IVIRMA Global

Biomedical Research Protocol

Title:

Embryo ploidy selection by Nuclear Magnetic Resonance: a fast, low cost and non-invasive technique to increase the success rate of ART

2004-LIS-037-SN

Version: 1.1

Version date: 20/Apr/2021

IVI Foundation

Project Title

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Project Code:	2004-LIS-037-SN
Research Area:	Non invasive embryo selection
Date:	20/04/2021

Keywords:

Embryo selection, metabolic profiling, nuclear magnetic resonance

Sponsor information

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Responsibilities and Signatures

By signing this protocol from the project entitled: *Title:* Embryo ploidy selection by Nuclear Magnetic Resonance: a fast, low cost and non-invasive technique to increase the success rate of ART

Those undersigning state that:

- This study respects the ethical and legal rules and follows good clinical practice in its implementation
- It has the material and human resources needed to carry out the study, without interfering in other studies or clinical tasks usually entrusted to them
- They are committed that each subject is treated and controlled according the approval granted by the Ethics Committee for Clinical Research, Institutional Review Board, remaining committees and the involved authorities
- Collaborators included in this study are adequately trained for its implementation, they will have an active participation, and they consent thereto.

Center and/or Laboratory Director

Dr. Sérgio Reis Soares IVI Lisboa Diretor	Date

Principal Investigator

Dra. Sofia Gouveia Nunes IVI Lisboa	Date

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Abstract

Assisted reproductive technologies (ART) refers to treatments used to assist people in achieving a pregnancy. Over the last years, ART have been developed with efforts to deliver a healthy baby. However, the selection of the embryo that most likely results in pregnancy remains a critical step in ART. Currently, this selection is based on morphological assessment of the embryos, but up to 70% of those embryos display an abnormal number of chromosomes. Preimplantation genetic test for aneuploidy (PGT-A) is used to assess the ploidy of embryos, although this is an invasive, expensive and time consuming technique. Alternatively, in order to predict the ploidy of pre-implantation embryos (euploidy vs aneuploidy), we suggest to characterise the metabolic profile of the embryo culture medium by Nuclear Magnetic Resonance (NMR), which is a non-invasive, low cost and fast technique.

The aim of this study is to assess whether, using multivariable analysis to all the collected NMR data, we are able to identify a differential metabolic pattern between aneuploid and euploid embryos which could improve embryo selection.

List of acronyms

ART	Assisted reproductive technologies
DNA	Deoxyribonucleic acid
GCP	Good clinical practice
ECM	Embryo culture medium
ICH	International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use
ICSI	Intracytoplasmic sperm injection
IEC	Independent ethics committee
IRB	Institutional review board
IVF	In vitro fertilization
IVI RMA	Instituto Valenciano de Infertilidade / Reproductive Medicine Associates
NMR	Nuclear magnetic resonance
PGT-A	Preimplantation genetic test for aneuploidy
SIVIS	Software for medical record management

AIM: main objective/research question

Main objective:

To evaluate whether nuclear magnetic resonance (NMR) spectroscopy can assist in the prediction of ploidy of pre-implantation embryos (euploidy vs aneuploidy).

Research question:

Is NMR able to identify a differential metabolic pattern between aneuploid and euploid embryos in order to improve embryo selection?

Is there a metabolic profile difference between an embryo with 3 days of culture and the same embryo after 5 or 6 days of culture?

Introduction

A. Background

Worldwide, infertility affects around 15% of the couples in reproductive age. The ability to procreate is largely a female-age limited process, with one in eight women experiencing infertility, a proportion that is even higher among women aged 35 or older. Assisted reproductive technologies (ART) are of paramount importance for these couples, and in many they are the only solution to constitute a family (1).

The optimal scenario in ART is a single-embryo transfer (SET) followed by the delivery of a healthy baby. For this reason, multiple laboratory technical approaches have been developed to improve the selection of the best embryo available. Currently, this selection process is largely based on morphologic assessment. This type of evaluation, however, appears to be insufficient since many apparently top quality embryos ultimately do not implant or eventually result in a miscarriage. One of the main reasons for this is the fact that up to 70% of the embryos considered viable from a morphological perspective present an abnormal number of chromosomes and are, therefore, generally not compatible with life (2).

One means to overcome this issue is to perform preimplantation genetic testing of embryos to detect aneuploidy (PGT-A), which entails the removal of a small number of cells for analysis by either next-generation screening or comparative genomic hybridization (3). However, this analysis has the disadvantage of being invasive, time-consuming and expensive. Thus, the search for an efficient alternative selection method during the preimplantation stage which could improve the success rate of ART while decreasing the cost and time required to select chromosomally normal embryos as best as possible continues.

A suitable alternative to PGT-A could rely on the characterization of the metabolic profile of the embryo culture medium (ECM) by NMR, a non-invasive, rapid, economic and sensitive technique which is already currently clinically applicable. The conceptual basis of this approach has been described in a previous publication performed after a day 3 embryo biopsy, which demonstrated that embryos with chromosomal abnormalities presented a different metabolic pattern and, consequently, a different ECM composition (4). However, this study is of limited value, since it has been demonstrated that embryos are able to self-correct chromosomal errors found by day 3, whereby the current recommendation is for PGT-A to be performed on day 5 (or day 6). Further studies are needed to assess the usefulness of NMR for prediction of the ploidy of pre-implantation embryos.

B. Justification and translational potential to the clinic and impact on patient's care improvement:

This research study aims to validate the ECM analysis by NMR spectroscopy, as a faster and less-costly alternative to PGT-A which could significantly enhance embryo selection and the success rate of ART.

Methodology

A. Study Design

We propose a single-center prospective sibling case-control pilot study. In summary, all consenting patients will undergo in vitro fertilization (IVF) followed by PGT-A according to normal clinical practice. While the overall procedures towards PGT-A will not change, we will request that they consent additionally to donate the embryo culture medium, which otherwise would be discarded, for investigational purposes. The study will be conducted in conformance with Good Clinical Practices (GCP).

B. Study period and context

Women who will undergo IVF followed by PGT-A at the Reproductive Medicine Clinic of IVI RMA Lisboa will be screened each day by a study collaborator and treating physician. Eligible women will be invited to participate in the study and provided written patient information on the trial (pre-screening visit). Those who accept to participate will be requested to sign written informed consent (screening/inclusion visit).

The on-study period is planned to have an overall duration of approximately 4 weeks with 2 visits for each subject, as detailed in Fig 1. The on-study period starts on the day that informed consent is signed, which can occur up to the day of patient oocyte retrieval, and ends when the results of the PGT-A procedure are known. More information regarding the overall recruitment plan, exposure, monitoring and data collection are described below.

Procedures		
Provide information regarding the study	■	
Verification of eligibility criteria		■
Informed consent form signature		■
Collection of demographic and anthropometric data		■
Collection of culture medium of embryos for assessment		■
Centre visits		
Visit number	0	1
	Pre- Screening	**Screening/Inclusion**

Figure 1 – overall on-study period scheme

C. Reference Population

Our target population are healthy women who will undergo a IVF followed by PGT-A. This study will evaluate embryo culture medium compositions of 50 aneuploid embryos, 50 euploid embryos, and 30 controls (no-embryo culture medium).

D. Subject Inclusion/exclusion criteria and case-control matching

The subject inclusion and exclusion criteria are summarized in Table .

Inclusion criteria	Exclusion criteria
▪ Age: ≥18 and <49 years old. ▪ Body Mass Index (BMI) ≥18.5 Kg/m2 and <30 Kg/m^2. ▪ Planned for IVF followed by PGT-A. ▪ Oocytes retrieval and fertilization by ICSI ▪ Six follicles over 14 mm on the day of the triggering. ▪ Signed and dated informed consent.	▪ Previous history of poor ovarian response (<4 oocytes retrieved) with a maximal dose of ovarian stimulation (≥300 IU/day). ▪ Presence of a medical condition which is known to affect ART outcome (e.g. thyroid dysfunction). ▪ Current use of anti-depressants, anti-psychotics, steroids, antiepileptics or chemotherapy. ▪ Those unable to comprehend the investigational nature of the proposed study.

Table 1 – Study inclusion and exclusion criteria

E. Intervention and Follow-up

Ovarian stimulation, oocyte retrieval, all embryology procedures and PGT-A will be performed according to how they are normally performed in IVI Lisboa. Women will start ovarian stimulation with either recombinant follicle-stimulating hormone (Gonal F, Puregon, Pergoveris, Bemfola, Ovaleap or Elonva) or highly purified human menopausal gonadotrophin (Menopur). Women will be submitted to pituitary down regulation, through daily administrations of either cetrorelix or ganirelix (GnRH antagonist) or oral progestogens (desogestrel) in either a fixed or flexible protocol. Cycles will be monitored by vaginal ultrasound scans and serum determination of estradiol and progesterone. Once three follicles of ≥17 mm are observed, oocyte maturation will be triggered with a GnRH agonist (0.3 mg decapeptyl). Cumulus-oocyte complexes will be collected by transvaginal aspiration 35-36 h after hCG administration (5). Oocytes will be denudated and ICSI will be performed at least 4 hours after the pick up to all mature oocytes. Fertilized oocytes will be cultured in the IVF laboratory incubators until they reach blastocyst stage. The ones presented good morphology will be biopsied, according to the ASEBIR embryo classification (6). After biopsy embryos will be frozen and the trophoectoderm cells will be analyzed by next generation sequencing (NGS) to identify aneuploidies. Culture media where each embryo developed will be analyzed via NMR Spectroscopy in order to evaluate the metabolomic profile and compare it with the chromosomic analysis.

The study period includes the following visits to the clinic and procedures:

VISIT 0 (PRE-SCREENING VISIT)

- The study coordinator will contact potential participants in our center. Information regarding the study will be provided followed by an invitation to participate in this study.

VISIT 1 (SCREENING AND STUDY INCLUSION)

- Participants will sign and date the informed consent.
- Collection of demographic and anthropometric data, medical and surgical history
- Verification of eligibility criteria check list.
- As described in more detail below, the culture medium from euploid and aneuploid embryos will be collected and then analyzed by NMR.

F. Study restrictions

During the study, patients are restricted to use any type of medication that may interfere with ovarian stimulation or embryology. Specifically, current use of anti-depressants, anti-psychotics, steroids, antiepileptics or chemotherapy constitute an exclusion criterion for study participation.

G. Participant withdrawal and protocol violation

Participants may withdraw from the study at any time. Eligible participants who gave adequate consent to the study and later withdraw will be replaced. Whenever a participant does not follow the planned protocol, this will be considered as a protocol violation. Given the pilot and explanatory nature of the study, these subjects will be excluded from the analysis with justification.

H. Potential advantages and financial incentives for the participating subjects

Beyond the potential clinical advantage of the study in the future, no other clinical or financial incentive will be provided for the participating subject.

I. Collection, selection, analysis and destruction of biological samples and information

BIOLOGICAL SAMPLE GATHER PROTOCOL AND OBJECTIVE OF SAMPLE COLLECTION

ECM collection

After embryo biopsy, a minimum quantity of 25 uL of embryo culture medium will be collected at Day 3 and Day 5 (or Day 6) of embryos development. Collection of the media, will be carried out by pipetting culture

media into Eppendorf tubes which will be immediately centrifugated in a mini centrifuge during 1 minute to remove cell contamination. Supernatant will be collected to a new tube with phosphate buffer, D20 and sodium azide to prevent bacterial and fungi development. Samples will be immediately anonymized, as described below, and frozen in liquid nitrogen and then stored at -20 ºC at IVI RMA Lisboa. Samples will be transferred in dry ice to ITQB, which is located at Av. da República, 2780-157, Oeiras, Portugal. Dr Sofia Nunes will be responsible for the collection and storage of samples at IVI Lisboa. This facility has a 24-hour security system in place and access to the storage area is restricted to authorized personnel only.

Selection process of case-control sibling embryo culture media

In order to minimize potential confounding, paired-sibling embryo culture media (two samples derived from the same patient and treatment cycle) will be allocated in pairs and in a 1:1 ratio to each study group. The pair-sibling allocation will be performing after the results of the PGT-A procedure are known. Pairing will be performed preferably using embryos with similar developmental staging and morphological grading.

Finally, each patient can include up to 3 pairs of sibling culture media in the analysis. The remaining samples will be immediately destroyed.

NMR procedure

ECM samples will be thawed at room temperature before analysis, will be transferred to a 1.7-mm NMR capillary shigemi tube for analysis. The NMR spectra will be acquired in a BrukerAvance II 500 spectrometer (BrukerBiospin) equipped with a prodigy TCI cryoprobe. All experiments will be acquired at 298 K. For each sample, two 1H-NMR spectra will be obtained: a Carr-Purcell-Meiboom-Gill (CPMG) spin echo pulse sequence, which generates spectra edited by T2 relaxation times to reduce signals from high molecular weight species and to facilitate the detection of low molecular weight species, and a 1D NOESY pulse sequence, which generates an unedited spectrum with improved solvent peak suppression (extremely useful for the observation of signals near the water resonance). The spectra will be processed by a Fourier Transform and multiplied by an exponential function using Topspin 4.0. Phase correction, baseline correction and spectra calibration (methyl group signal of the lactate at a chemical shift 1.32 ppm) will be performed before further analysis. Metabolites will be quantified with ChenomxNmrSuite 8.0.2 using the Human Metabolites Database as reference. This software allows the deconvolution of the NMR spectra and the determination of the

metabolite concentrations. From the previous studies it is expectable that we will be able of determine around 20 to 30 metabolites including aminoacids, fatty acids and carbohydrates like sucrose, glucose and pyruvate.

The samples analysed in this study will be destroyed immediately upon analysis.

SAMPLE IDENTIFICATION PROTOCOL

All consenting subjects will be given a unique screening number (eg. 037-SN-0X). Each participant will be assigned only one screening number. Screening numbers will not be re-used for different participants.

Samples (ECM) will be collected and identified immediately using sticker labels. These labels contain a single anonymized identifying code with the following format: <STUDY CODE> <SUBJECT NUMBER> <TYPE OF SAMPLE> <SUBJECT YEAR OF BIRTH> <SAMPLE DATE>. For example, the identifying code 2004-LIS-037-SN-01-ECM_D3-1983-30042020 provides the following information:

- <STUDY CODE>: internal reference code of study (2004-LIS-037-SN)
- <SUBJECT NUMBER>: 0X
- <TYPE OF SAMPLE>: ECM_DX (X = day 3 or day 5/ day 6)
- < SUBJECT YEAR OF BIRTH>: coded as YYYY;
- <SAMPLE DATE>: date the sample was collected, coded as DDMMYYYY.

Statistical methodology

A. Data base

Data will be collected in a secure and encrypted case report form system created specifically for the study hosted on a dedicated server firewall-protected serve. The database will be rigorously defined with the variables destined to be analyzed according to the described study objectives and will have inbuilt validation procedures to ensure the correct introduction of data and avoid cases of missing information where such is not applicable. The study team collaborating in the study will be responsible for the data collection. These anonymized data will be stored for at least an additional 25 years.

In accordance with Portuguese law, all data exported for statistical analysis will be anonymized to protect the clinical and personal information of the subjects. Finally, and prior to the statistical study, an exploratory data analysis will be carried out to review the quality of the information extracted.

B. Study Variables

MAIN-OUTCOME MEASURES (DEPENDENT)

The primary outcome of this study is the assessment of different metabolic profiles between culture medium of euploid versus aneuploid embryos.

EXPOSURE VARIABLES (INDEPENDENT)

The metabolic profile of culture medium of euploid and aneuploid embryos will be compared to culture medium without embryo (control).

CONTROL VARIABLES

The following variables will be recorded during the analysis (as continuous variables, unless mentioned otherwise):

- Female subject age (in years).
- Male subject age (in years)
- Female body mass index.
- Menstrual cycle length.
- Number of preceding ART cycles.
- Duration of infertility (in years).
- Infertility diagnoses (tubal, ovulatory, endometriosis, male factor or otherwise unexplained).
- Baseline AMH.
- Baseline AFC.
- Number of follicles >11 mm on the day of ovulation triggering.
- Total dose of exogenous gonadotropins.
- Total duration of OS.
- Number of oocytes retrieved.
- Number of fertilized oocytes.
- Number of embryos available for biopsy.
- Quality of each embryo available for biopsy.
- Number of euploid and aneuploid embryos.

C. Sample Size

Owing to the exploratory nature of the study, we are unable to determine an accurate sample size for this study hypothesis. Hence, we elected to recruit a pre specified number of a maximum of 50 subjects (donating a minimum of a pair of sibling culture media).

D. Statistical data analysis

EXPLORATORY DATA ANALYSIS

Basic demographic characteristics will be compared amongst the study who did and did not have a spontaneous miscarriage using the t-student/Mann-Whitney test (for continuous variables, according to the normality of the distribution) or Fisher's Exact test (for categorical variables).

HOMOGENEITY ANALYSIS

In order to minimize potential confounding, paired-sibling embryo culture media (two samples derived from the same patient and treatment cycle) will be allocated in pairs and in a 1:1 ratio to each study group. The pair-sibling allocation will be performing after the results of the PGT-A procedure are known.

OBJECTIVES ASSESSMENT

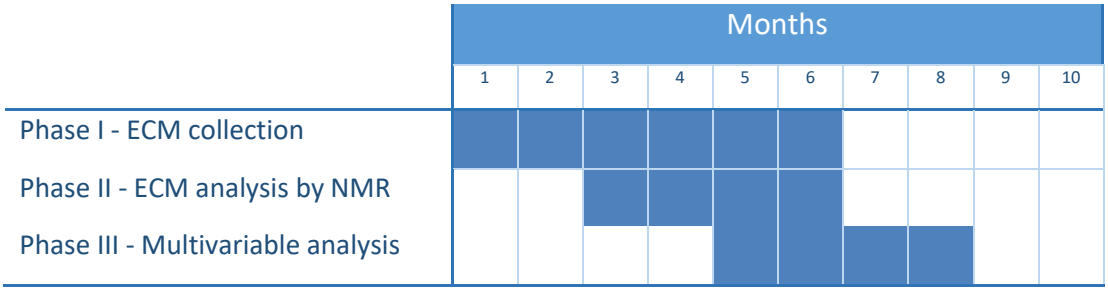
Considering that we will be able to identify around 20 to 30 metabolites, the minimum number of samples per group for a False Discovery Rate (FDR) of 5% is 40 samples, this value was calculated using the default parameters of MetSizeR (7), a software that allows sample size calculation for metabolomic studies. Assuming that a pre-specified proportion of at least 10% metabolites will be predictive of the outcome, the estimated power to detect a statistically significant difference among the groups is >90%.

Metabolite concentration will be used for univariate and multivariate analysis. In the univariate analysis, data normality will be checked by Shapiro test. Two-sample t-test, if the data has normal behavior, or Wilcoxon-rank test, if the data has not a normal behavior. Comparisons will be performed to confirm the metabolite differences significance. In multivariate analysis, sample classification will be performed by Principal Component Analysis (PCA), Partial Least Squares Discriminant Analysis (PLS-DA), and Orthogonal Projections to Latent Structures (OPLS-DA) using R and Simca.

Pathway analysis will be performed in Metaboanalyst (www.metaboanalyt.ca) to identify which metabolic pathways are affected in aneuploid embryos. Pathway Analysis combines pathway enrichment analysis with pathway topology analysis, allowing identification of the most relevant pathways involved in aneuploidy. For that, it will be used the metabolite concentrations determined previously for the aneuploid embryos and controls; and human pathway library as reference.

Work plan

The pilot study workplan of the pilot phase includes three phases (ECM collection, ECM analysis by NMR and multivariable analysis), as detailed below.



The responsible persons for each phase are: I- Sofia Nunes; II and III – Luis Gonçalves

Ethical issues

A. Ethical conduct of the study

This study will be submitted for approval by the Local Ethics Committee of IVI Lisboa. For the inclusion of all patients in the study, a signed informed consent is required, which must first be approved by this Ethics Committee.

This study respects the fundamental principles of the Declaration of Helsinki, the Council of Europe Convention on Human Rights and Biomedicine, the UNESCO Universal Declaration on the Human Genome and Human Rights, as well as the requirements of Portuguese law in the field of biomedical research, the protection of personal data and bioethics.

This study will be conducted in accordance with the ethical principles that have their origins in the Declaration of Helsinki and its most recent amendments, in compliance with the approved protocol, ICH-GCP guidelines, the EU Clinical Trial Regulation, and other national law(s) or regulatory requirements applicable in the countries where the study takes place.

B. Independent ethics committee /Institutional review board

Prior to the start of the study, the investigator is responsible for ensuring that the protocol and consent form have been reviewed and approved by a relevant IEC/IRB. The IEC/IRB shall be appropriately constituted and perform its functions in accordance with ICH-GCP guidelines and local requirements as applicable.

The IEC/IRB shall approve all protocol amendments (except for logistical or administrative changes), written informed consent documents updates, patient recruitment procedures (e.g. advertisements), written information to be provided to the patients, available safety information, information about payment and compensation available to patients, the investigator curriculum vitae and/or other evidence of qualification and any other documents requested by the IEC/IRB and Regulatory Authority (Competent Authority) as applicable.

C. Subject information and consent

The investigator will obtain a freely given written consent from each subject after an appropriate explanation of the aims, methods, anticipated benefits, potential hazards, and many other aspects of the study, which are relevant to the subject´s decision to participate. The subject must be given ample time to consider participation in the study, before the consent is obtained. The informed consent form must be signed and dated by the subject and the investigator who has provided information to the subject regarding the study before the subject is exposed to any study-related procedure.

The investigator will explain that the subject is completely free to refuse to enter the study or to withdraw from it any time, without any consequences for her further care and without the need to justify her decision.

The subject will receive a copy of the informed consent documents.

The subjects will be informed about a new information and re-consent will be obtained.

D. Data Handling and Record Keeping

CASE REPORT FORMS/SOURCE DATA HANDLING

The study data must be verifiable with the source data, which requires access to all original records, laboratory reports and subject records. Therefore, the investigator must agree to allow access to subject records and the source data must be available for all study data. The subjects or their legal representatives must also allow access to the medical records of the subjects and they will be informed of this need and will express their agreement when they provide the informed consent.

The principal investigator will be responsible of maintaining the accuracy and authenticity of the clinical and laboratory data entered in the eCRF. Subject´s source data will be data recorded by the embryologist, which will be kept in the clinical center where the study is carried out.

The investigator/center must allow direct access to the data / source documents (originals) for the activities related to the monitoring study, audit, review by the ethics committees (IEC/ IRB) and inspection by the regulatory authorities.

RETENTION OF ESSENTIAL DOCUMENTS

All records relating to the conduct of this study are to be retained by the investigator according to ICH, local regulations, or as specified in the clinical study agreement, whichever is longer. Prior to transfer or destruction

of these records, the Sponsor must be notified in writing and given the opportunity to further store such records. The investigator will allow representatives of Sponsor´s monitoring members (and of the applicable regulatory authorities) to inspect all study records, eCRFs, and corresponding portions of the study patient´s office and/or hospital medical records at regular intervals across the study. These inspections are for the purpose of verifying the adherence to the protocol, the completeness and accuracy of the data being filled in the eCRF, and compliance with applicable regulations.

E. Quality Assurance and Confidentiality

CONDUCT OF THE STUDY

The sponsor or designee will perform the quality assurance and quality control activities of this study, however, responsibility for the accuracy, completeness, and reliability of the study data presented to the Sponsor lies with the principal investigator generating data.

CONFIDENTIALITY

Any material, information (oral or written), unpublished documents provided by the investigators, including this protocol are owned by the sponsor, and will be regarded as confidential, even when used for review and for exclusive use in this study. This confidentiality clause, as well the obligation to comply during the study, applies to both the investigator and any collaborators. The investigator shall guarantee that the confidentiality of the data by the collaborators will be maintained. The data and/or any material shall not be disseminated, totally or partially, by the principal investigator and/or his/her team, to any unauthorized party, without previous written consent by the sponsor and it is not intended for any use other than the purposes of this study. The investigator and/or his/her team will treat as confidential all documents and results arising during the study, except for the information classified as releasable, pursuant to the law. The investigator and/or his/her team will ensure that anyone involved with the study will adhere to the same confidentiality obligations concerning the information about the participants. All parties involved with the study will maintain the strictest confidentiality so that they do not violate any confidentiality rights of the participants and their families. They will ensure that access to the research data is protected from unauthorized individuals. Considering the confidential nature, the data of a personal nature on subjects included in the study shall comply with the General Data Protection Regulation effective as of 25 May 2018 (Regulation (EU) 2016/679) and other national law(s) or regulatory requirements applicable in Portugal. Furthermore, the Investigator and the Sponsor agree to adhere to the principles of personal data confidentiality in relation to the subjects, Investigator and its collaborators involved in the study.

Funding

Needs funding	Yes

Externally Funded project	No	Funding Institution	Sponsor
External Funding plan (if no funding is available yet)	None		
IVI RMA Funding requested	Yes	Internally Funded project	Yes
Funding time range	Start: 03/05/2021	Finish: 31/01/2022	
Budget	1085,28 Eur		

Insurance

IVI Lisboa has an Insurance Policy in force that conforms to current legislation and with coverage to compensate and indemnify cases of ill health or injury, which may arise in connection with their participation in the study, within routine clinical practice.

Publication and diffusion

Upon conclusion, the results of the study will be submitted for presentation at the annual congress of the European Society of Human Reproduction and Embryology (ESHRE). Moreover, the study will be publicly disseminated following submission in peer-reviewed scientific journals, targeting the upper quartile in terms of impact factor according to Web of Science in Q1 of the preceding year in the field of Obstetrics and Gynecology, such as in the “Molecular Human Reproduction”.

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