Study Protocol

Comparison of hhCG with hCG+ß in the early prediction of ongoing pregnancy after in-vitro fertilization and embryo transfer. (hyperPOC)

A monocentric, prospective, open, biomedical study

Code: 1701-VLC-011-EB (CIM RD002786)

May 09, 2017

Responsibilities and Signature:

Dr. Ernesto Bosch, Principal Investigator of the biomedical study "Comparison of hhCG with hCG+ß in the early prediction of ongoing pregnancy after in-vitro fertilization and embryo transfer (hyperPOC)",

- confirms that this study respects the ethical and legal norms applicable to this type of study and will fulfill the norms of Good Clinical practices throughout the study.
- has the necessary material and human resources to carry out the study, and this will not interfere with other studies or other practices which take place habitually.
- is committed to assure that all the subjects in the study are treated according to that which is established in the study protocol which has approval from the corresponding Ethics Committee.
- confirms that all the collaborators partaking in the undertaking of this study are qualified and trained to do so.

Principal Investigator:

Dr. Ernesto Bosch
Medical Director IVI Valencia

Date

	Name/Function	Date	Signature
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REVISION HISTORY

Version	Changes	Date	Responsible
1	New document	Jan 31, 2017	Ernesto Bosch, MD, PhD Martin Hund, PhD Reinhard van der Does, MD
1.1	Shift of documentation of number of embryos transferred to Screening assessment Definition of clinical pregnancy on day 35 (+3) as detection of embryo sac and subsequent adaptations including the definitions of pregnancy categories	March 10, 2017	Ernesto Bosch, MD, PhD Martin Hund, PhD Reinhard van der Does, MD
2.0	Changed recruitment time to: June 1, 2017 to February 28, 2018 Visit dates: now counted from date of embryo transfer instead of date of oocyte pick-up, leading to a reduction of figures by 5 for all visit dates, as day-5 blastocysts are being transferred. New visit (Visit 4) with US inserted at day 21 (+4/-3), range of visit at day 28 (Visit 5) changed to (+4/-3), at Visit 6 documentation of pregnancy category. Inclusion criteria: Also frozen day-5 blastocysts and egg donation permitted, maximum age increased to 45 years. Subsequently for exclusion criteria: egg donation deleted; term 'gestational surrogacy' specified.	May 09, 2017	Ernesto Bosch, MD, PhD Martin Hund, PhD Reinhard van der Does, MD

GLOSSARY OF ABBREVIATIONS

AE	Adverse Event
AUC	Area Under the Curve
eCRF	Electronic Case Report Form
ET	Embryo Transfer
EU	European
hhCG	Hyperglycosylated human chorionic gonadotropin
IVF	In Vitro Fertilization
ROC	Receiver Operator Curve
US	Ultrasound

SYNOPSIS OF PROTOCOL

ACRONYM	HyperPOC (hyperglycosylated hCG Proof Of Concept study)
TITLE	Comparison of hhCG with hCG+ß in the early prediction of ongoing pregnancy after in-vitro fertilization and embryo transfer
STUDY NUMBER	1701-VLC-011-EB (CIM RD002786)
TRIAL CLASSIFICATION	Biomedical study
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SUPPORT FUNCTIONS	Biostatistics: Silke Ahlers, Prometris GmbH, Mannheim, Germany
PRODUCTS	hhCG (Quest Diagnostics), Elecsys® hCG+ß
INDICATION	Prediction of ongoing pregnancy in women undergoing IVF
STUDY DESIGN	Monocentric, prospective, open, biomedical study
STUDY PURPOSE SUMMARY	Proof of concept for the prediction of ongoing pregnancy in women undergoing IVF using hhCG

PRIMARY PURPOSE	Utility of the hhCG assay (Quest Diagnostics), early after In-Vitro Fertilization and fresh or frozen Embryo Transfer (IVF-ET), as aid in the prediction of ongoing pregnancy
STUDY OBJECTIVES	<u>Primary objective:</u> To determine, based on the AUCs of the ROC curves, whether serum hyperglycosylated human chorionic gonadotropin (hhCG) measured on day 4 (+1) after ET* is superior to hCG+ß measured on day 4 (+1) in predicting clinical pregnancy on day 21 (+4/-3) (=6 weeks after last menstruation, with detection of embryo sac by US). *ET= Embryo Transfer; all days mentioned in the following are counted from ET (=Day 0).
	<u>Secondary objectives*:</u> 1. To determine, based on the AUC of the ROC curves, whether hhCG measured on day 7 (+1) and day 11 (+2) is superior to hCG+ß measured on the same days in predicting clinical pregnancy on day 21 (+4/-3) 2. To determine, based on the AUC of the ROC curve, whether hhCG measured on day 4 (+1), day 7 (+1) and day 11 (+2) is superior to hCG+ß measured on the same days in predicting ongoing pregnancy on day 65 (+7) . 3. To determine, based on the AUC of the ROC curve, whether hhCG measured on day 4 (+1) and day 7 (+1) is superior to hCG+ß measured on the same days in predicting pregnancy on day 11 (+2) defined by serum hCG+ß. 4. To compare the sensitivity and specificity and the positive and negative predictive value of hhCG with hCG+ß measured on days 4 (+1), 7 (+1) and 11 (+2) for clinical pregnancy on day 21 (+4/-3) and ongoing pregnancy on day 65 (+7) and to identify cut-off values. 5. To compare the sensitivity and specificity and the positive and negative predictive value of hhCG with hCG+ß measured on days 4 (+1) and 7 (+1) for pregnancy on day 11 (+2) defined by serum hCG+ß and to identify cut-off values. 6. To determine, based on the AUCs of the ROC curves, whether changes in hhCG on day 7 (+1) vs. day 4 (+1) and day 11 (+2) vs. day 7 (+1) are superior to corresponding

	changes in hCG+ß in predicting clinical pregnancy on day 21 (+4/-3) and ongoing pregnancy on day 65 (+7). 7. To determine, based on the AUCs of the ROC curves, whether changes in hhCG on day 7 (+1) vs. day 4 (+1) are superior to corresponding changes in hCG+ß in predicting pregnancy on day 11 (+2) defined by serum hCG+ß. 8. To compare the sensitivity and specificity and the positive and negative predictive value of changes in hhCG with those of hCG+ß on day 7 (+1) vs. day 4 (+1) and day 11 (+2) vs. day 7 (+1) for clinical pregnancy on day 21 (+4/-3) and ongoing pregnancy on day 65 (+7). 9. To compare the sensitivity and specificity and the positive and negative predictive value of changes in hhCG with those of hCG+ß on day 7 (+1) vs. day 4 (+1) for pregnancy on day 11 (+2) defined by serum hCG+ß. 10. To determine the course of hhCG and hCG+ß from day 4(+1) through day 11(+2) 11. To determine the usefulness of absolute values and changes in hhCG over time in distinguishing between the 5 disjunct pregnancy categories of 1) negative pregnancy, 2) ectopic pregnancy, 3) 1st trimester miscarriage 4) ongoing pregnancy on day 65 (+7) and 5) biochemical pregnancy. 12. To investigate into differences in the course of hhCG values between single and multiple pregnancies (if warranted by the number of multiple pregnancies) 13. To determine incidents and indirect harms. *All days mentioned above are counted from ET (=Day 0).
TARGET STUDY POPULATION	Inclusion criteria: • Written informed consent • Patients undergoing their first or second IVF-ET cycle • Age 18-45 years • Use of Ovitrelle for final follicular maturation and luteinization in the case of fresh ET • Fresh or frozen day-5 blastocysts transfer (autologous or egg donation)

	Exclusion criteria: • Gestational surrogacy (patient's eggs used for pregnancy in a surrogate mother) • Canceled IVF cycles • GnRH agonist triggering cycles in the case of fresh ET
STUDY DURATION PLANNED	Clinical part of the study: from June 2017 to April 2018 Patient recruitment time: from June 1, 2017 to Dec 31, 2017
STUDY POPULATION & TOTAL NUMBER OF PATIENTS	A sample size of n= 136 patients eligible for the per-protocol analysis has been calculated of whom n=61 are expected to present with clinical pregnancy on day 21* (+4/-3). Based on an assumed rate of 10% patients enrolled but ineligible for the per-protocol analysis, n=152 patients are planned to be included in the study. *Counted from ET (=Day 0).
NO. OF SITES	Monocentric study
SCHEDULE OF ASSESSMENTS	See Schedule of Assessments below
SAFETY	As the only impact on patient safety by the study is from taking two additional blood samples, a general AE collection and assessment will not be carried out. Any AEs occurring in temporal relationship to drawing the two blood samples in addition to the study site's clinical routine will be documented observing the rights of patients' compensation for damage through participation in the study and the requirements of the insurance policy taken out. Incidents in connection with the diagnostic test will be documented following EU and local regulations and guidelines. Indirect harms to the patients are not possible to occur as the test result will only be known after individual study end. Hence, any incorrect results of the test can have no consequence for the further course of the patient during the study.
COMPLIANCE STATEMENT	The study will be conducted in compliance with the Guidelines for Good Pharmacoepidemiology Practices (GPP) and applicable regulatory requirements.

Schedule of Assessments

	Screening	Visit 1	Visit 2	Visit 3	Visit 4	Visit 5	Visit 6
Days from ET[1]		Day 4 (+1)	Day 7 (+1)	Day 11 (+2)	Day 21 (+4/-3)	Day 28 (+4/-3)	Day 65 (+7)
Patient information	●						
Informed consent [2]	●						
Demogr. data, Med. hist.[3]	●						
Body weight and height	●						
Concomitant diseases	●	●	●	●	●	●	
Concomitant medication	●	●	●	●	●	●	
Incl. / excl. criteria	●						
Number of embryos transferred	●						
Serum estradiol, progesterone		●	●	●			
(Serum) hhCG, hCG+ß [4]		●	●	●			
Ultrasound[5]					●	●	
Pregnancy category							●
Incidents and indirect harms							●
Final documentation[6]							●

1 Embryo Transfer = day 0
2 Leaving the patient ample time for consideration after patient information has taken place
3 Including previous pregnancies and complications/outcomes
4 Determination of hCG+ß by study site and asservation of aliquots (0.5 mL of serum for determination of hhCG by Quest Diagnostics, 0.5 mL for potential future biomarker analysis by IVI and 2.0 mL for Roche Diagnostics) (aliquots to be kept frozen at -80^{0}C and shipped in batches of patients having all of their samples completed). hhCG to be analyzed by Quest Diagnostics in batches per patient, i.e. same lot of test used for all samples of a patient.
5 Detection of embryo sac, heart beat or ectopic pregnancy.
6 Final documentation may be earlier in case of premature termination. Centrally measured hhCG values will be entered as soon as available.

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1 INTRODUCTION

1.1 hhCG Method Description

Serum samples will be collected and shipped on dry ice to Quest Diagnostics (Quest Diagnostics, Nichols Institute, San Juan Capistrano, CA) for measurements of hhCG. The ultrasensitive hhCG assay will be performed using a procedure described by Pandian et al. [1]. hhCG will be measured using an electrochemiluminescence (ECL) technique on 96-well plates from Meso Scale Discovery. This immunometric assay uses the hhCG specific antibody B-152 as the coating antibody and the hCG+ß beta specific antibody B-207 as the labeled antibody with Sulfo-Tag (Meso Scale Discovery). The light signal (relative light unit, RLU) generated by the antibody-antigen and antibody reaction will be measured by a luminometer (Meso Scale Discovery). The RLU is directly proportional to the concentration of hhCG.

1.2 Background and Study Rationale

1.2.1 Background

Hyperglycosylated human chorionic gonadotropin (hhCG) as it was later called was first isolated from the urine of women with invasive trophoblastic disease [2]. It is a hyperglycosylated isoform of human chorionic gonadotropin (hCG) with more extensive and more complex carbohydrate moieties than hCG during the initial 3 weeks of pregnancy hhCG is the primary hCG isoform [3–9]. It is synthesized primarily by cytotrophoblasts, whereas hCG is produced by syncytiotrophoblasts [12, 13]. hhCG is supposed to be of relevance during implantation into the uterine wall [13–15]. The monoclonal antibody against hhCG, B152, has a specificity for hhCG of >99 % relative to hCG [4, 8, 10], whereas many other antibodies against hCG show significant cross reactivity with hhCG [11].
Urinary hhCG levels during pregnancy were shown to rise 3 days sooner on average than those of hCG. The ratio of hhCG to hCG may allow to differentiate between clinical and biochemical pregnancy [5].
In a retrospective monocenter evaluation in 112 women with in vitro fertilization (IVF), fresh embryo transfer (ET) and day-9 and day-16 after egg retrieval (equals days 4 and 11 after ET) serum samples carried out by Chuan et al. it was found that a day-9 hhCG level of >110 pg/mL was 96% specific for ongoing pregnancy, yielding a positive predictive value of 94%. Compared with the day-9 hCG level, hhCG was more sensitive and had a larger area under the curve (0.87 vs. 0.67, respectively). In contrast, the diagnostic test characteristics were similar between the day-16 hhCG and hCG levels [16]. In a prospective blinded clinical trial by Strom et al. enrolling 58 IVF-ET patients a single serum or urine hhCG measurement identified pregnancies (both biochemical and clinical) on day 6 post ET with 100% sensitivity and specificity, whereby it was possible to identify biochemical pregnancies by their lower hhCG values [17].

1.2.2 Study Rationale

Proceeding on the results of previous clinical studies and particularly of the two studies by Chuan and Strom mentioned above [16, 17], it is a logical further step to try and validate the hypothesis of a superior predictive value of hhCG vs. hCG early after IVF-ET for clinical and ongoing pregnancy in a prospective study sufficiently powered based on existing data for the two diagnostic tests. The nature of the study is therefore that of a proof of concept trial.

1.3 Study Objectives

See Study Synopsis.

2 STUDY DESIGN AND ASSESSMENTS

2.1 Overview of Study Design

The study has been planned considering the principles of evidence based medicine using the PICO criteria [18]. It is a monocenter, open, biomedical study in patients undergoing in-vitro fertilization. The purpose of proof of concept requires keeping variability within very narrow limits. Accordingly, there is a strict definition of the selection criteria for patients to be enrolled, and the primary analysis will be carried out in the per protocol population.

It is planned to include a total of 152 patients. The study protocol is based on routine procedures at the site, with the only additional measures stipulated being the taking of 6-mL venous blood samples on visits 1 and 2 and the asservation of another 6-mL of venous blood on visit 3 when blood is taken anyhow within the scope of clinical routine. The three 6-mL blood samples will be processed for central assessment of hhCG. Neither the patient nor the investigator will know the result of the centralized analysis prior to individual study completion. Apart from the two blood samples mentioned there are only two further procedures involving the patients directly. This refers to the ultrasound assessments at visit 4 and the final visit for the assessment of clinical and ongoing pregnancy (both part of the study site's routine measures).

2.1.1 Blinding of Study

The study will be conducted in an open fashion. However, results of the hhCG assessments will only become available after study end.

2.1.2 Scope of Study

The study data will serve for a proof-of-concept evaluation.

2.2 In-/Exclusion Criteria

See Study Synopsis.

2.3 Patient Recruitment

Patients will be screened in a consecutive, unselected manner, and, after written informed consent has been obtained, will be eligible to be enrolled in the study if all inclusion and none of the exclusion criteria are met.

2.4 Data Collection

For an overview see Schedule of Assessments in the Protocol Synopsis.

Patients will only be included into the study after they have given their informed consent in writing. The study will begin with a Screening phase starting with Patient information and Patient's Informed Consent (leaving the patient ample time for consideration after Patient Information) and followed by the documentation of general patient data. On the basis of available data the decision as to the patient's eligibility to participate in the study will then be made considering the inclusion/exclusion criteria. In the case of eligible patients a total of five visits (planned in accordance with clinical routine) will follow during a period of a maximum of 68 days starting with day 4(+1) after ET (= Day 0).

As compared with clinical routine an additional 6 mL of blood is planned to be taken on each visit 1-3 (on Visit 3 a blood sample is drawn anyhow following clinical routine). Hence, the total amount of blood taken for the purpose of the study will be 18 mL (not considering the small amounts of blood needed for measuring serum estradiol and progesterone by IVI for visits 1 and 2). During visits 1 - 3 an aliquot of 0.5 mL of serum will be stored for the central assessment of hhCG by Quest Diagnostics, 0.5 mL for potential future biomarker analyses by IVI and another 2.0 mL for shipment to Roche potentially to be used for repeat measurements or measurements within the scope of developing a new hhCG immunoassay. Apart from this, there will be no further study-related measures with a potential impact on the patient's health and well-being. The US assessments at Visit 4 and 5 are part of clinical routine assessments.
Therefore, a general AE collection and assessment will not be carried out. Any AEs occurring in temporal relationship to drawing the two blood samples in addition to the study site's clinical routine will be documented on paper forms observing the rights of patients' compensation for damage through participation in the study and the requirements of the insurance policy taken out. Concomitant diseases and medication will be documented at each visit. Any changes from previous visits found, however, will not be followed up as a possible indication of an adverse event. Incidents in connection with the diagnostic test will be documented following EU and local regulations and guidelines. This will be done on a paper form and the information will not be entered into the eCRF. Indirect harms to the patients are not possible to occur as results of the central hhCG analyses will only be known to the study site after study end. Hence, any incorrect results of the hhCG test can have no consequences for the further course of the patient during the study.

Screening Phase

- Patient information
- Patient's informed consent
- Demographic data (race, ethnicity, age)
- Medical history including history of previous pregnancies and their outcomes and complications
- Body weight and height
- Concomitant diseases and medication
- Check of inclusion and exclusion criteria
- Number of embryos transferred and dates of oocyte pick-up, fertilization and ET

Visit 1

At Visit 1 the following will be done and documented in the eCRF:

- Measurement and documentation of serum estradiol, progesterone and hCG+ß values by the study site
- Taking of 6 mL of venous blood for asservation of 0.5 mL of serum (to be kept in the freezer at -80^{0}C with temperature log and shipped in batches for the central analysis of hhCG), of a further 0.5 mL for IVI kept in the freezer at -80°C and another 2.0 mL serum kept in the freezer at -80^{0}C to be shipped to Roche at the end of the study.
- Concomitant diseases and medication.

Visit 2

At Visit 2 the following will be done and documented in the eCRF:

- Measurement and documentation of serum estradiol, progesterone and hCG+ß values by the study site
- Taking of a further 6 mL of venous blood for asservation of 0.5 mL of serum (to be kept in the freezer at -80^{0}C with temperature log and shipped in batches for the central analysis of hhCG), of a further 0.5 mL for IVI kept in the freezer at -80^{0}C and of another of 2.0 mL kept in the freezer at -80^{0}C for to be shipped to Roche at the end of the study.
- Concomitant diseases and medication.

Visit 3

At Visit 3 the following will be done and documented in the eCRF:

- Measurement and documentation of serum estradiol, progesterone and hCG+ß by the study site
- Separation of 6 mL of blood taken in addition to routine blood sampling for asservation of 0.5 mL of serum (to be kept in the freezer at -80^{0}C with temperature log and shipped in batches for the central analysis of hhCG), of a further 0.5 mL for IVI kept in the freezer at -80^{0}C and of another 2.0 mL kept in the freezer at -80^{0}C for to be shipped to Roche at the end of the study.
- Concomitant diseases and medication.

Visit 4

At Visit 4 the following will be done and documented in the eCRF:

- Concomitant diseases and medication
- Ultrasound for detection of embryo sac, (heart beat) or ectopic pregnancy.

Visit 5

At Visit 4 the following will be done and documented in the eCRF:

- Concomitant diseases and medication
- Ultrasound for detection of embryo sac, heart beat or ectopic pregnancy.

Visit 6

At Visit 5 the following will be done and documented in the eCRF:

- Concomitant diseases and medication
- Documentation of pregnancy category: negative pregnancy, ectopic pregnancy, 1st trimester miscarriage, ongoing pregnancy (single/multiple) on day 65 (+7), biochemical pregnancy (see section 5.1)
- Incidents and indirect harms
- Final documentation.

2.5 Endpoints of the Study

2.5.1 Primary Endpoint

AUC of the ROC curve for hhCG and hCG+ß on day 4 (+1) after ET for the prediction of clinical pregnancy on day 21 (+4/-3) after ET (=6 weeks after last menstruation, detection of embryo sac by US).

2.5.2 Secondary Endpoints

See also Section 5 for definitions. Secondary endpoints of the study are*:

1. AUC of the ROC curve for hhCG and hCG+ß on days 7 (+1) and 11 (+2) for the prediction of clinical pregnancy on day 21 (+4/-3)
2. AUC of the ROC curve for hhCG and hCG+ß on days 4 (+1), 7 (+1) and 11 (+2) for the prediction of ongoing pregnancy on day 65 (+7)
3. AUC of the ROC curve for hhCG and hCG+ß on days 4 (+1) and 7 (+1) for the prediction of pregnancy on day 11 (+2) defined by serum hCG+ß.
4. Sensitivity and specificity and positive and negative predictive value of hhCG and hCG+ß on days 4 (+1), 7 (+1) and 11 (+2) for clinical pregnancy on day 21 (+4/-3)
5. Sensitivity and specificity and positive and negative predictive value of hhCG and hCG+ß on days 4 (+1), 7 (+1) and 11 (+2) for ongoing pregnancy on day 65 (+7)
6. Sensitivity and specificity and positive and negative predictive value of hhCG and hCG+ß on days 4 (+1) and 7 (+1) for pregnancy on day 11 (+2) defined by serum hCG+ß
7. AUC of the ROC curve for changes in hhCG and hCG+ß on day 7 (+1) vs. day 4 (+1) and on day 11 (+2) vs. day 7 (+1) for the prediction of clinical pregnancy on day 21 (+4/-3)

8. AUC of the ROC curve for changes in hhCG and hCG+ß on day 7 (+1) vs. day 4 (+1) and on day 11 (+2) vs. day 7 (+1) for the prediction of ongoing pregnancy on day 65 (+7)
9. AUC of the ROC curve for changes in hhCG and hCG+ß on day 7 (+1) vs. day 4 (+1) for the prediction of pregnancy on day 11 (+2) defined by serum hCG+ß
10. Sensitivity and specificity and positive and negative predictive value of changes in hhCG and hCG+ß on day 7 (+1) vs. day 4 (+1) and on day 11 (+2) vs. day 7 (+1) for the prediction of clinical pregnancy on day 21 (+4/-3)
11. Sensitivity and specificity and positive and negative predictive value of changes in hhCG and hCG+ß on day 7 (+1) vs. day 4 (+1) and on day 11 (+2) vs. day 7 (+1) for the prediction of ongoing pregnancy on day 65 (+7)
12. Sensitivity and specificity and positive and negative predictive value of changes in hhCG and hCG+ß on day 11 (+2) vs. day 7 (+1) for the prediction of pregnancy on day 11 (+2) defined by serum hCG+ß
13. Absolute values and changes vs. previous visits of hhCG and hCG+ß for course of the variables during the study
14. Absolute values and changes vs. previous visits of hhCG and hCG+ß for the distinction of outcomes 1) negative pregnancy, 2) ectopic pregnancy, 3) 1st trimester miscarriage 4) ongoing pregnancy on day 65 (+7) and 5) biochemical pregnancy.
15. Absolute values and changes vs. previous visits of hhCG and hCG+ß for the distinction between single and multiple pregnancies
16. Course of serum estradiol and progesterone during the study
17. Any AEs with a temporal relationship with drawing the two blood samples in addition to the site's clinical routine
18. Incidents involved with hhCG testing.

*All days mentioned in this section are counted from ET (=Day 0).

2.5.3 Safety Variables

AEs are only recorded if they occur in a temporal relationship with drawing of the two blood samples on visits 1 and 2 as these are the only measures carried out in addition to the routine procedures at the site and, hence, for the sole purpose of the study (interventions). A causal relationship of any adverse events with hhCG testing is impossible as a patient's test results will not be made known during the course of the study. Hence, the patients' course during and after the study cannot be influenced by any inaccurate test values or the interpretation of the hhCG values (accurate or inaccurate).

Incidents will be documented following the guidance provided in MEDDEV 2.12.-1 rev. 6 and local regulations and guidelines. Indirect harms are not possible to occur as discussed in section 4.3.2.

2.6 Known and Potential Risks and Benefits

Apart from two additional blood drawings on visits 1 and 2 there will be no further measures (interventions) during the study in addition to such procedures as are part of the clinical

routine at the site. Except for the known and generally accepted risks involved with drawing blood samples, no further risks nor any benefits for the patients are involved.

3 MATERIALS

The project is supported by Roche Diagnostics Centralised and Point of Care Solutions within the scope of a research agreement between IVI Valencia and Roche Diagnostics Centralised and Point of Care Solutions.

4 EVALUATION REQUIREMENTS

4.1 Data Management Plan

Clinical, laboratory and test result data will be entered into an eCRF. A limited range of validation checks will be detailed in a manual and implemented into the eCRF as appropriate. For data analysis SAS software will be used. The study database shall be made available to Roche Diagnostics and the study site in an agreed upon format and data transfer mode.

4.2 Safety of Specimen Handling

For specimen handling the local safety regulation will apply to rule out any possible biological hazard.

4.3 Safety Assessment

4.3.1 Adverse Events

AEs are only recorded if they occur in a temporal relationship with the blood drawings on visits 1 and 2 as these are the only measures carried out in addition to the routine procedures at the site and, hence, for the sole purpose of the study (interventions). A causal relationship of any adverse events with hhCG testing is impossible as a patient's test results will not be made known during the course of the study. Hence, the patients' course during and after the study cannot be influenced by any inaccurate test values or the interpretation of the hhCG values (accurate or inaccurate).

4.3.2 Incidents and Indirect Harms

The documentation and reporting of incidents will follow the guidance provided in MEDDEV 2.12.-1 rev. 6 and local regulations and guidelines.

An incident is any malfunction or deterioration in the characteristics and/or performance of a device, as well as any inadequacy in the labelling or the instructions for use which, directly or indirectly, might lead to or might have led to the death of a subject, or user or of other persons or to a serious deterioration in their state of health.

Indirect harms to the patients are not possible to occur as the test result will only be known after individual study end. Hence, any incorrect results of the test can have no consequence for the further course of the patient during the study.

All incidents are to be recorded on a separate paper form. A description of the event, including the start date, resolution date, action taken, and the outcome should be provided. As a general principle, there should be a pre-disposition to report rather than not to report in case of doubt on whether or not to report an incident. Any report should not be unduly delayed because of incomplete information.

4.4 Documentation of Medication

Concomitant medication will be documented at each visit. Any changes from previous visits found, however, will not be followed up as a possible indication of an adverse event.

5 Diagnostic Criteria and Definition of Analysis Groups

5.1 Pregnancy Endpoints

The study will have to use various pregnancy endpoints for various analyses. For the analysis of the primary objective and some of the secondary objectives the endpoint will be clinical pregnancy on day 21* (+4/-3) defined as detection of embryo sac by US, regardless of whether or not there will be a miscarriage during the later course of the 1st trimester. For some other secondary objectives the endpoint will be ongoing pregnancy on day 65 (+7) defined as detection of fetal heart beat by US at this time point or later). Detection of embryo sac/fetal heart beat on day 65 (+7) replaces missing detection of embryo sac on day 21 (+4/-3) for the definition of clinical pregnancy.

For pregnancy on day 11 (+2) as an individual endpoint and for the allocation of patients to categories as described below (negative and biochemical pregnancy in particular) a cut-off value for hCG+ß on day 11 (+2) of >10 mIU/mL will be used as per practice at the study site.

For the purposes of the analysis with respect to secondary objective no. 11 there will be 5 disjunct pregnancy categories:

1) Negative pregnancy

hCG+ß on day 11 (+2) ≤10 mIU/mL, unless clinical pregnancy, ectopic pregnancy, ongoing pregnancy or 1st trimester miscarriage are detected at a later stage

2) Ectopic pregnancy

Rules out the remaining categories 3), 4) and 5)

3) 1st trimester miscarriage

Rules out (supersedes) categories 4) and 5)

4) Ongoing pregnancy on day 65 (+7)

5) Biochemical pregnancy

hCG+ß >10 mIU/mL on day 11 (+2) and no clinical/ongoing pregnancy at any stage and no ectopic pregnancy and no signs or symptoms of a 1st trimester miscarriage.

The following patients are ineligible for the analysis with respect to secondary objective no. 11: hCG+ß on day 11 (+2) >10 mIU/mL, missing data on ectopic pregnancy/ presence of embryo sac, fetal heart beat and no 1st trimester miscarriage.
1st trimester miscarriage will always be assumed in cases with detection of embryo sac on day 21 (+4/-3) and no ongoing pregnancy on day 65 (+7). It should be noted that per-protocol patients with this constellation will be included in the group of subjects with clinical pregnancy on day 21 (+4/-3) for the primary analysis of the study.
*All days mentioned in this section are counted from ET (=Day 0).

5.2 Patient Group for the Primary Analysis

The primary analysis of the study will be carried out in the per-protocol group. This will include patients who have met all inclusion criteria, violated no exclusion criterion and for whom the data for the evaluation of the primary objective have been determined in compliance with the stipulations of this protocol (i.e. valid results for hhCG and hCG+ß on day 4 (+1)* and for US on day 21* (+4/-3).Ongoing pregnancy on day 65 (+7) replaces missing US data for day 21 (+4/-3) as to the question of ongoing pregnancy on this day.
*Days mentioned in this section are counted from ET (=Day 0).

6 Patient Insurance

IVI Valencia undertakes to insure liability for potential damage caused to a subject in connection with the trial and provides insurance policy for all study subjects.

7 SAMPLE SIZE AND STATISTICAL ANALYSIS

7.1 Sample Size Calculation

The two tests hhCG and hCG+β will be compared using receiver operating characteristics (ROC) curves by analyzing the area under the curve (AUC). Based on the data by Chuan S et al. [16], an AUC of 0.67 is assumed for the hCG+β test and an AUC of 0.79 for the hhCG test. Under the assumption that 45% of all patients with IVF will present with clinical pregnancy on day 21* (+4/-3), 136 patients are necessary to achieve 80% power for a one-sided z-test with significance level alpha = 2.5%.

Power	N+ *	N- **	AUC1	AUC2	Correlation
0.8022	61	75	0.6700	0.7900	0.6

* Pregnancies

** No Pregnancies

The results are for tests with continuous values and are based on the methodology of Hanley and McNeil (1983). The calculations are further based on the binormal model and the assumption of a correlation of 0.6 between the two tests for both the positive and negative groups (N+ and N-). Allowing for 10% of patients being non-evaluable, 152 patients are planned to be enrolled.

*Counted from ET (=Day 0).

7.2 Targeted Accrual

The 152 patients are planned to be enrolled over a period of 9 months, from June 1, 2017 at the earliest to February 28, 2018.

7.3 Groups for Biostatistical Analysis

The population of **patients enrolled** will include all patients who gave informed consent.

The **patients treated** population will consist of all patients for whom the IVF-ET was performed.

The per-protocol population will consist of all eligible patients treated, i.e. will be restricted to those patients of the patients treated population who did not violate any eligibility criteria and who have available measurements for both hhCG and hCG+β on day 4* (+1) and an assessment of clinical pregnancy on day 21* (+4/-3). A missing assessment of clinical pregnancy on day 21 (+4/-3) can be replaced by an assessment of ongoing pregnancy on day 28 (+4/-3) and/or 65 (+7).

In case of sufficiently high numbers of multiple pregnancies, subgroup analyses may be performed for patients with multiple and single pregnancies. Further subgroups of interest, if any, will be defined in the Statistical Analysis Plan.

*All days mentioned in this section are counted from ET (=Day 0).

7.4 General Biostatistical Approaches

For the pregnancy tests based on hhCG and hCG+β levels, ROC analyses will be performed. The predicted results of the test (positive or negative, i.e. pregnant or not pregnant) will be compared to the actual presence/absence of pregnancy as observed (e.g. clinical pregnancy on day 21* (+4/-3) or ongoing pregnancy on day 65 (+7)). There are the following possibilities for the test results and observed results:

		Observed	
		Positive	**Negative**
Test	**Positive**	True positive (TP)	False positive (FP)
	Negative	False negative (FN)	True negative (TN)

For the test concerned, the rate of true positives and true negatives can be regarded in two different ways: in relation to the observed positives and negatives and in relation to the predicted positives and negatives.
Based on the observed positives and negatives, the rate of true positives is called the **sensitivity** and the rate of true negatives is called **specificity**, i.e.:
Sensitivity = TP / (TP + FN)
Specificity = TN / (TN + FP)

Based on the predicted positives and negatives, the rate of true positives is called the **positive predictive value (PPV)**, and the rate of true negatives is called the **negative predictive value (NPV)**, i.e.:
PPV = TP / (TP + FP)
NPV = TN / (FN + TN)

Sensitivity, specificity, positive and negative predictive values are all dependent on the definition of the test concerned. Receiver operating characteristics (ROC) curves are used to visualize sensitivity and specificity for various thresholds for the pertinent test: Sensitivity is plotted against 1-specificity and thus displays the test's characteristics for various cut-offs. Tests can be compared by looking at summary measures for the ROC curves, e.g. the area under the curve (AUC). Since high sensitivity and specificity are wanted in a test, a bigger area under the curve is desirable.
To define a test for practical use, a threshold has to be chosen, i.e. a cut-off that defines that values below are considered as negative test results and values above as positive test results. A threshold should be chosen as a point that optimizes the combination of sensitivity and specificity which can be done via the ROC curve by choosing the point with the least distance to the upper left corner which stands for specificity = 100% and sensitivity = 100%.

All other data will be summarized by descriptive statistics in case of continuous variables or by absolute and relative frequencies. Laboratory data will be displayed over time for each visit as will ultrasound results. Concomitant diseases and medications will be listed for the complete

duration of the study. Incidents and indirect harms will be summarized by absolute and relative frequencies.

*Counted from ET (=Day 0).

7.5 Descriptive Statistics

Continuous variables will be summarized by descriptive statistics (mean, standard deviation, median, Q1, Q3, minimum, maximum, number of available data); categorical variables will be summarized by absolute and relative frequencies and number of available data. The relative frequencies will be based on the number of available data if not mentioned otherwise.

7.6 Analysis by Study Objectives

7.6.1 Primary study Objective

The primary study objective is to determine, based on the AUCs of the ROC curves, whether serum hyperglycosylated human chorionic gonadotropin (hhCG) measured on day 4 (+1) after embryo transfer (ET) is superior to hCG+ß measured on day 4* (+1) in predicting clinical pregnancy on day 21* (+4/-3) (=6 weeks after last menstruation, detection of embryo sac by US).

The empirical AUCs, pertinent standard errors and 95% confidence intervals will be estimated for both the hhCG test and the hCG+β test. Furthermore, the empirical AUCs of the two tests will be compared using a logistic regression model and the difference in AUCs will be estimated including 95% confidence intervals and chi-square test for the contrast. hhCG measured on day 4 (+1) after ET is considered superior to hCG+ß if the null hypothesis

$$H0: \quad AUC(hhCG) \leq AUC(hCG+\beta)$$

can be rejected at an one-sided significance level of 0.025.

The empirical ROC curves will be plotted to provide a visualization that supports the comparisons.

*Counted from ET (=Day 0).

7.6.2 Secondary Study Objectives

In addition to the comparison of the AUCs under the ROC curves for day 4*, the same analyses will be performed for the hhCG and hCG + β tests at days 7 and 11 with respect to predicting clinical pregnancy on day 21*; at days 4, 7 and 11 with respect to predicting pregnancy on day 65; at days 4 and 7 with respect to predicting pregnancy on day 11. The same methods as for the analysis of the primary objective will be applied here.

For the comparisons of sensitivity and specificity and the positive and negative predictive values at various time points with respect to pregnancy at various later time points, the following approach will be used:

Optimal thresholds for the two tests and the various time points will be identified from the pertinent ROC curves for the time point (day 4, 7 or 11) and endpoint (pregnancy at day 65,

21 or 11) considered. The optimal threshold for the time point and test at hand will be found using Youden's index (sensitivity + specificity -1), choosing the point for which the index is maximal.
The thresholds and the corresponding sensitivities and specificities will be summarized for each time point, pregnancy endpoint and test. Pertinent positive and negative predictive values will be shown for the same thresholds for the various time points.

The course of hhCG and hCG + β over time will be summarized by descriptive summary statistics (mean, standard error of the mean, median, Q1, Q3, minimum and maximum) for the disjoint pregnancy outcome categories as defined in 5.1. If there are sufficiently many multiple pregnancies, the same analysis will be performed for the categories of multiple and single pregnancies (irrespective of pregnancy outcome).

Incidents and indirect harms will be summarized by absolute and relative frequencies.
*All days mentioned in this section are counted from ET (=Day 0).

8 SAMPLE COLLECTION AND PROCESSING

Sample collection and processing for estradiol, progesterone and hCG+ß will be done according to local study site procedures and regulations. Serum obtained for the assessment of hhCG (0.5 mL at each occasion) will be stored in a freezer at -80^{0}C till shipped kept in dry ice for analysis to Quest Diagnostics (Quest Diagnostics, Nichols Institute, San Juan Capistrano, CA) in batches of complete samples of patients with the provision that in any particular patient the same batch of the diagnostic will be used. Another 0.5 mL of serum will be kept frozen for future potential biomarker analyses by IVI and 2.0 mL of serum from each occasion will be stored in a freezer at -80^{0}C and shipped kept on dry ice to Roche.

9 SECRECY AGREEMENT

Secrecy agreement:

The Principal Investigator agrees to handle all information and documentation he may have received from Roche Diagnostics confidentially and ensures that all other persons involved in this project will keep secrecy, too.

Principal Investigator of the study:

Location:	Name:
IVI Clinic, Valencia.	Ernesto Bosch, MD, PhD, Gynecologist
Plaza de la Policía Local, 3	Medical Director of IVI Clinic Valencia
46015 Valencia	Date / Signature:

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