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Reproduction

Ovarian Stimulation Effects on Ghrelin Secretion and Reproductive Potential

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ABSTRACT

Objective: Finely regulated Ghrelin (Ghrl) secretion is essential during early pregnancy, as infra or supraphysiologic levels can be detrimental. Since oestrogens stimulate Ghrl synthesis, ovarian stimulation (OS) might increase ghrelinemia, thus being detrimental for fertility. The aim of this work was to evaluate whether OS increases ghrelinemia and associates with maternal endocrine and immune biomarkers and reproductive success.

Design: The 97 women undergoing assisted reproduction were grouped as follows: OS: undergoing OS and fresh embryo transfer ($n = 35$); FET: undergoing frozen embryo transfer in a cycle different from that of OS ($n = 25$) and, OD: undergoing embryo transfer in oocyte donation cycles ($n = 37$). At embryo transfer day, several endocrine and immune biomarkers were assessed.

Results: OS patients showed significantly higher serum estradiol, progesterone and Ghrl, than those not stimulated. Patients that suffered miscarriage showed significantly lower concentrations of sex-hormones, with a similar trend for Ghrl, that deserves further investigation. Moreover, OS patients showed decreased frequencies of circulating T cells and reduced ratios of uNK/NK cells, which significantly associated with serum levels of sex-hormones. Besides, ROC curves identified cut-off values predictive of clinical pregnancy and/or miscarriage for peripheral counts of uNK cells, T cells, and uNK/NK cells ratio.

Conclusions: As hypothesised, OS significantly increased serum Ghrl in correlation with sex-hormone levels. These last, significantly associated with maternal immune response and reproductive outcome. Although Ghrl exhibited a similar profile, it did not reach statistical significance, indicating the need for further investigation. Additionally, the identification of maternal immunological cut-off values holds significant clinical relevance.

1 | Introduction

Ghrelin (Ghrl) is a gut polypeptide originally described by its ability to stimulate growth hormone secretion. Nevertheless, since its discovery in 1999, Ghrl and its active receptor (GHS-R1a) have been described to be ubiquitously expressed and to exert several endocrine and paracrine effects reviewed in [1]. Indeed, Ghrl has been shown to play different roles in

reproduction and gestation. Several studies indicate that Ghrl is involved in embryo implantation and development, placentation, decidualization and angiogenesis [2–7]. Tanaka et al. (2003) demonstrated that the endometrial expression of *Ghrl* and its receptor markedly increases during the implantation period, and that Ghrl enabled the in vitro decidualization of endometrial stromal cells. Tawadros et al. (2007) found that Ghrl and GHS-R mRNA levels peaked in the secretory phase of the menstrual cycle and that Ghrl stimulates the decidualization process through the production of prolactin and IGFBP-1. In addition, we recently reported data from a mice model indicating that the administration (s.c.) of a Ghrl antagonist [(D-Lys3)GHRP-6] to naturally pregnant dams during the peri-implantation period (Days 3–8) significantly impaired early embryo development, implantation and placenta formation/function [3], being these effects related to a hostile uterine environment associated with inflammation, altered immune response and nitrosative stress [2].

Nevertheless, either local or systemic, supraphysiologic levels of Ghrl have also been associated with implantation failure [3, 8], in agreement with the fact that this hormone acts as a food scarcity signal prioritising individual survival rather than reproduction reviewed in [9]. Concurring, Zwierzchowska et al. (2018) reported that women with recurrent miscarriage show higher endometrial expression of Ghrl and VEGF-A than healthy control individuals. Therefore, the evidence so far point to a tightly regulated Ghrl secretion as essential for a successful early pregnancy, since supra or infraphysiological concentrations of the hormone have deleterious effects on reproduction [2–4, 7].

Besides, Ghrl has emerged as a potent anti-inflammatory and immunomodulatory mediator reviewed in [10], which suggest it may also be involved in the maternal immunoregulation necessary for ensuing allogeneic embryo receptivity and healthy pregnancy. Interestingly, GHSR is widely expressed in thymus, several lymphoid organs and several leucocyte subsets including T and B cells, monocytes and dendritic cells, that upon activation can produce high concentrations of Ghrl and down regulate the secretion of several pro-inflammatory cytokines [10, 11].

In humans, gastric and circulating Ghrl levels are higher in females than in males, suggesting that the hormone secretion might be under control of sex hormones [12–14]. Consistently, it has been shown that oestrogens markedly stimulate *GHRL* expression on stomach cells [12]. These evidence, together with the fact that OS increases estradiol (E_2) levels supraphysiologically [15, 16], suggest that systemic Ghrl concentrations could be significantly altered in women undergoing OS in assisted reproduction treatments (ART). Moreover, OS and particularly high sex-hormone levels have been related with negative reproductive outcomes due to impaired decidua's physiology, advanced implantation window, and altered uterine immune profile [17], but the possible responsibility of hyperghrelinemia in these effects are almost unknown, since reported studies on the field are scarce and their results inconsistent [18–20].

To shed light on the matter, we herein analysed whether OS alters Ghrl serum levels in women undergoing assisted reproduction and associates with endocrine and immune biomarkers, and reproductive success.

2 | Material and Methods

2.1 | Ethical Approval

The experimental protocol was reviewed and approved by two external review boards (CIEIS del Hospital Nacional de Clínicas and Consejo de Evaluación Ética de la Investigación en Salud, Córdoba, Argentina).

2.2 | Patients

This prospective cohort study included 97 female patients who underwent ART between July 2018 and December 2019 at the Centro Integral de Ginecología, Obstetricia y Reproducción de Córdoba (Argentina). Inclusion criteria were women undergoing ART with OS for IVF/ICSI with fresh embryo transfer (OS group; $n = 35$), or undergoing frozen embryo transfer in a cycle different from that of stimulation (FET group; $n = 25$), or embryo transfer in oocyte donation cycles (OD group; $n = 37$). Although FET and OD groups function as our "control" group (i.e., patients without OS), they were considered separately because of the expected differences in oocytes quality that might modify reproductive outcomes (since FET included oocytes from the patient and OD included donated oocytes).

Exclusion criteria were as follows: Oocyte age ≥ 40 years; severe male factor (globozoospermia, severe cryptozoospermia and testicular sperm retrievals); low response to OS (< 5 oocytes); and patients with endometriosis using their own oocytes.

For ovarian stimulation, a combination of recombinant follicle stimulating hormone (rFSH) and human menopausic gonadotrophin (HMG) was given to each patient starting on the third day of cycle, with starting doses between 150 and 300 IU/day acoording to body mass index (BMI) and ovarian reserve. Subsequent doses were adjusted according to ovarian response.

2.3 | Clinical Evaluation

Recording of age, BMI [weight (kg)/height(m)^2], infertility duration and evaluation of its aetiology.

2.4 | Quantitation of Hormones and IL6 Plasma Levels

E_2 and P_4 plasma levels were assessed by electrochemiluminescence (ECLIA) at embryo transfer (ET) day, using the Architect Estradiol and Progesterone kits (sensitivity: 25 pg/mL and 0.1 ng/mL, respectively) and following the manufacturer's instructions. Peak E_2 levels (i.e., E_2 level on the trigger day-day of human chorionic gonadotrophin for retrieval-) were also assessed. Follicle stimulating hormone and anti-Müllerian hormone (AMH) serum levels were assessed at

cycle Day 3 by ECLIA, using Elecsys Cba-Roche kits (sensitivity: 0.1 mUI/mL and 0.01 ng/mL respectively). On ET day, Ghrl and IL6 plasma levels were quantitated using specific ELISA kits (Human Ghrelin Cat. Duo Set ELISA, R&D Systems, MN, USA, sensitivity of 31.3 pg/mL, and Elecsys-Roche ECLIA Human IL6 kit, sensitivity of 1.5 pg/mL, respectively). As a standard procedure, before obtaining blood samples, patients had fasted for at least 12 h.

2.5 | Analysis of Circulating Leucocyte Subsets

On ET day, peripheral blood specimens were obtained and peripheral blood mononuclear cells (PBMCs) were isolated using BD-Vacutainer-CPT tubes after centrifugation at 1500 *g* for 20 min. PBMCs were washed with PBS, counted, and re-suspended in cryoprotectant solution (85% of foetal bovine serum inactivated by heat plus 15% dimethylsulphoxide) at ~5×10^6 cells/vial (~4 vials/patient). Vials were then slowly cooled and frozen until −80°C for 24 h, and then stored in liquid nitrogen until use. After thawing, samples showed a cell viability > 93%.

As previously described [21, 22], PBMCs were stained with different fluorescent-labelled monoclonal antibodies and analysed by flow cytometry to assess circulating natural killer T cells (NKT: $CD3^+/CD56^+$), natural killer cells (NK: $CD3^-/CD56^{dim}/CD16^+$), uterine natural killer (uNK: $CD3^-/CD56^{bright}/CD16^-$), T cells ($CD3^+$), activated T helper (Th) cells ($CD3^+/CD4^+/CD62L^-$), naïve Th cells ($CD3^+/CD4^+/CD62L^+$), activated cytotoxic T (Tc) cells ($CD3^+/CD8^+/CD62L^-$), naïve Tc cells ($CD3^+/CD8^+/CD62L^+$), and regulatory T (Treg) cells ($CD3^+/CD4^+/CD25^{bright}/CD127^{dim}$).

2.6 | Assessment of Reproductive Outcome

Pregnancies were confirmed with a positive quantitative β-hCG (human chorionic gonadotrophin) on Day 9 after blastocyst transfer and the presence of foetal heartbeat on Week 7. Clinical pregnancy rates were calculated as a percentage of pregnancies/number of ET procedures. Miscarriage rate (calculated as % of miscarriages/number of clinical pregnancies), and live birth rate (calculated as % of live born children/number of ET procedures) were also recorded.

2.7 | Statistics

Variables were expressed as percentage or Mean ± SEM as appropriate. Variables expressed as percentage were evaluated by the Chi-square or Fisher exact test as appropriate. Variables expressed as Mean ± SEM were evaluated by ANOVA or Kruskall Wallis tests as appropriate. Age was used as a co-variable in ANOVA since differences among groups were observed. ROC curves were built to define cut-off values for hormonal and immune variables. Multiple logistic regression analysis was also performed between these variables (age, body mass index, endocrine values and immune profile) and outcome variables, using forward conditional analysis. A $p < .05$ was considered statistically significant.

3 | Results

3.1 | General Characteristics of the Study Patients

Characteristics of the patients included in this study are shown in Table 1 and, as expected, OD patients were older and had a longer history of infertility than OS and FET patients ($p < 0.01$). In consequence, age was included as a co-variable in the statistical analyses. However, age does not appear to be associated with reproductive success in these patients, as logistic regression slopes were not statistical significant (slope for age and clinical pregnancy rate = 0.07; miscarriage rate = 0.08 and live birth rate = 0.03; $p > 0.05$) and the values for clinical pregnancy, miscarriages, and live births in the OD group were not lower than OS or FET, which were composed of younger women (Table 1; more details below). Besides, body mass index showed comparable values among groups (Table 1).

In agreement with patients' characteristics, most prevalent infertility etiologies in OS and FET patients (who underwent ART with their own oocytes) were male factor (65.7% and 56.0%, respectively), tubal infertility (20.0% and 12.0%, respectively), and idiopathic infertility (5.7% and 12.0%, respectively). On the contrary, most frequent infertility etiologies in OD patients were ovarian factor (51.4%) and/or advanced age (48.7%).

3.2 | Serum Levels of Ghrl and Sex-Hormones and Their Association With Reproductive Outcome

On ET day, serum levels of Ghrl and sex-hormone were analysed. Interestingly, OS patients showed higher levels of Ghrl than FET and OD patients (Figure 1–panel A, $p < 0.01$). In addition, and as expected, OS patients showed also significantly increased levels of E_2 and P_4 than FET and OD patients (Figure 1–panel A, $p < 0.001$). Concurrently, a significant positive correlation was found between levels of Ghrl at ET day and those of E_2 and P_4 ($r = 0.28$ and $r = 0.28$ with E_2 and P_4, respectively; $p < 0.001$), and between levels of Ghrl and trigger day E_2 ($r = 0.42$, $p < 0.05$). Furthermore, a significant positive correlation between levels of Ghrl and AMH was found ($r = 0.71$; $p < .001$), whereas Ghrl concentrations inversely correlated with patient's age ($r = -0.22$, $p < 0.05$). Ghrelin levels did not correlate with body mass index and the adjustment of the statistical model for this variable did not change the differences between groups found in ghrelinemia.

After calculating E_2/P_4 serum ratio, OD patients showed significantly increased values with respect to OS and FET patients (Figure 1–panel A, $p < 0.01$). Interestingly, multiple logistic regression analysis selected this variable as the most assertive to predict miscarriage (Cox & Snell's $r = 51\%$; $p < 0.05$). Noteworthy, E_2/P_4 ratio positively correlated with age ($r = 0.20$, $p < 0.05$). These findings are in agreement with the fact that OD patients were older than OS and FET patients (Table 1).

TABLE 1 | General characteristics of the patients included in this study, and quantity/quality of embryos transferred and ART success.

Parameters	OS ($n = 35$)	FET ($n = 25$)	OD ($n = 37$)
Age (years)	35.5 ± 0.57	35.2 ± 0.68	41.7 ± 0.56*
Body mass index (kg/m^2)	24.6 ± 0.84	23.9 ± 1.00	24.2 ± 0.82
Infertility history (years)	3.74 ± 0.50	3.56 ± 0.59	5.65 ± 0.48#
Number of transferred embryos	1.26 ± 0.07	1.12 ± 0.07	1.11 ± 0.05
Good quality embryo rate (%)	84.6	77.3	90.9
Clinical pregnancy rate (%)	34.3	44.0	56.8
Miscarriage rate (%)	0.0	54.5&	28.6
Live birth rate (%)	34.3	16.0	40.5

Note: Values are expressed as Mean ± SEM or percentage. Good quality embryos are considered those that are category 3BB or higher. Miscarriages were calculated as percentage in relation to clinical pregnancies. Live births were calculated as percentage in relation to embryo transfers (i.e., general success of ART). n = number of patients.

Abbreviations: FET, frozen embryo transfer; OD, oocyte donation cycles; OS, controlled ovarian hyperstimulation.

*$p < 0.001$ OD versus OS and FET.

#$p < 0.01$ OD versus OS and FET.

&$p < 0.05$ FET versus OS (ANOVA and LSH Fisher as poshoc or Kruskal Wallis as appropriate).

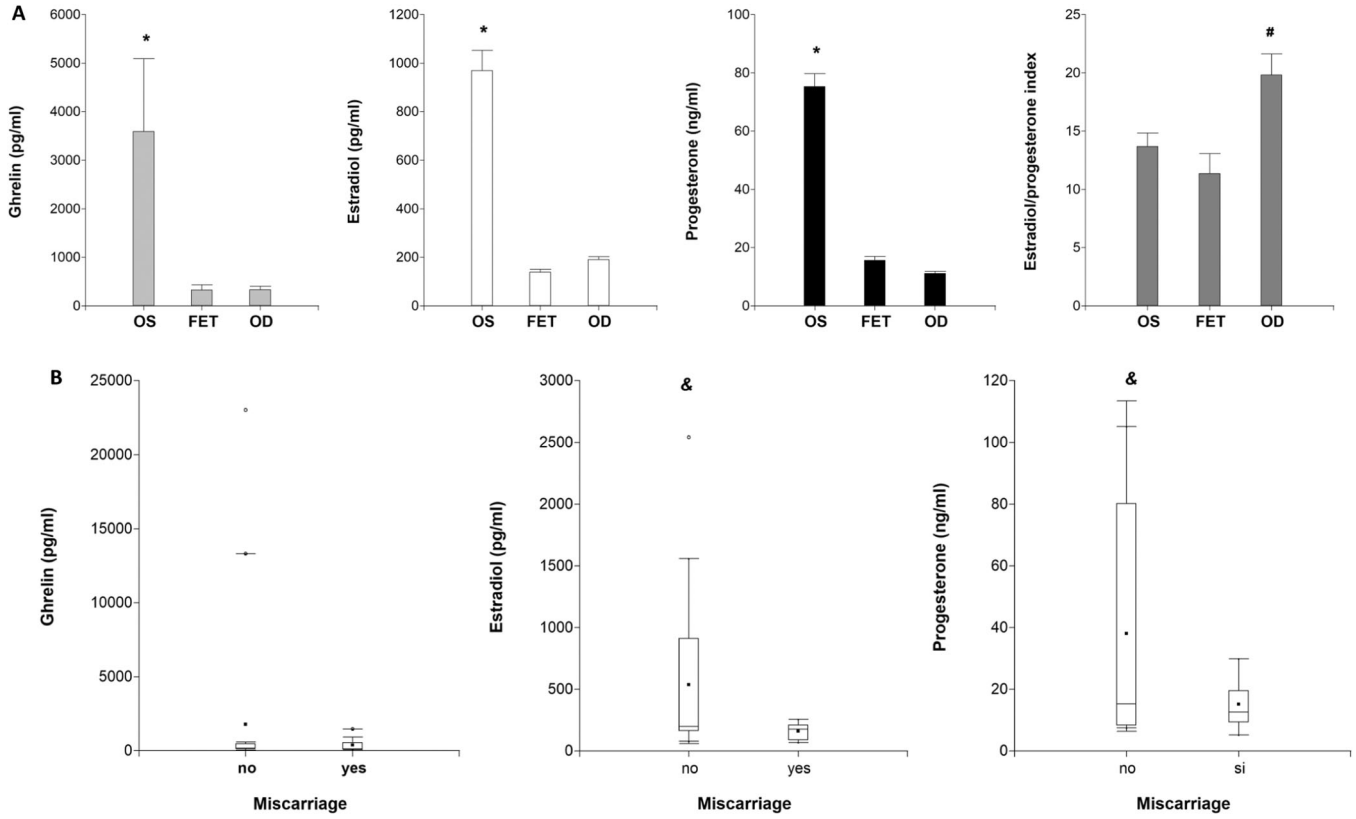

FIGURE 1 | Serum concentrations at embryo transfer day of ghrelin, sex-hormones and estradiol/progesterone index (panel A); and serum concentrations of ghrelin, and sex-hormones from patients that suffered (yes) or not (no) a miscarriage considering all the study patients (panel B). In panel A values are expressed as Mean ± SEM and on panel B, values are expressed as Mean, Q05–Q95 and 2 SD. OS, ovarian stimulation ($n = 35$); FET, frozen embryo transfer (n = 25), and OD, oocyte donation cycles ($n = 37$). *$p < 0.001$ OS versus FET and OD; #$p < 0.01$ OD versus FET and OS and &$p < 0.05$ no versus yes (Panel A: ANOVA and LSH Fisher as poshoc or Kruskal Wallis as appropriate; Panel B: Chi-square test).

Table 1 shows also ART outcomes from patients groups under analysis. As shown, no significant differences were found in the number of transferred embryos, as well as in the rates of good quality transferred embryos and clinical pregnancy rate among OS, FET and OD patients. On the contrary, FET patients showed significantly increased miscarriage rates than OS patients ($p < 0.05$), whereas no differences with respect to OD patients were observed. Finally, overall ART outcome (measured as live birth rate) showed no significant differences among OS, FET and OD patients, although FET patients showed a clear trend to present decreased values.

When assessing whether serum hormone concentrations associated with the occurrence of miscarriage in the overall patient

population, those who suffered a miscarriage showed significantly lower concentrations of E_2 and P_4 on ET day than those with successful pregnancies (Figure 1–panel B, $p < 0.05$). A similar trend was observed for serum Ghrl (Figure 1–panel B). Noteworthy, no patient with Ghrl concentrations above the sample Mean value (2228.55 ± 693.26 pg/mL) suffered miscarriage. Besides, no differences were found in ET day ghrelinemia between patients achieving clinical pregnancy or not (Ghrl: 1083.3 ± 994.7, $n = 40$, vs. 1689.2 ± 794.5, $n = 50$, respectively), or between those that delivered a live baby or not (Ghrl: 1782.9 ± 1043.3, $n = 29$, vs. 1036.4 ± 935.5, $n = 61$, respectively).

3.3 | Immune Biomarkers and Their Association With Reproductive Success

Figure 2 shows flow cytometry gating strategies (panel A) and the analysis of peripheral blood NKT cells ($CD3^+/CD56^+$; panel B) and total NK cells ($CD3^-/CD56^+$, panel C), differentiating uNK ($CD3^-/CD56^{bright}/CD16^-$) from NK ($CD3^-/CD56^{dim}/CD16^+$) cells (panel D). As shown, no significant differences in the frequencies of NKT cells and total NK were found among patients groups under study. Nevertheless, when evaluating the NK cell subsets (NK or uNK, panel D), a significant decrease in the frequencies of uNK cells together with an increase in that of NK were found in OS with respect to FET patients ($p < 0.05$). In agreement, significantly lower uNK/NK ratios were observed in OS patients than in FET (0.04 ± 0.004 vs. 0.05 ± 0.010, respectively, $p < 0.05$). These differences are related to the sex-hormones concentrations specific to each group, since when E_2 and P_4 levels are included in the statistical model, the differences disappear.

The flow cytometry gating strategies and the analysis of peripheral blood T lymphocytes are shown in Figure 3 (panels A–E). As shown, OS patients had significantly lower frequencies of T cells than FET and OD patients (panel B, $p < 0.05$), showing comparable proportions of Th ($CD3^+/CD4^+$), Tc ($CD3^+/CD8^+$) (panel C), and Treg cells ($CD3^+/CD4^+/CD25^+/CD127^+$, panel D). Moreover, no significant differences in the frequencies of naïve ($CD62L^+$) or effector ($CD62L^-$) Th and Tc cells were observed among groups (panel E). This difference is not attributed to the sex-hormones profile of each group, since when E_2 and P_4 levels are included in the statistical model, the difference persist.

When analysing possible correlations between immune and endocrine biomarkers, serum levels of sex-hormones (E_2 and P_4), but not those of Ghrl, significantly correlated with several immune biomarkers (Table 2). As shown, E_2 concentrations, either inversely correlated with blood frequencies of uNK among NK cells (i.e., uNK/total NK and uNK/NK, $p < 0.05$), or directly correlated with NK/total NK cell ratio ($p < 0.05$). In addition, concentrations of E_2 inversely correlated with circulating total T lymphocytes (Table 2, $p < 0.05$). The same correlation profile was detected for P_4, but this hormone correlated also with the percentage of NKT cells (Table 2, $p < 0.05$). On the contrary, the E_2/P_4 index positively correlated with peripheral NKT cells frequencies, and also with the percentage of peripheral Tregs (Table 2, $p < 0.05$). Ghrelin serum levels did not show significant correlation with any of the immune biomarkers analysed.

Finally, peripheral T cells frequencies associated with ART success, since ROC curves identified T lymphocyte cut-off values predictive for clinical pregnancy achievement and miscarriage. In detail, total $CD3^+$ T cell above 71.80% of the peripheral mononuclear cells resulted predictive of clinical pregnancy ($AUC = 0.68 \pm 0.06$, $p < 0.01$); 67% of the patients that did not achieve pregnancy showed levels below this percentage (Figure 4, panel A, $p < 0.01$). Furthermore, total T cells above 76.68% were predictive of miscarriage ($AUC = 0.69 \pm 0.10$, $p < 0.029$), since 64% of the patients that suffered a miscarriage showed T levels higher than this number (Figure 4, panel B, $p > 0.05$). Moreover, NK cell subsets were also related with reproductive outcome. The proportion of uNK cells and uNK/NK cell index showed cut-off values predictive of clinical pregnancy: a value of uNK higher than 4.08% ($AUC = 0.62 \pm 0.07$, $p < 0.029$) or a uNK/NK index higher than 0.0427 ($AUC = 0.63 \pm 0.06$, $p < 0.018$) resulted predictive of clinical pregnancy achievement, since 57% of the patients that achieved pregnancy showed values higher than those (Figure 4, panels C and D respectively, $p > 0.05$).

Finally, serum levels of IL6 on ET day showed neither significant differences among patients groups under study (OS = 9.09 ± 0.72 pg/mL, FET = 8.62 ± 0.42 pg/mL and OD = 8.77 ± 0.24 pg/mL), nor significant association with the concentrations of any of the hormones analysed.

4 | Discussion

As hypothesised, in this study we found that patients with OS showed significantly increased serum levels of Ghrl (evaluated on ET day), in correlation with those of E_2 and P_4. This increase in sex-steroids showed a positive association with pregnancy progression and outcome, with a similar trend for Ghrl that did not achieve statistical significance. In addition, there were differences in the leucocyte subsets between stimulated and non-stimulated patients, the former showing significantly lower frequencies or circulating CD3 + T cells, without differences in the proportions of CD4+ helper T (Th), CD8+ cytotoxic T (Tc), or regulatory T (Treg) cells. Moreover, OS patients showed lower proportion of uNK and higher of NK cells (with respect to the FET group), whereas comparable levels of NKT frequencies among groups. Furthermore, and irrespective of the patient group analysed, ROC curves identified cut-off values in the levels of circulating T cells, uNK cells and uNK/NK ratios that showed to be predictive for the achievement of clinical pregnancy and/or the occurrence of miscarriage.

To the best of our knowledge, there are two studies that evaluated the possible variation of ghrelinemia in women undergoing IVF/ICSI protocols [18, 19]. In the first one, the authors analysed the possible variation in Ghrl serum level from Day 2 of the IVF/ICSI cycle to Day 12 post-embryo transfer in 20 women undergoing ART. In contrast to our findings, authors found neither significant changes in ghrelinemia throughout the study period, nor a significant

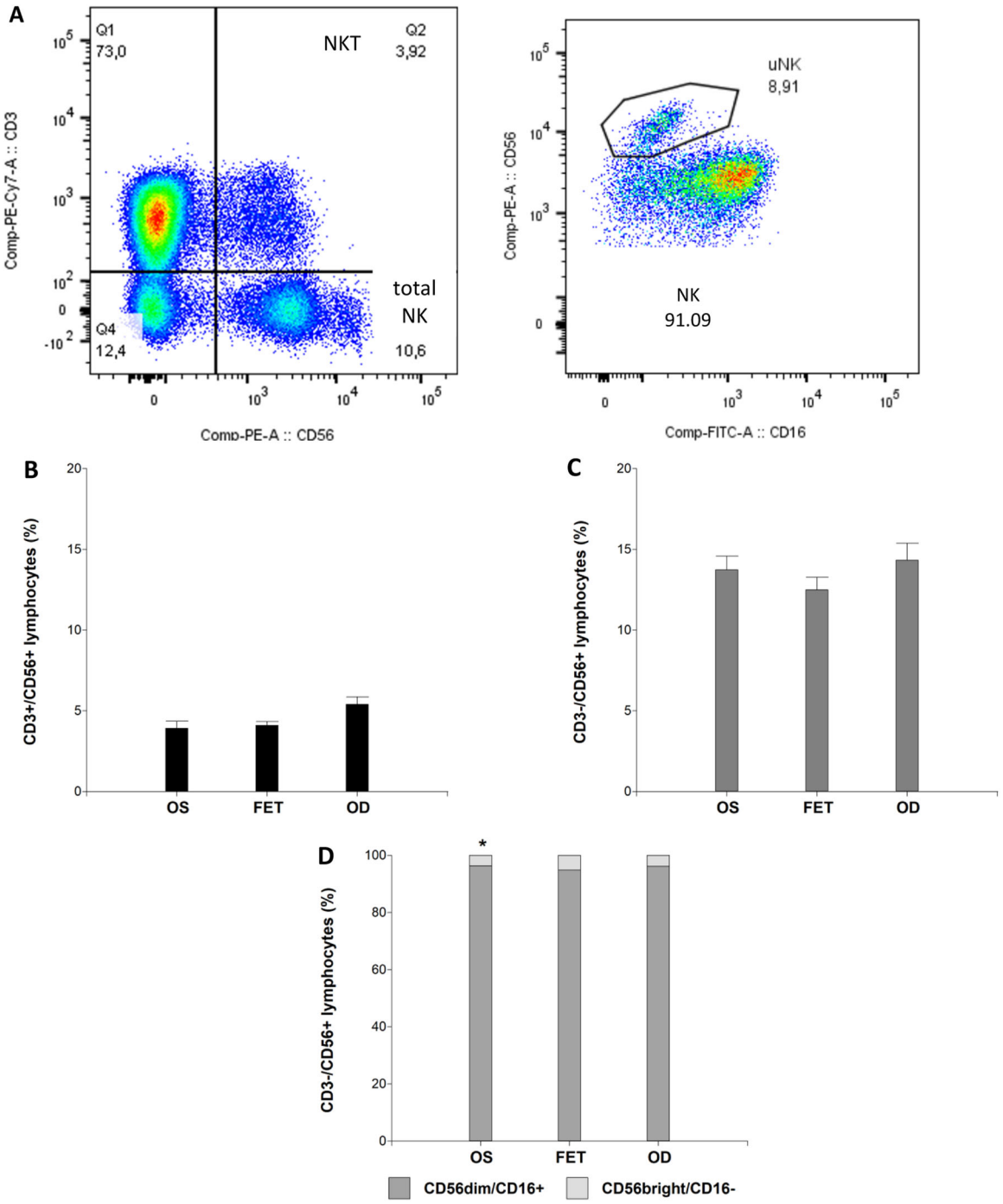

FIGURE 2 | Flow cytometry gating strategies (panel A) and analysis of frequencies of peripheral NKT cells($CD3^+/CD56^+$; panel B), NK cells ($CD3^-/CD56^+$; panel C), and NK cell subsets (uNK: $CD56^{bright}/CD16^-$ and NK: $CD56^{dim}/CD16^+$; panel D) on embryo transfer day. Values are expressed as Mean ± SEM. OS, ovarian stimulation ($n = 35$); FET, frozen embryo transfer ($n = 25$) and OD, oocyte donation cycles ($n = 37$).*: $p < 0.05$ OS versus FET (ANOVA and LSH Fisher as poshoc or Kruskal Wallis as appropriate).

correlation between Ghrl and E_2 or P_4 serum levels (except on Day 7 postembryo transfer, in which levels of Ghrl correlated inversely with those of E_2) [18]. This lack of correlation (or even further, the inverse correlation found on Day 7) is intriguing since other studies have found a positive correlation between oestrogens and Ghrl [12, 14]; in fact, Ghrl stimulating effects have been attributed to E_2 [13]. Nevertheless, it is important to mention that the stimulation protocol used in that study consisted in a short agonist coflare protocol, which is not frequently used in assisted reproduction.

In the second mentioned study, Li et al. (2011) assessed a group of normal-weight women ($n = 31$) that underwent IVF cycles. Authors found no differences between plasma levels of Ghrl on Day 2 of the cycle and those on aspiration day. In contrast to our findings, they did not find a correlation between serum Ghrl levels and age, trigger day E_2, or baseline AMH levels [19].

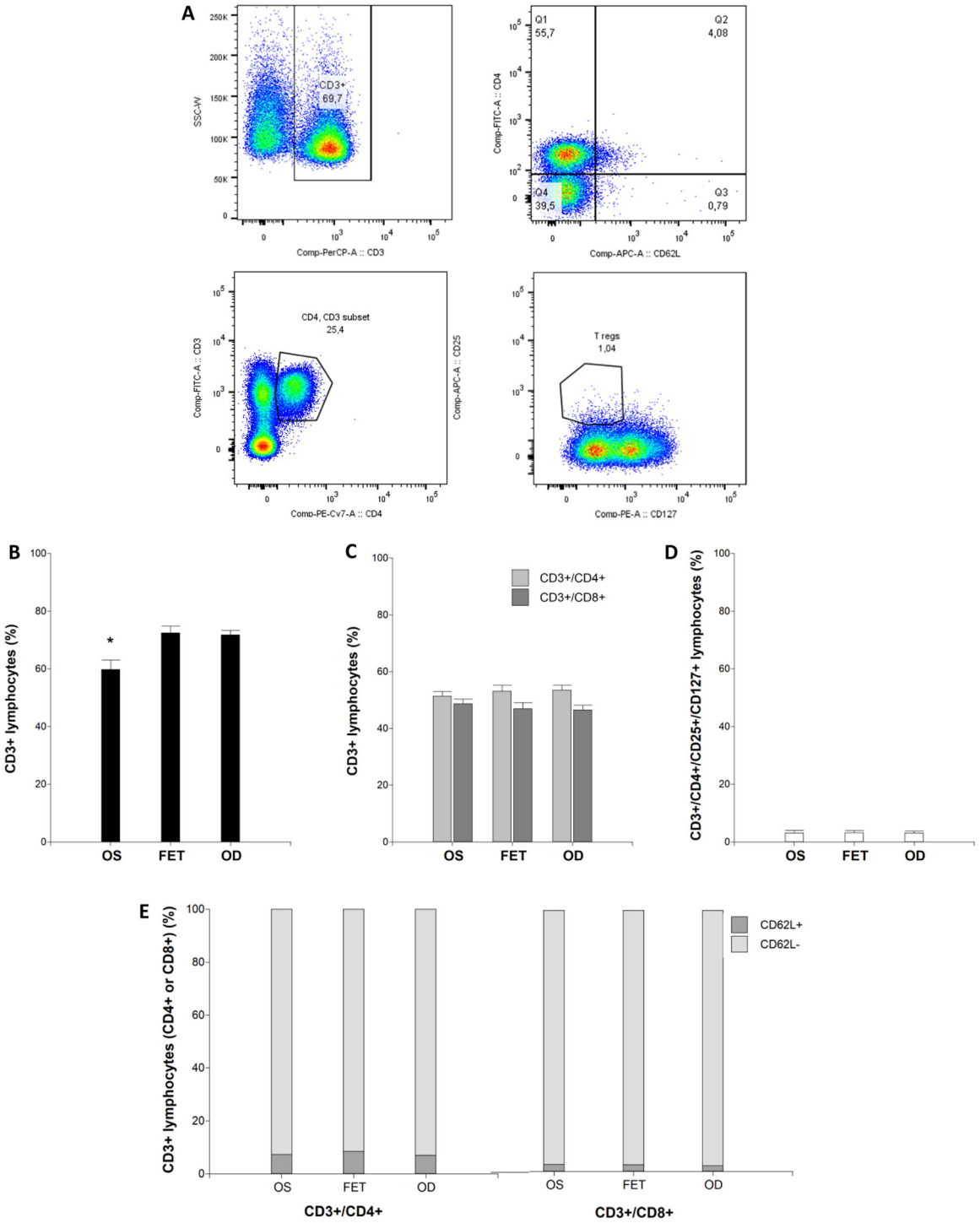

FIGURE 3 | Flow cytometry gating strategies (panel A) and analysis of frequencies of peripheral total T cells ($CD3^+$; panel B), and T cell subsets [T helper (Th): $CD3^+/CD4^+$ and T cytotoxic (Tc): $CD3^+/CD8^+$; panel C], T regulatory (Tregs: $CD3^+/CD4^+/CD25^+/CD127^+$; panel D)], and naïve ($CD62L^+$) or activated ($CD62L^-$) Th and Tc lymphocytes (panel E), on embryo transfer day. Values are expressed as Mean ± SEM. OS, ovarian stimulation ($n = 35$); FET, frozen embryo transfer ($n = 25$) and OD, oocyte donation cycles ($n = 37$).*: $p < 0.05$ OS versus FET and OD (ANOVA and LSH Fisher as poshoc or Kruskal Wallis as appropriate).

Interestingly, the authors found a significant correlation between Ghrl and E_2 levels in follicular fluid, but not with P_4 concentrations. They also found that intrafollicular Ghrl concentrations negatively associated with the oocyte's ability to develop into a viable embryo in the cleavage stage [19]. Differences with our findings could be related with dissimilarities in the experimental setting, such as the cycle day in which Ghrl was evaluated (oocytes retrieval day vs. ET day). In that regard, it is worth mentioning that the endocrine function of corpora lutea on the day of ET is much better established than that on the day of retrieval. However, it is important to mention that a limitation of our study is that we did not evaluate baseline Ghrl levels in the OS group. The control patients of our study were those non-stimulated.

TABLE 2 | Correlations between peripheral immune markers and endocrine profile.

	Plasma hormones			
	E_2 (pg/mL)	P_4 (ng/mL)	E_2/P_4 index	Ghrl (pg/mL)
Peripheral immune markers				
NKT ($CD3^+/CD56^+$)	—	−0.21	0.21	—
total NK ($CD3^-/CD56^+$)	—	—	—	—
uNK ($CD3^-/CD56bright/CD16^-$)	—	—	—	—
NK ($CD3^-/CD56^{dim}/CD16^+$)	—	—	—	—
uNK/total NK	−0.24	−0.23	—	—
NK/total NK	0.24	0.23	—	—
uNK/NK index	−0.23	−0.22	—	—
total T cells ($CD3^+$)	−0.22	−0.20	—	—
Th ($CD3^+/CD4^+$)	—	—	—	—
Tc ($CD3^+/CD8^+$)	—	—	—	—
Tregs ($CD3^+/CD4^+/CD25^+/CD127^+$)	—	—	0.49	—

Note: Only those *r* values from correlations statistically significant ($p < 0.05$) were included in the Table (Spearman's coefficient).

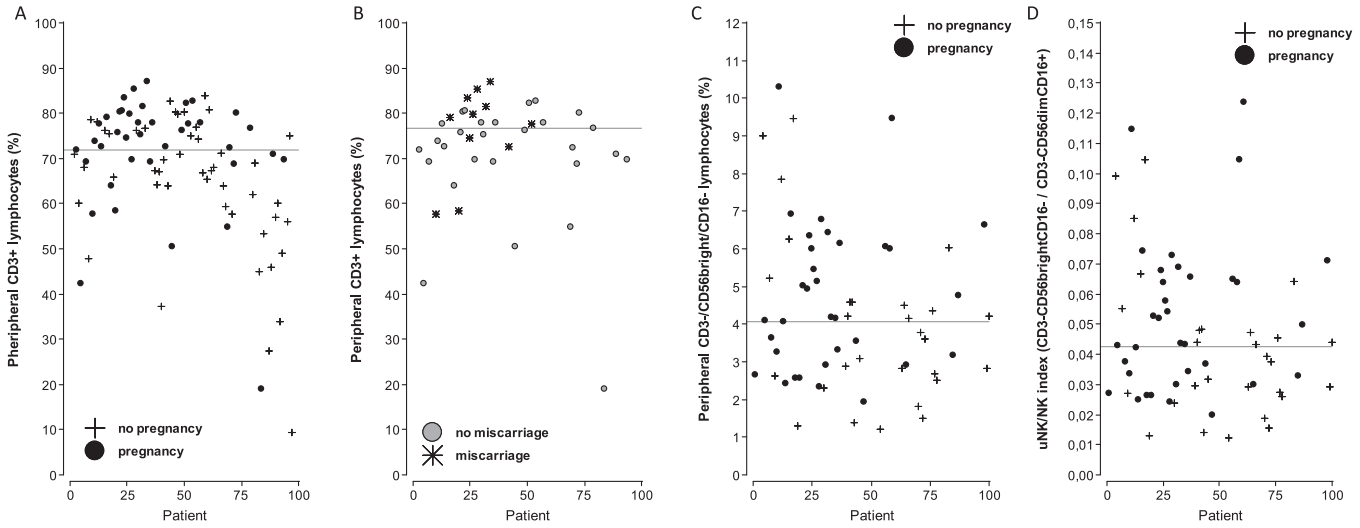

FIGURE 4 | Calculated cut-off values of the frequencies of circulating total T cells ($CD3^+$; panels A and B), uNK cells ($CD3^-/CD56^{bright}/CD16^-$; panel C), and uNK/NK cell index (NK: $CD3^+/CD56^{dim}/CD16^+$; panel D), predictive of clinical pregnancy achievement and/or miscarriage. Points and arrows/asterisks in each panel represent each one of the patients. The line in each panel indicates the cut-off value for this parameter. AUC values of ROC curves were 0.68 ± 0.06 ($p < 0.01$) and 0.69 ± 0.10 ($p < 0.05$) for T cells panels A and B, respectively; 0.62 ± 0.07 ($p < 0.05$) for uNK (panel C) and 0.63 ± 0.06 ($p < .05$) for uNK/NK cell index (panel D), respectively (ROC curves).

The rationale of studying a possible association of ghrelinemia with ART success is that Ghrl has been comprehensively associated with embryo development and implantation [2–7]. As stated above, various studies indicate that Ghrl concentrations may regulate mammalian pre-implantation embryo development, suggesting an inverted U shape response. Lower and higher concentrations appear detrimental, while median levels seem to be beneficial [4, 7]. These findings are supported by data previously reported by us from an in vivo experimental model [2, 3]. Furthermore, Ghrl has been linked with embryo implantation since it has been shown that Ghrl stimulates decidualization of human stromal cells in vitro [5, 6]. Moreover, the in vivo treatment with a Ghrl antagonist induced a hostile uterine environment, characterised by the induction of inflammation and nitrosative stress [2]. All these evidence prompted us to wonder whether a potential variation in ghrelinemia secondary to OS could alter reproductive outcomes, and in which direction these modifications might lead. Specifically, would they have harmful or beneficial effects on reproductive success?

Our results suggest that the hyperghrelinemia secondary to OS does not have a direct effect on maternal immune response or the success of assisted reproduction, since ET day Ghrl concentrations did not correlate with maternal immune biomarkers or reproductive outcomes (i.e., clinical pregnancy rate, miscarriage rate and live birth rate). Nevertheless, we found that patients that suffered miscarriage showed lower levels of Ghrl than those with an ongoing pregnancy. Moreover, no patient with a serum Ghrl concentration higher than the sample mean,

suffered miscarriage. Although these results only showed a trend without reaching statistical significance, we consider that this aspect warrants further investigation including larger patient populations. Although that trend might be related to the positive correlation of Ghrl levels with those of E_2 and P_4 (hormones that significantly associated with pregnancy progression), we consider that Ghrl might be exerting some independent effects. Sabatini et al. (2009), studying normal weight and underweight patients undergoing ART, showed that the hyperghrelinemia associated to low weight affected reproductive outcomes. Although they found reduced rates of clinical pregnancy in these patients, they also found reduced rates of miscarriage [23], supporting that Ghrl may exert protective effects on some pregnancy early events. Furthermore and besides of the afore mentioned evidence regarding the effects of Ghrl on stromal cells decidualization, Tawadros et al. (2007) hypothesised that Ghrl might enhance progesterone-induced decidualization by sensitising endometrial stromal cells to the action of P_4. Interestingly, in an murine model assessing OS reproductive effects, we found that stimulation induced a four-fold increase in plasma Ghrl levels and delayed embryo development, which was reversed by the coadministration of a Ghrl antagonist [(D-Lys3)GHRP-6] [24]. Hence, it would be interesting to differentiate between the effects of OS that are related to the increase in sex-hormones and those that are linked to Ghrl increase.

Furthermore, the inverse association between sex-hormones serum levels and miscarriage found in our study was somewhat expected, since the protective effects of E_2 and P_4 are widely recognised reviewed in [25]. Nevertheless, supraphysiological levels of sex-hormones secondary to OS has also been proposed as the main cause of reproductive failure in FIV/ICSI patients [15, 16]. In that regard, the degree of increase in P_4, but especially E_2 serum levels seems to be critical [15].

It is important to mention that in our study, no patient subjected to OS suffered miscarriage; this was somehow unexpected. Nevertheless, consisting findings were observed in this group, when assessing reproductive results, endocrine and immune biomarkers profile, and their correlations. In the same way, OD patients accounted for nearly 30% of reported miscarriages. Although this difference is not statistically significant with that of OS patients, and although this value is similar to the results reported by other reproductive centres of our country (Registro argentino de fertilización asistida – RAFA. Resultados 2018. http://www.samer.org.ar/pdf/Datos_rafa_2018.pdf), such a difference with OS patients caught our attention. Noteworthy in our study, OD patients showed significantly higher E_2/P_4 indexes, being this parameter identified by the logistic regression analysis as the most assertive to predict miscarriage. We do not know exactly the underlying aetiology for this higher E_2/P_4 index in OD patients; however, it might be related with patient's age since age and E_2/P_4 index correlated significantly. Certainly, an increase in the sample size will bring the results closer to the values more commonly found in TRA populations.

When analysing leucocyte subsets in peripheral blood, OS patients showed significantly lower frequencies of $CD3^+$T lymphocytes. This decrease should not be attributed to the increase in sex-hormones, since when E_2 and P_4 levels are included in the statistical model, the difference persist. An alternative explanation could be the raise in Ghrl concentrations observed in these patients, since it has been shown that Ghrl inhibits T cell proliferation in a dose-dependent manner [26]. Moreover, this effect was found to be GHSR-specific, since it was reversed by the treatment with the Ghrl antagonist (D-Lys3)GHRP-6 [11]. In addition, it has been shown that Ghrl inhibits the secretion of the pro-inflammatory cytokines IL1A, IL1B, IL6 and TNF by PBMCs [11]. However, we herein did not find significant differences in IL6 concentrations among stimulated or non-stimulated patients.

Although the frequencies of total T cells were reduced in OS patients, no significant differences in the levels of different T cell subsets (i.e., Tregs, Th and Tc –naïve or activated- cells) were observed among patient groups. Svoboda et al. [27] reported that GnRH-antagonist/hCG protocols induce changes in peripheral blood immune cell subsets that are detrimental for embryo implantation, which were characterised by decreased CD4/CD8 T cell ratios and increased proportions of $CD56^{dim}$ NK cells. In contrast, we found no differences in CD4 Th/CD8 Tc ratios among OS, OD and FET patients. Nevertheless, ROC curves identified a cut-off value for peripheral blood T cells (above 76.68%) as predictive of miscarriage, which could be related with the lower miscarriage rate found in OS patients. Moreover, cut-off value of circulating T cells was also identified as predictive of clinical pregnancy.

NK cell subsets have been strongly associated with pregnancy progression. Indeed, uNK cells ($CD56^{bright}/CD16^-$) have been largely associated with successful implantation and placental maturation [21, 28]. In fact, they are supposed to play a key role during implantation by controlling vasculature remodelling for trophoblast invasion and producing immunoregulatory cytokines to promote allogeneic embryo receptivity [29, 30]. Interestingly, it has been proposed that endometrial uNK survival depends on P_4, since endometrial uNK cells dramatically increase in number during the secretory phase of the menstrual cycle (when P_4 levels increase) and they undergo apoptosis at the end of the menstrual cycle (when P_4 levels drop) [22]. Noteworthy, OS patients showed the highest levels of sex-hormones and the lowest miscarriage rate. Nevertheless, these patients showed a reduced uNK/NK cell index with respect to FET patients; the latter showing the highest miscarriage rate. In that regard, and contrary to the proposed negative effects of OS on the maternal immune response [16, 17], Lukassen et al. (2004) reported data indicating that the resulting supraphysiological concentrations of sex steroids do not associate with deleterious effects on embryo implantation. Indeed, they reported that OS patients showed increased endometrial $CD56^{bright}/CD56^{dim}$ NK cell ratios, resultant from a decrease in the cytotoxic $CD56^{dim}CD16^+$ NK cell subset [21]. However, the authors did not find the same profile in peripheral blood, suggesting that circulating immune cell profile (at least for NK cell subsets) does not mirror the uterine one. Supporting these data, similar findings were obtained in a pilot study developed in our reproductive centre comparing uterine lavage and peripheral blood uNK and NK cell frequencies [31]. Nevertheless, the crucial role of balanced endometrial and/or peripheral uNK and NK cells for pregnancy [32] is once again supported by our findings, since ROC curves identified cut-off

values for these biomarkers (% of uNK and uNK/NK index) as predictors of clinical pregnancy.

Reported studies on reproductive immunology are commonly divided into those that evaluate immune biomarkers in peripheral blood, from those that evaluate them in endometrial tissue. Although the latter might better and more accurately reflect the particular characteristics and embryo implantation potential of a women, endometrial biopsy or uterine lavage are not recommended to be routinely performed in the context of ART. Instead, the assessment of these biomarkers in peripheral blood emerges as the most convenient option because it is easier, safer, and possible to be performed in the same cycle in which the embryos will be transferred. In that regard, we consider that results presented herein are novel, useful and clinically important.

Finally, in the OS patients we found an interestingly direct correlation between Ghrl and AMH serum levels, the latter being a known biomarker of ovarian reserve [33]. Although available reported evidence in that regard is very scarce, our results are in line with those reported in obese patients with polycystic ovarian syndrome [34]. In addition, a significantly direct correlation between Ghrl levels and the number of mature oocytes retrieved was reported by a study analysing Ghrl serum levels at the beginning of the IVF cycle in patients with clomiphene resistance [35]. These data suggest that primordial follicles could be a major source of Ghrl during OS. This could perhaps be a/the reason for the high variability in the Ghrl variable within the OS group, since, as explained in the corresponding section, the stimulation protocol must be adjusted according to the clinical characteristics and the patients' response.

In conclusion, in the present study we provide novel data indicating that OS significantly increases Ghrl levels in correlation with that of sex-hormones, and associates with decreased frequencies of circulating T cells and reduced uNK/NK cell ratios. Although Ghrl concentrations did not significantly correlate with maternal immune biomarkers or ART success, a role of Ghrl in promoting successful pregnancy development and outcome cannot be excluded. Given the observed trend suggesting an inverse relationship between Ghrl levels and miscarriage, together with the observed reduced frequencies of CD3 T cells in OS patients, further studies encompassing larger patient populations are needed to verify our results and to unveil other effects of Ghrl on reproduction. Furthermore, we herein identified cut-off values for peripheral T and uNK cell frequencies/proportions predictive of clinical pregnancy achievement and/or miscarriage. Currently, a new study with a greater number of enroled patients is undergoing to confirm the clinical predictive validity of these immunological cut-off values and seeking an algorithm which might be predictive of pregnancy success and/or miscarriage based on endocrine (E_2, P_4 and Ghrl) and immune (peripheral blood NK and T cell levels) variables. That would provide a useful tool for improving patient care, since it would help predicting patient suitability for embryo transfer in assisted reproduction.

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Ethics Statement

The experimental protocol was reviewed and approved by two external review boards, one of Cordoba National University (CIEIS del Hospital Nacional de Clínicas, Córdoba, Argentina), and the other from the "Consejo de Evaluación Ética de la Investigación en Salud" (COEIS, Córdoba, Argentina), in accordance with Helsinki Declaration. Written consent has been obtained from each patient after full explanation of the purpose and nature of all procedures used.

Conflicts of Interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Data Availability Statement

The data underlying this article will be shared on reasonable request to the corresponding author.

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