Universitat de Girona

THYROID HORMONE TREATMENT IN EUTHYROID WOMEN WITH UNEXPLAINED STERILITY UNDER INTRACYTOPLASMIC SPERM INJECTION

A pilot study

FINAL DEGREE PROJECT

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November 2017

ACKNOWLEDGMENTS

I would like to thank my tutor, Dr. Ramon Brichs, for all the knowledge acquired during the protocol writing and for being a guide in this project.

I would also like to thank Dr. Eva López for her valuable guiding and help in the realization of this protocol. I also thank Dr. José Manuel Fernández-Real, Dr. Josep Garre-Olmo and Dr. Teresa Puig for clearing out my doubts.

Last but not least, I would like to thank the whole Girexx team for getting me into the assisted reproduction field by allowing me to observe the daily practice.

TABLE OF CONTENTS

1. ABSTRACT

INTRODUCTION: Thyroid hormones have several implications in the human body. One of thyroid actions has been demonstrated to be in the ovaries. Oocytes respond to T3 as T3, T4, deiodinases and thyroid receptors have been discovered to exist in human oocytes. Thyroid influence in oocytes is not well-known but it seems that thyroid hormone is needed for a good oocyte development.

An inhibitory thyroid receptor, TRα2, is increased in granulosa cells of sterile women compared to fertile women. As sterility is known to be age-related, it could be a possible cause of sterility as TRα2 has been proven to increase with age.

OBJECTIVE: To test whether the administration of levothyroxine in euthyroid women between 30 and 40 years old with unexplained sterility increases their fertilization rate in ICSI compared to placebo.

DESIGN: Longitudinal, prospective, randomized, double-blinded, controlled clinical trial (pilot study).

POPULATION: Euthyroid women between 30 and 40 years old with unexplained sterility.

SAMPLE SELECTION: Consecutive non-probabilistic.

SAMPLE SIZE: 40 patients equally and randomly distributed into 2 groups: the intervention group (treatment with levothyroxine) and the control group (placebo).

METHODS: Patients included in the study will take a 3 months treatment with either levothyroxine or placebo. After that, they will undergo to an ICSI cycle. They will then start a COS antagonist protocol using a GnRH agonist as ovulation triggering. The resulting oocytes will be aspired and inseminated by ICSI once isolated and denudated. The injected oocytes will be conserved in an Embryoscope and observed after 16-20 hours to check which ones have been successfully fecundated.

Keywords: *thyroid hormone, intracytoplasmic sperm injection, oocyte, ovarian stimulation, fertilization rate.*

2. ABBREVIATIONS

AI- Artificial Insemination

AMH- Anti-Mullerian Hormone

ART- Assisted Reproduction Technique(s)

ASEBIR- Asociación para el Estudio de la Biología de la Reproducción

BMI- Body Mass Index

C-AMP- Cyclic adenosine monophosphate

CC- Clomiphene Citrate

CEIC- Comité de Ética para la Investigación Clínica

COS- Controlled Ovarian Stimulation

CPA- Cryoprotective Agents

CRH- Corticotropin-Releasing Hormone

CUN- Clínica Universidad de Navarra

D1, D2, D3- Deiodinase 1, 2 and 3

DIT- Diiodo-tyrosines

DMSO- Dimethyl Sulfoxide

DNA- Deoxyribonucleic Acid

E2- Oestradiol

EG- Ethylene Glycol

FET- Frozen-thawed Embryos Transfer

FSH- Follicle-Stimulating Hormone

GnRH- Gonadotropin-Releasing Hormone

HBV- Hepatitis B Virus

HCG- Human Chorionic Gonadotropin

HCV- Hepatitis C Virus

HIV- Human Immunodeficiency Virus

HMG- Human Menopausal Gonadotropin

HRE- Hormone Response Element

ICSI- Intracytoplasmic Sperm Injection

IMSI- Intracytoplasmic Morphologically-selected Sperm Injection

IVF- In Vitro Fertilization

LDL- Low Density Lipoprotein

LH- Luteinizing Hormone

MIT- Monoiodo-tyrosines

mRNA- Messenger Ribonucleic Acid

NIS- Na^+/I^- Symporter

OC- Oral Contraceptives

OHSS- Ovarian Hyperstimulation Syndrome

P- Progesterone

PB- Polar body / Polar bodies

PCOS- Polycystic Ovarian Syndrome

PGD- Preimplantation Genetic Diagnosis

PN- Pro-nucleus / Pro-nuclei

PROH- 1,2-propanedial

PRL- Prolactin

PVP- Polyvinylpyrrolidone (Povidone)

RPR- Rapid Plasma Reagin

rT3- Reverse T3

SCH- Subclinical Hypothyroidism

SEF- Sociedad Española de Fertilidad

SER- Smooth endoplasmic reticulum

T3- Triiodothyronine

T4- Thyroxine

TBG- Thyroxine-Binding Globulin

Tg- Thyroglobulin

TH- Thyroid Hormone(s)

TPO- Thyroid Peroxidase

TR- Thyroid Receptor

TRα- Thyroid Receptor α

TRα1- Thyroid Receptor α1

TRα2- Thyroid Receptor α2

TRβ- Thyroid Receptor β

TRβ1- Thyroid Receptor β1

TRβ2- Thyroid Receptor β2

TRE- Thyroid-Hormone Response Element

TRH- Thyrotropin-releasing Hormone

TSH- Thyroid-Stimulating Hormone

TSHr- Thyroid-Stimulating Hormone Receptor

TTR- Transthyretin

WHO- World Health Organization

ZP3- Zona pellucida sperm-binding protein 3

3. INTRODUCTION

3.1 Sterility

Sterility is defined as the inability to conceive after 1 year of sexual relations without any contraceptive method. It approximately affects 10-15% of couples(1). The causes of sterility among couples can broadly be subdivided into 4 categories: **female sterility** (35%), **male sterility** (30%), **a combination of both** (20%) and **unexplained or idiopathic sterility** (15%). Female causes of sterility comprise endometriosis, tubal damage and ovulatory dysfunction. In presence of a normal spermogram and a normal female work-up, the cause of a couple's sterility is considered idiopathic. Woman's age and smoking habit also constitute important prognostic factors(2).

Sterility has increased over the last 30 years. The main reason for this increase is the **adjournment of motherhood** that our society has shown, which leads to a **minor biological fertility** in these women. Another reason is the constant epidemic of venereal diseases, some of which are associated with an increased risk of posterior sterility(1).

3.2 Assisted reproduction techniques

Although sterility has increased, also did its treatment. The variety of treatments to solve fertility issues have experimented a spectacular improvement over the last 25 years as interest in sterility has increased. Over time, these techniques have been perfected and became more accessible(1).

The different options in assisted reproduction techniques (ART), which can be with own gametes or donors, are:

- **Artificial insemination (AI).** It consists of the introduction of sperm in the uterine cavity with the aim of pregnancy by natural fertilization of an oocyte in the fallopian tube. To achieve capacitated spermatozoa, 2 lab methods can be used: **density gradients**, where mobile spermatozoa cross different gradient media while a centrifugation process and rest in a *final pellet*, or **swim-up**, where mobile spermatozoa swim to the top while being incubated at 37ºC. With these techniques, the best spermatozoa are selected and used in the AI(1).
- **In vitro fertilization (IVF).** It is the process of fertilization by extracting oocytes by follicular aspiration, retrieving a sperm sample, and then manually combining an oocyte and sperm (previously treated) in a laboratory dish until they become fused on their own. Then, in case of fertilization, embryo evolution is observed until it is transferred to the uterus(3). IVF indications are

tubal damage, bilateral salpingectomy, endometriosis, mild male sterility and idiopathic sterility, as well as age-related sterility(1,2).

- **Intracytoplasmic sperm injection (ICSI).** It consists of choosing a spermatozoon with both good mobility and morphology, aspire it with a microneedle and then inject it directly inside the oocyte. ICSI indications are sperm abnormalities, failure of IVF, ejaculatory disorders and PGD(2).
- **Intracytoplasmic morphologically-selected sperm injection (IMSI).** It consists of conducting a previous selection of spermatozoa without anomalies before the microinjection procedure. It uses a much powerful microscopy lens than the one used in ICSI. Selecting morphological alteration-free spermatozoa may increase the probability of success, but it needs more studies to demonstrate its efficacy(4).

3.3 Hypothalamic-pituitary-gonadal axis

The aim of reproductive process is to achieve a mature oocyte by ovulation at the middle of every menstrual cycle to make it capable to be fertilized, as well as prepare the endometrium for a possible pregnancy. This process depends on the hypothalamic-pituitary-gonadal axis and other afferent stimulus from other systems, such as dopamine, serotonin, PRL, endogenous opioids and CRH (inhibitors) and noradrenaline (activator)(5).

The interaction of hormones is controlled by negative feedback. This means that oestrogens and progesterone, released by ovaries, will lower FSH and LH releasing in the pituitary gland, as well as GnRH levels, which is released by hypothalamus.

In general terms, this axis consists of:

- **Hypothalamus.** It secretes gonadotropin-releasing hormone (**GnRH**), also known as gonadoliberin. This neurohormone is transported by the portal circulation to the pituitary gland, where it has its receptors. To activate the axis, GnRH releasing must be **pulsatile**, since it has been demonstrated that continuous infusion of GnRH has an inhibitory effect among gonadotropins(6).
- **Pituitary gland.** It responds at GnRH through its receptors, with the consequent response of releasing follicle-stimulating hormone (**FSH**) and luteinizing hormone (**LH**), also known as gonadotropins. If frequency of GnRH pulses is increased, LH production is higher than FSH's; if frequency is decreased, LH production is lowered and predominates FSH production(6). In that way, 2 different gonadotropins are regulated as needed by only one hormone(1).

- **Ovary.** Responding to FSH, the follicle-growth is stimulated, and **oestrogens** are secreted by granulosa cells. LH controls the follicle-maturation, the ovulation and the **progesterone** secretion by the corpus luteum(5).

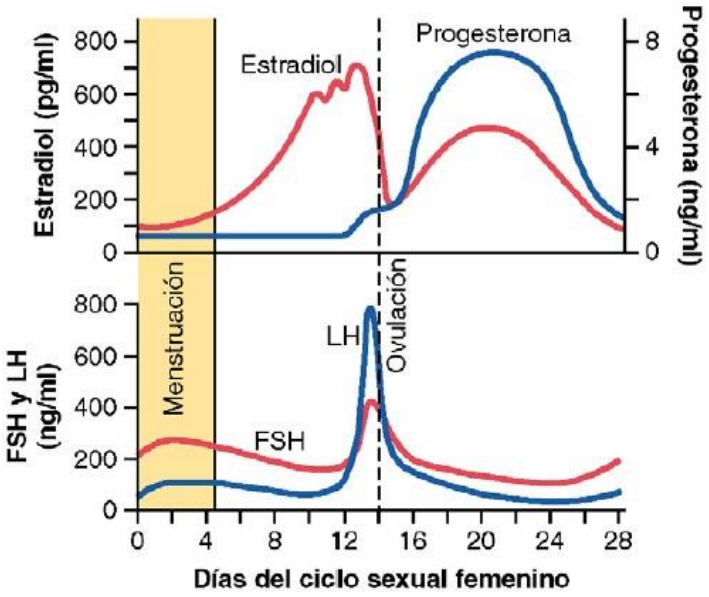

Figure 1. *Plasmatic concentrations of gonadotropins and ovarian hormones during feminine sexual cycle*(7).

3.4 Gametogenesis

It is the process through which masculine and feminine sexual cells turn to mature gametes capable of fertilize and being fertilized, respectively(8). This process can be divided in 4 phases:

- Extraembryonic origin and cell migration to gonads.
- Mitosis phase.
- Meiosis phase.
- Spermatozoa and oocyte maturation.

We will only refer to the feminine sexual cells process.

3.4.1 Extraembryonic origin and cell migration to gonads

Cells that will turn into gametes originate in ectoderm at 4th week of development. Then, these cells separate from the ectoderm and start a **migration process** through ameboid movements to the endoderm of yolk sac. These cells constitute the germinal line and are named **primordial cells**. They have a big size, a clear cytoplasm and contain a high concentration of alkaline phosphatase.

Between week 4 and 6, primordial germinal cells migrate again from the yolk sac to the digestive tube wall. From there, they migrate to the dorsal wall of the body, occupying the adjacent region of the 10th dorsal vertebra, where gonads will form(8).

3.4.2 Mitosis phase

Once in gonads, the germinal cells get differentiated into oogonias and start the mitotic proliferation. In every mitotic division, **each cell produces 2 genetically identic diploid cells**. After several mitotic divisions, primordial cells number exponentially increases.

Oogonias suffer an intense mitosis from 2nd to 5th month of embryonic development, until arriving to 7 million cells. Afterwards, many of them will degenerate by atresia(8).

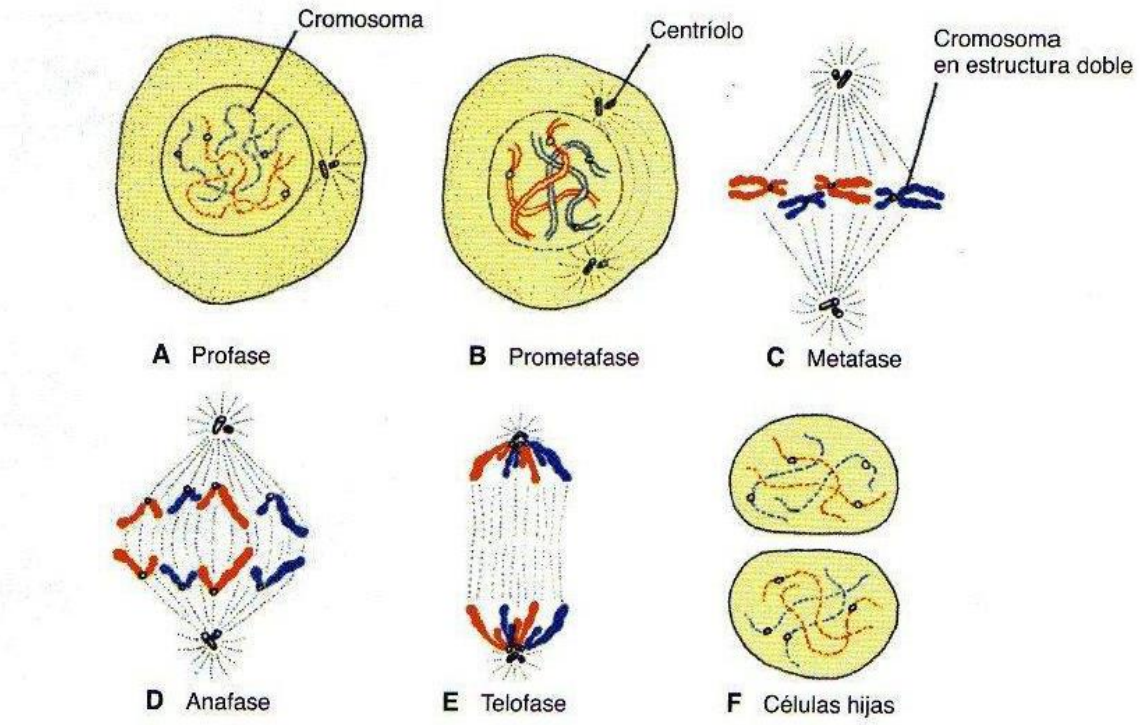

Figure 2. *Mitosis phases.* ***A:*** *chromosomes are uncondensed and cannot be identified under an optic microscope.* ***B:*** *Chromatids can be identified.* ***C:*** *Chromosomes get aligned and are clearly visible as individual units.* ***D:*** *Each centromere divides, and chromatids migrate towards opposite poles.* ***E:*** *Chromosomes become unrolled and elongated, nuclear wrapper is re-established and cytoplasm is divided.* ***F:*** *Each daughter cell gets half of the duplicated chromosomic material, maintaining the same chromosome number than the mother cell*(9).

3.4.3 Meiosis phase

Meiosis is a cell division process through which a **diploid cell (2n)** goes through **2 successive divisions** with capacity to generate **4 haploid cells (n).** This process is the responsible of maintaining the chromosome number in each specie(10).

Oogonias initiate the first meiotic division in fetal period. This meiotic process remains stopped in diplotene phase until puberty. At this moment, oogonias are known as **primary oocytes**. During prenatal development, lots of oogonias and primary oocytes degenerate and become atretic. During the fertile life of a women, a little number of surviving primary oocytes, between 15 and 20, start to develop with each menstrual cycle, but only 1 will complete the 1st meiotic division and reach ovulation; the others will become atretic. The rest of primary oocytes will remain as follicular reserve in diplotene phase waiting to start developing in another menstrual cycle.

After completing the 1st meiotic division before ovulation, the result is a big size cell, named **secondary oocyte**, and another smaller one with no function, named **1st polar body**.

Secondary oocytes initiate the **2nd meiotic division**, but this will remain in metaphase and will only continue if the oocyte is fertilized by a spermatozoon. If this does not happen, meiotic division will not conclude(8).

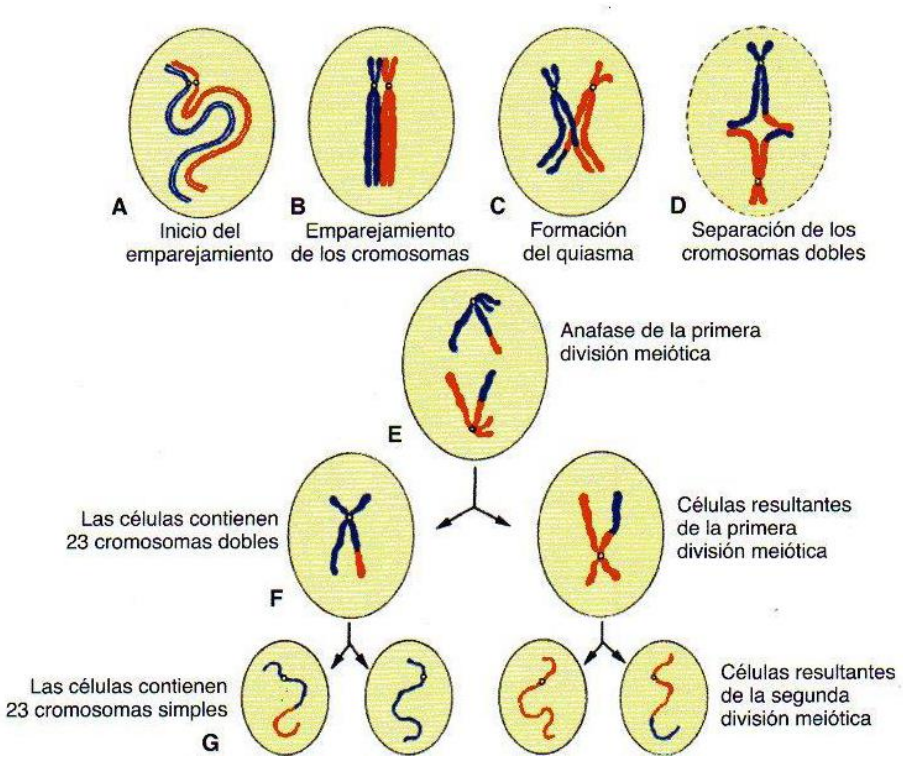

Figure 3. *First and second meiotic division.* ***A:*** *Homologous chromosomes get closer.* ***B:*** *Homologous chromosomes become paired. Each chromosome is composed by 2 chromatids.* ***C:*** *Homologous chromosomes exchange chromatic*

fragments, setting up a chiasm. ***D:*** *Chromosomes separate.* ***E:*** *Anaphase from first meiotic division.* ***F, G:*** *During second meiotic division, chromosomes get divided by the centromere. Once the division is completed, chromosomes from the 4 daughter cells are different from each other*(9).

3.4.4 Oocyte maturation/folliculogenesis

At birth-time, ovaries contain 2 million **primordial follicles** which are composed by a primary oocyte and a layer of flat epithelial cells. These cells do not express FSH receptors yet. The primordial follicles, which constitute the follicular reserve, will remain at 1st meiotic division until puberty, when some of them will be selected and continue their growth and maturation(11).

Since embryonic development and over years, the follicle reserve decreases drastically, going from 7-8 million of germinal cells at 5th month of intrauterine development down to 1-2 million of primordial follicles at birth-time. During life, this reserve decreases, especially from 35 years on(11).

Reproductive life starts with approximately 40.000 follicles at menarche. 99,9% of these follicles will start their growth, but only 400 throughout life will complete their maturation and will reach ovulation. The other follicles will experience atresia due to lack of adequate gonadotropic stimulus(9,11).

- **Follicle-development onset.** Since puberty, regularly and continuously, some of the quiescent primordial follicles from the ovarian reserve will be stimulated to grow by still unknown gonadotropin-independent mechanisms. As mentioned before, primordial follicles are composed by a primary oocyte surrounded by a layer of flat epithelial cells, which **do not respond to FSH** as they do not have FSH receptors yet. Paracrine signals between oocytes may be responsible of this selection and initial growth. **AMH** has also a role in this phase, as it regulates the amount of follicles that will progress from the ovarian reserve and which will become the dominant one, and acts as an indicator of follicular reserve(11).
- **Follicle-development progression.** Primordial follicles will experience atresia unless follicular growth continues. In this case, the layer of flat cells starts to divide to constitute cubic granulosa cells, and turn from monolayer into multilayer. The cells which are closer to the oocyte are known as **cumulus cells**, and have specific functions that the other granulosa cells do not have(10). The granulosa cells also secrete mucopolysaccharides that will form the **pellucid zone** surrounding the oocyte. Connections between the oocyte and the follicular cells are maintained through this pellucid zone. These junctions allow the exchange of amino acids and glucose metabolites, essential for the oocyte growth(8). At this moment, the primordial follicle is referred to as **primary or pre-antral follicle**. Outside the basement membrane and from the stroma, a layer of cells is differentiated forming the **theca**. These cells produce **androgens** (in response to LH), which will be converted into **oestrogens** in granulosa cells by **aromatase**. When the

theca is formed, and the granulosa cells are arranged in several layers, the pre-antral follicle is known as **secondary or antral follicle**, as it starts to be **gonadotropin-dependent** and by FSH action stimulates liquid formation. At the beginning, there are several spaces filled with liquid, and as more liquid appears, these spaces converge forming the **follicle antrum**.

Once the antral follicles start to be sensitive to FSH, **dominant-follicle selection** starts. This follicle will be selected based on its response to **FSH**. Thus, the follicle with most FSH receptors will generate more oestrogens by granulosa cells. FSH also induces **inhibin** secretion, which acts synergistically with oestrogens in the negative feedback on FSH secretion, lowing its concentration. In this way, those follicles with less response-capacity to FSH will become atretic, while the dominant one will continue secreting oestrogens and growing (11).

- **Dominant follicle.** Under FSH stimulus, LH receptors appear progressively in theca cells, making them to produce more androgens and consequently, more oestrogens by granulosa cells. Then, as the follicle growths, **oestrogens level increases**. Plus, FSH stimulates granulosa cells to produce LH receptors. This sensitization-increase to FSH and LH results in increased aromatase activity in granulosa cells. This dramatic increase of oestrogens, beside progesterone (which starts being secreted by luteinized granulosa cells), is responsible for **LH-peak** by a positive feedback on pituitary gland. This LH-peak suppresses granulosa-cells division and activates proteolysis, oocyte releasing and corpus luteum formation. A little after LH-peak, a less powerful **FSH-peak** occurs. Its aim is to release the oocyte from its follicular unions, as well as to stimulate granulosa cells to produce LH receptors in order to ensure progesterone secretion by corpus luteum once ovulation has occurred(11).
- **Corpus luteum.** After ovulation, granulosa cells and theca cells, beside its surrounding stroma, constitute the corpus luteum. From **LDL**, the corpus luteum will produce **progesterone**, with the aim of increasing endometrial thickness, forming glandular structures and increasing its vascularization(8). If fertilization does not occur, in 14 days the corpus luteum will become **corpus albicans** and progesterone levels will decrease. In this case, **LH** is responsible for maintaining the corpus luteum until its regression. If fertilization does occur, **hCG** secreted by the embryo is the hormone which maintains the corpus luteum and stimulates it to produce progesterone during the firsts months of pregnancy(11).

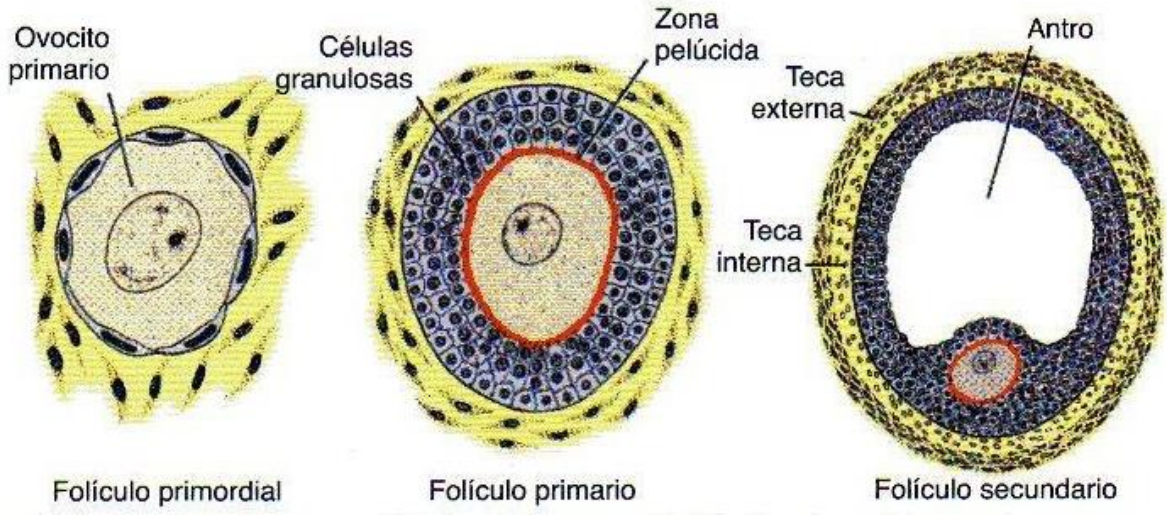

Figure 4. *Every day, some follicles from the FSH-independent primordial follicles reserve start to grow and become primary (or preantral) follicles, which start to respond to FSH. Later, FSH rescue some primary follicles to make them grow and become secondary (or antral) follicles. Oestrogen produced by follicular cells stimulate the pituitary gland to increase LH production. This hormone will make the follicle to get into the preovulatory phase, complete meiosis I and start meiosis II, remaining in metaphase II*(9).

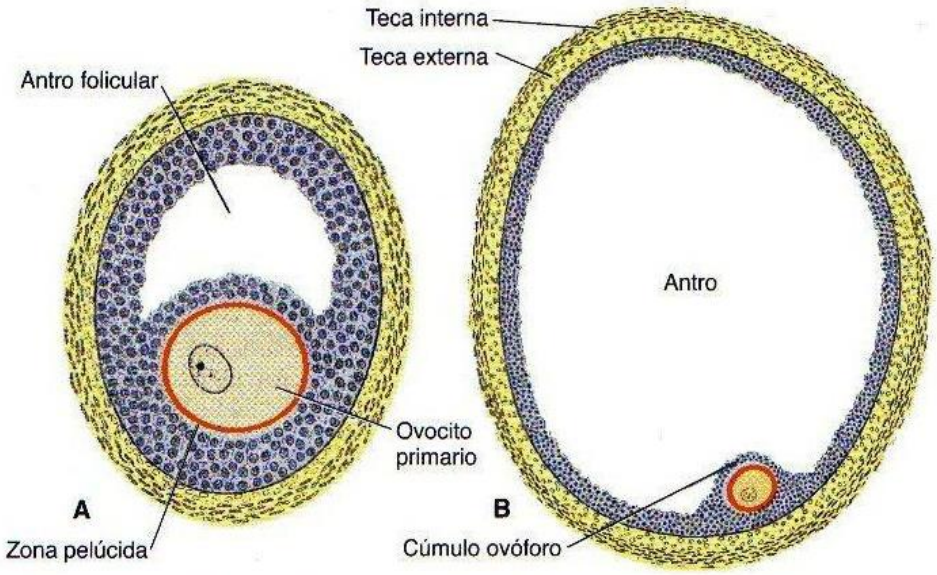

Figure 5. A: *Secondary follicle. The oocyte is in eccentric position. The antrum is developed from accumulated liquid in intercellular spaces.* ***B:*** *Mature secondary follicle (De Graaf, preovulatory). The antrum has become greater, is full of follicular fluid and surrounded by a stratified layer of granulosa cells. The oocyte is immersed into the cumulus cells*(9).

3.5 Fertilization

- **Phase 1: Corona radiata penetration.** Just 1% of spermatozoa deposited in the vagina will cross the cervix. Then, after a 2-7 hours route, spermatozoa arrive to the fallopian tube where they become capacitated, an indispensable process for being able to fertilize the oocyte. Capacitated spermatozoa cross the corona radiata cells with freedom(9).
- **Phase 2: Pellucid zone penetration.** This zone is composed by **glycoproteins** that facilitate and maintain the spermatozoon union. The **acrosomal reaction** takes place after spermatozoon union to pellucid zone, and it is induced by ZP glycoproteins. This reaction culminates in acrosomal enzymes releasing which are needed to penetrate the pellucid zone. **ZP3** participate in both the union and the acrosomal reaction. When spermatozoon's head gets in touch with oocyte's surface, pellucid zone's permeability changes. This provoke lysosomal enzymes releasing from cortical granules that coat the oocyte's plasmatic membrane. These enzymes alter pellucid zone properties to avoid other spermatozoa to get into the oocyte(9).
- **Phase 3: Oocyte and spermatozoon's cellular membrane union.** Once adhered, plasmatic membranes from the oocyte and spermatozoon become fused. When the spermatozoon gets into the oocyte, it responds in 3 ways(9):
    1. **Zone reaction and cortical reaction.** Cortical granules releasing containing lysosomal enzymes make the membrane impenetrable for other spermatozoa and pellucid zone structure gets modified. These changes avoid polyspermy.
    2. **2nd meiotic division resume.** The oocyte extrudes the **2nd polar body**, which barely contains cytoplasm. Chromosomes from the definitive oocyte get disposed in a vesicular nucleus known as **feminine pro-nucleus**.
    3. **Oocyte metabolic activation.** Probably, the activator factor is carried by the spermatozoon. Its nucleus grows and forms the **masculine pro-nucleus**, located next to the feminine one. Finally, they get in touch and lose their nuclear membrane. Each pro-nucleus must replicate its DNA. If not, each cell from the bicellular zygote will only contain half of the normal amount of DNA. After DNA synthesis, normal mitotic division occurs.

Fertilization results in:

- Chromosomes diploid number restoration
- Zygote gender determination
- Segmentation onset

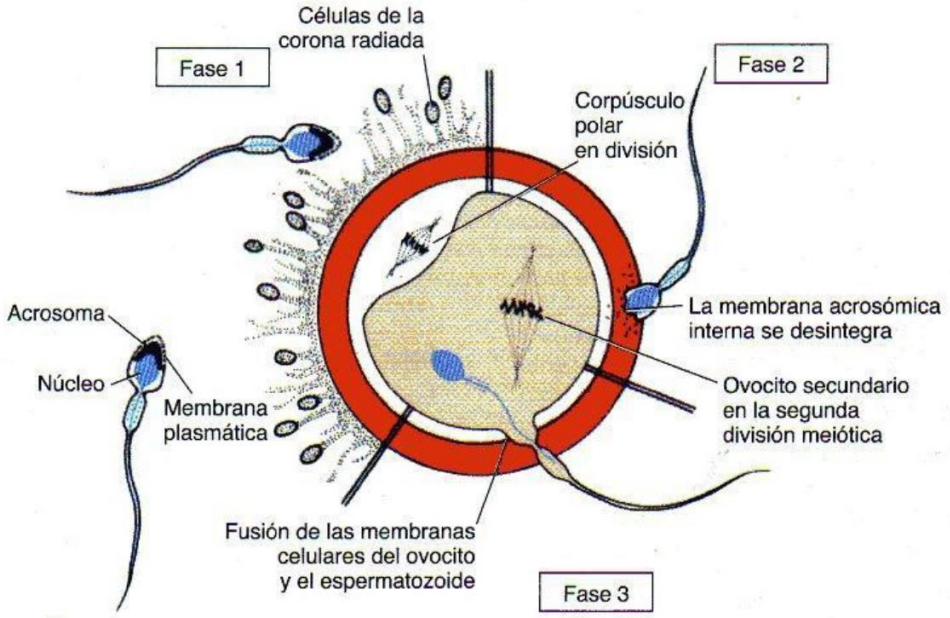

Figure 6. *Oocyte's penetration phases*(9).

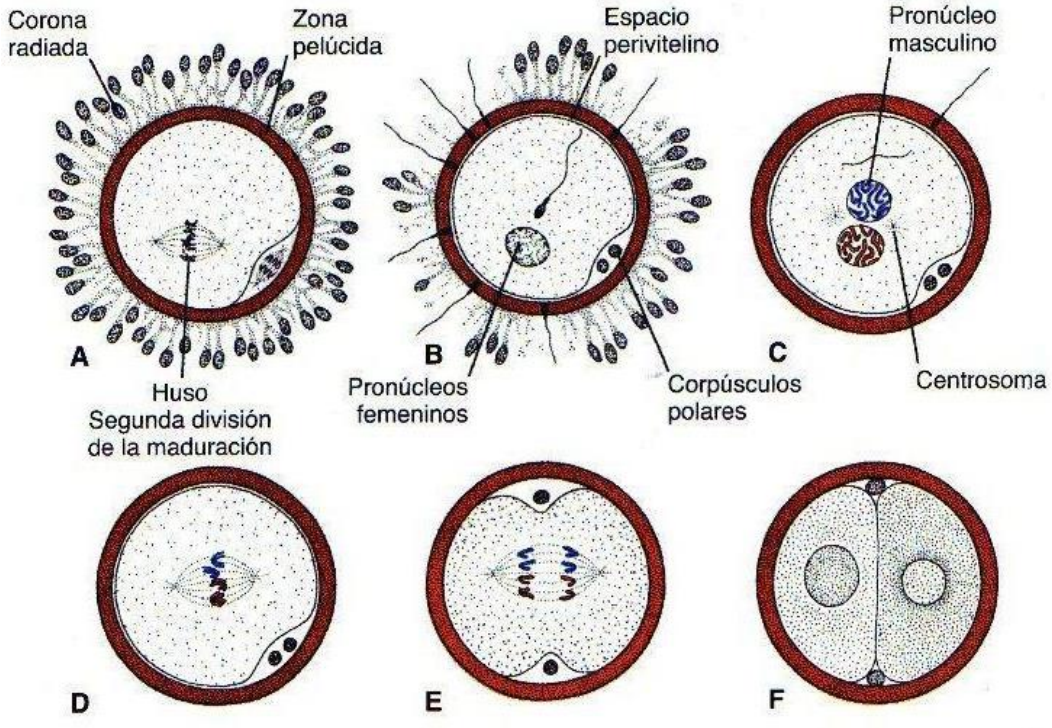

Figure 7. A: *oocyte just after ovulation. It can be observed the spindle apparatus.* ***B:*** *A spermatozoon has penetrated the oocyte, which has ended the second meiotic division. The oocyte chromosomes are arranged in a vesicular nucleus, the feminine pro-nucleus.* ***C:*** *Masculine and feminine pro-nucleus.* ***D, E:*** *Chromosomes are arranged in the spindle apparatus, are divided longitudinally and move to opposite poles.* ***F:*** *Bicellular phase*(9).

3.6 IVF/ICSI procedure

3.6.1 Controlled ovarian-stimulation protocols[1]

Since Edwards and Steptoe achieved the first pregnancy by IVF in 1978(10), a lot has changed in reproductive medicine. The first IVF was performed with an oocyte which had been achieved by a natural cycle. Over time, different COS protocols have been developed to make ART easier and more successful. They are recommended based on the type of patient:

- **Natural modified cycle.** It consists of ultrasonography control to see the single follicular development and then monitor its growth. Once the follicle achieves 21-23mm in diameter, the ovulation is pharmacologically induced by hCG, which is LH-like. It is mandatory to the patient to have regular ovarian cycles. The main advantage of this protocol is that the probability of a twin pregnancy is quite reduced as there is a unique follicle(12), and there is no risk of OHSS(13).
- **Weight loss.** It is not a stimulation protocol itself, but patients with overweight usually have irregular ovarian cycles, which are mostly anovulatory, and this reduces the probability of getting pregnant in a natural way. Furthermore, patients with overweight have worse results in ART. In obese patients (BMI >30), according to WHO, a 5-10% weight-loss may improve their menstrual cycles. Besides, those who require an ART will have better results(12).

  The patient weight is also important because the dose changes according to weight. Patients with more weight require more dose, which increases the risk of developing OHSS(12).
- **Clomiphene citrate.** It is a **selective modulator of estrogenic receptors** that acts blocking estrogenic receptors in pituitary gland. A low estrogenic blood-concentration is detected by the pituitary gland, and in response it secretes more FSH to stimulate ovaries. In ART patients, this treatment results in recruitment and development of a major number of follicles and eventually, a superovulation(12).

  Its first indication is in patients with ovulatory dysfunction type II from WHO and PCOS.

  Due to its anti-estrogenic effects, it can be administrated with exogenous oestrogen treatment(14). Even so, it is important to monitor the endometrial growth while CC is used, since some studies have reported a reduction in endometrial density in patients treated with clomiphene citrate(14).
- **Aromatase inhibitors.** They act by **blocking the androgen-oestrogen conversion**. It results in an increase of intra-follicular androgens, as well as FSH-receptors(12). These effects stimulate gonadotropins production, without the peripheric anti-estrogenic adverse effects of clomiphene citrate(1), as it does not act in estrogenic receptors.

[1] In this section all COS protocols that may be used in ART are explained, not only those used in IVF/ICSI.

- **Gonadotropins.** They are used more frequently in high-complexity ART as IVF/ICSI, because of the multiple follicular development they produce.
  - Human Menopausal Gonadotropin (hMG). Derives from menopausal-women urine. With a purification process, contained hormones (FSH and LH) are obtained. FSH recruits a major number of follicles and stimulates their growth, while LH is responsible of their final maturation without arriving at ovulation. This is achieved because LH is administrated constantly, avoiding the LH-peak(12).
  - Follicle-stimulating hormone (FSH). It is also obtained by urine, but it goes through a purification process which eliminates LH. It is recommended in patients with high LH-basal levels, which do not need this hormone during COS, such as patients with polycystic ovarian syndrome (PCOS)(12).
  - Recombinant FSH and LH. They are obtained from Chinese hamsters. The main advantage of recombinant gonadotropins compared to hMG is the absence of urinary proteins(1). For this reason, rFSH is the most used gonadotropin(15).

All COS medications may be associated with a **GnRH analogue**, which can be agonist or antagonist. They are used to prevent spontaneous LH-peak and ovulation in order to control ovulation when needed for follicular aspiration(12).

- **GnRH agonists.** They act by **desensitization**, phenomena that determines the loss of the secretive capacity of the pituitary gonadotropic cells due to continuous stimulation by GnRH. They link to the GnRH-receptors at the anterior pituitary gland. Being an agonist determines that a **flare-up effect** will occur at the beginning of the treatment. This effect consists of an increase of gonadotropins (FSH and LH) because of its agonists effects, with their consequent action on the folliculogenesis. For this reason, the agonist is given 1-2 weeks before the previous menstrual cycle ends. The disadvantage of this treatment is the necessity of injecting the product during many days, as it is a drug that acts by desensitization(12). Nevertheless, there exists a short protocol using GnRH agonists; it consists of initiating the drug between days 1-3 of the cycle, taking advantage of its flare-up effect for the follicular recruitment. After 2 days, ovarian stimulation with gonadotropins is initiated. It is used in low-responder patients(15).

  GnRH agonists can be used either as daily low-dose injections or through a single injection containing higher doses of the drug (depot)(16).

  However, GnRH agonist **can also be used to provoke LH-peak** due to its flare-up effect, so it is also given before follicle aspiration to promote follicle maturation(1).

- **GnRH antagonists.** They act by **competitively blocking the GnRH receptors** at the anterior pituitary gland. In this way, gonadotropins production rests directly and reversibly blocked. Unlike GnRH agonists, they do not require desensitization: their action is immediate. Thus, **there is no flare-up effect**. The main advantage of this drug is its rapid action, which avoids the LH-peak. Furthermore, it has to be injected less days than the GnRH agonists, what makes them a friendlier and more accepted protocol(12). As GnRH agonists, it can be administrated on a daily basis until final oocyte maturation, or with a unique dose only when the biggest follicle reaches 13-14mm(12,15).

  Also, oral contraceptives can be administered during the previous cycle. It allows to program cycles which turns into a better organization for the patient and the medical team(15).

  Antagonist protocol has been demonstrated to be **more cost effective than agonists**, as less gonadotropin is consumed and less stimulation days have been observed. Plus, it allows high-responder patients with OHSS risk not to cancel the cycle, as an agonist can be given to trigger ovulation instead of hCG (which is not possible in large agonist protocols)(15).

After the ovarian stimulation, it is needed to **induce ovulation** with the aim of obtaining mature oocytes. Initially, LH endogenous peak was tried to be identified, but it was very difficult and ineffective. Nowadays, LH-peak is tried to be reproduced(12). To make it possible, 2 medications can be used:

- **hCG.** Since its constitution is very similar to LH, it can **act over LH-receptors**, doing the same effect. hCG produce ovulation after 37-39h since its application, so the follicular aspiration must be performed between 35-36h, after hCG application but before ovulation(12). hCG is mostly used in **large-agonist protocols**, in which GnRH agonist can no longer be used as it has already reached des-sensitization when administered at the beginning of the protocol, and in **IVF/ICSI fresh cycles**, as GnRH agonists alter endometrial receptivity due to its luteolytic effect(15). hCG disadvantage is that l**asts more than 24 hours**, compared to the physiological LH duration of less than 60 minutes, increasing **OHSS risk**(15).
- **GnRH agonist.** Taking advantage of its flare-up effect, it can be used to provoke LH-peak and its consequent follicle maturation. It is a more physiological way to induce ovulation compared to hCG, since **it decrease OHSS risk**(1,17). Current evidence may show that in the context of GnRH antagonist protocol for IVF patients, the ovulation triggering with GnRH agonist have comparable results in terms of embryological laboratory results but a possible **lack in the patient's luteal phase** that ends up in lower pregnancy rates compared to hCG. On the other hand, in the context of an oocyte donation programme, where patients are not going to get pregnant, GnRH agonist triggering provide a simple, safe and effective treatment protocol(17).

There are 3 types of women based on their response to COS(15): 1-**High-responders,** women who obtain **>15 oocytes;** 2-**Normal-responders** (70%), women who obtain **4-15 oocytes**; 3-**Low-responders,** women who obtain **< 4 oocytes**. High-responders have a higher risk of developing OHSS.

Table 1. *Summary of agonist or antagonist protocol depending on patient's profile*(15).

	Antagonist + triggering with hCG	Antagonist + triggering with agonist	Large agonist	Short agonist
High response		X		
Normal response	X		X	
Low response	X		X	X
Oocyte donation		X		
Fertility preservation		X		

3.6.2 Follicular aspiration

Initially, laparoscopic follicular aspiration was the standard method for oocyte retrieval. Nowadays, **ultrasonography-directed techniques** have replaced the endoscopic approach. Transvaginal follicular aspiration with a vaginal ultrasonography transducer has become the prevalent technique at most centres.

The patient is placed in dorsal lithotomy position and the vagina is prepared with a copious saline irrigation. Intravenous sedation is adequate in most of cases.

A transvaginal ultrasonography is introduced with a fixed needle guide, and the collapse of every follicle is visualized on the ultrasonography screen as they are aspired one by one.

A typical oocyte retrieval will be completed within 15 to 45 minutes, depending on the number of follicles to be aspired and the speed with which oocytes can be identified(18). The oocyte is collected in the named **Cumulus-Corona-Oocyte complex**, composed by granulosa cells and the oocyte itself(19).

3.6.3 Semen collection and preparation

The sperm sample is usually collected by ejaculation, but it can also be obtained by testicular biopsy(3). The seminal fluid is allowed to liquefy at room temperature and then sperm is prepared for IVF/ICSI(18), as explained before, by **density gradients** and/or **swim-up**.

3.6.4 Insemination

Spermatozoon and oocyte become fused by themselves, in IVF, or through microinjection, in ICSI/IMSI.

Becoming fused by themselves in IVF implies that the spermatozoon must have both vitality and mobility, as it has to cross the pellucid zone, arrive at the nucleus of the oocyte and become fused with it. If spermatozoa are not of good quality, this process will not occur. In this case, it would be better to do an ICSI. Nowadays, however, most of fertility centres use ICSI by protocol. For this reason, we will refer to ICSI from now on.

In ICSI, unlike IVF, another procedure is performed before microinjection: **denudation**, where cumulus cells are eliminated to facilitate the introduction of the spermatozoon in the oocyte(20). After denudation, they must be classified in immature or mature oocytes. A **mature oocyte** is defined as such when **the 1st PB can be observed**, indicating that the 1st meiotic division is completed(21). Immature oocytes cannot be fertilized. Nevertheless, it is important to emphasize that having a mature oocyte does not mean it will get fertilized(19). Actually, 60 to 70% of oocytes collected from COS cycles have 1 or more morphologically abnormal features, such as SER accumulation, cytoplasmic inclusions, vacuolization or giant or fragmented 1st PB(21).

ICSI procedure consists of(22):

1. The mature oocyte is held with a specialized holding pipette.
2. A very delicate, sharp and hollow needle is used to immobilize and pick up a single spermatozoon.
3. This needle is then carefully inserted through the pellucid zone and into the cytoplasm of the oocyte.
4. The spermatozoon is injected in the cytoplasm and the needle is removed.

To evaluate if oocytes have been successfully fertilized, the most important parameter is to visualize the **PN and PB between 16-20 hours after ICSI**(21). Nowadays, with real time continuous monitoring introduction (*Time-Lapse*), the knowledge about embryonic development has increased(21).

Table 2. ASEBIR recommendations according to the relation between the number of pro-nucleus and polar bodies(21).

	IVF	ICSI
2 PN + 2 PB	Continue	
1 PN + 1 PB	Discard	
1 PN + 2 PB	If it evolves, 80% diploidy	Discard (If it evolves, 20% diploidy)
2 PN + 1 PB	Discard	
> 2 PN	Discard	

3.6.5 Embryo culture

Once the oocytes have been fertilized (day 0), the embryos are left in an incubator that maintains the optimal conditions for their development. They are observed until they are transferred, which will be realized on day +3 or +5-6, or until they are vitrified. Non evolutive embryos are discarded(23).

Embryos are classified, according to number of cells, division rhythm, percentage of cellular fragmentation, cellular symmetry and multinucleation, in 4 grades: **A, B, C, and D.** Grade A indicate the best quality, while grade D indicate the worst and implies discarding(21).

3.6.6 Endometrial preparation

Depending on the procedure, the endometrial preparation will vary:

- **AI and IVF/ICSI with own oocytes in fresh.** It is only administered progesterone since the follicular aspiration or the AI.
- **IVF/ICSI with frozen oocytes/embryos (cryotransfer) and IVF/ICSI with oocyte donation.** The treatment consists of oestrogens from the 1st day of the menstrual cycle and progesterone from the day 0 or the equivalent in frozen embryos.

In both cases, progesterone (plus oestrogens in case of cryotransfer or oocyte-donation) will be administrated until week 12-20 of pregnancy, or until negative pregnancy test.

The optimal endometrial thickness is considered between 7-10 mm, but for successful pregnancy the embryo quality and the endometrial receptivity are also important (24).

3.6.7 Embryo transfer

It can be done in fresh or frozen cycles. **Freeze-all strategy** consists of the cryopreservation of all embryos from an ART cycle and delayed embryo transfer in a natural cycle or a programmed hormone replacement cycle to prepare the endometrium. It is considered the preferred way to avoid potential deleterious effects of COS during fresh embryo transfer on endometrium receptivity, and consequently on embryonic implantation(25).

COS is associated with **negative effects** on endometrial receptivity during ART cycles, probably due to **high levels of oestrogen and progesterone** during the follicular phase compared to natural cycles. Because of subtle elevations of progesterone during COS, there could be a consequent asynchrony between the endometrium and the transferred embryos; the endometrial development may be at an advanced stage at

the moment of embryonic implantation(25). This progesterone increase would be conditioned by the sum of all the progesterone produced by the follicles developed as a result of COS in the preovulatory phase. Nevertheless, more studies are required to clarify if progesterone levels affect pregnancy rates in IVF/ICSI(15).

Therefore, the best results in ART considering pregnancy rates are found in oocyte donation cycles and cycles using frozen-thawed embryos transfer (FET). In these cases, there is no COS nor supraphysiologic hormonal levels at the time of embryo transfer(25).

3.6.8 Embryo vitrification

Vitrification is an ultrafast freezing procedure which use **cryoprotectants**, molecules which protect the embryo internal structures from low temperatures and icing. Vitrification has demonstrated better survival after thawing than the before-used slow congelation method(15).

There are two types of cryoprotective agents (CPAs): 1- **permeating agents** have small molecules that cross the cell's membrane, displace intracellular water and balance intracellular solutes and 2- **nonpermeating**, large-molecule CPAs that maintain an extracellular osmotic gradient that aids in further cell dehydration.

The examples of permeating CPAs include 1,2-propanedial (PROH), dimethyl sulfoxide (DMSO), ethylene glycol (EG), and glycerol. Sugars serve as the nonpermeating CPAs, with low molecular-weight disaccharides such as sucrose and trehalose typically selected for this role. The combination of the two types of CPAs creates an antifreeze cocktail that **reduces intracellular ice formation** by removing water from inside the cell and depresses the freezing point of water remaining in the cell(26).

Reasons to vitrify embryos:

- Not being able to do the transfer in the same cycle.
- After a IVF/ICSI cycle, with remaining embryos.
- To preserve fertility.
- Waiting for PGD results.

Embryos remain frozen at -196ºC in liquid nitrogen, and they can be used months or years after without losing quality.

3.6.9 Pregnancy test

The patient will be able to do an hCG test from 10-15 days after embryo transfer. The success of the treatment will vary depending on different factors, being the age the most important one.

It must be considered that successfulness of an IVF/ICSI cycle is not a positive pregnancy test, as some pregnancies may be non-evolutive. Real IVF/ICSI success is measured by a **healthy child born**(3).

3.7 Hypothalamic-pituitary-thyroidal axis

To maintain a normal metabolic activity in organism, an adequate level of thyroid hormone is required. In order to achieve it, there are specific feedback mechanisms which control thyroid secretion.

Nerve endings from hypothalamus middle eminence secrete thyrotropin-releasing hormone (**TRH**). Portal vessels transport TRH from hypothalamus to the pituitary gland; TRH interacts with its receptors and induces releasing of substances as phospholipase C, calcium ions and diacylglycerol which ultimately induces TSH liberation(7). **TSH** secretion stimulated by TRH is pulsatile, with an amplitude average of 0.6 mU/L every 2 hours, with a maximum between midnight and 4 AM(27). TSH is a glycoprotein composed of an α- and β-subunits. The α-subunit is also shared with other hormones such as LH, FSH and hCG(27,28).

Activation of TSH receptor (**TSHr**) by TSH leads to an increase in intracellular cAMP and stimulation of protein kinase A-mediated pathways. Some thyroid genes, including Na^+/I^- symporter (NIS), thyroglobulin (Tg), and thyroid peroxidase (TPO), are stimulated by TSH and promote the synthesis of TH.

The THs, T4 and the more potent T3, are synthesized in the thyroid gland. **Iodine** is actively transported and concentrated into the thyroid gland by **NIS**. NIS action allows the thyroid gland to maintain a iodine concentration 30-40 fold higher than plasma's(27). The trapped iodine is oxidized by **TPO** in the presence of hydrogen peroxide and incorporated into the tyrosine residues of **Tg**, once being transported by another protein called **pendrin**, which carries iodine to the colloid-membrane interphase(27). This iodination of specific tyrosines located on Tg yields **monoiodinated and diiodinated residues** (MIT, monoiodo-tyrosines; DIT, diiodo-tyrosines) that are enzymatically coupled to form **T4 and T3**(28). Then lysosomes, which contain proteolytic enzymes, become fused with the colloid vesicle. This release T4 and T3 and inactivated iodotyrosines, peptides and individual amino acids. Biologically active T3 and T4 enter circulation and DIT and MIT are deiodinated and its iodine is conserved(27).

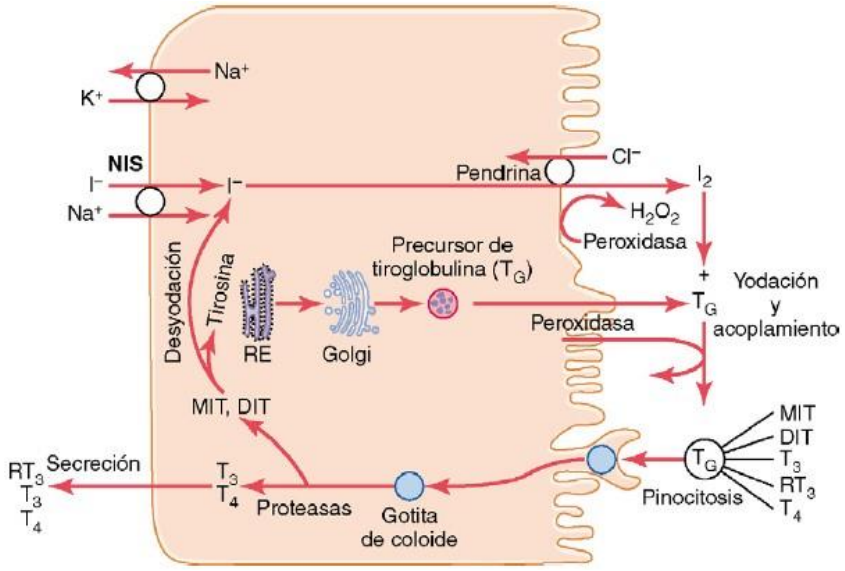

Figure 8. *Thyroid cell mechanisms for iodine transport, thyroxine (T4) and triiodothyronine (T3) formation and releasing towards bloodstream*(7).

About 93% of secreted hormone by thyroid gland is T4, and the remaining corresponds to T3(7). Nevertheless, **almost all T4 becomes T3 in tissues by a deiodination process**, as T3 is the biologically active hormone(29,30). This deiodination mainly occurs in liver, kidney and skeletal muscle(27).

As hypothalamus-pituitary-gonadal axis, this one is also regulated by negative feedback. Thyroid-hormone increase in blood reduces TSH releasing by the pituitary gland, as well as TRH releasing by hypothalamus, although to a lesser extent(7).

Additionally, other secondary modulators exist. For example somatostatin, as well as dopamine, have an inhibitory control of thyrotrophic cells(10,28), while alpha-adrenergic pathways activate them(10).

Table 3. *Normal TSH, T4 and T3 blood values*

	Normal blood values
TSH	0.34-4.25 mU/L
T4	Total: 5,4-11.7 µg/dL (70-151 nmol/L) Free: 0,7-1,24 µg/dL (9-16 nmol/L)
T3	Total: 77-135 ng/dL (1.2-2,1 nmol/L) Free: 2,4-4,2 pg/mL (3,7-6,5 pmol/L)

3.8 Thyroid-hormone transport and regulation

Around 75% of T4 concentration is transported by Thyroxine-Binding Globulin (**TBG**), as well as 10-15% is transported by transthyretin (**TTR**) and 10-15% by **albumin**. The rest, only 0,02% of T4 and 0,2% of T3 freely circulate in blood. Only the free fraction is biologically available for tissues(10).

Formerly, it was thought that thyroid hormones diffused passively across plasma membranes. Currently, it is known that thyroid hormone enters the cell by thyroid hormone transporters(28,29).

The intracellular availability of the biologically active T3 is the net result of a finely tuned system of 3 distinct deiodinases (**D1, D2 and D3**). According to CUN(31), a deiodinase is the enzyme which catalyse the loss of an iodine atom from the thyronine molecules. D1 and D2 can convert inactive T4 to active T3, whereas D3 is able to inactivate T4 and T3 converting them to reverse T3 (rT3) and T2, respectively(10). Their local activity contributes to increase or decrease both T3 blood and tissue levels. Their expression oscillates depending on the tissue:

- **D1.** It is found in peripheral tissues such as **liver and kidney** and is responsible for the conversion of most of T4 to T3 **in circulation**(10,28). T4 activates D1, contributing in hyperthyroid patients to have high circulating T3 levels(27).
- **D2.** It is found in **brain**, **pituitary** and **brown adipose tissue**, and primarily converts T4 to T3 **for intracellular use**(10,28). Excess of plasmatic T4 inactivates D2, with the main objective of protecting brain of excessive T3(27).
- **D3.** It is localized in **skin, placenta, liver, bowel and brain**, but also in brain tumours and vascular anomalies(10).

Deiodinases are regulated by multiple hormones, grow factors and environmental and nutritional factors; specially thyroid hormones as thyroid dysregulations induce deep changes in enzymatic activity(10).

Table 4. *Main characteristics of each deiodinase, adapted from*(27).

Deiodinase	D1	D2	D3
Substrates	rT3>T4>T3	T4>T3	T3>T4
Tissue distribution	Liver, kidney, skeletal muscle, thyroid gland	Brain, pituitary gland	Brain, placenta, fetal tissues
Function	Plasmatic T3 production	Local T3 production	T3 degradation
Hypothyroidism	Decreases	Increases	Decreases
Hyperthyroidism	Increases	Decreases	Increases

Biologically-active T3 finally enters the nucleus and exerts its function through the nuclear thyroid receptors, **TR alpha and TR beta**(29). TRs function as hormone-dependent transcription factors that repress target gene expression in the absence of hormone and stimulate gene transcription in response to T3 binding(10).

Unlike steroid receptors, TRs constitutively bind to thyroid-hormone response elements (**TREs**) independently from ligand occupancy(32). A HRE is a small DNA sequence specific for selective transcription factors that controls the expression of genes regulated by these factors(33). Unliganded TR represses the basal transcription, while ligand binding causes de-repression and enhances transcriptional activation. Thus, the biological significance of repression is to turn off target genes in the absence of hormone and to increase the magnitude of transcriptional activation by hormone ligand(32).

There are many TR isoforms, which are product of 2 genes denominated TRα and TRβ:

TRα gene is encoded on chromosome 17 and codifies for 2 proteins: **TRα1 and TRα2**. Only TRα1 is capable of binding T3 and activate gene transcription. **TRα2 is an "orphan" receptor**, homologous to viral oncogene c-erb-A, capable of antagonize T3 effects(10,30). It acts as a negative dominant transcriptional factor that binds to a specific TRE in thyroid hormone-regulated genes, but without generating transcriptional activity(34). This antagonist receptor has found to **increase with age**(10).

TRβ gene is encoded on chromosome 3 and also codifies for different proteins, **TRβ1 and TRβ2**. Both TRβ1 and TRβ2 are authentic receptors as they bind TREs and T3 with high affinity and specificity and can mediate T3-dependent transcription(28).

TR isoforms are expressed in different tissues; for example, TRβ is involved in TSH secretion (TRβ2), hepatic metabolism (TRβ1), hearing (TRβ1) and specific wavelength photoreceptors (TRβ2)(30). About TRα, it is mostly expressed on heart, brain, skeletal muscle and brown fat, where is involved in body temperature regulation(10).

3.9 Thyroid influence on reproduction

It has become clear that adequate levels of circulating T3 are of primary importance for normal female reproductive functions, as **changes in T3 levels result in menstrual disturbances, impaired fertility and altered pituitary gonadotropin secretion**(35). Some authors noted that the mean duration of sterility was significantly longer in patients with thyroid disorders compared to those without(2). Plus, iodine concentration in ovaries is the higher one after thyroid gland, and NIS expression in ovaries has been discovered, which may be the possible mechanism for this iodine catchment(10). In particular, small and

growing follicles take up more iodine than large ones indicating that the presence of this molecule is crucial for follicular development(36).

Hypothyroidism, defined as an increase in serum TSH with decrease in free T4 values(37), may affect the gonadotropic axis at different levels, determining changes in the hypothalamic-pituitary unit, gonadal function and the peripheral metabolism of sex steroids. Both hyperprolactinaemia, due to increased TRH production, and altered GnRH pulsatile secretion, leading to a delay in LH response and inadequate corpus luteum, have been reported(2). Joshi *et al.* found 68% of menstrual abnormalities in 22 women with hypothyroidism compared to only 12% in 49 controls(2). Recently, Cramer *et al.* demonstrated that serum TSH levels were significant predictor of failure of IVF, as TSH levels were significantly higher among women who produced oocytes that failed to be fertilized(2).

Subclinical hypothyroidism is defined as elevated TSH level with normal free T4 and no or very few symptoms(37). In a study, SCH rate was found higher in patients with reproductive disorders than women with proven fertility(38).

About **hyperthyroidism**, Joshi *et al.* found menstrual irregularities in 65% of hyperthyroid women, compared to 17% in healthy controls. Also, Krassas *et al.* observed irregular cycles in 22% of hyperthyroid women compared to 8% in the control population(2). Nevertheless, the precise impact of hyperthyroidism on fertility remains ill-defined, as most studies on the prevalence of hyperthyroidism in sterility are derived from uncontrolled and retrospective cohort studies. However, it is clear that women with increased thyroid hormones and sterility should treat and normalize their thyroid function specially before an ART procedure is planned(2).

Thyroid responsivity by the ovaries could be explained by the **presence of thyroid hormone receptors in human oocytes**. Steroid hormone synthesis by oocytes is dependent on adequate levels of thyroid hormones for normal reproductive function. **T3 modulates FSH and LH action on steroid biosynthesis** and T3 binding sites have been identified in mouse and human oocytes. Thyroid hormones enhance the action of oestrogen and potentiate oestrogen responses such as PRL production in the pituitary, as well as modulate progesterone production(2).

Plus, **free thyroid hormones** are present in follicular fluid(39) and **TR mRNA and proteins** are expressed in granulosa cells(36,39–41), cumulus cells and oocytes(36,40,41), suggesting that human oocytes may be directly responsive to T3. T4 and T3 were found in levels within the normal range for serum, while TSH was found in concentrations similar to, or above, than in serum(36). Plus, **TSHr** was found in oocytes, granulosa

cells, surface epithelial cells and stromal cells of human ovaries, with a particularly strong expression in the epithelium surface (34).

Aghajanova et al. performed a more detailed study proving that TRα1, TRα2, and TRβ1 proteins are not expressed in granulosa cells of primordial and primary follicles, while antral follicle granulosa cells express low amounts of TRα1 and moderate amounts of TRβ1, and granulosa cells of secondary follicles express TRβ1. This suggests a very fine regulation of thyroid hormone responsivity in various stages of follicle maturation. Positive faint-to-moderate immunostaining was found for TRα1 and TRβ1 proteins in human oocytes. These findings together allow to hypothesize that T3 may influence oocyte maturation by acting directly upon the oocyte itself or by influencing granulosa cell activity(36).

About **deiodinases** in ovaries, some studies have found that D1 is not expressed in human granulosa cells(34) nor epithelial ovarian cells(40), while another study have found D1 in granulosa cells but in small amounts(10). Despite of this, D2 and D3 were found in human ovarian epithelial cells by these studies. The fact of finding deiodinases at this level indicates a **local regulation** of thyroid hormone in ovaries(10,34,36).

4. JUSTIFICATION

Thyroid axis has several implications in reproduction. As mentioned before, many studies have shown that hypothyroidism and SCH, as well as hyperthyroidism, have negative effects on women fertility(2,37,38). Thyroid-function correction in these patients has already been demonstrated to improve their fertility(2), as euthyroidism normalizes PRL and LH levels, reverses menstrual abnormalities and increases spontaneous fertility(2).

Plus, thyroid hormones discovered in follicular fluid and thyroid receptors in granulosa cells(39) and ovarian surface epithelium(34) show the direct role of thyroid hormone in ovarian physiology. Also deiodinases have been described to be in granulosa cells(10) and ovarian epithelium(34), indicating a local thyroid regulation.

A recent study of thyroid receptors, fertility-linked genes and deiodinases differences between fertile and sterile women has contributed with new information that can improve and facilitate assisted reproduction. Concretely, it has demonstrated, among other things, that there exists a **TRα2 overexpression** in granulosa cells in sterile women compared to fertile women(10). TRα2, as explained before, is an orphan receptor that antagonizes T3. This suggests that having an overexpression of this receptor might be a cause of unexplained sterility because it would lead to a lack of T3 in oocyte development. There exists a direct correlation between TRα2 expression in granulosa cells and woman's age. This might suggest that the recognized age-associated increase in sterility is due, at least in part, to decreased intracellular thyroid hormone action in granulosa cells of sterile euthyroid women(42).

Plus, granulosa cells from sterile women whose oocytes did not result in pregnancy had increased TRα2 expression levels compared to granulosa cells from fertile women whose oocytes resulted in pregnancy. The unsuccessful IVF sterile cohort expressed more TRα2 mRNA in granulosa cells than the fertile cohort whose oocytes resulted in pregnancy after IVF, even after being adjusted for age and smoking. These findings suggest a relationship between intracellular thyroid function and success in ART(42).

Women with unexplained sterility are a difficult population to treat, since their sterility cause is unknown, so ART in these patients using their own oocytes have poorer results, with the psychological issues that this can cause to them. It would be important to find more solutions to these patients to facilitate their treatment. As a TRα2 overexpression in sterile women has been found, it would be a possible cause of unexplained sterility and in consequence, a possible door to their treatment.

Due to all these data, is important to do a pilot study of thyroid hormone treatment in middle-aged patients to prove if this treatment can improve their success (measured as fertilization rate) in ICSI and, if there is a

positive result, be able to make a clinical trial to consolidate its result. In this way, women with unexplained sterility may have better results in IVF/ICSI.

In this study, the drug used will be **levothyroxine 50µg**, because as the patients included in the study are euthyroid it is important to avoid a possible hyperthyroidism and its effects, so the dose cannot be high.

The result variable will be measured as **fertilization rate** since, as explained before, thyroid hormone treatment may improve oocyte's quality in these patients and, in consequence, increase oocyte's fertilization rate among the treatment group compared to placebo. We did not use pregnancy rate as the result variable because in that case, endometrial factors would also have been involved.

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6. HYPOTHESIS

Thyroid hormone treatment with levothyroxine in euthyroid women with unexplained sterility may improve their success in assisted reproduction techniques.

7. OBJECTIVE

To test whether the administration of levothyroxine in euthyroid women between 30 and 40 years old with unexplained sterility can increase their fertilization rate in ICSI compared to placebo.

8. METHODOLOGY

8.1 Study design

The best way to confirm or refuse that thyroid hormone treatment is effective increasing fertilization rate in women with unexplained sterility under ICSI is to do a longitudinal, prospective, randomized, double-blinded, controlled clinical trial (pilot study).

The total length of the study period will be approximately of 21 months and it will be conducted in Girexx Girona clinic.

8.2 Population

The study population will consist of women between 30 and 40 years old with unexplained sterility diagnosis who want to initiate an ICSI cycle to get pregnant.

As it has been proven that TRα2 overexpression in granulosa cells is directly proportional to age, we assume that our population will contain a higher percentage of women with this altered factor compared to a younger population.

8.2.1 Inclusion criteria

- Women between 30 and 40 years old with BMI <25
- Unexplained sterility diagnosis
- Failure in a previous ICSI cycle
- Normal ultrasonography, gynaecologic and mammary exploration
- Normal biochemical analytics, hemogram, coagulation and serologic and hormonal study
- Ability to comprehend and sign the informed consent

8.2.2. Exclusion criteria

- Indication of ICSI due to masculine factor
- HIV, HBV and/or HCV positive serologies
- Endocrinological alterations:
    - Diabetes Mellitus
    - Hyper or hypothyroidism, subclinical or stablished, with or without medical treatment, taking as normal rank: TSH 0,2-4,9 UI/mL and free-T4 0,8-1,9 ng/dL
    - Cushing syndrome

    - Congenital adrenal hyperplasia
- Women with any hormonal treatment
- Medical contraindications for pregnancy: autoimmune, cardiovascular, respiratory and renal severe diseases
- Contraindications for levothyroxine treatment: hypersensibility to the active principle, adrenal or pituitary insufficiency, non-treated thyrotoxicosis, acute myocardial infarction, acute myocarditis, acute pancarditis, coronary insufficiency, angina pectoris, arteriosclerosis, hypertension.

8.3 Sample selection

The sampling method will be consecutive, non-probabilistic; patients that fulfil the inclusion criteria will be asked to participate in the study when they come at the clinic until we complete our sample size.

8.4 Sample size

This will be a pilot study. We have no previous references regarding the expected differences. However, assuming a common standard deviation of 30% in fertilization rate, in an independent samples t-test means comparison with 20 subjects per group, we will have a statistical power of 75% to detect a difference equal or higher than 25% in the fertilization rate between groups. For this reason, the sample size used in this study will consist of 40 patients, equally distributed in 2 groups: 20 in thyroid hormone treatment and 20 in placebo group.

8.5 Analysed variables

- **Input variable.**
    - Thyroid hormone treatment. Levothyroxine 50µg will be provided. Patients included in the treatment group must take a pill once a day, preferable in the morning, at least 30 minutes approximately before breakfast, with empty stomach and preferably with a half-full glass of water. This treatment will last 3 months. A hormonal determination of TSH and free-T4 will be done at the beginning and end of treatment, in order to control whether the patients are taking the medication or not.
    - Placebo. Patients included in this group will take a placebo pill once a day, in the same conditions than the thyroid hormone. A hormonal determination will also be done in this group before and after the treatment.
- **Outcome variable.** Fertilization rate. It will be expressed as the percentage of fertilized oocytes (by ICSI) obtained by follicular aspiration in each group (treatment and placebo). A fertilized oocyte will

be considered when 2 pro-nuclei and 2 polar bodies can be determined by the Embryoscope microscope (400x).

Mature oocytes with abnormalities such as SER accumulation, severe central organelle clusters, excessive vacuolization, giant 1st PB or size >200µm will be discarded as well as zygotes with an abnormal fertilization such as 1 PN, >2 PN or 2 PN + ≥1 micronuclei.

- **Co-variables:**
  - Age: expressed as number of years
  - Smoking habit: expressed as smoker/non-smoker
  - TSH basal levels (mU/L)
  - TSH post-treatment levels (mU/L)
  - free-T4 basal levels (ng/dL)
  - free-T4 post-treatment levels (ng/dL)
  - Dose of FSH received (UI)
  - Dose of LH received (UI)
  - Number of oocytes obtained
  - Mature oocytes obtained: expressed as percentage
  - Mature oocyte's quality, according to ASEBIR parameters: organelle clusters, vacuolization, cytoplasmic inclusions, cellular remains in perivitelline space, pellucid zone anomalies, increased perivitelline space, 1st PB disturbances (fragmentation and size changes). Expressed as percentage of oocytes without any dysmorphism and percentage of oocytes with ≥1 dysmorphisms.
  - Discarded mature oocytes due to serious abnormalities: SER accumulation, severe central organelle clusters, excessive vacuolization, giant 1st PB, size >200µm: expressed as percentage. Those discarded mature oocytes will not be included in mature oocyte's quality variable.
  - Discarded zygotes due to abnormal fertilization: expressed as percentage.

8.6 Procedures

8.6.1 1st visit

To select the candidate patients for the study, we have to split patients into 2 groups:

1. Patients who arrive for the first time at the clinic with sterility (no pregnancy in more than 1 year of sexual relations without contraceptive methods).

2. Patients who have already done sterility tests and have already been diagnosed of unexplained sterility, with failure of previous ART.

In the first group, sterility protocol will have to be applied to see if there is any known cause. In these patients, the next studies will be required:

- Medical record with complete anamnesis (family and personal background, toxic habits, known diseases, obstetric and gynaecologic background, allergies)
- General analysis with ovarian function study
    - Hemogram, biochemistry, coagulation
    - Hormonal determination in the first phase of the cycle: AMH, FSH, LH, E2, PRL; TSH and free-T4
    - Serologies: RPR, HIV, HBV, HCV
- Transvaginal ultrasonography
- Hysterosalpingography post menstruation (to discard tubal obstruction and to assess endometrial morphology)
- Gynaecologic and mammary exploration
- Seminogram of patient's husband/partner

With these tests, we will be able to know which of them fulfil the inclusion criteria. Plus, they will serve to discard patients based on exclusion criteria.

In the second group, if the tests have been done in the last 6 months, no other evaluations will have to be applied. If any of the tests has been done earlier than 6 months ago or is missing, we will proceed to effectuate it.

Once we have arrived at unexplained sterility diagnosis and exclusion criteria has been applied, those patients will be eligible to be included in the study.

The next step will be to inform these patients of the possibility of participating in the study. We will have to give all the information of the procedures, so if the patient is interested in participating, we will give her the informed consent to be signed **(ANNEX)**.

The age and smoking habit variables, and BMI, will be evaluated in the 1st visit. To calculate BMI, patients will be weighted without shoes using a weighting machine located in the consult, and the doctor will measure their height using a measuring rod.

The doctor will proceed to gather all relevant patient data, avoiding to introduce any personal information that could identify the patient. All data will be stored in SPSS 22.0.0. 0 ® software.

8.6.2 Thyroid hormone/placebo treatment

The 40 patients included in the study will be randomly divided in 2 groups. For this aim, a statistical will prepare a table of random numbers and will randomly assign them levothyroxine or placebo treatment. As patients get included in the study, they will be randomly assigned to a number. The pharmacy associated with the study will prepare 40 sealed cardboard boxes containing the medication or placebo, identical in appearance, size and colour, and numbered according to the table of random numbers. Only the pharmacist responsible of the preparation will be aware of the contents.

Once the patients have been distributed, they will attend to the responsible doctor's consult in order to start the treatment. According to the random number assigned to each patient, the cardboard box marked with the correspondent number will be given to the patient.

Once the patients have completed 3 months of treatment, they will attend to the responsible doctor's consult in order to programme an ICSI cycle.

An analysis of TSH and free-T4 levels will be done in patients included in the study before and after the treatment period to ensure adherence to treatment. These analyses will be done by Centre d'Anàlisis Girona.

8.6.3 COS protocol

In this study, GnRH antagonist protocol will be used, with rFSH for follicular stimulation and GnRH agonist as ovulation triggering. The embryo transfer will have to be done in an asynchronic cycle as GnRH agonist cannot be used as ovulation trigger in fresh cycles because of its alterations on endometrial receptivity and embryo implantation.

The beginning of the COS will be determined by the follicular aspiration day (to simplify COS scheduling, in this study we will carry out follicular aspirations on Monday mornings). Women will start oral contraceptives since the first day of the previous menstruation, and then stop them 5 days before starting stimulation to provoke a menstruation. The 2nd day of period, the patient will start with subcutaneous self-administration of rFSH adjusting the dose depending on her predictable response (150-450 UI minimum and maximum doses, respectively). After a week, 3 transvaginal sonographic examinations will be performed to observe follicular development, coinciding with the 6th, 8th and 10th day of stimulation. These sonographies will serve

us to modify the rFSH dose according to the patient's response. Patients >35 years old will also take menotropin 75UI, a combination of urinary FSH and LH, in prevision of a low-response. After a follicular development of maximum 14-15mm (usually the day of 2nd ultrasonography) she will start with the subcutaneous injectable GnRH antagonist ganirelix 0,25 mg for 3-4 days to avoid spontaneous ovulation until the day of ovulation triggering. On Saturday night before, in a specific hour that will depend on the time of follicular aspiration, the patient will have to inject herself with GnRH agonist triptorelin 0,2 mg (36h before follicular aspiration). After that, no medication will be needed on Sunday, and the follicular aspiration will take place the next Monday morning.

The COS treatment and follicular aspiration will be cancelled in those patients with no response (<2 preovulatory follicles). As the ovulation triggering in the study will be performed by a GnRH agonist, patients with high response will not have to be cancelled.

All doses used during COS will remain written in the patient's medical record. When needed, this information will be used during the statistical analysis.

Table 5. *Schematic schedule of COS procedure in this study*

Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
OC	OC	OC	OC	OC	OC	OC
OC	OC	OC	OC	OC	-	-
-	Day 1 (menstruation)	Start rFSH +/- FSH+LH	rFSH +/- FSH+LH	rFSH +/- FSH+LH	rFSH +/- FSH+LH	rFSH +/- FSH+LH
1st ultrasonography rFSH +/- FSH+LH	rFSH +/- FSH+LH	**2nd ultrasonography** rFSH + ganirelix +/- FSH+LH	rFSH + ganirelix +/- FSH+LH	**3rd ultrasonography** rFSH + ganirelix +/- FSH+LH	Triptorelin	-
Follicular aspiration						

8.6.4 Follicular aspiration

The procedure will be conducted under sedation, via vaginal and controlled with transvaginal ultrasonography. The equipment used will be an ultrasonography machine with vaginal probe and guide, a puncture needle and a vacuum pump with continuous regulation and a thermic block to maintain the aspiration tubes at 37ºC.

All follicles will be aspired by the doctor in presence of the assigned biologist.

The aspired follicular liquid will be taken to the laboratory in optimal temperature and asepsis conditions. Then, the biologist will proceed to oocyte isolation and denudation and will classify them depending on their nuclear maturation and appearance. Oocytes without the 1st polar body will be discarded, as well as those mature oocytes with serious abnormalities mentioned before.

8.6.5 Laboratory procedures

- Oocyte denudation

Oocytes obtained by follicular aspiration will be conserved inside the incubator in culture medium until denudation. Denudation process will be performed under a laminar flux cabin with heated surface using a stereoscopic microscope at approximately 20x. 2 denudation steps will be done:

  - **Enzymatic digestion**. Oocytes are put in Hepes medium and hyaluronidase, which will eliminate a considerable part of cumulus cells.
  - **Mechanical denudation**. Using Flexiplet® or similar with decreasing diameters (300, 170 and 140 µm), oocytes will end up losing all the remaining cumulus cells by consecutively aspiring them.

- Insemination

Once the oocytes have been isolated and denudated, the biologist will proceed to classify them according to their quality. While waiting for insemination, oocytes will be conserved inside the incubator in culture medium. Then, the biologist will inseminate the oocytes with the ICSI method. An inverted microscope using Hoffman optics with a mineral oil micromanipulation system will be used. The to-be-fecundated oocytes will be put in the periphery of an ICSI plate in Hepes medium, one oocyte per drop. Sperm will be put in the centre of the plate, submerged in PVP in order to diminish spermatozoa mobility to facilitate ICSI. All the plate will be then submerged in mineral oil.

- Embryo culture

The resulting injected oocytes will be conserved in culture medium in *Embryoscope® Time-lapse Incubator* and observed by the *Embryoviewer® Software*. They will be conserved under the following conditions: O_2 5%, CO_2 6% and 37ºC. The biologist will evaluate the oocytes of each patient 16-20 hours after ICSI to check how many oocytes have been successfully fecundated.

8.7 Statistical analyses

The data collected will be analysed using the Statistical Package of the Social Science (SPSS) software.

In all analyses, **p ≤ 0,05** will be considered statistically significant and **p ≤ 0,001** will be considered as highly significant. Confidence intervals will be **95%.**

8.7.1 Univariate analysis

The results will be expressed as mean and standard deviation (SD) for normally distributed quantitative variables, as median and interquartile range for non-normally distributed quantitative variables and as frequencies for categorical variables.

8.7.2 Bivariate analysis

The input variable is dichotomous (treatment with levothyroxine or placebo) and the outcome one is continuous (fertilization rate). For this reason, comparison between these variables will be done with t-Student test assuming a normal distribution; otherwise, U-Mann-Whitney test will be used.

A bivariate analysis will also be performed on the co-variables to observe if they might be confounding or interaction variables for the outcome variable. Comparisons between the treatment and placebo groups will be carried out with:

- t-Student test for dichotomous normal distributed categorical variables, or U-Mann-Whitney test if they are non-normally distributed
- Pearson correlation coefficient for continuous quantitative variables
- Spearman rank for discrete quantitative variables

These analyses will allow to check if randomization is uniformly distributed in each population group in order to avoid randomization bias.

8.7.3 Multivariate analysis

As this study has 1 continuous outcome variable, we need to do a Multiple Lineal Regression model in order to see if the co-variables controlled in this study may explain or not the relation among the input and outcome variables.

We will use the outcome variable (fertilization rate) as a dependent variable and the input variable and the co-variables as independent variables.

9. WORK PLAN AND TIMETABLE

- **Phase 0** (September 2017 – December 2017):

    - Study protocol development (September 2017 – November 2017). The investigation team (**investigators 1 and 2**) will conduct the bibliographic research, define a hypothesis and objective and identify the variables of interest.
    - Presentation and approval of the CEIC (December 2017).

- **Phase 1** (January 2018 – December 2018):

    - Patients' selection and inclusion (January 2018 – June 2018), performed by **investigators 1 and 2.**
    - 1st TSH and free-T4 analysis by blood extraction (January 2018 – June 2018), performed by **Centre d'Anàlisis Girona.**
    - Levothyroxine/placebo treatment onset (January 2018 – June 2018), performed by **investigators 1 and 2.**
    - 2nd TSH and free-T4 analysis by blood extraction (April 2018 – September 2018), performed **by Centre d'Anàlisis Girona.**

As patients get included in the study, a blood extraction will be requested in Centre d'Anàlisis Girona to analyse TSH and free-T4 basal levels. Then, they will start the 3 months levothyroxine or placebo treatment depending on the patient's random group. Once the treatment is completed, another TSH and free-T4 analysis by blood extraction in Centre d'Anàlisis Girona will be performed to ensure adherence to treatment.

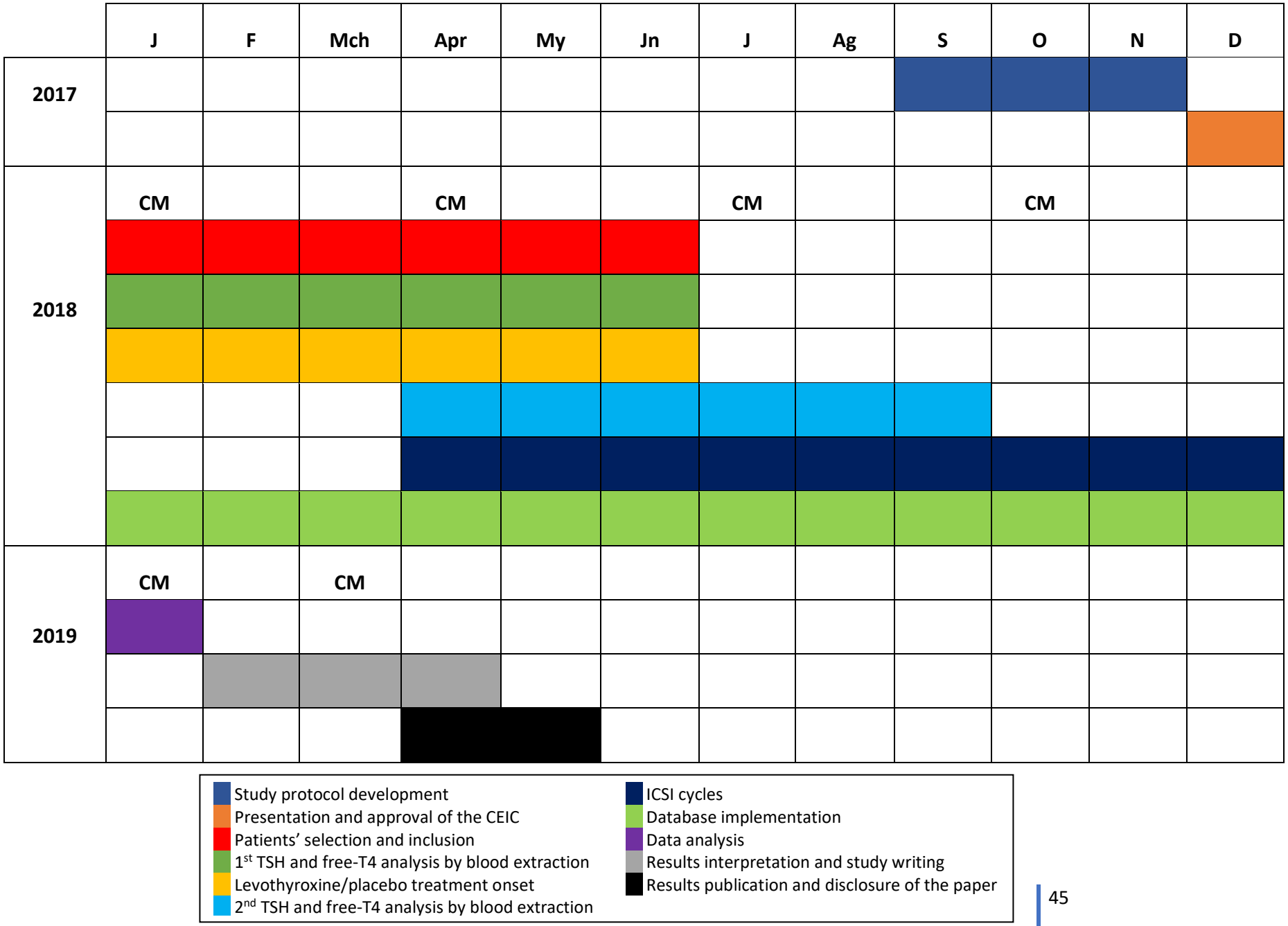
    - ICSI cycles (April 2018 – December 2018). Once the treatment is completed, patients will start COS followed by follicular aspiration. After that, the biologist will inseminate the mature oocytes obtained and will follow them up until fertilization is confirmed 16-20 hours after the insemination. Performed by **investigators 1 and 2 and the Girexx biologist.**
    - Database implementation (January 2018 – December 2018). All clinical and laboratory information of patients participating in the study will be stored in SPSS 22.0.0. 0®software as the information is obtained. Performed by **investigators 1 and 2.**

- **Phase 2** (January 2019): Data analysis. Once all clinical and laboratory data is gathered and entered in SPSS 22.0.0. 0 ® software, an **expert statistic** will analyze all the information.

- **Phase 3** (February 2019 – April 2019). Results interpretation and study writing. Performed by **investigators 1 and 2.**

- **Phase 4** (April 2019 – May 2019). Results publication and disclosure of the paper. Performed by **investigators 1 and 2.**

A **coordination meeting (CM)** will take place every 2-3 months starting on January 2018 until March 2019.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Presentation and approval of the CEIC
Patients' selection and inclusion
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Levothyroxine/placebo treatment onset
2nd TSH and free-T4 analysis by blood extraction
ICSI cycles
Database implementation
Data analysis
Results interpretation and study writing
Results publication and disclosure of the paper

10. BUDGET

		QUANTITY	COST	TOTAL
Personnel cost	Statistician consultant	10h	40 €/h	400 €
Material expenses	Levothyroxine 50µg pills	2.000 (20 boxes)	0,5 €/unit	1.000 €
	Placebo pills	2.000 (20 boxes)	0,43 €/unit	860 €
Subcontracts	Centre d'Anàlisis Girona	-	1.600 €	1.600 €
Insurance policy	-	1	6.000 €	6.000 €
Presentation cost	Inscription to a National Congress	-	1.500 €	1.500 €
Travel and meals	Transport	2	180 € pp	360 €
	Accommodation	2	150 € pp	300 €
	Meals	2	200 € pp	400 €
Publishing expenses	Submission fee	1	70 €	70 €
	Publication in *Thyroid Journal*	10 pages	65 €/page	650 €
Printing	Information sheet	80 pages	0,04 €/page	3,2 €
	Informed Consent	40 pages	0,04 €/page	1,6 €
TOTAL				**13.144,8 €**

11. ETHICAL ASPECTS

This research protocol has been conducted according to human's rights and ethical principles for Medical Research involving human subjects. Outlined in the World Medical Association's Declaration of Helsinki, last time reviewed in October 2013.

This research protocol will be presented to the appropriate ethics committee, "Comisión de Ética para la Investigación Clínica (CEIC)" in Hospital Dr. Josep Trueta, located in Girona (Spain). Once the authorization of the committee is obtained, it will be presented to the Girexx direction management for approval.

Written Informed Consent **(ANNEX)** will be obtained from each patient. The patients enrolled in the study will find all the information about the purpose of the study, the potential risks and benefits of the study, how the investigation will be conducted and their rights as participants in the Informed Consent. All the information will also be thoroughly explained by the investigators. The patient information will not be used without previous consent and it will be necessary to sign it before taking any action.

The investigators guarantee that this research protocol and the Informed Consent have been written as dictated by the following state laws:

- "Ley orgánica 15/1999, de 13 de diciembre, de Protección de Datos de Carácter Personal".
- "Real Decreto Legislativo 1/2015, de 24 de julio, por el que se aprueba el texto refundido de la Ley de garantías y uso racional de los medicamentos y productos sanitarios".
- "Real Decreto 1090/2015, de 4 de diciembre, por el que se regulan los Ensayos Clínicos con Medicamentos, los Comités de Ética de la Investigación con Medicamentos y el registro español de estudios clínicos".

The patient's data will be stored and maintained confidential in a secured system, just accessible for the investigators, and anonymization will be applied to every data introduced in this system.

This research protocol will also be presented to the "Agencia Española del Medicamento y Productos Sanitarios" (AEMPS) to obtain its authorization.

As the use of thyroid hormone in euthyroid women is not considered as a specific indication, it is mandatory to contract a liability insurance for patients participating in this study.

The authors declare that they have no conflict of interest.

12. LIMITATIONS

- This protocol is designed for a pilot study, so the sample size is small and may be non-representative of the studied population. Furthermore, the fact of having a small sample size increases the probability of a type II error. For this reason, it would be necessary to do a clinical trial with more patients and medical centres involved to be able to extrapolate the results to the studied population.

- The sampling method used is consecutive, which is a non-probabilistic sampling method. This fact has its own limitations (it might be non-representative of the population), but it is useful for small sample sizes and it is the best for non-so common pathologies such as unexplained sterility.

- As no TRα2 overexpression in granulosa cells has been demonstrated in our sample, we do not know how many women included in the study really have this overexpression. For this reason, some women may be receiving thyroid hormone while not having this alteration. This could lead to a selection bias, but we chose this study design to avoid extra invasive procedures (such as follicular aspiration) and annoying procedures (such as COS) to the patients. Analysing granulosa cells would imply having to do 2 COS and 2 follicular aspirations to each patient, increasing the inherent risks of these procedures and the risk of patients quitting from the study.

- One exclusion criteria for our population selection is all those couples with male sterility factor. The limitation in this fact is that, in the same way that a woman can be sterile with all tests in the normality, a man can be sterile with a normal spermogram as well. For this reason, a few women included in the study for unexplained sterility might actually be for a male factor. However, when a spermatic sample fulfils all normality parameters (morphology, mobility and quantity), it is unlikely to have DNA alterations that produce reproductive difficulties.

- The variable oocyte's quality may be subjective because it depends on the biologist discretion. For this reason, it is difficult to standardize this variable and it might differ from one measurement to another. With the aim of minimizing this factor, we will use the parameters mentioned in the ASEBIR criteria. However, there is not a stablished consensus about how dysmorphisms affect oocyte's fertilization due to lack of information.

ANNEXES

STUDY INFORMATION SHEET

Name of the study: **Thyroid hormone treatment in euthyroid women with unexplained sterility under intracytoplasmic sperm injection: a pilot study**

Lately, it has been observed that thyroid function has a relevant importance in fertility. We propose you to participate in this study to test if thyroid hormone treatment may improve fertility in patients with unexplained sterility under assisted reproduction techniques.

You may be a candidate for this study as you have been diagnosed with unexplained sterility. It means that your sterility cause has not been discovered after all the pertinent studies. A recent study concluded that this diagnosis might be related to a specific inhibitory thyroid hormone receptor in ovarian follicles (the sac that contain oocytes) which increases with age.

If you decide to participate, you will be requested to take a 3 months treatment with thyroid hormone or placebo before the ICSI cycle. You will have to take 1 pill every day, 30 minutes before breakfast with empty stomach, preferably with a half-full glass of water. A thyroid analysis will be conducted before and after the treatment period by Centre d'Anàlisis Girona. This analysis will be performed by blood extraction; complications derived from this procedure do not differ from a normal blood analysis.

You will be randomly distributed in one group, and you will not know If you are taking thyroid hormone or placebo. After the ICSI cycle, we will evaluate your fertilization rate. This study will take place in Girexx Girona clinic.

Thyroid hormone treatment may have some adverse effects typical of hyperthyroidism when the individual levothyroxine tolerance limit is exceeded: cardiac arrhythmias (i.e. auricular fibrillation and extrasystoles), tachycardia, palpitations, angina pectoris, headache, muscular weakness and cramps, blush, fever, vomits, menstrual alterations, pseudotumor cerebri (idiopathic intracranial hypertension), tremor, agitation, insomnia, hyperhidrosis, weight loss and diarrhoea. For this reason, **the thyroid hormone dose you might take in this study is low to avoid all these effects.**

In case you think you may be suffering from a side effect from the treatment, please get in touch with Girexx Girona (you will find a contact number at the end of the document). Plus, in that case, you will be economically compensated.

This study may be important to find solutions to patients with unexplained sterility with gestational desire that cannot achieve a pregnancy because of this issue. This would allow these patients to use their own oocytes before appealing to other options such as oocyte donation.

You must know that, according to "Ley Orgánica 15/1999 de 13 de diciembre de Protección de Datos de Carácter Personal", your personal data obtained during the trial will be confidentially treated.

Participating in this trial is completely volunteer, so you will not receive any economical compensation for your participation.

If you decide to not participate in this study or if you want to quit from the study in any moment, you will have the same assistance quality than patients participating in the study and no benefits loss.

You have the right to ask for the future results of the study. If you have any question, you can address to:

972 226 004 (Girexx Girona) or girona@girexx.cat

Thank you for your collaboration.

INFORMED CONSENT

Name of the study: **Thyroid hormone treatment in euthyroid women with unexplained sterility under intracytoplasmic sperm injection: a pilot study**

Reference centre: **Girexx Girona**

I, (name and surname)……., have received enough information about the study. I was able to ask about the study and my questions have been resolved satisfactorily. I have spoken to (investigator's name)……. I understand that my participation in this study is completely volunteer. I comprehend that I can back out from the study:

1. In the moment I desire it
2. Without having to give any kind of explanation
3. Without that supposing any difference in my medical assistance

Thereby, I give my conformity to participate in this study.

X _____ X _____

Participant's signature Investigator's signature

Date: / /