIGURE 1 | Flow chart according to LH urine testing results.

TABLE 2 | General characteristics of the patients with abnormal results in the urine LH test or failed oocyte retrieval after a positive LH test in urine.

Patients	BMI (kg/m^2)	Duration of stimulation (days)	Total gonadotrophin dose (IU)	Urine LH test	Serum LH (mIU/mL)	Progesterone (ng/mL)	Estradiol (pg/mL)	N°Follicles >11 mm	Re-trigger	# oocytes retrieved	# MII
1	20.2	10	1,500	+−	18.6	27	1,280	27		22	18
2	28.2	11	2,225	−	7.3	14	1,403	25	hCG+GnRHa	24	19
3	25.3	13	2,925	+++			2,380	20		0	0
4	21.4	11	2,000	−	12.1	20.2	3,423	36	hCG+GnRHa	32	22

+++, positive; −, negative; +−, slightly positive.

DISCUSSION

In this prospective cohort study performed in a total of 359 oocyte donors triggered with a bolus of GnRHa for final oocyte maturation, was explored the predictability of urine LH self-testing 12 h after trigger to avoid failure of oocyte retrieval. In 356 patients (99%) the LH urine assay was positive, leading to oocyte retrieval with normal mature oocyte ratio. One patient had a false-positive result, with a positive urinary LH test but no oocytes were retrieved. In three patients LH testing in urine was negative, however, only two patients had serum LH levels requiring re-trigger and re-scheduling.

This new approach of self-testing of the LH surge in urine and communication/confirmation via cell phone photo provides a simple, safe, convenient, and economical method to confirm that the LH surge was adequately induced by the GnRHa trigger. This model could be used in all patients undergoing oocyte retrieval after a GnRHa trigger, helping to detect errors in the administration and/or inadequate responses to the trigger, improving patient compliance and minimizing failure to retrieve oocytes in IVF.

The use of GnRHa for triggering of final oocyte maturation is the new paradigm as a safer alternative to hCG to prevent OHSS. The mechanism by which the GnRHa trigger is effective seems to be dependent solely on the resulting LH activity (12). After the administration of GnRHa, the immediate LH surge is followed by receptor down-regulation (23). Although data has demonstrated efficacy and safety of GnRHa trigger, the administration may not always result in an adequate oocyte yield in a subset of patients (6, 8, 12, 14). As a consequence of an inadequate action, fewer oocytes may be retrieved than expected from the number of mature follicles visible on the day of triggering (14). Failure

TABLE 3 | Summary of parameters of urinary LH test: true-positive rate (sensitivity) 355/356 = 99.7%; false-positive rates (1-specificity) 1/3 = 33.3%; false-negative rate (type 2 error) 1/356 = 0.28%; specificity 2/3 = 66.7%.

	Serum LH>15 IU/l (or oocytes retrieval uneventful)	Serum LH<15 IU/L (or no oocytes retrived)	
Urine LH	TP	FP	356
+	355	1	
Urine LH	FN	TN	3
–	1	2	
	356	3	359

TN, true negative; FN, false negative; FP, false positive; TP, true positive.

to retrieve oocytes is a rare and frustrating event that results in cycle cancellation. The complete failure to retrieve oocytes when final oocyte maturation trigger is administered correctly –either using GnRHa or hCG- is considered a "genuine" EFS, whereas "false" EFS is a condition of failed oocyte retrieval when the trigger was not administered correctly (2, 9). Although the exact origin of the failure to retrieve oocytes remains elusive, dysfunctional foliculogénesis in older patients has been suggested as a cause, probably secondary to altered apoptotic inhibition of the granulosa cell (24). Furthermore, the age of the patient has been reported to be an important factor to increase the recurrence of EFS after hCG trigger, considering that between the age of 35–39 years the recurrence rate was 24%, reaching 57% for patients above 40 years (25). The debate on the causes of retrieval failure in the young population, excluding errors of administration, remains open. However, among the possible causes, molecular mechanisms underlying slow or insufficient follicular cell response to the LH stimulus is not yet understood. However, it could involve LH receptor polymorphisms, the presence of variant LHβ or the in-efficiency of post-receptor signal-transduction pathways (2).

In young patients triggered with GnRHa, a 'borderline' hypothalamic-pituitary dysfunction with levels of gonadotropins above the hypogonadotropic/hypogonadal level was hypothesized (2, 8). A temporary state of hyposensitivity of the pituitary gland could also explain EFS. Low LH release after the trigger in borderline hypogonadotropic/hypogonadal patients would allow follicular development, and initial luteinization of the follicle; however, it would be insufficient for the completion of cumulus expansion and detachment from the follicular wall (6).

Interestingly, Christopoulos et al. (17) reported six EFS patients after GnRHa trigger, having a mean age of 32.6 years, a mean antral follicle count of 27 and a mean basal FSH level of 4.4 UI/l, rather far from the LH value defining the hypogonadotropic hypogonadal patient.

Although the reasons for failed oocyte retrieval in healthy and older patients may be different, the diagnosis and treatment are still the same. Urinary LH test could be helpful for patients undergoing IVF treatment using GnRHa trigger. The use of urine LH test in oocyte donors has several important advantages, allowing to mitigate the risk of failure at oocyte retrieval, making the whole donation process more efficient, eliminating unnecessary costs for the clinic, avoiding blood testing in the clinic, and reducing psychological stress from cycle cancellation, not only for the donor but also for the recipient.

The strategy to schedule a re-trigger after failed oocyte aspiration in six or seven mature follicles has been proposed as a more efficient and cost-effective strategy compared to laboratory analysis alone (26). Rather than routine screening strategy, maybe we could consider identifying risk factors –such as low initial LH levels suggesting hypothalamic hypofunction- for an inadequate response as more useful in a clinical setting. However, this strategy might increase the costs, unless hormonal screening is performed routinely at the initiation of an IVF cycle, as in some academic programs. There are two limiting factors for this novel approach: firstly, urine LH test have not the same sensitivity, so universal application should be suggested with caution; secondly, the need to rely on patients performing the urine test themselves, which leaves room for human error, emphasizing the need of training. However, our approach in which the result of the test was confirmed by a trained nurse via a cell phone picture reduces the risk of error.

Given the low number of patients who did not respond with an LH surge ($n = 3$), the specificity of testing was understandably low at 66.7%, but the sensitivity was 99.7%. Urinary LH test could be a optimal test to confirm the LH surge in those patients triggered with GnRHa, and reasonably good for those patients without LH surge after triggering. Considering the low incidence of failed LH surges after GnRHa trigger, probably testing in blood might be more precise, but the inconvenience of traveling to the clinic one more day during the treatment cycle in addition to the costs make urine LH self-testing a very cost-effective test. In fact, as shown, it would be a significant cost-saving strategy, as blood testing would have costed 14,840€ vs. only 185,50€ in urine LH kits. Also, the strategy to schedule a re-trigger after failed oocyte aspiration once six or seven mature follicles have been aspirated could be interesting in terms of cost savings. However, patients going by through reiterated oocyte retrievals are subjected to psychological and emotional stress.

The main limitation of our study is that it was conducted in oocyte donors, most of whom presumably have a well-functioning pituitary-ovarian axis. It is unknown whether these results would translate to older women in general IVF practice. Also, the lack of a control group where no testing was performed represented a limitation of the study, but difficult to justify in a real-life clinical setting.

The use of a home urinary LH assay kit to detect ovulation in a GnRHa triggered cycle has several advantages over blood LH testing. Although IVF has increased worldwide, access to fertility care remains restricted for many patients. Increased attention to developing low-cost, effective, and accessible treatment alternatives must be prioritized (27). In this sense, the use of urine LH assay could reduce the stress and costs of fertility care program (27). In a randomized controlled study in patients undergoing donor IUI that used urinary LH assay, was observed a significant reduction in the number of visits to the clinic per cycle, without a significant impact on monthly fecundity rate or cumulative conception rates compared to serum LH assay (28). Also, the use of a urinary LH test could detect patients without

LH surge after a GnRHa trigger that could benefit from a "rescue trigger" with a low-dose hCG.

In conclusion, this study validated the efficacy of LH urine surge testing after the GnRHa trigger in oocyte donors. A total of 99% of donors had a positive LH urine surge 12 h after the trigger, resulting in good oocyte retrieval rates, and, importantly, false positive and false negative rates of urine testing were very low; importantly, this novel concept used after GnRHa trigger allowed the identification of donors who would benefit from a re-trigger. Urinary LH surge testing is easy, safe, convenient, and reliable. Thus, this approach may constitute an alternative for clinicians and patients to minimize the failure of oocyte retrieval. Patients can safely perform the test at home, take a cell phone picture of the stick and communicate directly with the clinic without having to spend time for a blood sampling. Only in the few patients with a negative result in urine, an additional blood sample is needed to plan the continued handling of the patient; thus, this concept fulfills the demands of modern IVF in terms of safety, efficacy and patient friendliness.

DATA AVAILABILITY STATEMENT

The datasets analyzed in this article are not publicly available because the data contains identifiable information. Requests to access the datasets should be directed to Dr. Mauro Cozzolino, mauro.cozzolino@ivirma.com.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Hospital Puerta de Hierro. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MC wrote the first and final drafts of the manuscript, performed the statistical analysis, interpreted results, and the literature search. SM, AA, MT, VL, and MPe were responsible of data setting and result collection. EH and MPi recruit the patients included in the study. PH designed the study and critically revised the manuscript. JG-V designed the study, interpreted results and critically revised the manuscript in the first and final drafts.

FUNDING

This study was funded by IVI RMA Madrid, Spain.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling Editor declared a past co-authorship with the author PH.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 15 May 2020
doi: 10.3389/fendo.2020.00307

Inhibin A—A Promising Predictive Parameter for Determination of Final Oocyte Maturation in Ovarian Stimulation for IVF/ICSI

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: 25 February 2020
Accepted: 22 April 2020
Published: 15 May 2020

Citation:
Lawrenz B, Depret Bixio L, Coughlan C, Andersen CY, Melado L, Kalra B, Savjani G, Fatemi HM and Kumar A (2020) Inhibin A—A Promising Predictive Parameter for Determination of Final Oocyte Maturation in Ovarian Stimulation for IVF/ICSI. Front. Endocrinol. 11:307. doi: 10.3389/fendo.2020.00307

The number of mature oocytes is a key factor in the success of Assisted Reproductive Techniques (ART). Exogenous gonadotropins are administered during ovarian stimulation in order to maximize the number of oocytes available for fertilization. During stimulation, monitoring is mandatory to evaluate individual response, to avoid treatment complications and assist in the determination of the optimal day for final oocyte maturation and oocyte retrieval. Routine monitoring during stimulation includes transvaginal ultrasound examinations and measurement of serum estradiol (E2). Due to multifollicular growth of follicles of varying size, serum E2 levels are commonly supraphysiological and often variable, rendering E2-measurement during ovarian stimulation unreliable as a determinant of oocyte maturity. In contrast to serum E2, serum Inhibin A levels increase once a minimum follicle size of 12–15 mm is achieved. Due to this fact, serum Inhibin A levels could present in combination with ultrasound monitoring a more reliable parameter to determine the optimal follicle size for final oocyte maturation, as only follicles with a size of 12 mm and beyond will contribute to the serum Inhibin A level. This prospective observational, cross-sectional study demonstrates, that on the day of final oocyte maturation serum Inhibin A is strongly correlated to the number of follicles ≥15 mm (0.72) and to the number of retrieved and mature oocytes (ρ 0.82/0.77, respectively), whereas serum E2 is moderately correlated to the parameters mentioned above (ρ 0.64/0.69/0.69, respectively). With an area under the curve (AUC) of 0.91 for Inhibin A, compared to an AUC of 0.84 for E2, Inhibin A can be regarded as a better predictor for the optimal timing of trigger medication with a threshold number of ≥10 mature oocytes. It can be concluded from this data that serum Inhibin A in combination with transvaginal ultrasound monitoring may be a more powerful tool in the decision making process on trigger timing as compared to E2.

Keywords: Inhibin A, Inhibin B, estradiol, ovarian stimulation, oocyte maturity

INTRODUCTION

The number of retrieved oocytes is critical to the success of IVF treatment. As a result, the aim of ovarian stimulation for IVF is to maximize the number of oocytes available for fertilization (1, 2). In order to achieve this goal, ovarian stimulation prior to IVF requires the administration of exogenous gonadotropins to support multi-follicular growth until the day of final oocyte maturation (3). Currently, ultrasonographic determination of the antral follicle count (AFC) and/or the measurement of Anti-Müllerian-Hormone (AMH) prior to stimulation start assist in the determination of the optimal gonadotropin dose to prescribe, the identification of patients at risk of developing ovarian hyperstimulation syndrome (OHSS) (4) or a low/no response during stimulation (5). Despite the assessment of these parameters as an indicator for the expected treatment response prior to cycle initiation, close monitoring of individual response to ovarian stimulation is mandatory to avoid treatment complications, facilitate individualization of treatment and assist in the determination of the optimal day for final oocyte maturation and oocyte retrieval.

Routine monitoring of ovarian stimulation for IVF/ICSI includes transvaginal ultrasound examinations (TVUS) and measurement of serum estradiol (E2) (6). With TVUS, ovarian response to gonadotropin administration is monitored by recording the size and number of developing antral follicles and serum E2 levels that reflect the collective hormonal capacity of the follicles.

In a natural cycle, aromatase activity begins to increase on cycle day 5–8 in follicles larger than 8 mm (7, 8). Upon selection, the dominant follicle in a natural cycle produces more E2 than the other follicles and the E2 level increases with the increasing size of the dominant follicle.

Whereas, in a natural cycle the serum E2 level can give an indication of follicle size and the maturation process of the oocyte, serum E2 levels in ovarian stimulation cycles are supraphysiological and often variable due to growth of multiple follicles of varying size. Therefore, E2-measurement is not reliable in ovarian stimulation cycles as the sole determinant when choosing the optimal time for administration of trigger medication. In contrast to E2, Inhibin A levels increase from a follicle size of 12–15 mm and beyond (9–11). As only follicles from these sizes onwards will contribute to the serum Inhibin A levels, the combination of serum Inhibin A measurement and TVUS could present a more reliable parameter for determining the optimal timing for administration of final oocyte medication, as compared to serum E2 plus TVUS.

Previous publications have described Inhibin A levels during stimulation (11) and correlated these levels with ART outcome (12). To date the potential of serum Inhibin A as a decision-making tool in determining the optimal timing of final oocyte maturation has never been studied.

Therefore, the aim of this prospective observational, cross-sectional study was to evaluate the role of serum Inhibin A as predictor of the number of retrieved and mature oocytes as compared to serial measurements of E2 in a routine setting of a private IVF center and to define a cut-off level of serum Inhibin A above which retrieval of $\geq$10 mature oocytes is likely. This study will determine if serum Inhibin A could potentially serve as decision making tool meriting inclusion of Inhibin A into routine cycle monitoring.

MATERIALS AND METHODS

This observational prospective, cross-sectional study was performed in IVIRMA Fertility Clinic, Abu Dhabi, UAE, between September 2018 and January 2019. All patients, independent of the AFC as a quantitative marker of the ovarian reserve, undergoing ovarian stimulation for IVF/ICSI due to primary or secondary infertility during this time period in a GnRH-antagonist-protocol and who consented to take part in this study, were included. Only one stimulation cycle was included from each patient.

On day 2 or 3 of the period of the planned treatment cycle, prior to initiation of stimulation, a vaginal ultrasound was performed to determine the AFC. All follicles with a diameter between 2 and 10 mm in each ovary were recorded and the numbers added to determine the total AFC-count (13). In keeping with routine clinical practice patients were monitored during ovarian stimulation for IVF/ICSI treatment with serial transvaginal ultrasound examinations. Transvaginal scans were performed using a Voluson 6 (GE Healthcare, Milwaukee, WI, USA) ultrasound machine, equipped with a 7–10 MHz two-dimensional transvaginal probe. The patients were asked to empty their bladders and were placed in the lithotomy position.

During ovarian stimulation, patients were seen two or three times for ultrasound monitoring of follicular response and serum hormonal measurement in accordance with ovarian response.

Follicle size and number was determined during the course of stimulation and on the day of final oocyte maturation by vaginal ultrasound, as previously described. Follicle diameter was determined by measuring two orthogonal diameters and the mean value was recorded as follicle size.

Blood samples for this study were taken in addition to routine hormonal measurements (FSH, LH, E2 and progesterone), used for stimulation monitoring, on cycle day 2 or 3 prior to initiation of stimulation and on the day of final oocyte maturation. The blood was centrifuged for 10 min at 2,688 g (relative centrifugal force) per minute and the supernatant was retrieved and frozen at −21°C. For the measurement of serum levels of E2, Inhibin A and Inhibin B, the samples were thawed by keeping them for maximum 90 min at room temperature (∼20–24°C) and analyzed the same day with the same batch of reagents.

The following demographic data per patient were recorded: age, Body Mass Index (BMI), number of infertility years and number of previous stimulations. On the day of final oocyte maturation, the total number of follicles, the number of follicles $<$15 and $\geq$15 mm and the cycle day were registered. Moreover, the number of stimulation days, the total gonadotropin dose

required and the number of retrieved and mature oocytes were recorded.

Ovarian Stimulation Protocols

Ovarian stimulations were performed in Gonadotropin-Releasing-Hormone (GnRH)-antagonist-protocols, using recFSH (recombinant Follicle-stimulating-hormone) or human-Menopausal-Gonadotropin (HMG) as stimulation medication. Stimulation medication dosage was individualized prior to stimulation start in accordance with the quantitative parameters, reflecting ovarian reserve (14). During ovarian stimulation, medication dose was adjusted in line with ovarian response as determined by ultrasound scan findings and measured serum levels of E2 and progesterone (P4). In order to avoid P4 elevation during the late follicular phase a reduction in medication dose may have been warranted as determined by serial monitoring of progesterone levels (15). Final oocyte maturation was achieved by administration of either 10.000 IU of hCG, 0.3 mg of GnRH agonist (Triptorelin) or dual trigger (hCG and GnRH-analog), as soon as $\geq$3 follicles $\geq$17 mm were present, depending on the ovarian response and clinician discretion. In low responders with <3 growing follicles, medication for final oocyte maturation was given when at least 1 follicle of $\geq$17 mm was present. Oocyte retrieval was carried out 36 h after administration of the trigger.

Ultrasound findings and serum E2 levels formed the basis on which decisions were taken regarding cycle monitoring and trigger timing. The blood samples, obtained for study-purposes, were later analyzed for serum levels of Inhibin A, B and again for E2 in order to avoid bias through batch-to-batch inconsistencies for the herein presented analysis.

Hormonal Analysis

Inhibin A Analysis

Commercially available hypersensitive and specific immunoassays from Ansh Lab, Texas, USA were used to detect levels of Inhibin-A (AL-123). All samples were run neat in Inhibin A ELISA. Samples reading higher than the highest Calibrator in the assay were diluted 1:10 in calibrator A/sample diluent of the kit and re-assayed. The coefficient of variation for the Inhibin A assay over 10 assay runs for two kit controls at 105 and 348 pg/mL and two serum controls at 47 and 135 pg/mL was 4.7, 3.4, 4.6, and 3.9%, respectively. The calibrators in the Inhibin A ELISA are traceable to WHO reference preparation (WHO 91/624). The traceability factor is reported as slope of observed WHO preparation w.r.t. known concentration when analyzed in the ELISA. Inhibin A = 1.68 (WHO 91/624). The assay is specific to Inhibin A and does not crossreact with closely related analytes such as Inhibin B, Activin A, Activin B, Activin AB, FST-315, and FSTL3 when spiked at 50 ng/mL in analyte free matrix.

The Inhibin A in serum is stable up to 3 freeze thaw cycles. The assay is designed to measure mature Inhibin A and does not detect Activin A and Inhibin alpha fragments.

Estradiol Analysis

Estradiol measurements were performed using commercially available kits from DRG International (EIA-2693, DRG ELISA). All samples were run neat. Samples reading higher than that Calibrator F in the assay were diluted 1:10 in the calibrator A/sample diluent of the kit and re-assayed. The coefficient of variation for the E2 assay over 10 assay runs for two spiked E2 controls at 111 and 423 pg/mL and one serum control at 796 pg/mL was 9.8, 3.9, and 6.3, respectively.

Inhibin B Analysis

Commercially available hypersensitive and specific Inhibin B immunoassay (AL-107) from Ansh Lab, Texas, USA were used to detect Inhibin-B levels in serum. All samples were run neat in inhibin B ELISA. Samples reading higher than the highest Calibrator in the assays were diluted 1:10 in calibrator A/sample diluent of the kit and re-assayed. The coefficient of variation for the inhibin B assay over 12 assay runs for two kit controls at 126 and 345 pg/mL and two serum controls at 87 and 218 pg/mL was 2.0, 2.3, 3.8, and 4.0%, respectively. The calibrators in the Inhibin B ELISA are traceable to WHO reference preparation. Inhibin B = 0.4 (WHO 96/784, the WHO preparation is a mixture of Inhibin A, Inhibin B, and Inhibin alpha). The assay is specific to Inhibin B and does not cross-react with closely related analytes such as Inhibin A, Activin A, Activin B, Activin AB, AMH, FST-315, and FSTL3 when spiked at 50 ng/mL in analyte free matrix.

The Inhibin B in serum is stable up to 3 freeze thaw cycles. The assay is designed to measure mature Inhibin B and does not detect Activin B and Inhibin alpha fragments.

Statistical Analysis

Continous data are presented as mean ± SD, 95%CI, minimum and maximum values when appropriate. The assumption of normality was checked using a proc univariate. Pearson's Fisher Z-Transformation test (ρ) was used to test the strength of the correlation between Inhibin A, Inhibin B, E2 serum levels, and different variables at given cycle times. One way ANOVA was used to analyze mean changes on serum levels of Inhibin A and B, E2, and number of follicles, number of retrieved and mature ocytes as category variables. Logistic regression was used to find the Area Under the Curve (AUC) to determine the capacity of Inhibin A and E2 serum levels to predict mature oocytes. ROC curve analysis was performed, using SAS studio™ software, with Inhibin A and E2 as the classifier to predict oocyte maturity. AUC and 95% confidence intervals around the AUC were computed.

To find the optimal treshold for serum Inhibin A level to predict mature oocytes, the Youden index was calculated (Youden index = sensitivity + specificity −1). For this analysis a cut-off of $\geq$10 mature oocytes was applied. All analyses were performed using SAS studio (Copyright © 2018 SAS Institute Inc., Cary, NC, USA.). For interpretation of the results, a $p < 0.05$ was considered to be statistically significant.

Interpretation of Pearson coefficient was done according to Schober et al. (16):

0.00–0.10 = Negligible correlation
0.10–0.39 = Weak correlation
0.40–0.69 = Moderate correlation
0.70–0.89 = Strong correlation
0.90–1.00 = Very strong correlation

TABLE 1 | Patients' characteristics and results of AFC, serum levels of Inhibin A, E2, and Inhibin B on cycle day 2 or 3.

Parameter	Number of samples	Mean	*SD*	95%CI mean		Min	Max
				Lower bound	Upper bound		
Infertility years	145	3.9	3.0	3.4	4.4	0	16
Previous stimulations	145	3.1	4.2	2.5	3.8	0	26
Age (years)	145	35.4	6.5	34.2	36.3	21	48
BMI (kg/m^2)	145	28.2	4.8	27.4	28.9	17.5	41.7
AFC (n)	145	12.2	7.3	11.0	13.4	0	30
Inhibin A (pg/ml)	145	7.7	3.9	7.0	8.3	4.9	24.7
E2 (pg/ml)	145	63.2	28.4	58.5	67.8	17.2	219.6
Inhibin B (pg/ml)	145	89.2	65.6	78.5	100.0	1.60	503.0

BMI, body mass index; E2, estradiol; SD, standard deviation.

For interpretation of the results, a $p < 0.05$ was considered to be statistically significant.

Interpretation of ROC curve was done according to Li and He (17):

0.90–1 = Excellent
0.80–0.90 = Good
0.70–0.80 = Fair
0.60–0.70 = Poor
0.50–0.60 = Fail.

Ethical Approval and Trial Registration Number

This study was approved by the ethics committee of the IVIRMA Abu Dhabi Fertility Clinic, Abu Dhabi, UAE (approval number: REFA019) and was registered with clinicaltrials.gov. under the number NCT03607409.

RESULTS

Results of blood samples and parameters from the ovarian stimulation treatment for IVF/ICSI were available from a total of 145 patients at the start of ovarian stimulation (cycle day 2/3) and from 136 patients on the day of final oocyte maturation. From nine patients there were no recorded follicle measurements and no blood test results available on the day of final oocyte maturation due to cycle cancellation for various reasons (no/low response, patient's decision to cancel treatment due to personal reasons).

The mean age (±SD) of patients evaluated was 35.4 ± 6.5 years and mean BMI was 28.2 ± 4.8 kg/m^2 with a history of 3.9 ± 3.0 years of infertility and a mean number of 3.1 ± 4.2 previous stimulations. Patients' characteristics, AFC, the serum levels of Inhibin A, E2, and Inhibin B on cycle day 2 or 3, are summarized in **Table 1**.

A correlation test was applied in order to obtain the probability values (*p*-value) and the Pearson's coefficient (ρ). For the probability values, a statistically highly significant correlation was found between AFC and Inhibin B ($p < 0.0001$) and statistically no significant correlation between AFC and Inhibin A/E2 (*p*-value 0.16/0.41, respectively). The Pearson's coefficient (ρ) was moderate between AFC and Inhibin B (ρ 0.40; CI95% [0.26; 0.53]), weak/negligible between AFC and Inhibin A/E2 (ρ 0.12; CI95% [-0.04; 0.27]/−0.07; CI95% [−0.22; 0.09]), respectively.

On the day of final oocyte maturation, blood samples for Inhibin A, E2, and Inhibin B measurement were available from 136 patients. The results of hormonal measurements as well of the stimulation parameters and outcomes are listed in **Table 2**.

All ultrasonographic stimulation parameters (total number of follicles, number of follicles < 15 mm, number of follicles ≥15 mm) showed a statistically highly significant correlation with hormonal serum levels of Inhibin A, E2, and Inhibin B ($p < 0.0001$). A strong correlation was found with Pearson's coefficient between the total number of follicles and serum levels of Inhibin A, E2, and Inhibin B (0.78; CI95% [0.70; 0.84]/0.71; CI95% [0.63; 0.79]/0.73; CI95% [0.64; 0.80], respectively) and between the number of follicles ≥15 mm and Inhibin A (0.72; CI95% [0.62; 0.79]). **Table 3** lists the correlations.

The stimulation parameters on the day of final oocyte maturation (total gonadotropin dosage, number of retrieved and mature oocytes) were statistically highly significant ($p < 0.0001$) correlated with the results of serum levels of Inhibin A, E2, and Inhibin B. A strong correlation was found between Inhibin A and number of retrieved oocytes and mature oocytes (ρ 0.82; CI95% [0.75; 0.86]/ρ 0.77; CI95% [0.69; 0.83]), E2 showed a moderate correlation (ρ 0.69; CI95% [0.60; 0.77]/ρ 0.69; CI95% [0.59; 0.77]), and Inhibin B a strong/moderate correlation (ρ 0.71; CI95% [0.62; 0.78]/ρ 0.65; CI95% [0.54; 0.74]), respectively. The correlations of the stimulation parameters with Inhibin A, estradiol and Inhibin B are summarized in **Table 4** and **Figure 1** depicts the correlations between serum Inhibin A and E2 levels and the number of retrieved and mature oocytes.

A multivariate analysis was applied to evaluate the capacity of serum Inhibin A as a marker for retrieval of mature oocytes, controlling for age, and AFC on the day of final oocyte maturation. On the day of final oocyte maturation, serum Inhibin A level was found to be a predictive marker for the timing of final oocyte maturation ($p < 0.0001$) and was highly significantly correlated with the AFC ($p < 0.0001$), but not with age ($p = 0.6473$). The AUC for Inhibin A

TABLE 2 | Patients' characteristics and results of AFC, Inhibin A, estradiol, and Inhibin B on the day of final oocyte maturation.

Parameter	Number of samples	Mean	*SD*	95%CI mean		Min	Max
				Lower bound	Upper bound		
Infertility years (*n*)	136	3.89	3.02	3.38	4.40	0	16
Previous stimulations (*n*)	136	3.21	4.25	2.49	3.93	0	26
Age (years)	136	35.2	6.5	34.0	36.3	21	48
BMI (kg/m^2)	136	27.9	4.8	27.1	28.8	17.5	41.6
AFC (*n*)	136	12.3	7.4	11.0	13.5	0	30
Inhibin A (pg/ml)	136	827.7	632.9	720.4	935.1	10.6	3859.5
E2 (pg/ml)	136	2105.3	1588.8	1835.9	2374.7	82.9	9005.1
Inhibin B (pg/ml)	136	1706.6	1997.5	137.9	2045.4	17.6	13972.1
Total number of follicles	136	14.24	7.68	12.9	15.5	1	32
Number of follicles <15 mm	136	7.1	5.1	6.3	8.0	0	24
Number of follicles ≥15 mm	136	7.0	5.2	6.1	7.9	1	25
Cycle day of trigger	136	12.1	1.7	11.8	12.4	8	16
Stimulation days (*n*)	136	10.1	1.6	9.8	10.4	6	14
P4 trigger day (ng/ml)	136	0.6	0.4	0.5	0.7	0.05	2.2
Total dosage gonadotropins (IU)	136	3363.7	1218.0	3157.2	3570.3	1125.0	6150.0
Number of retrieved oocytes	136	12.0	9.0	10.5	13.5	0	40
Number of mature oocytes	136	9.1	7.1	7.9	10.3	0	32

SD, standard deviation; CI, confidence interval; BMI, body mass index; AFC, antral follicle count; E2, estradiol; P4, progesterone.

TABLE 3 | Correlations on the day of final oocyte maturation between total numbers of follicles/numbers of follicles <15 mm/numbers of follicles ≥15 mm and Inhibin A, estradiol, and Inhibin B.

Follicle number on the day of final oocyte maturation	Inhibin A	E2	Inhibin B
Total number of follicles	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
Pearson coefficient	0.78	0.71	0.73
	CI95% [0.70; 0.84]	CI95% [0.63; 0.79]	CI95% [0.64; 0.80]
Number of follicles <15 mm	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
Pearson coefficient	0.43186	0.40936	0.52855
	CI95% [0.28; 0.55]	CI95% [0.25; 0.54]	CI95% [0.39; 0.63]
Number of follicles ≥15 mm	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
Pearson coefficient	0.72	0.64	0.55138
	CI95% [0.62; 0.79]	CI95% [0.53; 0.73]	CI95% [0.42; 0.65]

E2, estradiol; CI95%, confidence interval.

as a predictor for ≥10 mature oocytes was ρ 0.91 (CI95% [0.87; 0.96]) and ρ 0.84 (CI95% [0.7769; 0.9124]) for E2. The AUCs for both parameters are presented in **Figure 2**. The threshold level of serum Inhibin A level to predict the number of mature oocytes ≥10 (with an equivalent ratio of Sensitivity and Specificity) was 668.1 pg/mL (sensitivity = 88.0%, specificity = 82.0%).

In order to describe the mean, the SD (standard deviation), the 95% Confidence Interval (95%CI) as well as the ranges of the serum levels of Inhibin A and E2 depending on the number of ultrasonographic visible follicles on the day of final oocyte maturation, the follicle numbers were divided into groups and the results are presented in **Supplementary Table 1**. **Supplementary Tables 2, 3** summarize the results of the descriptives of the serum levels of Inhibin A and E2, when analyzed according to the number of retrieved oocytes and mature oocytes.

DISCUSSION

To the best of our knowledge, this is the largest prospective observational, cross-sectional study, measuring the levels of Inhibin A in blood samples, obtained during ovarian stimulation for IVF/ICSI, in order to evaluate the efficacy of serum Inhibin A levels as a predictive parameter for the timing of final oocyte maturation in ovarian stimulation for IVF/ICSI, in comparison to serum E2 levels.

The findings of our study demonstrate, that on the day of final oocyte maturation Inhibin A is strongly correlated to the number of follicles ≥15 mm (ρ 0.72) and to the number of retrieved and mature oocytes (ρ 0.82/0.77, respectively), whereas E2 has only moderate correlations to the parameters mentioned (ρ 0.65/0.69/0.69, respectively). With an AUC of 0.91 for Inhibin A, compared to an AUC of 0.84 for E2, serum Inhibin A levels can be regarded as a better predictor of the optimal

TABLE 4 | Correlations on the day of final oocyte maturation between the stimulation parameters and Inhibin A, estradiol, and Inhibin B.

	Inhibin A	E2	Inhibin B
Cycle day (*n*)			
p-value	$p = 0.5933$	$p = 0.0949$	$p = 0.0137$
Pearson coefficient	0.04628	0.14381	−0.04921
	CI95% [−0.12; 0.21]	CI95% [−0.03; 0.30]	CI95% [−0.21; 0.12]
Number of stimulation days			
p-value	$p = 0.3056$	$p = 0.0466$	$p = 0.8333$
Pearson coefficient	0.08850	0.17095	−0.01821
	CI95% [−0.1; 0.25]	CI95% [0.0; 0.32]	CI95% [−0.18; 0.15]
P4 level			
p-value	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
Pearson coefficient	0.65165	0.62761	0.50450
	CI95% [0.54; 0.73]	CI95% [0.51; 0.71]	CI95% [0.36; 0.62]
Total gonadotropin dosage			
p-value	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
Pearson coefficient	−0.60	−0.47	−0.62
	CI95% [−0.69; −0.47]	CI95% [−0.60; −0.33]	CI95% [−0.72; −0.51]
Number of retrieved oocytes			
p-value	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
Pearson coefficient	0.82	0.69	0.71
	CI95% [0.75; 0.86]	CI95% [0.60; 0.77]	CI95% [0.62; 0.78]
Number of mature oocytes			
p-value	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
Pearson coefficient	0.77	0.69	0.65
	CI95% [0.69; 0.83]	CI95% [0.59; 0.77]	CI95% [0.54; 0.74]

E2: estradiol; P4, progesterone; CI95%, confidence interval.

time for administration of trigger medication with a number of ≥10 mature oocytes. As a consequence of these data it can be concluded, that serum levels of Inhibin A may represent in combination with TVUS a more accurate hormonal indicator for retrieval of mature oocytes and may be a more powerful tool in the decision making process on trigger timing as compared to serum levels of E2. Hence, it has to be stated that the clinical significance of this difference will have to be demonstrated in future studies.

Inhibin B, which is also used as a quantitative ovarian reserve parameter (18), was confirmed to be highly statistically significantly ($p < 0.0001$) correlated with AFC on cycle day 2 or 3 of the cycle confirming its role in assessing the existing follicle pool. Inhibin B levels were seen to increase during hormonal stimulation as a result of multifollicular growth, which is in keeping with previous reports (19). A moderate correlation was seen between Inhibin B, as well as between E2, and the number of mature oocytes. On this basis it can be concluded that the introduction of Inhibin B monitoring into routine clinical practice on the day of final oocyte maturation will not be of added benefit.

These findings do not concur with the study of Eldar-Geva et al. (20), who described a better correlation of Inhibin B with oocyte number, compared to Inhibin A and E2. The differences in the study findings may be explained by a larger sample size in our study (136 vs. 38) and a different patient population as our patients were older and had a higher BMI. Stimulation durations were approximately the same in both studies. Eldar-Geva et al. (20) stimulated either with a daily dosage of 100 or 200 IU, whereas in our population the mean stimulation dosage was 333 IU/day. Analyzing the differences in stimulation parameters in both studies, it can be assumed, that the patient population in the study of Eldar-Geva et al. (20) was predominantly composed of "good-prognosis" patients, whereas our study included patients undergoing ovarian stimulation for IVF/ICSI regardless of their ovarian reserve, a study population more closely reflecting "daily life" in an IVF center.

When serum levels of Inhibin B, E2, and Inhibin A were measured on day 5 of ovarian stimulation in a GnRH-agonist protocol, results indicated that Inhibin B may be a useful marker of follicular activity from smaller follicles at the time of recruitment and selection. On stimulation day 5, Inhibin B, but not E2, was shown to directly correlate with ovarian response. In addition, day 5 Inhibin A serum level correlated with the number of mature follicles in the late follicular phase. These study findings may be explained by the increased sensitivity of Inhibin B to the FSH-stimulus in the early follicular phase of ovarian stimulation as compared to serum levels of Inhibin A and E2, leading to an earlier rise of Inhibin B in serum (21).

Cycle monitoring is, as previously mentioned, an essential part of ART treatment and serum E2 level monitoring has been the cornerstone of ovarian stimulation cycles with gonadotropins since IVF inception. In 1990, Hardiman et al. (22) critically analyzed the correlation between serum E2 levels and ultrasonographic follicular development and demonstrated that the concentration of E2 in serum as well as in saliva correlates better with the total follicle number rather than with the number of mature follicles. They concluded that E2 serum level is a poor indicator of follicular maturity compared to ultrasound findings. In the following years, more studies investigated the

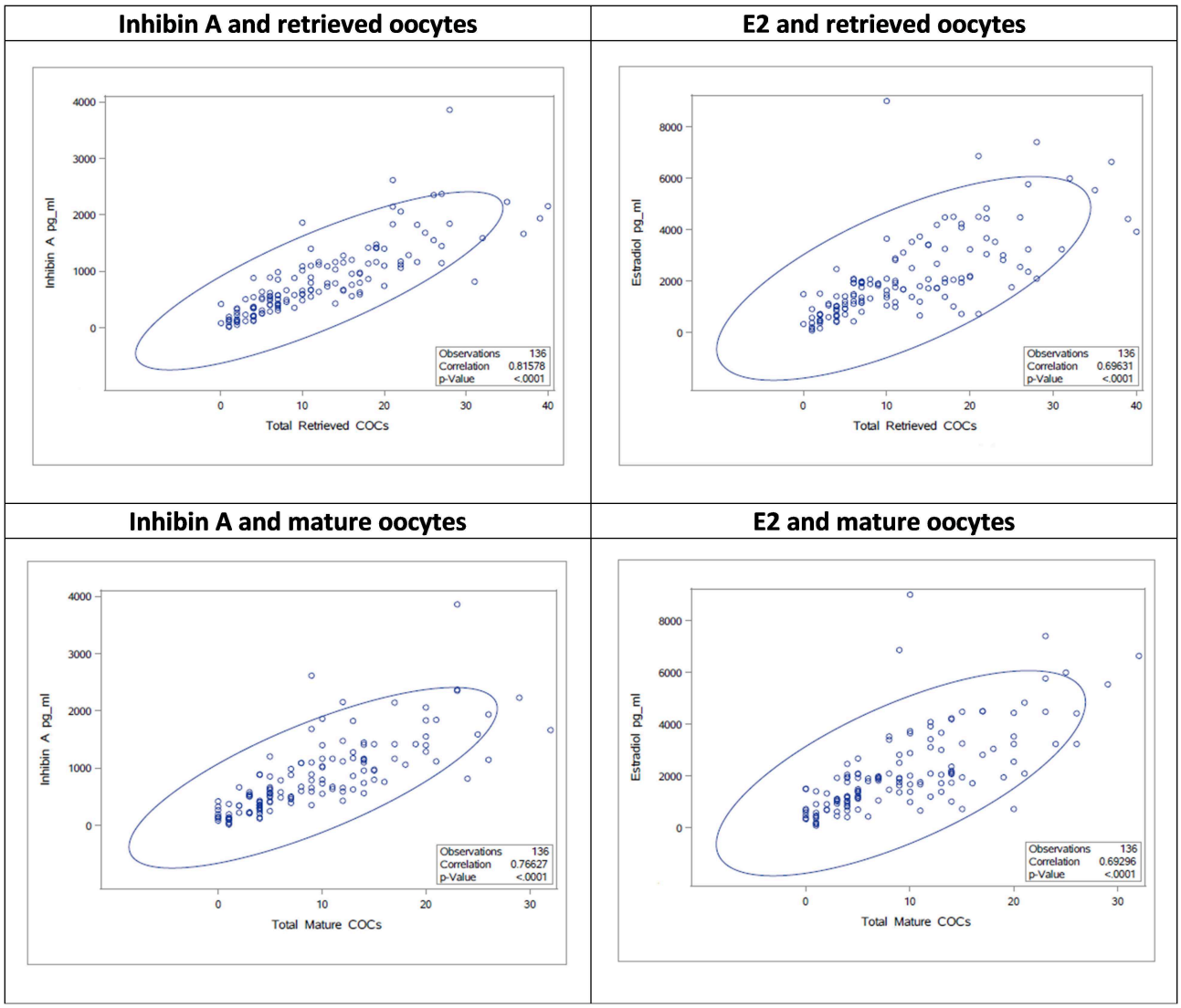

FIGURE 1 | ROC curve analysis of Inhibin A and E2 according to the number of ≥10 mature oocytes.

added benefit of E2 measurements to the outcome of IVF treatment: In an RCT (randomized controlled trial), Golan et al. (23) monitored patients, undergoing HMG stimulation in a GnRH-agonist protocol, either with ultrasonography and serum E2 measurements or with ultrasound in isolation. No significant differences were found between the two groups in terms of HMG stimulation duration, the total gonadotropin dosage, the number of oocytes retrieved and embryos transferred and the pregnancy rates. This study concluded that addition of E2 serum level measurements to ultrasound findings during cycle monitoring does not increase the number of mature oocytes retrieved. A further study by Vandekerckhove et al. (24) concurred with this study conclusion. Moreover, the theory that a certain E2-to-oocyte ratio at the time of hCG administration would result in improved implantation, pregnancy and live-birth (25) was refuted by Lass and UK Timing of hCG Group (26), who did not find any clinical benefit to including E2 measurement as a factor to consider when deciding when to administer hCG (human Chorionic Gonadotropin) for final oocyte maturation. The updated (6) Cochrane review (27), based on 6 instead of only 2 RCTs, did not find evidence for a better outcome by the addition of E2 in cycle monitoring. Despite the absence of conclusive evidence of a benefit to E2 monitoring, the Cochrane review recommended that a combined monitoring protocol including both, TVUS and serum E2, may need to be retained as precautionary good clinical practice.

Serum levels of E2 levels are often referred to as being an important marker for the prediction of ovarian hyperstimulation syndrome (OHSS) (28), which is a severe and possibly life-threatening complication of ovarian stimulation for IVF (29). However, OHSS can also occur in patients who conceived

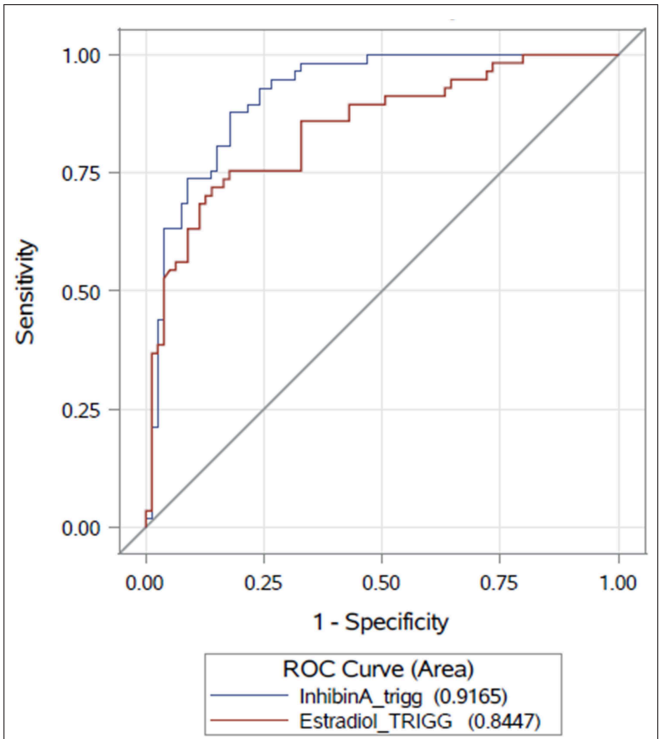

FIGURE 2 | Correlations between Inhibin A/E2 and number of retrieved/mature oocytes as scatter plots.

spontaneously or in patients with low serum E2 levels on the day of final oocyte maturation, thereby challenging the "E2 myth" in OHSS prediction (30). Further studies demonstrated that the number of developing follicles (31, 32) are superior to the E2 concentration on day of final oocyte maturation for identifying patients at risk for OHSS.

Crucial to the success of ART outcome is the number of mature oocytes, available for fertilization. Hence, cycle monitoring tools should consist of parameters with the capability of identifying the optimal time for final oocyte maturation.

Ultrasonographic measurement of the follicle number and size, is an essential part of cycle monitoring and several studies have been conducted to evaluate the "optimal" follicle size for triggering ovulation. Current literature suggests that follicles of sizes 16–22 mm on the day of oocyte retrieval contribute the most to the number of oocytes retrieved and that oocytes, derived from medium size follicles (13–23 mm), have equal maturity rates, fertilization rates, and blastocyst development compared to oocytes from large size follicles (≥23 mm). These rates were significantly lower for oocytes obtained from small follicles (8–12 mm) (33–35). Follicle growth is assumed to occur at a pace of ∼1.7 mm/day, theoretically resulting in a follicle size at the time of trigger administration, 34–38 h prior oocyte retrieval procedure, of 12–18 mm. Abbara et al. (36) confirmed the follicle size of 12–19 mm on the day of trigger as the size which is most likely to yield mature oocytes.

Inhibins are produced by granulosa cells and exist as heterodimeric glycoproteins, composed of an α-subunit linked to either a ßA-subunit (Inhibin A) or a ßB-subunit (Inhibin B) (37). Together with E2, Inhibin A, and Inhibin B play a role in restraining FSH in order to ensure the development of a single dominant follicle (38). Inhibin B is mainly secreted by the smaller, non-selected cohort follicles, whereas Inhibin A is mainly produced by the dominant follicle and in a natural cycle, Inhibin A levels start to rise in the late follicular phase with the presence of larger size follicles of a size of ∼15 mm (11, 39). Oocytes, derived from medium size follicles have equal maturity-, fertilization-, and blastocyst development rates as compared to oocytes from large size follicles.

An Inhibin A serum level of 668.1 pg/ml could be identified as a minimum threshold above which it is likely to retrieve ≥10 mature oocytes. In light of previous publications, evaluating the association between the number of retrieved oocytes and live birth (2)/cumulative live birth rates (40) and describing a number of retrieved oocytes to maximize live birth rate as ∼15 (2)/and a relatively unchanged live birth probability between seven and 20 oocytes retrieved (40), respectively, the number of ≥10 mature oocytes presents a robust marker for a successful ART outcome. The only publication referring to the number of mature oocytes in the context of live birth rates derived their data from oocyte donation cycles (41). They described the threshold number as >10 oocytes retrieved, mature oocytes, zygotes, and cleaved embryos, as significant predictors of live birth as compared to <10 of each of these variables. Therefore, Inhibin A serum level represents an excellent parameter for the planning of trigger administration and oocyte retrieval in combination with TVUS.

The strength of our study lies in the sample size and the inclusion of patients independent of their quantitative ovarian reserve parameters, representing the "real life scenario" of an IVF center. Inhibin A has the potential to serve as a decision making tool when deciding the optimal time to trigger. Limitations of the study are that some of the included patients had previously performed stimulation cycles which may have influenced the decision when to administer medication for final oocyte maturation and that Inhibin A results were not taken into account when deciding on the trigger timing and therefore Inhibin A serum level was not used as a decision-making tool in this setting.

CONCLUSIONS

The herein presented data revealed that serum levels of Inhibin A correlates better with the number of follicles ≥15 mm on the day of final oocyte maturation as well as with the number of retrieved and mature oocytes, compared to serum levels of E2. Therefore, serum Inhibin A levels may represent in combination with TVUS a promising tool for the planning of oocyte retrieval procedures. Currently there are no other studies demonstrating the predictive value of serum levels of Inhibin A on the number of mature oocytes. Large, prospective randomized studies are required to confirm our study findings and before the introduction of serum Inhibin A monitoring into routine clinical practice.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics committee of IVIRMA Middle East Fertility Clinic Abu Dhabi, United Arab Emirates. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

BL: conceptualization of the study, data analysis, drafting, and review of paper. LD: statistical analysis and review of paper. CC: review of paper and linguistic revision of paper. CA and LM: review of paper. BK: immunoassay design and hormone measurements and review of paper. GS: immunoassay design and review of paper. HF and AK: conceptualization of the study and review of paper.

FUNDING

This study was supported by Ansh Labs, Webster, Texas, United States. The funder provided support in the form of performing the hormonal analysis of Inhibin A, B, and E2 and salary for author BK, GS, and AK.

ACKNOWLEDGMENTS

We thank Prof. Leif Bungum for support in conceptualization of this study and Ms. Shieryl Digma and Ms. Jonalyn Edades for the data registration.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2020.00307/full#supplementary-material

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Conflict of Interest: BK, GS, and AK are employees of Ansh Labs. They participated in the blinded analysis of the samples, however the final data collection and statistical analysis of the results, as well as the decision to publish was taken by BL, CA, and HF. The funder did not have any additional role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. The commercial affiliation of BK, GS, and AK did not play any role in the study.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 22 May 2020
doi: 10.3389/fendo.2020.00182

Stop GnRH-Agonist Combined With Multiple-Dose GnRH-Antagonist Protocol for Patients With "Genuine" Poor Response Undergoing Controlled Ovarian Hyperstimulation for IVF

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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: 29 November 2019
Accepted: 13 March 2020
Published: 22 May 2020

Citation:
Orvieto R, Kirshenbaum M, Galiano V, Elkan-Miller T, Zilberberg E, Haas J and Nahum R (2020) Stop GnRH-Agonist Combined With Multiple-Dose GnRH-Antagonist Protocol for Patients With "Genuine" Poor Response Undergoing Controlled Ovarian Hyperstimulation for IVF. Front. Endocrinol. 11:182. doi: 10.3389/fendo.2020.00182

Objective: To examine whether the Stop GnRH-agonist combined with multiple-dose GnRH-antagonist protocol may improve conventional IVF/intracytoplasmic sperm injection (ICSI) cycle in poor ovarian response (POR) patients.

Design: Cohort historical, proof of concept study.

Setting: Tertiary, University affiliated Medical Center.

Patient(s): Thirty POR patients, defined according to the Bologna criteria, who underwent a subsequent Stop GnRH-agonist combined with multiple-dose GnRH-antagonist controlled ovarian hyperstimulation (COH) protocol, within 3 months of the previous failed conventional IVF/ICSI cycle, were included. For the purposes of this study, we eliminated a bias in this selection by including only "genuine" poor responder patients, defined as those who yielded up to 3 oocytes following COH with a minimal gonadotropin daily dose of 300 IU.

Main Outcome Measure(s): Number of oocytes retrieved, number of top-quality embryos, COH variables.

Result(s): The Stop GnRH-agonist combined with multiple-dose GnRH-antagonist COH protocol revealed significantly higher numbers of follicles >13 mm on the day of hCG administration, higher numbers of oocytes retrieved, and top-quality embryos (TQE) with an acceptable clinical pregnancy rate (16.6%). Moreover, as expected, patients undergoing the Stop GnRH-agonist combined with multiple-dose GnRH-antagonist COH protocol required significantly higher doses and a longer duration of gonadotropins stimulation.

Conclusion(s): The combined Stop GnRH-ag/GnRH-ant COH protocol is a valuable tool in the armamentarium for treating "genuine" poor ovarian responders. Further, large prospective studies are needed to elucidate its role in POR and to characterize the

appropriate patients subgroup (before initiating ovarian stimulation) that may benefit from the combined Stop GnRH-ag/GnRH-ant COH protocol.

Keywords: poor responders, COH, Bologna criteria, stop protocol, GnRH-antagonist

INTRODUCTION

Controlled ovarian hyperstimulation (COH) is a crucial step in the success of *in vitro* fertilization-embryo transfer (IVF-ET), enabling the recruitment of multiple oocytes and subsequently, the vitrification of all surplus embryos (1). However, due to the extreme heterogeneity in ovarian response to COH in some patients, referred to as "low/poor-responders," COH may only yield a few follicles, if any (2).

Until 2011, there was no one single definition for patients with poor ovarian response, though the most accepted criterion was a decreased response to COH, which, in IVF cycles, correlates to the reduced quantity of oocytes retrieved. The controversy surrounding the diagnosis of patients with poor ovarian response (POR) to ovarian stimulation resulted in a systematic standardization of the definition by the European society of Human Reproduction and Endocrinology (ESHRE), known as the Bologna criteria. According to the Bologna criteria, in order to define POR, "at least two of the following three features must be present: (i) Advanced maternal age ($\geq$40 years) or any other risk factor for POR; (ii) A previous POR ($\leq$3 oocytes with a conventional stimulation protocol); and (iii) An abnormal ovarian reserve test (3). In the absence of advanced maternal age or abnormal ovarian reserve tests, two previous maximal stimulation attempts with POR are sufficient to define a patient as a poor responder."

Several treatment strategies are offered to patients with POR to COH. These include reducing or stopping the dose of GnRH-agonist (GnRH-ag), the ultrashort, short and microdose GnRH-ag ("flare" protocols), the use of GnRH-antagonist (GnRH-ant), the combined ultrashort GnRH-ag with the multiple-dose GnRH-ant, the co-administration of letrozole, the modified natural-IVF cycle (2, 4–8), or the use of different doses and types of gonadotropin preparations (9, 10). Nevertheless, despite the multiplicity of strategies, no clear conclusion has been established on which regimen would be the ideal COH protocol for patients defined as POR (11).

In 1998, Faber et al. were the first to introduce the Stop protocol aiming to improve treatment outcome in patients with POR. The Stop protocol combines down-regulation with GnRH-ag starting at the luteal phase, cessation of GnRH-ag therapy with the onset of menstruation and high-dose gonadotropin administration. This short-term ovarian suppression, which begun in the luteal phase and discontinued with the onset of menses, followed by a high-dose stimulation with gonadotropins, was demonstrated to yield favorable pregnancy results in low responders (12). Although promising, a Cochrane review by Maheshwari et al. assessing the most effective GnRH-ag protocol as an adjuvant to gonadotropins in ART cycles, could not demonstrate any evidence of a difference in any of the outcome measures for continuation vs. stopping of GnRH-ag at the beginning of stimulation and follicular vs. luteal start of GnRH-ag (13).

Several years ago, our group demonstrated that combining the ultrashort flare GnRH-ag and GnRH-ant protocols in POR patients, who previously failed several IVF treatments cycles, yielded a 14.3% clinical pregnancy rate (7). This protocol, "which combines the benefit of the stimulatory effect of GnRH-ag flare on endogenous FSH with the benefit of immediate LH suppression of the GnRH antagonist," was therefore suggested as a valuable new tool for treating poor responders.

Based on the valuable addition of the ultrashort flare GnRH-ag combined with GnRH-ant to the COH protocols armamentarium (14), in the Chaim Sheba Medical Center, we started offering POR patients the combined Stop GnRH-ag with multiple-dose GnRH-ant protocol. In the present study, we sought to examine the role of Stop GnRH-ag combined with multiple-dose GnRH-ant in POR patients undergoing conventional IVF/intracytoplasmic sperm injection (ICSI) cycle. Assessing a new potentially promising treatment protocol will aid both fertility specialists' counseling and POR patients in adjusting their appropriate treatment strategy.

PATIENTS AND METHODS

We reviewed the computerized files of all consecutive women admitted to our IVF unit at the Chaim Sheba Medical Centre between January and November 2019. Inclusion criteria included patients with POR to conventional multiple-dose GnRH-antagonist IVF/ICSI cycles, defined according to the Bologna criteria (3), who underwent a subsequent COH using the combined Stop GnRH-ag with multiple-dose GnRH-ant protocol within 3 months of the previous *failed* conventional IVF/ICSI cycle. By only including a subgroup of "genuine" poor responder patients, those who fulfilled 2 out of 3 Bologna criteria and yielded up to 3 oocytes following COH with a minimal gonadotropin daily dose of 300 FSH IU, we eliminated potential selection bias. The study was approved by the institutional research ethics board of Sheba Medical Center.

In the initial conventional COH, gonadotropins were started on day 2–3 of the menstrual cycle (corresponding to stimulation day 1) in variable doses, with a minimal daily dose of 300 IU, depending on the patient's age and/or ovarian response in previous cycles. The continuing dose was adjusted according to serum E2 levels and vaginal ultrasound measurements of follicular diameter obtained every 2 or 3 days. GnRH-antagonist treatment (0.25 mg/day, Cetrorelix, Cetrotide, Serono

Abbreviations: COH, Controlled ovarian hyperstimulation; GnRH-ag, GnRH-agonist; GnRH-ant, Gonadotropin-releasing hormone-antagonist; ICSI, intracytoplasmic sperm injection; IVF-ET, *in vitro* fertilization-embryo transfer; POR, poor ovarian response; TQE, top-quality embryos.

International SR, Geneva, Switzerland or Orgalutran; NV Organon, Oss, The Netherlands) was started when a follicle reached 13 mm and/or E2 levels exceeded 400 pg/mL.

In the combined Stop GnRH-ag with multiple-dose GnRH-ant protocol, patients received triptorelin (Lapidot, Netanya, Israel) 0.1 mg/day, started in the midluteal phase and discontinued with the onset of menses and after confirmation of down-regulation by serum E2 levels and vaginal ultrasound measurements. Gonadotropins were initiated after two wash-out days, with maximal doses. Once the leading follicle had reached a size of 13 mm, and/or E2 levels exceeded 400 pg/mL, co-treatment with the GnRH antagonist 0.25 mg/day was initiated and continued up to, and including, the day of HCG administration.

Routine IVF or ICSI was performed, as appropriate. All patients received luteal support with progesterone. Embryos classification was based on the individual embryo scoring parameters according to pre-established definitions (15). A top-quality embryo (TQE) was defined as three or more blastomeres on day 2 and seven or more blastomeres on day 3, equally-sized blastomeres and <20% fragmentation. All other characteristics defined poor embryo quality.

Data on patient age and infertility-treatment-related variables were collected from the computerized clinical files. Outcome was assessed in terms of COH characteristics, cancellation rates, amount of gonadotropin required to COH, duration of stimulation, number of retrieved oocytes, number of TQE, number of embryos transferred, and pregnancy rates and compared between the previous conventional (Conventional-group) and the combined Stop GnRH-ag with multiple-dose GnRH-ant IVF/ICSI cycles.

Results are presented as means ± standard deviations. Comparison of continuous variables between the two groups was conducted using a Mann–Whitney *U* test or student *t*-test, as appropriate. Chi-square or a Fisher exact test were used for comparison of categorical variables. Significance was accepted at a probability value of <0.05.

RESULTS

Thirty "genuine" poor responder patients (age 37.4 ± 7.8 years) during a conventional IVF/ICSI cycle, who underwent a subsequent combined Stop GnRH-ag with multiple-dose GnRH-ant cycle, were evaluated. The clinical characteristics of the IVF cycles in the two study groups are shown in (**Table 1**).

As expected, the conventional IVF/ICSI cycles preceding the combined Stop GnRH-ag with multiple-dose GnRH-ant cycles were characterized by a significantly shorter COH (8.4 ± 2.1 vs. 10.7 ± 2.8, $p < 0.001$, respectively) and significantly lower requirement of gonadotropin doses (3,842 ± 1,702 vs. 5,372 ± 1,572, $p < 0.001$, respectively). Patients undergoing the combined Stop GnRH-ag with multiple-dose GnRH-ant cycles achieved significantly higher peak estradiol levels compared to those in the conventional cycles (3,033 ± 2,003 vs. 1,841 ± 1,580, $p < 0.001$, respectively) and higher numbers of follicles >13 mm in diameter on the day of triggering final follicular maturation (3.53 ± 1.90 vs. 1.76 ± 1.13, $p < 0.001$, respectively). Moreover, other COH outcomes were improved in the combined Stop GnRH-ag with multiple-dose GnRH-ant cycles compared to the conventional cycles, such as the number of oocytes retrieved (3.93 ± 2.91 vs. 1.33 ± 1.12, $p < 0.001$, respectively), MII oocytes (3.43 ± 2.69 vs. 1.08 ± 0.99, $p < 0.001$, respectively), TQE (1.6 ± 1.40 vs. 0.53 ± 0.73, $p < 0.01$, respectively) and the number of embryos transferred (1.13 ± 0.77 vs. 0.53 ± 0.77, $p < 0.001$, respectively) (**Table 1**).

Cancellation rates were 56.7% in the preceding conventional IVF/ICSI cycles, as compared to 20.0% in the combined Stop GnRH-ag with multiple-dose GnRH-ant cycles ($p < 0.002$). Of the six patients canceled in the combined Stop GnRH-ag with multiple-dose GnRH-ant cycles, five were also canceled in the previous conventional cycle. No patients conceived following the previous conventional IVF/ICSI cycles, while five pregnancies (16.6%) were recorded in the Stop GnRH-ag with multiple-dose GnRH-ant group.

DISCUSSION

In the present cohort historical, proof of concept study of "genuine" POR patients, according to the Bologna criteria, who achieved ≤3 oocytes following COH with conventional IVF/ICSI, the combined Stop GnRH-ag with multiple-dose GnRH-ant cycle provided significantly higher numbers of oocytes retrieved, as well as higher numbers of embryos transferred, as compared to their previous IVF attempt. Five clinical pregnancies (pregnancy rate, 16.6%) were recorded. However, it should be emphasized that this reasonable pregnancy rate in the combined Stop GnRH-ag with multiple-dose GnRH-ant cycle is biased due to the study design, which offered this protocol to poor-responder patients who had failed a previous IVF attempt.

When considering the additional benefit of increasing the oocyte yield in POR, it has been demonstrated in all age groups, that the retrieval of merely one more oocyte (2 instead of 3 oocytes) increases the cumulative live birth rate (LBR) per cycle by ∼25% (16). Moreover, a retrospective study by Drakopoulos et al. (17), evaluating the cumulative LBR deriving from one stimulation cycle (following fresh and frozen-thawed transfers), has demonstrated that low response patient (1–3 oocytes) achieved a significantly lower cumulative LBR compared to suboptimal response patient (4–9 oocytes). Therefore, the additional two oocytes retrieved and one TQE in the present study of genuine POR undergoing the combined Stop GnRH-ag with multiple-dose GnRH-ant cycle, may explain the observed improvement in the IVF outcome with a reasonable live birth rate.

The rationale behind the sequential treatment of the combined Stop GnRH-ag with multiple-dose GnRH-ant protocol stems from the advantages of its components. The long GnRH-ag protocol pretreatment results in better synchronized response and a scheduled cycle (18, 19). Moreover, since continuing the

TABLE 1 | Clinical characteristics of the IVF cycles in the two study groups.

	Control cycles	Study cycles	*p*-values
Number of cycles	30	30	
Cancellation rate (%)	56.7%	20.0%	0.002
Total dose of gonadotropin used (IU)	3,842 ± 1,702	5,372 ± 1,572	0.001
Length of stimulation (days)	8.4 ± 2.1	10.7 ± 2.8	0.001
Peak E2 levels on day of hCG administration (pmol/L)	1,841 ± 1,580	3,033 ± 2,003	0.01
Number of follicles >13 mm on day of hCG administration (range)	1.76 ± 1.13 (0–5)	3.53 ± 1.90 (1–9)	0.001
Number of oocytes retrieved (range)	1.33 ± 1.12 (0–3)	3.93 ± 2.91 (0–10)	0.001
Number of MII oocytes (range)	1.2 ± 1.06 (0–3)	3.43 ± 2.71 (0–9)	0.001
Number of TQE (range)	0.53 ± 0.73 (0–3)	1.65 ± 1.4 (0–4)	0.001
Number of embryos transferred (range)	0.53 ± 0.68 (0–2)	1.13 ± 0.77 (0–3)	0.001

GnRH-ag during COH is often associated with a significant increase in the number of gonadotropin ampoules required for achieving adequate follicular development, its cessation might improve ovarian response and avoid the need of increasing the gonadotropin daily dose. GnRH-ag causes suppression of pituitary LH secretion for as long as 10 days after the last dose of the agonist (20), which, together with the immediate LH suppression provided by the GnRH-ant, will eliminate premature LH surge and may improve the quality of the embryos generated. In POR, GnRH-ant down-regulation has an additional advantage in that final oocyte maturation may be triggered by GnRH agonist together with hCG (Double trigger), with an improved IVF outcome (21).

In our previous observation in this subgroup of "genuine" poor responders, we demonstrated that clinical pregnancy was observed in 4% in their subsequent IVF cycle using conventional COH (8). Moreover, according to a recently published study by our group, the reported live birth rates per cycle for poor responder patients using a daily gonadotropin dose of 450 IU resulted in 7.7% (9). These figures are in accordance with the present study, reflecting a reasonable IVF outcome using the combined Stop GnRH-ag with multiple-dose GnRH-ant protocol in this frustrating group of "genuine" POR.

A limitation of our analysis is its retrospective design and the small sample size. However, based on our patients' selection process, we enrolled only consecutive patients fulfilling the inclusion criteria, therefore, considerably decreasing the likelihood of selection bias. In addition, the combined Stop GnRH-ag with multiple-dose GnRH-ant cycle outcomes were compared to the previous COH-IVF of the same patients, thus aiming to eliminate any matching hurdles.

In conclusion, we chose to concentrate on a specific population among all POR (according to the Bologna criteria) with ≤3 oocytes following conventional COH for IVF with a high (>300 IU) daily dose gonadotropins, because these patients are most challenging. In the present study, the combined Stop GnRH-ag/GnRH-ant COH protocol was demonstrated to be a valuable tool in the armamentarium for treating "genuine" poor ovarian responders. Further, large prospective studies are needed to identify the specific characteristics of POR patients (before initiating ovarian stimulation) who may benefit from the combined Stop GnRH-ag/GnRH-ant COH protocol.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by IRB Sheba Medical Center. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

RO was the principal investigator, designed the study, performed the statistical evaluations, assisted in writing the paper, and edited it in all its revisions. MK, TE-M, EZ, and JH participated in designing the study, assisted in writing the paper and edited it, proof read the paper, and took part in discussions regarding the results. VG retrieved the data, assisted in writing the paper and edited it, proof read the paper, and took part in discussions regarding the results. RN participated in designing the study, retrieved the data, assisted in writing the paper, and edited it in all its revisions. All authors read and approved the final manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 29 May 2020
doi: 10.3389/fendo.2020.00318

Impact of Gonadotropin-Releasing Hormone Agonist Pre-treatment on the Cumulative Live Birth Rate in Infertile Women With Adenomyosis Treated With IVF/ICSI: A Retrospective Cohort Study

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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: 11 January 2020
Accepted: 24 April 2020
Published: 29 May 2020

Citation:
Chen M, Luo L, Wang Q, Gao J, Chen Y, Zhang Y and Zhou C (2020) Impact of Gonadotropin-Releasing Hormone Agonist Pre-treatment on the Cumulative Live Birth Rate in Infertile Women With Adenomyosis Treated With IVF/ICSI: A Retrospective Cohort Study. Front. Endocrinol. 11:318. doi: 10.3389/fendo.2020.00318

Introduction: Although pre-treatment with a GnRH agonist can reduce the size of adenomyosis lesions, the supra-physiological hormone level induced by controlled ovarian hyperstimulation (COH) may negate the usefulness of the GnRH agonist in patients with adenomyosis lesions, leading to continued poor outcomes in fresh embryo transfer cycles during *in vitro* fertilization (IVF). It is unclear whether GnRH agonist pre-treatment before starting the long GnRH agonist protocol for IVF/ICSI (intracytoplasmic sperm injection) can improve cumulative live birth rate (CLBR) of infertile women with adenomyosis.

Method: In this retrospective cohort study, a total of 374 patients diagnosed as adenomyosis (477 cycles) underwent IVF/ICSI with long GnRH agonist protocol with or without GnRH agonist pre-treatment between January 2009 and June 2018. Logistic regression was used to assess the association between GnRH agonist pre-treatment and pregnancy outcome after adjusting for confounding factors.

Results: The live birth rate in fresh embryo transfer cycles was higher in the non-pre-treatment group than in the GnRH agonist pre-treatment group (37.7 vs. 21.2%, $P = 0.028$); the adjusted odds ratio (OR) for the long agonist protocol without pre-treatment was 1.966 (95% CI: 0.9–4.296, $P = 0.09$). The CLBR was higher in the non-pre-treatment group than in the GnRH agonist pre-treatment group (40.50 vs. 27.90%, $P = 0.019$); the adjusted OR for the long agonist protocol without pre-treatment was 1.361 (95% CI: 0.802–2.309, $P = 0.254$).

Conclusion: Our results indicated that GnRH agonist pre-treatment before starting the long GnRH agonist protocol does not improve the live birth rate in fresh embryo transfer cycles or CLBR in infertile women with adenomyosis after IVF/ICSI treatment when compared to that in non-pre-treated patients. A subsequent prospective randomized controlled study is needed to confirm these results.

Keywords: GnRH agonist, ovarian stimulation, adenomyosis, *in vitro* fertilization, live birth rate

INTRODUCTION

Uterine adenomyosis is a common gynecological disorder characterized by the presence of ectopic endometrial glands and stroma surrounded by hyperplastic smooth muscle within the myometrium. Clinical manifestations include pelvic pain, abnormal uterine bleeding, and infertility. Results from two recent meta-analyses have revealed that adenomyosis has a detrimental effect on *in vitro* fertilization (IVF) outcomes, resulting in a reduced implantation rate, reduced pregnancy rate, reduced live birth rate, and an increase in miscarriage risk (1, 2).

Although the pathogenic mechanisms underlying the development of adenomyosis are unclear, it is well-understood that adenomyosis grows and declines in an estrogen-dependent manner. Adenomyotic tissue contains estrogen receptors (ER), progesterone, and androgen receptors. In addition, aromatase and sulfatase enzymes—which catalyze the conversion of androgens to estrogens—can be found in adenomyotic tissues. Together with circulating estrogens, locally produced estrogens stimulate the growth of tissue through interactions with the ER (3). Therapy with an agonist for GnRH decreases the expression of aromatase cytochrome P450 in the eutopic endometrium; this protein is overexpressed in women with adenomyosis (4). In addition, administration of a GnRH agonist leads to a hypo-estrogenic status by suppression of the hypothalamic-pituitary axis. Therefore, it is foreseeable that treatment with a GnRH agonist can reduce the size of adenomyosis lesions (3). Successful spontaneous pregnancies following treatment with a GnRH agonist in infertile women with adenomyosis have been reported (5–7). Recently, a retrospective study compared patients with and without long-term GnRH agonist pre-treatment before the preparation of the endometrium with hormone-replacement therapy (HRT). In this study, long-term pre-treatment with the GnRH agonist significantly improved the implantation rate, clinical pregnancy rate, and on-going pregnancy rate of patients with adenomyosis in frozen embryo transfer (FET) cycles (8).

Although pre-treatment with a GnRH agonist can reduce the size of adenomyosis lesions, the supra-physiological hormone level induced by controlled ovarian hyperstimulation (COH) may negate the usefulness of the GnRH agonist in patients with adenomyosis lesions, leading to continued poor outcomes in fresh embryo transfer cycles during IVF. A retrospective study compared fresh embryo transfer cycles with or without GnRH agonist pre-treatment and showed no group difference in the clinical pregnancy rates of patients with adenomyosis (9). With the increasing use of embryo freezing-thawing, the cumulative live birth rate (CLBR) has been suggested as a suitable mode of reporting the success of an IVF program, which incorporates both fresh and freeze-thawed embryo transfer (10). It is unknown whether GnRH agonist pre-treatment can improve the CLBR in patients with adenomyosis after *in vitro* fertilization treatment. To answer this question, we devised this retrospective study.

MATERIALS AND METHODS

Patients Population

This is a retrospective, single-center cohort study. Our patient population consisted of women with ultrasound-diagnosed adenomyosis who underwent IVF or ICSI, using the long GnRH agonist protocol, both with and without pre-treatment with a GnRH agonist between January 2009 and June 2018 at the Reproductive Medicine Center of the First Affiliated Hospital of Sun Yat-sen University. The sonographic diagnosis criteria of adenomyosis included: heterogeneous myometrial area, globular asymmetric uterus, irregular cystic spaces, myometrial linear striations, poor definition of the endometrial myometrial junction, myometrial anterior posterior asymmetry, thickening of the anterior and posterior myometrial wall, and increased or decreased echogenicity (11). The diagnosis was made by a single doctor in condition that the patients were in non-menstrual period and did not receive hormone therapy within 3 months. Cycles involving oocyte donation, oocyte sharing, oocyte cryopreservation, and/or frozen oocyte thawing were excluded from the analysis.

Controlled Ovarian Stimulation Protocol

The patients were allocated to the GnRH agonist pre-treatment group and non-pre-treatment group by doctors' preference. In the GnRH agonist pre-treatment group, GnRH agonist pre-treatment was initiated at the early follicle phase by administration of up to three injections of 3.75 mg of triptorelin acetate (Ipsen Pharma Biotech, France). The uterine anteroposterior diameter was measured 28 days after each injection and if it was more than 70 mm, injection of the same dose of GnRH agonist would be repeated up till the third injection. COH was induced using the long GnRH agonist protocol with a long-lasting formulation of triptorelin acetate depot (1.0–1.8 mg) or a daily dose (0.05–0.1 mg) of triptorelin acetate for pituitary downregulation. GnRH agonist administration for pituitary downregulation initiated 28 days after the last injection of 3.75 mg of triptorelin acetate in the GnRH agonist pre-treatment group and in the mid-luteal phase of the previous cycle in the non-pre-treatment group (12, 13). Gonadotropin stimulation with recombinant FSH (150-300 IU; Gonal-F, Merck Serono, Darmstadt, Germany) was initiated 14 days after GnRH agonist downregulation. The dose of recombinant FSH was determined on the basis of the patient's age, weight, and ovarian reserve, with or without human menopausal gonadotropin (hMG; Livzon, Zhuhai, China). Final oocyte maturation was induced by administering human chorionic gonadotropin (hCG, Ovidrel 250 mg; Merck Serono, Darmstadt, Germany) when at least one follicle $\geq$18 mm in diameter or two follicles $\geq$17 mm in diameter could be visualized on ultrasonography. Oocyte retrieval was performed 34–36 h after hCG administration. Fertilization was performed using either standard insemination or intracytoplasmic sperm injection (ICSI). Embryo transfer was performed on either day 3 or 5. No more than three embryos were transferred. An intramuscular

injection of progesterone (40 mg/day) was administered as luteal support.

Vitrification and Preparation of the FET Cycle

Supernumerary embryos were cryopreserved if they met the following criteria: day 3 embryos with at least six blastomeres and ≤20% fragmentation or day 5–6 blastocysts with at least expansion stage 3, inner cell mass score A or B, and trophectoderm score A or B (according to the Gardner grading system) (14). The protocols for FET included the natural cycle and the hormone replacement therapy (HRT) cycle with endometrial preparation with exogenous estrogen and progesterone, with or without GnRH agonist pre-treatment (8). FET was performed 2 months after failure of fresh embryo transfer.

Outcome Measures

The primary outcome measure was the CLBR per ovarian stimulation cycle. The secondary outcome was the live birth rate per fresh embryo transfer cycle. A clinical pregnancy was defined as the presence of at least one intrauterine gestational sac, as visualized by ultrasonography. A miscarriage was defined as the loss of a clinical pregnancy before 12 weeks of gestation. A live birth was defined as any birth event in which at least one baby was born alive. The CLBRs were calculated by including the first live births generated during the IVF/ICSI cycles as the numerator and censoring additional live births. The denominator was defined as all ovarian stimulation cycles.

Statistical Analysis

Continuous data were assessed for normality using the Shapiro–Wilk test, and the data were expressed as mean (±SD) or median (interquartile range), depending on the distribution. Categorical data were presented as frequency and percentage within each study group. Inter-group differences were assessed using Student's *t*/Mann–Whitney tests and chi-squared tests for continuous and categorical data, respectively. Fisher's exact test was applied when the expected values in any of the cells of a contingency table were below 5; in all other cases, Pearson's chi-squared test was applied. The association between GnRH agonist pre-treatment and pregnancy outcome was evaluated by multivariable logistic regression analysis while adjusting for potential confounders. Statistical significance was set at $P \leq 0.05$. Analyses were performed using IBM SPSS statistics (version 25; IBM, Chicago, US).

RESULTS

Study Population

Between January 2009 and June 2018, a total of 374 patients diagnosed as adenomyosis (477 cycles) underwent IVF/ICSI with long GnRH agonist protocol with or without GnRH agonist pre-treatment. Among the 374 patients, 313 adenomyosis patients (410 cycles) were included in the analysis of the CLBR and the other 61 patients (67 cycles) who did not have live birth but still had frozen embryos remaining were excluded. Ninety-seven patients (111 cycles) received GnRH agonist pre-treatment, whereas the remaining 216 patients (299 cycles) did not receive pre-treatment. Among the 374 patients, 188 patients (214

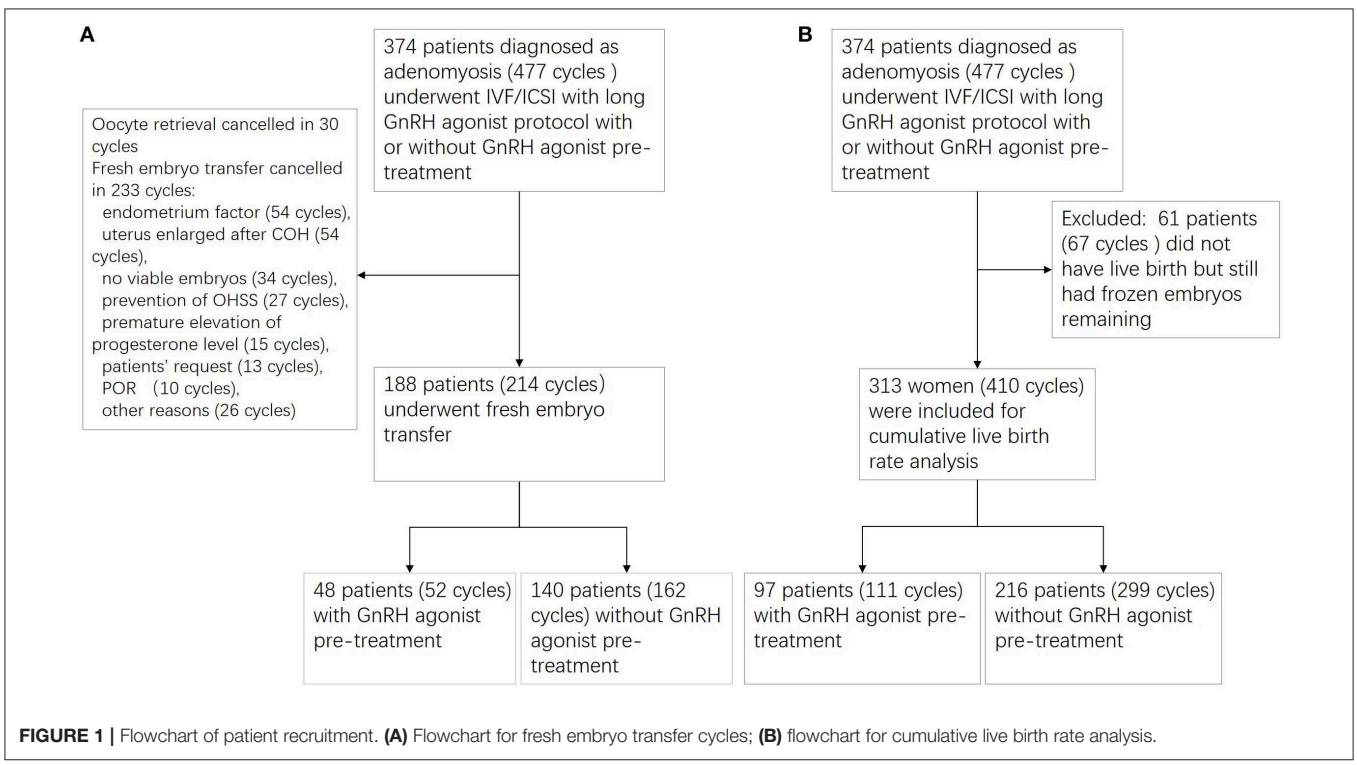

FIGURE 1 | Flowchart of patient recruitment. **(A)** Flowchart for fresh embryo transfer cycles; **(B)** flowchart for cumulative live birth rate analysis.

cycles)underwent fresh embryo transfer; 48 patients (52 cycles) in the pre-treatment group and 140 patients (162 cycles) in the non-pre-treatment group. Oocyte retrieval was canceled in 30 cycles; fresh embryo transfer was canceled in 233 cycles for the reasons included: endometrium factor (54 cycles), uterus enlarged after COH (54 cycles), no viable embryos (34 cycles), prevention of OHSS (27 cycles), premature elevation of progesterone level (15 cycles), patients' request (13 cycles), poor ovarian response (10 cycles), other reasons (26 cycles) (**Figure 1**).

Baseline Characteristics and Treatment Characteristics in Fresh Embryo Transfer Cycles

In fresh embryo transfer cycles, LH level and progesterone level on day of hCG administration were higher in the non-pre-treatment group ($P < 0.001$ and $P = 0.032$). The number of oocytes retrieved, mature oocytes, oocytes fertilized, viable embryos, high-quality embryos were higher in the non-pre-treatment group ($P = 0.007$, 0.010, 0.001, 0.004, and 0.004). The remaining baseline characteristics and treatment characteristics were comparable between the two groups (**Table 1**).

Pregnancy Outcome in Fresh Embryo Transfer Cycles

The live birth rate in the fresh embryo transfer cycles was higher in the non-pre-treatment group than in the GnRH agonist pre-treatment group (37.7 vs. 21.2%, $P = 0.028$). The clinical pregnancy rate, miscarriage rate, and preterm labor rate were comparable in the two groups (**Table 1**). To identify potential confounders that may interfere with the association analysis between the GnRH agonist pre-treatment and live birth, we compared baseline and treatment characteristics between the live birth and non-live birth groups. There were significant differences in female age, endometrial thickness at mid-luteal phase, number of oocytes fertilized, viable embryos, high-quality embryos, and high-quality embryos transferred between the two groups (**Table 2**). The adjusted odds ratio (OR) for the long agonist protocol without pre-treatment was 1.966 (95% CI: 0.9–4.296, $P = 0.09$) after adjustment for potential confounders (**Table 2**).

Baseline Characteristics and Treatment Characteristics in CLBR Analysis

In the analysis of CLBR, the proportion of patients complicated with endometriosis and the proportion of primary infertility were lower in the non-pre-treatment group (42.10 vs. 53.20%, $P = 0.046$; 42.80 vs. 56.80%, $P = 0.012$). The antral follicle count was higher in the non-pre-treatment group ($P = 0.039$). The number of oocytes retrieved, mature oocytes, oocytes fertilized normally, viable embryos, and high-quality embryos were higher in the non-pre-treatment group ($P = 0.003$, 0.005, 0.005, 0.019, and 0.019). The remaining baseline characteristics and treatment characteristics were comparable between the two groups (**Table 3**).

TABLE 1 | Patient characteristics and pregnancy outcomes in fresh embryo transfer cycles by stimulation protocol.

	Non-pre-treatment group	GnRH agonist pre-treatment group	*P*-value
No. of cycles	162	52	
Female age (years)	33.5 (30–36.75)	33.5 (31–36.75)	0.727[b]
Male age (years)	36 (32–39)	36 (33–38)	0.838[b]
Duration of infertility (years)	4 (2–6)	4 (2–6.75)	0.851[b]
Body mass index (kg/m^2)	20.80 (19.5–23.3)	20.55 (18.95–23.375)	0.637[b]
Complicated with endometriosis	63 (38.9%)	24 (46.2%)	0.353[c]
Type of infertility			0.266[c]
primary	76 (46.9%)	29 (55.8%)	
secondary	86 (53.1%)	23 (44.2%)	
Gravidity	1 (0–2)	0 (0–2)	0.511[b]
Parity	0 (0–0)	0 (0–0)	0.845[b]
Times of previous miscarriage	0 (0–0)	0 (0–0)	0.561[b]
Insemination method			0.915[d]
IVF	127 (78.4%)	42 (80.8%)	
ICSI	29 (17.9%)	8 (15.4%)	
IVF + ICSI	6 (3.7%)	2 (3.8%)	
Basal FSH (mIU/mL)	5.74 (4.88–6.74)	6.19 (4.99–7.39)	0.113[b]
Antral follicle count	9 (6–12)	7.5 (5–11.75)	0.053[b]
Endometrial thickness at mid-luteal phase	11.26 (10–13)	11.00 (9–12)	0.163[b]
Anteroposterior diameter of uterus at mid-luteal phase	55 (47–62)	55.97 (43–60)	0.612
Stimulation duration (day)	11 (10–12)	11 (10–12)	0.851[b]
Total dosage of gonadotropin (IU)	2,647.44 (801.886)	2,609.81 (742.989)	0.809[a]
LH level on day of hCG administration (mIU/mL)	0.77 (0.55–1.11)	0.38 (0.30–0.57)	<0.001[b]*
Estrogen level > 3,000 ng/mL on day of hCG administration	62 (38.3%)	11 (21.2%)	0.893[c]
Progesterone level on day of hCG administration (ng/mL)	0.6 (0.4–0.9)	0.5 (0.33–0.71)	0.032[b]*
Number of oocytes retrieved	10 (7–15)	8 (5.25–12)	0.007[b]*
Number of mature oocytes	9 (6–12)	7 (4–11)	0.010[b]*
Number of oocytes fertilized	6.5 (4–9)	4.5 (3–7)	0.001[b]*
Number of viable embryos	3.5 (2–6)	3 (2–4)	0.004[b]*
Number of high-quality embryos	3 (1–5)	2 (1–3)	0.004[b]*
Number of fresh embryos transferred	2 (2–3)	2 (2–3)	0.875[b]
Number of high-quality embryos transferred	2 (1–2)	2 (1–2)	0.674[b]
Type of embryo transferred			0.457[d]
Cleavage embryo	153 (94.4%)	51 (98.1%)	
blastocyst	9 (5.6%)	1 (1.9%)	
Clinical pregnancy	69 (42.6%)	16 (30.8%)	0.130[c]
Miscarriage	8 (11.6%)	5 (31.3%)	0.063[d]
Preterm labor	7 (11.5%)	0 (0.0%)	0.585[d]
Live birth	61 (37.7%)	11 (21.20%)	0.028[c]*

[a] *Two-sample t-test. Values are means (SD).*
[b] *Two-sample Mann–Whitney test. Values are medians (interquartile range).*
[c] *Pearson chi-squared test. Values are number (percentage).*
[d] *Fisher exact probability test. Values are number (percentage).*
**Statistical significance.*

TABLE 2 | Patient characteristics by live birth or no live birth in fresh embryo transfer cycles.

	Live birth ($n = 72$)	No live birth ($n = 142$)	*P*-value	Adjusted ORs	*P*-value
Female age (years)	32.96 (3.847)	34.2 (4.579)	0.050[a]	0.94 (0.872–1.013)	0.105
Male age (years)	36.13 (5.604)	35.7 (4.976)	0.576[a]		
Duration of infertility (years)	4 (2–7)	4 (2–6)	0.752[b]		
Body mass index (kg/m^2)	20.7 (19.5–23.3)	20.8 (19.25–23.35)	0.578[b]		
Complicated with endometriosis	30 (41.70%)	57 (40.10%)	0.830[c]		
Type of infertility			0.628[c]		
Primary	37 (51.40%)	68 (47.90%)			
Secondary	35 (48.60%)	74 (52.10%)			
Gravidity	0 (0–2)	1 (0–2)	0.828[b]		
Parity	0 (0–0)	0 (0–0)	0.17[b]		
Times of previous miscarriage	0 (0–0)	0 (0–0)	0.813[b]		
Insemination method					
IVF	57 (79.20%)	112 (78.90%)	0.962[c]		
ICSI	12 (16.70%)	25 (17.60%)			
IVF + ICSI	3 (4.20%)	5 (3.50%)			
Basal FSH (mIU/mL)	5.74 (4.6–6.98)	5.87 (4.955–6.985)	0.38[b]		
Antral follicle count	9 (6–13)	9 (6–11.5)	0.310[b]		
Endometrial thickness at mid-luteal phase	12 (10–13)	11 (9–12)	0.012[b*]	1.106 (0.987–1.24)	0.083
Anteroposterior diameter of uterus at mid-luteal phase	54 (46.75–59.25)	55.97 (46.25–62.75)	0.297[b]		
Stimulation protocol			0.028[c*]		
Non-pre-treatment	61 (84.70%)	101 (71.10%)		1.966 (0.9–4.296)	0.09
GnRH agonist pre-treatment	11 (15.30%)	41 (28.90%)		Reference	
Stimulation duration (day)	11 (10–12)	11 (10–12)	0.518[b]		
Total dosage of gonadotropin (IU)	2700 (2000–3300)	2600 (2075–3062.5)	0.714[b]		
LH level on day of hCG administration (mIU/mL)	0.77 (0.51–1.13)	0.64 (0.4–0.96)	0.056[b]		
Estrogen level > 3,000 ng/mL on day of hCG administration	25 (34.70%)	48 (33.80%)	0.893[c]		
Progesterone level on day of hCG administration (ng/mL)	0.6 (0.4–0.8)	0.6 (0.4–0.9)	0.428[b]		
Number of oocytes retrieved	10 (8–15)	9 (6–13.5)	0.092[b]		
Number of mature oocytes	9 (6–12)	8 (6–11)	0.082[b]		
Number of oocytes fertilized	7 (5–9)	5 (3–8.5)	0.009[b*]	1.052 (0.937–1.18)	0.389
Number of viable embryos	4 (3–6)	3 (2–5.5)	0.024[b*]	0.915 (0.731–1.146)	0.441
Number of high-quality embryos	3 (2–5)	2 (1–4)	0.007[b*]	1.1 (0.846–1.429)	0.477
Number of fresh embryos transferred	2 (2-3)	2 (2–3)	0.441[b]		
Number of high-quality embryos transferred	2 (1–2)	2 (1–2)	0.011[b*]	1.485 (0.974–2.262)	0.066
Type of embryo transferred			0.736[d]		
Cleavage embryo	68 (94.40%)	136 (95.80%)			
blastocyst	4 (5.60%)	6 (4.20%)			

[a] *Two-sample t-test. Values are means (SD).*
[b] *Two-sample Mann–Whitney test. Values are medians (interquartile range).*
[c] *Pearson chi-squared test. Values are number (percentage).*
[d] *Fisher exact probability test. Values are number (percentage).*
**Statistical significance.*

Cumulative Live Birth and COH Protocol

The CLBR was significantly higher in the non-pre-treatment group than in the GnRH agonist pre-treatment group (40.50 vs. 27.90%, $P = 0.019$, **Table 3**). To identify potential confounders that may interfere with the association analysis between the GnRH agonist pre-treatment and cumulative live birth, we compared baseline and treatment characteristics between the cumulative and non-cumulative live birth groups. There were significant differences in female and male ages, proportion of patients complicated with endometriosis, basal FSH, antral follicle counts, endometrial thickness at mid-luteal phase, number of oocytes retrieved, mature oocytes, oocytes fertilized normally, viable embryos, and high-quality embryos between the two groups (**Table 4**). The adjusted odds ratio (OR) for the long agonist protocol without pre-treatment was 1.361 (95% CI: 0.802–2.309,

TABLE 3 | Patient characteristics and pregnancy outcomes by stimulation protocol for cumulative live birth rate analysis.

	Non-pre-treatment group	GnRH agonist pre-treatment group	*P*-value
No. of cycles	299	111	
Female age (years)	34 (31–37)	34 (31–37)	0.895[a]
Male age (years)	36 (32–40)	37 (33–39)	0.393[a]
Duration of infertility (years)	4 (2–6)	4 (2–6)	0.715[a]
Body mass index (kg/m^2)	21 (19.5–23.3)	21.3 (19.28–23.11)	0.715[a]
Complicated with endometriosis	126 (42.10%)	59 (53.20%)	0.046[b]*
Type of infertility			0.012[b]*
Primary	128 (42.80%)	63 (56.80%)	
Secondary	171 (57.20%)	48 (43.20%)	
Gravidity	1 (0–2)	0 (0–2)	
Parity	0 (0–0)	0 (0–0)	
Times of previous miscarriage	0 (0–0)	0 (0–0)	
Insemination method			0.716[b]
IVF	240 (80.30%)	93 (83.80%)	
ICSI	53 (17.70%)	16 (14.40%)	
IVF + ICSI	6 (2.00%)	2 (1.80%)	
Basal FSH (mIU/mL)	5.69 (4.76–7.02)	5.73 (4.76–7.11)	0.994[a]
Antral follicle count	8 (5–12)	7 (4–10)	0.039[a]*
Endometrial thickness at mid-luteal phase	11.26 (9–13)	11 (9–12)	0.052[a]
Anteroposterior diameter of uterus at mid-luteal phase	55.97 (49–62)	55.97 (45–60)	0.226[a]
Cycles canceled			0.722[b]
Cancel of oocyte retrieval	22 (7.40%)	8 (7.20%)	
Cancel of fresh embryo transfer	127 (42.50%)	52 (46.80%)	
Number of oocytes retrieved	9 (5–15)	7 (3–12)	0.003[a]*
Number of mature oocytes	8 (4–13)	6 (3–11)	0.005[a]*
Number of oocytes fertilized normally	6 (3–9)	4 (2–7)	0.005[a]*
Number of viable embryos	3 (2–6)	2.5 (1–4)	0.019[a]*
Number of high quality embryos	2 (0–5)	1 (0–3)	0.019[a]*
Cycles with supernumerary embryos	206 (68.9%)	70 (63.1%)	0.263[b]
Cumulative Live birth	121 (40.50%)	31 (27.90%)	0.019[a]*

[a] *Two-sample Mann–Whitney test. Values are medians (interquartile range).*
[b] *Pearson chi-squared test. Values are number (percentage).*
**Statistical significance.*

$P = 0.254$) after adjustment for potential confounders (**Table 4**).

DISCUSSION

To date, no studies have elucidated whether GnRH agonist pre-treatment is beneficial in improving the CLBR in patients with adenomyosis. In this study, our data showed that the live birth rate in fresh embryo transfer cycles and CLBR of infertile women with adenomyosis after IVF/ICSI treatment is higher among patients undergoing the GnRH long agonist protocol without GnRH agonist pre-treatment than in the group with pre-treatment. However, after adjustment for confounding factors such as female ages, antral follicle counts, endometrial thickness, number of oocytes retrieved, mature oocytes, oocytes fertilized normally, viable embryos, and high-quality embryos, we show that GnRH agonist pre-treatment status is not associated with the live births or cumulative live births. A previous retrospective study showed GnRH agonist pre-treatment did not improve the clinical pregnancy rate of women with adenomyosis after fresh embryo transfer (9). Our results indicate that GnRH agonist pre-treatment also does not improve the live birth rate in fresh embryo transfer cycles or the CLBR of women with adenomyosis after IVF/ICSI.

Evidence from a systematic review suggests that administration of a GnRH agonist for 3–6 months before IVF or ICSI in women with endometriosis increases the odds of clinical pregnancy four-fold; this analysis included randomized controlled trials using any GnRH agonist before IVF or ICSI to treat women with any degree of endometriosis diagnosed by laparoscopy or laparotomy (15). Although adenomyosis and endometriosis share many diagnostic, symptomatic, and molecular similarities, the two conditions are distinct entities—many differences have been observed in their pathogenesis, risk factors, and clinical presentation (16). These differences may explain the differential impact of GnRH agonist pre-treatment on pregnancy outcome in IVF for patients with adenomyosis vs. endometriosis.

A recent retrospective study identified that long-term GnRH agonist pre-treatment significantly improved the implantation rate, clinical pregnancy rate, and on-going pregnancy rate of patients with adenomyosis in FET cycles (8). The authors suggested that this may have resulted from the observation that the GnRH agonist can induce a hypo-estrogenic effect by suppressing the hypothalamus–pituitary–ovary axis with a resultant reduction in adenomyosis and subsequent symptomatic relief (8). Moreover, exogenous treatment with a GnRH agonist significantly suppressed the proliferation of cells derived from the endometrium and the expansion of pathologic lesions in patients with adenomyosis (17). Nevertheless, COH following GnRH agonist pre-treatment will undoubtedly result in a supra-physiological elevation of estrogen levels, leading to re-enlargement of the uterus; therefore, this may offset the expected uterine shrinkage with GnRH agonist pre-treatment. Our results show that after adjustment for confounding factors, GnRH agonist pre-treatment status is not associated with live births.

A prospective study of 74 infertile patients with surgically proven endometriosis showed no significant differences in the implantation rate, miscarriage rate, and clinical pregnancy rate following IVF/ICSI between women with and those without adenomyosis (18). Our results showed that there was no significant difference in proportion of adenomyosis with endometriosis between live birth group and no live birth group in fresh embryo transfer cycles. Besides, adenomyosis with endometriosis was not associated with cumulative live birth after

TABLE 4 | Patient characteristics by pregnancy outcome for cumulative live birth rate analysis.

	No cumulative live birth ($n = 258$)	Cumulative live birth ($n = 152$)	*P*-value	Adjusted ORs (95% CI)	*P*-value
Female age (years)	35 (32–38)	33 (30–36)	<0.001[a]*	0.897 (0.831–0.968)	0.005*
Male age (years)	37 (32.75–40)	35 (32–39)	0.029[a]*	1.024 (0.964–1.088)	0.434
Duration of infertility (years)	4 (2–6)	4 (2–6)	0.841[a]		
Body mass index (kg/m^2)	21.21 (19.5–23.3)	20.76 (19.4–23.05)	0.11[a]		
Complicated with endometriosis	129 (50.00%)	56 (36.80%)	0.010[b]*	1.584 (0.986–2.545)	0.057
Type of infertility			0.807[b]		
Primary	119 (46.10%)	72 (47.40%)			
Secondary	139 (53.90%)	80 (52.60%)			
Gravidity	1 (0–2)	1 (0–2)	0.637[a]		
Parity	0 (0–0)	0 (0–0)	0.704[a]		
Times of previous miscarriage	0 (0–0)	0 (0–0)	0.445[a]		
Insemination method			0.580[b]		
IVF	213 (82.60%)	120 (78.90%)			
ICSI	41 (15.90%)	28 (18.40%)			
IVF + ICSI	4 (1.60%)	4 (2.60%)			
Basal FSH (mIU/mL)	5.91 (4.88–7.36)	5.49 (4.71–6.67)	0.007[a]*	0.897 (0.8–1.006)	0.064
Antral follicle count	7 (4–10)	9 (6–13)	<0.001[a]*	0.996 (0.938–1.057)	0.891
Endometrial thickness at mid-luteal phase	11 (9–12)	11.26 (10–13)	<0.001[a]*	1.098 (1.007–1.196)	0.034*
Anteroposterior diameter of uterus at mid-luteal phase	55.97 (48–60.25)	55.97 (48–62)	0.891[a]		
Stimulation protocol			0.019[b]*		
Non-pre-treatment	178 (69.00%)	121 (79.60%)		1.361 (0.802–2.309)	0.254
GnRH agonist pre-treatment	80 (31.00%)	31 (20.40%)		Reference	
Number of oocytes retrieved	6 (3–12)	11 (8–17)	<0.001[a]*	0.962 (0.848–1.09)	0.539
Number of mature oocytes	6 (2–11)	10 (7–15)	<0.001[a]*	0.993 (0.834–1.183)	0.938
Number of oocytes fertilized	4 (1–7)	7 (5–11)	<0.001[a]*	1.043 (0.9–1.21)	0.574
Number of viable embryos	2 (1–3)	5 (3–7)	<0.001[a]*	1.149 (0.977–1.352)	0.093
Number of high-quality embryos	1 (0–3)	3 (2–5)	<0.001[a]*	1.213 (1.015–1.449)	0.033*

[a] *Two-sample Mann–Whitney test. Values are medians (interquartile range).*
[b] *Pearson chi-squared test. Values are number (percentage).*
**Statistical significance.*

adjustment for confounding factors. In addition, female age, endometrial thickness, and the number of high-quality embryos were associated with the cumulative live birth of women with adenomyosis treated with IVF/ICSI—this finding is consistent with results of previous research (19–22).

Our study has notable strengths. It is the first study that has demonstrated the impact of GnRH agonist pre-treatment on the CLBR of infertile patients with adenomyosis after IVF treatment. Furthermore, the association between ovarian stimulation protocols and cumulative live births was evaluated by multivariable logistic regression models with adjustment for potential confounders. Our study has some limits. Firstly, it is a retrospective cohort study and therefore selection bias may exist. The patients were allocated to the GnRH agonist pre-treatment group and non-pre-treatment group by doctors' preference. Even so, the most baseline characteristics in the two groups are comparable. Although the complete data of indicators for severity of adenomyosis as uterus volume or serum CA125 were not available, anteroposterior diameters which can partly reflect the uterus volume were comparable between the two groups. Secondly, information of severity of the disease is lacking. However, at present there is no consistent standard for grading the severity of adenomyosis. Thirdly, data of cycle regimen for frozen-thawed embryo transfer was not analyzed. A meta analysis showed that there was no evidence of a difference between natural FET cycle and HRT FET cycle in the clinical pregnancy rate or a difference between natural FET cycle and HRT plus GnRH agonist suppression FET cycle in live birth rate (23). Therefore, the association analysis between GnRH agonist pre-treatment and pregnancy outcome may not be interfered by FET regimen status. Above all, a subsequent prospective randomized controlled study is needed to confirm our results in the future.

In conclusion, our results indicated that GnRH agonist pre-treatment before the long agonist protocol does not improve the live birth rate in fresh embryo transfer cycles or CLBR after IVF/ICSI among infertile women with adenomyosis.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the study was approved by the Institutional Ethics Committee from the First Affiliated Hospital of Sun Yat-sen University. Owing to the retrospective nature of the study, written informed consent was not required from the participants. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

MC and LL contributed equally to design of the study, acquisition, analysis and interpretation of data, drafting, and revising the manuscript, and provided final approval of the manuscript prior to submission. QW contributed to the conception and design of the study, revising the manuscript, and provided final approval of the manuscript prior to submission. JG, YC, and YZ were involved in data acquisition and provided final approval of the manuscript prior to submission. CZ contributed to the conception and design of the study and provided final approval of the manuscript prior to submission.

FUNDING

The authors were supported by grants from the National Natural Science Funds of China for Young Scholars (81601239).

ACKNOWLEDGMENTS

We would like to thank Saihong Wan and Xi Yuan for help with data processing.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 28 August 2020
doi: 10.3389/fendo.2020.00584

Mildly Higher Serum Prolactin Levels Are Directly Proportional to Cumulative Pregnancy Outcomes in *in-vitro* Fertilization/Intracytoplasmic Sperm Injection Cycles

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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *02 December 2019*
Accepted: *17 July 2020*
Published: *28 August 2020*

Citation:
Zhang D, Yuan X, Zhen J, Sun Z, Deng C and Yu Q (2020) Mildly Higher Serum Prolactin Levels Are Directly Proportional to Cumulative Pregnancy Outcomes in in-vitro Fertilization/Intracytoplasmic Sperm Injection Cycles. Front. Endocrinol. 11:584. doi: 10.3389/fendo.2020.00584

Hyperprolactinemia has long been considered detrimental to fertility due to irregularity of ovulation. Whether mild hyperprolactinemia should be corrected before initiating an *in-vitro* fertilization/intracytoplasmic sperm injection cycle (IVF/ICSI) has not been determined; this study aimed to examine how different levels of prolactin affect IVF outcomes. A total of 3,009 patients with basal prolactin level <50 ng/mL undergoing IVF/ICSI cycles for tubal or male factors were recruited in this study. Patients diagnosed with anovulation owing to polycystic ovarian syndrome or hyperandrogenism were ruled out. Pregnancy outcomes were compared between patients with basal prolactin levels higher or lower than the median level of prolactin (16.05 ng/mL). Multifactor analyses were carried out among four subgroups depending on different prolactin levels. Repeated-measures analysis of variance was used to explore the relationship between the ascending trend of prolactin levels over ovarian stimulation and the corresponding cumulative pregnancy outcomes. There were significantly higher numbers of oocytes (9 vs. 8, $P = 0.013$) and embryos (6 vs. 5, $P = 0.015$) in patients with basal prolactin higher than 16.05 ng/mL. Basal prolactin higher than 30 ng/mL was positively related to cumulative clinical pregnancy, and a level higher than 40 ng/mL was a good indicator for the cumulative live birth rate. Throughout ovarian stimulation, the prognosis of pregnancy improved with increasing prolactin levels. Patients with better cumulated pregnancy outcomes had significantly higher prolactin levels as well as a profoundly increasing trend during the stimulating process than those who did not conceive. For patients who underwent the gonadotropin-releasing hormone agonist long protocol IVF/ICSI treatment, a slightly higher prolactin level during the controlled ovarian hyperstimulation protocol was a positive indicator for cumulated pregnancy/live birth rates.

Keywords: prolactin, GnRH agonist protocol, IVF, ICSI, cumulated clinical pregnancy rate, cumulated live birth rate

INTRODUCTION

Prolactin (PRL) is known as a stimulator of the proliferation and differentiation of mammary cells for lactation. The primary regulator for PRL pituitary secretion is dopamine via hypothalamic inhibitory signals, and this constitutes the pharmacological basis for hyperprolactinemia treatment (1). In addition, PRL, as a stress hormone, is actively involved in metabolism, electrolyte transport, angiogenesis, and immunity (1).

Serum PRL is ordinarily under 25 ng/L; a level above the normal upper limit is diagnosed as hyperprolactinemia as long as the sample is obtained without excessive stress challenges before venipuncture. Hyperprolactinemia is a well-established cause of hypogonadotropic hypogonadism (2); PRL acts on kisspeptin-1 neurons expressing the PRL receptor (PRL-R) and is responsible for decreased kisspeptin-1 and GnRH secretion, leading to anovulation (3). Dopamine agonists are widely used for suppression of serum PRL and resumption of ovulation in infertile women with hyperprolactinemia seeking to conceive naturally. However, follicle genesis in women undergoing *in-vitro* fertilization/intracytoplasmic sperm injection (IVF/ICSI) treatment depends on exogenous gonadotropins, instead of endogenous ones, and luteal phase support is always ensured by sufficient progesterone (P) administration. Is it really necessary to suppress the slightly higher PRL? Or is there a proper PRL range to optimize IVF outcomes? We hypothesize an isolating mildly increasing PRL level if these women have no organic lesions such as prolactinoma would not negatively affect cumulated IVF pregnancy outcomes.

MATERIALS AND METHODS

This retrospective study included all women who underwent IVF/ICSI treatment for tubal or male infertility with the gonadotropin-releasing hormone agonist (GnRHa) long protocol at Peking Medical College Hospital (PUMCH) between 1st July 2014 and 31st March 2018. Patients diagnosed with anovulatory diseases like polycystic ovarian syndrome (PCOS) or hyperandrogenism were not included. The study was approved by the Ethics Committee of PUMCH (No. S-K601). Exclusion criteria were: Patients with serum P level $\geq$ 1.5 ng/mL during a controlled ovarian hyperstimulation protocol (COH), patients undergoing a freeze-all strategy, egg-donating cycles, basal PRL level $\geq$ 50 ng/mL, previous diagnosis of pituitary lesions, or abnormal thyroid functions. All patients included had to have used up all fresh or vitrified embryos generated from the stimulating cycle by the time of the study in order to analyze the cumulative pregnancy outcomes.

Sexual hormone levels were tested at three individual times for each patient. The first basal one was on the 2nd day of the menstrual cycle before pituitary downregulation by GnRHa, which we marked as T0. On the 2nd day of the next menstrual cycle patients started receiving recombinant human follicle-stimulating hormone (rFSH; Gonal-F, Merck-Serono) at an individualized dose adjusted based on patient ovarian response. Final oocyte maturation was triggered by intramuscular injection of 250 μg recombinant human chorionic gonadotropin (hCG; Ovitrelle, Merck-Serono) and the 2nd hormone sample taken on that morning was defined as T1. The third hormone samples were collected on the morning after administering hCG, and that time was defined as T2. After that, oocytes were retrieved by ultrasound-guided transvaginal aspiration at around 36 h after hCG trigger. Intramuscular injection of 40 mg P was administered daily for luteal phase support. Embryo development was evaluated daily until the fresh transfer of cleavage stage embryos (Day 3). Embryos were evaluated following a standardized scoring system (4). After fresh embryo transfer, the remaining embryos were cultured to blastocysts (Day 5 or 6) before vitrification. Frozen-thawed embryo transfer may be applied to either artificial or natural cycles.

Serum FSH, luteinizing hormone (LH), PRL, estrogen (E_2), and P levels were measured by the automated Elecsys Immunoanalyzer (Beckmann, USA). The inter-assay coefficients of variation were <5 and <10% for E_2 and P and <8% for FSH, LH, and PRL, respectively.

Clinical pregnancy was defined as intrauterine pregnancy with at least one fetus with a positive heartbeat at 6 weeks of gestation or later. Live birth was defined as the delivery of a live-born child at >28 weeks of gestation. The clinical pregnancy rate (CPR) and live birth rate (LBR) referred to the cumulated outcome after transferring all embryos from the studied stimulating cycle. Secondary outcomes included the number of oocytes retrieved, mature oocytes, two-pronuclear zygotes, and embryos.

The data analysis was carried out using SPSS 24.0 statistical analysis software (IBM Inc., USA). The normality of distribution of continuous variables was assessed using the Kolmogorov-Smirnov test (cutoff at $P = 0.01$). Descriptive statistics for continuous variables are reported as the mean ± standard deviation (SD). Categorical variables were described using frequency distributions and are presented as frequency and percentage (%). The *t*-test for independent samples or the Mann-Whitney *U*-test were used as appropriate to compare continuous variables by group. The chi-squared test was used to compare categorical variables by group. Repeated-measures analysis of variance was used for measuring repeated longitudinal data. A logistic regression model of the two groups (PRL $\leq$ 16.05 vs. PRL > 16.05 ng/mL) was developed to additionally adjust for age, body mass index (BMI), basal FSH, basal E_2, and duration of infertility. Odds ratios were estimated with 95% confidence intervals. All tests were two-sided and considered significant at $P < 0.05$.

Abbreviations: BMI, body mass index; CPR, clinical pregnancy rate; COH, controlled ovarian hyperstimulation; FSH, follicle stimulating hormone; GnRHa, gonadotropin releasing hormone agonist; hCG, human chorionic gonadotropin; LBR, live birth rate; ICSI, intracytoplasmic sperm injection; IVF, *in vitro* fertilization; PRL, prolactinl; rFSH, recombined human FSH.

RESULTS

A total of 3,009 patients fulfilling the criteria were recruited in the study, of whom, 2098 underwent IVF cycles and 911 received

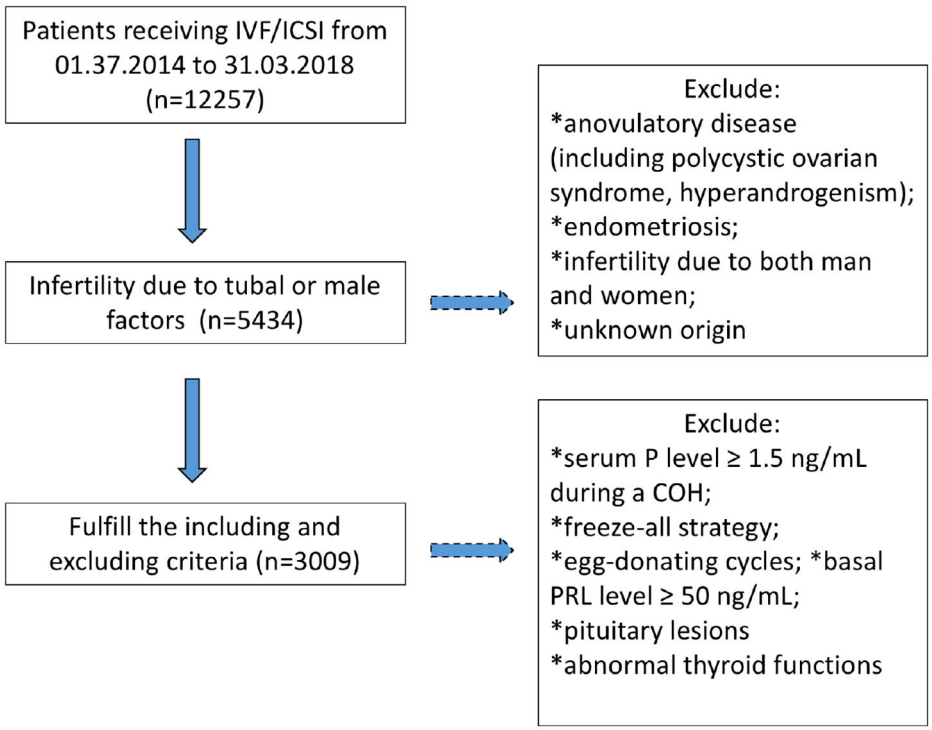

FIGURE 1 | Flowchart regarding patients' inclusion and exclusion. COH, controlled ovarian hyperstimulation; P, progesterone; PRL, prolactin.

ICSI (**Figure 1**). Their demographic characters were shown in **Table 1**. To explore the relationship between basal PRL (T0) and pregnancy outcomes, we divided patients into two groups by median PRL level (≤16.05 vs. >16.05 ng/mL). The two groups were compared in terms of baseline characteristics and pregnancy outcomes (**Table 1**). Patients with basal PRL >16.05 ng/m had slightly but significantly more oocytes retrieved, MII oocytes, fertilization, and embryos ($P < 0.05$). No statistically significant differences in cumulated CPR and LBR were detected between the two groups.

We further divided all patients into five groups according to different basal PRL levels: Group I with PRL 0–9.9 ng/ml, Group II, 10–19.9 ng/ml; Group III, 20–29.9 ng/ml; Group IV, 30–39.9 ng/ml; and Group V, 40–49.9 ng/ml (**Table 2**). Nearly half of the patients were distributed in Group II. Therefore, we applied Group II as a dummy variable. Other factors including age, basal FSH, rFSH starting dose, total consumption of rFSH, basal E_2, and BMI were entered into the multifactor analysis. It turned out the last two factors (basal E_2 and BMI) were not statistically significant. The results revealed that a higher basal PRL was related to a better rate of cumulated clinical pregnancy and live birth.

In order to analyze the fluctuation of PRL levels through COH in the GnRHa long protocol cycle and to examine whether the change in PRL level is related to IVF pregnancy outcomes, we compared the PRL levels between patients with positive and negative pregnancy results on T0 (basal status), T1 (end of follicular stage), and T2 (early initiation of luteal phase) (**Tables 3**, **4**). There were 1,585 cases with positive cumulated clinical pregnancy and 1,381 cases with cumulated live birth. The PRL levels of patients with positive pregnancy outcomes were significantly higher at all measurement points than those of patients with negative results. Moreover, a sharper spike was observed in groups with positive clinical pregnancy or live birth.

DISCUSSION

In this retrospective study, we analyzed the relationship between basal PRL levels, as well as their increasing tendency, and pregnancy outcomes of IVF/ICSI treatments for tubal/male factor infertility. Hyperprolactinemia has long been considered detrimental to fertility due to its effect on blocking LH secretion, leading to anovulation, or luteolysis (2). However, in IVF, oocyte maturation is induced by hCG trigger, and sufficient luteal phase support is guaranteed by progesterone supplements. Therefore, IVF procedures provide an ideal opportunity to observe the potential effect of PRL on reproduction in comparison to suppression of gonadotropins. This study was designed to answer two main questions: [1] Are cumulative pregnancy outcomes better in women with higher basal PRL levels when it is under 50 ng/mL?; [2]. In cycles with better pregnancy outcomes, will there be greater increase of PRL throughout ovarian stimulation (basal state, hCG day, and the day after hCG triggering)?

Around 85% of PRL molecules in circulation are 23 kDa monomers, which is the major bioactive form of PRL. Approximately a quarter of patients with hyperprolactinemia are shown to have macroprolactinemia. Women with

TABLE 1 | Comparisons of baseline characteristics and pregnancy outcomes between patients with PRL ≤ 16.05 or > 16.05 ng/mL.

	PRL ≤ 16.05 ng/mL (1506)		PRL > 16.05 ng/mL (1503)		*P*
Age (years)	35.215	4.391	34.613	4.257	0.079
BMI (kg/m^2)	22.364	3.186	21.835	3.063	0.102
Duration of infertility (years)	4	2, 6	4	3, 6	0.978
Basal sexual hormone					
FSH (IU/L)	7.130	5.770, 9.000	7.190	5.930, 9.010	0.343
LH (IU/L)	3.655	2.520, 5.093	3.840	2.730, 5.230	0.193
E_2 (pg/mL)	45.910	34.985, 58.730	46.240	35.320, 60.260	0.344
rFSH starting dose (ampoule)	4	3, 4	4	3, 4	0.608
Oocytes retrieved	8	5, 11	9	5, 12	0.013
MII oocytes	6	3, 10	7	4, 10	0.008
Zygotes	5	3, 9	6	3, 10	0.012
Embryos	5	3, 9	6	3, 10	0.015
Cumulative CPR	50.1%	755/1506	53.9%	810/1503	0.097
Cumulative LBR	44.5%	670/1506	47.3%	711/1503	0.065

Continuous variables following the normal distribution are presented as the mean (SD); non-normal distribution parameters are presented as the median (quartile); categorical variables are presented as percentages (with their frequencies).

TABLE 2 | Multifactor analysis of the relationship between basal PRL and pregnancy outcomes.

Outcomes	Group	Basal PRL (ng/mL)	%	Frequency (3,009 in total)	*P*	OR	95% CI
Cumulative clinical pregnancy	I	0–9.9	13.5	407	0.047	0.858	0.683, 0.879
	II	10–19.9	56.1	1,689	–	1.000	–
	III	20–29.9	21.8	655	0.569	0.997	0.823, 1.207
	IV	30–39.9	6.4	192	0.046	1.281	1.030, 1.764
	V	40–49.9	2.2	66	0.039	1.639	1.247, 2.837
Cumulative live birth	I	0–9.9	13.5	407	0.047	0.871	0.691, 0.997
	II	10–19.9	56.1	1,689	–	1.000	–
	III	20–29.9	21.8	655	0.354	1.030	0.850, 1.247
	IV	30–39.9	6.4	192	0.341	1.139	0.830, 1.562
	V	40–49.9	2.2	66	0.008	1.916	1.115, 3.290

Categorical variables are presented as percentages (with their frequencies), OR, and 95% CI. The clinical pregnancy rate and live birth rate are additionally adjusted for age, bFSH, rFSH starting dose, and total consumption of rFSH.

TABLE 3 | Comparison of serum PRL levels between different pregnancy outcomes at different time points by repeated-measures analysis of variance.

Pregnancy outcomes	T0 (ng/mL)	T1 (ng/mL)	T2 (ng/mL)
Cumulative clinical pregnancy	16.21 (12.34, 22.05)	32.7 (23.92, 43.86)	33.16 (24.38, 46.69)
No clinical pregnancy	15.85 (11.62, 21.15)	27.46 (19.29, 38.76)	30.12 (20.31, 42.06)
P-Value	0.011	< 0.001	< 0.001
Cumulative live birth	16.25 (12.34, 22.08)	33.06 (24.31, 43.82)	33.45 (24.61, 46.475)
No live birth	15.85 (11.74, 21.23)	27.84 (19.56, 39.47)	30.31 (20.67, 42.42)
P-Value	0.020	< 0.001	< 0.001

Non-normal distribution parameters are presented as median (quartile).

macroprolactinemia may have no symptoms despite their elevated serum PRL levels due to inactive macroprolactin (5). That is to say, some asymptomatic hyperprolactinemia may be caused by macroprolactinemia; thus, such patients may not need dopamine agonist administration before IVF treatment. However, macroprolactin was not measured in our study. Future research should study macroprolactin and the proportion of active PRL levels.

Kamel et al. found that women who conceived had a remarkable increase of PRL compared to women who did not conceive, supporting the variation we found between T2 vs. T1 and T1 vs. T0. Additionally, higher PRL levels were

TABLE 4 | Absolute difference of serum PRL between different time points.

Time point for comparison	Group	Positive outcomes (ng/mL)	Negative outcomes (ng/mL)	*P*
Δ_1PRL (T1–T0)	Cumulative clinical pregnancy	13.297 (15.096)	17.416 (15.959)	< 0.001
	Cumulative live birth	13.719 (15.330)	17.528 (15.852)	< 0.001
Δ_2PRL (T2–T1)	Cumulative clinical pregnancy	2.534 (9.962)	1.720 (10.565)	0.030
	Cumulative live birth	2.572 (9.990)	1.555 (10.611)	0.007

Continuous variables following the normal distribution are presented as the mean (SD).

associated with higher embryo quality (6). In our study, PRL levels were noted to increase throughout the COH. Unlike the high PRL levels because of ovarian stimulation, pre-existing hyperprolactinemia before IVF/ICSI treatment puzzles physicians the most. Doldi et al. prescribed cabergoline as pretreatment to women with hyperprolactinemia until egg-retrieval. Thus, the PRL levels were significantly lower than those of the control group who did not receive cabergoline. However, there was no improvement of CPR in patients treated with cabergoline adding the effect of rFSH consumption (38.1 ± 18.2 vs. 43.9 ± 28.5 ampoule; $P < 0.05$), lower MII oocyte rate (87.9 vs. 80.4%; $P < 0.05$), and fertilization rate (70.8 vs. 60.8%; $P < 0.03$) (7).

It is known that better IVF/ICSI outcomes are observed in patients with higher PRL levels in either the basal state or during COH. According to the present findings, the group with basal PRL level > 16.05 ng/mL experienced a surge in the numbers of oocytes, MII oocytes, zygotes, and embryos. Previous research by Mendoza et al. discovered that higher basal PRL levels are related to larger numbers of mature oocytes and good quality embryos (8), suggesting that PRL plays a role in oocyte maturation as well as embryonic development. Oogenesis is a complicated process involving oocytes and the granular cell cumulus actively exchanging signals within the circulating body fluid. Nakamura et al. reported that PRL receptor-knocked-out mice can only produce eggs with intact germinal vesicles (9). In contrast, higher mature rates were found when exogenous PRL was added to pre-antral follicle cultures of the IVF system (10). It could be a possible hypothesis that a certain PRL level guarantees the accomplishment of meiosis. Moreover, in the PRL receptor in deprived mice, there was a sharp decrease of the fertilization rate; most of the zygotes underwent retardation, and only 19% developed to blastocysts (11). PRL participates in embryo implantation via BRCA1, a protein expressed on the surface of the trophoblast cells. As the PRL concentration gradually increased in the pre-antral follicle culture (0, 10, 20 mIU/mL), BRCA1 expression also increased (12). Although there was no statistical significance, there was an increasing trend of the implantation rate from 47.0% in the control population to 56.1% when cultured with 20 mIU/mL PRL (12). Since PRL improved oogenesis and embryonic development, some researchers have tried to improve the IVF outcomes by prescribing bromocriptine to patients with a history of recurrent implantation failure until the initiation day of rFSH. Therefore, PRL rebounded to a higher level, and the CPR did improve compared to that in the controls (10.1 vs. 27.2% $P < 0.05$) owing to the significantly increased PRL (13).

In addition to its effect on oogenesis and embryogenesis, PRL also boosts other physiological reproductive activities. When either PRL genes or PRL-receptor genes were knocked out, a profound decrease in progesterone levels was noticed in the luteal phase of mice (11); moreover, the corpus luteum underwent early degradation 2 days after mouse intercourse (14). We revealed that a dramatic surge of PRL after luteinization was associated with better cumulated IVF/ICSI outcomes. This is consistent with the promoting effect of PRL on luteal function (15). PRL stimulates the long chain receptor in the luteinized cells to activate the Jak2/STAT5 pathway and suppress 20-α-hydroxysteroid dehydrogenase, subsequently spurring progesterone production. Meanwhile, multiple vascular endothelial growth factors are secreted into the ovaries to accelerate the vascularization of the corpus luteum when the PRL short chain receptor is stimulated. A human study by Daly et al. raised the concern that mid-luteal PRL levels were the lowest (15.0 ± 11.7 ng/mL) in women with early pregnancy loss compared to those who were infertile or expected to conceive normally (16). Furthermore, PRL acts on the adaptive immune system. PRL receptors are widely expressed on the surface of CD4+ T cells and B cells. Once stimulated, inflammatory factors such as interleukin-2 and interferon-gamma would be suppressed (17). This process might allow an immune privilege status between the maternal-fetal interface leading to a smoother pregnancy.

The major limitation of our study is that no causal relationship between PRL and IVF pregnancy outcomes could be inferred due to the study's retrospective nature. In multifactor regression, we found that the pregnancy outcomes became better as PRL increased. Nevertheless, the power of the test may be compromised due to the significantly different number of patients in each subgroup and the number of patients with hyperprolactinemia decrease with increasing basal PRL levels. Reasonably, the beneficial effect of PRL cannot continue permanently and constantly rising, and there should be an inflection point of PRL level beyond which, the advantageous effect on IVF/ICSI pregnancy outcomes would become harmful. However, in clinical practice, physicians are prone to prescribe dopamine agonists to patients with high PRL level > 50 ng/mL before entering a cycle; thus, we could not recruit such patients. Consequently, the inflection point could not be illustrated by our recruited sample. In this study, we targeted mainly at tubal or male factor infertility. Particularly we avoided including anovulation or endometriosis because these diseases possibly interfere with ovarian reserve or HPO axis and, in turn, affect the PRL status. For example, PCOS or hyperandrogenemia was both sorted to anovulatory disorders in our center and excluded. However, if a patient had not meet the full diagnosis criteria of PCOS, but merely demonstrated either a polycystic ovarian morphology or very mild hyperandrogenemia which appear not to interfere with regular ovulation, she could still be included as long as she was sorted as tubal or male factor infertility. This could lead to a potential bias since PCOS or hyperandrogenemia will slightly increase the PRL level. Another drawback of this study is we merely employed

basal FSH as the major ovarian reserve indicator. As we know, ovarian reserve markers are closely related to the number of oocytes retrieved as well as the CCPR and CLBR. However, our center has not initiated universal AMH test until 2019, and the data of AFC are not uniformly documented. Luckily we will have had enough AMH data to analyze in foreseeable future.

In conclusion, for patients receiving IVF/ICSI treatment with a basal PRL level within the range of 0–50 ng/mL, higher PRL levels were associated with higher numbers of oocytes, mature oocytes, zygotes, and embryos. Both the cumulative CRP and LBR increased with increasing PRL levels. There was a remarkable surge of PRL level from the basal status to the next day after hCG injection. The beneficial effect of PRL on pregnancy outcomes may be attributed to the facilitation of oogenesis and embryonic development, as well as the improvement of luteal function. Hence, in clinical settings, when physicians encounter a patient with asymptomatic hyperprolactinemia planning IVF/ICSI treatment, the serum PRL level may be not suppressed to an extremely low level if organic lesions are excluded.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of PUMCH (No. S-K601). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

DZ analyzed the data and drafted the manuscript. XY designed the study and revised the manuscript. DZ and XY did a major and equal contribution to this work. ZS was responsible for the data acquisition. CD analyzed and interpreted the data. QY edited the manuscript. JZ provided final approval for the submitted version and is responsible for the whole work. All authors have critically reviewed and approved the final submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 06 October 2020
doi: 10.3389/fendo.2020.537205

Endocrine Requirements for Oocyte Maturation Following hCG, GnRH Agonist, and Kisspeptin During IVF Treatment

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Objective: The maturation of oocytes to acquire competence for fertilization is critical to the success of *in vitro* fertilization (IVF) treatment. It requires LH-like exposure, provided by either human chorionic gonadotropin (hCG), or gonadotropin releasing hormone agonist (GnRHa). More recently, the hypothalamic stimulator, kisspeptin, was used to mature oocytes. Herein, we examine the relationship between the endocrine changes following these agents and oocyte maturation.

Design: Retrospective cohort study.

Methods: Prospectively collected hormonal data from 499 research IVF cycles triggered with either hCG, GnRHa, or kisspeptin were evaluated.

Results: HCG-levels (121 iU/L) peaked at 24 h following hCG, whereas LH-levels peaked at ~4 h following GnRHa (140 iU/L), or kisspeptin (41 iU/L). HCG-levels were negatively associated with body-weight, whereas LH rises following GnRHa and kisspeptin were positively predicted by pre-trigger LH values. The odds of achieving the median mature oocyte yield for each trigger were increased by hCG/LH level. Progesterone rise during oocyte maturation occurred precipitously following each trigger and strongly predicted the number of mature oocytes retrieved. Progesterone rise was positively associated with the hCG-level following hCG trigger, but negatively with LH rise following all three triggers. The rise in progesterone per mature oocyte at 12 h was greater following GnRHa than following hCG or kisspeptin triggers.

Conclusion: The endocrine response during oocyte maturation significantly differed by each trigger. Counter-intuitively, progesterone rise during oocyte maturation was negatively associated with LH rise, even when accounting for the number of mature

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *22 February 2020*
Accepted: *09 September 2020*
Published: *06 October 2020*

Citation:
Abbara A, Hunjan T, Ho VNA, Clarke SA, Comninos AN, Izzi-Engbeaya C, Ho TM, Trew GH, Hramyka A, Kelsey T, Salim R, Humaidan P, Vuong LN and Dhillo WS (2020) Endocrine Requirements for Oocyte Maturation Following hCG, GnRH Agonist, and Kisspeptin During IVF Treatment. Front. Endocrinol. 11:537205. doi: 10.3389/fendo.2020.537205

oocytes retrieved. These data expand our understanding of the endocrine changes during oocyte maturation and inform the design of future precision-triggering protocols.

Keywords: trigger, oocyte maturation, fertility, progesterone, *in vitro* fertilization treatment

INTRODUCTION

The World Health Organization recognizes subfertility as the fifth most serious global disability, affecting 1 in 6 couples (1). *In vitro* fertilization (IVF) is a supraphysiological process that simulates many of the physiological processes apparent during the natural menstrual cycle (2). The number of IVF cycles carried out annually is increasing (3), however 11.8% of cycles commenced did not progress to oocyte retrieval (3). Indeed, a recent international priority setting partnership designated the variation in oocyte number following IVF treatment as one of the top ten unresolved research uncertainties (4).

The "trigger" of oocyte maturation replicates the function of the mid-cycle ovulatory luteinizing hormone (LH) surge of the natural cycle (2) and provides LH-like exposure such that oocytes resume meiosis and advance to the metaphase II stage of development to acquire competence for fertilization (5). This LH-like activity can be provided by human chorionic gonadotropin (hCG), gonadotropin releasing hormone agonist (GnRHa), or kisspeptin. These agents have distinct mechanisms of action; namely hCG acts directly at ovarian LH receptors, GnRHa stimulates gonadotropin release from the pituitary gland, and kisspeptin stimulates the hypothalamus to induce release of endogenous GnRH (2). The hormone used to provide this LH-like exposure plays a determinant role in key outcomes affecting the success and safety of IVF treatment, including the ability to retrieve mature oocytes, luteal phase characteristics (impacting on pregnancy rates) and the occurrence of "ovarian hyperstimulation syndrome" (OHSS) (2).

HCG is the most widely used trigger in current practice, being applied in more than three quarters of cycles (6). As it shares the same alpha subunit, and has 85% homology of the beta subunit as native LH, hCG activates the LH receptor (7). HCG has a greater affinity for the LH receptor than native LH and activates distinct intracellular signaling, with a five-fold increased potency for cAMP activity in granulosa cells, whereas LH preferentially activates extracellular signal-related kinase 1/2 and protein kinase B (8). Overall, hCG has a greater steroidogenic action consistent with a critical role in supporting pregnancy, whereas LH has a stronger anti-apoptotic signal (2). Furthermore, hCG ($t_{1/2}$ 28–29 h) has a longer half-life than native LH ($t_{1/2}$ ~20 min), and thus also risks OHSS (2). GnRHa activates the pituitary gland to induce a shorter duration of LH exposure and thus has a lower risk of OHSS than hCG (9). Thus, GnRHa is usually reserved for patients at increased risk of OHSS, as more intensive luteal phase support is required to maintain pregnancy rates (9, 10).

More recently, kisspeptin has been used to safely induce oocyte maturation even in women at increased risk of OHSS (11–13). Kisspeptin stimulates hypothalamic GnRH neurons to release endogenous GnRH (14). Not all GnRH receptors on pituitary gonadotrophs are contiguous with GnRH neuronal terminals, and thus kisspeptin induces a tempered LH rise compared to equimolar doses of GnRH (15). Indeed, kisspeptin induced even fewer signs and symptoms of OHSS in women at increased risk of OHSS (16). This may in part be mediated through an additional direct ovarian action to reduce ovarian vascular endothelial growth factor (VEGF) production (13).

The ability to accurately quantify oocyte maturation facilitates the assessment of the minimum endocrine requirements for oocyte maturation. Different measures have been used to quantify oocyte maturation, including the absolute number of mature oocytes and the "oocyte maturation rate" (proportion of oocytes retrieved that are mature). However, suboptimal oocyte maturation can lead to fewer oocytes being retrievable, leading to a reduction in the denominator as well as the numerator, and thus the "oocyte maturation rate" is often preserved even in the context of suboptimal oocyte maturation. Consequently, we advocate the use of the "mature oocyte yield" defined by the number of mature oocytes expressed as a proportion of the number of follicles on the day of trigger most likely to yield a mature oocyte (17). While estimates for this follicle size have ranged from 10 to 14 mm (18–20), we have determined that follicles with a diameter of 12 to 19 mm on day of trigger are most likely to yield a mature oocyte (17). In the present study, we used this measure to evaluate the minimal endocrine requirements for LH-like activity (either LH or hCG level) for oocyte maturation following hCG, GnRHa and kisspeptin triggers.

MATERIALS AND METHODS

We conducted a comprehensive retrospective analysis of endocrine profiles prospectively collected from 499 IVF cycles triggered with either hCG, GnRHa, or kisspeptin. These cohorts were chosen as detailed endocrine data were collected following each trigger. The primary objective was to investigate the relationship between LH-like exposure after each trigger and the efficacy of oocyte maturation as quantified by the mature oocyte yield (MOY). The secondary objective was to assess determinants of the level of LH-like activity following each trigger and of the rise in progesterone during and after oocyte maturation.

Study Participants

Participants were aged 18 to 42 years, body mass index (BMI) 18 to 30 kg/m^2, and antral follicle count (AFC) 4 to 87. HCG and GnRHa-triggered cycles were oocyte donation research cycles

conducted at My Duc Hospital in Vietnam, whereas kisspeptin-triggered cycles were conducted at Hammersmith Hospital in the UK. Data on kisspeptin have been published in (11–13). Data on GnRH agonist triggered cycles have been published in (21). Data on hCG/hormonal levels during the luteal phase have been published in (22, 23) and reviewed in (24).

Study Approvals

All subjects gave written informed consent in accordance with the Declaration of Helsinki and Good Clinical Practice. Data from GnRHa triggered IVF cycles were obtained from a single-center randomized controlled trial conducted at My Duc Hospital, Ho Chi Minh City, Vietnam (21). The Institutional Review Board (IRB) reference number was NCKH/CGRH_01_2014 and ClinicalTrials.gov registration was NCT02208986. For the hCG case-series, the IRB reference number was NCKH/CGRH_09_2017, ethical approval reference number: 10/17/DD-BVMD and ClinicalTrials.gov Identifier: NCT03174691. For the kisspeptin trial, ethical approval was granted by the Hammersmith and Queen Charlotte's Research Ethics Committee, London, UK (reference: 10/H0707/2), undertaken at the IVF Unit at Hammersmith Hospital under a license from the UK Human Fertilization and Embryology Authority (11–13) and registered on the National Institutes of Health Clinical Trials database (NCT01667406).

Stimulation Protocol

Follicular stimulation was conducted using a GnRH antagonist co-treated cycle. FSH stimulation was in the form of 150 to 300 IU of follitropin-β for hCG-triggered, corifolliotropin alfa (Elonva; Merck Sharp & Dohme, UK) for GnRHa-triggered, and recombinant FSH (112.5–150 IU Gonal F, Merck Serono, Geneva, Switzerland) for kisspeptin-triggered cycles. The triggers recombinant hCG (250 μg) or GnRHa triptorelin (0.2–0.4 mg) were administered as soon as two follicles reached a size of ≥17 mm. The trigger kisspeptin-54 (6.4–12.8 nmol/kg as a single subcutaneous bolus or 19.2 nmol/kg as a split bolus over 10 h, Bachem Holding AG, Bubendorf, Switzerland) was administered once three follicles reached ≥18 mm. Patients who received two doses of kisspeptin were not included in analyses of parameters potentially affected by the second dose (outcomes after 10 h).

Hormonal parameters (LH, FSH, estradiol, and progesterone) were measured at regular intervals following hCG (0,12, 24, 36, 60, 84, 108, 132, 156, 180 h), GnRHa (0, 4, 12, 24, 36, 84, 132, 180, 228 h), and kisspeptin (0, 4, 10, 12, 14, 20, 36 h). HCG and GnRHa triggers were used in oocyte donation cycles allowing full examination of endocrine profiles during the luteal phase, whereas timepoints after 36 h following kisspeptin-triggered cycles were not examined due to contamination from luteal phase support. Only patients who had an ultrasound scan on the day of trigger were included in the analyses of the "mature oocyte yield."

Assays

GnRHa/hCG group: all samples were processed immediately and stored at −20°C. Serum hormone levels were determined using electrochemiluminescence immunoassay (ECLIA; Roche Cobas E 801, Roche Diagnostics, Germany). Lower level of quantification, inter-assay variability and intra-assay variability were LH 0.1 iU/L, 2–5% and 2–5%; hCG 0.1 iU/L, 2–5% and 2–5%; progesterone 0.5 ng/ml, 2–6% and 2–4%; and estradiol 5 pg/ml, 2–6%, and 2–4%, respectively. Kisspeptin group: serum LH, FSH, estradiol, and testosterone were measured using automated chemiluminescent immunoassays (Abbott Diagnostics, Maidenhead, UK). Interassay coefficients of variations were as follows: LH, 3.4%; FSH, 3.5%; estradiol, 3.4%; testosterone, 3.6%. Limits of detectability for each assay are as follows LH, 0.07 iU/L; FSH, 0.05 iU/L; estradiol, 70 pmol/L (19 pg/ml); testosterone, 0.08 nmol/L.

Statistical Analysis

Normality was assessed by D'Agostino and Pearson test and equality of variances by F-test for two groups or the Brown-Forsythe test for multiple groups. Parametrically distributed continuous variables were analyzed by t-test for two groups, or one-way ANOVA with *post hoc* Tukey's test for multiple groups, and non-parametrically distributed data by the Mann Whitney U test for two groups, or the Kruskal Wallis test with *post hoc* Dunn's test for multiple groups. Simple linear regression was used to analyze the relationship between two continuous variables. Categorical variables were analyzed by logistic regression. Neural net and random forest models were used to quantify (a) the accuracy of the data when used to predict mature eggs and (b) the relative importance of LH/hCG as a predictor variable. Data were analyzed using GraphPad Prism version 8.0 and STATA version 14. P-value < 0.05 was regarded as indicating statistical significance.

RESULTS

Baseline characteristics are presented in **Supplementary Table 1**.

Endocrine Profiles Following hCG, GnRHa, and Kisspeptin

Mean LH levels peaked at ~4 h following GnRHa (140.4 iU/L) and kisspeptin (41.4 iU/L), whereas hCG levels (121.0 iU/L) peaked at ~24 h following hCG trigger (**Figure 1**). The initial progesterone rise (**Figure 1D**) peaked at 24 h following GnRHa (69.4 nmol/L) and hCG (51.4 nmol/L), and at ~14 h following kisspeptin (22.0 nmol/L).

Serum LH/hCG Levels Following Trigger

Body-weight negatively predicted hCG levels at 24 h following hCG trigger ($r = -4.2$, $r^2 = 0.22$, $P < 0.0001$; **Figure 2A**). LH rise at 4 h (but not 12 h) was negatively predicted by BMI after GnRHa ($r = -3.3$, $r^2 = 0.05$, $P = 0.008$), whereas LH rise at 12 h was greater in women with body-weight >75 kg following kisspeptin (**Figure 2B**). Kisspeptin was dosed using a weight-based regimen, which may in part explain this result. However, higher pre-trigger LH levels with increased body-weight could also contribute to the increased rise in LH in those with increased body weight (**Figure 2C**). Indeed, the pre-trigger LH level was

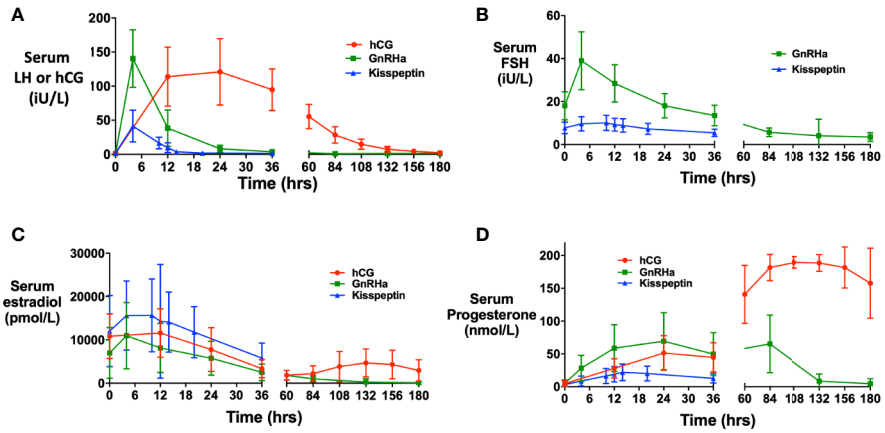

FIGURE 1 | Hormonal responses after each trigger of oocyte maturation. **(A)** Mean (± SD) of change in serum human chorionic gonadotropin (hCG) levels (iU/L) over time in response to hCG (red), and change in serum LH after GnRH agonist (green) and kisspeptin (blue). **(B)** Mean (± SD) of change in serum FSH (iU/L) over time in after GnRHa (green) and kisspeptin (blue). **(C)** Mean (± SD) of change in serum estradiol (pmol/L) over time after hCG (red), GnRHa (green) and kisspeptin (blue). **(D)** Mean (± SD) of change in serum progesterone (nmol/L) over time after hCG (red), GnRHa (green) and kisspeptin (blue).

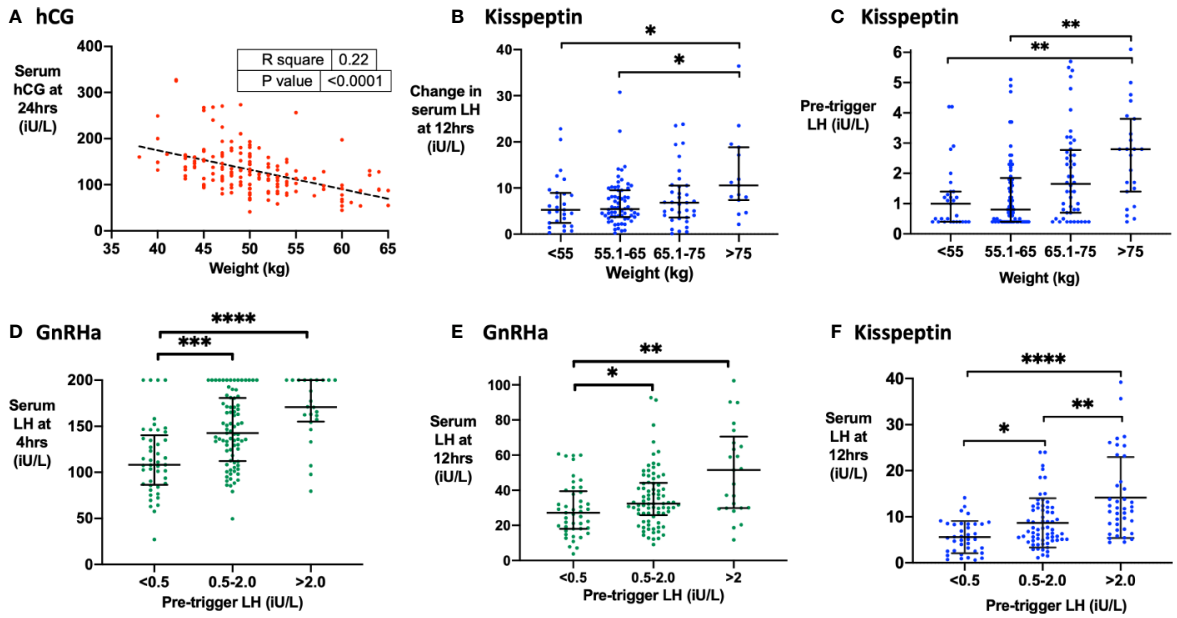

FIGURE 2 | Factors that determine hCG levels after hCG trigger and the LH level after GnRHa or kisspeptin. **(A)** Pre-treatment weight (kg) negatively predicted serum hCG level at 24 h (iU/L) after hCG trigger by simple linear regression (n = 161). Serum hCG at 24 h (iU/L) = –4.21 X body weight (kg) + 343.1, $r^2 = 0.22$, $P < 0.0001$. **(B)** Median (IQR) of change in serum LH at 12 h (iU/L) by categories of body weight (kg) after kisspeptin (n = 141). Categories were compared by the Kruskal-Wallis test with post-hoc Dunn's multiple comparison test. **(C)** Median (IQR) of pre-trigger serum LH (iU/L) by categories of weight (kg) after kisspeptin (n = 173). Categories were compared by the Kruskal-Wallis test with post-hoc Dunn's multiple comparison test. Three outliers were not shown on the graph. **(D)** Median (IQR) of serum LH at 4 h (iU/L) by categories of pre-trigger LH after GnRHa (n = 150). Categories were compared by the Kruskal-Wallis test with post-hoc Dunn's multiple comparison test. **(E)** Median (IQR) of serum LH at 12 h (iU/L) by categories of pre-trigger LH after GnRHa is presented (n = 151). Categories were compared by the Kruskal-Wallis test with post-hoc Dunn's multiple comparison test. Two outliers were not shown on the graph. **(F)** Median (IQR) of serum LH at 12 h (iU/L) by categories of pre-trigger LH after kisspeptin (n = 142). Categories were compared by Kruskal-Wallis test with post-hoc Dunn's multiple comparison test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

the strongest predictor of LH rise following both GnRHa and kisspeptin. Specifically, LH rise at 4 h and 12 h following GnRHa (**Figures 2D, E**) and at 12 h following kisspeptin (**Figure 2F**) were positively predicted by pre-trigger LH level.

Endogenous LH Levels Following hCG Trigger

HCG levels at 24 h were negatively associated with endogenous LH levels at 24 h ($r = -5.46$, $r^2 = 0.035$, $P = 0.018$), but there was no significant association between hCG and LH levels at any later timepoint. Women with higher endogenous LH levels prior to hCG trigger continued to have higher LH levels after hCG-levels started to fall following hCG trigger (i.e. pre-trigger LH was positively associated with LH at 24 h following hCG; r 0.49, r^2 0.35, $P < 0.0001$).

Effect of LH/hCG on Oocyte Maturation

Notably, there was little to no association between levels of LH-like activity and the number of mature oocytes after all three triggers. Indeed, neither hCG levels at 12 h, 24 h (**Supplementary Figure 1A**) or 36 h after hCG, nor LH rise at 4 h **(Supplementary Figure 1B)**, 12 h (**Supplementary Figure 1C**), or 24 h following GnRHa were associated with the number of mature oocytes retrieved. After kisspeptin, LH rise at 12 h ($P = 0.048$) (but not at 4 h or 36 h) was weakly associated with the number of mature oocytes retrieved (**Supplementary Figure 1D**).

We analyzed the cumulative "mature oocyte yield" (MOY; number of mature oocytes divided by the number of follicles of 12–19 mm on the day of trigger) to identify the threshold of hCG/LH level, beyond which there was unlikely to be any significant additional benefit to oocyte maturation from higher levels. Binary thresholds were ~80 iU/L for hCG at 24 h, ~25 iU/L at 12 h following GnRHa and ~10 iU/L at 12 h following kisspeptin (**Supplementary Figures 1E–H**). MOY was not significantly associated with the peak hCG level at 24 h (**Figure 3A**). LH at 4 h (**Figure 3B**) following GnRHa was associated with the mature oocyte yield (MOY), but not at 12 h (**Figure 3C**), or later timepoints. LH > 10 iU/L at 12 h following kisspeptin (but not at 4 h or 36 h) was associated with increased MOY (**Figure 3D**).

We investigated the reliability of triggering oocyte maturation at different hCG/LH thresholds by assessing the proportion of patients achieving the median MOY for each trigger. The odds of achieving the median MOY for hCG was increased by 3.4-fold (95% CI 0.97–11.7) in those with an hCG level >160 iU/L vs <80 iU/L (**Figure 3E**). For GnRHa, the odds of achieving the median MOY was increased by 4.2-fold (95% CI 1.4–13.0) if 4 h LH >199 iU/L vs <100 iU/L (**Figure 3F**). The proportion achieving the median MOY following GnRHa was 43% if LH at 12 h was <25 iU/L, 52% if 25 to 49.9 iU/L, and 61% if >50 iU/L ($P = 0.29$) (**Figure 3G**). For kisspeptin, the odds of achieving the median MOY was increased by 4.6-fold (95% CI, 1.4–14.6) if 12 h LH >10 iU/L vs <5 iU/L (**Figure 3H**).

Random forest models outperformed neural network models when predicting the number of mature oocytes from baseline characteristics, hormone levels and number of 12 to 19 mm follicles (88% accuracy of prediction to within 3 mature eggs for random forests; 57% accuracy for neural networks). Random forest accuracy fell from 88% to 83% when data on LH/hCG levels were not included.

We evaluated the area under the curve over 36 h for hCG levels following hCG trigger, or for LH following GnRHa or kisspeptin triggers, however this measure was not associated with either the number of mature oocytes, nor the MOY *($P>0.14$* for all*)*. There was no significant difference in "oocyte maturation rate" or "fertilization rate" by category of hCG/LH following any trigger (**Supplementary Figure 2**).

Progesterone Rise During Oocyte Maturation

Progesterone peaked at 24 h to 51.2 nmol/L following hCG trigger consistent with granulosa cell luteinization/oocyte maturation, before a subsequent greater rise at 108 h (4.5 days post-hCG) to 190.8 nmol/L corresponding to secretion from corpora lutea. Similarly, progesterone peaked to 69.4 nmol/L at 24 h following GnRHa, before a secondary rise to 66.4 nmol/L at 84 h (3.5 days) post GnRHa. Following kisspeptin, progesterone peaked to 22.3 nmol/L at 14 h (luteal progesterone levels were not assessed due to contamination from luteal phase support).

Endogenous LH rise at 12 h following hCG was negatively associated with serum hCG (**Figure 4A**). Progesterone rise at 12 h was positively predicted by hCG level following hCG trigger (**Figure 4B**) but was negatively predicted by LH rise following all triggers (**Figures 4C–F**).

The strongest biochemical predictor of the number of mature oocytes retrieved was the rise in serum progesterone. Progesterone rise at 12 h predicted the number of mature oocytes following hCG ($r^2 = 0.23$, $P < 0.0001$), GnRHa ($r^2 = 0.29$, $P < 0.0001$) and kisspeptin (r^2 0.18, $P < 0.0001$) (**Figures 5A–D**). The relationship between MOY and progesterone rise at 12 h was much weaker (r^2 hCG 0.07, GnRH 0.02, KP 0.02). To assess, whether each oocyte produced the same amount of progesterone during maturation after the different triggers we assessed progesterone rise per mature oocyte. Accordingly, progesterone rise at 12 h per mature oocyte was greater after GnRHa (3.18 nmol/L) than hCG (1.7 nmol/L) or kisspeptin (1.99 nmol/L) (**Figure 5H**). Similarly, the progesterone rise per mature oocyte was greater with hCG level following hCG trigger, but lesser with LH rise at 12 h following other triggers (**Figures 5E–G**).

Data comparing luteal estradiol and progesterone rises are presented in Supplemental Data.

DISCUSSION

We examined the hormonal responses following three triggers of oocyte maturation with distinct mechanisms of action to provide an insight into the endocrine requirements for oocyte maturation. HCG levels peaked at 24 h following hCG (albeit similar levels were encountered by 12 h), whereas LH-levels peaked sooner at 4 to 6 h following both GnRHa and kisspeptin. It is likely that a threshold level for LH-like activity needs to be

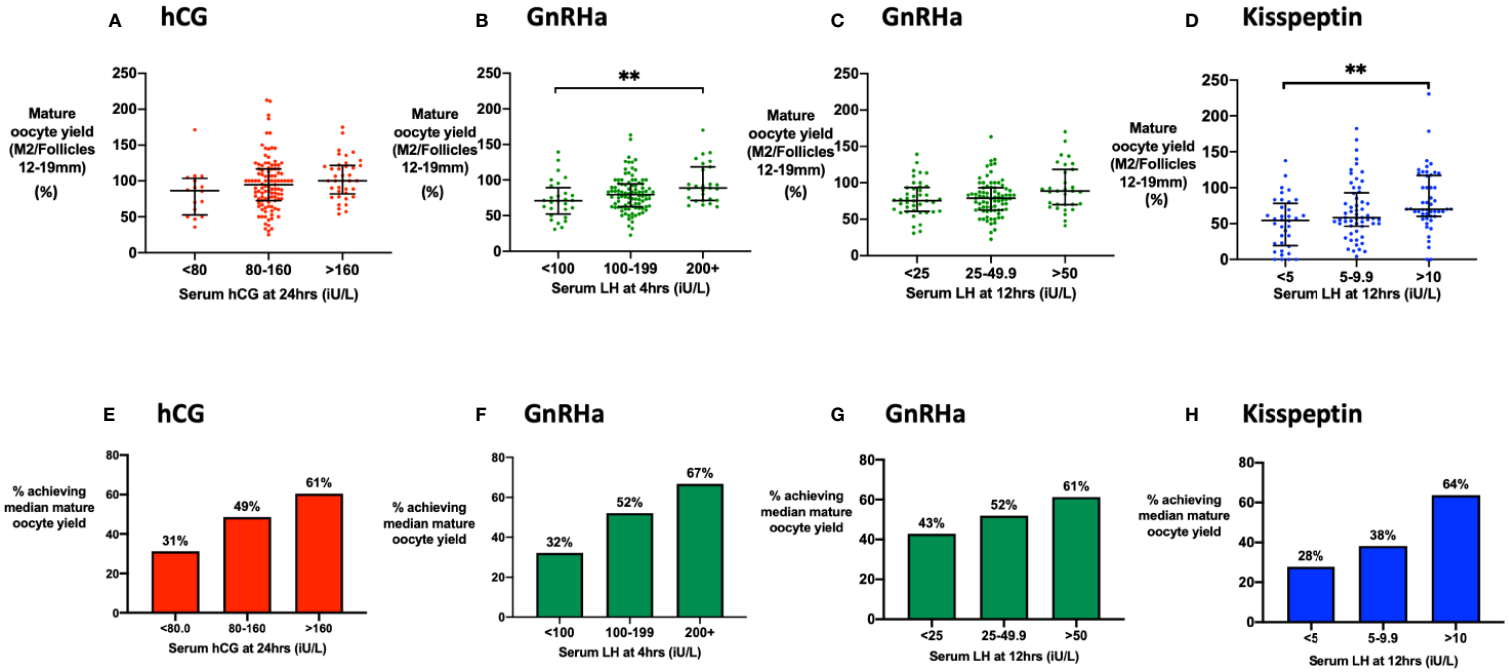

FIGURE 3 | Mature oocyte yield by level of LH-like activity. **(A)** Median (IQR) of the mature oocyte yield (number of mature oocytes divided by the number of follicles of 12 to 19 mm in diameter on day of trigger) by categories of serum hCG at 24 h (n = 161). Categories were compared by the Kruskal-Wallis test with post-hoc Dunn's multiple comparison test. **(B)** Median (IQR) of the mature oocyte yield by categories of serum LH at 4 h after GnRHa (n = 151). Categories were compared by Kruskal-Wallis test with post-hoc Dunn's multiple comparison test. **(C)** Median (IQR) of the mature oocyte yield by categories of serum LH at 12 h after GnRHa is presented (n = 152). Categories were compared by Kruskal-Wallis test with post-hoc Dunn's multiple comparison test. **(D)** Median (IQR) of the mature oocyte yield by categories of serum LH at 12 h after kisspeptin (n = 142). Categories were compared by the Kruskal-Wallis test with post-hoc Dunn's multiple comparison test. **(E)** The proportion of women achieving the median mature oocyte yield with hCG used as a trigger were compared by categories of serum hCG at 24 h: <80 (n = 16), 80–160 (n = 107), > 160 (n = 38). Categories were compared by logistic regression: $P = 0.13$. **(F)** The proportion of women achieving the median mature oocyte yield with GnRHa were compared by serum LH at 4 h: <100 iU/L (n = 28), 100–199 iU/L (n = 96), >200 iU/L (n = 27). Categories were compared by logistic regression: The odds of achieving the median mature oocyte yield was increased by 4.22-fold (95% CI 1.37–13.02; P = 0.012) if LH at 4 h following GnRHa was at least 200 iU/L compared to <100 iU/L. **(G)** The percentage achieving the median mature oocyte yield with GnRH used as a trigger were compared by serum LH at 12 h: <25 iU/L (n = 42), 25–49.9 iU/L (n = 79), >50 iU/L (n = 31). Categories were compared by logistic regression $P = 0.29$. **(H)** The percentage achieving the median mature oocyte yield after kisspeptin by serum LH at 12 h: <5 iU/L (n = 18), 5–9.9 iU/L (n = 34), >10 iU/L (n= 58) was compared by logistic regression. The odds of achieving the median mature oocyte yield was increased by 4.6-fold (95% CI 1.4–14.6; $P = 0.010$) if LH at 12 h following kisspeptin was >10 iU/L compared to <5 iU/L. $^{**}P < 0.01$.

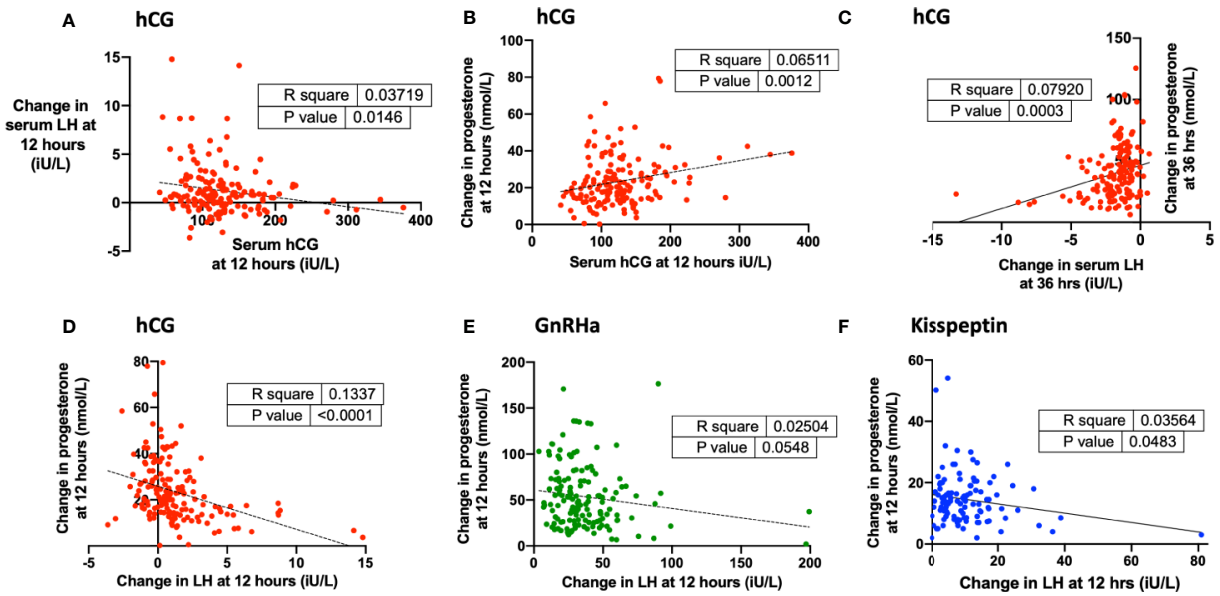

FIGURE 4 | Endogenous LH levels after each trigger and relationship with progesterone rise during oocyte maturation. **(A)** Change in serum hCG at 12 h (iU/L) was negatively associated with change in serum LH at 12 h (iU/L) after hCG by simple linear regression (n = 161). Change in serum hCG at 24 h = –0.010 × change in serum hCG at 12 h + 2.47, r^2 = 0.04, *P* = 0.01. **(B)** Serum hCG at 12 h (iU/L) was negatively associated with change in progesterone at 12 h (nmol/L) after hCG by simple linear regression (n = 159). Change in progesterone at 12 h (nmol/L) = 0.065 x serum hCG at 12 h (iU/L) + 15.3, r^2 = 0.065, *P* = 0.001. **(C)** Change in serum LH at 36 h (iU/L) negatively predicted change in progesterone at 36 h (nmol/L) after hCG by simple linear regression (n = 160). Change in progesterone at 36 h (nmol/L) = 3.52 X change in serum LH at 36 h + 46.4, r^2 = 0.08, *P* = 0.0003. **(D)** Change in LH at 12 h (iU/L) was negatively associated with change in progesterone at 12 h (nmol/L) after hCG by simple linear regression (n = 160). Change in progesterone at 12 h (nmol/L) = –1.86 × change in serum progesterone at 12 h + 25.85, r^2 = 0.133, *P* < 0.0001. **(E)** Change in LH at 12 h (iU/L) was weakly negatively associated with change in progesterone at 12 h (nmol/L) after GnRHa by simple linear regression (n = 148). Change in progesterone at 12 h (nmol/L) = –0.202 × change in serum progesterone at 12 h + 60.98, r^2 = 0.025, *P* = 0.05. **(F)** Change in LH at 12 h (iU/L) was negatively associated with change in progesterone at 12 h (nmol/L) after kisspeptin by simple linear regression (n = 110). Change in progesterone at 12 h (nmol/L) = –1.522 × change in serum progesterone at 12 h + 16.16, r^2 = 0.036, *P* = 0.05.

reached to initiate the process of oocyte maturation. The timing of oocyte retrieval is precisely controlled to occur following oocyte maturation but prior to ovulation (2). Consequently, it is noteworthy that the same interval between trigger and oocyte retrieval is ordinarily used (36–37 h) following all three triggers despite the different times of peak LH-like activity. Although near maximal levels of hCG are achieved by 12 h, it is conceivable that the threshold to initiate oocyte maturation is exceeded even sooner after hCG administration, closer to the 4 h peak observed following GnRHa and kisspeptin to permit a similar duration for oocyte maturation to occur after each trigger.

We investigated the threshold for LH-like activity required for oocyte maturation. A lower level of LH-like activity was sufficient for efficacious oocyte maturation in some women, however oocyte maturation was more reliable with higher levels. Indeed, the proportion exceeding the median mature oocyte yield for each trigger was increased by the level of LH-like activity achieved. Although the LH surge following kisspeptin was of lower amplitude than GnRHa (15), it is possible that kisspeptin could enhance oocyte maturation *via* an additional direct action at ovarian kisspeptin receptors to compensate for the lower LH rise (25). Indeed, kisspeptin enhances *in vitro* maturation of ovine (26) and porcine (27) immature oocytes.

GnRHa and kisspeptin triggers must be used in the context of a GnRH antagonist co-treated stimulation protocol. Thus, the GnRH antagonist must be competitively displaced from the GnRH receptor in order to generate a gonadotropin rise. Consequently, a low LH level prior to the trigger could reflect increased suppression from the GnRH antagonist, and there are data to suggest that escape from GnRH antagonist suppression varies between individuals, occurring more precipitously with greater BMI (28). Thus, the attenuated LH rise following GnRHa and kisspeptin with lower pre-trigger LH values, could reflect more pronounced suppression by the GnRH antagonist. However, it is also recognized that the LH value prior to native GnRH determines subsequent LH rise even in the absence of GnRH antagonist pre-treatment (29). Indeed, the risk of encountering an LH <15 iU/L at 12 h following GnRHa (a threshold commonly used to denote a suboptimal LH rise), occurred in 17.3% of those with a pre-trigger LH <0.5 iU/L but only 3.8% if pre-trigger LH was >2 iU/L. Thus, adjusting the timing and dose of the GnRH antagonist to avoid excessive suppression could further optimize the efficacy of the trigger (30).

Similarly, the hCG level following hCG trigger was negatively associated with body weight consistent with published reports (26). The cohort of women who received hCG in this study was from Vietnam and had a lower body weight in comparison to

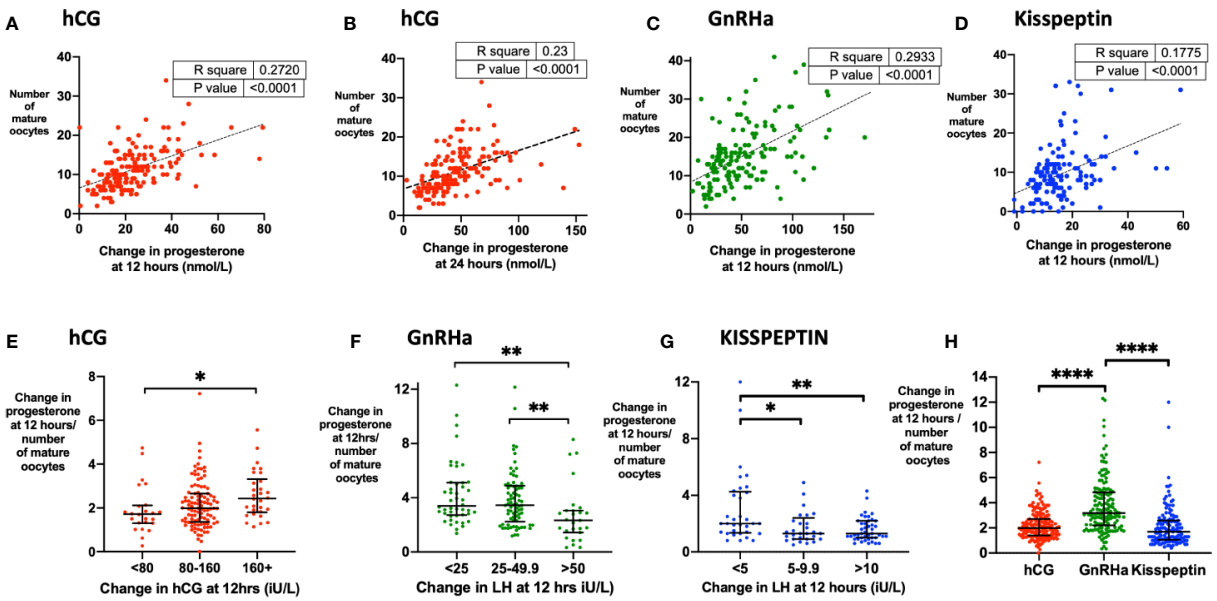

FIGURE 5 | Progesterone rise during oocyte maturation. **(A)** Change in progesterone at 12 h (nmol/L) after hCG trigger predicted the number of mature oocytes retrieved by simple linear regression (n= 159). Number of mature oocytes retrieved = 0.204 x change in progesterone at 12 h (nmol/L) + 6.60, $r^2 = 0.27$, $P < 0.0001$. **(B)** Change in progesterone at 24 h (nmol/L) after hCG trigger predicted the number of mature oocytes retrieved by simple linear regression (n= 161). Number of mature oocytes retrieved = 0.098 x change in progesterone at 24 h (nmol/L) + 6.8, $r^2 = 0.23$, $P < 0.0001$. **(C)** Change in progesterone at 12 h (nmol/L) after GnRHa trigger predicted the number of mature oocytes retrieved by simple linear regression (n= 151). Number of mature oocytes retrieved = 0.133 x change in progesterone at 12 h (nmol/L) + 8.37, $r^2 = 0.29$, $P < 0.0001$. One outlier has not been shown on the graph. **(D)** Change in progesterone at 12 h (nmol/L) after kisspeptin trigger predicted the number of mature oocytes retrieved by simple linear regression (n = 143). Number of mature oocytes retrieved = 0.297 x change in progesterone at 12 h (nmol/L) + 4.83, $r^2 = 0.18$, $P < 0.0001$. **(E)** Median (IQR) of change in progesterone at 12 h (nmol/L) divided by number of mature oocytes after hCG by categories of change in serum hCG at 12 h (iU/L) (n = 159). Categories were compared by the Kruskal-Wallis test with post-hoc Dunn's multiple comparison test. **(F)** Median (IQR) of change in progesterone at 12 h (nmol/L) divided by number of mature oocytes after GnRH by categories of change in LH at 12 h (iU/L) (n = 151). Categories were compared by the Kruskal-Wallis test with post-hoc Dunn's multiple comparison test. One outlier has not been shown on the graph. **(G)** Median (IQR) of change in progesterone at 12 h (nmol/L) divided by number of mature oocytes after kisspeptin by categories of change in LH at 12 h (iU/L) (n = 151). Categories were compared by the Kruskal-Wallis test with post-hoc Dunn's multiple comparison test. **(H)** Median (IQR) of change in progesterone at 12 h divided by number of mature oocytes after each trigger is presented: hCG (n = 159); GnRHa (n = 151), kisspeptin (n = 137). Categories were compared by Kruskal-Wallis test with *post hoc* Dunn's multiple comparison test. Four outliers were not shown on the graph. $^*P < 0.05$, $^{**}P < 0.01$,$^{****}P < 0.0001$.

western women. Thus, it is likely that western patients with higher body weights could encounter lower serum hCG levels more often, with a more detrimental impact on oocyte maturation than observed in the present study. Indeed, obese women have been reported to have an increased chance of encountering an hCG level <50 iU/L, with a subsequent increased risk of suboptimal oocyte maturation (31).

Interestingly, progesterone rose immediately following administration of the trigger and the level by 12 h was strongly predictive of the number of mature oocytes that would be retrieved. This was the case after all three triggers notwithstanding the fact that oocytes would not be mature had they been collected at this time-point. Logically, one would hypothesize that a greater LH rise following the trigger would result in increased oocyte maturation and a greater rise in progesterone. In fact, we observed that the LH rise after the trigger was inversely associated with progesterone rise. Progesterone is known to exert negative feedback on GnRH and LH during the luteal phase of the natural cycle (32, 33). As progesterone increased almost immediately following administration of the trigger, it is conceivable that progesterone tempered the LH rise following the trigger through negative feedback. Progesterone rise was strongly associated with the number of mature oocytes retrieved; therefore we examined the "progesterone rise per mature oocyte" and observed that this was greater for GnRHa than for either hCG or kisspeptin. This increased progesterone production during oocyte maturation may represent a specific characteristic of GnRHa triggering as the LH rise following GnRHa exceeds that by kisspeptin, which was associated with lower progesterone production.

Insufficient LH-like exposure increases the risk of "empty follicle syndrome"—a condition where no oocytes are retrieved (2). Early assessment of biochemical parameters to predict whether a mature oocyte is likely to be retrieved is therefore of value, as this can allow for re-administration of the trigger and rescheduling of oocyte retrieval to prevent a failure to retrieve oocytes. Logically, LH rise would be the measure that should confirm the successful deployment of the trigger. However, due to the inverse relationship of LH rise with progesterone rise and the closer relationship between progesterone rise and the number of mature oocytes retrieved, in fact progesterone rise

appears to be the most reliable predictive biochemical marker of successful oocyte maturation.

Peak progesterone levels after hCG occurred at 3 days following oocyte retrieval, however 89% of women still exceeded the limit of detection for progesterone at 156 h (day 5 post oocyte retrieval) when a blastocyst transfer is most usually conducted, whereas progesterone levels after GnRHa were already low by this timepoint. The mid-luteal rise in progesterone after hCG far exceeded that generated during oocyte maturation by ~4-fold, whereas estradiol rose during the luteal phase to ~2.5-fold higher levels than trough levels at 60 h post administration. Nevertheless, this suggests that both estradiol and progesterone can be used to monitor corpora luteal function (34).

Strengths of the study include access to detailed endocrine data following each trigger. Limitations include the heterogenous nature of the study population. Further prospective study with direct comparison in the same population is indicated to verify the findings presented.

In summary, we evaluated the endocrine profile following each trigger and assess its impact on oocyte maturation. An unexpected negative association between LH and progesterone rises was observed during oocyte maturation. Moreover, progesterone rise appears to be the most reliable biochemical predictive marker for oocyte maturation following all triggers and the progesterone rise per oocyte was higher following GnRHa trigger compared to other agents. These findings further explicate our understanding of the endocrine changes during induction of oocyte maturation.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Institutional Review Board (IRB) at My Duc Hospital, Ho Chi Minh City, Vietnam and Hammersmith and Queen Charlotte's Research Ethics Committee, London. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AA, TH, PH, LV, and WD designed the study. AA, TH, VH, SC, AC, CI-E, TH, GT, RS, PH, LV, and WD contributed to collection of the data. AA, TH, AH, and TK analyzed the data. All authors were responsible for the reporting of results and writing of the manuscript. LV and WD provided overall oversight. All authors contributed to the article and approved the submitted version.

FUNDING

The study was designed, conducted, analyzed and reported entirely by the authors. Data from clinical trials using kisspeptin was funded by independent research grants from the MRC, and NIHR and supported by the NIHR/Wellcome Trust Imperial Clinical Research Facility and Imperial Biomedical Research Centre. The views expressed are those of the author(s) and not necessarily those of the MRC, the NHS, the NIHR or the Department of Health. The Section of Endocrinology and Investigative Medicine is funded by grants from the MRC, BBSRC, NIHR, an Integrative Mammalian Biology (IMB) Capacity Building Award, an FP7-HEALTH-2009-241592 EuroCHIP grant and is supported by the NIHR Biomedical Research Centre Funding Scheme. Data from trials of GnRH agonist/hCG data were funded by My Duc Hospital, Ho Chi Minh City, Vietnam. AA is funded by an NIHR Clinician Scientist Award (CS-2018-18-ST2-002). CI-E is funded by an MRC Clinical Research Training Fellowship (MR/M004171/1). TK is supported by EPSRC EP/P015638/1. WD is funded by an NIHR Professorship (CDF-2009-02-05) and MRC grant (G1000455).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2020.537205/full#supplementary-material

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Conflict of Interest: AA and WD have undertaken consultancy work for Myovant Sciences Ltd who are developing a kisspeptin receptor agonist.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer AC declared a past co-authorship with one of the authors PH to the handling editor

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 05 November 2020
doi: 10.3389/fendo.2020.536100

The Relationship Between Serum Delta FSH Level and Ovarian Response in IVF/ICSI Cycles

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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *18 February 2020*
Accepted: *22 September 2020*
Published: *05 November 2020*

Citation:
Hu L, Sun B, Ma Y, Li L, Wang F, Shi H and Sun Y (2020) The Relationship Between Serum Delta FSH Level and Ovarian Response in IVF/ICSI Cycles. Front. Endocrinol. 11:536100. doi: 10.3389/fendo.2020.536100

Background: When ovarian response to FSH stimulation for IVF/ICSI is unsatisfactory, the FSH dose is often adjusted in the treatment cycles, thereby assuming that hormone status and follicular development were insufficient for optimal stimulation.

Objectives: To evaluate whether serum delta FSH levels between D6 of gonadotrophin use and basal serum FSH or between D6 of gonadotrophin use and D1 of gonadotrophin use predict ovarian response in IVF/ICSI cycles.

Method: The participants of this retrospective study were chosen from the Reproductive Medicine, The First Affiliated Hospital of Zhengzhou University between August 2015 and December 2017 (n = 3,109), and during the COS, each participant was given a fixed dose of rFSH in the first 6 days. Delta FSH1: The difference of serum FSH between D6 of gonadotrophin use and basal serum FSH. Delta FSH2: The difference of serum FSH between D6 of gonadotrophin use and D1 of gonadotrophin use. Logistic regression was used to analyze the association between delta FSH1 level and delta FSH2 level and ovarian response. Besides, we also use the tertile statistics to divide the groups.

Results: Part I: Delta FSH1 levels (mean: 1.41 ± 3.46) in normal responders were higher than delta FSH1 levels (mean: 1.07 ± 23.89) in hyper responders (P = 0.0248). The tertile of delta FSH1 is dif ≤ 0, 0 < dif ≤ 2.25 and dif > 2.25. Compared with the hyper responder, the delta FSH1 (0 < dif ≤ 2.25 and dif > 2.25) in the normal responder has a higher ratio and is statistically significant. Part II: Delta FSH2 levels (mean: 4.90 ± 2.84) in normal responders were similar with delta FSH2 levels (mean: 4.74 ± 2.09) in hyper responders (P = 0.103). The tertile of delta FSH1 is dif ≤ 3.91, 3.91 < dif ≤ 5.69 and dif > 5.69. Compared with the hyper responders, the delta FSH2 (3.91 < dif ≤ 5.69 and dif > 5.69) in the normal responders has a higher ratio and is statistically significant.

Conclusions: There is a weak relationship between ovarian response and serum delta FSH levels.

Keywords: IVF, ICSI, controlled ovarian stimulation, recombinant FSH, serum delta FSH level, ovarian response

INTRODUCTION

In the controlled ovarian stimulation (COS) for IVF/ICSI, the approach to obtain the optimal ovarian response is still an important topic to be discussed. Although more oocytes were considered to be better over the past few decades, we now aim for an optimal range of 8-15 oocytes (1, 2). Too few oocytes or a poor response is associated with higher rates of treatment cycle cancellation and lower pregnancy rates (3), but too many oocytes or a hyper response is also associated with higher rates of cycle cancellation and an increased risk of ovarian hyperstimulation syndrome (OHSS) (4, 5). To find a direct and non-invasive way to count the primordial follicles before the COS, many studies have been conducted to correctly predict the ovarian response to hyperstimulation (6). Until now, the antral follicle count (AFC) and circulating anti-Müllerian hormone (AMH) were the most accurate methods (7–10). In addition to ovarian reserve status (i.e., antral follicle number), antral follicle sensitivity, and FSH pharmacokinetics influence ovarian response (11), the focus of treatment individualization has been mainly on FSH dose adjustment.

The gonadotropin follicle stimulating hormone (FSH) plays a central role in the regulation of the menstrual cycle and the development of antral follicles (12, 13). For multi-follicular growth, high amounts of exogenous FSH are administered daily to achieve the FSH threshold (14, 15). Currently, recombinant FSH (rFSH) is the most widely used (16, 17), and it has shown bioavailability after single administration of 63–66% (18, 19). Additionally, it will reach the steady state after 5–7 days of repeated administration (20). FSH dose individualization based on an ovarian reserve test could theoretically improve IVF/ICSI treatment outcome. Although we adjusted the FSH doses according to body weight, oestrogen levels, and follicle condition, a range from 100 to 600 IU per day has been used in practice (21), without clear evidence suggesting that such extraordinarily high dosages are effective (22). There is an urgent need for substantiation of this concept by evaluating whether serum FSH levels during stimulation with a fixed FSH dose indeed differ between women with different ovarian responses.

Serum FSH levels measured during controlled ovarian stimulation with rFSH are an adequate reflection of the *in vivo* serum FSH levels to which the ovaries are exposed (23). Because the basal serum FSH level was relatively stable and the rFSH dose was unchanged in the first 6 days of FSH stimulation, the serum delta FSH levels (between D6 of Gn and basal serum FSH or between D6 of Gn and D1 of Gn) could be a more reliable marker for FSH dose adjustment. To our knowledge, no studies have been performed that directly evaluate the relationship between serum delta FSH levels during FSH stimulation and the ovarian response to COS for IVF/ICSI in GnRH agonist cycles. If we assume that serum delta FSH levels can be used to evaluate follicle sensitivity and FSH pharmacokinetics to a certain extent, then we have a better strategy to obtain to ideal number of retrieved oocytes. Moreover, avoiding the use of unnecessary gonadotropin reduces the cost of IVF/ICSI treatment. The aim of this study was therefore to assess whether serum delta FSH levels differ significantly between poor, normal, and hyper responders to a fixed daily dose of rFSH protocol in GnRH agonist cycles.

MATERIAL AND METHODS

Study Population

From the 23,667 women, 3,109 women were included in this study (**Figure 1**). The present study only includes patients who underwent the first IVF/ICSI-ET cycles in the Reproductive Medicine Center at the First Affiliated Hospital of Zhengzhou University between August 2015 and December 2017. The inclusion criteria were as follows: women aged ≤40 years with a regular menstrual cycle (average cycle length between 21 and 35 days) and an indication for IVF of ICSI. Patients were treated with an early follicular phase long-acting protocol. The rFSH starting dose was 112.5 IU. The exclusion criteria were as follows: body mass index (BMI) > 32 kg/m2, patients with ovarian surgical history, infertility induced by ovarian factors (including PCOS, POI, and endometriosis), and patients using urofollitropin (Livzon, China) in the stimulation cycles. Part I: According to the exclusion of missing basal serum FSH, 3,040 patients were included in the data analysis. Part II: According to the exclusion of missing serum FSH on D1 of gonadotrophin, 1,872 patients were included in the data analysis.

IVF/ICSI-ET Protocols

After reaching downregulation criteria, all patients were given a 112.5 IU rFSH starting dose. The dose was unchanged for the first 6 days of Gn cycles during ovulation induction. After that, the Gn will be increased or decreased in a timely manner according to the number, size, and growth of follicles. The researchers will determine the increase and decrease in Gn based on the hormone status and follicular development. The criteria for HCG injection include the following: when the diameter of one primary follicle is ≥20 mm and the diameter of the other follicles is ≥18 mm or the quantity of follicles with a diameter of ≥14 mm accounts for more than 2/3 of the follicles. The trigger drug and HCG exposure time will be determined by the researchers according to the patient's body weight, oestrogen levels, and follicle condition. Eggs will be retrieved 36–37 h after HCG administration.

Outcome Measure

Part I: Delta FSH1 level (the difference between serum FSH level on D6 of Gn use and basal serum FSH level) was the primary outcome measure.

Part II: Delta FSH2 level (the difference between serum FSH level on D6 of Gn use and serum FSH level on D1 of Gn use) was the primary outcome measure.

Grouping Method

Step 1: In parts I and II, we used definitions of ovarian response based on GnRH agonist protocols. In accordance with the Bologna criteria, poor response was defined as the retrieval of

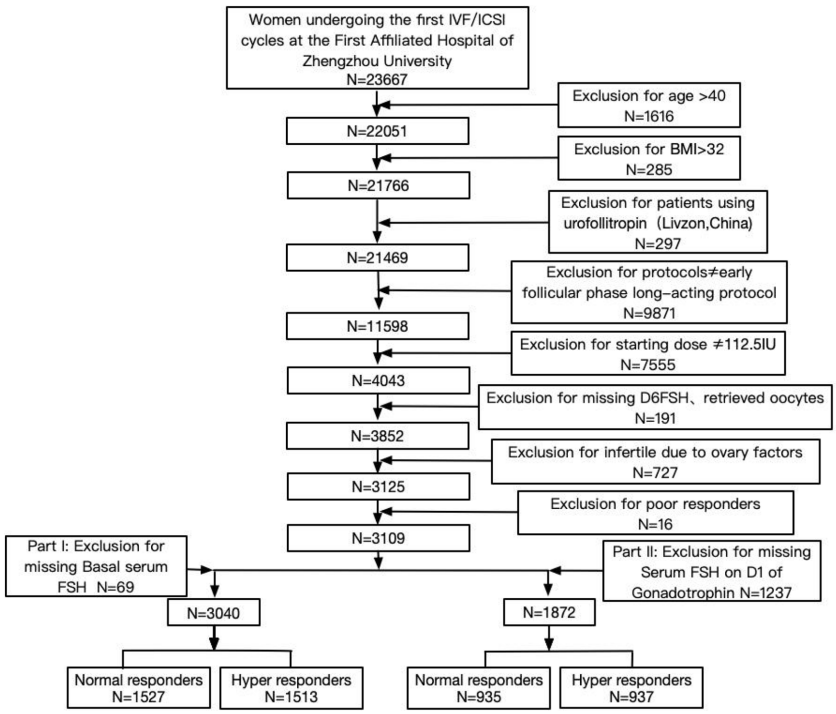

FIGURE 1 | Patient selection flowchart.

less than four oocytes (irrespective of oocyte maturity) or cancellation due to poor ovarian response (less than three dominant follicles of >12 mm). Normal response was defined as the retrieval of 4–15 oocytes, and hyper response was defined as the retrieval of more than 15 oocytes or cancellation due to an anticipated risk of OHSS.

Step 2: Because no consensus exists on delta FSH levels, we used tertile statistics in Part I and Part II.

Statistical Analysis

Data were analysed using the SPSS statistical package (SPSS version 13.0, Chicago, IL). Measurement data are described as the means ± sd, and differences between the groups were compared using Student's t-test and Wilcox's test. The categorical variables were compared using a Chi square test or Fisher test, where appropriate. Multivariable statistical analysis was used to assess the relationship between serum delta FSH levels and the number of retrieved oocytes. All data are reported as the mean with their associated standard deviations, and all tests were two-tailed. $P < 0.05$ was considered statistically significant.

RESULTS

Baseline Information and Outcomes of Patients

Of the 23,667 women included, 3,109 women were included in this study (**Figure 1**). Of these women, 16 were categorized as poor responders. Compared to the hyper responders and normal responders, the sample size of poor responders was too small to be discussed. We just analyzed only the hyper and normal groups.

Part I

According to the exclusion criteria for missing basal serum FSH, 3,040 patients were included in the data analysis. Of these women, 1,513 were hyper responders, and 1,527 were normal responders. Baseline characteristics for the two response groups are listed in **Table 1**. The ages of the two groups differed significantly. The analysis showed that this result was due to a difference between normal and hyper responders (29.32 ± 3.71 versus 29.00 ± 3.69 years, respectively; $P = 0.0087$). A significant difference was also found in male age, duration of infertility, BMI, and AFC. Hyper responders had a significantly higher BMI compared to normal responders (22.80 ± 2.56 versus 22.54 ± 2.65, $P = 0.0036$), and a significantly higher AFC (16.81 ± 5.43 versus 15.12 ± 5.33, $P < 0.0001$). Hyper responders had a significantly lower total gonadotrophin dose (2058.87 ± 649.98 versus 2141.97 ± 683.31, $P = 0.0005$) but a significantly higher Gn duration (13.92 ± 1.97 versus 13.56 ± 2.04, $P < 0.0001$), total oocytes obtained (21.46 ± 5.13 versus 11.17 ± 2.85, $P < 0.0001$), no. of 2PN oocytes (12.66 ± 5.00 versus 6.87 ± 3.03, $P < 0.0001$), no. of MII oocytes (16.26 ± 6.38 versus 8.43 ± 3.73, $P < 0.0001$), and no. of 2PN cleavage embryos (15.11 ± 5.82 versus 8.15 ± 3.26, $P < 0.0001$).

Part II

According to the exclusion criteria for missing serum FSH on D1 of gonadotrophin, 1,872 patients were included in the data

TABLE 1 | Characteristics and outcomes of patients during August 2015 to December 2017.

Variable	PartI		P value	PartII		P value
	Normal responders	Hyper responders		Normal responders	Hyper responders	
N	1527	1513		935	937	
Female age	29.32 ± 3.71	29.00 ± 3.69	0.0087*	29.34 ± 3.65	29.08 ± 3.69	0.0974
Male age	30.59 ± 5.03	30.09 ± 4.73	0.006*	30.57 ± 4.92	30.12 ± 4.48	0.0682
Duration of infertility (years)	3.62 ± 2.52	3.58 ± 2.47	0.9813	3.58 ± 2.47	3.58 ± 2.55	0.9647
Body mass index (kg/m2)	22.54 ± 2.65	22.80 ± 2.56	0.0036*	22.67 ± 2.62	22.97 ± 2.59	0.0124*
Antral follicle count (n.)	15.12 ± 5.33	16.81 ± 5.43	<0.0001*	15.43 ± 5.43	16.97 ± 5.59	<0.0001*
Infertility factors						
Secondary infertility	631 (41.32)	650 (42.96)	0.36851	382 (40.86)	390 (41.62)	0.7362
Primary infertility	895 (58.61)	863 (57.04)		553 (59.14)	547 (58.38)	
Treatment						
ICSI	348 (22.79)	388 (25.64)	0.06619	193 (20.64)	222 (23.69)	0.11208
IVF	1179 (77.21)	1125 (74.36)		742 (79.36)	715 (76.31)	
Total Gonadotrophin dose (IU)	2141.97 ± 683.31	2058.87 ± 649.98	0.0005*	2159.19 ± 677.14	2044.97 ± 645.96	0.0001*
Gonadotrophin duration (d)	13.56 ± 2.04	13.92 ± 1.97	<0.0001*	13.53 ± 2.02	13.87 ± 1.93	<0.0001*
Total oocytes obtained (n.)	11.17 ± 2.85	21.46 ± 5.13	<0.0001*	11.17 ± 2.86	21.45 ± 5.08	<0.0001*
No. of 2PN oocytes	6.87 ± 3.03	12.66 ± 5.00	<0.0001*	6.86 ± 3.05	12.43 ± 4.78	<0.0001*
No. of MII oocytes	8.43 ± 3.73	16.26 ± 6.38	<0.0001*	8.92 ± 3.28	16.89 ± 5.62	<0.0001*
No. of 2PN cleavage embryos	8.15 ± 3.26	15.11 ± 5.82	<0.0001*	8.26 ± 3.26	15.06 ± 5.80	<0.0001*
Outcomes			<0.0001*			<0.0001*
Whole embryo freezing	146 (9.57)	602 (39.79)		87 (9.32)	346 (36.93)	
No cleavage	4 (0.26)	1 (0.07)		3 (0.32)	1 (0.11)	
Cancellation	1 (0.07)	0		0	0	
No insemination	12 (0.78)	3 (0.20)		8 (0.85)	3 (0.32)	
No transfer	12 (0.79)	26 (1.72)		7 (0.75)	10 (1.07)	
No transferred embryo	50 (3.28)	15 (0.99)		30 (3.22)	8 (0.85)	
Transfer	1296 (84.98)	864 (57.11)		796 (85.32)	568 (60.62)	
Abnormal fertilization	4 (0.26)	2 (0.13)		2 (0.21)	1 (0.11)	
Missing	2			2		

*$P < 0.05$.

analysis. Of these women, 937 were hyper responders, and 935 were normal responders. Baseline characteristics for the two response groups are listed in **Table 1**. There were no significant differences in female age, male age, and duration of infertility. A significant difference was found in BMI and AFC. Hyper responders in comparison to normal responders had a significantly higher BMI (22.97 ± 2.59 versus 22.67 ± 2.62, P = 0.0124) and a significantly higher AFC (16.97 ± 5.59 versus 15.43 ± 5.43, P < 0.0001). Compared to normal responders, hyper responders had a significantly lower total Gn dose (2044.97 ± 645.96 versus 2159.19 ± 677.14, P = 0.0001) but significantly higher Gn duration (13.87 ± 1.93 versus 13.53 ± 2.02, P < 0.0001), total oocytes obtained (21.45 ± 5.08 versus 11.17 ± 2.86, P < 0.0001), no. of 2PN oocytes (12.43 ± 4.78 versus 6.86 ± 3.05, P < 0.0001), no. of MII oocytes (16.89 ± 5.62 versus 8.92 ± 3.28, P < 0.0001), and no. of 2PN cleavage embryos (15.06 ± 5.80 versus 8.26 ± 3.26, P < 0.0001).

Serum Hormone Levels

Part I

Basal serum FSH levels were significantly lower in normal responders than in hyper responders (P < 0.0001). For normal and hyper responders, the means of serum FSH on D1 of Gn were 3.20 ± 2.50 and 2.96 ± 1.20 mIU/ml (P = 0.0002), respectively. Additionally, the mean of serum FSH on D6 of Gn was significantly higher in normal versus hyper responders (8.03 ± 1.83 versus 7.69 ± 1.87 mIU/ml, P < 0.0001). Hyper responders also had a significantly higher basal serum E2 (44.44 ± 249.38 versus 41.26 ± 43.46 pg/ml, P = 0.0267) and serum E2 on D6 of Gn (165.66 ± 185.35 versus 135.20 ± 156.72 pg/ml, P < 0.0001). No significant differences were found for basal serum LH, basal serum PRL, serum LH on D1 of Gn, serum E2 on D1 of Gn, serum P4 on D1 of Gn, serum LH on D6 of Gn, and serum P4 on D6 of Gn (**Table 2**).

Part II

Basal serum FSH levels were significantly higher in normal responders than in hyper responders (P < 0.0001). For normal and hyper responders, the means of serum FSH on D1 of Gn were 3.20 ± 2.47 and 2.96 ± 1.19 mIU/ml (P = 0.0002), respectively. Additionally, the mean of serum FSH on D6 of Gn was significantly higher in normal versus hyper responders (8.10 ± 1.90 versus 7.70 ± 1.68 mIU/ml, P < 0.0001). Hyper responders also had a significantly higher serum E2 level on D6 of Gn (128.44 ± 130.97 versus 168.72 ± 188.31 pg/ml, P < 0.0001). No significant differences were found for basal serum LH, basal serum E2, basal serum PRL, serum LH on D1 of Gn, serum E2 on D1 of Gn, serum P4 on D1 of Gn, serum LH on D6 of Gn, and serum P4 on D6 of Gn (**Table 2**).

Serum Delta FSH

Part I

Delta FSH1 levels (mean: 1.41 ± 3.46) in normal responders are higher than delta FSH1 levels (mean: 1.07 ± 23.89) in hyper

TABLE 2 | Hormonal levels of patients during August 2015 to December 2017.

Variable	Part I		P value	Part II		P value
	Normal responders	Hyper responders		Normal responders	Hyper responders	
N	1527	1513		935	937	
Basal serum FSH	6.62 ± 3.26	6.62 ± 23.85	<0.0001*	6.55 ± 3.05	5.94 ± 1.34	<0.0001*
Basal serum LH	5.43 ± 9.51	5.31 ± 2.96	0.0755	5.66 ± 12.11	5.25 ± 2.97	0.5419
Basal serum E2	41.26 ± 43.46	44.44 ± 249.38	0.0267*	41.07 ± 44.67	38.27 ± 27.17	0.2945
Basal serum P4	0.74 ± 1.33	0.73 ± 1.03	0.0433*	0.79 ± 1.57	0.73 ± 1.07	0.3311
Basal serum PRL	20.39 ± 27.31	22.27 ± 66.11	0.6051	20.59 ± 32.28	21.35 ± 45.96	0.1097
Serum FSH on D1 of Gn	3.20 ± 2.50	2.96 ± 1.20	0.0002*	3.20 ± 2.47	2.96 ± 1.19	0.0002*
Serum LH on D1 of Gn	0.63 ± 0.99	0.57 ± 0.36	0.4677	0.62 ± 0.97	0.57 ± 0.36	0.5455
Serum E2 on D1 of Gn	8.31 ± 7.56	7.52 ± 6.30	0.0643	8.26 ± 7.49	7.52 ± 6.28	0.0908
Serum P4 on D1 of Gn	0.48 ± 0.22	0.49 ± 0.21	0.3024	0.48 ± 0.22	0.49 ± 0.21	0.2399
Serum FSH on D6 of Gn	8.03 ± 1.83	7.69 ± 1.87	<0.0001*	8.10 ± 1.90	7.70 ± 1.68	<0.0001*
Serum LH on D6 of Gn	0.51 ± 0.77	0.47 ± 0.95	0.0936	0.49 ± 0.54	0.47 ± 0.54	0.0438*
Serum E2 on D6 of Gn	135.20 ± 156.72	165.66 ± 185.35	<0.0001*	128.44 ± 130.97	168.72 ± 188.31	<0.0001*
Serum P4 on D6 of Gn	0.15 ± 0.20	0.15 ± 0.17	0.4136	0.14 ± 0.19	0.13 ± 0.16	0.7113
Delta FSH1	1.41 ± 3.46	1.07 ± 23.89	0.0248*	/	/	/
Delta FSH2	/	/	/	4.90 ± 2.84	4.74 ± 2.09	0.103

Delta FSH1 = FSH on D6 of gonadotrophin -Basal serum FSH Delta FSH2 = FSH on D6 of gonadotrophin -FSH on D1 of gonadotrophin.
Gn = Gonadotrophin, FSH (mIU/ml), LH (mIU/ml), E2 (pg/ml), P4 (ng/ml), PRL (ng/ml).
**P < 0.05.*

responders (P = 0.0248) (**Table 2**). The tertile of delta FSH1 is dif ≤ 0, 0 < dif ≤ 2.25 and dif > 2.25. Compared with the normal responder, the delta FSH1(0 < dif ≤ 2.25 and dif > 2.25) has a higher ratio than the hyper responder and is statistically significant (**Table 3**).

Part II

Delta FSH2 levels (mean: 4.90 ± 2.84) in normal responders were higher than delta FSH2 levels (mean: 4.74 ± 2.09) in hyper responders (P = 0.103) (**Table 2**). The tertile of delta FSH1 is dif ≤ 3.91, 3.91 < dif ≤ 5.69 and dif > 5.69. Compared with the hyper responder, the delta FSH2 (3.91 < dif ≤ 5.69 and dif > 5.69) has a higher ratio than the normal responder and is statistically significant (**Table 4**).

Correlation

Part I

We found a weak but significant correlation between delta FSH1 levels (0 < dif ≤ 2.25) and the number of retrieved oocytes. After adjusting for female age, there was a relationship between ovarian response and the delta FSH1 (0 < dif ≤ 2.25) (OR 1.45; 95% CI, 1.19–1.76). After adjusting for female age, infertility year, basal serum FSH and AFC, there was a relationship between ovarian response and delta FSH1 (0 < dif ≤ 2.25) (OR 1.46; 95% CI, 1.19–1.79) (**Table 5**).

Part II

We found a weak but significant correlation between delta FSH2 levels (3.91 < dif ≤ 5.69) and the number of retrieved oocytes. After adjusting for female age, there was a relationship between ovarian response and the delta FSH2 (3.91 < dif ≤ 5.69) (OR 0.73; 95% CI, 0.59–0.91). After adjusting for female age, infertility year, basal serum FSH and AFC, there was a relationship between ovarian response and the delta FSH2 (3.91 < dif ≤ 5.69) (OR 0.81; 95% CI, 0.63–1.03) and delta FSH2 (dif > 5.69) (OR 1.17; 95% CI, 0.91–1.51) (**Table 5**).

DISCUSSION

This study demonstrates that slightly higher delta FSH1 levels and delta FSH2 levels are present in normal responders compared to hyper responders undergoing the first IVF/ICSI cycles at the Reproductive Medicine Center. When performing a multivariable statistical analysis for the number of retrieved oocytes, infertility year, basal serum FSH level and AFC did

TABLE 3 | Tertile of Delta FSH1.

Variable	Part I		P value
	Normal responders	Hyper responders	
N	1527	1513	
Delta FSH1			
dif ≤ 0	326 (21.35)	245 (16.19)	0.00094*
0 < dif ≤ 2.25	700 (45.84)	760 (50.23)	
dif > 2.25	501 (32.81)	508 (33.58)	

Delta FSH1 = FSH on D6 of gonadotrophin -Basal serum FSH.
**P < 0.05.*

TABLE 4 | Tertile of Delta FSH2.

Variable	Part II		P value
	Normal responders	Hyper responders	
N	935	937	
Delta FSH2			
dif ≤ 3.91	289 (30.91)	336 (35.86)	0.0268*
3.91 < dif ≤ 5.69	336 (35.94)	288 (30.74)	
dif > 5.69	310 (33.16)	313 (33.4)	

Delta FSH2 = FSH on D6 of gonadotrophin -FSH on D1 of gonadotrophin.
**P < 0.05.*

TABLE 5 | Association of Delta FSH with ovarian response among patieiits during August 2015 to December 2017.

	case/contiol	OR (95%CD)*	OR (95%a)**
Delta FSHI			
dif ≤ 0	326/245	1.00 (reference)	1.00 (reference)
0 < dif ≤ 2.25	700/760	1.45 (1.19–1.76)	1.46 (1.19–1.79)
dif > 2.25	501/508	1.36 (1.10–1.67)	1.37 (1.10–1.70)
Delta FSH2			
dif ≤ 3.91	289/336	1.00 (reference)	1.00 (reference)
3.91 < dif ≤ 5.69	336/288	0.73 (0.59–0.91)	0.81 (0.63–1.03)
dif > 5.69	310/313	0.86 (0.69–1.08)	1.17 (0.91–1.51)

OR, odds ratio; CI, confidence interval.

**Adjusted for female age.*

***Adjusted for female age, infertility year, basal serum FSH, and Antral follicle count.*

not appear to play a more important role in determining the response to rFSH stimulation than serum delta FSH levels. A recent study by S.C. Oudshoorn et al. concluded that there is no consistent relationship between ovarian response and serum FSH levels on the day of hCG trigger in a 150 IU fixed dose treatment protocol. Because the studies only focused on serum FSH levels on the day of hCG trigger, it is difficult to directly compare these results to our study (23).

In our study, we divided the women undergoing IVF/ICSI cycles into Part I (missing the basal serum FSH level) and Part II (missing the serum FSH level on D1 of Gn). Normal responders have higher serum FSH levels on D1 of Gn and serum FSH levels on D6 of Gn either in Part I or Part II. One explanation for these results is that normal responders could have antral follicles that can be explained by a different sensitivity or insensitivity of follicles to FSH. FSH stimulates follicular growth by binding to its receptors localized in the granulosa cells of the follicles (24–26). The analysis of patient-specific genotypes might lead to an individualized pharmacogenomic approach to controlled ovarian stimulation (COS). However, no consensus has been established regarding if the genetic variations of these receptors influence serum FSH levels and the degree of ovarian response to stimulation (27–30). Therefore, variation in FSH receptor genotypes is unlikely to be the main reason for the difference in the number of oocytes retrieved in response to standard FSH dose stimulation. Another explanation for this result in normal responders could be that the hyper responders have a higher BMI compared to the normal responders in both Part I and Part II. Several studies have shown that serum FSH levels after administration of rFSH are influenced by the route of administration (intravenous or subcutaneous), body weight and the administered rFSH dose (31–33). Hence, higher BMI in hyper responders could explain why there was no increase in serum FSH level on D1 of Gn and serum FSH level on D6 of Gn in those women.

Currently, there is no research regarding the relationship between serum delta FSH levels and retrieved oocytes. Therefore, there is no consensus for us to reference when grouping the patients by serum delta FSH levels and then to tripartite the women according to the statistical scheme. In Part I, hyper responders have a higher ratio to higher delta FSH1 levels. This finding is inconsistent with the conclusions that normal responders have higher delta FSH1. This result may be due to the inconsistency caused by the individualized differences. However, normal responders have a higher ratio to higher delta FSH2 levels, which is consistent with the conclusions that normal responders have higher delta FSH2. Serum delta FSH levels differed conversely between the response groups than we had hypothesized when designing the study. This finding may seem to be in contrast to these studies, suggesting that a higher dose of FSH leads to higher serum FSH levels and a slightly greater oocyte yield (22, 34). We must draw a conclusion carefully because this study lacks data from poor responders.

Most previous studies illustrate that 8–15 oocytes give a pregnancy rate plateau, and there seems to be no benefit of creating an excessive response with only higher risks of jeopardizing the patients' health and increasing costs of IVF/ICSI treatment (35). Even though doctors have paid attention to the problem, approximately 20% of women undergoing IVF/ICSI experience an excessive response (36), and up to 7% may develop OHSS. In the stimulation protocol, researchers will determine the increase and decrease in Gn based on the hormone status and follicular development after D6 of Gn. Even if the hyper responders use a lower total gonadotrophin dose, 40% of patients still underwent whole embryo freezing because of the high ovarian response. The serum delta FSH level can be an additional marker to guide the adjustment of the rFSH dose in the agonist cycles.

The present study has several strengths. First, to increase the reliability of the results, all blood samples from patients were collected at approximately the same point in the experiments. Moreover, the rFSH used was the same, and the rFSH dose was unchanged in the first six days of FSH stimulation. Additionally, several limitations should be mentioned. While we excluded women with a different FSH starting dose, a dose adjustment during the treatment cycle could possibly affect the number of oocytes obtained. Compared to the normal and hyper responders, the sample size of the poor responders was too small to be discussed.

In general, the results of this study show that there is a weak relationship between ovarian response and serum delta FSH levels in the rFSH fixed dose treatment protocol. This finding may imply that decreasing the dose of rFSH in women who respond highly will lead to a more ideal oocyte yield and improve the safety of IVF/ICSI treatment. However, these issues should be studied in a larger trial with poor responders and a true dose comparison design.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Research Ethics Committee of the First Affiliated

Hospital of Zhengzhou University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LH performed the original trial. LH and BS designed the study. LH carried out all necessary data analyses and in collaboration with BS wrote the article. YM, FW, and HS collected all data. LL revised the article. All authors participated in the interpretation of the data and provided significant revisions. YS read and approved the final version of the article. All authors contributed to the article and approved the submitted version.

FUNDING

This work was funded by the Chinese Medical Association Clinical Medical Research Special Fund Project (FDN-17020190688), the Medical Science and Technology Research Project Joint Co-construction Project of Henan Province (FDN-2018020116), and the Henan Provincial Higher Education Key Research Project Plan (FDN-19A320056).

ACKNOWLEDGMENTS

The authors thank the staff of Reproductive Medicine Center, First Affiliated Hospital of Zhengzhou University for their cooperation and support.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 06 September 2021
doi: 10.3389/fendo.2021.734638

Insulin Resistance Adversely Affect IVF Outcomes in Lean Women Without PCOS

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OPEN ACCESS

Edited by:
Ye Chun Ruan, Hong Kong Polytechnic University, Hong Kong, SAR China

Reviewed by:
Alessandro D. Genazzani, University of Modena and Reggio Emilia, Italy
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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *01 July 2021*
Accepted: *18 August 2021*
Published: *06 September 2021*

Citation:
Wang H, Zhang Y, Fang X, Kwak-Kim J and Wu L (2021) Insulin Resistance Adversely Affect IVF Outcomes in Lean Women Without PCOS. Front. Endocrinol. 12:734638. doi: 10.3389/fendo.2021.734638

Objective: To investigate the effects of insulin resistance (IR) on IVF outcomes and a potential underlying mechanism in lean women without PCOS.

Design: A prospective cohort study at the University Clinic.

Setting: IVF center at the University setting.

Patients: A total of 155 lean women (body mass index <25) without PCOS undergoing IVF cycle.

Intervention: Patients were allocated to IR and non-IR groups based on HOMA-M_{120}.

Main Outcome Measure(s): IVF outcomes, including egg quality, the percentage of mature oocytes, fertilization rate, blastocyst formation rate, advanced embryo rate, and cumulative live birth rate were investigated. Auto-immune parameters, peripheral blood immunophenotypes, thyroid hormone, homocysteine, and 25-OH-vitamin D_3 (25-OH-VD_3) levels were analyzed.

Results: The percentage of mature oocytes and blastocyst formation rate were significantly lower in the IR group as compared with those of the non-IR group ($p<0.05$, respectively). The proportion of peripheral blood $CD19^+$ B cells was significantly higher in the IR group than those of the non-IR group ($p<0.05$). Homocysteine, 25-OH-VD_3, and auto-immune parameters were the same between the two groups.

Conclusion: In lean infertile women without PCOS, IR is associated with the decreased percentage of mature eggs and poor embryo quality in which B cell immunity may play a role.

Keywords: insulin resistance, non-PCOS, lean women, IVF outcome, B cell immunity

HIGHLIGHTS

Lean non-PCOS women with insulin resistance have adverse IVF outcomes with the decreased percentage of mature oocytes and blastocyst formation rate and increased peripheral blood B cell levels.

INTRODUCTION

Insulin resistance (IR) is typically defined as decreased sensitivity or responsiveness to the metabolic action of insulin (1). IR is caused by a primary defect in insulin receptor signaling and a reduced insulin clearance rate, resulting from decreased hepatic insulin extraction (2). It is commonly associated with obesity, hypertension, cardiovascular disease, and typically type 2 diabetes. It is reported that about 50% to 70% of women with PCOS have IR (3). Infertile women with PCOS had higher levels of fasting insulin, and the resultant hyperinsulinemia plays a role in the pathogenesis of reproductive disorders (4–6).

Hyperinsulinemia disrupts the intrafollicular microenvironment during folliculogenesis and reduces the rate of fertilization and embryonic development potential during the natural and ovarian stimulation cycles (7, 8). Insulin signaling in the uterus controls gene expression and glucose utilization and affects the decidualization process to facilitate implantation (9). IR and free androgen index correlate with total ovarian follicle count in non-PCOS women who underwent IVF-ET treatment. It is suggested that the higher level of IR and androgen can positively affect short-term follicle development and benefit responses to exogenous gonadotrophin stimulation while increasing the risk of OHSS in non-PCOS women (10).

There is increasing evidence at the cellular level showing that inflammation is a critical factor for obesity-induced IR. The tissue-resident immune cells, especially adipose-tissue resident cells, play a major role in regulating obesity-induced inflammation. On the other hand, cellular and molecular factors in adipose tissue regulate obesity-induced inflammation and IR (11). Elevated inflammatory cytokines, including interleukin (IL)-17 and IL-6, could cause a subclinical inflammatory state for a prolonged time. Abnormal inflammatory responses decrease glycolipid metabolism, increase IR, and affect ovulation and fertilization, resulting in polycystic ovary syndrome (PCOS) characterized by oligomenorrhea and irregular ovulation (12).

Obesity is a global epidemic related to numerous health concerns, including reproductive disorders. Central obesity is considered an independent risk factor for early miscarriage (13). Obese individuals exhibit increased estrogen concentrations due to aromatase overexpression in the adipose tissue. Consequently, higher estrogen level causes anovulation via the hypothalamus-pituitary-ovary axis (14). On the other hand, weight loss through lifestyle changes or bariatric surgery positively affects hormonal parameters and ovulation rates (15, 16).

Most of the studies found the effect of IR on IVF outcome in women with overweight/obese PCOS, using homeostasis model assessment for insulin resistance (HOMA-IR) as the index to assess IR. The HOMA index was calculated as HOMA = (fasting insulin uIU/ml * fasting glucose mmol/l)/22.5.HOMA-IR has been reported to effectively predict IR in the overweight-obese PCOS population with a cutoff of 2.62 or more (AUC 84.1%) (17). However, HOMA-IR was found not to be reliable or predictable in detecting IR in lean women, who had neither fasting hyperinsulinemia nor increased basal hepatic glucose production (18). Recently, the HOMA-M_{120} was reported as a reliable and straightforward measure of IR for lean European and Asian women with PCOS (17, 19). HOMA-M_{120} was calculated as (post-load 2-hour insulin uIU/ml * post-load 2-hour glucose mmol/l)/22.5. IR was diagnosed when HOMA-M_{120}>12.8. Lean women with HOMA-M_{120}>12.8 were considered as IR group, and the others were non-IR group (17). Previous studies have focused on the adverse effect of IR on IVF outcome in women with overweight/obese PCOS. IR's impact on reproductive outcome in lean PCOS has also been reported (20). Therefore, doctors pay more attention to the diagnosis and treatment of IR in obese and non-obese PCOS patients, metformin for example, to improve IVF outcome.It is noteworthy that IR can also occur in infertile women who had a regular menstrual cycle without polycystic ovaries, which has been reported before (10). But there are no researches about whether IR in lean patients without PCOS adversely affects IVF outcomes. Hence, in this study, we determined to investigate IR by HOMA-M_{120} in lean women without PCOS and analyze the IR effect on IVF outcomes and related cellular and endocrine factors.

MATERIALS AND METHODS

Study Population

This was a prospective observational study done at Reproductive Medicine Center, Department of Obstetrics and Gynecology, the First Affiliated Hospital of University of Science and Technology of China (USTC), Division of Life Sciences and Medicine, USTC, Hefei, Anhui, China, from May 2016 to July 2018. Ethics approval was given by the Ethical Committee of the First Affiliated Hospital of USTC, and all study candidates signed the informed consent form prior to entering the study.

Inclusion criteria were couples with only tubal factor infertility, women's age from 18 to 40 years old and all underwent a standard long-protocol agonist IVF cycle as the first treatment cycle. Tubal etiology was diagnosed if hysterosalpingography or laparoscopy showed evidence of bilateral tubal obstruction.

Exclusion criteria were women with polycystic ovarian syndrome (PCOS) according to the Rotterdam criteria, high BMI (>25), male infertility, and other endocrine and systemic diseases (including type 1 or 2 diabetes), without familial diabetes history. Patients undergoing ICSI for fertilization failure were also excluded.

A total of 190 women were asked to participant, but only 170 women agreed to participant and were subjected to an oral glucose tolerance test (OGTT) before the IVF cycle. 5 participants were excluded from the study for taking metformin before the cycle which is known to affect glucose metabolism and insulin sensitivity. Four cases were lost during the follow-up. 6 women were excluded for fertilization failure.Finally, a total of 155 lean infertile women were included in the study. The flow chart is shown in **Figure 1**.

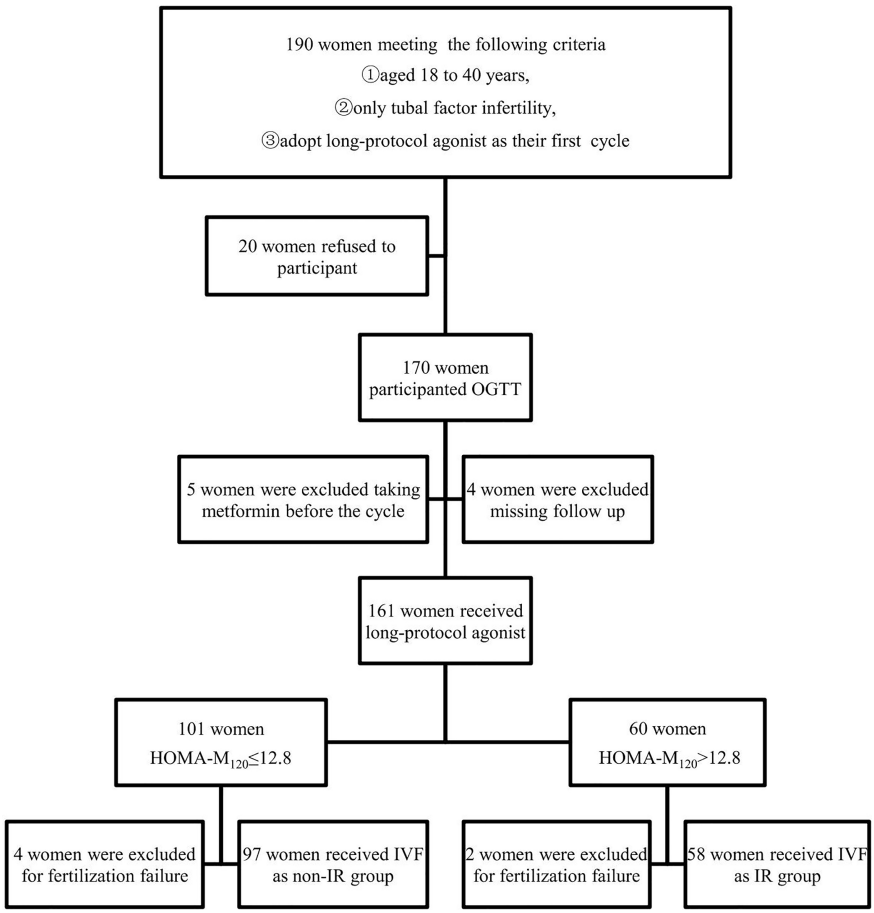

FIGURE 1 | Flow diagram of literature search.

Before the IVF cycle, the BMI, hormonal, biochemical, immunological, and ultrasound parameters were investigated. All underwent a standard long-protocol agonist IVF cycle. Based on the BMI of patients, 0.9-1.5mg Triptorelin was initiated on day 21 to achieve adequate ovarian suppression. After 14 days, pituitary desensitization was checked by estradiol (E_2) level and B ultrasound scan. Once the criteria for desensitization were satisfied (E 2 ≤ 50 pg/ml, diameter of follicle≤ 5 mm and endometrial thickness ≤ 5 mm), Patients was daily injected 150-225 IU of recombinant FSH (Gonal-F®, Merck Serono, Switzerland). Injection of recombinant hCG (Ovitrelle®, Merck Serono, Switzerland) 250μg were given when diameter of 2-3 follicle ≥17 mm or at least diameter of one follicle ≥18 mm. Oocytes retrievals were performed 36 h after hCG administration. All patients received conventional IVF as fertilization method regardless of their ages.

During the 2 hour OGTT, fasting blood glucose (FBG), 2-h blood glucose (2-h BG), and 2-h insulin levels were measured. HOMA-M_{120} was used to assess IR in this study, which was calculated as (post-load 2-hour insulin uIU/ml x post-load 2-hour glucose mmol/l)/22.5. IR was diagnosed when HOMA-M_{120}>12.8. Lean women with HOMA-M_{120}>12.8 were considered as IR group (n=58), and the others were non-IR group (n=97) (17). OHSS has been classified on severity (mild, moderate, severe, critical) according to RCOG classification, a guideline with the title" The Management of Ovarian Hyperstimulation Syndrome" published in 2016.The percentage of mature oocytes, blastocyst formation rate, pregnancy rate, implantation rate, chemical pregnancy rate, abortion, and delivery rates were analyzed. Good-quality embryos mean embryos that reached 6 to 8-cell stage with cytoplasmic fragmentation occupying less than 10% of the embryo surface and had equal size blastomeres. Cumulative live birth was defined as all subsequent embryo transfers during a single oocyte retrieval cycle within 18 months of treatment, including both fresh and frozen-thawed embryo.

Hormonal Evaluation

All baseline blood sampling including E_2, follicle-stimulating hormone (FSH), luteinizing hormone (LH), total testosterone (T) and prolactin (PRL) were done during cycle day 2-4 of the menstrual cycle. The oral glucose tolerance test (OGTT) was performed at the same time after overnight fasting and the administration of an oral hypertonic glucose solution (75g). Serum glucose and insulin were measured at 0 and 120 min. Body mass index (BMI) was calculated according to the formula,

weight/height^2. Serum E_2, FSH, LH, T and PRL were measured employing commercial RIA kits (bioMErieux, Charbonnirres les Bains, France).The serum glucose level was determined by an automatic analyzer employing an enzymatic-colorimetric assay (Gemon, Lindau, Germany). Insulin level was measured by RIA using a commercial kit (CIC bio international, Gif-sur-Yvette, France), and homocysteine (HCY) level was determined using an enzyme conversion immunoassay kit (Axis-Shield, Dundee, UK). The 25-OH-VD_3 level was measured using the liquid chromatography/tandem mass spectrometry (LC/MS/MS) 155 method at the reference laboratory. Thyroid-stimulating hormone (TSH) and free thyroxine (FT4) were measured by electrochemiluminescence immunoassay (Roche, Germany). The cut-off values for the reference range was 0.4–4.0 mIU/L for TSH and 11–25pmol/L for FT4 (21). Subclinical hypothyroidism was determined when TSH was 2.5-4.0 mIU/L with normal FT4 levels (22).

Autoantibodies

Antiphospholipid antibodies (APA) and anti-β2GP1 were tested by enzyme-linked immunosorbent assay (ELISA). Anti-nuclear antibodies (ANA) were performed by indirect immunofluorescence using a commercially available kit (Immunoconcepts, Sacramento, CA, USA). Anti-thyroglobulin antibody (ATG) and anti-thyroperoxidase (Anti-TPO) were tested by ELISA (Inova Diagnostic, San Diego, CA, USA).

Peripheral Blood Immunophenotype

Peripheral blood immune effectors were analyzed by the flow cytometry analysis. Briefly, whole blood samples were labeled using fluorochrome-conjugated monoclonal antibodies (mAb) against CD45-PC5, CD3-FITC, and CD56-PE, or CD45-PC5 and CD19-FITC (Beckman Coulter, Fullerton, CA, USA), and samples were analyzed by a FC 500 flow cytometer using CXP software (Beckman Coulter). Lymphocytes were gated based on side scatter characteristics and CD45 expression. Within lymphocytes, T cells were identified as $CD3^+$ cells, NK cells as $CD3^-CD16^+CD56^+$ cells, and B cells as $CD19^+$ cells.

Statistical Analysis

Statistical analyses were performed using the Statistical Package for the Social Sciences software (SPSS 19, Armonk, NY, USA). Student's t-test and chi-square test were applied to determine the differences between the means or the distributions of the study groups. Results were presented as mean ± SD for each group, and p-value <0.05 was considered to be significant.

RESULTS

Endocrine Profiles

Based on the OGTT and HOMA-M_{120} index, 58 women had IR (37.4%), and 97 women had normal insulin sensitivity (62.6%). Women with IR had a significantly higher incidence of subclinical hypothyroidism (37.93% *vs.* 20.62%, p=0.019). However, age, BMI, antral follicle count, and baseline hormone levels, including FSH, LH, E2, PRL, and T, were similar between IR and non-IR groups. As expected, FBG, 2h BG, FI, 2-h insulin, HOMA-IR were significantly different between the two groups. There were no differences in HCY (7.15 ± 2.67 *vs.* 7.26 ± 2.28, p=0.814) and 25-OH-VD_3 (18.86 ± 5.40 *vs.* 18.93 ± 8.30, p=0.957) levels in women with IR as compared with those of the non-IR group (**Table 1**).

Immune Parameters

There was no significant difference in auto-immune parameters between IR and non-IR groups, including ATA, ATG, APA, ANA, and a-β2GP1(p>0.05, respectively) (**Table 2**). The proportion of peripheral blood $CD19^+$ B cells in women with IR (12.73 ± 4.37%) was significantly higher than that of women with non-IR groups (11.03 ± 4.37%, p=0.032) (**Figure 2**). However, T (70.14 ± 6.35% *vs.* 70.79 ± 6.66%, p=0.581) and NK cell (14.32 ± 5.92% *vs.* 15.13 ± 7.38%, p=0.513) populations in the peripheral blood were not different between the two groups.

IVF Outcome

In the IR group, the duration of induction (13.83 ± 3.06 *vs.* 12.70 ± 3.26, p=0.035),the percentage of mature oocytes per oocytes retrieved (85.17 ± 15.16% *vs.* 90.53 ± 12.91%, p=0.02), and freezable blastocysts per residual embryos (39.15% *vs.* 46.46%, p=0.023) were significantly different between the two study groups. However, there were no differences in the number of retrieved oocytes (14.28 ± 9.72 *vs.* 12.13 ± 7.82, p=0.135), the incidence of Ovarian Hyperstimulation Syndrome (20.69% *vs.*16.49%, p=0.511,the number of mature oocytes (12.19 ± 8.93 *vs.* 10.96 ± 7.10, p=0.347), the number of fertilized oocytes (9.98 ± 8.15 *vs.* 8.93 ± 6.54, p=0.221), fertilization rate (81.90% *vs.* 81.46%, p=0.820), the percentage of fresh ET cycles in total ET cycles(39.62% *vs.* 45.98%, p=0.462) and the percentage of good-quality embryos per cleaved embryos (56.50% *vs.* 56.97%, p=0.869). In non-IR group, 14 patients were diagnosed with mild OHSS and 2 patients with moderate OHSS. In IR group, 10 patients were diagnosed with mild OHSS and 2 patients with moderate OHSS. No severe or critical OHSS happened in either non-IR group or IR group. The small sample size limited the stratification analysis. But we found there was no difference in the incidence of mild OHSS (20.83% *vs.*16.86%, p=0.64).

In the first ET cycle, chemical pregnancy rate(41.51% *vs.* 28.74%, p=0.121),pregnancy rate (32.08% *vs.* 26.44%, p=0.474), chemical pregnancy loss rate(22.73% *vs.* 8%, p=0.157),clinical pregnancy loss rate (35.29% *vs.* 13.67%, p=0.111) and pre-term births rate (18.18% *vs.* 10.53%, p=0.552) between IR and non-IR group were not different.Besides, an ectopic pregnancy occurred in non-IR group in the first ET cycle. Moreover, cumulative live birth rate per oocyte retrieval cycle (46.55% *vs.* 50.52%, p=0.633) was not different between the two study groups (**Table 3**).

DISCUSSION

In this study, we found that IR is associated with a slower response to ovulation induction, poor oocyte maturation, and decreased proportion of freezable embryos in lean and non-PCOS women who underwent IVF treatment, with increased B cell levels as compared to non-IR women.

TABLE 1 | Clinical characteristics and endocrine profiles of insulin resistant (IR) and non-insulin resistant infertile women (Non-IR).

	Non-IR (lean) (n = 97)	IR (lean) (n = 58)	P-value
Age (years)	31.43 ± 4.56	30.67 ± 4.65	0.320
Antral follicle number	12.60 ± 6.05	14.43 ± 6.99	0.070
BMI (kg/m^2)	21.43 ± 1.96	22.07 ± 1.70	0.239
FSH (U/L)	7.93 ± 3.09	7.25 ± 2.87	0.175
LH (U/L)	4.92 ± 3.42	4.74 ± 2.87	0.740
E2 (pg/ml)	49.43 ± 26.92	43.73 ± 31.25	0.232
PRL (ng/ml)	14.46 ± 6.56	15.21 ± 9.03	0.551
Testosterone (ng/ml)	0.45 ± 0.26	0.47 ± 0.36	0.645
FBG (mmol/L)	4.89 ± 0.41	5.11 ± 0.64	0.014[a]
2-h BG (mmol/L)	5.32 ± 0.90	6.88 ± 1.68	0.006[b]
FI (mU/L)	60.17 ± 27.80	97.09 ± 58.41	0.000[b]
2-h Insulin (mU/L)	174.08 ± 83.28	545.17 ± 230.38	0.000[b]
HOMA-IR	1.90 ± 0.93	3.23 ± 2.38	0.000[b]
HOMA-M$_{120}$	6.09 ± 3.27	21.85 ± 8.45	0.000[b]
Sc-Hypothyroidism (%)	20.62	37.93	0.019[a]
HCY	7.26 ± 2.28	7.15 ± 2.67	0.814
25-OH-VD3	18.93 ± 8.30	18.86 ± 5.40	0.957

BMI, Body mass index; FSH, follicle stimulating hormone; LH, luteinizing hormone; E2, estradiol; PRL, prolactin; FBG, fasting blood glucose;2-h BG, 2 hour blood glucose; FI, fasting insulin; HOMA-IR, homeostasis- model assessment of insulin resistance; HOMA-M$_{120}$ was calculated as (post-load 2-hour insulin uIU/ml x post-load 2-hour glucose mmol/l)/ 22.5.Sc-Hypothyroidism, subclinical hypothyroidism.
P < 0.05 was considered statistically significant; [a]P < 0.05, [b]P < 0.01.

TABLE 2 | Auto-immune parameters between insulin resistant (IR) and non-insulin resistant (Non- IR) infertile women.

Autoantibodies	Non-IR (lean)	IR (lean)	P-value
ATA/ATG, n (%)	15 (22.06)	9 (18.00)	0.588
APA, n (%)	0 (0)	0 (0)	–
ANA, n (%)	18 (19.78)	11 (20.75)	0.888
anti-β2GP1, n (%)	5 (5.62)	2 (4.00)	0.675

ATA/ATG, anti-thyroid antibody/Anti-thyroglobulin; APA, any IgG or IgM antibodies to phospholipids; ANA, anti-nuclear antibody.

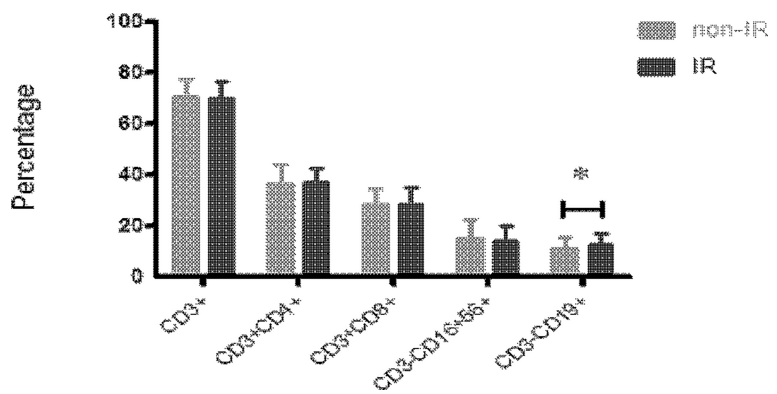

FIGURE 2 | The peripheral blood immunophenotypes of immune effectors, including CD3+, CD3+CD4+, CD3+CD8+, CD3+CD16+CD56+ and CD3+CD19+ cells, were analyzed in the samples of 58 women with IR and 97 women without IR in lean women (16<BMI<25). The proportion of peripheral blood CD19$^+$ B cells was significantly higher in lean patients with IR as compared with those without IR. Data are represented as the mean ± SD. Significance was determined using the two-tailed Student t-test. *$P < 0.05$.

In this study, the prevalence of subclinical hypothyroidism was significantly higher in the IR group than the non-IR group among lean women without PCOS, which is consistent with the previous study demonstrating that the HOMA-M$_{120}$ index was significantly increased in women with subclinical hypothyroidism (23). There are several possible mechanisms to explain the observed relation between low-normal thyroid function and IR. Insulin might influence thyrotropin-releasing hormone (TRH) and TSH when modulating glycemic status (24), and subclinical hypothyroidism is associated with decreased insulin sensitivity and glucose

TABLE 3 | Oocyte and embryological data after IVF-ET treatment between insulin resistant (IR) and non-insulin resistant (Non-IR) infertile women.

	Non-IR (n = 97)	IR (n = 58)	P-value
Duration of induction (days)	12.70 ± 3.26	13.83 ± 3.06	0.035[a]
No. of retrieved oocytes	12.13 ± 7.82	14.28 ± 9.72	0.135
No. of mature oocytes	10.96 ± 7.10	12.19 ± 8.93	0.347
No. of fertilized oocytes	8.93 ± 6.54	9.98 ± 8.15	0.221
% of mature oocytes per oocyte retrieved	90.53 ± 12.91	85.17 ± 15.16	0.02[a]
% of fertilized oocytes among the mature oocytes	81.46 (866/1063)	81.90 (579/707)	0.820
% of good-quality embryos per cleaved embryos	56.97 (429/753)	56.50 (291/515)	0.869
% of freezable blastocysts per residual embryos	46.46 (269/579)	39.15 (157/401)	0.023[a]
% of fresh ET cycles in 1st ET cycles	45.98 (40/87)	39.62 (21/53)	0.462
% of chemical pregnancy in 1st ET	28.74 (25/87)	41.51 (22/53)	0.121
% of pregnancy in 1st ET	26.44 (23/87)	32.08 (17/53)	0.474
% of chemical pregnancy loss in 1st ET	8 (2/25)	22.73 (5/22)	0.157
% of clinical pregnancy loss in 1st ET	17.39 (4/23)	35.29 (6/17)	0.196
% of pre-term births in 1st ET	10.53 (2/19)	18.18 (2/11)	0.552
Cumulative live birth rate,%	50.52 (49/97)	46.55 (27/58)	0.633

P < 0.05 was considered statistically significant, [a]P < 0.05.

tolerance, partially due to a decreased insulin ability to increase glucose utilization mainly in the muscle (25).

We have demonstrated that the retrieved number of oocytes was higher in the IR group than the non-IR group, although the difference did not reach a significant level, which is consistent with the previous study (10). Insulin promotes primordial to primary follicle transition (26). Additionally, the response of granulosa cells to FSH during the gonadotropin-dependent stage of folliculogenesis can be enhanced by insulin growth factors (27). Therefore, in the IVF setting, the risk of developing multifolliculogenesis or ovarian hyperstimulation syndrome to exogenous gonadotropin stimulation is higher in IR than non-IR patients (28).

In our study, the percentage of mature oocytes per oocytes retrieved and the percentage of freezable blastocysts per residual embryos were significantly lower than those of women in the non-IR group. Previous study found insulin could stimulate theca cell androgen production, elevating serum free testosterone levels, so hyperinsulinemia will increase the local production of androgens (29). It is proved that high level of androgen can interfere with fertilization and cleavage rates of in vitro–matured oocytes, which decrease the number of mature oocytes and blastocyst formation rate (30). In our study, the percentage of mature oocytes per oocytes retrieved and the percentage of freezable blastocysts per residual embryos were significantly lower than those of women in the non-IR group. This is consistence with previous study. But the direct mechanisms of hyperinsulinemia adverse mature oocytes and blastocyst need further study.

As metformin often induces side effects, new integrative strategies have been proposed to treat insulin resistance, such as the use of inositols. Myo-inositol (MYO) and d-chiro-inositol (DCI) are two inositol stereoisomers in humans. MYO is the precursor of inositol triphosphate, a second messenger that regulates thyroid-stimulating hormone (TSH) and FSH as well as insulin. Several preliminary studies suggest that a deficiency of D-chiro-inositol (DCI) containing IPG might be at the basis of insulin resistance (31). In fact Genazzani et al. reported that MYO administration can not only decrease fasting insulin plasma levels in obese patients (32) but also improve insulin sensitivity in non-obese PCOS patients (33).

Finally, in contrast to the non-IR group, the IR group had a significantly higher level of peripheral blood B lymphocytes. Since there is no difference in autoimmunity between the IR and non-IR groups, increased B cells in the IR group may reflect the expansion of the functional subset of B cells. Previous study found B cells can worsen glucose tolerance by production of IgG antibodies and activation of proinflammatory macrophages and T cells in mice model. Depletion of B cells in mice can ameliorate glucose tolerance and fasting insulin. However, the return of B cells will exert their detrimental effects on glucose tolerance again. These results indicates B cells play a role in insulin resistance (34). In our study, we found B cells were increased in IR group, this was consistence with previous studies. Further studies are required to confirm the mechanism of the speculation.

Previously, we reported that women with low vitamin D levels had higher peripheral blood B-cell proportion and T helper/T cytotoxic cell ratios than those of normal vitamin D (35). In this study, vitamin D levels were not different between the two study groups. Maybe the IR in lean women with non-PCOS didn't have vitamin D deficiency tendency.

The study is limited since the underlying mechanism of higher B lymphocyte count in the peripheral blood of women with IR was not explored further and auto-antibodies to insulin and its receptors were not investigated. Since the genetic study of the embryos were not made in all cases, IR and genetic abnormalities of the embryos were not thoroughly investigated.

Currently, the independent role of IR in IVF outcomes is not defined well (30, 36). Our study demonstrated the association between IR and adverse IVF outcomes in the lean non-PCO population. Possible use of anti-glycemic agent during the IVF cycle should be investigated in the future in women with IR without obesity or PCOS.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethical Committee of the First Affiliated Hospital of USTC. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

All authors qualify for authorship by contributing substantially to this article. HW and LW were responsible for study design and conception, data analysis, the first draft of the article, review, and approval of revisions, and the final article. JK-K interpreted the data and revised the manuscript. XF and YZ were responsible for recruitment, data collection, and article review. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by the National Natural Science Foundation (No. 81601353, No.82071650).

ACKNOWLEDGMENTS

The authors acknowledge all the Chinese patients and ART clinics for their contributions to this study.

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