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Coordinator: Prof. Claudio Pignata

PhD Thesis

Identification of ultrasound and genetic variables for the development of algorithms able to optimize the outcome of assisted conception pregnancies and to manage the correlated obstetric risk

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To the Sense of my life, Davide

INDEX

INTRODUCTION

This is an exciting time for Medicine with the development of new biomarkers, and the realization that their potential role extends beyond the diagnosis, the staging, and the monitoring of a phenomenon. This is the time of biomarker-guided strategies, which may allow more refined risk stratification and lead to improved patient care and outcomes prediction and individualizing treatment strategies.

Combining biomarkers and baseline phenotypes is already widely used in Obstetrics, for example in Fetal Medicine field: first trimester screening comprises age, past history, smoking status, uncial translucency, free b-human chorionic gonadotrophins and pregnancy associated plasma protein to calculate the risk for trisomy. The inclusion of these additional markers all improved the detection rate while reducing the false positives.

The traditional "Pyramid of care" has definitely been turned upside-down: screening by biophysical and biochemical markers are developing and aim to predict early in pregnancy huge obstetric complications such as premature delivery and pre-eclampsia (PE) that affected both spontaneous and in vitro pregnancies.

At present, algorithms for Reproductive Medicine are lacking; however, combining patient's anthropometric features, ovarian biomarkers, and ultrasound measurements would theoretically be expected to perform better in predicting ovarian response after stimulation protocols than each biomarker alone.

This PhD thesis takes in account the lesson from the past decades in Fetal Medicine field in terms of personalized approach to the patient and tries to apply this model to Reproductive Medicine field in order to obtain a standardized flowchart of procedure.

The overall framework is laided out in three different research lines.

The first research line has been focused on the estimation of assisted conception's impact on pregnancies outcome at the time of first trimester screening; in particular, it has been studied the impact of assisted reproduction technologies (ART) on the early pre-eclampsia risk.

The second research line has been focused on ART population's features, in particular, on the estimation of genetic variables' impact on ovarian response within controlled ovarian stimulation (COS) protocols.

The third line focused on two topics: on Reproductive Medicine side, the research aims to explore the potential role of some genetic and ultrasound information in a combined algorithm which can be used as an objective tool for personalizing the management of COS. protocols.

On Prenatal Care side, it time to move from personalized risk prediction to prevention: it is here reported the preliminary phase of a double- blind placebo controlled III phase study named ASPRE study, one of the biggest multicentre trial ever thought in Obstetric which is currently on going (and the candidate is working on) and aims to prevent pre-eclampsia in high risk pregnancies through the prophylactic use of low dose aspirin before 16 weeks.

CHAPTER 1 - THE ROLE OF ULTRASOUND IN REPRODUCTIVE HEALTH

o *BACKGROUND*

Role of ultrasound in Assisted Reproduction Technologies (ART)

Over the last 25 years, the advances in Ultrasound have paralleled advances in Assisted Reproductive Technology (ART). ART could not even be practiced or considered today without imaging. Ultrasound has become the most important and widely used tool in the diagnosis and treatment of infertility. Ultrasound evaluation is one of the first steps to assess the cause of infertility; the three areas of evaluation are the ovaries, uterus, and fallopian tubes. Ultrasound allows physicians to diagnose ovarian reserve but also pathologies such as polycystic ovarian syndrome, endometriosis, or other ovarian cysts that can impact fertility. The results of this initial exam immediately affect the decisions in the management of the patient's condition. When fertility treatments begin, ultrasound is used in almost any interaction with the patient in order to monitor follicular development and endometrial response; ultrasound guidance is also vital for embryo retrieval and transfer (ET).

The ability of ultrasonography to measure endometrial thickness in addition to detecting uterine masses gives it an edge over laparoscopy/hysteroscopy as a diagnostic procedure in uterine cavity assessment, although hysteroscopy has the advantage of therapeutic potential. Similarly, ultrasonography is superior to biochemical methods for follicular monitoring because of its ability to demonstrate the number and sizes of follicles, and guide preparations for oocyte retrieval. The relative ease of ultrasound guided oocyte retrieval; its less technical demands and the possibility of conducting the procedure under local anaesthesia have made ultrasound guided oocyte retrieval more popular across the world. Randomized controlled trials show that ultrasound-guided transfer techniques have better outcomes than the clinical touch technique in terms of on-going pregnancies and clinical pregnancies. Ultrasonography is now the key instrument for diagnosing and monitoring pregnancy following embryo transfer, biochemical methods being complimentary. Ultrasonography is now the single most important instrument in in-vitro fertilization (IVF) .programmes and gynaecologists with interest in reproductive medicine need necessarily to obtain a formal training in its use.

Emergent role of three dimensional transvaginal sonography in ART

The use of ovarian reserve markers responds to the need of individualization in Reproductive Medicine.To predict the number of oocytes that a woman has remaining within her ovary, means potentially counsel her appropriately regarding the duration of her reproductive lifespan, the likely response to assisted conception and the likelihood of success with both natural and assisted conception[1].

Ovarian reserve is estimated by combining certain clinical parameters and a variety of endocrinological or ultrasonographic measurements [2-6.] There is debate as to which combination of parameters has the best predictive value and there is no consensus at present. The relative contribution of each individual measure of ovarian reserve is clearer, and most authors agree that antral follicle counts (AFCs) and serum anti-Müllerian hormone (AMH) levels have the best discriminative potential [7-9.]

Serial ultrasound (US) examinations are performed to assess the number and size of follicles during controlled ovarian stimulation (COS) for in vitro fertilization (IVF).

However, AFC assessment has been limited by the lack of consensus regarding how these should be measured and what size of follicle should be classed as an antral follicle[10].

AFCs are typically performed using real-time, two dimensional (2D) ultrasound but may be estimated from three-dimensional (3D) ultrasound data. Both methods involve the identification of follicles measuring between 2 and 10 mm as the observer manipulates the ultrasound transducer or scrolls through a stored dataset. It has been shown that manual measurement of follicles with 2D US is often inaccurate and subject to significant intra- and interobserver variability [11-13]: this technique takes time and is associated with a degree of measurement error as follicles can be missed or counted more than once.

Automated measurement of the number of antral follicles has the potential to address both issues [14-15.]

The Automatic Volume Calculation is software that identifies and quantifies hypoechoic regions within a 3D dataset and provides automatic estimation of their absolute dimensions, mean diameter and volume[16]. Each individual volume is given a specific color and the automated measurements of its mean diameter (relaxed sphere diameter), its maximum dimensions (x, y, z diameters), and its volume are displayed in descending order from the largest to the smallest. An unlimited number of volumes can theoretically be quantified, which makes it an ideal tool for follicle tracking. It also identifies small volumes and can theoretically be used to count antral follicles. To date it has been used to assess stimulated ovaries mainly: some studies have shown that it provides automatic measurements of follicular diameter and volume that are more reliable and more

accurate than comparable estimations made from 2D data [15-16]. Other data regarding the agreement between 3D and 2D measurements in women undergoing IVF suggest good agreement between the two methods [17-21].

The 3D US software automatically identifies hypoechogenic follicles within the captured ovarian volume and generates a set of measurements for each follicle. These measurements include the largest diameters in three orthogonal planes, the mean follicular diameter (MFD), the volume of the follicle and the volume-based diameter (dV) of the follicle. The volume calculation is based on the voxel count within the identified follicle. It therefore represents a true measure of follicular volume. Although the MFD is the arithmetic mean of the three longest orthogonal diameters, dV is the diameter of a perfect sphere with the same volume as the follicle that is measured. After calculation of the actual volume of a follicle the software calculates the diameter of a perfect sphere which has the same volume as the follicle by using the relaxed sphere diameter formula.

This measurement has been showed to equate more closely to the volume of follicles aspirated at the time of oocyte retrieval than the mean diameter obtained by averaging the three perpendicular (x, y, z) diameters [22].

Upon completing automated analysis and prior to generating the final report which provides many post-processing options.

Recently the applicability and reproducibility of this automated follicle measurement method in an IVF programme have been evaluated [23-27]: 3D ultrasound significantly improves the inter-observer reliability of AFCs and and have also been shown to provide highly reliable measures of AFC [26] and their relative sizes [27].

However, postprocessing of the data is often required. The technique is best considered as "semi-automated" therefore; although the additional processing adds to the assessment time, it is straightforward and the overall time required for analysis has been reported still significantly less than that for measurements made during 2D ultrasound examination when the size of follicle size was also assessed. Some Authors [26-27] reported that 3D US software identifies and measures significantly fewer follicles than 2D ultrasound imaging, resulting in a consistently lower total antral follicle count regardless of the upper and lower size limits used to define the antral follicle population. This may be a true result or reflect fundamental differences in the measurement techniques. An obvious limitation of these studies is the inability to confirm the accuracy of these measurements, but histological work [28] suggests that the automated measurements are more valid than those derived using 2D ultrasound imaging.

If we accept that the "semi-automated" 3D ultrasound measurements are more valid than those made by 2D ultrasound imaging, we need to consider why this new technique is better. It may relate to the image being displayed in a 3D ultrasound multiplanar view, which allows cross-checking of each follicle in three different

planes, thus improving spatial orientation, but is more likely to reflect the fact that each follicle is color coded which prevents repeat measurements of the same follicle[15].

As regards the objective assessment of follicle size, the 2D ultrasound technique derives the follicular diameter through estimation of the mean of two perpendicular linear measures, whereas 3D US software uses volumetric information to define a 'relaxed sphere'. This might explain the differences seen between the two techniques as 2D ultrasound imaging is more likely to disregard small irregularities in the follicles. Volumetric measurements have been shown to provide a more reliable and valid assessment of the estimated diameter of larger follicles than both 2D ultrasound imaging and manual assessment of 3D ultrasound datasets, regardless of the number of linear measurements taken to define the size of the follicle16. A variable number of procedures are needed to reach proficiency in 3D ultrasound, even for trained 2-dimensional sonographers. Assessment of learning curves should be implemented when incorporating 3D ultrasound in reproduction units [30].

What improvements can this system offer ?

It is providing a means of standardization of follicle measurements when scans are performed by several sonographers. In busy IVF units, some patients are scanned by different sonographers in the course of one treatment cycle. Data suggest very good reproducibility of 3D measurements.

It saves time during follicle tracking for both the patient and the sonographer. This is particularly important for patients with many follicles as it not only saves time but also reduces the discomfort of a prolonged US examination. In fact, studies comparing time required for follicle measurements with 3D and with the 2D method have invariably reported substantial time saving provided by SonoAVC [17-18]. It has been reported that the average time saving reached 7.6 min for women who had ≥ 10 follicles to measure [29]. Time saving can be further increased by using an off-line system: the ovarian volumes can be captured and transferred to a network by the sonographer, and the patient can leave the examination room without spending any time for measurements. The sonographer can start scanning the next patient while another operator located elsewhere can retrieve the volumes from the network and analyse them with the software.

3D software introduces a chance to implement a quality control system for follicle measurements: if it is implemented in routine practice it is possible to save raw ovarian volumes that are available for retrieval and analysis at later time. This enables virtually repeating the US examinations in the absence of the patient should the need arise. Such re-examinations can be periodically repeated for a sample of patients as a method of

quality control. Moreover, this gives a chance to monitor results generated by junior staff or trainees in academic units.

Potentially follicle volume-based criteria may prove to be a better indicator of oocyte maturity and increase mature oocyte yield. Using follicular volume measured with 3D as the measure of follicular growth combined with volume-based HCG criteria may improve treatment outcome over that achieved with conventional monitoring with follicular diameter and using follicular diameter-based HCG.

It can provide an option for out-of-town women who need to travel several times for follicle monitoring and other procedures for IVF treatment. If they are monitored by local sonographers with less experience in IVF, results can sometimes cause suboptimal management of treatment cycle. If 3D volumes of their ovaries can be captured by local sonographers and transmitted to the IVF centre, they can be analysed with 3D software at the treating centre.

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CHAPTER 1 - THE ROLE OF ULTRASOUND IN REPRODUCTIVE HEALTH

- o *PAPERS*

1A) Contribution of method of conception on pregnancy outcome after the 11-13 weeks scan

Running head: Method of conception and pregnancy outcome

Key words: Ovulation induction, In-vitro fertilization**,** First trimester screening, Miscarriage, Stillbirth, Preeclampsia, Gestational diabetes mellitus, Preterm delivery, Birth weight, Cesarean section.

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Conflict of interest: No

Abstract

Objective: To examine the effect of method of conception on adverse pregnancy outcome after the 11-13 weeks scan.

Methods: Prospective screening study for adverse obstetric outcomes in women with singleton pregnancies and live fetus with no obvious defects at 11^{+0}-13^{+6} weeks. The method of conception was recorded as spontaneous, in-vitro fertilization (IVF) and assisted by ovulation induction (OI) drugs without IVF. Regression analysis was performed to examine the association between the method of conception and pregnancy outcome after adjustment for maternal characteristics.

Results: In the study population of 41,577 pregnancies, conception was spontaneous in 40,261 (96.9%), by IVF in 634 (1.5%) and by OI in 682 (1.6%). In the pregnancies conceived by assisted reproductive technology (ART), compared to spontaneous conceptions, there was a higher risk of stillbirth, preeclampsia, gestational hypertension, gestational diabetes mellitus, delivery of small for gestational age (SGA) neonates and cesarean section. However, multiple regression analysis showed that after taking into account maternal characteristics the only significant contributions of IVF were for preeclampsia and elective cesarean section and the contributions of OI were for miscarriage, spontaneous early preterm delivery and SGA.

Conclusions: Conception by IVF and OI is associated with increased risk for adverse pregnancy outcome.

Introduction

Effective screening for fetal aneuploidies is provided in the first-trimester of pregnancy by a combination of maternal age and the findings of ultrasonographic examination of the fetus and biochemical analysis of maternal blood [1]. Recent evidence suggests that at the same hospital visit at 11-13 weeks data from the maternal history can be combined with the results of biophysical and biochemical tests to estimate the patient-specific risk for a wide variety of pregnancy complications, including miscarriage, stillbirth, preeclampsia (PE), gestational hypertension (GH), gestational diabetes mellitus (GDM), preterm delivery, and birth of small for gestational age (SGA) or large (LGA) neonate [2]. Early estimation of risks for these pregnancy complications could potentially improve pregnancy outcome by shifting antenatal care from a series of routine visits to a more individualized patient and disease-specific approach both in terms of the schedule and content of such visits. In this respect the 11-13 weeks assessment is likely to be the basis for a new approach to antenatal care [2].

An increasing proportion of pregnancies are conceived by assisted reproductive technology and several studies have reported that the incidence of adverse outcomes in such pregnancies may be higher than in spontaneous conceptions [3-5]. However, there are contradictory or insufficient data to allow an accurate estimate of the effect of in-vitro fertilization (IVF) and the use of ovulation induction (OI) drugs without IVF on a wide range of pregnancy complications which could be incorporated in the 11-13 weeks assessment of risks.

The aim of this study is to estimate the effect of the method of conception on a series of adverse pregnancy outcomes after adjustment for confounding factors in obstetric history and maternal characteristics assessed at 11-13 weeks' gestation.

Methods

Screening study population

This was a prospective screening study for adverse obstetric outcomes in women attending for their routine first hospital visit in pregnancy at King's College Hospital, London, UK and Medway Maritime Hospital, Kent, UK. This visit, which is held at 11^{+0}-13^{+6} weeks of gestation, includes recording of maternal demographic characteristics and previous obstetric and medical history, measurement of maternal weight and height and calculation of the body mass index (BMI), and ultrasound examination for the measurement of the fetal crown-rump length (CRL) to determine gestational age [6], measurement of the fetal nuchal translucency (NT) thickness as part of screening for aneuploidies [7] and examination of the fetal anatomy for the diagnosis of major fetal defects [8]. Written informed consent was obtained from the women agreeing to participate in the study, which was approved by King's College Hospital Ethics Committee.

A second ultrasound examination for fetal biometry and examination of the fetal anatomy is carried out at 20-24 weeks. In the neonatal period all babies are examined by a pediatrician. Data on pregnancy outcome are collected from the hospital maternity records or the general medical practitioners of the women.

Maternal characteristics and obstetric history

Patients complete a questionnaire on maternal age, racial origin (Caucasian, African, South Asian, East Asian and mixed), method of conception (spontaneous, IVF, use of ovulation induction drugs (OI) without IVF or intrauterine insemination), cigarette smoking during pregnancy (yes or no), history of chronic hypertension (yes or no), history of type 1 or 2 diabetes mellitus (yes or no) and obstetric history including the outcome of each previous pregnancy. The questionnaire was then reviewed by a doctor together with the patient.

Outcome measures

In this study we examined the relationship between method of conception (spontaneous, IVF, OI without IVF) with first, miscarriage, second, stillbirth, third, development of PE or GH, fourth, development of GDM, fifth, spontaneous early preterm delivery, sixth, delivery of SGA or LGA neonate, and seventh, delivery by elective or emergency cesarean section.

We excluded pregnancies conceived by intrauterine insemination because we did no have data whether or not they had received OI drugs, those with fetal aneuploidies or major defects diagnosed either prenatally or in the neonatal period and pregnancies ending in termination for psychosocial reasons.

Miscarriage and stillbirth

Miscarriage included spontaneous miscarriage and fetal death before 24 weeks. Stillbirths were fetal deaths at or after 24 weeks.

Preeclampsia and gestational hypertension

The definitions of PE and GH were those of the International Society for the Study of Hypertension in Pregnancy [9]. In GH the diastolic blood pressure should be 90 mm Hg or more on at least two occasions, at 4 h apart, developing after 20 weeks of gestation in previously normotensive women in the absence of significant proteinuria and in PE there should be GH with proteinuria of 300 mg or more in 24 h or two readings of at least ++ on dipstick analysis of midstream or catheter urine specimens if no 24-h collection is available. In PE superimposed on chronic hypertension significant proteinuria (as defined above) should develop after 20 weeks of gestation in women with known chronic hypertension (history of hypertension before conception or the presence of hypertension at the booking visit before 20 weeks of gestation in the

absence of trophoblastic disease). In the investigation of the relationship between method of conception and PE or GH we excluded pregnancies ending in miscarriage or fetal death before 24 weeks.

Gestational diabetes

Screening for GDM in our hospitals is based on a two-step approach. In all women a random plasma glucose is measured at 24-28 weeks of gestation and if the concentration is more than 6.7 mmol/L an OGTT is carried out within the subsequent 2 weeks. The diagnosis of GDM is made if the fasting plasma glucose level is at least 6 mmol/L or the plasma glucose level 2-hours after the oral administration of 75 g glucose is 7.8 mmol/L or more [10]. In women with normal random blood sugar an OGTT is performed if they have persistent glucosuria, they develop polyhydramnios, or the fetus becomes macrosomic. Women with the diagnosis of GDM are given dietary and exercise advise and are encouraged to test capillary blood glucose before and 1-hour after each meal. If during a period of 1-2 weeks the pre-meal or 1-hour post meal blood glucose level is higher than 5.5 mmol/L and 7 mmol/L, respectively, the women are treated with insulin. In the investigation of the relationship between method of conception and GDM we excluded pregnancies with pre-pregnancy diabetes mellitus type 1 or 2 and those ending in miscarriage or delivery before 30 weeks because they may not have had screening and diagnosis of GDM.

Spontaneous preterm delivery

Spontaneous preterm deliveries included those with spontaneous onset of labor and those with preterm pre-labor rupture of membranes occurring before 34 completed weeks (238 days). In the investigation of the relationship between method of conception and spontaneous preterm delivery we excluded pregnancies ending in miscarriage or fetal death and those with iatrogenic delivery before 34 weeks.

Small and large for gestational age

The definitions of SGA and LGA were delivery of neonates with birth weight below the 5th centile or above the 95th centile for gestation, respectively [11]. In the investigation of the relationship between method of conception and SGA or LGA we excluded pregnancies ending in miscarriage or fetal death before 24 weeks.

Elective or emergency cesarean section

Emergency cesarean section included all cases where such delivery was undertaken after the onset of labor, usually for failure to progress, fetal distress or intrapartum hemorrhage. This group also included cases of antepartum hemorrhage requiring cesarean section. Elective caesarean section was performed before the onset of labor for obstetrical or medical indications or at the request of the mother. In the investigation of the relationship between method of conception and elective or emergency cesarean section we excluded pregnancies ending in miscarriage or fetal death before 24 weeks.

Statistical analysis

First, a univariate logistic regression analysis was performed to examine the association between method of conception and each of the adverse pregnancy outcomes. Second, the risk for each of the pregnancy outcomes was calculated from the formula: odds / (1+odds), where odds=e^Y, and Y was derived from the univariate logistic regression analysis. Third, we performed a multivariate logistic regression analysis for the prediction of each pregnancy outcome from method of conception, maternal age, racial origin, smoking, history of chronic hypertension or diabetes, and previous history of adverse pregnancy outcome or family history of PE.

The statistical software package SPSS 16.0 (SPSS Inc., Chicago, IL) was used for data analyses.

Literature search

We searched MEDLINE and EMBASE from 1978, when the first child conceived by IVF was born, to November 2010 to identify English language articles reporting on the outcome of pregnancies conceived by IVF and OI without IVF. We included all case-control and cohort studies which reported data from singleton pregnancies regarding the primary outcome measures including miscarriage, stillbirth, PE, GH, GDM, early preterm delivery, birth of SGA or LGA neonates and delivery by elective or emergency cesarean section. We only included and used the reported data in each paper. We excluded duplicate publications.
Two independent reviewers extracted the data from each article and these were then examined by a third reviewer. Odds ratio (OR) with 95% confidence intervals (CI) were calculated for each outcome in each study. Forrest plots were constructed and a random-effects model, which takes into account the random variation within studies [41], was used to calculate weighted summary ORs by taking into account the weight of each study.

Forrest plots and summary ORs were generated using Medcalc software version 9.6.2.0 (MedCalc Software, Mariakerke, Belgium).

Results

Study population

During the study period we carried out an ultrasound examination at 11-13 weeks in 45,191 singleton pregnancies with a live fetus and CRL of 45-84 mm. We excluded from further analysis 77 (0.2%) cases because they conceived by intrauterine insemination, 2,739 (6.1%) because there were no or incomplete data on pregnancy outcome and 682 (1.5%) because of the prenatal or postnatal diagnosis of aneuploidies or major defects and 116 (0.3%) because of pregnancy termination.

The maternal and pregnancy characteristics of the 41,577 cases included in the study are shown in Table 1. In the OI and IVF groups, compared to the spontaneous conception group, the maternal age was higher, more women had pre-existing diabetes mellitus and less were cigarette smokers . In the OI group maternal BMI was increased and in the IVF group there were more Caucasians and fewer women were parous.

Pregnancy complications

Univariate logistic regression analysis demonstrated that use of OI drugs was associated with an increased risk of subsequent miscarriage, GDM, spontaneous delivery before 34 weeks, delivery of SGA neonate and elective cesarean section, whereas IVF was associated with an increased risk of subsequent development of PE, GH and GDM, iatrogenic delivery before 34 weeks and both emergency and elective cesarean section (Table 2).

The results of multivariate logistic regression analysis for the prediction of pregnancy complications from method of conception and maternal characteristics and previous obstetric history are summarized in Tables 3-7. Significant contributions, independent of maternal characteristics were provided by IVF only for early-PE and elective cesarean section and by OI only for miscarriage, spontaneous delivery before 34 weeks and delivery of SGA neonates (Figure 1).

Literature search

Forest plots of ORs from previous reports and our study for second trimester miscarriage, stillbirth, PE, GH, GDM, early preterm delivery before 32 or 34 weeks, delivery of SGA neonate with birth weight below the 5th and 10th centiles and delivery by caesarean section are shown in figures 2-9 [12-40]. In the case of early delivery in our study we selected spontaneous delivery before 34 weeks, but in most previous studies the outcome measure was total delivery before 32 weeks. In our study we had data on both elective and emergency caesarean section but most previous studies provided data only for total caesarean section.

Discussion

The combined data from this study and previous reports indicate that in ART pregnancies there is an increase in the rate of subsequent stillbirth, PE, GH, GDM, early preterm delivery, birth of SGA neonates and cesarean section. There was also a non-significant increase in risk of second trimester miscarriage. The results demonstrated that essentially there were no qualitative differences between the women undergoing IVF and those receiving OI drugs but only small differences in the magnitude of risk for the same pregnancy complications. These findings raise the possibility that the risk may be the consequence of subfertility itself rather than the interventions involved in ART. Unfortunately we did not have the necessary data to examine the various pregnancy complications in relation to the causes of subfertility or the different regimes for OI.

Our study has shown that there were significant differences in maternal characteristics between the ART and spontaneously conceived pregnancies. We therefore performed multivariate logistic regression analysis and demonstrated that after taking into account certain maternal characteristics and obstetric history, firstly, ART did not contribute to increased risk for most pregnancy complications and secondly, there were clear qualitative differences between IVF and OI in their effects on complications. In the IVF group, compared to spontaneous conceptions, there was a higher risk of early-PE and elective cesarean section. In the OI group, compared to spontaneous conceptions, there was a higher risk of miscarriage, spontaneous delivery before 34 weeks and delivery of SGA neonates. We did not record detailed data on the possible causes of subfertility requiring ART and it is therefore not possible to conclude that the observed effects are the consequence of the ART rather than maternal characteristics.

The inability to distinguish between ART and maternal factors that predated such therapy as the cause of the observed association with pregnancy complications is further reinforced by the pattern of such complications which can not be explained by a single pathophysiological mechanism. In normal pregnancy the spiral arteries in the placental bed are invaded by trophoblast, which becomes incorporated into the vessel wall and replaces the endothelium, muscular layer and neural tissue [42-45]. These physiological changes convert the spiral arteries from narrow muscular vessels to wide non-muscular channels independent of maternal vasomotor control. In early onset PE this process is impaired. In IVF there was a three fold increase in risk for early PE, independent of maternal characteristics, suggesting that the procedure could somehow result in impaired placentation. However, had this been the case it would be expected that in this group in addition to the increased risk of PE there would have been a higher incidence of miscarriage, stillbirth and delivery of SGA neonates. Similarly, if in a high proportion of the OI group there was underlying polycystic ovarian syndrome (PCOS) it would be expected that in this group there would have been a higher incidence of GDM. Previous studies examining the outcome of pregnancies in women with polycystic ovarian disease reported increased incidence of GDM, PE, GH and preterm delivery together with a paradoxical decrease in LGA [46]. In this respect it was suggested that in PCOS the presence of placental insufficiency mitigates against the development of fetal macrosomia due to the increased glucose load associated with GDM.

The finding of increased rate of elective cesarean section in the IVF group is likely to be the consequence of parental and medical anxiety rather than the result of any specific pregnancy complication, which would in any case be reflected in increased rate of emergency rather than elective cesarean section.

In conclusion, ART pregnancies are at increased risk of several pregnancy complications, including fetal death, hypertensive disorders, GDM, preterm delivery, birth of SGA neonates and caesarean section. However, most of these complications are the consequence of the characteristics of the subfertile women undergoing IVF or OI which predate the interventions involved in ART. Further focused investigations of specific complications, including early-PE in IVF pregnancies and second-trimester miscarriage, spontaneous early delivery and birth of SGA neonates in OI pregnancies, will determine whether these are truly the consequence of ART or the type of women undergoing such therapy.

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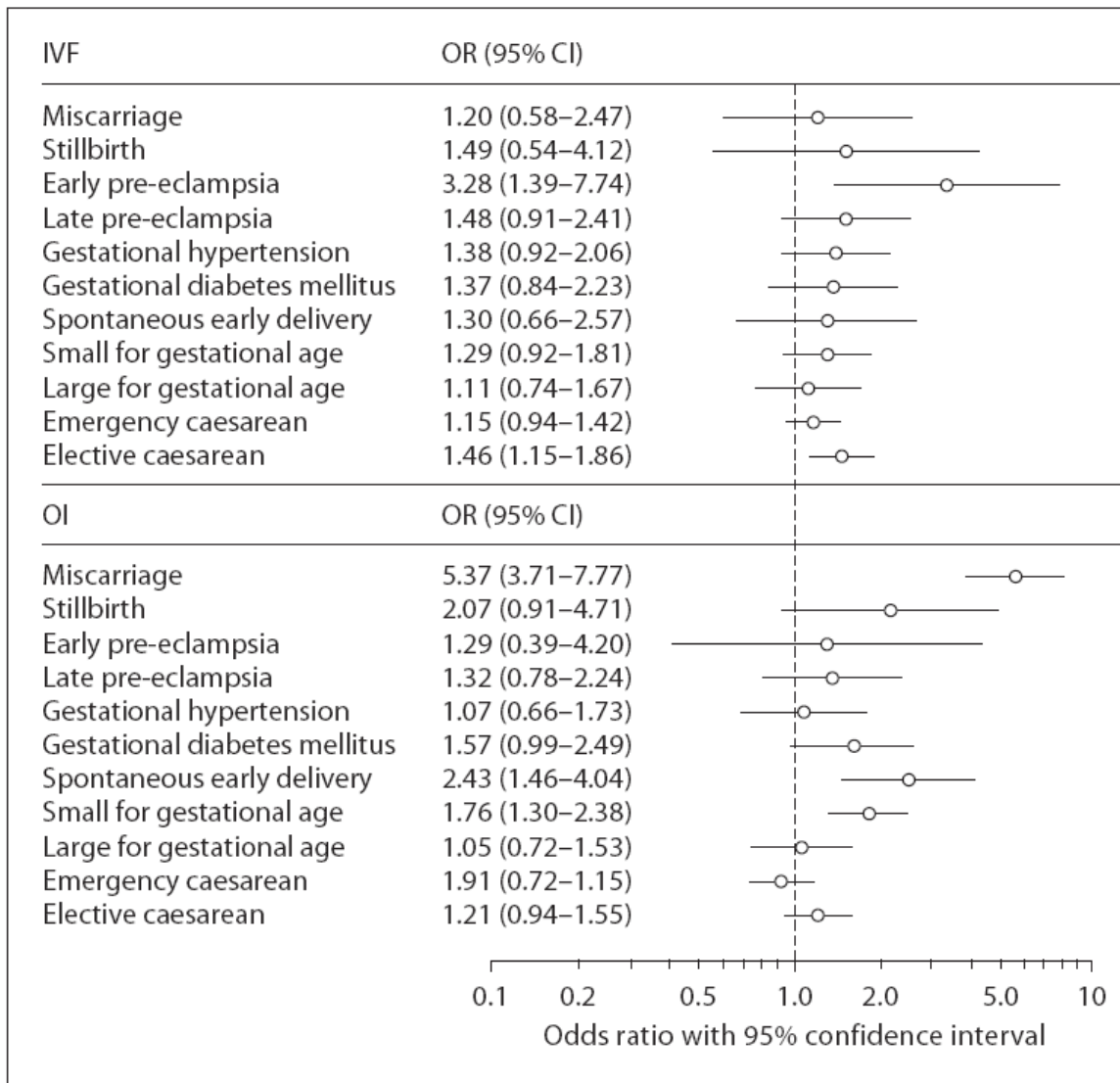
Fig. 1.3 Forest plot of ORs, after adjustment for maternal characteristics and obstetric history, for the risk of complications in pregnancies conceived by IVF and after OI compared to SCPs

Fig 1.4 - Forest plot of ORs from previous reports and current study for the risk of PE in pregnancies conceived by IVF and after OI compared to SCPs

Table 1. Maternal characteristics in the study population.

Independant variable	Spontaneous conception (n=40,261)	Ovulation induction drugs (n=682)	In vitro-fertilization (n=634)
Maternal age in years, median (IQR)	31.1 (26.4-35.1)	32.5 (28.1-36.8)*	36.3 (33.2-39.3)*
Body mass index in kg/m^2, median (IQR)	24.4 (22.0-28.0)	24.6 (22.2-28.7)*	24.4 (21.9-27.4)
Racial origin			
Caucasian, n (%)	30,367 (75.4)	515 (75.5)	531 (83.3)*
African, n (%)	6,526 (16.2)	110 (16.1)	46 (7.3)*
South Asian, n (%)	1,638 (4.1)	31 (4.5)	25 (3.9)
East Asian, n (%)	706 (1.8)	10 (1.5)	17 (2.7)
Mixed, n (%)	1,024 (2.5)	16 (2.3)	15 (2.4)
Cigarette smoking, n (%)	4,498 (11.2)	46 (6.7)*	10 (1.6)*
History of chronic hypertension, n (%)	421 (1.0)	9 (1.3)	12 (1.9)
History of pre-existing Diabetes, n (%)			
Type I	177 (0.4)	6 (0.9)	9 (1.4)*
Type II	118 (0.3)	6 (0.9)*	1 (0.2)
Parity			
Nulliparous, n (%)	19,045 (47.3)	322 (47.2)	482 (76.0)
Parous, n (%)	21,216 (52.7)	360 (52.8)	152 (24.0)*

Comparison between outcome groups by Mann Whitney U-test with post-hoc Bonferroni correction; * p<0.025.

Adverse outcome	Ovulation induction drugs		In-vitro fertilization	
	Odds ratio (95% CI)	***P* value**	**Odds ratio (95% CI)**	***P* value**
Miscarriage	5.20 (3.61-7.48)	<0.0001	1.30 (0.64-2.64)	0.462
Intrauterine death	2.17 (0.96-4.91)	0.064	1.49 (0.55-4.04)	0.430
Hypertensive disorders				
Early-preeclampsia	1.47 (0.47-4.63)	0.512	3.13 (1.38-7.13)	0.007
Late- preeclampsia	1.36 (0.81-2.28)	0.243	1.74 (1.08-2.80)	0.022
All preeclampsia	1.32 (0.82-2.12)	0.249	1.95 (1.29-2.95)	0.002
Gestational hypertension	1.17 (0.73-1.87)	0.520	1.87 (1.26-2.76)	0.002
Gestational diabetes	1.68 (1.07-2.63)	0.025	1.62 (1.01-2.61)	0.046
Preterm delivery				
Iatrogenic	1.29 (0.48-3.47)	0.619	2.99 (1.52-5.85)	0.001
Spontaneous	2.35 (1.42-3.89)	0.001	1.36 (0.70-2.65)	0.364
Growth disorders				
Large for gestation	1.19 (0.83-1.72)	0.346	1.01 (0.68-1.50)	0.960
Small for gestation	1.61 (1.20-2.16)	0.002	1.30 (0.93-1.80)	0.122
Cesarean section				
Emergency	1.02 (0.81-1.28)	0.859	1.89 (1.55-2.30)	<0.0001
Elective	1.38 (1.10-1.73)	0.005	1.96 (1.58-2.44)	<0.0001

Table 2. Univariate regression analysis demonstrating the odds ratios with 95% confidence intervals (CI) for adverse pregnancy outcomes in pregnancies conceived by in-vitro fertilization and by use of ovulation induction drugs without in-vitro fertilization compared to spontaneous conceptions.

Table 3. Logistic regression analysis for the prediction of miscarriage and stillbirth by maternal factors and obstetric history. Note that the classification of parity used for the two outcome measures was different.

Independant variable	Miscarriage		Stillbirth	
	OR (95 %CI)	*P* value	OR (95% CI)	*P* value
Maternal age	1.04 (1.02-1.05)	<0.0001	1.01 (0.99-1.04)	0.401
Body mass index	1.03 (1.01-1.05)	<0.0001	1.05 (1.02-1.07)	<0.0001
Racial origin		<0.0001		0.005
Caucasian	1.00	-	1.00	-
African	3.41 (2.75-4.23)	<0.0001	1.95 (1.37-2.76)	<0.0001
South Asian	1.22 (0.69-2.15)	0.488	1.52 (0.74-3.12)	0.260
East Asian	1.09 (0.45-2.65)	0.857	0.91 (0.22-3.69)	0.891
Mixed	2.59 (1.61-4.17)	<0.0001	1.36 (0.55-3.33)	0.506
Cigarette smoking	1.45 (1.06-1.98)	0.019	2.08 (1.39-3.11)	<0.0001
Conception		<0.0001		0.174
Spontaneous	1.00		1.00	-
Ovulation induction drugs	5.37 (3.71-7.77)	<0.0001	2.07 (0.91-4.71)	0.084
In-vitro-fertilization	1.20 (0.58-2.47)	0.620	1.49 (0.54-4.12)	0.439
History of chronic hypertension	0.94 (0.47-1.89)	0.863	2.92 (1.43-5.98)	0.003
History of pre-existing Diabetes	1.57 (0.74-3.30)	0.239	2.97 (1.28-6.93)	0.012
Parity		<0.0001		0.031
Nulliparous without previous miscarriage	1.00	-		
Nulliparous with previous miscarriage at <16 w	1.71 (1.27-2.29)	<0.0001		
Nulliparous with previous miscarriage at 16-23 w	10.27 (5.85-18.0)	<0.0001		
Parous without previous miscarriage	1.03 (0.80-1.34)	0.808		
Parous with previous miscarriage at <16 w	1.09 (0.74-1.59)	0.663		
Parous with previous miscarriage at 16-23 w	4.15 (2.40-7.16)	<0.0001		
Nulliparous			1.00	-
Parous without previous stillbirth			0.70 (0.51-0.95)	0.024
Parous with previous stillbirth			1.63 (0.59-4.55)	0.347

Table 4. Logistic regression analysis for the prediction of preeclampsia and gestational hypertension by maternal factors and obstetric history.

Independant variable	Early-preeclampsia		Late-preeclampsia		Gestational hypertension	
	OR (95 %CI)	P value	OR (95 %CI)	P value	OR (95% CI)	P value
Maternal age	1.00 (0.97-1.03)	0.970	1.02 (1.01-1.04)	0.002	1.03 (1.02-1.04)	<0.0001
Body mass index	1.06 (1.03-1.09)	<0.0001	1.08 (1.06-1.09)	<0.0001	1.07 (1.06-1.08)	<0.0001
Racial origin		<0.0001		<0.0001		<0.0001
Caucasian	1.00	-	1.00	-	1.00	-
African	3.50 (2.40-5.10)	<0.0001	2.59 (2.19-3.08)	<0.0001	1.47 (1.25-1.73)	<0.0001
South Asian	2.52 (1.20-5.30)	0.015	1.95 (1.38-2.77)	<0.0001	1.09 (0.77-1.53)	0.627
East Asian	0.70 (0.10-5.05)	0.719	1.73 (1.00-2.98)	0.049	1.13 (0.68-1.88)	0.629
Mixed	1.88 (0.68-5.20)	0.222	1.32 (0.80-2.16)	0.274	1.06 (0.70-1.61)	0.778
Cigarette smoking	0.68 (0.33-1.42)	0.301	0.83 (0.62-1.11)	0.211	0.56 (0.43-0.74)	<0.0001
Conception		0.024		0.179		0.284
Spontaneous	1.00	-	1.00	-	1.00	-
Ovulation induction drugs	1.29 (0.39-4.20)	0.678	1.32 (0.78-2.24)	0.303	1.07 (0.66-1.73)	0.780
In-vitro-fertilization	3.28 (1.39-7.74)	0.007	1.48 (0.91-2.41)	0.116	1.38 (0.92-2.06)	0.117
History of chronic hypertension	6.95 (3.87-12.50)	<0.0001	3.32 (2.36-4.66)	<0.0001	0.76 (0.45-1.27)	0.288
History of pre-existing diabetes mellitus	1.27 (0.37-4.34)	0.706	1.15 (0.62-2.17)	0.655	1.20 (0.67-2.14)	0.538
Parity		<0.0001	<0.0001			<0.0001
Nulliparous	1.00	-	1.00	-	1.00	-
Parous without previous preeclampsia	0.35 (0.23-0.54)	<0.0001	0.30 (0.25-0.36)	<0.0001	0.34 (0.29-0.40)	<0.0001
Parous with previous PE	2.06 (1.18-3.62)	0.012	1.95 (1.51-2.52)	<0.0001	1.87 (1.48-2.36)	<0.0001
Family history of PE in mother	1.95 (1.09-3.50)	0.025	1.64 (1.24-2.19)	0.001	2.00 (1.59-2.51)	<0.0001

Table 5. Logistic regression analysis for the prediction of small and large for gestational age neonates and gestational diabetes by maternal factors and obstetric history.

Independant variable	Small for gestation		Large for gestation	
	OR (95 %CI)	P value	OR (95% CI)	P value
Maternal age	1.01 (1.00-1.02)	0.033	1.01 (1.00-1.02)	0.068
Body mass index	0.97 (0.96-0.98)	<0.0001	1.08 (1.07-1.08)	<0.0001
Racial origin		<0.0001		<0.0001
Caucasian	1.00	-	1.00	-
African	2.21 (1.97-2.48)	<0.0001	0.64 (0.56-0.75)	<0.0001
South Asian	3.08 (2.60-3.64)	<0.0001	0.49 (0.35-0.70)	<0.0001
East Asian	2.04 (1.54-2.70)	<0.0001	0.69 (0.43-1.11)	0.124
Mixed	1.64 (1.27-2.12)	<0.0001	0.73 (0.51-1.04)	0.079
Cigarette smoking	2.76 (2.44-3.11)	<0.0001	0.61 (0.50-0.74)	<0.0001
Conception		<0.0001		0.858
Spontaneous	1.00	-	1.00	-
Ovulation induction drugs	1.76 (1.30-2.38)	<0.0001	1.05 (0.72-1.53)	0.805
In-vitro-fertilization	1.29 (0.92-1.81)	0.134	1.11 (0.74-1.67)	0.617
History of chronic hypertension	2.04 (1.44-2.89)	<0.0001	0.94 (0.62-1.42)	0.764
History of pre-existing diabetes mellitus	0.99 (0.53-1.83)	0.968	4.88 (3.66-6.50)	<0.0001
Parity		<0.0001		
Nulliparous	1.00	-	1.00	-
Parous without previous SGA	0.44 (0.40-0.49)	<0.0001		
Parous with previous SGA	1.82 (1.56-2.13)	<0.0001		
Parous without previous LGA			1.25 (1.11-1.40)	<0.0001
Parous with previous LGA			5.01 (4.32-5.82)	<0.0001

Table 6. Logistic regression analysis for the prediction of gestational diabetes and spontaneous preterm delivery by maternal factors and obstetric history.

Independant variable	Gestational diabetes		Preterm delivery	
	OR (95 %CI)	P value	OR (95% CI)	P value
Maternal age	1.06 (1.05-1.08)	<0.0001	1.01 (1.00-1.03)	0.109
Body mass index	1.11 (1.10-1.12)	<0.0001	1.00 (0.98-1.02)	0.756
Racial origin		<0.0001		0.006
Caucasian	1.00	-	1.00	-
African	1.43 (1.19-1.72)	<0.0001	1.54 (1.21-1.95)	<0.0001
South Asian	2.88 (2.16-3.83)	<0.0001	1.45 (0.93-2.25)	0.098
East Asian	3.58 (2.43-5.29)	<0.0001	1.06 (0.50-2.25)	0.888
Mixed	1.19 (0.74-1.92)	0.480	0.95 (0.50-1.79)	0.863
Cigarette smoking	1.02 (0.78-1.33)	0.904	1.89 (1.46-2.45)	<0.0001
Conception		0.077		0.002
Spontaneous	1.00	-	1.00	-
Ovulation induction drugs	1.57 (0.99-2.49)	0.055	2.43 (1.46-4.04)	0.001
In-vitro-fertilization	1.37 (0.84-2.23)	0.206	1.30 (0.66-2.57)	0.443
History of chronic hypertension	1.00 (0.61-1.66)	0.989	1.03 (0.47-2.24)	0.941
History of pre-existing diabetes mellitus	-	-	2.09 (1.05-4.16)	0.036
Parity		<0.0001		<0.0001
Nulliparous	1.00	-	1.00	-
Parous without previous LGA	0.80 (0.68-0.94)	0.006		
Parous with previous LGA	1.68 (1.31-2.14)	<0.0001		
Nulliparous, miscarriage < 16 weeks			1.30 (0.97-1.73)	0.083
Nulliparous, miscarriage 16-23 weeks			5.87 (3.78-9.12)	<0.0001
Parous, delivery 24-30 weeks			5.78 (3.52-9.50)	<0.0001
Parous, delivery 31-36 weeks			3.32 (2.29-4.82)	<0.0001
Parous, iatrogenic preterm delivery			1.77 (0.71-4.37)	0.219
Parous, delivery > 37 weeks			0.79 (0.62-1.01)	0.058

Table 7. Logistic regression analysis for the prediction of elective and emergency cesarean section by maternal factors and obstetric history.

Independant variable	Emergency cesarean section			Elective cesarean section	
	OR (95%CI)		P value	OR (95% CI)	P value
Maternal age	1.05	(1.05-1.06)	<0.0001	1.07 (1.07-1.08)	<0.0001
Body mass index	1.06	(1.06-1.07)	<0.0001	1.05 (1.04-1.06)	<0.0001
Racial origin			<0.0001		0.005
Caucasian	1.00		-	1.00	-
African	1.23	(1.14-1.34)	<0.0001	0.93 (0.84-1.03)	0.161
South Asian	1.33	(1.15-1.54)	<0.0001	1.29 (1.09-1.53)	0.003
East Asian	1.12	(0.89-1.40)	0.325	1.32 (1.03-1.68)	0.028
Mixed	0.93	(0.77-1.13)	0.445	0.90 (0.72-1.14)	0.384
Cigarette smoking	1.18	(1.07-1.30)	0.001	0.92 (0.82-1.05)	0.213
Conception			0.290		0.003
Spontaneous	1.00		-	1.00	-
Ovulation induction drugs	0.91	(0.72-1.15)	0.433	1.21 (0.94-1.55)	0.141
In-vitro-fertilization	1.15	(0.94-1.42)	0.179	1.46 (1.15-1.86)	0.002
History of chronic hypertension	1.32	(1.01-1.72)	0.045	1.71 (1.31-2.24)	<0.0001
History of pre-existing diabetes mellitus	3.67	(2.76-4.89)	<0.0001	3.41 (2.47-4.70)	<0.0001
Parity			<0.0001		<0.0001
Nulliparous	1.00		-	1.00	-
Parous with one or more vaginal deliveries (VD) only	0.26	(0.24-0.28)	<0.0001	0.96 (0.88-1.03)	0.258
Parous with one caesarean section (CS) only	2.77	(2.46-3.11)	<0.0001	9.89 (8.78-11.15)	<0.0001
Parous with one CS plus one or more VD	0.51	(0.38-0.69)	<0.0001	3.75 (3.00-4.69)	<0.0001
Parous with two or more CSs only	2.48	(1.48-4.17)	0.001	72.28 (49.22-106.13)	<0.0001
Parous with two or more CSs plus one or more VD	1.04	(0.38-2.80)	0.946	20.47 (10.82-38.72)	<0.0001

1.B) Assisted conception and placental perfusion assessed by uterine artery Doppler at 11-13 weeks' gestation

Running title: Assisted conception and placental perfusion

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Abstract

BACKGROUND: Pregnancies conceived by in-vitro fertilization (IVF) are at increased risk of preeclampsia (PE). This study examines the potential mechanism for such association by examining the effect of method of conception on placentation as assessed by uterine artery Doppler at 11-13 weeks' gestation.

METHODS: Prospective screening study at 11^{+0}-13^{+6} weeks for PE in singleton pregnancies by a combination of maternal history and uterine artery pulsatility index (PI). Regression analysis was performed to examine the association between the method of conception and both uterine artery PI and development of PE, after adjustment for maternal characteristics and obstetric history.

RESULTS: In the study population of 27,461 pregnancies, conception was spontaneous in 26,538 (96.6%), by IVF in 426 (1.6%), and by use of ovulation induction (OI) drugs in 497 (1.8%). Conception by IVF was associated with a 3.9-fold increase in risk for early-PE, requiring delivery before 34 weeks, but not late-PE. In the OI group the risk of early- and late-PE was not increased. Significant contributions in explaining $\log_{10}$ uterine artery PI were provided from maternal characteristics but not from method of conception. The median uterine artery PI multiple of the median (MoM) in the IVF group (1.02 MoM) and OI group (1.03 MoM) was not significantly different from the spontaneous conception group (1.01 MoM; p=0.870 and p=0.296, respectively).

CONCLUSIONS: Conception by IVF is associated with increased risk for early-PE but the underlying mechanism is unlikely to be impaired placental perfusion.

Key words: In-vitro fertilization, Ovulation induction, First trimester screening, Preeclampsia, Uterine artery Doppler.

Introduction

An increasing proportion of pregnancies are conceived by assisted reproduction technology (ART). Several studies have reported that in ART pregnancies the incidence of preeclampsia (PE) is higher than in spontaneous conceptions (Jackson *et al.,* 2004; Helmerhorst *et al.,* 2004; McDonald *et al.,* 2009; Chaveeva *et al.,* 2010). Preeclampsia affects approximately 2% of pregnancies and is a major cause of maternal and perinatal morbidity and death (ACOG, 2002; Högberg, 2005). There is evidence that PE is not a homogeneous condition and the underlying mechanism for early onset severe disease requiring delivery before 34 weeks (early-PE) is thought to be impaired placentation because of inadequate trophoblastic invasion of the maternal spiral arteries (Brosens *et al.,* 1967; Khong *et al.,* 1986; Meekins *et al.,* 1995; Brosens *et al.,* 2010), whereas late-PE is thought to be a manifestation of an underlying metabolic disorder with increased insulin resistance (Egbor *et al.,* 2006; Moldenhauer *et al.,* 2003; Kaaja *et al.,* 1999; Bosio *et al.,* 1999; D'Anna *et al.,* 2006*).*

In normal pregnancy the spiral arteries in the placental bed are invaded by trophoblast, which becomes incorporated into the vessel wall and replaces the endothelium, muscular layer and neural tissue (Brosens *et al.,* 1967; Khong *et al.,* 1986; Meekins *et al.,* 1995; Brosens *et al.,* 2010). These physiological changes convert the spiral arteries from narrow muscular vessels to wide non-muscular channels independent of maternal vasomotor control. This process of placentation is reflected in the measurement of impedance to flow in the uterine arteries which can be investigated by the measurement of uterine artery pulsatility index (PI) (Yu *et al.,* 2005; Plasencia *et al.,* 2007).

The aim of this study is to examine the possible effect of in vitro fertilization (IVF) and the use of ovulation induction drugs without IVF (OI) on placentation as documented by the measurement of uterine artery PI at 11-13 weeks' gestation.

Methods

Screening study population

This was a prospective screening study for adverse obstetric outcomes in women attending for their routine first hospital visit in pregnancy at King's College Hospital, London, UK and Medway Maritime Hospital, Kent, UK. This visit, which is held at 11^{+0}-13^{+6} weeks of gestation, includes recording of maternal demographic characteristics and previous obstetric and medical history, measurement of maternal weight and height and calculation of the body mass index (BMI), and ultrasound examination for the measurement of the fetal crown-rump length (CRL) to determine gestational age (Robinson and Fleming, 1975), measurement of the fetal nuchal translucency (NT) thickness as part of screening for aneuploidies (Snijders *et al.,* 1998), examination of the fetal anatomy for the diagnosis of major fetal defects (Syngelaki *et al.,* 2010) and Doppler assessment of the uterine arteries with measurement of PI (Plasencia *et al.,* 2007). Written informed consent was obtained from the women agreeing to participate in the study, which was approved by King's College Hospital Ethics Committee.

A second ultrasound examination for fetal biometry and examination of the fetal anatomy is carried out at 20-24 weeks. In the neonatal period all babies are examined by a pediatrician. Data on pregnancy outcome are collected from the hospital maternity records or the general medical practitioners of the women.

In this study we examined the relationship between method of conception (spontaneous, IVF, OI without IVF) with uterine artery PI and PE. We excluded pregnancies conceived by intrauterine insemination because we did not have data on whether or not they had received OI drugs, those with fetal aneuploidies or major defects diagnosed either prenatally or in the neonatal period and pregnancies ending in termination for psychosocial reasons.

Maternal characteristics and obstetric history

Patients complete a questionnaire on maternal age, racial origin (Caucasian, African, South Asian, East Asian and mixed), method of conception (spontaneous, IVF, use of ovulation induction drugs (OI) without IVF or intrauterine insemination), cigarette smoking during pregnancy (yes or no), history of chronic hypertension (yes or no), history of diabetes mellitus (yes or no), family history of PE in the mother of the pregnant woman (yes or no) and obstetric history including PE in a previous pregnancy (yes or no). The questionnaire was then reviewed by a doctor together with the patient.

Uterine artery pulsatility index

Uterine artery Doppler was performed transabdominally by sonographers who had received the Certificate of Competence in Doppler of the Fetal Medicine Foundation (www.fetalmedicine.com). A sagittal section of the

uterus was obtained and the cervical canal and internal cervical os were identified. The transducer was then gently tilted from side to side and color-flow mapping was used to identify each uterine artery adjacent to the cervix and uterus at the level of the internal os. Pulsed wave Doppler was used with the sampling gate set at 2 mm to cover the whole vessel and care taken to ensure that the angle of insonation was less than 30°. When three similar consecutive waveforms were obtained, the PI was measured and the mean PI of the left and right arteries was calculated.

Preeclampsia

The definition of PE was that of the International Society for the Study of Hypertension in Pregnancy [9]. The diastolic blood pressure should be 90 mm Hg or more on at least two occasions at 4 h apart, developing after 20 weeks of gestation in previously normotensive women, together with proteinuria of 300 mg or more in 24 h or two readings of at least ++ on dipstick analysis of midstream or catheter urine specimens if no 24-h collection is available. In PE superimposed on chronic hypertension significant proteinuria (as defined above) should develop after 20 weeks of gestation in women with known chronic hypertension (history of hypertension before conception or the presence of hypertension at the booking visit before 20 weeks of gestation in the absence of trophoblastic disease).

Statistical analysis

Maternal and fetal characteristics in the IVF, OI and spontaneous conception groups were compared using the χ^2-test or Fisher's exact test for categorical variables and Mann-Whitney U-test with *post hoc* Bonferroni correction for continuous variables. The data on uterine artery mean PI were made Gaussian after logarithmic transformation. Univariate and multivariate regression analysis was performed to examine whether method of conception had a significant contribution in predicting $\log_{10}$ uterine artery PI. In the spontaneous conception group multivariate regression analysis was performed to derive a model for the prediction of $\log_{10}$ uterine artery PI and the measured PI in each patient was expressed as a multiple of the expected median (MoM) calculated from this model. The median MoM uterine artery PI in the IVF and OI groups was compared to the spontaneous conception group using -Whitney U-test with *post hoc* Bonferroni correction. Multivariate logistic regression analysis was carried out to estimate the contribution of method of conception in addition to maternal characteristics and obstetric history in the prediction of early- and late-PE.

The statistical software package SPSS 16.0 (SPSS Inc., Chicago, IL) was used for data analyses.

Results

Study population

During the study period we carried out an ultrasound examination at 11-13 weeks which included measurement of uterine artery PI in 29,810 singleton pregnancies with a live fetus and CRL of 45-84 mm. We excluded from further analysis 50 (0.2%) cases because they conceived by intrauterine insemination, 1,810 (6.1%) because there were no or incomplete data on pregnancy outcome and 417 (1.4%) because of the prenatal or postnatal diagnosis of aneuploidies or major defects and 64 (0.2%) because of pregnancy termination.

In the 27,461 cases included in the study, conception was spontaneous in 26,538 (96.6%), by IVF in 426 (1.6%), and by OI in 497 (1.8%). The maternal and pregnancy characteristics of the study population are shown in Table 1. In the OI and IVF groups, compared to the spontaneous conception group, the maternal age was higher and fewer women were cigarette smokers. In the OI group more women had diabetes mellitus and in the IVF group there were fewer women of African racial origin, more were nulliparous and had chronic hypertension. The incidence of early-PE was increased in the IVF but not in the OI group.

Uterine artery pulsatility index

Univariate regression analysis demonstrated that in the prediction of $\log_{10}$ uterine artery PI there was a significant contribution from conception by IVF, but not from conception by OI (Table 2). Multiple regression analysis demonstrated that significant contributions in explaining $\log_{10}$ uterine artery PI were provided from fetal CRL, maternal age, weight, racial origin, smoking status, previous history of PE, but not from method of conception, family history of preeclampsia in the mother or history of chronic hypertension and diabetes mellitus.

The median uterine artery PI MoM in the IVF group and OI groups was not significantly different from the spontaneous conception pregnancies (1.02 MoM, 1.03 MoM and 1.01 MoM, respectively; p=0.870 and p=0.296).

Early and late preeclampsia

The incidence of PE was 2.4% (663 of 27,461), including 101 (0.4%) with early-PE and 562 (2.0%) with late-PE. Logistic regression analysis demonstrated that significant contributions in the prediction of early-PE were provided from conception by IVF, maternal weight, height, racial origin, previous and family history of PE and history of chronic hypertension, but not from conception by OI, maternal age, smoking or history of diabetes mellitus.

Significant contributions in the prediction of late-PE were provided from maternal age, weight, height, racial origin, previous and family history of PE and history of chronic hypertension, but not from conception by IVF or OI, smoking status or history of diabetes mellitus.

Discussion

The findings of this study demonstrate that conception by IVF is associated with a four fold increase in risk for early-PE which is unlikely to be mediated by impaired trophoblastic invasion of the maternal spiral arteries.

We have recently reported on the combined results of seven studies on the outcome of a total of 4,238 IVF pregnancies, compared to 638,416 spontaneously conceived pregnancies, that the summary odds ratio for PE was 1.76 (95% CI 1.34-2.31) (Tanbo *et al.,* 1995; Zadori *et al.,* 2003; Kallen *et al.,* 2005; Shevell *et al.,* 2005; Chen *et al.,* 2009; Sun *et al.,* 2009; Chaveeva *et al.,* 2010). In most of these studies the data were provided for total PE, rather than early and late onset disease. In our study we used multiple regression analysis of all the maternal characteristics known to affect the risk for PE and demonstrated that IVF had a significant additional contribution in increasing substantially the risk for early-PE. In the IVF pregnancies there was also an increase in late-PE but presumably because of the small number of cases examined this did not reach statistical significance. In contrast to IVF in the OI pregnancies the risk of PE was not significantly increased in either this or the previous studies (Tanbo *et al.,* 1995; Zadori *et al.,* 2003; Kallen *et al.,* 2005; Shevell *et al.,* 2005; Chen *et al.,* 2009; Sun *et al.,* 2009; Chaveeva *et al.,* 2010).

There is extensive evidence from histological and Doppler studies that a high proportion of pregnancies developing early-PE have impaired trophoblastic invasion of the maternal spiral arteries with consequent increase in the impedance to flow in the uterine arteries manifested in increased PI (Papageorghiou *et al.,* 2001; 2004; Yu *et al.,* 2005; Plasencia *et al.,* 2007; Brosens *et al.,* 2010). It was therefore hypothesized that the observed association between IVF and increased risk for early-PE would be mediated by impaired placentation manifested in increased uterine artery PI.

Uterine artery PI normally decreases with gestational age and is affected by maternal characteristics including a decrease in PI with age and weight and higher PI in women of African racial origin, in cigarette smokers and those with previous history of PE. Consequently, in investigating the potential effect of IVF on uterine artery PI we performed multiple regression analysis to take into account these maternal characteristics and found that after adjustment for these factors there was no significant difference in uterine artery PI between the IVF and spontaneously conceived pregnancies. A previous case-control study comparing 31 IVF and 62 spontaneously conceived pregnancies has also reported no significant difference between the groups in uterine artery PI at 11-13 weeks (Prefumo *et al.,* 2007).

Low-dose aspirin, which inhibits vasoconstriction and platelet aggregation, may improve the development of placental vasculature and reduce the risk for PE. Randomized studies reported that the prophylactic use of aspirin reduces the risk of developing PE by about 10% (Askie *et al.,* 2007). Recent evidence suggests that in pregnancies with increased impedance to flow in the uterine arteries the prophylactic use of low-dose aspirin started before 16 weeks' gestation may reduce the risk of PE by more than 50% (Bujold *et al.,* 2009). A small randomized study in women undergoing IVF reported that use of low-dose aspirin commenced at the time of ovarian stimulation and continued until delivery did not reduce the incidence of PE compared to women receiving placebo (Haapsamo *et al.,* 2010). The implications of these studies are that firstly, the effect of prophylactic low-dose aspirin appears to be greatest when given early in pregnancy during the process of placentation to women with documented evidence of impaired placental perfusion, and secondly, in IVF pregnancies aspirin may not be effective in preventing PE because the underlying mechanism for this disease in such pregnancies is not impaired placental perfusion.

In conclusion, conception by IVF increases substantially the risk for early-PE though a mechanism unrelated to clinically measurable impairment in placental perfusion.

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33. **Table 1.** Maternal and pregnancy characteristics in the study population.

Independant variable	Spontaneous conception (n=26,538)	Ovulation induction drugs (n=497)	In vitro-fertilization (n=426)
Maternal age in years, median (IQR)	30.9 (26.2-34.9)	32.2 (27.9-36.7)*	36.3 (33.1-39.5)*
Maternal weight in kg, median (IQR)	66.0 (59.0-76.0)	66.0 (60.0-78.0)	65.7 (60.0-75.0)
Maternal height in cm, median (IQR)	164.0 (160.0-168.0)	163.0 (159.0-167.8)	165.1 (160.0-170.2)*
Racial origin			
Caucasian, n (%)	18,763 (70.7)	366 (76.6)	344 (80.8)
African, n (%)	5,509 (20.8)	86 (17.3)	39 (9.2)*
South Asian, n (%)	1,067 (4.0)	25 (5.0)	19 (4.5)
East Asian, n (%)	450 (1.7)	6 (1.2)	12 (2.8)
Mixed, n (%)	749 (2.8)	14 (2.8)	12 (2.8)
Cigarette smoking, n (%)	2,933 (11.1)	32 (6.4)*	7 (1.6)*
History of chronic hypertension, n (%)	316 (1.2)	8 (1.6)	11 (2.6)*
History of diabetes mellitus, n (%)	191 (0.7)	9 (1.8)*	7 (1.6)
Parity			
Nulliparous, n (%)	12,627 (47.6)	240 (48.3)	323 (75.8)*
Parous with previous preeclampsia, n (%)	13,104 (49.4)	243 (48.9)	98 (23.0)*
Parous without previous preeclampsia, n(%)	807 (3.0)	14 (2.8)	5 (1.2)
Family history of preeclampsia, n (%)	1,089 (4.1)	27 (5.4)	14 (3.3)
Incidence of preeclampsia in this pregnancy	628 (2.4%)	15 (3.1%)	20 (4.8%)*

Comparison between outcome groups by Mann Whitney U-test with *post-hoc* Bonferroni correction; * p<0.025.

Table 2. Parameter estimates with 95% confidence intervals from univariate and multivariate regression analysis demonstrating factors from maternal characteristics and obstetric history in the prediction of $\log_{10}$ uterine artery pulsatility index

Independant variable	Univariate analysis		Multivariate analysis	
	Parameter estimate (95 %CI)	**P value**	**Parameter estimate (95% CI)**	**P value**
Fetal crown-rump length in mm	$2.2e^{-03}$ ($2.4e^{-03}$ to $2.1e^{-03}$)	<0.0001	$2.2e^{-03}$ ($2.4e^{-03}$ to $2.1e^{-03}$)	<0.0001
Maternal age in years	-0.001 (0.002 to -0.001)	<0.0001	$6.0e^{-04}$ ($8.7e^{-04}$ to $3.4e^{-04}$)	<0.0001
Maternal weight in kg	-0.002 (-0.003 to -0.001)	<0.0001	-0.002 (-0.002 to -0.001)	<0.0001
(Maternal weight in kg)^2	$9.4e^{-006}$ ($5.4e^{-006}$ to $1.4e^{-005}$)	<0.0001	$8.0e^{-006}$ ($3.9e^{-006}$ to $1.2e^{-005}$)	<0.0001
Maternal height in cm	$7.4e^{-04}$ ($9.4e^{-04}$ to $5.0e^{-04}$)	<0.0001	$1.7e^{-04}$ ($4.0e^{-04}$ to $6.5e^{-005}$)	0.157
Racial origin				
Caucasian	(Reference)		(Reference)	
African	0.026 (0.022 to 0.030)	<0.0001	0.040 (0.034 to 0.046)	<0.0001
South Asian	0.011 (0.003 to 0.018)	0.006	0.009 (-0.002 to 0.020)	0.123
East Asian	2.0e-03 (-0.011 to 0.012)	0.973	-0.003 (-0.019 to 0.014)	0.729
Mixed	0.022 (0.013 to 0.031)	<0.0001	0.024 (0.012 to 0.037)	<0.0001
Cigarette smoking	0.005 ($-8.5e^{-005}$ to 0.010)	0.054	0.005 ($1.4e^{-04}$ to 0.010)	0.044
Conception				
Spontaneous	(Reference)		(Reference)	
Ovulation induction drugs	2.8e-03 (-0.011 to 0.011)	0.962	0.017 (-0.002 to 0.035)	0.073
In-vitro-fertilization	-0.014 (-0.027 to -0.002)	0.019	0.003 (-0.012 to 0.019)	0.659
History of chronic hypertension	0.009 (-0.004 to 0.023)	0.177	0.009 (-0.005 to 0.023)	0.149
History of diabetes mellitus	-0.015 (-0.033 to 0.002)	0.079	-0.011 (-0.028 to 0.006)	0.194
Parity				
Nulliparous	(Reference)		(Reference)	
Parous without previous preeclampsia	-0.007 (-0.010 to -0.004)	<0.0001	0.001 (-0.002 to 0.005)	0.493
Parous with previous preeclampsia	0.013 (0.004 to 0.022)	0.003	0.023 (0.012 to 0.034)	<0.0001
Family history of preeclampsia	0.006 (-0.001 to 0.014)	0.103	0.004 (-0.003 to 0.012)	0.274

Table 3. Multivariate logistic regression analysis demonstrating odds ratios and 95% confidence intervals in the prediction of early- and late-preeclampsia from maternal characaeristics and obstetric history

Independant variable	Early-preeclampsia		Late-preeclampsia	
	OR (95 %CI)	P value	OR (95 %CI)	P value
Maternal age	1.01 (0.97-1.05)	0.622	1.03 (1.01-1.04)	<0.0001
Maternal weight	1.02 (1.01-1.03)	0.007	1.03 (1.03-1.04)	<0.0001
Maternal height	0.94 (0.91-0.97)	<0.0001	0.96 (0.95-0.97)	<0.0001
Racial origin		<0.0001		<0.0001
Caucasian (Reference)	1.00	-	1.00	-
African	2.90 (1.86-4.53)	<0.0001	2.39 (1.98-2.89)	<0.0001
South Asian	2.66 (1.21-5.85)	0.015	1.82 (1.20-2.75)	0.005
East Asian	0.66 (0.09-5.00)	0.690	1.48 (0.77-2.85)	0.237
Mixed	2.28 (0.81-6.43)	0.118	1.45 (0.86-2.42)	0.160
Cigarette smoking	0.84 (0.38-1.86)	0.660	0.84 (0.60-1.18)	0.325
Conception		0.016		0.178
Spontaneous (Reference)	1.00	-	1.00	-
Ovulation induction drugs	1.61 (0.48-5.41)	0.442	1.26 (0.70-2.28)	0.439
In-vitro-fertilization	3.94 (1.51-10.27)	0.005	1.61 (0.94-2.76)	0.086
History of chronic hypertension	6.45 (3.28-12.67)	<0.0001	2.97 (2.03-4.34)	<0.0001
History of diabetes mellitus	1.43 (0.39-5.21)	0.585	1.09 (0.54-2.23)	0.805
Parity		<0.0001	<0.0001	
Nulliparous	1.00	-	1.00	-
Parous without previous preeclampsia	0.39 (0.24-0.64)	<0.0001	0.30 (0.25-0.37)	<0.0001
Parous with previous preeclampsia	2.74 (1.47-5.11)	0.002	2.01 (1.54-2.75)	<0.0001
Family history of preeclampsia in mother	2.47 (1.28-4.73)	0.007	1.89 (1.36-2.61)	<0.0001

CHAPTER 1- THE ROLE OF ULTRASOUND IN REPRODUCTIVE HEALTH

o *COMMENT*

Ultrasound has become an essential tool for the assessment and management of women undergoing assisted reproduction treatment. Ultrasound permits the screening of women prior to treatment and prediction of ovarian reserve and endometrial receptivity, allows for direct monitoring of response to controlled ovarian stimulation, and facilitates oocyte retrieval and embryo transfer.

Recent advances in three-dimensional ultrasound (3-D US) have made accurate non-invasive measurements of the follicular, ovarian, and endometrial volumes feasible. Storage capacities, reconstruction of the volume images, and simultaneous viewing of all three orthogonal planes are main advantages of this method in the field of infertility. 3-D US is useful in patients scheduled for serial ovarian monitoring in whom planar reformatted sections allow more accurate and objective volumetric assessment of the leading follicles, which are not always spherical. Ovarian volume measurements by 3-D US contribute to accurate diagnosis of polycystic ovarian syndrome and prediction of the response to stimulation and estimation of the risk of ovarian hyperstimulation. Transvaginal ultrasound directed follicular aspiration and embryo transfer under 3-D US guidance may improve the operator's spatial evaluation and allows precise follicular and/or catheter tip location during the course of interventional procedures.. By providing multiple tomographic sections of the uterine cavity, uterine causes of infertility such as congenital uterine anomalies, submucous leiomyoma, and/or adhesions become easily visible. Quantification of the endometrial volume by 3-D US in combination with blood flow studies contributes to assessment of the endometrial receptivity and may have the potential to predict pregnancy rates in ART technique.

In case of achievement of a pregnancy (in both spontaneous conception or in vitro fertilization cases), the use of 2D / 3D ultrasound allows to check markers for chromosomal defects and for placenta dysfunction which can be used in a predictive multimarkers model at the time of the first trimester visit.

CHAPTER 2 - THE ROLE OF GENETICS IN REPRODUCTIVE HEALTH

- o *BACKGROUND*

As medicine has evolved over the last century, medical genetics has grown from nonexistence to one of the most visible aspects of how we understand and treat disease. This increased role of genetics within medicine will only increase in the coming years, and its role in reproductive medicine will be significant. Genetics has emerged as a primary focus of research with translational applications within reproductive medicine.

"Reproductive genetics", a field of medical genetics integrated with reproductive medicine, are conducted with the intent of informing individuals about the possible outcomes of current or future pregnancies. The tests themselves can include the analysis of chromosomes, DNA, RNA, genes, and/or gene products to determine whether an alteration is present that is causing or is likely to cause a specific disease or condition.

Moreover, many genetic association studies have been performed to investigate disorders of female reproduction, such as polycystic ovary syndrome, premature ovarian failure and endometriosis. These disorders typically manifest heterogeneously, and their pathogeneses are influenced by polygenic and environmental factors. Researchers evaluating these genetic associations have chosen candidate genes related to hormone action, steroid biosynthesis, inflammatory cytokines and autoimmune factors. Several of these genes have yielded statistically significant associations with female reproductive disorders; however, few associations have been robust and reproducible. Whole-genome association studies generate more reliable and unbiased results and represent a breakthrough in genetic studies of female reproduction. Nevertheless, to date only a very small fraction of the overall heritability has been identified and so further studies are needed.

Genetic association studies aim to identify candidate genes or genome regions that contribute to a specific trait or disease by identifying a correlation between disease status and genetic variation1. This strategy is particularly applicable to complex and polygenic diseases. During the past decade, the genetic association study has emerged as a popular and powerful tool for deciphering the link between a genetic locus and a disease2 .

In genetic association studies, single-nucleotide polymorphisms (SNPs) are the most widely tested markers, and most studies use the case–control design. The detection of a higher frequency of an SNP allele or genotype in a series of individuals affected with a disease indicates an association with that disease. Initially genetic association studies employed candidate-gene strategies. To date, candidate genes have been confirmed for many different diseases and traits3 However, candidate-gene studies have limitations. Most of these studies

have evaluated small sample populations with limited statistical power, and few have yielded sufficiently robust results that could be replicated in different populations or by different investigators4 .

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CHAPTER 2 - THE ROLE OF GENETICS IN REPRODUCTIVE HEALTH

- o *PAPERS*

2A) Investigation of the association between polymorphisms of follicle-stimulating hormone receptor gene (FSH-R) and ovarian response in controlled ovarian hyperstimulation (COH)

The FSH-R genotype affects responsiveness to controlled ovarian stimulation in normogonadotrophic women treated with GnRH-a long protocol plus recombinant FSH

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Introduction

Recently, the European Society for Human Reproduction and Embryology (ESHRE) published criteria for definition of " poor ovarian response" to controlled ovarian stimulation (COS) in IVF/ICSI cycles (Ferraretti *et al.*, 2011). According to these criteria, at least two of the following characteristics should be present: advanced maternal age (≥40 years) or any other risk factor for poor ovarian response; previous poor ovarian response (≤3 oocytes with a conventional stimulation protocol); abnormal ovarian reserve test. Nevertheless, further "low prognosis" patients subgroup who do not fit neither with these criteria nor with classical "normal responder" profile has been identified. More specifically, it has been reported that 10-15% of young, normogonadotrophic women show sub-optimal response to standard GnRH-a long protocol (Ferraretti *et al.*, 2004). These patients, despite an apparently normal ovarian response (i.e., recovery of at least 5 oocytes), require higher doses of exogenous FSH than would be expected based on age, anthropometric variables and ovarian reserve tests (Devroey *et al.,* 2009, Alviggi *et al.,* 2011, 2013), thus the definition of hypo-responders has been suggested. Evidences indicate that this phenomenon could be associated with genetic characteristics

(Huhtaniemi *et al.,* 2009). We recently reported that the frequency of an allelic variant of the luteinizing hormone beta-subunit (v-βLH [Haavisto *et al.*, 1995]) is higher in women with a hypo-response to recombinant FSH (r-hFSH) than in the general population (Alviggi *et al.*, 2009; 2011; 2013). This observation is consistent with clinical trials demonstrating that recombinant LH (r-hLH) significantly increases both ovarian response and implantation rate in hypo-responders to monotherapy with r-hFSH (De Placido *et al.,* 2004; Ferraretti *et al.*, 2004; Alviggi *et al.,* 2006). However, the LH variant has been found in only 32% of IVF candidates with a hypo-response profile, thus the pathogenetic mechanism underlying the hypo-response to FSH in about 2/3 of women remains to be established.

Two polymorphisms of the of FSH receptor (FSH-R), Thr307/Asn680 and Ala307/Ser680 have been associated with a higher requirement of exogenous gonadotrophins during COS (Perez-Mayorga *et al.*, 2000; Mohiyiddeen and Nardo, 2010; Yao *et al.*, 2012), suggesting higher ovarian threshold compared with wild type. FSH-R is a member of G-protein-coupled receptor which mediate FSH intracellular signals through cAMP pathway (Simoni *et al.*, 1997).

In addition, FSH receptor might explain inter-individual differences in menstrual pattern. In fact, in homozygotes Ser680/Ser680 of FSH-R a higher ovarian threshold to FSH as well as decreased negative feedback of luteal secretion to the pituitary during the inter-cycle transition and longer menstrual cycles has been described (Greb *et al.*, 2005).

The aim of the this study is to explore FSH-R allelic frequency in a population of 42 normal responders stratified according to r-hFSH consumption, focusing on the impact of these polymorphisms on COS outcome.

Material and methods

A retrospective study on 42 normogonadotrophic patients undergoing a standard IVF/ ICSI cycle in our Department (Federico II University), from October 2011 to April 2012, has been carried out.

Enrollment criteria were: age <37 years; menstrual cycle lasting 24-35 days (intra-individual variability ± 3 days) 6 months before the onset of IVF cycle; FSH <11 IU/l, LH <8 IU/L and prolactin (PRL) <30 ng/ml (measured from the 2nd to 4th day of spontaneous menstrual cycle); at least 5 oocytes retrieved for each patient.

Patients with one or more of the following criteria were excluded from the study: polycystic ovary syndrome (ESHRE/ASRM 2004); stage III-IV endometriosis (revised classification-American Fertility Society, 1985); autoimmune disorders; chromosomal abnormalities.

All patients received a standard GnRH-agonist-long protocol using triptorelin (Decapaptyl 0.1, Ipsen, Italy) at the daily dose of 0.1 mg s.c. starting on the 21st day of the cycle preceding IVF treatment. Pituitary desensitization was confirmed by transvaginal ultrasound (no evidence of ovarian activity, endometrial thickness < 5 mm) and circulating estradiol assessment (<50 pg/ml). Patients with pituitary down-regulation started r-hFSH treatment (Gonal F, MerckSerono, Rome, Italy). A starting dose of 225 IU/day s.c. was administered for the first four days of stimulation. This dose was reduced to 150 IU/day in women who had, on day 5 of stimulation, serum estradiol >160 pg/ml. In other cases, the daily dose was not changed or was increased to 150 IU. In all patients, the ovulatory dose of 10,000 IU i.m. hCG was administered in the presence of at least one follicle reached a mean diameter of 17 mm. All patients had received luteal phase supplementation with a daily dose of 50 mg progesterone intramuscularly (Prontogest, AMSA srl, Rome Italy) from the day of oocyte retrieval. Ongoing pregnancy rate was defined as the presence of fetal heart activity had been detected at 12 weeks of gestation.

Based on the total r-hFSH dose administered, the study population was divided into 2 groups: hypo-responders, namely 17 women who required a cumulative dose of r-hFSH >2500 IU (Group A) and a control group constituted by 25 patients who received a cumulative dose ≤ 2500 IU (Group B).

A blood sample was collected from each patient. Genomic DNA was extracted from peripheral blood leukocytes with a Cell Culture DNA kit (QIAGEN, Dusseldorf, Germany) according to the manufacturer's instructions. A fragment of the FSH-R gene from exon 10 (from 10D to 10G) was amplified by the polymerase chain reaction (PCR) and analyzed by electrophoresis on 2% agarose gel. Then the segment was extracted with phenol chloroform. The purified fragment was digested by Bsrl1 (Biolabs, Schwabach, Germany) and fragments were analyzed by electrophoresis on 2.5% agarose gel. The unpurified fragment, indicating homozygosity for Asn, measures 755 bp. The purified fragment indicating homozygous Ser, generates two fragments measuring respectively 612 and 143 bp. The presence of all three fragments indicates the status of homozygosity.

The serum concentrations of estradiol and LH were measured using an enzyme-linked fluorescent assay (Vidas oestradiol and Vidas LH II, respectively; Bio Mérieux SA, Lyon, France). The sensitivity of the method, defined as the lowest concentration that is significantly different from zero with a probability of 95%,

was 0.03 pg/ml for estradiol and 0.1 IU/l for LH. The coefficient of variations (CVs) intra-and inter-assay was <8% for both estradiol and LH. Serum levels of FSH were determined by an immunoassay based on luminescence (Amerlite FSH Assay, Amersham International plc, Amersham Pharmacia Biotech, Buckinghamshire, UK). The detection threshold was 0.5 IU/l. The intra-and inter-assay CVs were <7%.

Statistical analysis

The results are reported in terms of means ± standard deviations (± SD). The data were analyzed with SPSS, version 12.0 (SPSS Inc., USA). One-way ANOVA was used to determine the effects of stimulation protocols on continuous variables. The Mann-Whitney U test was applied to evaluate inter-groups differences with respect to continuous nonparametric variables. The χ^2 test was used to compare categorical data. A p value <0.05 was considered as statistically significant.

Results

We retrospectively reviewed the outcome of 42 cycles of IVF/ICSI.

Demographic, anthropometric, hormonal characteristics did not differ significantly between the two groups. On the other hand, we observed that the duration of infertility status was significant different among patients with higher r-hFSH consumption versus normal responders (4.15 ± 1.2 years versus 3.2 ± 0.9, $p = 0.0055$) (Table 1).

Indications for assisted reproduction were comparable in the two groups (Table 1). The outcome of ART cycles in the two groups is illustrated in Table 2. The mean number of r-hFSH vials (36.3 ± 7.5 versus 28.6 ± 4.5, $p = 0.0001$) and number of days of stimulation (12.7 ± 2.4 days versus 10.8 ± 2.8, $p = 0.03$) were significantly lower in group B. The number of oocytes retrieved was significantly lower in group A compared with group B (7.1 ± 1.5 versus 9.6 ± 2.4; $p = 0.0003$). The average number of embryos transferred was significantly higher in group B than in group A (2.7 ± 0.4 versus 2.1 ± 0.7; $p = 0.001$). There were no statistically significant differences regarding cumulative pregnancy rates, abortion rates and rates of ongoing pregnancy. Serum levels of estradiol, measured on the day of hCG administration, were significantly lower in group A than in group B (997.8 ± 384.9 pg/ml versus 1749.1 ± 644.4; $p = 0.0001$).

In group A, the Ser/Ser genotype was identified in 10 patients (58.8%), the Asn/Ser genotype in 4 patients (23.5%) and the Asn/Asn genotype in 3 patients (17.6%) (Table 1). In group B the Ser/Ser genotype occured in 5 women (20%), Asn/Ser genotype in 15 women (60%) and Asn/Asn genotype in 5 patients (20%) (Table

1). The incidence of Ser/Ser genotype was higher in patients who showed higher r-hFSH consumption (Group A) compared with control group (Group B) ($p = 0.02$). On the contrary, the Asn/Ser genotype was more frequent in the control group ($p = 0.04$)

Discussion

This study confirms that the r-hFSH genotype may interfere with physiological responsiveness of the target organ to FSH stimulation.

The presence of the FSH-R Ser680 variant seems to result in a significant decrease in ovarian response to r-hFSH during ART cycles and, therefore, is reflected in a significant increase of drug consumption.

More specifically, among patients requiring a higher cumulative dose of r-hFSH (Group A), the expression of Ser/Ser genotype was significantly higher compared to the subgroups carrying variants Asn/Ser or Asn/Asn of FSH-R (Table 1).

An interesting evidence emerged from our results is that among patients with higher r-hFSH consumption and among the FSH-R Ser680 variant carriers the duration of infertility condition was higher (Table 1). The increased resistance to endogenous FSH, observed in FSH-R Ser680 carriers (Greb *et al.*, 2005) may influence female fertility, delaying pregnancy occurrence. As a matter of fact even in our study, a higher basal levels of FSH was detected in hypo-responder group (Table 2); nonetheless this hypothesis need to be confirmed by larger population trials.

The frequency of FSH-R polymorphism in our study population differed from that reported by other Authors. We compared our results with those published by Perez-Mayorga *et all.* in 2000 considering the ethnicity (Caucasian) and the number of patients involved ($n = 161$). As shown in Fig. 3, the prevalence observed were: 26% for Ser/Ser genotype, 45% for heterozygous Asn/Ser and 29% for homozygous Asn/Asn, whereas prevalence in our study population were respectively 36%, 45% and 19%.

The difference observed in the frequency of FSH-R polymorphism could reflect a particular pattern of distribution in Campania region. Alternatively, it could be due to a main difference in the two study designs: more specifically, our recruitment was not random but we chose patients from a selected pool of women affected by reproductive problems. Moreover, 17 of our 42 patients showed "resistance" to the protocols of ovarian stimulation, and 10/17 were homozygous carries of the Ser680 variant. In other words, the high frequency of Ser/Ser variant found in group A may have significantly affected the distribution of allelic

frequency in the whole study population, which in turn would account for the discrepancy between our patients and data reported by Perez-Mayorga.

Our results highlight the existence a subgroup of patients (hypo-responders) who require a higher cumulative dose of r-hFSH to obtain a reproductive outcome in comparison with normal responders. As a matter of fact, in all our patients at least ≥ 3 oocytes had been retrieved with peak estradiol levels > 500 pg/ml; accordingly, on the basis of the new criteria, they were classified as "normal responders". However, when the two groups were divided on the basis of the cumulative dose of r-hFSH, the average number of oocytes retrieved, the serum estradiol peak and the number of embryo replaced, were statistically higher in the group receiving the lowest cumulative dose of gonadotrophin. This observation suggest an "intermediate" prognostic category of patients which, although not fitting the criteria of "poor responders", still have a marked resistance to ovarian stimulation and a less favorable prognosis compared to "normal responders".

The association between FSH-R Ser680 variant and ovarian resistance to exogenous gonadotrophins, observed in our study, is in agreement with a recent meta-analysis that showed a higher consumption of exogenous gonadotrophins in homozygous carriers of Ser680 (Ser/Ser) genotype compared to Asn/Asn variant carriers (Yao *et al.* ,2011). The main difference between our study and other publications on this topic (Perez-Mayorga *et al.*, 2000; De Castro *et al.,* 2003; Behre *et al.*, 2005; Loutradis *et al.*, 2006), lies in the study design and in the parameters evaluated.

In fact, we stratified the study population on the basis of gonadotrophins consumption rather than on the FSH-R genotype expression, in order to figure out the mechanism underlying the hypo-response phenomenon. Thus, we were able to identify a significant difference in the frequency of Ser/Ser genotype between hypo-responder patients and control group (58.8% versus 20.0%).

The decision to use a cumulative r-hFSH dose of 2500 IU as a cut-off to define the two profiles of response is based on our clinical experience, in which it has been observed that young normogonadotrophic women with BMI <27 kg/m^2 achieve an adequate ovarian response with cumulative doses of FSH not exceeding 2000-2225 IU.

Although none of patients enrolled showed a poor responder profile, a decreased number of oocytes retrieved (Figure 1) and embryos transferred have been noted in the hypo responder group where the incidence of FSH-R Ser680 variant was higher (58.8% of cases) (Table 1). A possible effect on ovarian stimulation outcome was argued by De Castro *et al* in 2003 (De Castro *et al .,* 2003). Specifically, they reported a higher

incidence of Ser/Ser carriers among poor responder patients in which the number of oocytes retrieved were less than three.

Interestingly, we found that the homozygous Ser680 variant seems to affect serum estradiol (Fig. 2): during stimulation, estradiol levels were lower in Ser680variant carriers compared to Asn/Asn ones. This observation is in line with another report showing lower serum estradiol in Ser/Ser carriers at the time of hCG administration (Jun *et al*., 2006). Despite of findings reported by Behre *et al.*, no melioration of estradiol levels were found if an increased amount of r-hFSH was administrated (Behre *et al*., 2006).

As mentioned before, another group of hypo-responder patients has been identified among common LH variant carriers. This polymorphism is quite common with a prevalence estimated around 42% in some countries of northern Europe, characterized by reduced bioactivity *in vivo* and low response to r-hFSH. Therapeutic behavior of both v-beta LH and FSH-R polymorphism carriers seems to be comparable in terms of cumulative r-hFSH dosage and number of oocyte retrieved.

Although the small sample size of our study, it appears that 58% (10/17) of patients with a hypo-response profile during CO, carried FSH-R homozygous Ser680 genotype.

Thus, we can admit an association between the investigated FSH-R polymorphisms and the risk of ovarian resistance to exogenous FSH.

The fact that the FSH-R genotype affects the ovarian response to FSH has implications for the choice of stimulation protocol. It is conceivable that a "tailored" FSH therapy can be opted on the basis of patient genetic profile, giving the chance to customize not only the dosage but also the timing of stimulation. Other possible benefits could be the reduction of the stimulation duration and of the amount of FSH needed. Moreover, the know-how of mechanisms regulating the ovarian sensitivity to FSH could be useful in the prevention of ovarian hyperstimulation syndrome.

The immediate implications would be a saving in costs and an increase in treatment acceptance.

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Table 1 Characteristics of group A and group B patients and indications for *in vitro* fertilization.

Characteristics	Group A Hypo-responders (*n* = 17)	Gruppo B Controls (*n* = 25)	P value
Age (years)	31.82 ± 4.08	29.32 ± 4.67	NS
BMI (kg/m^2)	25.0 ± 3.4	23.6 ± 3.2	NS
Years of infertility	4.15 ± 1.2	3.2 ± 0.9	0.0055
Baseline LH (IU/l)	4.2 ± 1.2	4.1 ± 1.8	NS
Baseline estradiol (pg/ml)	48.75 ± 16.9	43.11 ± 19.4	NS
Indications for IVF			
Tubal factor (%)	5 (29.4%)	5 (20%)	NS
Male factor (%)	7 (41.2%)	8 (32%)	NS
Combined (%)	3 (17.6%)	5 (20%)	NS
Other (%)	2 (11.8%)	7 (28%)	NS
Distribution of the FSH-R genotypes			
Ser/Ser (%)	10 (58.8)	5 (20)	0.02
Asn/Ser (%)	4 (23.5)	15 (60)	0.04
Asn/Asn (%)	3 (17.6)	5 (20)	NS

Data are showed as means ± standard deviation or

NS: not significant; BMI: body mass index; LH Luteinizing hormone; IVF: *in vitro* fertilization

Table 2 Outcome of cycles of assisted reproduction in groups A and B.

	Group A Hypo-responders (*n* = 17)	Group B Controls (*n* = 25)	*P value*
Baseline FSH	6.9 ± 1.9	5.6 ± 1.9	0.02

Baseline LH	4.2 ± 1.2	4.1 ± 1.8	NS
Duration of stimulation (days)	12.7 ± 2.4	10.8 ± 2.8	0.03
N. of r-hFSH vials	36.3 ± 7.5	28.6 ± 4.5	0.0001
Estradiol on hCG day	997.8 ± 384.9	1749.1 ± 644.4	0.0001
N. oocytes retrieved	7.1 ± 1.5	9.6 ± 2.4	0.0003
N. embryo transferred	2.1 ± 0.7	2.7 ± 0.4	0.001
Implantation rate (%)	11.1	16.2	NS
Pregnancy rate (%)	17.6	36.0	NS
Rate of ongoing pregnancy rate (%)	11.7	32.0	NS

Data are showed as mean ± standard deviation

NS, not significant; FSH: follicle stimulating hormone; hCG: human chorionic gonadotrophin; LH Luteinizing hormone; r-FSH: recombinant FSH;

Figure 1 Mean number of oocytes retrieved in hypo-responders (Group A) and in controls (Group B). *p* = 0.0003.
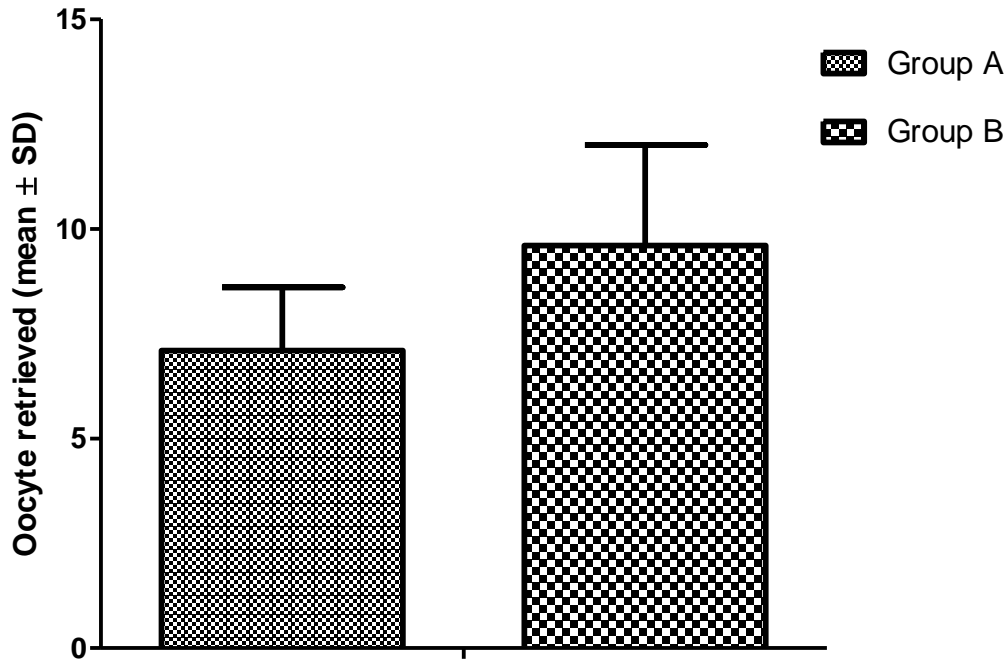

Figure 2 Serum estradiol levels on the day of hCG administration in hypo-responders (Group A) and in normal responders (Group B). *p* = 0.0001.
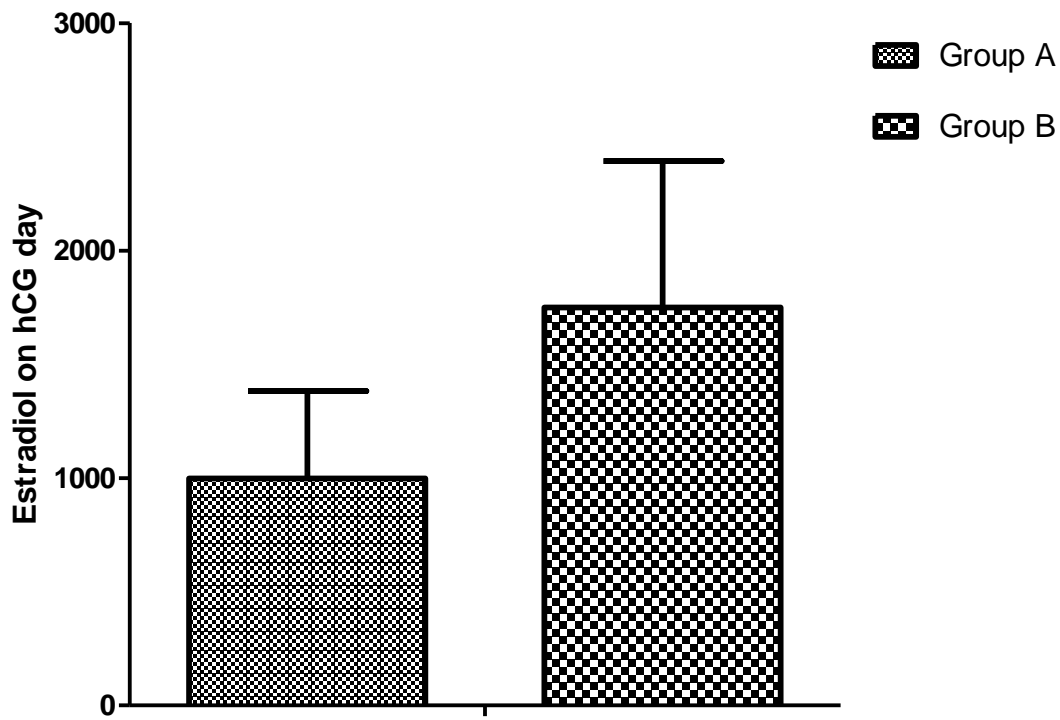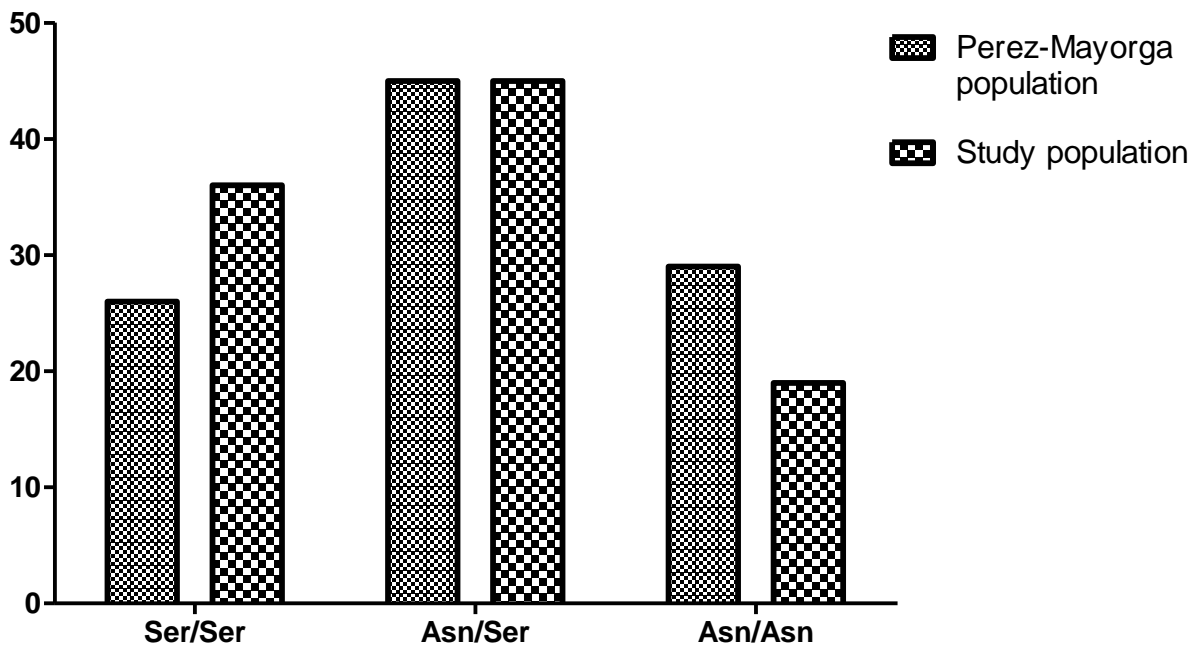
Figure 3 Prevalence of FSHR polymorphisms: comparison between population study and data reported by Perez-Mayorga.

2B) Evaluation of ovarian polymorphisms' effect on ovarian response to the recombinant gonadotrophins treatment according the clinical practice in normogonadotrophic women undergone to a gn-rh long down-regulation protocol.

Evaluation of the effects of the polymorphism of gonadotropins and of their receptors on ovarian response to recombinant gonadotropin treatment according to clinical practice in normo-gonadotropic women undergoing IVF / ICSI, in GnRH-a long down-regulation protocol

Aims	The aim of this interventional non pharmacological study is to assess the effects of polymorphisms, evaluated by sequencing the entire genes of FSH, FSH-R, LH and LH-R, on ovary response to recombinant gonadotropin treatment according to clinical practice in normo-gonadotropic women undergoing IVF/ICSI cycles. These patients are suppressed with GnRH-a long down-regulation protocol.
Pstients	A total of 150 IVF/ICSI cycles from three IVF Units will be evaluated. Inclusion criteria: 1. Caucasian 2.Eumenorrhea-normogonadotrophism 3.Age ≥20 and <35 years 4.Body Mass Index (BMI) ≥20 and ≤27 kg/m2 5.Basal FSH ≤10 IU/1 6.Indication for ART treatment 7.GnRH-a long protocol 8.All other criteria according to SmPC 9.Patient non presenting PCOS 10.Patient non presenting endometriosis
Endpoints	Key primary endpoint(s): 1.Efficacy endpoint will be the cumulative gonadotrophins dose/mature oocyte retrieved (MII oocytes). 2. Key secondary endpoint(s): 3.Estradiol levels on the days 1, 5, 8 of stimulation and on the day of hCG 4.Mean number of oocytes retrieved 5.Percentage of mature oocytes; fertilisation rate; number of embryos transferred 6.Implantation rate 7.Pregnancy rate per cycle 8.Pregnancy rate per transfer 9.Clinical Pregnancy rate for started cycle (presence of embryo with heartbeat) 10.Clinical Pregnancy rate per transfer (presence of embryo with heartbeat)

Evaluation of the effects of the polymorphism of gonadotropins and of their receptors on ovarian response to recombinant gonadotropin treatment according to clinical practice in normo-gonadotropic women undergoing IVF / ICSI, in GnRH-a long down-regulation protocol

Duration of the treatment	One IVF/ICSI cycle for each patient
Type of Study	Interventional non pharmacological study
Study Design	150patients who meet the criteria normoresponsiveness will be included in the evaluation and subjected to suppression with a GnRH -a in the normal clinical practice (buserelin acetate 0.5 mg sc per day luteal phase from the average for 12-14 days , after which the dose will be reduced to 0.2 mg) . Samples of blood and serum will be collected for each patient as required by clinical practice and an aliquot will be sent to laboratories that perform tests for the analysis of polimorfirmo and their receptors and for "immunofluorometric assay" (IFMA). The patients included in the observation will be women normoresponsive which will be submitted to the following type of stimulation : 150 IU daily dose of r- hFSH , the dose of gonadotropin will be reduced according to clinical practice in women who show serum E2 > 180 pg / ml on day 5 of stimulation. the dose of r- FSH will be doubled compared to the initial dose based on ovarian response measured on day 8 of stimulation (at least 6 follicles between 6-10 mm , but no follicle > 10 mm). to patients with at least 3 follicles > 17 mm, hCG is administered . For which patients will require a reduction of the daily dose of gonadotropins on day 5 of stimulation and which will require a further increase on day 8 of stimulation will be excluded from the evaluation. Similarly, women are excluded from the assessment that will require a temporary suspension of administration of gonadotropins (coasting) to reduce the risk of OHSS . All primary and secondary endpoints will be evaluated and the patients will be stratified according to the polymorphisms identified

Background

Controlled ovarian stimulation (COS) for in vitro fertilisation (IVF) is usually performed in a low endogenous LH environment due to pituitary desensitisation with a GnRH analogue (a). The standard GnRH-a long protocol associated with exogenous FSH stimulation still represents the most common regimen for COS in normogonadotrophic women. In daily clinical practice, the ovarian response to this protocol is optimal in about 85% of patients, with more than 6 mature oocytes recruited. In about 12-15% of cases, however, an initial low response is seen, leading to an increase in the daily dose of FSH, resulting in a higher total FSH consumption (e.g. >2500 IU). These observations lead to the development of the concept of "hypo-response" to COS to identify normogonadotrophic women who have normal estimated ovarian reserves but require high amounts of FSH to obtain an adequate number of oocytes retrieved (De Placido et al., 2001, 2004, 2005; Ferraretti et al., 2004; Mochtar et al., 2007; Devroey et al., 2008). These women seem to be distinct from classical poor responders because they have normal ovarian reserve, but unexpectedly show sub-optimal response when stimulated with standard regimens. Conversely, specific adjustments of classical protocols seem to optimise ovarian response.

On the basis of the current literature, it is possible to argue that hypo-response seen could be related to genetic characteristics. More specifically, two lines of evidence indicate that this clinical condition may be related to polymorphisms of the genes of LH, FSH and their receptors.

2.1 LH and hypo-response to FSH

The hypothesis that inadequate LH activity is responsible for hypo-response to FSH is related to both basic and clinical data. More specifically, it has been found that LH receptors are also detectable on the granulosa compartment at the intermediate follicular phase (Erickson et al., 1979; Shima et al., 1987; Hillier et al., 1994; Filicori et al., 2003). Therefore, it appears that LH regulates both granulosa and theca cells.

FSH and LH cooperate in inducing the local production of the soluble molecule inhibin B, and growth factors. Among these, insulin growth factors (IGF)-I and II, which are expressed by both granulosa and theca cells throughout folliculogenesis, are important in promoting follicular maturation (Zhou et al., 1993; Huang et al. 1994). Locally produced peptides, rather than estrogens, are known to be the key factor regulating primate

follicle growth and development (Rabinovici et al., 1989; Pellicer et al., 1991; Zelinski-Wooten et al., 1993, 1994; Shetty et al., 1997; Park et al., 2004; Panigone et al., 2008). In light of these finding, we can conclude that 1) both gonadotrophins contribute (via granulosa) to maintain the autocrine-paracrine system governing dominant/s follicle/s growth and 2) LH is crucial in sustaining FSH activity in the granulosa during intermediate-late stages of folliculogenesis. On this basis it is possible to argue that lack of each gonadotrophin can be counteracted by higher levels of the other. This hypothesis is partially confirmed by the observation that FSH activity can be totally substituted by LH once granulosa cells express adequate amounts of LH receptors (Zeleznik and Hillier, 1984; Filicori et al., 2003). Interestingly, observations from animal models indicate that FSH can compensate low LH levels but not absence of LH receptors. More specifically, FSH alone can induce ovulation in hypophysectomised rodents (Tapanainen et al., 1993), but not in LHR knockout mice (Pakarainen et al., 2005). Data from clinical-IVF practice seem to confirm that, higher exogenous FSH doses during COS seem to be able to compensate GnRH-a related reduction of LH.

Taken together, these lines of evidence support the concept that fall of LH concentration (and/or activity) below a hypothetical threshold may lead to impairment of granulosa paracrine activities, which in turn can determine the requirement of higher FSH doses.

Several authors have investigated the role of exogenous LH in women with sub-optimal ovarian response to FSH. In 2001, Lisi et al. conducted a "self-control study" of the effect of recombinant LH (r-hLH) supplementation in 12 patients who, during a previous stimulation with r-hFSH , required >3000 IU to reach follicular maturity. Patients had a mean basal FSH of 12.2 IU/l and a mean age of 36.1 years. Re-stimulation entailed the addition of 75 IU of r-hLH from day S7 to the standard r-hFSH regimen. As in the first cycle, all women received triptorelin 0.1 mg daily, from the mid-luteal phase (GnRH-a long protocol). There was no difference in the total consumption of r-hFSH, days of stimulation or number of M2 oocytes per patient between the two cycles of the study. However, fertilization (86.0 versus 60.9%) and clinical pregnancy rates (50.0 versus 5.9%) were significantly higher (P <0.05) in r-hLH supplemented cycles. Despite the small sample size and the methodological limitations of un-randomised trials, this study provided the first clinical evidence that a sub-optimal response to r-hFSH may be significantly improved by LH supplementation.

The role of LH supplementation in hypo-responders was evaluated in at least three controlled randomised trials. De Placido et al. (2001) suggested to detect hypo-response during the initial phases of r-hFSH administration. The authors noticed that in about 10−12% of normogonadotrophic patients, an initial response (i.e., at least five 2−9 mm follicles in each ovary) during the first days of stimulation is followed by a plateau

in which there is no significant increase in follicular size or E2 production during the next 3−4 days of stimulation. This profile of initial ovarian response to r-hFSH usually leads physicians to increase the r-hFSH dose. On this basis, De Placido and colleagues (2001) conducted a RCT to determine whether this clinical condition could be reverted by LH administration. Women (age <37 years, basal FSH ≤10 IU/l) who had no follicle with a mean diameter >10 mm and E2 serum levels ≤180 pg/ml on day S8 were randomised to receive LH supplementation (n = 20) in the form of hMG (150 IU/day) or an increase in the r-hFSH daily dose (maximum daily dose of 375 IU; n = 23). In order not to modify the daily FSH administration, the r-hFSH dose was reduced to 150 IU in women of the hMG group. Forty women matched for age and body mass index (BMI) and with an initial adequate response to r-hFSH (i.e., a tripling of serum E2 concentration between days S5−8 in association with >4 follicles >10 mm on day S8) served as a not randomised control population. All women received triptorelin 3.75 mg (depot preparation) on the first day of spontaneous menstruation. After pituitary desensitisation, a starting dose of 300 IU of r-hFSH was administered. First dose adjustment was performed on day S5. The mean number of oocytes retrieved was significantly higher in women treated with hMG supplementation than in those who received r-hFSH 'step up'. Moreover, the ovarian outcome of the hMG group was comparable with that observed in 'normal responders', suggesting that LH supplementation was able to 'rescue' this apparently abnormal response to r-hFSH. Following these findings, De Placido et al. (2004; 2005) evaluated the efficacy of r-hLH supplementation in women displaying an initial ovarian resistance to r-hFSH. With this purpose, they performed a multicentre RCT (De Placido et al., 2005) with the r-hFSH 'step up protocol' as reference standard. A total of 229 IVF/ICSI cycles performed in seven Italian units were analysed. In all patients (age <37 years, basal FSH ≤10 IU/l), triptorelin 3.75 mg (depot preparation) was administered on the first day of spontaneous menstruation. The starting dose of r-hFSH was 225 IU/day. Hypo-responders were identified on day 8 (E2 serum levels <180 pg/ml and at least 6 follicles ranging between 6 and 10 mm, but no follicle with a mean diameter >10 mm) and randomised to receive either an r-hLH supplementation of 150 IU/day (n = 59), or an increase of 150 IU in the daily r-hFSH dose (n = 58; r-hFSH 'step-up' protocol). Also in this case, an age/BMI-matched population of 'normal responders' (tripling E2 levels between days S5 and S8, more than 4 follicles >10 mm on day S8) was selected as a control group (n = 112). The number of cumulus-oocyte complexes (primary end-point) and mature oocytes retrieved was significantly higher in women receiving r-hLH than in those treated with the r-hFSH step up protocol. Moreover, the mean number of mature oocytes of r-hLH group was similar to that observed in 'normal responders'. Although power analysis was not performed for these categorical variables, it is noteworthy that

also implantation and ongoing pregnancy rates were similar in 'hypo- responders' treated with r-hLH and 'normal responders' (14.2 and 32.5% versus 18.1 and 40.2%, respectively). Conversely, both parameters were significantly lower (P <0.05) in 'hypo-responders' treated with step-up r-hFSH (10.0 and 22.0%) than in 'normal responders'.

Moreover, Ferraretti et al. (2004) conducted a RCT on 184 patients (age <38 years) undergoing the GnRH-a long protocol. Patients with normal initial follicular recruitment (>10 antral follicles ≥8 mm in diameter and E2 ≥100 pg/ml) with the fixed starting dose of recombinant FSH (150-300 IU), but showing a plateau in follicular growth between days S7 and S10 (no increase in follicle size or in E2 level) were randomised as follows: group A (n = 54) received an increase in the daily dose of r-hFSH; group B (n = 54) received 75–150 IU of r-hLH in addition to the increased dose of FSH; group C (n = 26) received hMG; group D consisted of 54 age-matched patients with an optimal response (no need to increase the FSH dose). The mean number of oocytes retrieved was significantly lower in group A (8.2) versus the other groups (11.1, 10.9, 9.8 in groups B, C, and D, respectively). Furthermore, the pregnancy-per-embryo transfer and implantation rates were significantly higher in group B than in groups A and C, and did not differ from normal responders.

Taken together, these studies reinforced the idea that hypo-responders benefit from LH supplementation.

Interestingly, all RCTs (De Placido et al., 2001, 2005; Ferraretti et al., 2004), clearly showed that hypo-responders who benefited from LH activity had endogenous levels of LH in the normal range. In addition, endogenous LH concentrations of these patients during early phases of COS were always comparable with those observed in women who had optimal response to FSH and who did not require any change of FSH dose during stimulation. This observation led to the hypothesis that hypo-response to r-hFSH is associated with a less bioactive LH. We have recently performed an observational trial (Alviggi et al., 2009) aimed to evaluate whether the presence of a common polymorphism of LH (v-LH), which has been shown to have altered in vitro and in vivo activities, is associated with different profiles of ovarian response to r-hFSH. V-LH was initially discovered by Petterson and Söderholm (1991) as an immunologically anomalous form of LH. V-LH is due to two point mutations in the β subunit gene, both altering the aminoacid sequence (Trp8Arg and Ile15Thr). V-LH has elevated bioactivity in vitro but significantly shorter (5-9 min) half life in circulation when compared with the wild type LH (wt-LH) (12-22 min). As the pulse frequency of the v-LH is normal, this results in an overall LH action that is more potent at the receptor site, but shorter in duration in vivo (Huhtaniemi and Themmen, 2000).

The v-LH is common worldwide, with carrier frequency varying from 0 to 52% in various ethnic groups. Its incidence in Italy ranges between 12-13%. The v-LH differs functionally from wt-LH, and it seems to predispose its carrier, both men and women, to mild aberrations of reproductive function. In our observational trial, sixty normogonadotrophic patients undergoing a GnRH-a long down-regulation plus r-hFSH for IVF/ICSI, and in whom at least 5 oocytes were retrieved were divided into three groups: 22 women requiring a cumulative dose of r-hFSH >3500 IU constituted group A, 15 patients requiring 2000-3500 IU were included in the group B, 23 women requiring <2000 IU severed as control group (group C). The presence of the v-LH was evaluated using specific immunoassays. Group A (Table 1) showed a significantly lower (p <0.05) number of oocytes retrieved when compared with group B and C (7.3 ± 1.5, 11.7 ± 2.4 and 14.7 ± 4.1 in the three groups, respectively). Seven carriers (32%) of v-LH were found in group A whereas only one variant (7%) was observed in group B; no variant was detected in group C. This study suggested, for the first time, an association between a less bioactive LH and a higher FSH requirement.

2.2 FSH-R and FSH-beta promoter polymorphisms: possible explanations for hypo-response to exogenous FSH

A polymorphic variant of the FSH receptor (FSH-R) where aminoacid asparagine (Asn), at position 680, is replaced by Serine (Ser), has been associated with higher FSH basal levels and increased number of antral follicles during the early follicular phase (Greb et al., 2005). In an observational trial, Perez-Mayorga et al. (2000) evaluated the relationship between the presence of the Ser/680 FSH-R variant and the ovarian response to COS in 161 norm-ovulatory women undergoing IVF. All women were below 40 years. The distribution of genotypes in the study population was 29% for the Asn/Asn, 45% for the Asn/Ser, and 26% for the Ser/Ser FSH- R variant. E2 levels at the day of human chorionic gonadotrophin (hCG) and number of retrieved oocytes were similar in the three groups. Conversely, basal FSH levels were significantly different among the three groups (6.4 ± 0.4 IU/l, 7.9 ± 0.3 IU/l, and 8.3 ± 0.6 IU/l for the Asn/Asn, Asn/Ser, and Ser/Ser groups, respectively, P <0.01). In addition, the mean number of FSH ampoules required for successful stimulation was significantly different among groups (31.8 ± 2.4, 40.7 ± 2.3, and 46.8 ± 5.0 for the Asn/Asn, Asn/Ser, and Ser/Ser groups, respectively, P <0.05). These clinical findings demonstrated that the ovarian response to FSH stimulation depends on the FSH-R genotype. Following these observations, Behre et al. (2000) tested whether the same daily dose of FSH results in lower levels of oestradiol in women homozygous for the Ser/Ser, and

whether the difference can be overcome by higher FSH doses. Fifty-nine women undergoing COS for in IVF or ICSI and homozygous for the FSH-R polymorphism Ser/680, were randomly allocated in the Group I (n = 24), to receive a daily FSH dose of 150 IU/day, or group II (n = 25), to receive an FSH dose of 225 IU/day. In the group III (Asn/Asn, n = 44), the FSH dose was 150 U/day. Age and basal FSH levels were not different between groups. Total FSH doses were comparable in group I (1631 ± 96 IU) and group III (1640 ± 57 IU) but significantly higher in group II (2421 ± 112 IU) (P <0.001). Peak E2 levels were significantly lower in group I (5680 ± 675 pmol/l) compared to group III (8679 ± 804 pmol/l) (P <0.05). Increasing the FSH dose from 150 to 225 IU/day overcame the lower E2 response in women with Ser/Ser (group II, 7804 ± 983 pmol/l). The authors concluded that the Ser/Ser FSH-R results in lower E2 levels following FSH stimulation. This lower FSH receptor sensitivity can be overcome by higher FSH doses. The study represented the first case of pharmacogenomic approach to COS.

Recently, we have evaluated the occurrence of the Ser/680 FSH-R variant among women classified as 'hypo-responders' (Alviggi et al., unpublished data). Forty-two normogonadotrophic patients undergoing a GnRH-a long down-regulation protocol followed by stimulation with r-hFSH for IVF/ICSI, and in whom at least 5 oocytes were retrieved were retrospectively included. On the basis of the total r-hFSH consumption, patients were divided into two groups: 17 women requiring a cumulative dose of r-hFSH >2500 IU constituted group A, whereas 25 patients requiring <2500 IU severed as control group (B). DNA was analysed to determine the FSH receptor genotype. E2 peak levels were significantly lower in group A (997 ± 385 pg/ml) when compared with group B (1749 ± 644; P <0.001). Group A also had a significantly lower (P <0.001) number of oocytes retrieved (7.1 ± 1.5 versus 9.6 ± 2.4). Homozygous Ser/Ser receptor variant at codon 680 was observed in 47.0% of women of the group A and in 28.0% of women of the control group. The homozygous Asn/Asn receptor variant was found in 23.6% and 20.0% of patients in the two groups, respectively. Heterozygosis Ser/Asn was detected in 29.4% of patients of the group A and in 52.0% of patients of the group B. These preliminary results reinforced that the Ser 680/variant of the FSH-R is more frequent in women with hypo-response to r-hFSH.

A common polymorphism was recently detected in the promoter region of FSHbeta (Grigorova et al. 2008). It has phenotypic effects on male reproductive characteristics, but its possible effects in the female have not yet been investigated.

Normogonadotrophic women with higher LH levels after GnRH-a administration

Recent clinical data led to identify another subgroup of normogonadotrophic patients who display higher-endogenous LH levels following the administration of GnRH-a. In particular, Humaidan et al. (2004) performed a RCT aimed to evaluate the efficacy of r-hLH supplementation during COS in normogonadotrophic women undergoing GnRH-a long protocol plus r-hFSH for IVF/ICSI cycles. The authors clearly demonstrated that among patients stimulated with r-hFSH monotherapy, those having endogenous LH levels >1.99 IU/l on day 8 of stimulation showed significantly lower implantation rates. In these patients LH levels usually increase or plateau during FSH stimulation despite the use of the GnRH-a, instead of showing a gradual decrease in response to the rising estradiol level. Interestingly, r-hLH supplementation significantly increased the implantation rate which was comparable to that observed in patients with low LH levels treated with r-hFSH alone. This evidence led to hypothesise that a less functional LH receptor is involved in the pathogenesis of sub-optimal ovarian response to FSH.

In light of the above mentioned studies the following scenario could be proposed:

About 15% of normogonadotrophic patients undergoing GnRH-a long protocol in association with r-hFSH for IVF programs show ovarian resistance to stimulation: the number of oocytes retrieved is apparently adequate but the consumption of FSH is higher. These patients have been defined hypo responders and should be distinguished by classical "poor responders" (women with few follicles and low number of oocytes retrieved irrespective of the dose of FSH used).

These patients seem to benefit from exogenous LH despite presence of apparently normal endogenous LH levels.

Preliminary data seem to suggest that the frequency of v-beta LH and FSH-R ser/680 are increased in this subgroup.

Another group of normogonadotrophic patients who benefit from exogenous LH during GnRH-a long protocol is represented by women showing higher LH levels (i.e. >1.99 IU/l) after GnRH-a suppression. In these patients the presence of a less performing LH receptor could be hypothesised.

Although intuitively less likely to explain inadequate response to exogenous FSH, the recently detected common polymorphism in FSHbeta promoter region is worth testing in the context of COS.

3. Objectives and study design

Assessment of the effects of polymorphisms of gonadotropins and their receptors, with sequencing of entire genes on ovarian response to treatment with recombinant gonadotropins according to clinical practice in normo-gonadotropic women undergoing IVF / ICSI,

following in a GnRH-a long protocol.

4. Endpoints

4.1 Key primary endpoint(s)

Efficacy endpoint will be the cumulative gonadotrophins dose/mature oocyte retrieved (MII oocytes).

4.2 Key secondary endpoint(s):

Estradiol levels on the days 1, 5, 8 of stimulation and on the day of hCG

Mean number of oocytes retrieved

Percentage of mature oocytes; fertilisation rate; number of embryos transferred

Implantation rate

Pregnancy rate per cycle

Pregnancy rate per transfer

Clinical Pregnancy rate for started cycle (presence of embryo with heartbeat)

Clinical Pregnancy rate per transfer (presence of embryo with heartbeat)

4.3 Safety Endpoint(s)

The safety endpoint according to literature and drug products indication is:

Incidence and severity of any adverse event

Incidence and severity of ovarian hyperstimulation syndrome (OHSS).

Laboratory parameters.

5. Materials and methods

5.1 Patients

A total of 150 IVF/ICSI cycles from three IVF Units will be observed.

5.2 Key inclusion criteria:

Caucasian

Eumenorrhea-normogonadotrophism

Age ≥20 and <35 years

Body Mass Index (BMI) ≥20 and ≤27 kg/m2

Basal FSH ≤10 IU/l

Indication for ART treatment

GnRH-a long protocol

All other criteria according to SmPC

Patient non presenting PCOS

Patient non presenting endometriosis

5.3 Key exclusion criteria:

Any anomalies of the uterine cavity

Age <20 and >35 years

Body Mass Index (BMI) <20 and >27 kg/m2

Endocrine, genetic, and systemic inflammatory-immunological disorders

Polycystic ovarian syndrome (Rotterdam criteria, 2004)

Endometriosis

Presence of one ovary

History of more than two previous IVF/ICSI cycles with normal ovarian response

History of previous stimulation cycle which had been cancelled for insufficient ovarian response or in which <4 oocytes had been retrieved

Women who will have their daily dose of gonadotrophins reduced on the fifth day of stimulation and who will require a new increase on day 8 will be treated according to single centre protocols and excluded from the study

Women who will require "coasting" for reducing the risk for OHSS will be treated according to single centre protocols and excluded from the study

5.4 Stimulation protocol

The stimulation protocol followed will be the standard clicical practice for this patient population. All patients will undergo a GnRH-a long down-regulation protocol with buserelin acetate (Suprefact) as follows: 0.5 mg s.c. daily from the mid-luteal phase for 12-14 days, after which the dose is reduced to 0.2 mg. After 14 days, patients will undergo transvaginal-ultrasonographic (TV-USG) evaluation and biochemical evaluations: subjects with serum E2 level ≤40 pg/ml, endometrial thickness ≤5 mm, and arrested follicular development will commence gonadotrophin administration. Women with delayed suppression (including subjects who develop ovarian cysts after the GnRH-a administration) will be excluded from the obserbvational study. All subjects observed will be whom that will receive a starting-daily dose of 150 of r-hFSH (Gonal-F®; Merck Serono s.p.A, Rome, Italy). The starting gonadotrophin dose will be maintained for four days. E2 serum levels will be measured on day five of stimulation. On that day, the daily dose of gonadotrophin will be modified only in women having E2 concentration >180 pg/ml. Should it be the case, according standard clinical practice, a daily dose of r-hFSH of 112.5 IU will be necessary, it will be adopted. Follicular growth will be evaluated by on day 8 of stimulation by TV-USG. Patients who will display at least 6 follicles ranging between 6 and 10 mm, but no follicle with a mean diameter >10 mm will receive an increase in the daily gonadotrophin dose. For these subjects the dose of FSH will be increased by 150 IU per day of r-hFSH , giving a cumulative daily dose of 300 IU. Women who will have their daily dose of gonadotrophin reduced on the fifth day of stimulation and who will require a new increase on day 8 will be excluded from the observation. Analogously, women who will require "coasting" for reducing the risk for OHSS will be also excluded from the observation. E2 serum levels will be measured on days 1, 5, 8 of stimulation and on the day of human chorionic gonadotrophin (hCG) administration. All the other determinations, including hormone measurements and polymorphism evaluation are described in Table 1. The ovulatory dose 10,000 IU of hCG or 250 mcg of r-hCG will be administered in the presence of three follicles with a mean diameter of at least 17 mm according to clinical practice. Oocytes will be retrieved by transvaginal ultrasound-guided aspiration 34-36 h after the hCG injection. Serum concentrations of LH are measured on the day of pituitary suppression assessment and on the eighth day of stimulation.

5.5 Sampling and polymorphisms analyses

Blood samples will be collected according to clinical practice, for evaluating the presence of different polymorphisms. The venous blood (10 ml) will be allowed to clot and centrifuged at 400 g for 10 min. Serum will be separated, divided into a maximum of four aliquots and frozen. Pellets will be also divided in four aliquots and stocked at -80°C to be successively evaluated. In Table 1 the timing for sampling, sites and methods of analyses are described.

Table 1. Overall study schedule, including timing of sampling, sites and methods of analyses

TIMING OF EVENT*	SAMPLE	SITE OF ANALYSIS	ANALYSES
Day 1 of r-hFSH administration	blood	Department of Reproductive Biology, Hammersmith Campus, Imperial College London, London, United Kingdom (Ilpo Huhtaniemi)	FSH-R polymorphisms (PCR) LH-R polymorphism (PCR) FSH polymorphism (PCR) LH polymorphism (PCR, and IFMA)
Day 1 of r-hFSH administration	serum	Department of Biotechnology, University of Turku, 20520, Turku, Finland (Kim Pettersson)	LH concentration (IFMA) V-LH polymorphism (IFMA) Estradiol concentration (IFMA) Delta-4-Androstenedione (IFMA)
Day 5 of r-hFSH administration	serum	Department of Biotechnology, University of Turku, 20520, Turku, Finland (Kim Pettersson)	LH concentration (IFMA) Estradiol concentration (IFMA) Delta-4-Androstenedione (IFMA)
Day 8 of r-hFSH administration	serum	Department of Biotechnology, University of Turku, 20520, Turku, Finland (Kim Pettersson)	LH concentration (IFMA)
Day of hCG	serum	Department of Biotechnology, University of Turku, 20520, Turku, Finland (Kim	LH concentration (IFMA) Estradiol concentration (IFMA) Delta-4-Androstenedione

		Pettersson)	(IFMA)
			Progesterone (IFMA)

5.5.1 V-LH measurements

The LH concentrations and the presence of the v-LH in all serum samples collected will be determined by two different IFMA as previously reported (Pettersson and Söderholm, 1990, 1991). In brief, total LH concentration will be determined using LHspec (Wallac Oy, Turku, Finland) as this assay also recognizes v-LH (Pettersson et al., 1992). The other assay, I3/A2, recognizes only the wt form of LH (Pettersson and Söderholm, 1991). A ratio of the two assays (wt-LHI3/A2 /total LHLHspec) will be used to determine the LH status of these individuals. Previous studies have shown that heterozygotes for v-LH have a wt/total LH ratio between 0.2 and 0.9 as the I3/A2 assay recognizes only about half of the total immunoreactive LH in heterozygote serum. The ratio is >0.9 for wt-LH homozygotes and <0.2 for homozygous variants (Nilsson et al., 1997).

5.5.2 Polymorphisms in FSHbeta, LHR and FSHR

The PCR-based Custom TacMan® DNP Genotyping Assay (Applied Biosystems) will be used to genotype the SNP's in FSHbeta (rs10835638), FSHR (rs6165/6166) and LHR (rs4539842).

6   Schedule of Assessments

The patients observed have to be tested, according standard clinical practice, for screening/baseline level of:

Date of birth,

Height,

Weight,

Medical history and concomitant diseases,

Prior and concomitant therapies (including changes during the trial)

Tubal Factor

Idiopathic sterility

Basal LH,

Basal FSH,

E2

Endometriosis

BMI

Male factors

Smoking

AMH

The patients observed in the study as per inclusion criteria are coming from previous failed or cancelled cycles. The investigator has to record for each patient:

N° of previous cycles

N° of oocytes retrieved in each previous failed cycle

Total and daily IU FSH used in each previous failed cycle.

According standard clinical practice, monitoring starts from day 8 (day 1 = the first day of stimulation), and consists of:

transvaginal ultrasound scan to evaluate the number and maximum diameter of ovarian follicles and the thickness of the endometrial rima;

rapid dosing of plasmatic 17β-extradiol.

total number of follicles on administration of hCG;

blood estradiol levels on the day hCG is administered;

number of gonadotrophin units used in the present cycle;

number of days stimulation;

Retrieving of the oocytes will take place transvaginally under ultrasound guidance approximately 36 hours after administration of hCG:

N° of oocyte retrieved from each stimulation cycle

number of mature oocytes (for ICSI cycles number of metaphase II oocytes);

oocyte morphology

number of fertilised oocytes;

total no. embryos

number of good quality and transferred embryos;

Intrauterine transfer of embryos: on the second day after retrieving of the oocytes.

Support of the luteal phase will be provided using Crinone 8 vaginal gel, 1 application a day from day +1 after harvesting of the oocytes.

Approximately 14 days after transfer the plasma ßhCG will be dosed and all the patients found to be pregnant will be subjected to pelvic ultrasound scan 34-36 days after administration of hCG.

The following parameters will be registered:

bio-chemical pregnancy

clinical pregnancy

implantation rates;

abortion rate

cycle cancellation: low stimulation, poor response, no oocytes retrieved, fertilization failure, no developed embryos.

Date of subject's end of trial

6.1   Assessment of Safety

The safety profile during standard clinical practice is assessed through the recording, reporting and analyzing of baseline medical conditions, adverse events, physical examination findings including vital signs and laboratory tests.

Comprehensive assessment of any apparent toxicity experienced by the subject will be performed throughout the course of the standard treatment. The site personnel will report any serious adverse event (SAE), whether observed by the Investigator or reported by the subject using the attached form (see section 8.3.3) A copy of this form should be sent as soon as possible to Merck Serono Global Drug Safety at the fax number + 41 22 414 30 95

6.1.2   Safety Reporting to Regulatory Authorities, Investigators and Ethics Committees/ Institutional Review Boards

The Sponsor will send appropriate safety notifications to regulatory authorities in accordance with applicable laws and regulations (European Directive 2001/20/EC, legislative decree 211/2003 and D.M. 17 december 2004).

The Investigator must comply with any applicable site-specific requirements related to the reporting of SAEs (and in particular deaths) involving his/her subjects to the Ethics Committee/Institutional Review Board (EC/IRB) that approved the trial.

In accordance with ICH GCP guidelines, Merck Serono will inform the Sponsor/Investigator of "findings that could adversely affect the safety of subjects, impact the conduct of the trial or alter the EC's/IRB's approval/favorable opinion to continue the trial."

The Sponsor will provide appropriate Safety reports directly to the concerned lead IEC/IRB and will maintain records of these notifications.

7   Statistics

7.1   Sample Size

As this is an interventional non-pharmacological study, no formal sample size calculation has been carried out. It is felt that 150 subjects will allow adequate observation of both the parameters and the the identified biomarkers on the population.

7.2   Description of Statistical Analyses

Description of the primary efficacy analysis and population:

It is expected that the patients showing the listed polymorphisms (one or more in association) will consume significantly higher amount of gonadotrophins (r-hFSH)/mature oocyte when treated with r-hFSH alone compared to patients not showing polymorphisms.

The results will be reported by descriptive statistics, such as, Number of observations, the mean ± SD, median and minimum/maximum. Data will be analysed with the SPSS version 12.0 (SPSS Inc., USA). Appropriate tests will be carried out to investigate the normality of the distribution of any continuous data. The effects of the polymorphisms will be analysed by means of analysis of variance (ANOVA) in a four- within 0-between design, having polymorphisms as the main factors and cumulative-gonadotrophins dose/mature oocyte retrieved as dependent variable. The "interaction" among main factors will be also estimated. If the data are found to be non-normally distributed, non-parametric statistics will be applied to analyse the data. ANOVA or the relevant non-parametric test will be utilised as required to compare baseline characteristics, hormonal

characteristics and all secondary endpoints within and between arms. Chi-squared ($X2$) statistics will be used to compare categorical data.

Post-hoc Fisher least-significant-difference (PLSD) to look for differences between each polymorphism and wt/wt genotype in each arm will also be carried out where felt appropriate.

All results will be tested at a p value of <0.05 for investigating statistical significance.

8   Subject Information and Informed Consent

An unconditional prerequisite for a subject's participation in the interventional non-pharmacological study is his/her written informed consent. The subject's written informed consent to participate in the observation must be given before any trial-related activities are carried out according to the dedicated form.

Adequate information must therefore be given to the subject by the Investigator before informed consent is obtained (a person designated by the Investigator may give the information, if permitted by local regulations). A subject information sheet in the local language and prepared in accordance with the Note for Guidance on Good Clinical Practice (ICH, Topic E6, 1996) will be provided by the Sponsor for the purpose of obtaining informed consent. In addition to providing this written information to a potential subject, the Investigator or his/her designate will inform the subject verbally of all pertinent aspects of the observation. The language used in doing so must be chosen so that the information can be fully and readily understood by laypersons. Depending on national regulations, a person other than the Investigator may inform the subject and sign the Informed Consent form.

The Informed Consent Form must be signed and personally dated by the subject and the Investigator. The signed and dated declaration of informed consent will remain at the Investigator's site, and must be safely archived by the Investigator so that the forms can be retrieved at any time for monitoring, auditing and inspection purposes. A copy of the signed and dated information and consent form should be provided to the subject prior to participation.

Whenever important new information becomes available that may be relevant to the subject's consent, the written subject information sheet and any other written information provided to subjects will be revised by the Sponsor and be submitted again to the IEC/IRB for review and favorable opinion. The agreed, revised information will be forwarded to each subject in the observation. The Investigator will explain the changes to the previous version.

9   Subject Identification and Privacy

A unique subject number will be assigned to each subject at inclusion in the interventional non-pharmacological study, immediately after informed consent has been obtained. This number will serve as the subject's identifier in the study as well as in the database.

The subject's data collected in the observation will be stored under this number. Only the Investigator will be able to link the subject's trial data to the subject via an identification list kept at the site. The subject's original medical data that are reviewed at the site during source data verification by the monitor, audits and authority inspections will be kept strictly confidential.

Data protection and privacy regulations will be observed in capturing, forwarding, processing, and storing subject data. Subjects will be informed accordingly, and will be requested to give their consent on data handling procedures in accordance with national regulations.

10.2   Investigator Site File and Archiving

The Investigator will be provided with an Investigator Site File upon initiation of the trial. This file will contain all documents necessary for the conduct of the interventional non-pharmacological study and will be updated and completed throughout the trial. It must be available for review by the Auditor. The documents to be thus archived include the Subject Identification List and the signed subject Informed Consent Forms.

All original subject files (medical records) must be stored at the site (hospital, research institute, or practice) for the longest possible time permitted by the applicable regulations.

10.3   Changes to the Protocol

Changes to the protocol will be documented in written Protocol Amendments. Major (substantial, significant) amendments will usually require submission to ethic committee.

Minor (nonsubstantial) protocol amendments, including administrative changes, will be filed by the Sponsor at the site.

Any amendment that could have an impact on the subject's agreement to participate in the trial requires the subject's informed consent prior to implementation.

Appendix 1

Study Flow Chart

	Screening	Pituitary suppression	Treatment phase	Treatment phase	Treatment phase	Trigger	OPU	ET	Pregnancy assessment	
		D 14 after GnRH-a	D Stim. 5	D Stim. 8	D Stim. X	hCG day	34-36 hours post hCG	Day 3 post OPU	12-18 day post - ET	12th week
Informed consent	X									
Demographic data	X									
Medical history	X									
Physical examination	X									
Gynecological/obstetrical history	X									
Gynecological examination	X									
Diagnostic transvaginal ultrasound	X	X	X	X	X	X	X	X		X
Haematology - Biochemistry	X									
Urinalysis	X									
Sperm analysis	X									
Hormone analysis:										
FSH	X									
PRL	X									
LH	X									
E2	X	X	X	X	X	X				
Pregnancy test (hCG)	X								X	
Treatments:										
Study treatment		X	X							
Adverse Events		X	X	X	X	X	X	X	X	X

FSH-R polymorphisms (PCR) LH-R polymorphism (PCR) FSH polymorphism (PCR) LH polymorphism (PCR, and IFMA)
LH concentration (IFMA) V-LH polymorphism (IFMA) Estradiol concentration (IFMA) Delta-4-Androstenedione (IFMA)
LH concentration (IFMA) Estradiol concentration (IFMA) Delta-4-Androstenedione (IFMA)
LH concentration (IFMA)
LH concentration (IFMA) Estradiol concentration (IFMA) Delta-4-Androstenedione (IFMA) Progesterone (IFMA)

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CHAPTER 2 - THE ROLE OF GENETICS IN REPRODUCTIVE HEALTH

- o *COMMENT*

Female reproductive disorders are typically heterogeneous and complex and originate from genetic and environmental factors. Candidate gene association studies have limitations such as small sample sizes and limited statistical power, and few positive results have been reproduced using this strategy.

Genome-wide association studies (GWASs) have changed the landscape of genetic research of polycystic ovarian syndrome (PCOS) and endometriosis and provide exciting insights into the genetic architecture of PCOS.

Different studies investigated the pathophysiological role of FSH receptor polymorphisms in ovarian dysfunction or ovarian response to stimulation: The ovarian response to follicle stimulating hormone (FSH) stimulation in assisted conception cycles is variable. Although it would be beneficial to predict accurately the response of patients to FSH, to date no absolute predictors of ovarian performance have been identified. Recently, there have been a number of studies on the effect of single-nucleotide polymorphisms (SNPs) in the FSH receptor gene and its predictability in ovarian response to FSH stimulation. Most of the available studies are retrospective, observational. Until now, there is no clear clinical benefit in the screening for SNP before IVF treatment. However, there is the prospect of devising mathematical models using a group of polymorphisms to provide an important tool for improving ovulation induction, especially in poor responders.

Future association studies need global collaboration to expand sample sizes, identify more susceptibility loci and ultimately to discover missing heritabilities. Based on these results, disease subtypes must be specified so that subtype-related markers can be explored. Besides an understanding of the genetic mechanisms, association studies are the key to establishing risk prediction models, developing less invasive methods of diagnosis and creating more effective and targeted treatments.

CHAPTER 3 - DEVELOPMENT OF ALGORITHMS ABLE TO OPTIMIZE THE OUTCOME OF ASSISTED CONCEPTION PREGNANCIES AND TO MANAGE THE CORRELATED OBSTETRIC RISK

o *BACKGROUND*

P4 (Personalized, Predictive, Preventive, Participatory) medicin promises to improve patient outcomes, and to empower both the patient and the physician. Having much more, and more accurate, information to be used by the patient and the doctor to make decisions about prevention and treatment is at the heart of this future medicine.

We must accelerate this transformation by promoting the necessary scientific research and at the same time dealing with the societal challenges presented by P4 medicine.

The healthcare industry, public policy sector, and consumer industries will of necessity be required to develop new and creative business models and products. There is a unique opportunity now to enable and accelerate change by eliminating the key technical and societal (ethical, societal, policy, legal, economic, etc.) barriers that will prevent the full realization of the revolution of P4 medicine.

A coordinated and integrated program can be envisioned, based on the following key objectives, to accelerate solving the technical challenges of this new scientific approach.

• Methods for determining individualized genomes – personalized genome sequencing – new methods and approaches are necessary, but to deploy its predictive power these data will have to be integrated with diagnostic measurement data.

• Methods for determining the levels of organ- specific proteins, microRNAs and other possible biomarkers, including cells, in the blood to assess the health or disease in all major human organ systems and thus monitor the earliest onset of disease with its implications for more effective treatment.

• Digitize medical records and create effective and secure databases for individual patient records (new, data intense records with gigabytes of data). When the diagnostic methods are available the medical interpretation of the patterns observed from blood data will become increasingly rich and informative as science and databases advance.

• Develop new mathematical and computational methods for extracting maximum information from molecular information on individuals (including their genomes), and from other clinical data and history. Develop new computational techniques for building dynamic and disease- predictive networks from massive amounts of integrated genomic, proteomic, metabolomic and higher level phenotypic data. This is the heart of the new medicine: new methods for interrogating and understanding the interaction between the environment and the genome of the individual.

• Drug perturbations of biological networks to be understood in a predictive sense. These problems are experimental and computational. Therapeutic perturbations of biological networks re- engineering of networks in higher organisms with drugs (diseased back to normal). • Among the great opportunities here are new methods for creating pluripotent cells (stem cells) from normal, differentiated cells, and then differentiating them to specific body cell types. The practical methods are developing rapidly, but the understanding will be complex and very important. The ability to create stem cells with a given individual's genome will be remarkable, understanding it will be revolutionary.

• Imaging molecular disease indications. New In vivo molecular imaging methods and analysis methods to follow disease, drug response, drug effectiveness, drug dosage determinations etc. . Integrating this kind of data with all the other measurement data to make predictions and diagnoses is an important challenge. • Handling the enormous personalized data sets—policies, security, quality control (validation), mining, reporting, modeling, etc. Technically challenging, requiring significant investment and effort, but completely achievable. The challenges here are large, but they include the challenge of making transparent interfaces for researchers, basic and clinical, to mine, analyze and visualize this data. All these are essential for its effective use in future. • Education of patients and physicians about P4 Medicine. For the full effect of the changes discussed here to be felt patients must be well informed about their personal choices in many different ways. At least as important is a profound change in the way that physicians understand the medical issues (therefore medical school education must be radically changed) and how they relate to their patients. The patient- physician relationship will undoubtedly change dramatically. These issues are key to how information can be presented effectively to physicians, practitioners and patients.

On the scientific front, a broad interdisciplinary approach , employing scientists and engineers of all types, is essential to this change – systems biology, including computational methods, technology development and global measurement and analysis of biological systems is essential. Much of the conventional medical research and practice will have to be replaced with systems approaches to catalyze this transformation. On the policy front, we must make sure that policies with respect to privacy, non- discrimination, and access to health insurance, all critical for any health care system, are aligned to maximize both the protections and the benefits to patients. The societal, ethical and healthcare policy issues attendant to the anticipated changes will be profound. These changes must also be planned for so

that the barriers to delivery of the benefits of the coming technical advances will not prevent their adoption.

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CHAPTER 3 - DEVELOPMENT OF ALGORITHMS ABLE TO OPTIMIZE THE OUTCOME OF ASSISTED CONCEPTION PREGNANCIES AND TO MANAGE THE CORRELATED OBSTETRIC RISK

o *PROJECTS ON GOING*

3A) *Controlled ovarian stimulation for IVF/ICSI: development of a new ultrasound guided algorithm to identify the most optimal starting dose of r-hFSH in patients co-treated with a GnRH-antagonist*

The aim of this research project is to develop an algorithm able to personalize the ovarian stimulation protocol for in-vitro fertilization and embryo transfer (IVF-ET).

The following research synopsis is the plan for this research project. It provides the rationale for the research, the research objectives, the proposed methods for data collection and recording formats and/or questionnaires and interview guides. The synopsis is based on the information provided by the supervisor and by secondary sources of information.

	Controlled ovarian stimulation for IVF/ICSI: development of new algorithm for identifying the starting dose of r-hFSH in GnRH-antagonist cycles

	STUDY SYNOPSIS

- **Investigators/Unit**	- **Prof. Carlo Alviggi** Department of Neuroscience, Reproductive Sciences and Dentistry - University "Federico II", Naples, Italy. - **Dr Ilma Floriana Carbone** Department of Neuroscience, Reproductive Sciences and Dentistry - University "Federico II", Naples, Italy. - **Dr Adolfo Allegra** Director of ANDROS Day Surgery Center –Palermo - **Dr Enrico Papaleo** Responsable of IVF center of San Raffaele Hospital- Milano - **Prof. Kypros Nicolaides** Harris Birthright Research Centre for Fetal Medicine– King's College Hospital, London, UK.
- **Aim and study design**	- - This is an observational longitudinal trial aimed to develop an algorithm for identifying the r-hFSH starting dose in women undergoing COS with GnRH-antagonist for IVF/ICSI cycles. -
Patients and Methods	**Sample size** About 500-800 patients enrolled in the three following centers : - Centro Day Surgery ANDROS –Palermo - Centro di Scienze della Natalità dell'Ospedale San Raffaele- Milano - Dipartimento Universitario di Neuroscienze, Scienze Riproduttive ed

Odontostomatologiche – University "Federico II", Napoli. Reproductive Medicine Centre "Desidera", Napoli.

Running Time

March 2015-March 2018

Inclusion criteria

Women aged 24 – 39 years, with both ovaries present and a normal endometrial cavity as assessed by TV-USG and/or hysteroscopy within 6 months before the enrolment will be included.

Exclusion criteria

- Age ≥40 years;

- AMH <5 ng/ml

- BMI >30

- Polycystic ovarian syndrome (PCOS) according The Rotterdam ESHRE/American Society for Reproductive Medicine (ASRM) Sponsored PCOS Consensus Workshop Group, 2004 [1]

- Previous ovarian surgery;

- Endometriosis;

- Abnormal endometrial cavity, including Müllerian malformations, polyps, myomas;

- Autoimmune disorders;

- Chromosomal abnormalities;

- History of previous "poor response" (POR) according ESHRE criteria[2] (At least two of the following three features must be present: (i) Advanced maternal age (≥40 years) or any other risk factor for POR; (ii) A previous POR (≤3 oocytes with a conventional stimulation protocol); (iii) An abnormal ovarian reserve test -i.e. AFC ,5–7 follicles or AMH ,0.5 –1.1 ng/m)

- Last ovarian stimulation less than 3 months before the beginning of this study

- Patients already undergone with a strating FSH dose of 150UI with an egg recruitment of less of 6 ooycite

Ovarian stimulation protocol

On the second day of the cycle , a TV-USG will be performed by an ultrasound machine able to perform an automated Antral Follicle Count (AFC). Should be the day 2 of the cycle on holiday, the AFC will be performed on the day 3. Both 2D and 3D assessments will be performed in order to allow comparative analyses and manage intra- and inter-operator variability.

	A starting dose of 150 IU of r-hFSH will be administered from the same day of the AFC. The starting dose will be fixed for four days and should thus not be changed until the fifth day of stimulation (S5). On S5, TV-USG will be performed in order to evaluate the number and the mean diameter of each follicle. On the same day, a blood sample will be taken for the measurement of Estradiol serum levels as well as cryopreservation for future diagnostics. The dose will be reduced to 100 IU if the FORT will be < 50%. In contrast the dose will be increased to 225 IU in the case of FORT >50 %. The GnRH antagonist at a daily dose of 0.25 mg will be administered on the day 5 of stimulation. The ovulatory dose of hCG will be administered as soon as three follicles >17 mm are present. Trans vaginal egg collection (TVOR) will be performed 34-36 h later. The luteal phase will be supported according the good clinical practice. Antral follicle responsiveness to FSH administration assessed by the FORT (preovulatory follicle PFCx100/AFC)[3].
Endpoints	The primary endpoint is the number of cycles during which the predetermined starting dose of gonadotropins (150 IU) can be maintained (yes) or not (no). Furthermore, the cumulative number of follicles, the number of follicles >10 mm and the mean diameter of each follicle will be recorded and analysed as dependent variables. The secondary endpoints are: - Cumulative r-hFSH dose (IU); - Mean stimulation length (days); - Mean number of oocytes retrieved; - Mean number and percentage of mature oocytes; - Mean levels of Estradiol on day S5 and the day of hCG triggering (pg/ml); - Fertilization rate; - Mean number of embryos transferred; - Cancellation rate; - Implantation rate (number of gestational sacs/number of embryos transferred); - Pregnancy rate (number of positive beta-hCG/started cycles); - Pregnancy rate per transfer; - Ongoing pregnancy rate (number pregnancies overcoming 12th week/started cycles); - Life birth rate. - OHSS rate
Independent (predictive) variables	For each patient, the following independent variables will be recorded (see

appendix 1)

- Age (years);
- Height (cm):
- Weight (Kg);
- Ethnicity;
- Smoking status
- Mean length of menstrual cycle;
- Basal FSH (IU/L);
- Basal LH (IU/L);
- Basal Estradiol (pg/ml);
- AMH (ng/ml);
- PCOS (Rotterdam, 2004);
- FSH-R Ser/Asn 680 polymorphism (Ser/Ser – Ser/Asn – Asn/Asn);
- LH beta unit polymorphism (v-beta LH [v-bLH/v-bLH – v-bLH/wt – wt/wt);
- 2D AFC;
- 3D AFC.

Statistics	Since the dependent variable is dychotomic, it can be described by a Bernoulli random variable in a PROBIT model. In order to estimate the cumulative dose of r-FSH it will be used a risk model.
Attended results	The aims of the study are to develop a software which on the basis of a combination of AFC, demographic, anthropometric, biochemical and genetic variables will give: 1) the probability that the dose of 150 IU needs to be changed or not on day S5. On the basis of this probability the system will be able to suggest the most appropriate starting dose of gonadotropins to guarantee the patient an optimal stimulation cycle; 2) the criteria for modifing the starting dose in a stimulation cycle- for the first time in literature; Moreover the results will help: 3) to verify whether carriers of common single-nucleotide polymorphisms (SNPs) of the FSH receptor (FSHR) show reduced responsiveness of antral follicles to FSH administration 4) to validate a method of changing the starting dose

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3B) *ASPRE trial: combined multi-marker screening & randomised patient treatment with aspirin for evidence.-based preeclampsia prevention*

Here it is reported the study protocol of ASPRE trial.

It provides the rationale for the research, the research objectives, the proposed methods for data collection and recording formats and/or questionnaires and interview guides. The synopsis is based on the information provided by the supervisor and by secondary sources of information.

ASPRE

Combined multi-marker screening and randomised patient treatment with aspirin for evidence-based pre-eclampsia prevention

Version	3.0
Date	21 October 2014
Sponsor	University College London (UCL)
UCL Clinical Trials Unit Trial #	CTU/2013/064
Trial registration	EudraCT Number: 2013-003778-29
	ISRCTN number: ISRCTN13633058
	WHO UTN number: U1111-1140-4837
CTA #	20363/0329/001-0001
MREC #	13/LO/1479

Authorisation: Chief Investigator

Name	Professor Kypros Nicolaides
Role	Professor of Fetal Medicine, Harris Birthright Research Centre for Fetal Medicine, King's College Hospital, London, SE5 9RS UK.
Signature	

Date	21 October 2014

Authorisation: Sponsor/UCL CTU Director Representative at UCL CTU

Name	Susan Tebbs
Role	UCL CTU Deputy Director
Signature	

Date	21 October 2014

Authorisation: Senior Operations Staff

Name	Leona Poon
Role	Co-Principal Investigator

Signature

Date   21 October 2014

Authorisation: Statistician
Name   David Wright
Role   Professor in Statistics, School of Computing and Mathematics,
                              Room 4, 5 Kirkby Terrace, Drake Circus, Plymouth, Devon, PL4
                              8AA.
Signature

Date   21 October 2014

Primary Registry and Trial Identifying Number	EudraCT Number – 2013-003778-29
Date of Registration in Primary Registry	04 September 2013
Secondary Identifying Numbers	WHO UTN Number - U1111-1140-4837 ISRCTN – ISRCTN13633058
Source of Monetary or Material Support	The European Commission Seventh Framework Programme FP7 (#601852).
Primary Sponsor	University College London
Secondary Sponsor	Sponsor responsibilities for Trial Management are delegated to UCL CTU by the regulator, primary sponsor UCL. Other partner roles and responsibilities are defined in the FP7 Technical Annex.
Contact for Public Queries	ctu.aspre@ucl.ac.uk
Contact for Scientific Queries	Prof Kypros Nicolaides Harris Birthright Research Centre for Fetal Medicine, King's College Hospital, London, SE5 9RS, UK. Telephone: +44 2032998256 Fax: +44 20 3299 3898 Email: kypros@fetalmedicine.com Miss Leona Poon Harris Birthright Research Centre for Fetal Medicine, King's College Hospital, London, SE5 9RS, UK. Telephone: +44 7795312884 Fax: +44 20 3299 3898 Email: leonapoon@nhs.net
Public Title	Screening and Prevention of Pre-eclampsia (High Blood Pressure): Randomised Trial of Aspirin Versus Placebo (ASPRE)
Scientific Title	Combined multi-marker screening and randomised patient treatment with aspirin for evidence-based pre-eclampsia prevention
Countries of Recruitment	United Kingdom, Spain, Belgium and Italy
Health Condition(s) or Problem(s) Studied	Pre-eclampsia (PET)
Screening	All women with singleton pregnancies undergoing routine screening for aneuploidies will be invited to participate in the screening study for PET.
Intervention(s)	Participants who are screened with a high-risk of developing PET will be randomised into one of 2 arms of the trial (on the same day or the follow day of the screening visit). The intervention group will be advised to start 150mg of Aspirin once per night within 24 hours of randomisation until 36 weeks' gestation or when signs of labour commence. The control group will receive an identically looking placebo tablet for the same duration as the intervention group. The maximum duration for aspirin or placebo intake will be 25 weeks.

Key Inclusion and Exclusion Criteria	**Inclusion criteria for participant selection for RCT** • Age $\geq$ 18 years, • Singleton pregnancies, • Live fetus at 11-13 weeks of gestation, • High-risk for preterm-PET at 11-13 weeks by the algorithm combining maternal history and characteristics, biophysical findings (mean arterial pressure and uterine artery Dopplers) and biochemical factors (pregnancy associated plasma protein-A and placental growth factor), • English, Italian, Spanish, French, or Dutch, speaking (otherwise interpreters will be used), • Informed and written consent. **Exclusion criteria** • Multiple pregnancies, • Women taking low-dose aspirin regularly, • Pregnancies complicated by major fetal abnormality identified at the 11-13 weeks assessment, • Women who are unconscious or severely ill, those with learning difficulties, or serious mental illness, • Bleeding disorders such as Von Willebrand's disease, • Peptic ulceration, • Hypersensitivity to aspirin or already on long term non-steroidal anti-inflammatory medication, • Age < 18 years, • Concurrent participation in another drug trial or at any time within the previous 28 days, • Any other reason the clinical investigators think will prevent the potential participant from complying with the trial protocol.

Study Type	Phase III two arm double-blinded randomised controlled trial to examine the effect of prophylactic low-dose aspirin from the first-trimester of pregnancy in women at increased risk for PET on the incidence and severity of the disease
	
	Allocation concealment mechanism and sequence generation:
	    a. Randomisation sequence using a simple permuted block, will be generated by Hananja plc. They will apply a randomisation code to each of the treatment pack (tablet bottle) as allocated.
	    b. Each tablet bottle will only be identified by a randomisation code and labelled: "contains 150 mg aspirin or placebo". When a participant enrols onto the study, a randomisation code will be generated via Sealed Envelope's website.
	
	The main trial will be preceded by a screening quality study.

Date of First Enrolment	Screening quality study: January 2015 Main ASPRE trial: May 2015
Target Sample Size	Main ASPRE trial Screening population 33,680 Randomised control trial 1,684
Primary Outcome(s)	Outcome name - preterm-PET (delivery <37 weeks)

	Metric/method of measurement – PET will be defined as per the International Society for the Study of Hypertension in Pregnancy (Brown, 2001). The systolic blood pressure should be 140 mm Hg or more and/or the diastolic blood pressure should be 90 mmHg or more on at least two occasions four hours apart developing after 20 weeks of gestation in previously normotensive women (blood pressure less than 140/90 mmHg) and there should be proteinuria of 300mg or more in 24 hours or two readings of at least ++ on dipstick analysis of midstream or catheter urine specimens if no 24-hour collection is available. Timepoint – Development of PET requiring delivery before 37 weeks
Key Secondary Outcomes	• Incidence of early-PET (<34 weeks) and total PET (at any gestation) • Neonatal birthweight below the 3rd, 5th and 10th centile • Stillbirth or neonatal death due to any cause • Stillbirth or neonatal death ascribed to PE or fetal growth restriction • Stillbirth or neonatal death in association with maternal or neonatal bleeding • Rate of neonatal intensive care unit admission • Composite measure of neonatal mortality and morbidity • Placental abruption (clinically or on placental examination) • Spontaneous preterm delivery <34 weeks and <37 weeks

1.4   Roles and responsibilities

1.4.1   Protocol contributors

Name	Affiliation	Role
Kypros Nicolaides	Fetal Medicine Unit, University College London Hospital. Harris Birthright Research Centre for Fetal Medicine, King's College Hospital.	Chief Investigator
Leona Poon	Harris Birthright Research Centre for Fetal Medicine, King's College Hospital. Fetal Medicine Unit, University College London Hospital.	Co-Principal Investigator
David Wright	School of Computing and Mathematics, Plymouth University, UK.	Trial statistician

1.4.2   Role of trial sponsor and funders

Name	Affiliation	Role
The European Commission Seventh Framework Programme FP7		Scientific peer review of the study proposal Provision of funds Study monitoring
University College London (UCL)		Regulatory sponsor

1.4.3   Trial Team

Name	Affiliation	Role and responsibilities
Kypros Nicolaides	Fetal Medicine Unit, University College London Hospital, UK. Harris Birthright Research Centre for Fetal Medicine, King's College Hospital, UK.	Chief Investigator Responsible for the concept and design of the study protocol, supervision of the research team for the measurement of biomarkers, coordination and management of the study, monitoring and statistical analysis of data, and writing up the scientific publications.
Leona Poon	Harris Birthright Research Centre	Co-Principal Investigator Responsible for the concept and design of the study

	for Fetal Medicine, King's College Hospital, UK. Fetal Medicine Unit, University College London Hospital, UK.	protocol, application for ethics and R&D approval, supervision of the research team for the measurement of biomarkers, coordination and management of the study, counselling and recruitment of participants, collection, monitoring and statistical analysis of data, and writing up the scientific publications.
David Wright	School of Computing and Mathematics, Plymouth University, UK.	Statistician Responsible for statistical analysis and monitoring of data.
Details of site PIs are provided in a separate document, outside of the study protocol.		

Name	Affiliation	Role and responsibilities
Kypros Nicolaides	Fetal Medicine Unit, University College London Hospital, UK. Harris Birthright Research Centre for Fetal Medicine, King's College Hospital, UK.	Chief Investigator Responsible for overall coordination and management of the study, monitoring of data.
Leona Poon	Harris Birthright Research Centre for Fetal Medicine, King's College Hospital, UK. Fetal Medicine Unit, University College London Hospital, UK.	Co-Principal Investigator Responsible for coordination and day-to-day clinical support of the study, monitoring of blinded data
David Wright	School of Computing and Mathematics, Plymouth University, UK.	Statistician Responsible for statistical analysis and monitoring of data.
Susan Tebbs	UCL CTU	Clinical Project Manager Responsible for the project management of the trial and oversight of the CTU team members
Kate Maclagan	UCL CTU	UCL CTU Trial Manager Responsible for the day to day management of the trial

Name	Affiliation	Role and responsibilities
Zarko Alfirevic	Institute of Translational Medicine University of Liverpool, UK.	Chair Independent of the trial team
Kypros Nicolaides	Fetal Medicine Unit, University College London Hospital, UK. Harris Birthright Research Centre for Fetal Medicine, King's College Hospital, UK.	Chief Investigator
Leona Poon	Harris Birthright Research Centre for Fetal Medicine, King's College Hospital, UK. Fetal Medicine Unit, University College London Hospital, UK.	Co-Principal Investigator
Bryony Jones	Fetal Medicine Unit, Imperial College Healthcare NHS Trust, UK.	Independent Specialist in Fetal Medicine and Obstetrics
George Attilakos	Fetal Medicine Unit, University College London Hospital, UK.	Independent Specialist in Fetal Medicine and Obstetrics
Ranjit Akolekar	Fetal Medicine Unit, Medway Maritime Hospital, UK.	Site Investigator representative

Name	Affiliation	Role and responsibilities
Mark Turner	Institute of Translational Medicine University of Liverpool, UK.	Chair Independent of the trial team
Christina Yu	Fetal Medicine Unit, Imperial College Healthcare NHS Trust, UK.	Independent Specialist in Fetal Medicine and Obstetrics
Ian Bradbury	Statistics at Frontier Science Scotland, UK.	Independent Statistician Responsible for monitoring and statistical analysis of data.

Name	Role and responsibilities
Hy Laboratories Israel	Hy Laboratories will be responsible for standardising blood drawing, processing and storage, making reagents and kits for measurement of PP13 for the prediction of PET. In project management Hy Laboratories will carry out the financial management of the project and assist the CI with dissemination and exploitation of the technological reports and administrative coordination of the project.
Hananja plc Iceland	Hananja will be responsible for: (1) Generation of the randomisation codes; (2) Quality Control (QC) protocols for all raw materials, actives and packaging; standard operating procedures (SOPs), manufacturing procedures and application for manufacturing permission; (3) manufacture aspirin and placebo trial batches and final batches and label them according to randomisation procedure; (4) shipment of drug/placebo to clinical centres; (5) code breaking via its website by authorised users.
Astraia Software Gmbh Germany	Astraia will be responsible for development and installation of the risk assessment software.
Perkin Elmer (Wallac Oy) Finland	Perkin Elmer will be responsible for the production of PAPP-A and PlGF reagents, participation in dissemination through webcasts and educational material, and development of major marketing efforts to exploit the study outcome.
Sealed Envelope	Sealed Envelope is responsible for the provision of patient randomisation online and code breaking by authorised users online and via telephone.

2 Trial Diagram

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┌─────────────────────────────────────────────────────────────────────────┐
│                          Screening Visit                                   │
│              11-13 wk or CRL 45-84mm (N=33,680)                            │
└─────────────────────────────────────────────────────────────────────────┘
                    ↙                                    ↘ 10%
┌──────────────────────┐          ┌─────────────────────────────────────────┐
│   Screen negative    │          │          Screen positive                 │
│     N=30,312         │          │            N=3,368                        │
└──────────────────────┘          └─────────────────────────────────────────┘
            │                                         │ 50%
┌──────────────────────┐          ┌─────────────────────────────────────────┐
│ Routine antenatal    │          │    Randomised aspirin/placebo            │
│       care           │          │       (11-14 wk) N=1,684                  │
└──────────────────────┘          └─────────────────────────────────────────┘
            │                              ↙                        ↘
            │              ┌──────────────────────┐  ┌──────────────────────┐
            │              │ Allocated to aspirin │  │ Allocated to placebo │
            │              │       N=842          │  │       N=842          │
            │              └──────────────────────┘  └──────────────────────┘
            │                         │                         │
            │              ┌──────────────────────┐  ┌──────────────────────┐
            │              │ 16 wk telephone      │  │ 16 wk telephone      │
            │              │ interview            │  │ interview            │
            │              │ Ensure compliance(EC)│  │ Ensure compliance(EC)│
            │              │ Address concern (AC) │  │ Address concern (AC) │
            │              └──────────────────────┘  └──────────────────────┘
            │                         │                         │
┌──────────────────────┐* ┌──────────────────────┐  ┌──────────────────────┐
│ 20-24 wk clinical    │  │ 20-24 wk clinical    │  │ 20-24 wk clinical    │
│ visit                │  │ visit                │  │ visit                │
│                      │  │ EC & AC              │  │ EC & AC              │
└──────────────────────┘  └──────────────────────┘  └──────────────────────┘
            │                         │                         │
            │              ┌──────────────────────┐  ┌──────────────────────┐
            │              │ 28 wk telephone      │  │ 28 wk telephone      │
            │              │ interview            │  │ interview            │
            │              │ EC & AC              │  │ EC & AC              │
            │              └──────────────────────┘  └──────────────────────┘
            │                         │                         │
┌──────────────────────┐* ┌──────────────────────┐  ┌──────────────────────┐
│ 30-37 wk clinical    │  │ 32-34 wk clinical    │  │ 32-34 wk clinical    │
│ visit                │  │ visit                │  │ visit                │
│                      │  │ EC & AC              │  │ EC & AC              │
└──────────────────────┘  └──────────────────────┘  └──────────────────────┘
            │                         │                         │
            │              ┌──────────────────────┐  ┌──────────────────────┐
            │              │ 36 wk clinical visit │  │ 36 wk clinical visit │
            │              │ EC & AC              │  │ EC & AC              │
            │              │ Discontinue medication│  │ Discontinue medication│
            │              └──────────────────────┘  └──────────────────────┘
            │                         │                         │
            │              ┌──────────────────────┐  ┌──────────────────────┐
            │              │ F/U telephone        │  │ F/U telephone        │
            │              │ interview            │  │ interview            │
            │              │ 30-d post last dose  │  │ 30-d post last dose  │
            │              └──────────────────────┘  └──────────────────────┘
            ↘                         ↘                ↙
┌─────────────────────────────────────────────────────────────────────────┐
│   Extraction of pregnancy outcome (anticipating 5% lost to follow up)     │
└─────────────────────────────────────────────────────────────────────────┘
```

* Clinical visits at 20-24wk and 30-37wk will only be performed on screen-negative participants at sites where a scan is performed as part of the routine clinical care pathway at either of these times.

	Screening Visit	Randomisation Visit	First telephone interview	First follow up visit	Second telephone interview	Second follow up visit	Third follow up visit	Third telephone interview
Gestation (weeks)	11-13 or CRL 45-84mm	11-14	16	20-24	28	32-34	36	30 days after the last dose of IMP
Patient information and characteristics	√							
Informed consent	√	√						
Measurement of weight and height	√			√		√	√	
Measurement of MAP	√			√		√	√	
Fetal ultrasound scan	√			√		√	√	
Measurement of uterine artery PI	√			√		√	√	
Measurement of biochemical markers (PAPP-A and PlGF)	√			√		√		
Check concomitant medications		√	√	√	√	√	√	
IMP dispensing		√		√				
Ensure compliance			√	√	√	√	√	
Check side effects/adverse events and review of diary card			√	√	√	√	√	√
Discontinue IMP							√	

CRL = Crown-Rump Length; MAP = mean arterial pressure; PI = pulsatility index; PAPP-A = pregnancy associated plasma protein-A; PlGF = placental growth factor; IMP = investigational medicinal product

AE	Adverse Event			Committee
AR	Adverse Reaction	NT	Nuchal translucency
ß-hCG	ß-human chorionic gonadotropin	PAPP-A	Pregnancy associated plasma protein-A
CI	Chief Investigator	PI	Principal Investigator
CI	Confidence interval	PlGF	Placental growth factor
CRF	Case Report Form	PIS	Participant Information Sheet
CRL	Crown-rump length	PET	Pre-eclampsia
CTA	Clinical Trial Authorisation	QA	Quality Assurance
CTU	Clinical Trials Unit	QC	Quality Control
DSUR	Development Safety Update Report	QP	Qualified person
EU	European Union	R&D	Research and Development
FDA	(US) Food and Drug Administration	REC	Research Ethics Committee
FGR	Fetal growth restriction	RR	Relative risk
FMF	Fetal Medicine Foundation	SAE	Serious Adverse Event
FWA	Federal Wide Assurance	SAP	Statistical Analysis Plan
GCP	Good Clinical Practice	SAR	Serious Adverse Reaction
ICH	International Conference on Harmonisation	SPC	Summary of Product Characteristics
IDMC	Independent data monitoring committee	SSA	Site Specific Approval
		SUSAR	Suspected Unexpected Serious Adverse Reaction
IMP	Investigational Medicinal Product	TMF	Trial Master File
IMPD	Investigational Medicinal Product Dossier	TMG	Trial Management Group
		TMT	Trial Management Team
IRB	Institutional Review Board	ToR	Terms of Reference
ITT	Intention to Treat	TSC	Trial Steering Committee
MAP	Mean arterial pressure	UCL	University College London
MCQ	Metacognitions Questionnaire	Uterine artery PI	Uterine artery pulsatility index
MHRA	Medicines and Healthcare products Regulatory Agency		
MoU	Memorandum of Understanding		
NREC	National Research Ethics		

5 Introduction

5.1 Background and Rationale

Background

Pre-clampsia (PET) is an important cause of maternal and perinatal mortality and morbidity.
There is extensive
evidence that the risk of adverse outcome in relation to PET is much higher when the disease is severe and of early onset requiring delivery before 37 weeks' gestation (preterm-
PET), than at term (Witlin et al., 2000; Irgens
et al., 2001; von Dadelszen et al., 2003; Yu et al., 2008). A major challenge in modern obstetrics is early identification of pregnancies at high-risk of preterm-PET and undertaking the necessary measures to improve placentation and reduce the prevalence of the disease.

Prediction of preterm pre-eclampsia

Extensive research in the last 20 years, mainly as a consequence of the shift in screening for aneuploidies from the second to the first-trimester of pregnancy, has identified a series of early biophysical and biochemical markers of impaired placentation (Akolekar et al., 2011). A combination of maternal demographic characteristics, including medical and obstetric history, uterine artery pulsatility index (PI), mean arterial press ure (MAP) and maternal serum pregnancy associated plasma protein-A (PAPP-A) and placental growth factor (PlGF) at 11-13 weeks' gestation can identify a high proportion of pregnancies at high-risk for PET (Akolekar et al., 2011).
A recent study involving more than 60,000 singleton pregnancies examined at 11-13 weeks' gestation has further refined the prediction algorithm for PET.
Using this algorithm the estimated detection rate of preterm-PET was 76%, at a
false positive rate of 10% (Akolekar et al., 2012).
Consequently, there is a well-described approach for effective screening for preterm-PET
at the time of routine screening for aneuploidies at 11-13 weeks' gestation.

Prevention of preterm pre-eclampsia

The prophylactic use of low-dose aspirin for prevention of PET has been an important research question in obstetrics for the last three decades. In 1979, Crandon and Isherwood observed that nulliparous women who h ad taken aspirin regularly during pregnancy were less likely to have PET than women who did not. Subsequently, more than 50 trials have been carried out throughout the world and a meta-analysis of these studies reported that the administration of low-dose aspirin in high-risk pregnancies is associated with a decrease in the rate of PET by approximately 10% (Askie et al., 2007).

Initiation of low-dose aspirin in early pregnancy

In most studies that evaluated aspirin for the prevention of PET the onset of treatment was after 16 weeks' gestation.

However, examination of a small number of randomised trials of low-dose aspirin in women at high-risk for PET suggests that the effectiveness of therapy is related to the gestational age at the onset of treatment. A meta-analysis by Bujold et al. (2010) reported that low-dose aspirin started at 16 weeks or earlier was associated with a significant reduction in the relative risk (RR) for PET (0.47, 95% confidence interval [CI] 0.34 – 0.65) and fetal growth restriction (FGR) (0.44, 95% CI 0.30 – 0.65; Bujold et al., 2010). In contrast, aspirin started after 16 weeks did not have a significant benefit (PET: RR 0.81, 95% CI: 0.63–1.03; FGR: RR 0.98, 95% CI: 0.87–1.10). More detailed analysis of the data on PET demonstrated that low-dose aspirin started at or before 16 weeks' gestation was particularly effective in preventing preterm-PET rather than term-PET (RR: 0.11, 95% CI: 0.04-0.33 vs. RR:0.98, 95% CI: 0.42-2.33; Roberge et al., 2012).

However, the small number and small size of individual trials preclude definitive conclusions to be drawn regarding the effectiveness of aspirin starting before 16 weeks and the results need to be examined in a prospective major randomised trial.

Low-dose aspirin is defined as less than 300 mg per day.

There is evidence that approximately 30%, 10% and 5% of pregnant women are "aspirin resistant" with dosage of 81 mg, 121 mg, and 162 mg, respectively (Caron et al.,2009).

Furthermore, a retrospective cohort study reported that women who were identified by the PFA-100 test as being resistant to 81 mg of aspirin were less likely to develop severe PET when the dose of aspirin was increased from 81 to 162 mg, compared to those who continued with 81 mg (Rey and Rivard, 2011). Consequently, a trial investigating the effectiveness of low-dose aspirin in the prevention of preterm-PET should use a dose closer to 160 mg rather than 80 mg.

Safety of low-dose aspirin

The relative safety of first-trimester use of low-dose aspirin has been demonstrated in large cohort and case-control studies, which reported that the drug is not associated with increase in risk of congenital heart defects or other structural or developmental anomalies (Slone et al., 1976; Klebanoff and Berendes, 1988; Werler et al., 1989; Norgard et al., 2005).

Potential risks associated with aspirin therapy during the third-trimester include haemorrhagic complications to the mother or fetus and premature closure of the ductus arteriosus for the fetus. Randomised studies reported that although approximately 10% of women receiving low-dose aspirin complained of gastro-intestinal symptoms there was no evidence of increase in any type of

maternal bleeding (Sibai et al.,1993; Caritis et al., 1998; Rotchell et al., 1998). Similarly, the best evidence suggests that low-dose aspirin started

before 16 weeks' gestation does not increase the risk of placental abruption (RR: 0.62, 95% CI: 0.08–5.03; Bujold

et al., 2010). No additional adverse effects related to epidural anaesthesia have been reported in women taking low-dose aspirin compared to those taking placebo (Sibai et al., 1995).

Prospective and case-control studies did not find an association between daily consumption of 60-150 mg of aspirin during the third-trimester and antenatal closure of the ductus arteriosus

(Di Sessa et al., 1994; Schiessl et al., 2005; Wyatt-Ashmead, 2011).
A meta-analysis including more than 26,000 women randomised to low-dose

(80-150 mg) aspirin or placebo/no treatment during pregnancy demonstrated that the use of aspirin was not associated with an increase in intra-ventricular haemorrhage or other neonatal bleeding (Duley et al., 2007).

In the trials evaluating the effect of aspirin for the prevention of PET in high-risk women, treatment was continued until a pre-specified gestation between 34 and 37 weeks or until delivery. On the basis of currently available evidence it would be reasonable to continue with low-dose aspirin well into the third-trimester of pregnancy.

5.1.1   Explanation for choice of comparators

In order to evaluate the effectiveness of low-dose aspirin in reducing the risk of PET,
the study will compare the results of the interventional group with the results of the placebo group.

5.2   Objectives

Aim: To examine if the prophylactic use of low-dose aspirin from the first-trimester of pregnancy in
women at increased risk for PET can reduce the incidence and severity of the disease.

Primary objective:
- Incidence of preterm-PET (<37 weeks)

Secondary objectives:
- Incidence of early-PET (<34 weeks) and total PET (at any gestation),
- Birthweight below the 3rd, 5th and 10th centile,
- Stillbirth or neonatal death due to any cause,

- Stillbirth or neonatal death ascribed to PET or FGR,
- Stillbirth or neonatal death in association with maternal or neonatal bleeding,

- ☐ Rate of neonatal intensive care unit admission,
- ☐ Composite measure of neonatal mortality and morbidity,
- ☐ Placental abruption (clinically or on placental examination),
- ☐ Spontaneous preterm delivery <34 weeks and <37 weeks.

5.3   Trial Design

Proposed Study

There are three components to the study: a screening quality study and a screening study followed by a double-blind randomised placebo-controlled trial.

The main trial will be preceded by a two-month pilot study, which will be undertaken at
King's College Hospital.
Approximately 1,000 participants will be consented into the screening study and an estimated 50 participants to the randomised control trial.

This study will be used to assess the following:
- ☐ The feasibility of recruitment to both the screening study and randomised controlled trial
- ☐ The ability of the centre to ensure successful compliance
- ☐ Determine whether assessment of compliance with study drug by biochemical testing is effective

The results of the pilot study will be reviewed by the ASPRE IDMC. Following this, a strategic decision on the way forward will be made by the ASPRE TSC.

A review of the two-month recruitment period of the pilot study at King's College Hospital demonstrated the study had been successful with respect to recruitment to both the screening study and randomised controlled trial; however it has also highlighted the complexity of the main ASPRE trial and confirmed the need for enhanced quality systems to be in place in advance of starting the trial in order to ensure the quality of pivotal data.

Therefore, a screening quality study, with a minimum recruitment period of three months at each site, is being introduced to precede the main ASPRE trial. The aim of this study is to establish systems that will monitor quality in a more detailed, formalised manner at sites and use these systems to assess quality,
 identify areas for improvement and, where required, implement strategies to improve quality e.g. re-training. This will be based on the DQASS system that has been successful for improving the quality of the ultrasound and biochemical measurements in the NHS fetal anomaly screening programme.

The following will be established at study sites to assess quality:

☐ Fetal Medicine Foundation accreditation/continuous monitoring programme for the acquisition of uterine artery PI and MAP

☐ A PAPP-A and PlGF quality monitoring system

Recruitment rates will also be monitored. Furthermore, an assessment of data quality will be made by the trial team at UCL CCTU and any site-specific operational issues, which could not have been foreseen by the site assessment process, will be identified, and addressed in advance of starting the main ASPRE trial.

Participants enrolled in the screening quality study will not be informed of their risk of developing pre-eclampsia
and will be managed according to routine standard of care at the site they attend.

The trial will be conducted in compliance with the protocol, the Declaration of Helsinki (1996), the principles of Good Clinical Practice (GCP) and applicable regulatory requirements.
The study will be reviewed and approved by the National Research Ethics Committee (NREC),
Medicine and Healthcare products Regulatory Agency (MHRA) and applicable Hospital Trusts.
 The University College London Clinical Trials Unit (UCL CTU) will manage the
sponsors' responsibilities and Quality Assurance to ensure compliance with the Clinical Trial Regulations.

This will be a multicentre trial in the UK, other countries in the European Union. In all the participating centres, all women attending for their routine first hospital visit in pregnancy at 11-13 weeks'
 gestation are offered routine combined screening for aneuploidies by measurement
of the fetal crown-rump length (CRL) and nuchal translucency (NT) thickness and maternal serum PAPP-A, free ß-hCG and PlGF
(Robinson and Fleming 1975;Snijders et al., 1998; Kagan et al., 2008, Pandya et al., 2012). The Patient Information Sheet (PIS) for the screening study for PET will be sent with the appointment letter
to all potential participants, with the exception of the site in Milan where appointment letters are not sent
 routinely.

All women undergoing routine screening for aneuploidies will be invited to participate in the screening study for PET.
In women who agree to participate in the screening study for PET, after obtaining informed consent, we also measure the maternal MAP by automated devices (Poon et al., 2012), use transabdominal colour Doppler ultrasound to visualise the left and right uterine artery and measure the PI in each vessel and calculate the
 mean PI (Plasencia et al., 2007).
The principal investigators for each site are doctors who received their training by
Professor Nicolaides and follow the Fetal Medicine Foundation (FMF) guidelines on how to undertake the

appropriate measurements.

6 Methods

6.1 Site Selection

The trial sponsor has overall responsibility for site and investigator selection and has delegated this role to UCL CTU.

6.1.1 Study Setting

This is a multicentre study that will be carried out in the Fetal Medicine Units in the UK, Belgium, Spain and Italy (that are within the FMF Research Network).

6.1.2 Site/Investigator Eligibility Criteria

Once a site has been assessed as being suitable to participate in the trial, the trial team will provide them with a copy of this protocol and relevant Summary of Product Characteristics (SPC), followed by a simplified Investigational Medicinal Product Dossier.

To participate in the ASPRE trial, investigators and trial sites must fulfil a set of criteria that have been agreed by the ASPRE Trial Management Group (TMG) and that are defined below.

Trial sites meeting eligibility criteria and that are accepted by the TMG as being suitable to recruit to the trial, will be issued with the ASPRE Trial Master File (TMF) documentation to use when applying for Site-Specific Approval (SSA).

6.1.2.1 Principal Investigator's (PI) Qualifications and Agreements

The investigator(s) must be willing to sign a UCL CTU Clinical Trial Agreement or an Investigator Agreement to comply with the trial protocol (confirming their specific roles and responsibilities relating to the trial, and that their site is willing and able to comply with the requirements of the trial). This includes confirmation of appropriate qualifications, familiarity with the appropriate use of any investigational products, agreement to

comply with the principles of GCP, to permit monitoring and audit as necessary at the site, and to maintain documented evidence of all staff at the site who have been delegated significant trial related duties.

6.1.2.2 Resourcing at site

The investigator(s) should be able to demonstrate a potential for recruiting the required number of suitable participants within the agreed recruitment period (ie the investigator(s) regularly treat(s) the target population) They should also have an adequate number of qualified staff and facilities available for the foreseen duration of the trial to enable them to conduct the trial properly and safely.

Sites will be expected to complete a delegation of responsibilities log and provide staff contact details.

The site should have sufficient data management resources to allow prompt data return to UCL CTU.

6.2   Site approval and activation

The Clinical Trial Authorisation (CTA) for the trial requires that the Medicines and Healthcare products Regulatory Agency (MHRA) is supplied with the names and addresses of all participating site Principal Investigators.

Trial staff at UCL CTU will perform this task.

On receipt of the signed Clinical Trial Agreement or Investigator Agreement, approved delegation of responsibilities log and staff contact details, written confirmation will be sent to the site PI. The trial manager or delegate will notify the PI in writing of the plans for site initiation.

The site must conduct the trial in compliance with the protocol as agreed by the Sponsor and, by the Regulatory authority(ies) (as appropriate), and which was given favourable opinion by the Research Ethics Committee (REC)and/or Institutional Review Board (IRB).

The PI or delegate must document and explain any deviation from the approved protocol, and communicate this to the trial team at UCL CTU.

A list of activated sites may be obtained from the Trial Manager.

6.3   Participants

6.3.1   Eligibility Criteria

6.3.1.1 Participant selection

There will be NO EXCEPTIONS (waivers) to eligibility requirements at the time of randomisation. Questions about eligibility criteria should be addressed PRIOR to attempting to randomise the participant.

The eligibility criteria for this trial have been carefully considered and are the standards used to ensure that only medically appropriate participants are entered.

Participants not meeting the criteria should not be entered into the trial for their safety and to ensure that the trial results can be appropriately used to make future treatment decisions for other people with similar diseases or conditions. It is therefore vital that exceptions are not made to these eligibility criteria.

Participants will be considered eligible for enrolment in this trial if they fulfil all the inclusion criteria and none of the exclusion criteria as defined below.

6.3.1.2 Participant Inclusion Criteria for RCT

☐ Age > 18 years*,

☐ Singleton pregnancies*

☐ Live fetus at 11-13 weeks of gestation*,

☐ High-risk for preterm-PE will be defined at 11-13 weeks by the algorithm combining maternal history and characteristics, biophysical findings (MAP and uterine artery Dopplers) and biochemical factors (PAPP-A and PlGF),

☐ English, German, Italian, Spanish and French speaking (otherwise interpreters will be used)*,

☐ Informed and written consent*.

6.3.1.3 Participant Exclusion Criteria for RCT

☐ Multiple pregnancies*,

☐ Women taking low-dose aspirin regularly,

☐ Pregnancies complicated by major fetal abnormality identified at the 11-13 weeks assessment*,

☐ Women who are unconscious or severely ill, those with learning difficulties, or serious mental illness*,

☐ Bleeding disorders such as Von Willebrand's disease,

☐ Peptic ulceration,

☐ Hypersensitivity to aspirin or already on long term non-steroidal anti-inflammatory medication,

☐ Age < 18 years*,

☐ Concurrent participation in another drug trial or at any time within the previous 28 days,

☐ Any other reason the clinical investigators think will prevent the potential participant from complying with the trial protocol.[1]

6.3.1.4 Eligibility Criteria for Individuals Performing the Interventions

All centres involved in the data collection will have staff who are appropriately trained in obstetric ultrasound and possess certificates of competence from the FMF.

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[1] Eligibility criteri afro screening quality study and main ASPRE screening study.

6.3.1.5 Co-enrolment Guidance

For parous women, participation in the trial in a previous pregnancy will be checked (as there will be a record in the electronic Source Data) in order to prevent participants from being enrolled more than once in this trial. Data of each participant should only be recorded as one entry in the database.

6.3.1.6 Screening Procedures and Pre-randomisation Investigations

Written informed consent will be obtained from all women agreeing to participate in the screening study for P ET.

Written informed consent will then be obtained from those who are found to be high-risk that agree to participate in the randomised trial, after explanation of the aims, methods, benefits and potential hazards of the trial and BEFORE any trial-specific procedures are performed or any blood is taken for the trial. The only procedures that may be performed in advance of written informed consent being obtained are those that would be performed on all participants in the same situation as a usual standard of care, such as the routine first- trimester combined screening for aneuploidies, which includes an ultrasound scan to measure fetal CRL, NT and assessment of fetal anatomy and blood draw for measurement of biochemical markers PAPP-A and free ☐-hCG.

6.4   Interventions

Women eligible to participate in this trial will provide informed consent and receive written information on the test drug.

Each participant will be provided with a participation number according to a predesigned randomisation code. The participation number will determine who receives placebo or investigational drug (aspirin 150 mg). Hananja plc will keep and store the randomisation code. All participants, the PI and clinical trial pharmacy will remain blind to trial drug allocation.

Randomisation will be performed using an online web based system. After enrolment and randomisation, the study participant will be given the first bottle (88 tablets). The second bottle will be given at their 20-24 weeks visit. The participants will be given instructions to swallow the whole tablet and to avoid aspirin-containing compounds and other non-steroidal anti-inflammatory drugs throughout the study.

Participants will take one tablet per night of either aspirin 150 mg or matching placebo. Participants will be asked to stop taking tablets at 36 weeks' gestation or in the event of early delivery, at the onset of labour (maximum duration of 25 weeks).

Intervention:

The aspirin tablets will be film-coated to be taken orally once per night from enrolment until 36 weeks' gestation.

Active substance – 150 mg acetylsalicylic acid (C9H8O4, CAS number 50-78-2),

having the following molecular structure:

Comparator:

Identical-appearing tablet of film coated placebo will be taken orally once per night from enrolment until 36 weeks' gestation.

Manufacturer:

The tablets (placebo and aspirin) will be manufactured by Actavis (Iceland):013, a global, integrated specialty pharmaceutical company and released by their internal QP. The active and placebo will then be distributed to Hananja plc who will coat and pack the products and distribute to the sites (see below)

Packaging and Labelling

88 tablets of active (aspirin) or placebo will be packaged in two bottles (total of 176 tablets per patient) with the approved Trial labelling.

All labelling will be compliant with the regulations and in the respective language of the

enrolling country, such as English, Spanish, Italian, German and French. The translation from English to other languages will be back translated and verified. The two bottles will be assembled together into a carton also labelled with approved trial labelling. Trial labels will ensure blinding of the trial medication. Hananja plc will hold a randomisation schedule and provide code breaking via its website by authorised site-PIs when un-blinding is appropriate.

Trial labels will denote a patient's randomisation number and the site PI name.

QP release

Actavis's QP will release manufactured aspirin and placebo tablets according to manufacturing protocol and International/European GMP. PharmArctica will pack, label, assemble and carry out QC analysis on the final product with oversight from Hananja plc. PharmArctica's QP will release the packed product. Then, Hananja's QP will release the final packed supplies to Distica ehf, that will distribute to packed supplies to the participating sites.

Given the stability and long shelf life of the products, the IMP will be sent to all participating sites in complete numbers.

6.4.1   Investigational Arm

Please note, all duties described below as being undertaken by pharmacy will be carried out by the site PI and delegated individuals in Ospedale Maggiore Policlinico, Milan, Italy because the hospital pharmacy do not providean out-patient service.

6.4.1.1 Products

Film-coated aspirin 150mg tablets will be packaged into two bottles (88 tablets per bottle).

6.4.1.2 Treatment Schedule

Participants are advised to start treatment within 24 hours of randomisation.

6.4.1.3 Dispensing

The first bottle with aspirin 150 mg tablets will be dispensed by the local clinical trial pharmacy according to a participation number.

The second bottle with aspirin 150mg tablets will be dispensed by the local clinical trial

pharmacy following the 20-24 weeks clinical visit.

The local clinical trial pharmacy will be blinded to who receives aspirin and the content of each bottle.

6.4.1.4 Dose Modifications, Interruptions and Discontinuations

One fixed dose will be used, 150 mg aspirin. This dosage has been carefully selected based on aspirin pharmacology and will not be changed, irrespective of indications.

Participants have the right to withdraw from the study at any time for any reason. If participant wishes to withdraw from the study, this will not affect their care. All efforts will be made to report the reason for withdrawal as thoroughly as possible. Should a patient withdraw from study drug only, efforts will be made to continue to obtain follow-up data, with the permission of the patient.

At all patient withdrawals a patient follow

up form will be completed. Withdrawn participants are not replaced as the analysis is based on intention to tre at.

6.4.2 Placebo Arm

Please note, all duties described below as being undertaken by pharmacy will be carried out by the site PI and delegated individuals in Ospedale Maggiore Policlinico, Milan, Italy because the hospital pharmacy do not pr ovide

an out-patient service.

6.4.2.1 Products

Matching placebo tablets will be identical to the intervention (aspirin) in such parameters as size, thickness, physical properties and appearance. A film coating will be applied to the placebo tablets for aesthetic and taste reasons.

6.4.2.2 Treatment Schedule

Participants are advised to start treatment within one week of randomisation.

6.4.2.3 Dispensing

The first bottle with placebo tablets will be dispensed by the local clinical trial pharmacy according to participation number. The second bottle with placebo tablets will be dispensed by the local clinical trial pharmacy following the 20-24 weeks clinical visit.

The local clinical trial pharmacy will be blind to who receives placebo and the content of each bottle.

6.4.2.4 Dose Modifications, Interruptions and Discontinuations

One placebo tablet will be used and its content will not be changed, irrespective of indications.

Participants have the right to withdraw from the study at any time for any reason. If a participant wishes to withdraw from the study, this will not affect their care during their pregnancy.

All efforts will be made to report the reason for withdrawal as thoroughly as possible.

Should a patient withdraw from study drug only, efforts will be made to continue to obtain follow-up data, with the permission of the patient.

At all patient withdrawals a patient follow up form will be completed.

Withdrawn participants are not replaced as the analysis is based on intention to treat.

6.4.3   Accountability

Trial medications will be dispensed under the supervision of a local hospital pharmacist. Study drugs will be supplied only to women participating in the study.

The responsible pharmacist, and delegated staff at each clinical site, are responsible for ensuring that all study drugs at the site are inventoried and accounted for throughout the study. The dispensing of study drugs will be documented and accounted for. Study drugs will be handled in strict accordance with the protocol and will be stored in a controlled access area within the pharmacy. Study drugs will be kept within appropriate humidity conditions and under 25°C. Temperature logs should be maintained and temperature excursions reported to Hananja plc, who will keep the CTU fully informed at any time via their secured data archive.

IMP will be prescribed using the appropriate trial prescription form, copies of which should be retained in the pharmacy site file. The IMP label will include randomisation number and PI name.

Unused, returned or expired study drugs will be available for verification by the study personnel, clinical IMP monitor (Hananja plc) and the trial monitor. Before proceeding to the destruction of any IMP, authorisation fr om CTU will be sought.

Once authorised, the destruction will be in accordance with the Local Pharmacy Standard

Operating Procedure (SOP) or by incineration.

6.4.4   Compliance and Adherence

Participants will be asked to bring their trial medication to each trial visit; IMP compliance will be assessed by study site staff by counting remaining tablets at each follow up visit and asking about compliance at telephone follow up. In addition, a random sample of participants (10%) will be selected for assessment of compliance via an appropriate biochemical assay.

Analysis of these samples will be performed by an independent laboratory and the trial team will be blinded to results. Results will be reviewed by the ASPRE IDMC.

Compliance with other aspects of the trial protocol will also be assessed. Participants will be encouraged to report any concerns or side effects in a diary for review at each trial visit.

6.4.5   Concomitant Care

Any other medication will be permitted concurrently with the study medication and recorded in the relevant case report form (CRF) at each trial visit.

We advise avoiding aspirin-containing compounds and other non-steroidal anti-inflammatory drugs.

We will collect information about this in CRF.

6.4.6   Overdose of Trial Medication

Participants will be advised to contact the designated trial doctor (telephone number will be provided at enrolment) should they have taken an overdose of the trial medication.

6.4.7   Protocol Treatment Discontinuation

In consenting to the trial, participants are consenting to trial treatments, trial follow-up and data collection.

However, an individual participant may stop treatment early or be stopped early for any of the following reasons:

- ☐ Unacceptable treatment toxicity or adverse event
- ☐ Inter-current illness that prevents further treatment
- ☐ Any change in the participant's condition that in the clinician's opinion justifies the discontinuation of treatment
- ☐ Withdrawal of consent for treatment by the participant

As participation in the trial is entirely voluntary, the participant may choose to discontinue trial treatment at any time without penalty or loss of benefits to which they would otherwise be entitled.

Although not obliged to give a reason for discontinuing their trial treatment, a reasonable effort should be made to establish this reason, whilst remaining fully respectful of the participant's rights.

Participants who discontinue protocol treatment, for any of the above reasons, should remain in the trial where

possible for the purpose of follow up and data analysis. Participants who wish to withdraw from study medication will be asked to confirm whether they are still willing to provide the following.

☐ Trial specific data at visits

☐ Data collected as per routine clinical practice at visits

The investigator also has the right to withdraw participants from the study drug in the event of inter-current illness, adverse events (AE), serious adverse events (SAE), suspected unexpected serious adverse reaction (SUSAR), protocol violations, cure, administrative reasons or other reasons.

All AE, SAE and SUSAR will be reported to the CTU. SAEs and SUSARs will be reported to the local Competent Authorities according to European Commission guidelines CT3 (2011/C, 172/01, June 2011).

It is understood by all concerned that an excessive rate of withdrawals can render the study uninterpretable; therefore, unnecessary withdrawal of participants should be avoided.

If participant wishes to withdraw from the study, this will not affect their care during their pregnancy.

All efforts will be made to report the reason for withdrawal as thoroughly as possible. Should a patient with draw from study drug only, efforts will be made to continue to obtain follow-up data, with the permission of the patient.

At all patient withdrawals a patient follow up form will be completed. Withdrawn participants are not replaced as the analysis is based on intention to treat.

Only data up to the point of withdrawal from the study will be used, unless the patient specifically requests that their data be withdrawn and this will be clearly explained in the consent form and information sheet.

6.5   Outcomes

6.5.1   Primary Outcomes

☐ Incidence of preterm-PET (<37 weeks)

PET will be defined as per the International Society for the Study of Hypertension in Pregnancy (Brown, 2001).

The systolic blood pressure should be 140 mm Hg or more and/or the diastolic blood pressure should be 90 mmHg or more on at least two occasions four hours apart developing after 20 weeks of gestation in previously normotensive women (blood pressure less than 140/90 mmHg) and there should be proteinuria of 300mg or more in 24 hours or urinary protein creatinine ratio (UPCR) of 30 mg/mmol or more or two readings of at least ++ on dipstick analysis of midstream or catheter urine specimens if no 24-hour collection is available.

The efficacy will be assessed by the development of PET at any gestation after 20 weeks of pregnancy

as defined above.

6.5.2 Secondary Outcomes

☐ Incidence of early-PET (<34 weeks) and total PET (at any gestation),

☐ Birthweight below the 3rd, 5th and 10th centile,

☐ Stillbirth or neonatal death due to any cause,

☐ Stillbirth or neonatal death ascribed to PET or FGR,

☐ Stillbirth or neonatal death in association with maternal or neonatal bleeding,

☐ Rate of neonatal intensive care unit admission,

☐ Composite measure of neonatal mortality and morbidity,

☐ Placental abruption (clinically or on placental examination),

☐ Spontaneous preterm delivery <34 weeks and <37 weeks.

Birthweight will be recorded in the participants' medical notes and birthweight percentile for gestational age at delivery is calculated using a normal range derived from our population (Poon et al., 2012).

Other secondary efficacy parameters will be assessed by the clinical team at the time of delivery and recorded in the participants' medical notes (all data is routine clinical practice) and will be transcribed into the trial CRF by the trial team.

Collection of pregnancy and neonatal outcomes
Data on pregnancy and neonatal outcomes will be collected from the hospital maternity records or their general medical practitioners.
The obstetric records of the randomised women with pre-existing or pregnancy associated hypertension will be examined to determine if the condition was chronic hypertension,
 PET or gestational hypertension.
In the event when the neonates are admitted to Special Care Baby Unit (SCBU), additional neonatal outcomes will be collected from the discharge summary of SCBU.

6.6 Participant Timeline

The trial procedure by visit
Screening Visit (11-13 week of pregnancy or CRL 45-84mm) – All consented participants

☐ Informed Consent

☐ Patient demographics

☐ Routine first-trimester scan

- ☐ Risk assessment for PET
- ☐ Height and Weight
- ☐ Maternal and family history
- ☐ MAP
- ☐ Uterine artery blood flow (transabdominal colour Doppler ultrasound)
- ☐ PAPP-A and PlGF measurements

Randomisation visit (11-14 week of pregnancy) – RCT participants

- ☐ Informed Consent
- ☐ Concomitant medications
- ☐ Randomisation
- ☐ IMP dispensing and first dose

Telephone interview 1: 16 weeks – RCT participants

- ☐ Concomitant medication
- ☐ Adverse events/side effects
- ☐ IMP compliance

Follow up visit 1: 20-24 week of pregnancy – RCT participants and all screen-negative participants at sites where a 20-24 week scan forms part of the routine clinical care pathway

- ☐ Routine anomaly scan
- ☐ MAP
- ☐ Uterine artery blood flow (transabdominal colour Doppler ultrasound)
- ☐ PAPP-A and PlGF measurements (RCT participants)
- ☐ Concomitant medications (RCT participants)
- ☐ Adverse events/side effects (RCT participants)
- ☐ Review diary card (RCT participants)
- ☐ IMP compliance (RCT participants)

Telephone interview 2: 28 weeks – RCT participants

- ☐ Concomitant medication
- ☐ Adverse events/side effects
- ☐ IMP compliance

Follow up visit 2: 32-34 weeks for RCT participants and 30-37 weeks for all screen-negative participants at siteswhere a 30-37 week scan forms part of the routine clinical care pathway

- ☐ Routine fetal growth scan
- ☐ MAP

- Uterine artery blood flow (transabdominal colour Doppler ultrasound)
- PAPP-A and PlGF measurements (RCT participants only)
- Concomitant medications (RCT participants)
- Adverse events/side effects (RCT participants)
- Review diary card (RCT participants)
- IMP compliance (RCT participants)

Follow up visit 3: 36 weeks – RCT participants
- Routine fetal growth scan
- MAP
- Uterine artery blood flow (transabdominal colour Doppler ultrasound)
- Concomitant medication
- Adverse events/side effects
- Review diary card
- IMP compliance
- Stop IMP

Telephone interview 3: 30 days after the last dose of IMP – RCT participants
- Adverse events

The period for adverse events reporting will be from the time of first dose until 30 days post final IMP administration. The participants will be followed up by a telephone interview 30 days after the last dose of IMP.

When the patient completes the trial or is withdrawn the patient status will be collected on a follow up form (part of the CRF). If the patient has a miscarriage or has delivered before 36 weeks, the trial co-ordinators will contact these women and ask them to return the trial medication separately.

Laboratory Tests

At the time of the 11-13 weeks scan, 20mL of maternal blood will be taken for the measurement of PAPP-A, free β-hCG and PlGF using automated machines that provide reproducible results (DELFIA Xpress system, PerkinElmer Life and Analytical Sciences, Waltham, USA) as part of the routine screening for Down's syndrome on sit e. Serum concentration of placental protein 13 (PP13; Hy Laboratories Ltd., Rehovot, Israel) will be measured by robotic ELISA.

The remaining serum and plasma will be stored at -80oC for future studies of potential biochemical markers for pregnancy complications.

6.6.1 Early Stopping of Follow-up

If a participant chooses to discontinue their trial treatment, they should continue to be followed up as closely as possible to the follow-up schedule defined in the protocol, providing they are willing.

They should be encouraged and facilitated not to leave the whole trial, even though they no longer take the trial treatment.

If, however, the participant exercises the view that they no longer wish to be followed up either, this view must be respected and the participant withdrawn entirely from the trial.

UCL CTU should be informed of the withdrawal in writing using the appropriate ASPRE trial documentation.

Data already collected will be kept and included in analyses according to the intention-to-treat principle for all participants who stop follow up early.

Participants who stop trial follow-up early will not be replaced.

6.6.2 Participant Transfers

If a participant moves from the area making continued follow up at their consenting centre inappropriate, every effort should be made for them to be followed at another participating trial centre. Written consent should be taken at the new centre and then a copy of the participant's CRFs should be provided to the new centre. Responsibility for the participant remains with the original consenting centre until the new consent process is complete.

6.6.3 Loss to Follow-up

For participants that are lost to follow up, every effort should be made to contact their general practitioners in order to acquire the pregnancy outcomes. We will also use NHS spine to trace them by NHS number in case they change their GP.

6.6.4 Trial Closure

The end of the study will be defined as the last visit of the last patient (n=1,684) with details of their complete pregnancy outcome. This will take approximately 18 months to complete.

The MHRA will be notified of the end of the trial within 90 days of its completion.

6.7 Sample Size

The sample size calculation is based on a 76% detection rate of the multi-parameter screening for preterm-PET at a screen positive rate of 10%.

With the aim to achieve a significant 50% reduction in the prevalence of preterm-PET from 7.6% in the placebo group to 3.8% in the aspirin group, with a power of 90%, and 5% significance level, it is necessary to randomise 1,600 high-risk pregnancies.

If we allow for 5% loss to follow up (Yu et al. 2008), it will be necessary to randomise a total of 1,684 high-risk pregnancies, 842 women in each of the aspirin and placebo arms.

On the assumption that 50% of high-risk pregnancies will agree to randomisation we need to identify 3,368 high-risk pregnancies (that will constitute 10% of the screened population). We will therefore have to recruit a total of 33,680 pregnancies to the screening study.

6.8 Recruitment and Retention

6.8.1 Recruitment

In all the participating centres, all women attending for first-rimester combined screening for aneuploidies will be invited to take part in the screening study for PET. The PIS for the screening study will be sent with the appointment letter to all potential participants, with the exception of Milan where appointment letters are not sent routinely. Following screening for PET, high-risk women will be invited to take part in the randomised controlled trial by the designated trial teams.

Recruitment rates will be actively monitored by the TMG. This will include analyses by centre of the number of women screened, the number of screen positive and the number randomised. Appropriate strategies will be implemented if recruitment falls below an acceptable level.

6.8.2   Retention

If women fail to attend their follow-up visits, the trial coordinators will contact them to arrange for another visit within 7 days.

6.9   Assignment of Intervention

6.9.1   Allocation

6.9.1.1 Sequence generation

Randomisation sequence using a simple permuted block will be provided by Hananja plc. They will apply a random number to each of the treatment packs (tablet bottle) and the appropriately numbered drug will be dispensed by each hospital. The randomisation code will only be revealed to the researchers after completion of the study or where clinically essential.

6.9.1.2 Allocation concealment mechanism

Each tablet bottle will only be identified by a randomisation code. When a participant enrols onto the study, a randomisation code will be generated via Sealed Envelope's website. She will bring the request form with the allocated randomisation code to the pharmacy, where the pharmacy will provide her with the tablet bottle matched to the randomisation code. Neither the pharmacy at each clinical site, or the clinical staff, will have access to the code, unless in case of emergency.

6.9.1.3 Allocation Implementation

The responsibility for who will enrol and receive IMP is the Principal Investigator (PI). S/he makes his/her eligibility decision according to the approved protocol.
 Other physicians, employed at the same Clinical Site may enroll participants as long as they appear on the ASPRE Trial Delegation log and are signed off by the PI.

6.9.2   Blinding

Hananja plc will provide labelling (for all bottles and boxes) ensuring complete blinding of the IMP to all investigators and participants in the study. That includes the PI, participating research doctors, pharmacists at the local clinical trial pharmacy, project managers and others involved in the trial.

They are all blinded to the IMP.

As mentioned previously, the tablets will be identical, so it will not be possible to distinguish between the active (aspirin) and placebo.

Hananja plc will keep the codes safe and locked. However, a secure website has been designed for code breaking in case of emergency.

6.9.3   Emergency Unblinding

In case of emergency, the code may be broken at any time during the study. Code break will be provided by Sealed Envelope through their web site (http://www.sealedenvelope.com/trials/). Only pre-authorised participants (CI, Co-PI and all site-PIs) will receive a login name therefore providing access control.

When registered, the designated PI will receive a password via e-mail.

This password will allow him/her to type in the randomisation number and receive information regarding aspirin or placebo. This website will be accessible at all times during the trial. In the event of the website not being accessible, Sealed Envelope will provide a back-up telephone service.

Please call Sealed Envelope on +44-20-3384-6368 if you are unable to access the website. The ASPRE study number is 5277.

If a site-PI breaks a code, an automated e-mail will be sent to him/her requesting a report to be filed. An email will also be sent to Prof. Kypros Nicolaides, Dr. Leona Poon and UCL CTU informing them that there has been a code break, but without revealing the allocation and at which site.

6.10 Data Collection, Management and Analysis

6.10.1 Data Collection Methods

Patient information for this study will be entered into an electronic CRF that will be printed and signed by the enrolling researcher. The CRF will be composed of 3 parts:

CRF part 1 This part will include patient demographics, medical and obstetric history, measurement of MAP, collection of blood samples for biochemical testing, performance of an ultrasound scan to confirm the GA by the measurement of fetal CRL and measurement of the uterine artery Doppler PI;
and calculation of a risk score for preterm-PET.
Participants will be identified on CRFs by initials, patient code and site name, date of enrolment and the enrolling site-PI or fellow.

CRF part 2 will record details of the routine clinical visits at 20-24 weeks and/or 32-34 weeks, where applicable, and 36 weeks including fetal growth assessment, uterine artery PI and MAP and details of the telephone interviews at 16 weeks and 28 weeks. Information on drug compliance and side effects will be collected.
CRF part 3 will record details of pregnancy outcomes, details of labour, development of PET, neonatal birth weight and outcomes.

All parts of CRFs should be signed by the site-PI and/or fellows for each participant enrolled, including those removed/withdrawn from the study for any reason. The reason for removal/withdrawal must be noted on the study conclusion CRF by the site-PI.
CRFs must be kept current to reflect the participant's status at each phase during the course of the study.

CRF part 1 and 3 will be used in the screening quality study.

The CRFs will be the source documents of the study that must be available at all times for inspection by the regulatory authorities.

A participant identification record will be kept by each site-PI that would allow linking of the participant Study number, participant name and date of birth for those included in the study along with participant contact information. This file will be kept locked at the site-PI cabinet.

6.10.2 Non-Adherence and Non-Retention

For non-adherers (as defined by poor attendance to clinical visits) we will record details of

pregnancy outcomes,details of labour, development of PET, neonatal birth weight and outcomes as

for adherers. Reasons for non-adherence and non-retention and those lost to follow up

 will be recorded in the CRF.

6.10.3 Data Management

The CTU will act as custodian for the trial data. The following guidelines will be strictly adhered to:

☐ Patient data will be pseudo-anonymised.

☐ All anonymised data will be stored on a password protected CTU computer.

☐ All trial data will be stored in compliance with the Medicines for Human Use (Clinical Trials) Amended

Regulations 2006 and the Data Protection Act and archived in line with the Medicines for Human Use (Cli

nical

Trials) Amended Regulations 2006 as defined in the Sponsor's Archiving SOP.

6.10.4 Statistical Methods

6.10.4.1Statistical Analysis Plan

A stand-alone Statistical Analysis Plan (SAP) detailing the analysis will be produced

during the recruitment stage of the study.

To ensure that the analysis can be validated and is reproducible, this document will include listings of

all program code used for the statistical analysis together with sample outputs, tables and figures.

The SAP may be informed by analyses of data blinded to outcome, but it will be finalised before the

blind is broken.

The SAP will be approved by the DMC and by the TSC.

Results will be reported according to the CONSORT statement. It is envisaged that the analysis will be undert

aken using R and WinBugs software.

6.10.4.2Statistical Methods – Outcomes

Primary Analysis:

The primary analysis will comprise an intention-to-treat comparison of the two groups with respect to the

proportion of high-risk pregnancies that develop preterm PET at the two tailed 5% level. 95% confidence intervals will be produced for the proportions developing preterm PET in each of the two groups and for the difference (active – placebo).

Planned secondary analysis of the primary outcome will include a survival analysis of the time to delivery with PET treating births for other causes as censoring.

Pre-specified baseline variables considered to be predictive will be included as appropriate.

Their interactions with the treatment effect will be investigated. Gestational age at

randomisation and its interaction with treatment will also be investigated.

This analysis of treatment interactions will be considered as exploratory.

Descriptive statistics: A full set of descriptive statistics for all variables, overall and by treatment group, will be produced. Graphical displays will be produced as appropriate.

Secondary Analysis: Secondary outcomes will be compared across treatment groups using appropriate tests. P values and 99% confidence intervals will be produced for treatment effects. No corrections will be made for multiplicity.

Safety: The incidence rates of adverse events and serious adverse events and their relationship to trial drugs will be summarized by treatment group.

The proportion of women discontinuing treatment will be summarized by reason and by treatment group.

All investigators, participants and clinicians will be unaware of the treatment groups. All outcomes will be determined before the randomisation code of the trial is broken.

6.10.4.2.1   Economic evaluations
Not applicable

6.10.4.3 Additional Analyses - Subgroup
Not applicable

6.10.4.4 Additional Analyses – Adjusted
Not applicable

6.10.4.5 Analysis Population and Missing Data
Not applicable

6.11 Data Monitoring

6.11.1 Data Monitoring Committee

The Data Monitoring Committee (DMC) is independent from the trial and is responsible for monitoring the progress of the trial including: recruitment, protocol adherence, serious adverse events and side effects of treatment as well as the difference between the trial treatments on the primary outcome measures. The data monitoring committee will be appointed and will meet at least annually to assess the safety data. They will provide a confidential trial progress report at the end of each meeting, which will be sent to the TSC.
The chief investigator (or their representative), trial statistician and other trial staff may be in attendance for the open session of the DMC meeting.

Further details of the roles and responsibilities of the Data Monitoring Committee (DMC), including members hip, relationships with other committees, decision making processes,
and the timing and frequency of interim analyses (and description of stopping rules and/or guidelines where applicable) are described in detail in the ASPRE DMC Terms of Reference (ToR).

6.11.2 Interim Analyses

Given that recruitment will be completed before outcome data are available on a sufficient number of patients, no interim analyses are scheduled. However, depending on factors such as the recruitment rate, the DMC may decide on the need for an interim analysis.

6.11.3 Data Monitoring for Harm

Any unfavourable and intended sign, symptom or illness that develops or worsens during the period of the stu dy will be classified as an adverse event (AE),
whether or not it is considered to be related to the study treatment.
Adverse events will include unwanted side effects, sensitivity reactions, abnormal laboratory results, injury or inter-current illnesses, and may be expected or unexpected. These will be recorded electronically on the CRF.
Aspirin at high doses may induce hypersensitivity and asthma, may cause urate kidney stones, chronic gastro-intestinal blood loss, tinnitus, nausea and vomiting, and has been reported (with high blood salicylate levels) t o prolong pregnancy and labour, with increased bleeding before and after delivery, decreased
birth weight and increased rate of stillbirth. However, several major randomised controlled trials using low-dose aspirin have shown no adverse effects to the mother or the fetus during pregnancy,

delivery or epidural anaesthesia. The Collaborative Perinatal Project prospectively monitored 50,282 mother-child pairs, 64% of which were exposed to aspirin at some point during pregnancy; it found no differences in outcomes between those exposed and those not exposed to aspirin. Furthermore, longer-term follow up studies are now emerging which attest to the safety of aspirin on the development of children exposed to aspirin in utero. In an exhaustive literature review on the use of low dose aspirin in pregnancy, Dekker and Sibai (1993) conclude that 'there is no evidence that low-dose aspirin carries any significant maternal or fetal risks'.

Safety evaluations will be conducted at each of their follow-up visits. Co-PI or site-PIs can be directly contacted by the participants if there are any concerns regarding their medication.

The period for adverse event reporting will be from the time of first dose until 30 days post final IMP administration. The participants will be followed up by a telephone interview 30 days after the last dose of IMP.

All events will be followed until resolution if that means beyond 30 days post-final IMP implementation.

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CHAPTER 3 - DEVELOPMENT OF ALGORITHMS ABLE TO OPTIMIZE THE OUTCOME OF ASSISTED CONCEPTION PREGNANCIES AND TO MANAGE THE CORRELATED OBSTETRIC RISK

- *COMMENT*

The Pan-European randomized multicentre trial-ASPRE - is on going and the results are not available yet.

All women undergoing routine screening for aneuploidies will be invited to participate in the screening study for PET.

The IVF singleton pregnancy will be included in the trial: it means the possibility to evaluate, in a prospective way, the impact of the method of conception on pregnancies outcome and, in particular, on the development of complications as early preeclampsia compared to spontaneus pregnancies. Moreover, the data collected in this randomized study will allow to compare the treatment (aspirin) vs placebo in the IVF subgroup at high risk for PET.

This means to complete the topic studied into the first research line and answer to the question marks previously presented.

Regarding the second project – i.e. the development of a multi markers algorithm for individualizing the starting dose of FSH in COS, it is at moment under EC's approval.

It will definitely permit to introduce for the first time in Reproductive Medicine a new "tailored" approach for stimulation and treatment of infertile patients.

CHAPTER 4 - CONCLUSIONS

Medicine is now undergoing a major revolution that will transform the nature of healthcare from reactive to preventive.

The changes will be catalyzed by a new systems approach to disease that will trigger the emergence of personalized medicine — a medicine that focuses on the integrated diagnosis, treatment and prevention of disease in individual patients.

This PhD thesis has demonstrated that:
- o ART pregnancies are associated at high risk of obstetric complications such as early-PE
- o Ultrasound alone is not able to predict the risk of early-PE in ART pregnancies
- o Using a combined multi markers algorithm, it is possible to screen patient at high risk of PE and hopefully-once the ASPRE trial will be concluded- to prevent it giving low dose of aspirin
- o Hopefully, it will be possible to combine genetic and ultrasound information to the endocrine, biochemical and anthropological variables in a new algorithm (on FMF model), introducing for the first time in Reproductive Medicine field a new approach.
- o The use of 3D ultrasound for antral follicle count (AFC) introduces an objective way to monitor the COS
- o The knowledge of ovarian gonadotrophins-receptor polymorphisms will help to optimize the dose and the timing of ovarian stimulation protocols

The convergence of systems approaches to disease, new measurement and visualization technologies, and new computational and mathematical tools can be expected to allow our current, largely reactive mode of medicine, where we wait until the patient is sick before responding, to be replaced over the next 10 to 20 years by a *personalized, predictive, preventive, and participatory* medicine that will be cost effective and increasingly focused on wellness.

Detect disease at an earlier stage, when it is easier and less expensive to treat effectively; Stratify patients into groups that enable the selection of optimal therapy; reduce adverse drug reactions by more effective early assessment of individual drug responses; improve the selection of new biochemical targets for drug discovery; reduce the time, cost, and failure rate of clinical trials for new therapies

This hopefully will shift the emphasis in medicine from reaction to prevention and from disease to wellness.