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ARTICLE

Soluble LH-HCG receptor and oestradiol as predictors of pregnancy and live birth in IVF

BIOGRAPHY

Subhasis Banerjee received his PhD from Pennsylvania State University and undertook his postdoctoral studies at Columbia University School of Medicine, New York, USA. He has published and reviewed numerous manuscripts. He is currently Director of Origin Biomarkers Ltd, and has developed diagnostic tests for pregnancy diseases and prediction of premature and multiple birth outcomes before the start of fertility treatment.

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KEY MESSAGE

A blood test that uses LH–HCG receptor (blood LHCGR) together with oestradiol augments current approaches to predicting pregnancy, miscarriage and live birth before embryo transfer in IVF treatment.

ABSTRACT

Research question: Circulating soluble LH-HCG receptor (sLHCGR) is a first-trimester marker for screening pregnancy pathologies and predicts premature or multiple births before fertility treatment. Oestradiol per oocyte at ovulation induction predicts IVF treatment outcomes. We asked whether sLHCGR levels are stable during fertility treatment and whether, alone or with oestradiol, they could improve prediction of fertility treatment outcomes.

Design: Serum sLHCGR, anti-Müllerian hormone [AMH] and oestradiol were measured in patients undergoing IVF. Antral follicle count before ovarian stimulation and oocyte yield were used to establish sLHCGR– oocyte ratio (SOR), sLHCGR– antral follicle ratio (SAR), oestradiol at trigger per oocyte (oestradiol–oocyte ratio [EOR]) and oestradiol at trigger per antral follicle (oestradiol–antral follicle ratio [EAR]).

Results: The relatively stable sLHCGR was negatively related to AMH when oocyte yield was high. The sLHCGR levels were proportional ($r = 0.49$) to oestradiol at early cycle (day-3). Pregnancy and live birth were highest at low sLHCGR (≤1.0 pmol/ml) and SOR (≤ 0.1 pmol/ml/oocyte). A total of 86–89% of live births in IVF treatment were within the cut-off parameters of SAR and SOR (0.5 pmol/ml) and EAR and EOR (380 pg/ml). For failed pregnancy, age, SOR and EOR together had positive and negative predictive values of 0.841 and 0.703, respectively.

Conclusions: sLHCGR levels are negatively related to AMH when oocyte yield is high. High early cycle sLHCGR is associated with elevated day-3 oestradiol. Low sLHCGR and SOR are indicators of increased clinical pregnancy and live birth rates. Patient age and SOR, combined with EOR, might improve prediction of IVF treatment outcomes.

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KEYWORDS

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INTRODUCTION

Both LH and HCG operate via the same receptor, LH–HCG-R (LHCGR). The fundamental importance of LHCGR in ovarian function and IVF treatment outcomes was established by recent genetic analysis of polymorphism (*Lindgren et al., 2016*), point mutations (*Bentov et al., 2012*) and molecular analysis of alternative splicing (*Papamentzelopoulou et al., 2012*). LHCGR is found in two forms: the active form, which is membrane bound, and a soluble form (sLHCGR) circulating in blood (*Chambers et al., 2011a; 2011b; 2012; 2014; Crovetto et al., 2015; Chambers et al., 2016*). As the soluble receptor binds both LH and HCG, it can incapacitate these hormones before they can interact with the membrane-bound cognate receptor, thus affecting their bioactivity (discussed in Chambers *et al*. 2011). The standard measurement of serum LH and HCG (immunoreactivity) for the diagnosis of various conditions does not establish receptor-bound versus free hormone levels, so the concentration of the bioactive hormone is unknown. Apart from Leydig cell assays, which measure the bioactivity of LH–HCG (*Ding and Huhtaniemi, 1989; Fauser et al., 1991; Galeraud-Denis et al., 1999; Camejo et al., 2003*), and are both expensive and time consuming, no simple diagnostic tests can measure the concentrations of bioactive forms of LH and HCG in any clinical condition.

Physiological pregnancy begins with implantation of the embryo followed by feto–placental development. A successful outcome in fertility treatment requires clinical pregnancy, progressing to live birth at term with a normal birth weight (*Legro and Wu, 2014; Silver, 2014; Anderson, 2015; Chambers et al., 2016*). Multiple factors affect the outcome of fertility treatment; however, endocrine regulation by reproductive hormones (LH, FSH, HCG, oestrogen, and progesterone) has been the subject of intense investigation. These hormones are key to fertility treatment, but their ability to predict pregnancy outcomes before fertility treatment and embryo transfer is limited. Therefore, a sLHCGR blood test capable of indicating the pregnancy outcome with improved accuracy before fertility treatment could be clinically useful.

We have shown that circulating sLHCGR, either unbound or bound to LH or HCG, could usefully indicate reproductive outcomes in fertility treatment (*Chambers et al., 2011a*) as well as outcomes in naturally conceived pregnancies tested in the first trimester (*Chambers et al., 2014; 2016; Crovetto et al., 2015*). On the basis of a series of studies involving first-trimester pregnancy and patients undergoing fertility treatment (*Chambers et al., 2011a; 2012; 2014; 2016; Crovetto et al., 2015*), we suggest that sLHCGR may act as a sink for LH–HCG, reducing the availability of the active form to the cognate membrane-bound receptor. Under this model, low concentrations of sLHCGR would be expected to lead to an excess of active hormones, whereas high concentrations of sLHCGR would be expected to suppress normal hormonal responses leading to poor oocyte yield and reduced probability of pregnancy.

In our initial study on human fertility treatment (*Chambers et al. 2011a*), the clinical relevance of pre-treatment sLHCGR and LH–LHCGR complex, in relation to ovulation in response to ovarian stimulation, oocyte yield and embryo implantation, were addressed. Patients with high pre-treatment sLHCGR and LH-LHCGR, irrespective of the ovarian response (oocyte yield), had poor treatment outcome or failed implantation, whereas in those with undetectable-to low serum sLHCGR and LH-LHCGR concentrations, clinical pregnancy was favoured in both low and high responders. The pre-treatment sLHCGR levels did not significantly affect the treatment outcome of intermediate ovarian responders.

Increased oestradiol is currently thought to be sub-optimal for IVF outcomes, but this is confounded by the fact that good responders, who typically exhibit increased oestradiol, tend to produce more eggs and have a better chance of pregnancy. About 20 years ago, in an attempt to examine the individual roles of LH and FSH on ovarian secretion of oestradiol and fertility outcomes, *Loumaye et al. (1997)* first reported that oestradiol levels per retrieved oocyte (EOR) significantly determined the pregnancy and live birth rate in IVF (*Loumaye et al., 1997*). This discovery was substantiated by independent and wider studies (*Yang et al., 2001; Orvieto et al., 2007; Ozdegirmenci et al., 2011; Var et al., 2011; Vaughan et al., 2016*). In the present study, the stability of the circulating LH–HCG receptor during fertility treatment, its correlation with oestradiol, oocyte yield and the treatment outcomes, were examined.

MATERIALS AND METHODS

Study participants and protocol

This study examined the potential association of sLHCGR and oestradiol concentrations with pregnancy outcome after embryo transfer in two IVF clinics: Glasgow Centre For Reproductive Medicine (GCRM) and University of Southern California (USC), between August 2013 and December 2014. The ethical committee of USC approved the study on 1 October 2014 (reference HS-14-00709) and the ethical committee of GCRM indicated on 1 October 2013 that approval was not required for the analysis of anonymized samples collected from patients who signed consent forms indicating their agreement to storage and subsequent analysis of serum. Blood samples were collected and the stored serum samples retrospectively analysed. For the GCRM study, follicular phase samples from 135 patients (average age 34.9 years, range 26–44 years) were analysed. In addition, samples taken on the day of embryo transfer were analysed for 67 (median age 36 years [± 4.39]) out of these 135 patients. For the USC study, paired blood samples from 80 patients (average age 37.5 yrs, range 29–47 years) at the start of the cycle (menses cycle day 2–3) and at trigger were collected. Each serum sample was collected and stored at –20°C until assayed in batches. Therefore, patient concentrations of sLHCGR and LH-LHCGR were unknown to the IVF clinic before the treatment plan, embryo transfer and clinical outcome.

All women (n = 215) underwent fresh embryo transfer, with prior ovarian stimulation, performed in both clinics. For ovarian stimulation, one clinic (USC) used typically one of three protocols, gonadotrophin releasing hormone (GnRH) agonist down-regulation gonadotropin stimulation 'long protocol', 'microdose GnRH agonistic flare' stimulation protocol and the GnRH antagonist gonadotrophin stimulation protocol. Briefly, the 'long protocol' uses oral contraceptive pill and GnRH agonist down regulation before starting gonadotrophin therapy. The 'flare

protocol' uses lower doses of GnRH agonist for an acute release of stored pituitary gonadotrophins. The GnRH antagonist gonadotropin stimulation protocol uses GnRH antagonist for immediate gonadotrophin suppression from the pituitary, and is started once the lead follicle reaches about 14 mm in diameter. Ovarian stimulation regimens were based on provider preference. In the second clinic (GCRM), one of three stimulation protocols was used based on anti-Müllerian hormone (AMH) concentration. When AMH was less than 8.3 pmol/l, a flare protocol was used: norethisterone 5 mg twice a day for 10 days in the luteal phase, leuprorelin 3.75 mg 5 days after cessation of norethisterone and Gonal-F (Merck) 225 IU/day or 300 IU/day (<80 kg weight >80 kg, respectively) commencing 2 days after the leuprorelin. If AMH was greater than 8.3 pmol/l and less than 30 pmol/l, a long down-regulation protocol was used: leuprorelin (Lupron (Abbott Healthcare Pvt. Ltd), 3.75 mg and menotrophin (Menopur, Ferring Pharmaceuticals Ltd., Saint-Prex, Switzerland) 200 IU/day commencing 2 days after onset of menses. If AMH was greater than 30 pmol/l, a GnRH-antagonist stimulation protocol was used: menotrophin 150 IU/day commencing 2 days after onset of menses and cetrorelix (Cetrotide; Merck Serono Ltd., Darmstadt, Germany) starting on the morning of stimulation day 4. All cycles were triggered with choriogonadotropin alfa 250 µg (Ovitrelle, Merck Serano Ltd., Darmstadt, Germany) and oocyte retrieval carried out about 37 h later.

A positive pregnancy test was determined 17 days after ovulation trigger or LH surge by serum beta HCG concentrations greater than 5 IU/l. A clinical pregnancy was defined as a fetal heartbeat seen on ultrasound scan after 8 weeks' gestation. Miscarriage was defined as any positive pregnancy test after which the pregnancy ended before 24 weeks' gestation and did not result in a live birth.

Assays

The sLHCGR and LH-sLHCGR assays were carried out as described previously (*Chambers et al. 2011a; 2012; 2014; 2016; Crovetto et al., 2015*) with the following modifications: 50 µl of five to 10-fold diluted serum was incubated in antibody-coated plates for 15 min before adding 100 µl of diluted horse radish peroxidase-labelled detection antibody for another 90 min. After six washes, the plates were further incubated with 100 µl of 3,30,5,50-tetramethylbenzidine for 15–30 min and the colour reaction was stopped by adding 100 µl 1M hydrogen chloride. Plates were read at 450–650 nm in a standard plate reader. The sensitivity of the sLHCGR assay was 0.15 pmol/ml.

Oestradiol was measured by direct chemiluminescent immunoassay on the Immulite analyzer (Siemens Healthcare Diagnostics, Deerfield, IL, USA). The assay sensitivity is 10 pg/ml. Anti-Müllerian hormone (AMH) was measured at follicular phase by enzyme-linked immunosorbent assay (ELISA) using the AMH Gen II ELISA kit (Beckman-Coulter, Brea, CA). It was carried out using the semi-automated programmed Evolis immunoassay system supplied by Bio-Rad Laboratories (Hemel Hempstead, UK). Kit instructions were followed and the AMH concentrations in the samples were interpolated from the calibration curve produced. The assay sensitivity had been previously established as 1.5 pmol/l (*Wallace et al., 2011*).

Data analysis

Treatment outcomes include the following: pregnancy rate (denominator is the total number of patients who underwent embryo transfer in the group); clinical pregnancy rate (the denominator is the total number patients who had embryo transfer in the group); and miscarriage rate (the denominator is total number of pregnancies in the group).

For fertility treatment studies, data on patient identifiers corresponding to age, body mass index (BMI), AMH, sLHCGR values, oestradiol on day-3 and at trigger, antral follicular count (AFC), number of oocytes and embryos produced, mode of treatment (fresh transfer), and treatment response (such as no pregnancy, pregnancy, miscarriage or clinical pregnancy) were recorded. The sLHCGR– oocyte ratio (SOR), sLHCGR–antral follicle ratio (SAR), measured in pmol/ml, and oestradiol–oocyte ratio [EOR]) and oestradio–antral follicle ratio (EAR), measured in pg/ml, were log transformed.

Statistical analyses

P-values reached the conventional significance level of $P \leq 0.05$ (at 5% level) and, in one, case $P \leq 0.1$ (at 10% level); a small sample size was considered as significant. Age, SOR and EOR were used for predicting IVF treatment outcomes, with live birth as the target.

The USC data was subjected to a Naive Bayes Classifier (NBC) as training set. Separately, we used AMH alone in a similar analysis to compare the predictive utility of the new variables with AMH, which is used as an established predictor of IVF outcomes in many IVF clinics. The NBC programme used was part of the R 'caret' package and the receiver operator characteristic (ROC) analysis was carried out using the 'pROC' package. The analyses were carried out using the R statistical environment and associated packages (*Team, 2008*), and graphs were plotted using 'ggplot2'; package (*Wickham, 2009*).

RESULTS

The sLHCGR levels remain relatively unaffected during fertility treatment

We first asked when should the blood samples be collected during fertility treatment for measuring serum sLHCGR concentrations and prediction of treatment outcomes? The blood samples were collected at four different time points in two IVF clinics (GCRM and USC). These included one from before treatment to the day of embryo transfer, which was around 6–8 weeks, and the other from cycle day-3 to trigger, which was at the most a month. The day of trigger and the day of embryo transfer can be at multiple days depending on the patient and variable response to the treatment. Therefore, the paired samples for this study were derived at multiple points during the 6–8 weeks of IVF treatment.

Contrary to our expectation, in both studies, the 'pre-treatment' and 'start of the cycle' sLHCGR concentrations correlated strongly with sLHCGR values at embryo transfer and at trigger (FIGURE 1). The observation that the sLHCGR levels did not alter significantly during fertility treatment suggested that the concentration of the soluble LH–HCG receptor was generally unaffected by the variety of ovarian stimulation protocols used, primarily based on ovarian reserve tests (AMH or antral follicular count). Results of LH bound to sLHCGR (LH-sLHCGR complex) analysis were similar to those of sLHCGR (data not shown).

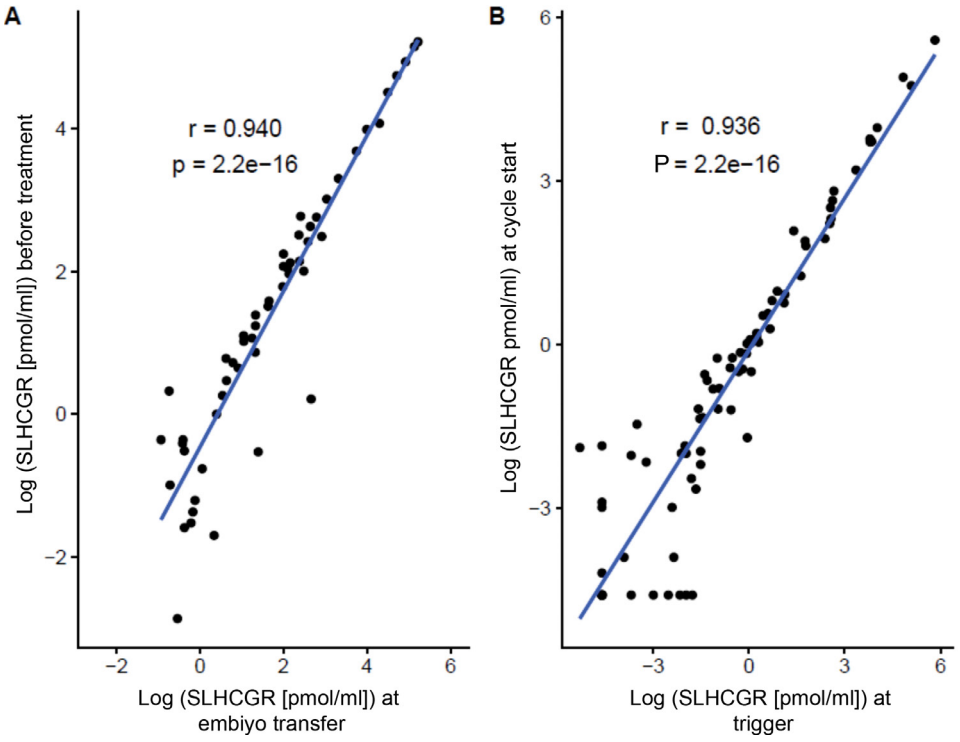

FIGURE 1 Correlation of soluble LH–HCG receptor (sLHCGR) concentrations at two time points during fertility treatment: (A) between pre-treatment and the day of embryo-transfer (clinic number 1; $n = 67$) with r = 0.94; $P = 2.2e^{-16}$; and (B) between the start of the cycle and on the day of trigger (clinic number 2; $n = 80$), with r = 0.936; and $P = 2.2e^{-16}$.

High oocyte yields were associated with low pre-treatment serum sLHCGR concentrations

The relationships between sLHCGR and AMH to high oocyte yield were plotted and the data were log-transformed to condense the scale of distribution of the data points with respect to oocyte numbers (FIGURE 2). The relative density of the distribution of data is shown above each plot where asterisk (*) above each plot represents the maximum value. For sLHCGR and AMH, the maximal values were 1 pmol/ml (log = 0.0) and 58 pmol/l (log = 3.90), respectively. Contrary to AMH (r = +0.36; $P = 0.036$), high oocyte yield was inversely associated with the serum sLHCGR (r = –0.24; $P = 0.064$) concentrations (FIGURE 2). Therefore, AMH and sLHCGR have broadly positive and negative correlations with high oocyte yields, respectively. The sLHCGR also negatively correlated with low oocyte yield. The correlation coefficients for oocyte yield of

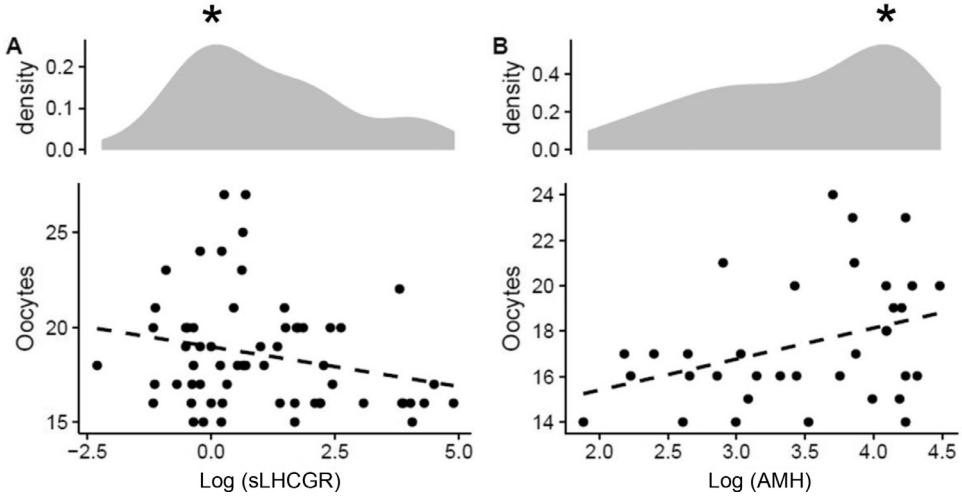

FIGURE 2 The correlations of high oocyte yield (≥14) with (A) soluble LH-HCG receptor (sLHCGR) and (B) anti-Müllerian hormone (AMH). The sLHCGR, pmol/ml ($n = 55$) and AMH, pmol/l ($n = 35$) were log transformed with respect to oocyte yields and plotted. The density of the distribution of values are shown above each plot and the highest density points of distribution shown by asterisk (*) in case of sLHCGR and AMH were 1 pmol/ml and 58 pmol/l, respectively. The correlation coefficients (r) for (A) sLHCGR and (B) AMH were –0.24 ($P = 0.0642$; significant at <10% level) and +0.36 ($P = 0.0361$), respectively.

four oocytes or more with sLHCGR were –0.25 (n =30) and –0.14 (n = 27), and were not significant (P > 0.1). The correlation between very high sLHCGR (≥10 pmol/ml) and AMH was not significant.

Inverse relationships between soluble LH–HCG receptor and Oestradiol during IVF treatment

The relationship between AMH and sLHCGR with oocyte yield (FIGURE 2) prompted similar analysis of the correlation between sLHCGR and oestradiol on day-3 and at trigger. First, we examined the correlation between oestradiol at day-3 and at trigger with oocyte yield. The oestradiol at trigger, unlike that of at day-3, was positively correlated with oocyte yield (r = 0.69; P < 0.0001). Therefore. oestradiol at trigger could be an indicator for ovarian response. Second, day-3 sLHCGR was directly proportional to oestradiol at early cycle or day-3 (r = 0.494; P < 0.0001), suggesting that high sLHCGR might be linked to elevated oestradiol at the beginning of the cycle. This is consistent with reduced oocyte yield on ovarian stimulation and poor pregnancy outcome in the high sLHCGR group (see below).

Unlike sLHCGR, oestradiol at trigger was significantly higher than at the start of the cycle (day-3) (FIGURE 3). Average oestradiol concentration at trigger was at least 50-fold higher than at day-3 of the cycle. To separate the data points in a plot with tight cluster, including a few outliers, the sLHCGR values were log transformed (FIGURE 3A and 3B). Correlation of day-3 oestradiol with sLHCGR (r = 0.2) did not achieve significance (FIGURE 3A). This correlation, however, became significant (r = 0.4; P = 0.04) when the top 30% of patients with high concentration of sLHCGR (>1.55 pmol/ml) was compared with day-3 oestradiol (FIGURE 3C). As shown below, this group of patients with extremely high sLHCGR have poor IVF outcome (FIGURE 4 and FIGURE 5). Unlike day-3 oestradiol, the oestradiol at trigger showed no significant correlation (r = –0.156) with sLHCGR at trigger (FIGURE 3B). This correlation, however, became stronger (r = –0.53) and significant (P = 0.005) when the bottom 50% of the patients with very low concentrations of sLHCGR (≤0.39 pmol/ml) was compared with oestradiol at trigger (FIGURE 3D). This negative correlation of low sLHCGR with oestradiol at trigger favoured the most successful IVF outcome (FIGURE 4 and FIGURE 5) suggesting that, for prediction of IVF outcomes, both sLHCGR and oestradiol should be measured.

High serum LHCGR and sLHCGR–oocyte ratio predict increased miscarriage, reduced pregnancy and live birth in IVF

The results shown in FIGURE 4 indicate that the risks of no pregnancy were low and high, respectively, at extreme sLHCGR–SOR (low and high) concentrations. To further substantiate these results, the pregnancy, live birth and miscarriage at three levels (low, intermediate and high) of sLHCGR and SOR were examined (FIGURE 4). This trend analysis revealed that the pregnancy and live births were highest at very low sLHCGR (A) and SOR (B) and lowest at very high sLHCGR (A) and SOR (B). Higher sLHCGR or SOR seem to be associated with increased miscarriages.

The circulating LHCGR per AFC and per oocyte (SAR and SOR) together with oestradiol at trigger per AFC and per oocyte (EAR and EOR) are predictors of live birth in IVF

We observed that, in addition to sLHCGR, the sLHCGR and oestradiol combination could also be an indicator

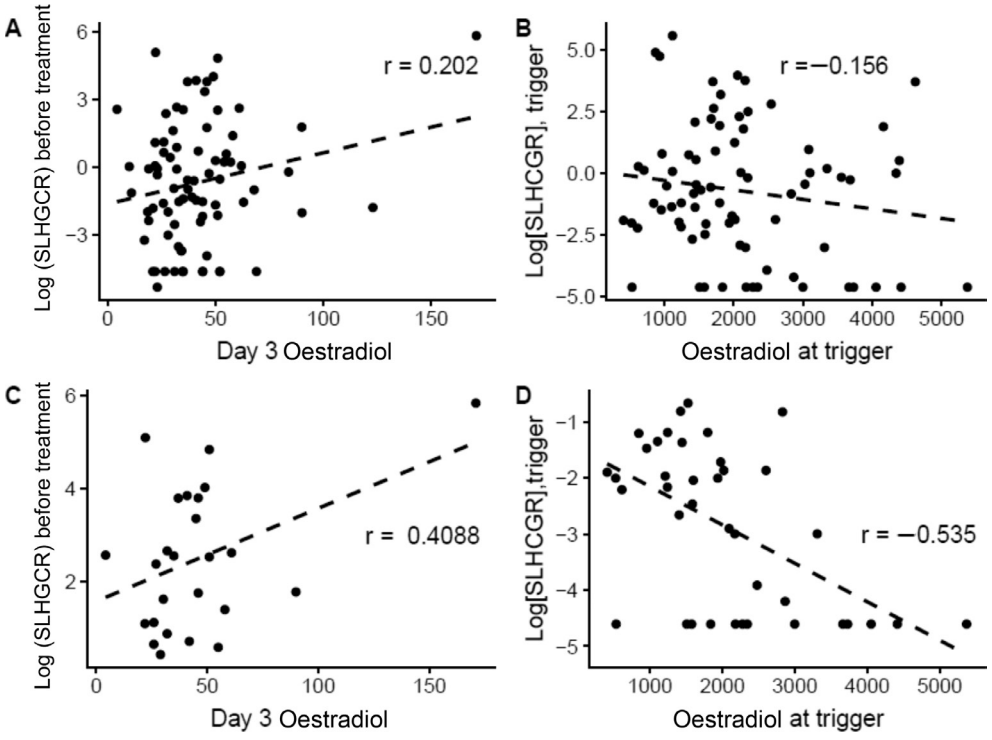

FIGURE 3 (A) Correlation between concentration of oestradiol on day-3 and soluble LHHCG receptor (sLHCGR) before treatment (n = 80); (B) correlation between oestradiol at trigger and sLHCGR at trigger (n = 78); (C), same as (A) except that top 25 patients with very high concentration of sLHCGR (>1.55 pmol/ml) correlated with corresponding oestradiol on day-3; (D) same as (B) except that the bottom 40 patients with very low concentration of sLHCGR (0.39 pmol/ml) were correlated with corresponding oestradiol at trigger. Unlike (A) and B), the correlation values in (C) (P = 0.0425) and (D) (P = 0.005), respectively were significant.

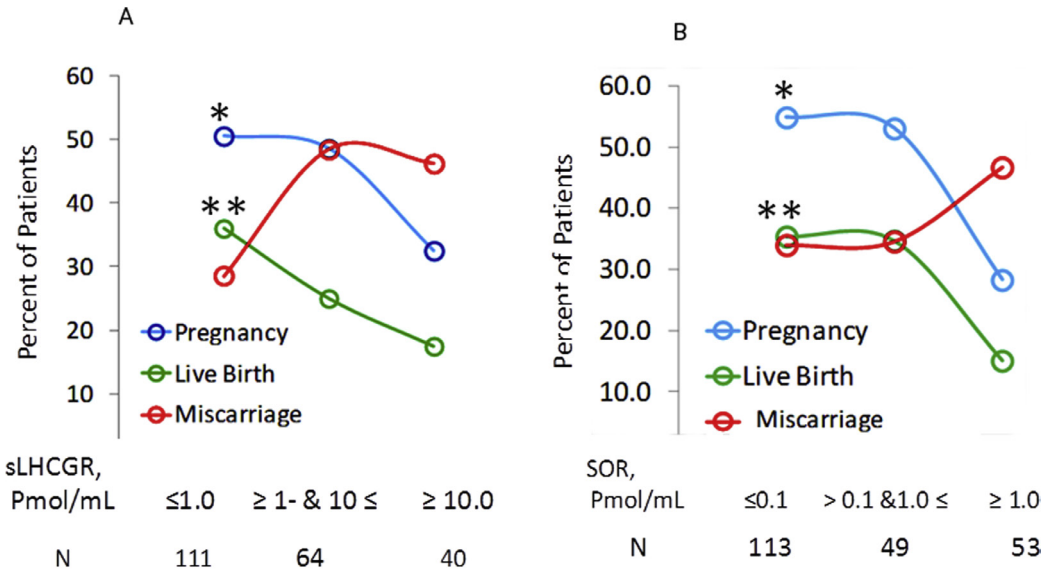

FIGURE 4 Differential effects of extreme concentrations (high and low) of soluble LH-HCG receptor (sLHCGR) and sLHCGR–oocyte ratio (SOR) on IVF outcomes. (A) Pregnancy, live birth and miscarriages with respect to pre-treatment serum sLHCGR in IVF. Pregnancy and live birth were highest at very low serum LH-HCG receptor (sLHCGR ≤1.0 pmol/ml) compared with that of the extremely high group (sLHCGR, ≥10.0 pmol/ml. *P = 0.0501; **P = 0.0302). Similarly, in (B), pregnancy and live birth were highest at lowest SOR values (≤0.1 pmol/ml) compared with the very high SOR group (≥1.0 pmol/ml); *P = 0.044; **P = 0.039. The number of patients in low (≤0.1 pmol/ml), intermediate (>0.1 and <1.0 pmol/ml) and very high (≥1.0 pmol/ml) groups are shown below each figure. Treatment outcomes included pregnancy and live birth, as well as miscarriage rates.

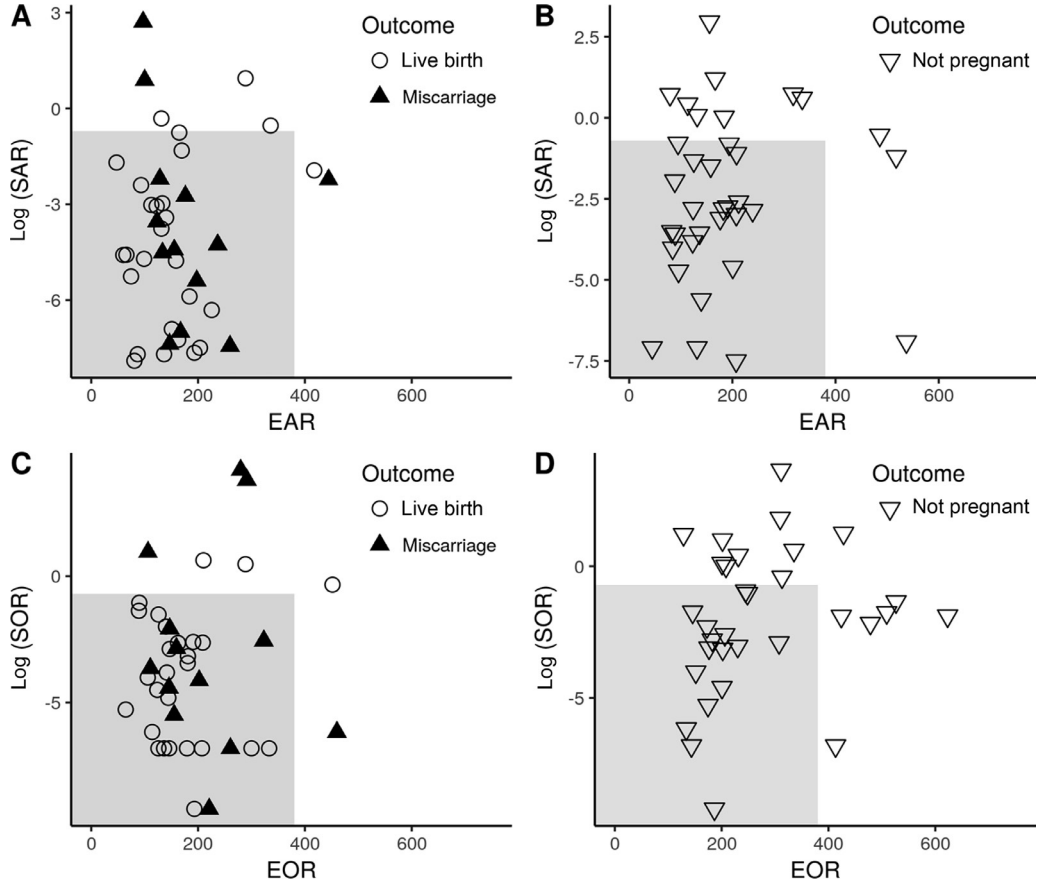

FIGURE 5 The pre-treatment soluble LH-HCG receptor (sLHCGR), oestradiol at trigger together with oocyte–antral follicle count can predict 85–89% of live births in IVF. The sLHCGR per antral follicle pmol/ml (SAR) and oestradiol at trigger per antral follicle pg/ml (EAR) were plotted in (A and B). Similarly, the sLHCGR per oocyte pmol/ml (SOR) and oestradiol at trigger per oocyte pg/ml (EOR) were plotted (C and D). The shaded area in each plot represents the cut-offs for SAR and SOR (0.5 pmol/ml) and EAR and EOR (380 pg/ml), which were used to calculate the frequency of pregnancy, live birth and miscarriages within the area. For clarity, the outcomes for live birth and miscarriage (A and C) were separated from the no pregnancy outcomes (B and D) in each case.

of IVF treatment outcomes, including miscarriage (FIGURE 4). Therefore, we attempted to combine the data sets sLHCGR; oocyte and oestradiol; and oocyte together, and created a cut-off based on published data for establishing the optimum IVF treatment outcomes. The results shown in FIGURE 5 demonstrate the distribution of patients and the outcomes (pregnancy, no pregnancy, miscarriage and live birth) within and outside the shaded areas defined by the cut-off values in each case. Because oestradiol–oocyte and sLHCGR–oocyte data were most reliable (compared with antral follicular counts), the clinical outcomes were compared.

For oestradiol–oocyte and sLHCGR–oocyte shaded area (FIGURE 5C and FIGURE 5D), out of 50 patients, 34 became pregnant, 16 did not become pregnant (10 miscarriages and 24 live births), representing the relative proportion of 68%, 32%, (20% and 48%) of the patients, respectively. Similar analysis of the outcomes outside the shaded area demonstrated that, out of 23 patients, seven became pregnant and 16 did not become pregnant (four miscarriages and three live births), representing the relative proportion of 30.4%, 69.6% (17.4% and 13%) of the patients, respectively. When the proportion of patients within and outside the shaded area were compared, the pregnancy, no pregnancy and live birth within the shaded area were significantly higher ($P < 0.05$) except miscarriage ($P > 0.1$). Therefore, we could predict that the outcomes with statistical significance were more likely for patients whose parameters lie inside the shaded area than for those outside the area.

For oestradiol–follicle count and sLHCGR–follicle count (FIGURE 5A and FIGURE 5B), out of 57 patients in the shaded area, 33 became pregnant and 24 did not become pregnant (10 miscarriages and 23 live births), representing the relative proportion of 57.9%, 42% (17.5% and 40.3%) of the patients, respectively. The area outside the shaded area (FIGURE 5A and FIGURE 5B), had 18 patients, out of which seven became pregnant and 11 did not become pregnant (three miscarriages and four live births), accounting for 38.9% and 61.1% (16.6% and 22.2%) of the patient population, respectively. When compared, none of the outcomes within the shaded area were significantly different to those outside.

We conclude that sLHCGR–oocyte ratio together with oestroadiol–oocyte ratio is a strong index of success rate, including live birth In IVF. Although this analysis is not an indicator of miscarriage, sLHCGR alone and sLHCGR-oocyte ratio together could provide such information as described in FIGURE 4.

Age, sLHCGR, and oestradiol as predictors of pregnancy and live birth

As an alternative approach to assess the importance of multiple variables for prediction, a Naive Bayes Classifier (NBC) from the R statistical environment was applied to the raw data to produce predictive models. As shown above, oestradiol–oocyte ratio and the sLHCGR–oocyte ratio and other variables were used for prediction in the current analysis (data not shown).

The NBC analysis using the three parameters age, sLHCGR–oocyte and oestradiol–oocyte resulted in a ROC plot with area under the curve (AUC) of 0.848 (FIGURE 6). This compared

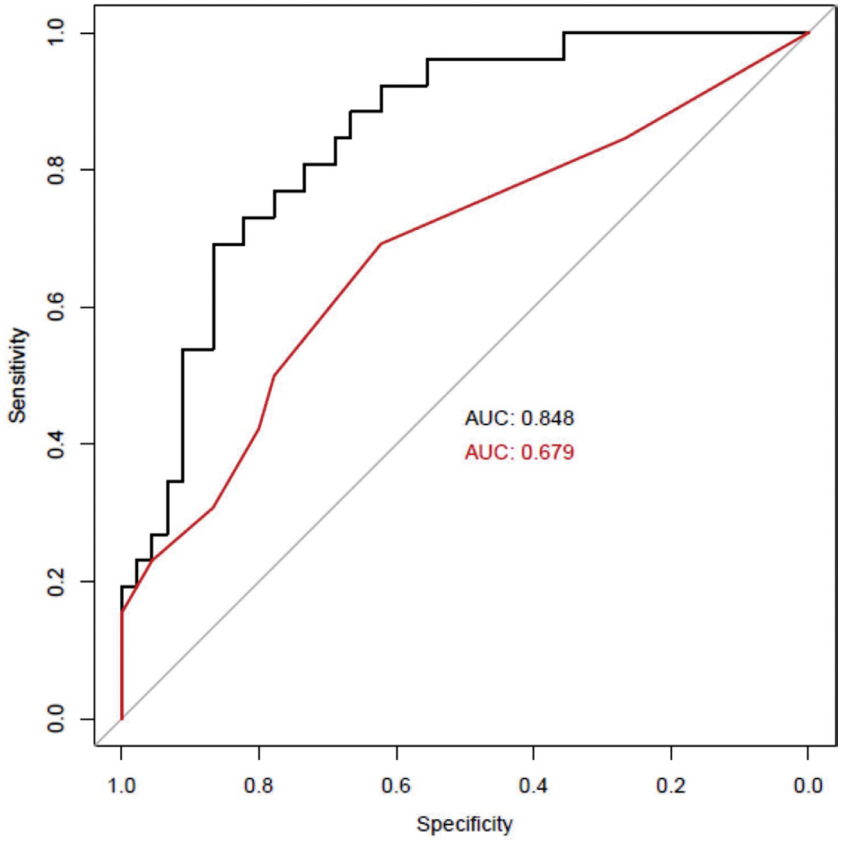

FIGURE 6 Naive Bayes Classifier analysis of age, soluble LH-HCG receptor and ostradiol as predictors of pregnancy and live birth. The area under the curve (AUC) is 0.848. For comparison, the red line in the plot shows the result for AMH alone.

favourably to the AUC of 0.679 obtained using AMH as predictor. Both positive predictive (0.841) and negative (0.703) predictive values could be calculated from the confusion matrix where 'no live birth' is a positive prediction and 'live birth' a negative prediction This classifier derived from NBC analysis using only the three variables, distinguishing between those patient who will have a live birth (19 out of 27) and those who will not (37 out of 44), resulted in an overall accuracy of 79%. Therefore, a combination of age, sLHCGR–oocyte and oestradiol– oocyte is potentially a useful set of predictors of pregnancy and live birth before embryo transfer in IVF clinics.

DISCUSSION

In the present study, we show that, despite the limited number of samples taken, it is clear that the relative concentration of serum sLHCGR did not change significantly during the critical stages of fertility treatment, suggesting that analysis of the serum sample at any time during fertility treatment (although preferably before stimulation) would be sufficient for future assessment of the association between sLHCGR concentration, ovarian function and pregnancy outcomes.

Very high concentrations of AMH (≥45 pmol/l) before ovarian stimulation have been used as a marker for ovarian hyperstimulation syndrome, and high oocyte yield after ovarian stimulation. In our previous study (*Chambers et al., 2011a*), patients with very high pre-treatment sLHCGR and LH-LHCGR, irrespective of the ovarian response (oocyte yield), had poor treatment outcome or no pregnancy, whereas in those with undetectable-to low serum sLHCGR and LH-LHCGR concentrations, pregnancy was favoured in both low and high responders. Moreover, women with very low serum sLHCGR have a predisposition to premature and multiple births when two or more embryos are transferred (*Chambers et al., 2016*).

Generally, patients with very high pre-treatment sLHCGR are expected to have high oestradiol at the start of the cycle. In fact, very high oestradiol on day-3 (>50–60 pg/ml) has been shown to be associated with poor ovarian response and pregnancy outcomes (*Licciardi et al. 1995; Smotrich et al., 1995; Prasad et al. 2014*). In the process of establishing the role of LH on follicular development in a GnRH-agonist protocol, *Loumaye et al. (1997)* first established that endogenous LH was sufficient for FSH-induced follicular development and also claimed that the oestradiol–oocyte ratio at trigger was a strong index of success rate in IVF. An extremely high serum LHCGR and SOR predict reduced probability of pregnancy and live birth in IVF. At a defined cut-off for SOR and SAR, about 89% of live births could be predicted. The results shown in **FIGURE 5** were an extension of the original report of *Loumaye et al. (1997)* as, in addition to the oestoradiol–oocyte ratio, the sLHCGR–oocyte ratio was used to establish the indices of success rate in IVF. It is difficult to understand why there are significant differences between the oestradiol–oocyte and sLHCGR–oocyte ratios but not in oestradiol–follicle count or sLHCGR–follicle count (**FIGURE 5**). *Loumaye et al. (1997)* observed a strong correlation between the oestradiol–oocyte ratio and the ratio of oestradiol to follicles 11 mm or wider. Further studies would be required to establish whether the correlation between EOR and EAR are affected by the average diameter of the follicles.

The most important clinical applications of the sLHCGR test together with that of oestradiol at trigger and oocyte ratio stem from their potential ability to predict live birth, identify the risk of no pregnancy and miscarriage after fertility treatment and in its therapeutic application so that patients can be counselled about their individual prognoses for pregnancy before embryo transfers. Clinicians could use it to identify a set of patients with potentially poor outcomes well before the start of treatment. On the basis of pre-treatment sLHCGR concentrations and SOR, both no pregnancy and miscarriage could be reduced by staggering the treatment cycle with frozen instead of fresh embryo transfer in the high sLHCGR and SOR groups if frozen embryos are available. Notably, this will be applicable primarily to high ovarian responders (*Weinerman and Mainigi, 2014; Ozgur et al., 2015; Casper and Yanushpolsky, 2016*) and not to those patients with normal ovarian response (*Shi et al., 2018*). When the first option is not available, however, owing to the lack of frozen embryos, an alternative might be to consider selective and extended luteal support (including additional HCG) after embryo transfer based on relative sLHCGR concentrations (discussed below).

A large proportion of pregnancies after embryo transfer end in first trimester miscarriage. Our data are consistent with emerging evidence that HCG together with progesterone and oestradiol are the major factors in establishing and maintaining immune tolerance of the embryo, preventing miscarriage. Recent studies have identified a novel role for HCG as a chemo-attractant of the regulatory T-cells (T-reg) around the trophoblasts, preventing miscarriage (*Tsampalas et al. 2010; Schumacher et al., 2013*), inducing proliferation of uterine natural killer cells and expansion of monocyte–macrophage derived dendritic cells, which prevent maternal rejection of the embryo (*Wan et al., 2008; Evans, 2016*). Therefore, suboptimal HCG functions as a result of very high sLHCGR serum concentrations before uterine transfer would be expected to reduce the implantation and clinical pregnancy rates.

Despite conflicting reports on the application of uterine HCG infusion or injection before or during embryo transfer (*Mansour et al., 2011; Hong et al., 2014; Santibañez et al., 2014; Zarei et al., 2014; Aaleyasin et al., 2015; Humaidan et al., 2015; Navali et al., 2016*), the practice of providing universal luteal support (*Hong et al., 2014*) remains prevalent in many clinics. Our data demonstrate that a wide range of concentrations of sLHCGR, capable of binding circulating HCG, exists in women presenting for fertility treatment. For individuals with high sLHCGR concentrations, luteal support may benefit, whereas, for those at the other end of the spectrum, it may provide no benefit or risk an adverse ovarian reaction to the hormone. Therefore, the universal therapeutic application of HCG during or before embryo transfer may not be appropriate for all patients and could lead to adverse reactions in those with very low serum sLHCGR. Further studies are necessary in order to establish the above precept. It is not difficult, however, to envisage that such selective luteal support might increase the chances of clinical pregnancy and reduce miscarriage. By measurement of serum sLHCGR concentrations, treatments such as luteal support might be tailored to the individual's requirements more accurately than is

possible at present, leading to higher clinical pregnancy rates and a reduction in miscarriage.

In conclusion, we recently reported that individuals with very low serum sLHCGR have a predisposition towards multiple births when two or more embryos are transferred (*Chambers et al., 2016*). As sLHCGR is a putative regulator of LH–HCG, the bioavailability of these hormones could be dependent upon the circulating receptor concentrations. During conception, undetectable or very low sLHCGR could translate to high free HCG and unregulated uterine activity promoting multiple implantation. High sLHCGR means reduced HCG bioactivity and poor implantation and reduced multiple birth. Therefore, in patients with undetectable or very low pre-treatment sLHCGR, multiple pregnancies could be reduced by avoiding transfer of two or more embryos without compromising the clinical success rate. We have also reported a risk of premature singleton birth (≤34 weeks) in women with very low serum sLHCGR concentrations (*Chambers et al., 2016*). By identifying individuals most at risk, fertility treatment plans could be implemented to reduce the risk of singleton prematurity well in advance of embryo transfer. To summarize, a single blood test measuring the maternal circulating LH–HCG receptor, sLHCGR together with oestradiol at trigger might help to predict embryo implantation, miscarriage, clinical pregnancy and birth outcomes in a cost-effective manner before fertility treatment.

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