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Optimization of Ovarian Stimulation protocols and IVF outcomes: new perspectives

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Table of content

Introduction 2

Chapter 1. Infertility: definition, epidemiology, and female causes 4

Definition 4

Epidemiology 5

Female causes 7

Focus on female age-related fertility decline 7

Focus on female disease-related infertility 9

Chapter 2. Assisted Reproductive Techniques and controlled ovarian stimulation 12

Assisted Reproductive Technologies: IVF/ICSI 12

Controlled Ovarian Stimulation 13

Controlled Ovarian Stimulation Monitoring 16

Controlled Ovarian Stimulation Outcomes 18

Chapter 3. Difficult to treat patients: PORs and POI 21

PORs 21

POI 23

Chapter 4. Most Relevant studies 26

Does the dose or type of gonadotropins affect the reproductive outcomes of poor responders undergoing modified natural cycle IVF (MNC-IVF)? 26

Impact of cell loss after warming of human vitrified day 3 embryos on obstetric outcome in single frozen embryo transfers 37

Impact of GnRH antagonist pretreatment on oocyte yield after ovarian stimulation: a retrospective analysis 52

Chapter 5. Timeline 67

Conclusions 70

Acknowledgement 70

References 71

Introduction

The success rates of in vitro fertilization embryo transfer (IVF-ET) have steadily risen thanks to advancements in controlled ovarian stimulation (COS).

COS plays a pivotal role in achieving the goal of a healthy newborn.

Recent research and clinical focus have shifted toward personalized cycle optimization, aiming to enhance IVF outcomes. While there is no consensus on the optimal ovarian stimulation protocol, as it is typically chosen on a case-by-case basis considering the couples' medical history and the individual characteristics of the women, some patients remain challenging to treat.

Among these are women with a diminished ovarian reserve due to age-related fertility decline, inadequate responses to ovarian stimulation, and premature exhaustion of fertility.

In response to these challenges, the past few decades have witnessed various efforts, including the development of innovative stimulation strategies and adjunct treatments to achieve optimal ovarian responses in such patients.

Additionally, scheduling of ovarian stimulation can be demanding, particularly when maintaining IVF success is crucial. Therefore, it is essential to explore approaches that offer flexibility in ovarian stimulation scheduling without compromising IVF outcomes.

This thesis comprises three studies conducted between 2021 and 2023, all aimed at improving IVF results. Particular attention is devoted to addressing issues related to poor ovarian response, embryo development, and ovarian stimulation. Furthermore, a brief overview on IVF and stimulation protocols precedes the

presentation of the studies. Towards the end of this manuscript, a timeline showcasing other completed studies and ongoing projects is provided.

Chapter 1.
Infertility: definition, epidemiology, and female causes

Definition

The most recent international glossary on infertility and fertility care provides a comprehensive definition of infertility as a condition characterized by the inability to achieve a clinical pregnancy after 12 months of regular, unprotected sexual intercourse or due to an individual's impaired capacity to reproduce, whether in isolation or in relation to their partner.

According to the World Health Organization's latest definition, infertility is considered a complex disease that can result in functional disability (Zegers-Hochschild et al., 2017).

Subfertility is a term that can be used interchangeably with infertility (Zegers-Hochschild et al., 2017) and is defined as any degree of reduced fertility in couples who have been unsuccessful in their attempts to conceive (Habbema et al., 2004). It's important to note that the definition of infertility is primarily time-based, whereas sterility denotes a permanent condition of infertility (Zegers-Hochschild et al., 2017). Furthermore, infertility can be categorized into primary and secondary forms. Primary female infertility refers to a woman who has never experienced a clinical pregnancy and meets the criteria for an infertility diagnosis. Secondary female infertility, on the other hand, applies to a woman who is unable to establish a clinical pregnancy after having previously

experienced one (Zegers-Hochschild et al., 2017). A similar distinction can be made for males concerning their ability to contribute to the initiation of a pregnancy.

Epidemiology

It is estimated that infertility impacts between 8 and 12% of couples of reproductive ages globally (Ombelet et al., 2008). Among these, secondary infertility, is the most prevalent form of female infertility worldwide (Nachtigall, 2006; Rutstein & Shah, 2004). Global demographic trends vary widely, with high fertility rates and rapid population growth observed in the poorest countries, such as some nations in sub-Saharan Africa. In contrast, population decline, aging, and low fertility rates pose concerns in many developed countries (Bongaarts, 2015).

The prevalence of infertility in Europe varies among countries and regions. Generally, it is estimated that around 1 in 6 couples experience infertility at some point in their reproductive years (Mascarenhas et al., 2012). This figure can fluctuate, with some countries reporting slightly lower or higher rates. These differences can be attributed to a variety of factors, including age, socioeconomic status, and access to healthcare.

1) Demographic Factors:

Age: Age is a crucial determinant of infertility prevalence. Europe, like many other developed regions, has experienced a trend of delayed childbearing. Women are choosing to start their families later in life, which significantly

affects infertility rates (Johnson & Tough S. 2012). As women age, the chances of experiencing infertility increase due to the natural decline in fertility with advancing age. This trend is particularly notable in Western European countries with more career-focused lifestyles.

Marital Status: Infertility rates also differ based on marital status. Couples in formal marriages tend to seek fertility evaluation and treatment more frequently than unmarried or cohabiting couples. However, this gap is narrowing as societal norms around family formation continue to evolve (Pinelli, 2020)

Reproductive History: Individuals who have previously given birth may experience secondary infertility, which has a distinct prevalence rate from primary infertility. The number of children a woman has already had can influence her risk of infertility (Whitley et al. 1999).

Socioeconomic factors:

Economic Status: Socioeconomic factors, including income and education, play a role in infertility prevalence. High-income countries in Western Europe may have more access to fertility treatments and higher rates of infertility diagnosis. Conversely, lower-income countries in Eastern Europe may face economic barriers to accessing infertility care (Imrie et al. 2023)

Urbanization: Urban areas in Europe often have higher infertility rates than rural areas due to differences in lifestyle, environmental exposures, and access to healthcare. Urbanization can be associated with increased stress, pollution, and sedentary lifestyles, all of which can impact fertility (Li et al.1994).

3) Lifestyle Factors:

Obesity: The prevalence of obesity in Europe has been on the rise. Obesity can have a detrimental effect on fertility in both men and women, leading to hormonal imbalances and ovulatory disorders. Countries with higher rates of obesity may experience elevated infertility rates (Sharma et al. 2013).

Smoking and Alcohol Consumption: Lifestyle choices, such as smoking and excessive alcohol consumption, are known risk factors for infertility. Rates of smoking and alcohol use vary across European countries, contributing to differences in infertility prevalence (Sharma et al. 2013).

4) Healthcare Access:

Access to Fertility Care: The availability and accessibility of fertility treatments, including assisted reproductive technologies (ART) like IVF, differ across European countries. Nations with more comprehensive healthcare systems may have higher rates of infertility diagnosis and treatment (Reindollar, 2015).

Insurance Coverage: The extent of insurance coverage for infertility treatments can also impact prevalence. Countries that provide more extensive insurance coverage for fertility treatments may have higher utilization rates (Reindollar, 2015).

Female causes

Focus on female age-related fertility decline

Since the 1960s, there has been a notable shift in how motherhood is perceived, transitioning from being primarily dictated by biology to becoming a matter of

personal choice (Van de Kaa, 1987). Women now have the option to pursue education and establish careers before considering starting a family, leading to a significant delay in childbearing in Western societies (Lutz et al., 2003).

In several European countries, the average age at which women have their first child is approaching 30 years, and many women are giving birth for the first time at the age of 35 or older (Eijkemans et al., 2014). However, a challenge arises with this delayed desire for children: fertility begins to decline as early as 25–30 years of age. Additionally, the median age at which women have their last child is typically around 40–41 years in most populations with natural fertility (Eijkemans et al., 2014). This suggests a relatively universal pattern of age-related fertility decline. **Figure 1** illustrates the age-related decline in fertility. Eijkemans et al. conducted an analysis of the distribution of female age at the time of their last childbirth in a natural fertility population, revealing that the age-related loss of fertility gradually increases from 4.5% at age 25, 7% at age 30, 12% at age 35, and 20% at age 38. Afterward, it accelerates rapidly, reaching about 50% at age 41, nearly 90% at age 45, and nearly 100% at age 50 (Eijkemans et al., 2014). The prevailing concept of fertility decline suggests that age-dependent fertility loss is a result of the gradual depletion of oocytes stored in both ovaries from fetal life, initially causing decreased fertility and eventually leading to its complete cessation a decade later during menopause (Te Velde & Pearson, 2002). Additionally, it is well-established that oocyte quality deteriorates with advancing age, along with premature follicle recruitment, increased ovulatory disorders, reduced ovulatory frequency, and impaired luteal phase, all contributing to decreased conception rates (Hart, 2016).

Several studies have shown that most women are not aware of the fact that delaying childbirth increases the risk of infertility (Schmidt, 2010). Furthermore, many women mistakenly believe that infertility treatments like IVF can effectively address the fertility decline associated with advancing age (Maheshwari et al., 2008).

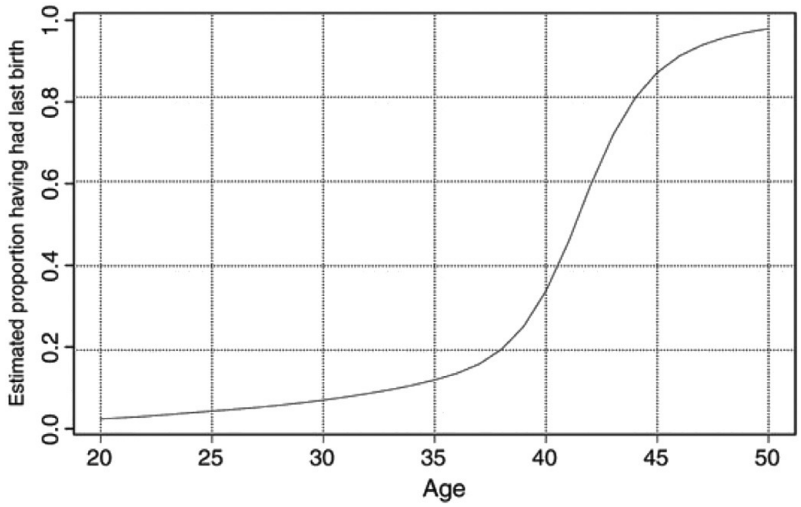


Fig.1. The biological age at last birth curve. Figure adapted from: Eijkemans MJ, Van Poppel F, Habbema DF, Smith KR, Leridon H, te Velde ER. Too old to have children? Lessons from natural fertility populations. Human Reproduction. 2014 Jun 1;29(6):1304-12.

Focus on female disease-related infertility

Disease-related infertility can affect both men and women, with certain conditions impacting one gender more than the other. Causes of female disease-related infertility are reported as follows.

Hypogonadotrophic Hypogonadism: It results from insufficient gonadal stimulation due to a lack of Gonadotropin-Releasing Hormone (GnRH)

production or secretion in the hypothalamus, leading to a decrease in luteinizing hormone (LH) and follicular stimulating hormone (FSH). GnRH insufficiency is often attributed to issues in the migration of GnRH-secreting neurons to the forebrain. It can be associated with anosmia (Kallmann syndrome) or not (normosmic idiopathic hypothalamic hypogonadism) (Inhorn & Patrizio, 2015).

Hyperprolactinemia: Excess prolactin negatively affects gonadotropin secretion, leading to anovulation in women and infertility (Inhorn & Patrizio, 2015).

Ciliary Function Disorders: Disorders affecting ciliary function contribute significantly to infertility in both genders. Cilia in the fallopian tubes play a vital role in sperm and embryo transport. Damage to these cilia by pathogens or inflammation, as well as primary ciliary dyskinesia, can impair tubal transport and increase the risk of ectopic implantation or subfertility (Inhorn & Patrizio, 2015).

Cystic Fibrosis: Cystic Fibrosis causes mucus dysfunction, which can hinder sperm penetration through thick cervical mucus. It may also affect sperm capacitation in the fallopian tube (Ahmad et al., 2013).

Infectious Agents: Infections like Chlamydia trachomatis, Neisseria gonorrhoea, or Mycoplasma hominis can negatively impact fertility. In women, these infections can lead to pelvic inflammatory disease and tubal obstruction (Inhorn & Patrizio, 2015).

Hydrosalpinxes: These fluid-filled sacs in the fallopian tubes can reduce embryonic implantation potential, as evidenced by IVF success rates (Strandell et al., 2009).

Systemic Diseases: Severe systemic illnesses like sepsis or severe renal disease can adversely affect embryonic implantation. Metabolic, endocrinological, and autoimmune diseases such as unstable diabetes, celiac disease, vitamin D insufficiency, and subclinical hypothyroidism are associated with reduced chances of conception in both genders (Tersigni et al., 2014; Inhorn & Patrizio, 2015).

Primary Ovarian Insufficiency (POI): POI, characterized by early cessation of menstrual cycles, is associated with reduced ovarian function and decreased fertility. It may have genetic, environmental, infectious, autoimmune, or treatment-related causes (Dewailly et al., 2014).

Polycystic Ovary Syndrome (PCOS): PCOS, the most prevalent endocrine disorder in women, can lead to infrequent or absent ovulation, ovarian abnormalities, and hyperandrogenism. Insulin resistance, obesity, and metabolic imbalances also contribute to PCOS (Franks, 2008).

Endometriosis: This chronic pelvic inflammatory condition reduces the chances of conceiving due to anatomical distortions, endocrine abnormalities, and immunological disturbances (Tanbo & Fedorcsak, 2017).

Uterine Fibroids and Polyps: Submucosal fibroids and endometrial polyps decrease the likelihood of spontaneous conception by affecting embryonic implantation potential and increasing the risk of early pregnancy loss (Jacoby et al., 2010; Ben-Nagi et al., 2009; Inhorn & Patrizio, 2015)

In summary, infertility linked to medical conditions involves a range of factors. Understanding these conditions and their impact on fertility is crucial for diagnosing and addressing infertility issues in individuals and couples.

Chapter 2.
Assisted Reproductive Techniques and controlled ovarian stimulation

Assisted Reproductive Technologies: IVF/ICSI

ART can be defined as any procedure that involves manipulation of oocytes and/or sperm to establish a pregnancy in treatment of infertility. Methods used to achieve this result included IVF, gamete intrafallopian transfer (GIFT) and zygote intrafallopian transfer (ZIFT) (Zegers-Hochschild et al., 2009) The first IVF treatment was used almost 40 years ago by Dr. P. Steptoe and Dr. R. Edwards, combining an oocyte retrieved during a natural ovulating cycle with sperm in laboratory and transferring an 8-cell stage embryo in the uterine cavity (Steptoe and Edwards, 1978). Since that moment, more than 6 million children have been conceived worldwide using IVF and many progresses and changes have been made in reproductive medicine (Dyer et al., 2009).

With regards to IVF's purpose, it was initially designed to bypass blocked tubes; however, nowadays, its use has been expanded to other diseases which cause infertility, as severe endometriosis, poor ovarian reserve, oligo-anovulation, male infertility, and failure of less aggressive infertility therapies. In 1992, Dr. Palermo reported the first pregnancy achieved after intracytoplasmic sperm injection (ICSI), which consists in the single injection of one sperm directly into the oocyte cytoplasm, bypassing with a micropipette the zona pellucida and the plasma membrane of the oocyte, overcoming many pivotal steps of the natural

fertilization. Since its introduction, ICSI represents a cornerstone among infertility treatments, determining a great revolution especially in the management of severe male infertility and improving the fertilization rate respect to conventional IVF cycles (Palermo et al., 1992). However, it is important to empathize that studies comparing conventional IVF to ICSI in absence of male infertility, demonstrated no advantage of ICSI over IVF in terms of pregnancy rate (Bhattacharya et al., 2001; Van Rumste et al., 2003). More recently it has been shown that there were no significant differences in the live birth rates (LBR) between fresh and cumulative cycles for both IVF and ICSI across various response categories, including poor, suboptimal, normal, and high responders (Drakopoulos, 2019).

Controlled Ovarian Stimulation

At the starting of reproductive medicine, IVF cycles were performed utilizing oocytes from natural unstimulated cycle. However, few times later, the introduction COS with adequate medications (purified and highly purified gonadotropin preparations, or recombinant FSH and LH [rFSH, rLH], and/or human Menopausal gonadotropins, hMG) led to the development of multiple follicles, and thus, subsequent embryos to transfer, resulting in lower cancellation rates, higher pregnancy, and birth rates. Greater number of ovarian follicles are today “picked-up” from bilateral ovarian puncturing resulting in the retrieval of numerous oocytes which are used for IVF. Several stimulation protocols for COS have been developed during the last twenty years.

The introduction of the GnRH agonist protocol in the mid-1980s successfully helped to control premature LH surge, preventing thus premature follicular luteinization (Porter et al., 1984; Wildt et al., 1986; Meldrum et al., 1989). The GnRH long agonist protocol starts with administration of a GnRH agonist (e.g. leuproreline, triptorelin) on day 21 of menstrual cycle followed by the injection of gonadotropins on day 2/3 of menstruation, using the "flare effect" of GnRH agonist for the initial follicular recruitment. Initial dose of gonadotropins can be chosen according to the woman phenotype characteristics; however, markers of ovarian reserve, eventual ovarian disorders, and ovarian response to previous COS, represent the main factors to be considered to choose and adjust gonadotrophins dosages (Sherestha et al., 2015). When at least three or more follicles reach the dimension of 17 mm or more, hCG trigger is administered **(Figure 2).** The use of the GnRH agonist long protocol may determinate main side effects which include longer treatment duration (average of 15 days), possibility of ovarian cyst formation, development of menopausal symptoms (hot flushes, vaginal dryness) and higher risk of ovarian hyperstimulation syndrome (OHSS) and necessity for more intensive luteal support compared to the antagonist protocol (Sherestha et al., 2015; Jungheim et al., 2015).

With regards to GnRH antagonist short protocol, it starts with spontaneous menstrual bleeding with gonadotropins administration from day 2 or 3 of menstrual cycle after an accurate assessing of basal hormonal levels and follicular size <10 mm. On day 6 of stimulation, when follicles sizes are ≥ 14 mm, a GnRH antagonist is administered with the aim of preventing spontaneous LH surge. When at least 3 follicles reach the dimension of 17 mm, hCG or GnRH

agonist trigger is administered and oocytes retrieval is performed approximately 36 hours from it (Devroey et al., 2009).

Considering natural cycle and modified natural cycle IVF (MNC-IVF), characterized by minimal stimulation, they have emerged as valuable therapeutic strategies for the treatment of poor ovarian responders (PORs). The primary objective of the "mild" approach is to retrieve a single oocyte with superior characteristics, potentially resulting in a single top-quality embryo that can be transferred to a more receptive endometrium. Additionally, MNC-IVF cycles, which avoid the use of high doses of gonadotropins for ovarian stimulation, may help to reduce the occurrence of premature progesterone elevation, a known factor that can have a detrimental effect on pregnancy rates. Furthermore, MNC-IVF may offer cost-effectiveness benefits due to reduced gonadotropin consumption (Di Guardo et al. 2022)

Other stimulation protocols may include the use of clomiphene citrate (CC), an estrogen receptor modulator with anti-estrogenic property, or letrozole (off-label), an aromatase inhibitor, in combination or not with gonadotropins and/or GnRH antagonist (Mohsen & El Din et, 2013; Ibrahim, 2014; Sherestha et al., 2015, Zhang et al., 2016).

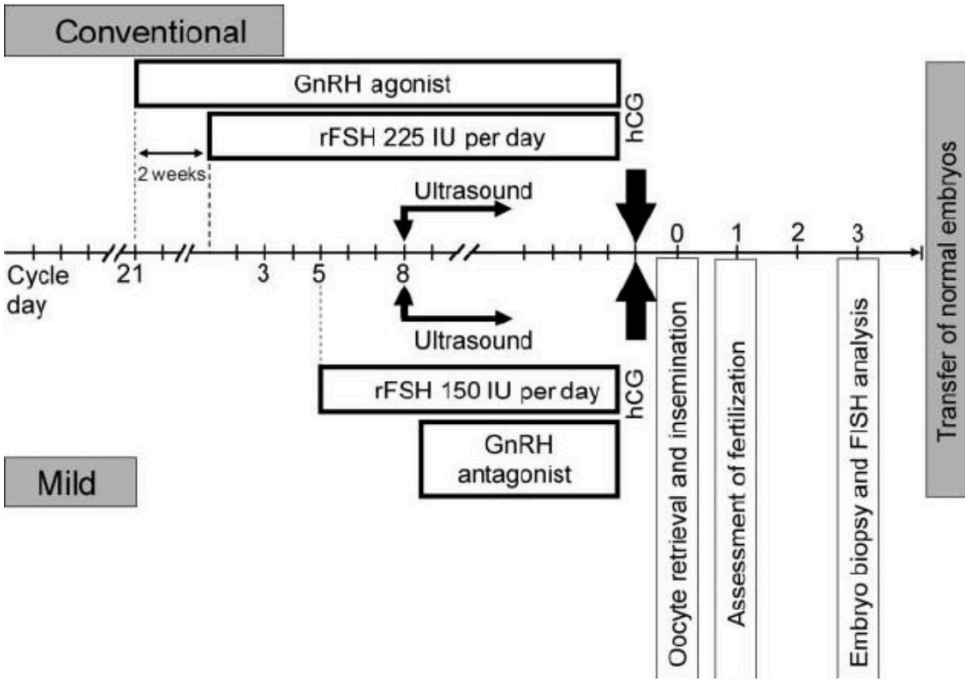


Fig 2. Schematic explanation of COS protocols. Image adapted from "Baart EB, Martini E, Eijkemans MJ, Van Opstal D, Beckers NG, Verhoeff A, Macklon NS, Fauser BC. Milder ovarian stimulation for in-vitro fertilization reduces aneuploidy in the human preimplantation embryo: a randomized controlled trial. Human Reproduction. 2007 Apr 1;22(4):980-8".

Controlled Ovarian Stimulation Monitoring

Ovarian stimulation is a crucial step in IVF treatment, aiming to stimulate the development of multiple follicles and subsequently induce final follicular maturation. Effective monitoring of COS is of paramount importance to ensure both the safety of the procedure and the optimal ovarian response required for assisted reproduction treatment (Rizk & Smitz, 1992; Thomas et al., 2002).

Two primary techniques, transvaginal ultrasound (TVUS) and the assessment of serum Estradiol (E2), Progesterone (P), LH, FSH levels are employed for monitoring COS. These methods play a pivotal role in reducing the incidence

and severity of OHSS while helping clinicians make informed decisions. Accurate monitoring allows for: 1) identifying cycles with insufficient ovarian response, leading to early cancellation, when necessary, 2) determining the optimal time for triggering final follicular maturation, 3) assessing the risk of OHSS and taking measures to prevent and minimize its occurrence.

Combining ultrasonography with hormonal assessment provides clinicians with comprehensive information, reducing the chances of errors associated with using a single monitoring method. However, it's important to note that there is currently no strong consensus regarding the frequency and timing of monitoring. These aspects are often chosen arbitrarily by clinicians and can vary significantly between different clinics.

The most recent Cochrane review on monitoring stimulated cycles in assisted reproduction, which included only 781 patients (Kwan et al., 2008), found insufficient evidence from randomized trials to support the idea that combined monitoring with TVUS, and serum hormonal levels is more effective than TVUS alone in terms of clinical pregnancy rates and the incidence of OHSS. However, it's worth mentioning that the review highlighted the low quality of the studies involved, as well as substantial heterogeneity and methodological variability among them.

Monitoring ovarian stimulation can be a challenging practice, especially considering the recent data emphasizing the importance of hormonal assessment during the follicular phase (Bosch et al., 2010; Venetis et al., 2013). Alternatively, it can be simplified, following a traditional approach to keep the monitoring process as straightforward as possible. The choice between these

approaches may depend on individual patient characteristics and clinical preferences (Bosch et al., 2010; Wiser et al., 2012; Venetis et al., 2013).

On the other hand, there is a common practice of administering the Oral Contraceptive Pill (OCP) as part of antagonist protocols to achieve a more flexible scheduling for the initiation of ovarian stimulation. However, it's essential to note that this practice has been associated with a decrease in ongoing pregnancy rates (OPR) (Griesinger et al., 2010; Farquhar et al., 2017), as well as reduced rates of fresh and cumulative LBR (Lu et al., 2020). Additionally, this approach has been linked to an extended duration of ovarian stimulation and higher consumption of gonadotropins (Griesinger et al., 2008).

In contrast, administering GnRH antagonist before commencing ovarian stimulation during the menstrual cycle may help to reduce the size variation and enhance the uniformity of antral follicles (Fanchin et al., 2003), nonetheless can contribute to an easy scheduling of ovarian stimulation.

Controlled Ovarian Stimulation Outcomes

The primary objective of controlled ovarian stimulation is to promote the development of multiple ovarian follicles. Within these adequately sized follicles are cumulus-oocyte complexes (COCs) that are retrieved through ovarian puncturing. The mature oocytes contained in COCs are crucial for achieving successful fertilization and development of more than one embryo. The determination of the ideal number of collected oocytes for achieving a

favorable cumulative live birth rate (CLBR) still needs to be established (Sunkara et al., 2011; Steward et al., 2014). CLBR is described as the occurrence of at least one live-born infant (who has reached a gestational age of over 24 weeks) in either the initial fresh cycle or subsequent frozen-thawed cycles, in relation to the total number of oocytes retrieved. Recent evidence shows that in the era of vitrification, CLBR consistently rises as the number of retrieved oocytes increases in patients with a favorable prognosis (Polyzos et al., 2018). On the other hand, newly discovered findings suggests that the use of blastocyst transfer, particularly in Frozen Embryo Transfer (FET) cycles and when combined with vitrification, plays a significant role in improving LBR over time. This offers the potential to decrease the necessary number of oocyte retrievals to achieve a live birth and expedite the time required to attain a live birth (Saket et al., 2021). With regards to ET, over the past few years, an ongoing debate has centered on determining the most effective approach for ET in IVF cycles: transferring embryos at the cleavage stage or waiting until they reach the blastocyst stage. The transfer of blastocyst-stage embryos is seen as an improvement in the selection process, as only the viable embryos are expected to progress to the blastocyst stage. However, before the routine adoption of vitrification as a laboratory procedure, cryopreserving blastocysts using slow-freezing techniques was considered challenging and less successful. With the introduction of vitrification cryopreservation techniques, the survival rate of thawed blastocysts now equals that of cleavage-stage embryos (Cobo et al., 2012; Rienzi et al., 2017).

Both fresh and frozen blastocyst-stage embryo transfer have become viable alternatives to cleavage-stage embryo transfer. However, prolonging the embryos' culture in the laboratory presents additional challenges and risks. Generally, on day 5, the number of embryos available for transfer or cryopreservation tends to be lower than on day 3 because some embryos may cease developing in vitro. The higher availability of embryos in cleavage-stage transfer results in more embryo transfers per oocyte retrieval and, potentially, a higher CLBR (Cornelisse et al.; 2021; Glujovsky et al., 2022). However, it seems there is a notable advantage in favor of day-5 embryos among women aged 36 and older, with a trend towards increased CLBR, when compared to transfer of day 3 embryos (Cornelisse et al.; 2021).

Chapter 3.
Difficult to treat patients: PORs and POI

A significant proportion of women undergoing in vitro fertilization (IVF), approximately 20%, experience what is known as poor ovarian response. This means that one out of every five patients face a challenging prognosis due to a limited response to ovarian stimulation (Rienzi et al., 2005; Vaiarelli et al., 2018). Poor ovarian response is a condition that often leads to high cancellation rates and low live birth rates, making it a crucial and difficult issue to address in the field of IVF.

PORs

Definition of Poor Ovarian Responder (POR) in assisted reproductive technology indicates "A woman treated with ovarian stimulation for ART, in which at least two of the following features are present: (1) Advanced maternal age (≥40 years), (2) A previous poor ovarian response (%3 oocytes with a conventional stimulation protocol aimed at obtaining more than three oocytes); and, (3) An abnormal ovarian reserve test (i.e. antral follicle count < 7 follicles or anti-Mullerian hormone <1.1 ng/ml (Bologna criteria); or other reference values obtained from a standardized reference population" (Zegers-Hochschild et al., 2017). However, the use of Bologna criteria (BC) has faced criticism on multiple fronts. Some of the key points of contention include the lack of clarity in defining risk factors and the failure to account for factors such as oocyte quality and other variables linked to diminished ovarian reserve (Frydman, 2011;

Younis, 2012; Papathanasiou, 2014; Boza et al., 2018). Nevertheless, the most significant concern expressed by experts is the persistence of substantial heterogeneity even within the population identified by BC. This heterogeneity becomes evident when various patterns or subgroups of patients with poor ovarian response emerge as a result of combining risk factors, ovarian reserve testing outcomes, and IVF attempts. These distinct patient subpopulations often exhibit differing baseline characteristics, such as age, leading to diverse prognoses (La Marca et al., 2015; Bozdag et al., 2017).

In light of these concerns, a revised definition of 'impaired ovarian response' has been put forward by the Poseidon Group, which stands for Patient-Oriented Strategies Encompassing IndividualizeD Oocyte Number (Alviggi et al., 2016). This updated classification aims to provide a more nuanced approach by considering four distinct subgroups, considering several factors: (1) Numerical and qualitative parameters such as the patient's age and the expected aneuploidy rate, (2) Markers of ovarian reserve, including antral follicle count (AFC) and/or anti-Mullerian hormone (AMH), (3) Ovarian response observed in previous stimulation cycles **(Figure 3)**. Additionally, the Poseidon Group has introduced a novel marker called the "number of oocytes needed" to assess the potential success of ART. This marker calculates the minimum number of oocytes required for a specific patient to obtain at least one euploid embryo suitable for transfer (Humaidan et al., 2016; Esteves et al., 2019).

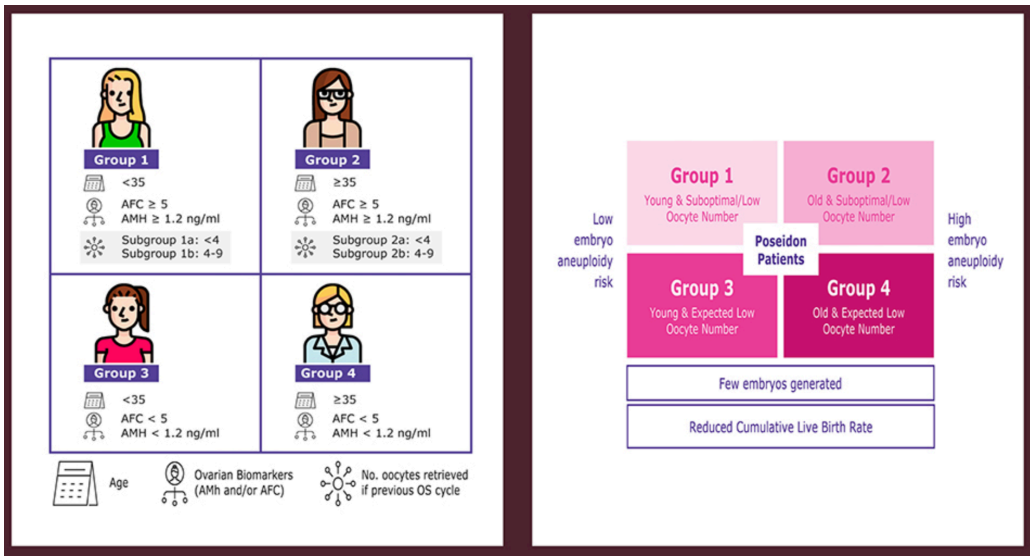


Fig 3. The Poseidon Classification for PORs. Image adapted from Roque M, Haahr T, Esteves SC, Humaidan P. The POSEIDON stratification-moving from poor ovarian response to low prognosis. JBRA Assisted Reproduction. 2021 Apr;25(2):282.

POI

POI is characterized by the disruption of normal ovarian function, leading to a significant reduction in the number of primordial follicles (Luisi et al., 2015). This condition affects approximately 1–2% of women, manifesting as either primary or secondary amenorrhea, reduced estrogen production, elevated levels of gonadotropins, and an increased risk of osteoporosis and cardiovascular disease.

Clinical symptoms of POI include alterations in the menstrual cycle, such as shorter or longer intervals between periods, irregular menstruation, dysfunctional uterine bleeding, oligomenorrhea, or complete amenorrhea. Additionally, women with POI often experience estrogen deficiency-related symptoms like hot flashes, mood disturbances, and atrophic vaginitis. Furthermore, individuals with POI may confront long-term consequences of low

estrogen levels, including osteoporosis, accelerated aging of the cardiovascular system, and neurocognitive disorders (McKinlay et al., 1992).

Despite being recognized as a heterogeneous condition, the exact cause of POI remains a topic of ongoing debate, highlighting the complex nature of its pathogenesis. As a multifactorial disorder, lifestyle factors such as the use of oral contraceptives, parity, and smoking appear to contribute to the development of POI. However, none of these factors alone can consistently explain the variation in the age at which menopause occurs (Luisi et al., 2015).

In this challenging context, various hypotheses have been proposed to elucidate the intricate mechanisms leading to POI. The involvement of multiple elements, including immunological, infectious, environmental, iatrogenic, hereditary, and genetic factors, seems to be responsible for the development of this condition (Beck-Peccoz & Persani, 2006). While there is strong scientific consensus regarding the role of the X-linked FMR1 gene premutation, which reduces the antral follicular pool in many women with X-fragile syndrome, similar conclusive evidence has not yet been reported for other candidate genes, located on both the X chromosome and autosomal chromosomes, which may contribute to the non-syndromic phenotype of POI. However, recent scientific findings suggest that genes such as BMP15, GDF9, NOBOX, FIGLA, and SALL4 may be involved in the pathogenesis of non-syndromic POI through various mechanisms that regulate early folliculogenesis, follicular growth, ovulation, follicular atresia, and oocyte maturation (Qin et al., 2015).

Additionally, a significant number of women with POI face fertility challenges during their reproductive years due to ovarian dysfunction and the depletion of

their ovarian reserve. The lack of consensus on diagnostic criteria for identifying POI in adolescents and young women often leads to delayed diagnosis, jeopardizing their reproductive potential (Qin et al., 2015).

Chapter 4.
Most Relevant studies

Does the dose or type of gonadotropins affect the reproductive outcomes of poor responders undergoing modified natural cycle IVF (MNC-IVF)?

Introduction

Poor ovarian response (POR) is defined as the failure to respond adequately to standard ovarian stimulation protocols. Due to the limited oocyte yield, high cycle cancellation rates (Polyzos et al., 2015) and low live birth rates (LBR) (Zhang et al., 2020), POR remains a core challenge in IVF clinical practice. It has been estimated that the prevalence of POR ranges from 6 % to 35 % (Oudendijk et al., 2012; Patrizio et al., 2015). Although predicted POR has usually been treated with high doses of gonadotropins (Papathanasiou et al., 2016), milder stimulation approaches have recently gained interest. This interest had been fueled by studies showing no benefit from high doses of gonadotropins in predicted poor responders (Youssef et al., 2017,Youssef et al., 2018).

In this context, IVF in a modified natural cycle (MNC-IVF) with mild gonadotropin stimulation has emerged as a therapeutic option for women with POR POR (Kadoch et al., 2011, Nargund et al., 2017, Moffat et al., 2020). This “mild” approach could offer several ad- vantages such as yielding of better quality oocytes (Weghofer et al., 2004) and embryos, that could be further

transferred to a more physiological endometrial milieu (Reyftmann et al., 2007). In mild stimulation MNC-IVF protocols, GnRH antagonists are used to block the spontaneous LH surge and gonadotropins are administered as an add-back therapy to counterbalance the suppressed endogenous FSH levels. However, there is currently no evidence base for a specific protocol or gonadotropin type for MNC-IVF. In addition, although the POSEIDON classification seems a step in the right direction for the classification of POR, validation, and conduction of new studies in different subgroups are warranted. The aim of the present study is to investigate whether the daily dose or the type of gonadotropin may affect the reproductive outcomes of predicted poor responders under- going MNC-IVF.

Material and methods

Study design

This was a retrospective, single-centre cohort study including consecutive subfertile patients undergoing MNC-IVF with mild ovarian stimulation using gonadotropins at our centre. The study was approved by the institutional Review Board of Universitair Ziekenhuis Brussel (approval B.U.N. 143201938863). A cycle was considered cancelled when the patient failed to respond to gonadotropins with no possibility to perform the oocyte retrieval.

Study population

Data were retrieved from all predicted poor responders (Group 3 and 4) according to POSEIDON criteria (AMH < 1.1 ng/ml) (Alviggi et al., 2016) undergoing at least one MNC-IVF cycle between 1st January 2017 and 1st

March 2020. MNC-IVF cycles with administration of clomiphene citrate were excluded from the study.

Treatment protocol

Ovarian stimulation was started as a follicle with a mean diameter of 12–14 mm was observed on ultrasound scan, followed by GnRH antagonists (0.25 mg/day) from the next day onwards. Gonadotropins used in doses < 75 IU/d or 75 to < 100 IU/d or ≥ 100 to 150 IU/d were recombinant FSH (rFSH) Gonal-F®, Merck Pharmaceuticals, Darmstadt, Germany; Ovaleap®, Theramex, Ireland Limited; Puregon®, Merck- Sharp&Dohme, Whitehouse Station, NJ, USA; urinary FSH (uFSH) Fos- timon®, IBSA, Switzerland or highly purified HMG (hpHMG) Menopur®, Ferring Pharmaceuticals, St. Prex, Switzerland. Cycle monitoring was performed through serum E2, P, FSH and LH assess- ments, and serial transvaginal ultrasound examinations (Popovic-Todorovic et al., 2018). Ovulation triggering was performed with the administration of hCG when a single follicle of 17 mm diameter was observed (Humaidan et al., 2013), followed by oocyte retrieval 34–36 h later. Collected mature oocytes were inseminated via intracytoplasmic sperm injection (ICSI). Embryos were cultured up to day cleavage stage or blastocyst stage following oocyte retrieval and the embryo transfer (ET) was performed under ultrasound guidance. Luteal phase support consisted of vaginal progesterone tablets of 200 mg three times daily, administered from the day after oocyte retrieval onwards until 7 weeks of pregnancy (Kyrou et al., 2011, Liu et al., 2012).

Main outcome measures

The primary outcome parameter was live birth rate (LBR) per started cycle. Secondary outcomes were clinical pregnancy rate (CPR) and cycle cancellation rate (no response at day 10–11 of the cycle).

Statistical analysis

Continuous data were presented as mean ± standard deviation (SD) and categorical data were described as numbers and percentages. Continuous variables were analyzed using the independent *t*-test or Mann–Whitney *U* test depending on the normality of the distribution. Normality was examined by the use of the Shapiro–Wilk test. Categorical variables were analyzed by Pearson's chi-squared test or Fisher's exact test, as appropriate. To account for the non-independent nature of the data (more than one cycle per patient), the association of the dose and type of gonadotropin with the reproductive outcomes (LBR and CPR) after adjusting for potential confounders, was examined by GEE multivariate regression analysis. All covariates (age, BMI, number of MII oocytes) were simultaneously entered into the GEE multivariate regression model. The assumptions for the final model were successfully tested. All statistical tests used a two-tailed α of 0.05. Analyses were performed using STATA 13.0. A p-value < 0.05 was considered as statistically significant.

Result

Cohort baseline characteristics

In total, 484 patients undergoing 1398 cycles were included. Mean (SD) age and serum AMH were 38.2 (3.7) years and 0.28 (0.26) ng/ml, respectively. The daily

dose of gonadotropins was either < 75 IU/d [11/ 1398 (0.8 %)] or 75 to < 100 IU/d [1303/1398 (93.2 %)] or ≥ 100 to 150 IU/d [84/1398 (6 %)]. Overall, rFSH was used for stimulation in 251/1398 (18 %) cycles, uFSH in 45/1398 (3.2 %) cycles and hp-hMG in 1102/1398 (78.8 %) cycles **(Table 1)**.

Table 1
Baseline characteristics of the cohort.

Cycles (n)	1398
Age (years)	38.2 ± 3.7
BMI (kg/m2)	24.3 ± 4.5
AMH (ng/ml)	0.28 ± 0.26
Daily gonadotropins' dose (IU/d)	
<75	11/1398 (0.8 %)
75 to < 100	1303/1398 (93.2 %)
≥ 100 to 150	84/1398 (6 %)
Type of gonadotropin	
rFSH	251/1398 (18 %)
uFSH	45/1398 (3.2 %)
hp-hMG	1102/1398 (78.8 %)

Note. Data are expressed as mean ± SD, number (n) and percentage (%).
BMI (Body Mass Index), rFSH (recombinant FSH), uFSH (urinary FSH), hp-hMG (highly purified HMG).

Reproductive outcomes

In total, CPR per started cycle was 119/1398 (8.5 %). Live birth was achieved in 80/1398 (5.7 %) of cycles. LBR was similar across different types and doses of gonadotropins [10 (4%) vs1(2.2%) vs 69(6.2%), p-value 0.3; 1(9%) vs 76 (5.8%) vs 3 (3.6 %), p-value 0.51, respectively] **(Tables 2 and 3)**. Similarly, CPRs did not differ significantly between the different type and doses of gonadotropins [17 (6.8 %) vs 2 (4.4 %) vs 100 (9.1 %), p-value 0.37; 1 (9 %) vs 113 (8 %) vs 5 (5 %); p-value 0.6, respectively] **(Tables 2 and 3)**. Moreover, the number of oocytes retrieved, and the number of oocytes mature (MII) was comparable between the different groups (0.86 ± 0.75 vs 0.75 ± 0.48 vs 0.83 ±

0.64, p-value 0.84; 1.18 ± 0.75 vs 0.82 ± 0.65 vs 0.96 ± 0.82, p-value 0.13; 0.7 ± 0.69 vs 0.64 ± 0.52 vs 0.72 ± 0.61, p- value 0.7; 0.8 ± 0.4 vs 0.71 ± 0.62 vs 0.84 ± 0.77, p-value 0.63). Similarly, the day of ET (D3 ET and D5 ET) did not differ significantly between the different type and doses of gonadotropins [92/95(96.8 %), 3/95(3.2 %) vs 16/17(94.1 %), 1/17(5.9 %) vs 451/457(98.7 %), 6/457 (1.3 %), p-value 0.08; 6/6(100 %), 0/6(0 %) vs 528/537(98.3 %), 9/537 (1.7 %) vs 25/26(96.1 %), 1/26(3.9 %), p-value 0.44]. The average number of embryos transferred was significantly different between the different dose of gonadotropins (1 ± 0 vs 1.01 ± 0.12 vs 1.15 ± 0.36, p value 0.001), while was comparable between the different type of gonadotropins (1.01 ± 0.1 vs 1 ± 0 vs 1.02 ± 0.16, p value 0.5) **(Tables 2 and 3)**. Cancellation rates were also similar [38 (15.4 %) vs 8 (17.8 %) vs 185 (16.8 %), p-value 0.8; 0 (0 %) vs 215 (16.5 %) vs 6 (7.1 %), p-value 0.3] **(Tables 2 and 3)**. Fertilization rate was significantly different between the different dose of gonadotropins (48.3 ± 47.4. vs 62.6 ± 46.5 vs 44.7 ± 45.3, p value 0.01) while was comparable between the different type of gonadotropins (57.2 ± 46.3 vs 50 ± 50 vs 62.8 ± 46.5, p value 0.1I).

Table 2
Reproductive outcomes per daily gonadotropins' dose (IU/day).

	<75 (n = 11)	75 to < 100 (n = 1303)	≥ 100 to 150 (n = 84)	*P* value
Number of oocytes	1.18 ± 0.75	0.82 ± 0.65	0.96 ± 0.82	0.13
Number of oocytes MII	0.8 ± 0.4	0.71 ± 0.62	0.84 ± 0.77	0.63
Fertilization rate[a]	48.3 ± 47.4	62.6 ± 46.5	44.7 ± 45.3	0.01
Average number of embryos transferred[b]	1 ± 0	1.01 ± 0.12	1.15 ± 0.36	0.001
Day of ET[b]	6/6(100 %)	528/537 (98.3 %)	25/26(96.1 %)	0.44
D3 ET				
D5 ET	0/6(0 %)	9/537 (1.7 %)	1/26(3.9 %)	
Cancellation rate	0 (0 %)	215 (16.5 %)	6 (7.1 %)	0.3
Clinical Pregnancy rate	1 (9 %)	113 (8 %)	5 (5 %)	0.6
Live Birth rate	1 (9 %)	76 (5.8 %)	3 (3.6 %)	0.51

Note. Data are expressed as mean ± SD, number (n) and percentage (%).
D3(Day3); D5(Day5); ET (embryo transfer).
[a] Calculated as number of oocytes fertilized divided by number of COC, multiplied by 100.
[b] Calculated for patients who had ET.

Table 3
Reproductive outcomes per type of gonadotropins.

	rFSH (n = 251)	uFSH (n = 45)	hp-hMG (n = 1102)	*P* value
Number of oocytes	0.86 ± 0.75	0.75 ± 0.48	0.83 ± 0.64	0.84
Number of oocytes MII	0.7 ± 0.69	0.64 ± 0.52	0.72 ± 0.61	0.7
Fertilization rate[a]	57.2 ± 46.3	50 ± 50	62.8 ± 46.5	0.11
Average number of embryos transferred[b]	1.01 ± 0.1	1 ± 0	1.02 ± 0.16	0.5
Day of ET[b]				
D3 ET	92/95 (96.8 %)	16/17 (94.1 %)	451/457 (98.7 %)	0.08
D5 ET	3/95(3.2 %)	1/17(5.9 %)	6/457(1.3 %)	
Cancellation rate	38 (15.4 %)	8 (17.8 %)	185 (16.8 %)	0.8
Clinical Pregnancy rate	17 (6.8 %)	2 (4.4 %)	100 (9.1 %)	0.37
Live Birth rate	10 (4 %)	1 (2.2 %)	69 (6.2 %)	0.3

Note. Data are expressed as mean ± SD, number (n) and percentage (%).
rFSH (recombinant FSH), uFSH (urinary FSH), hp-hMG (highly purified HMG).
D3(Day3); D5(Day5); ET (embryo transfer).
[a] calculated as number of oocytes fertilized divided by number of COC, multiplied by 100.
[b] calculated for patients who had ET.

Multivariable regression analysis

The GEE multivariate regression analysis adjusting for relevant confounders (age, BMI, number of embryos transferred, day of embryo transfer) showed that the type of treatment strategy (rFSH/uFSH/hp- hMG) and the dose of gonadotropins (<75 UI/d, 75 to < 100 UI/ d and ≥ 100 to 150 IU/d) were not significantly associated with LBR (p value 0.08 and 0.8, respectively) **(Table 4)**.

Table 4
Multivariable logistic regression. Outcome LBR, predictors: maternal age, BMI, type and dose of gonadotropins, number of Embryo transferred and Day of ET.

	Coefficient	95 % C.I.	P value
Type of gonadotropin			
rFSH (ref)	–	–	0.08
uFSH	-0.03	-0.20 to 0.14	
hp-hMG	0.07	-0.00 to 0.15	
Dose of gonadotropins			
<75	–		0.8
75 to < 100	-0.094	-0.37 to 0.19	
≥ 100 to 150	−0.99	-0.41 to 0.21	
Age	-0.021	-0.029 to -0.013	0.00
BMI	-0.002	-0.008 to 0.004	0.5
n of Embryo transferred	0.030	-0.15 to 0.21	0.7
Day of ET	0.08	-0.020 to 0.19	0.1

Note. C.I. (confidence interval), rFSH (recombinant FSH), uFSH (urinary FSH), hp-hMG (highly purified HMG), BMI (Body Mass Index), n (number), ET (Embryo Transfer).

Discussion

Our large retrospective study is the first to demonstrate that the type (rFSH/uFSH/hp-hMG) and the daily dose (<75 UI/d, 75 to < 100 UI/ d and ≥ 100 to 150 IU/d) of gonadotropins were not associated with LBR in predicted poor ovarian responders treated with MNC-IVF using GnRH-antagonists. The term “mild (or minimal) stimulation” refers to the use of low doses of

gonadotropins for a short period of time in a gonadotropin- releasing hormone (GnRH) antagonist co-treatment cycle (Nargund et al., 2017), either from the early or the mid-follicular phase onwards. The main benefit of mild stimulation regimens, including MNC-IVF, is that they are more cost-effective than conventional ovarian stimulation in some groups of patients, reducing gonadotropins consumption and thus the overall cost of treatment (Datta et al., 2020; Nargund et al., 2017).

So far, the scientific community had mainly focused on the efficiency of IVF in a (modified) natural cycle compared to IVF after conventional ovarian stimulation in women with predicted POR, without investi- gating whether the choice of type and dose of gonadotropin could have an impact on the clinical outcome of MNC-IVF cycles. In particular, two previous RCTs have shown similar pregnancy rates when comparing MNC-IVF to the microdose flare-up (Morgia et al., 2004) and the GnRH antagonist protocol (Kim et al., 2009). However, the findings of these studies were flawed by the absence of specific criteria for the selection of poor ovarian responders. In a retrospective study, Lainas *et al. (*Lainas *et al.*, 2015)found significantly higher LBR after MNC–IVF in predicted POR women selected according to Bologna criteria, when compared to women stimulated with high-dose gonadotropins (HDOS); albeit, several methodological issues about the statistical approach have been raised (Polyzos et al., 2016), questioning the robustness of the findings. On the contrary, a retrospective study conducted by Kedem et al. (Kedem et al., 2014) on a cohort of one hundred eleven poor responders selected according to the Bologna Criteria, concluded that MNC-IVF is of no benefit for genuine poor

ovarian responders due to the fact that LBR was < 1 %. Lastly, a recent retrospective study by Drakopoulos *et al. (*Drakopoulos *et al.*, 2019) showed similar ongoing pregnancy rate (OPR) in MNC-IVF and HDOS treated Bologna POR women of more than 40 years, suggesting that MNC-IVF may be an option in advanced age women.

Another group of patients in which mild stimulation has gained ground is oncofertility women who wish to preserve their fertility (Koch and Ledger, 2013). The aim of mild stimulation in that case is to reduce the duration of stimulation and gonadotropins total dosage consumption, decreasing thus the time of exposure to high estrogen concentrations (Meirow et al., 2014). In this regard, ultra-mild approaches have also been developed, including the administration of solely letrozole or tamoxifen for ovarian stimulation (Oktay et al., 2005).

Finally, it has to be mentioned that high doses of gonadotropins have been used over the last decades in predicted poor responders (POSEIDON groups 3 and 4), resulting in increased cost and treatment burden with low chance to alter their destiny (Van Tilborg et al., 2017). There is evidence that stimulation doses over 300 IU daily are highly unlikely to increase ovarian response and improve reproductive outcomes in this difficult group of women (Bastu et al., 2016; Berkkanoglu and Ozgur, 2010).

A major strength of this study relies on its large sample size. More- over, this is the first study evaluating MNC-IVF protocol outcomes in POSEIDON group 3 and 4 poor responders, who represent expected low prognosis women. The

POSEIDON classification of poor responders is a relatively recent one and validation is mandatory (Esteves et al., 2019, Humaidan et al., 2016). Nonetheless, a number of limitations should be considered when interpreting the results. The retrospective nature and the small sample size of some subgroups are factors inherent to risk of bias. Although a significant effort has been made to eliminate all known sources of systematic error through multivariable analysis, there might still exist non apparent sources of bias. Moreover, as this was the first study investigating the effect of gonadotropins dose on clinical outcomes in MNC cycles, a proper sample size calculation could not be applied, given the lack of available evidence. Furthermore, the choice of the type and/or dose of gonadotropin used in the MNC-IVF cycles was not standardized and based on the physician's discretion. For all the reasons above, attention is warranted before drawing firm conclusions.

In conclusion, in women with predicted POR according to the Poseidon classification treated with MNC-IVF, the type and dose of gonadotropin add-back stimulation seems to not influence live birth rates. Based on our data we considering the use of MNC-IVF with 75 IU of gonadotropins daily as add-back therapy a feasible option in predicted poor responders. Larger prospective randomized trials are war- ranted to validate these findings.

Impact of cell loss after warming of human vitrified day 3 embryos on obstetric outcome in single frozen embryo transfers

Introduction

The application of frozen embryo transfer (FET) has progressively increased during the last two decades due to advances in the efficacy and safety of cryopreservation strategies (Belva et al., 2008; Loutradi et al., 2008; Shapiro et al., 2014). Indeed, vitrification has replaced the slow-freezing technique as a result of improved survival rates and higher implantation rates. Although today FET is widely used in assisted reproductive technology, several concerns have recently emerged regarding its safety in terms of pregnancy, obstetric, and perinatal outcomes. Following slow freezing, blastomere loss impairs embryo post- thawing in vitro development (Edgar *et al.*, 2000; Archer *et al*., 2003; Rienzi *et al.*, 2005). Moreover, compared to fully intact embryos, day 3 vitrified embryos with cell loss after warming also show lower overnight cleavage (Van Landuyt et al., 2013). However, the latter study also reported that when a damaged embryo under- went overnight cleavage, similar implantation rates were found between intact and damaged embryos. Likewise, Edgar et al. emphasized the importance of further cleavage after warming for implantation rate, irrespective of blastomere survival (Edgar *et al*., 2007). Early reports initially based on slow freezing concluded that transfers of embryos with cell

loss negatively affect implantation and conception rate (Van den Abbeel *et al.*, 1997; Burns *et al.,* 1999; El-Toukhy *et al.* 2003). Other studies showed that transfers of embryos with blastomere loss are not associated with lower implantation rates compared to those with intact blastomeres (Zheng *et al.*, 2008; Capodanno *et al.*, 2016; O'Shea *et al.*, 2016). In addition, a recent retrospective study compared obstetrical outcomes of neonates born after a transfer of an intact embryo with those deriving from an embryo with cell loss, highlighting an increased risk to deliver small for gestational age babies or with transient tachypnea at birth (Wu et al., 2018). In contrast, recent evidence concluded that blastomere loss is not associated with an increased risk of any adverse neonatal out- come in the singletons, describing comparable neonatal conditions between embryos derived from blastomere loss embryos and intact embryos (Jiang et al., 2022).

In clinical practice, embryos with blastomere loss are generally transferred because they can implant and further develop; nevertheless, the possibility of increased adverse neonatal outcomes remains a concern for clinicians. In this scenario, the present study aims at investigating whether transfers of single vitrified day 3 embryo with blastomere loss impact on pregnancy, live birth rate, and obstetric outcome.

Materials and methods

Study design

This is a retrospective, single-center cohort study including day 3 vitrified–warmed embryos that were transferred between 2011 and 2018 at the Centre for Reproductive Medicine, Universitair Ziekenhuis Brussel, Belgium. The study was approved by the Ethics Committee of the Universitair Ziekenhuis Brussel (B.U.N.143201940782).

Study population

The analysis included all vitrified/warmed day 3 single embryo transfers obtained from consecutive treat- ments with standard in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI). Each woman was included only once in our analysis. Patients undergoing in vitro maturation (IVM) procedures, who were oocyte donors or recipients or had undergone embryo biopsy for pre-implantation genetic diagnosis, were not included. Embryos were divided into two groups, namely group A (intact embryo after warming) and group B (embryo with blastomere loss after warming). An intact embryo was defined as an embryo that remained fully intact after warming, not showing any blastomere loss. Conversely, an embryo with blastomere loss was defined as an embryo that lost one or more blasto- meres after thawing, referred to as partially damaged, but with at least 50% of the blastomeres intact.

Stimulation protocol

Ovarian stimulation was started on day 2 or 3 of the men- strual cycle with daily injections of gonadotrophins, fol- lowed by a daily dose of 0.25 mg of GnRH antagonist in a fixed protocol, starting 6 days after the gonadotrophin intake. Cycle monitoring was performed through serum estradiol (E2), progesterone (P), and luteinizing hormone (LH) assessments, and serial transvaginal ultrasound examinations (Popovic-Todorovic et al., 2018). Ovulation triggering was performed with the administration of hCG or GnRH agonist in case of risk for OHSS (Humaidan et al., 2013), as soon as three follicles reached 17 mm of diameter. Oocyte retrieval was performed 36 h later. Collected oocytes were inseminated via either conventional IVF or ICSI.

FET preparation protocol

FET preparation methods used included hormonal replacement therapy (HRT) and natural cycle (NC) protocol with spontaneous or triggered ovulation. In the HRT protocol, estradiol valerate 6 mg daily (Progynova, Bayer Health- Care Pharmaceuticals) was administered orally from day 1 or 2 of the menstrual cycle onwards. Embryo transfers were planned on the fifth day of progesterone intake (Utrogestan, Vifor Pharma, 400 mg twice daily). Exogenous hormonal supplementation was continued for 14 days until a blood B-hCG test was performed. Patients with a positive test continued with hormone supplementation until 12 weeks of gestation. Conversely, the use of NC with spontaneous ovulation did not require any pharmacological intervention (Mackens et al., 2017). Serial blood test for hormonal workup and ultrasound

monitoring during the proliferative phase were exclusively performed to identify the presence of a dominant follicle as well as the LH surge, in order to schedule the transfer when the endometrium was synchronized to the developmental stage of the embryo. Embryo transfers were planned on day 5 from the LH peak. In case of NC with triggered ovulation protocol, hCG 5000 IU was administered as soon as a dominant follicle of > 16 mm was observed. Embryos were warmed 1 day before embryo transfer, cultured overnight, and transferred on the sixth day from the hCG administration.

Embryo selection before vitrification

On day 3 in the morning, embryos with at least 6 cells and ≤ 20% fragmentation were selected for vitrification.

Vitrification method

The cryopreservation method used for all the embryos ana- lyzed in the study was closed vitrification using CBS-VIT High-Security straws (Cryo Bio System, L'Aigle, France) using DMSO–ethylene glycol (EG)–sucrose(S) as the cryoprotectants (Irvine ScientificR Freeze kit, Newtownmount- kennedy, County Wicklow, Ireland) (Van Landuyt et al 2013). The device and vitrification media did not change during the study period; neither did the protocol for day 3 vitrification change over time.

Cell loss assessment after warming and quality assessment at transfer

Day 3 embryos were warmed 1 day prior to transfer and transferred as day 4 embryos after overnight culture. The number of surviving cells was indicated on the total number of cells that were visible at evaluation immediately after warming (e.g., 5/8 or 8/8). If a single ET was planned, one embryo (the best according to quality at freezing) was put in culture for overnight cleavage when at least 50% of the cells were surviving. If more than 2 cells were damaged and other embryos were available, a second embryo was thawed, since we know that further cleavage is more likely in embryos without or with minimal damage (Van Landuyt et al 2013). On day 4, further cleavage was assessed and could be taken into consideration to determine the final embryo quality at transfer. Embryo quality at transfer was categorized into 4 qualities depending on the degree of further cleavage and final cell stage. Quality 1 embryos were embryos that were already compacting after overnight cleavage or even reached the blastocyst stage; embryo quality 2 included embryos with > 8 cells and with at least further cleavage of 2 blastomeres. Embryo quality 3 included embryos with at least 8 cells and cleavage of 1 blastomere. Embryo quality 4 was defined as embryos which had < 8 blastomeres and/or no signs of further cleavage. An extra embryo thawing on day 4 was never realized to avoid embryo–endometrial asynchrony. Assisted hatching was not performed after warming.

Main outcome measures

The primary aim of this retrospective cohort study was to evaluate the possible association between cell loss after warming and live birth rate (LBR). The secondary endpoint was to evaluate the potential association between embryo

cell loss after warming and neonatal measurements (length, weight, and head circumference at birth).

Statistical analysis

Continuous data were presented as mean ± standard deviation (SD), and categorical data were described as number and percentages. Continuous variables were analyzed using the independent *t* test or Mann–Whitney *U* test depending on the normality of the distribution. Normality was exam- ined using the Shapiro–Wilk test. Categorical variables were analyzed by Pearson's chi-squared test or Fisher's exact test, as appropriate. To study the association between blastomere loss and pregnancy as well as neonatal outcomes (length, weight, and head circumference at birth), odds ratios were calculated for each outcome, after adjusting for potential confounders, using multivariate logistic regression. All covariates (age of the patient at the moment of embryo cryo-preservation, and intact or no embryos) were simultaneously entered into the multivariable logistic regression model. The assumptions for the final model were successfully tested. All statistical tests used a two-tailed α of 0.05. A p value < 0.05 was considered as statistically significant. The analyses were exploratory. No formal sample size calculation was performed. Analyses were performed using STATA 15.0.

Results

Demographic characteristics

Demographic characteristics such as maternal age at cryopreservation, maternal age at the embryo transfer, cause of infertility, insemination procedure (ICSI\IVF), and semen origin were comparable between the two groups **(Table 1)**.

Table 1 Couples' demographic characteristics between groups A and B

	Grouping		
	Group A ($n=1953$)	Group B ($n=374$)	*p* values
Age at the time of embryo cryopreservation	34 ± 4.8	34 ± 4.9	0.98
Age at ET	34.8 ± 4.8	34.6 ± 4.9	0.557
Cause of infertility			0.583
1. Male factor	782 (45.2)	150 (43)	
2. Endometriosis	109 (6.3)	29 (8.3)	
3. Idiopathic	451 (26.1)	94 (26.9)	
4. Ovarian insufficiency	82 (4.7)	16 (4.6)	
5. PCO	118 (6.8)	18 (5.1)	
6. Tubal factor	188 (10.9)	42 (12)	
Procedure for injection			0.899
ICSI	1888 (94.7)	366 (94.6)	
IVF	105 (5.3)	21 (5.4)	
Procedure semen extraction			0.441
Ejaculate	1950 (97.8)	381 (98.5)	
Testicular biopsy	43 (2.2)	6 (1.5)	

Data are expressed as mean ± SD, number (*n*) and percentage (%)

Group A intact embryo after warming, *Group B* blastomere loss after warming, *ET* embryo transfer, *PCO* polycystic ovarian syndrome, *ICSI* intracytoplasmic sperm injection, *IVF* in vitro fertilization, *n* number

Embryo characteristics

A total of 2327 vitrified/warmed day 3 transferred embryos were included in the analysis, of which 1953 (83.9%) embryos were fully intact after warming (group

A), and 374 (16.1%) presented with cell loss (group B). Characterization of all frozen–thawed embryos according to cell stage at freezing and cell loss after thawing is displayed in **Table 2**. The majority of embryos presented 8 cells and/or more than 8 cells at the time of vitrification (897, 38.4%, and 1027, 44.3%, respectively). With regard to embryo qual- ity at transfer, defined by the number of cells that further cleaved after warming and final cell stage at transfer, a total of 2071 (89.0%) embryos were of quality 1 and 2 (1558 and 513, respectively) **(Table 3)**. Considering embryo quality at ET, the percentage of embryos represented in each quality class (1–4) was not significantly different between the two groups (class 1 = 67.7% vs 64.2, class 2 = 21.8% vs 23.2%, class 3 = 3.7% vs 7.6%, class 4 = 6.8% vs 5%, respectively; $p = 0.198$) **(Table 3)**. In only a minor number of transfers, embryos without further cleavage were transferred.

Table 2 Characterization of 2327 frozen–thawed embryos according to cell stage at vitrification and cell loss after warming

Cell stage at vitrification	Number thawed	Number of damaged cells				
		0	1	2	3	4
6 cells	120 (5.1)	107	9	4	–	–
7 cells	283 (12.2)	242	32	7	2	–
8 cells	897 (38.4)	780	83	25	8	1
> 8 cells	1027 (44.3)	824	108	56	29	10
Total	2327	1953 (83.7)	232 (9.9)	92 (3.9)	39 (1.6)	11 (0.5)

Data are expressed as number (*n*) and percentage (%)

Table 3 Presence of the different embryo qualities at transfer for the two groups of embryos with (B) and without (A) cell loss

	n	Grouping		
		Group A (*n* = 1953)	Group B (*n* = 374)	*p* values
Embryo quality at ET		1.5 ± 0.9	1.5 ± 0.8	0.198*
1	1558	1320 (67.7)	238 (64.2)	
2	513	426 (21.8)	87 (23.2)	
3	104	74 (3.7)	30 (7.6)	
4	152	133 (6.8)	19 (5)	

Data are expressed as mean ± SD, number (*n*), and percentage (%)

Group A intact embryo after warming, *Group B* blastomere loss after warming, *ET* embryo transfer, *n* number

*Wilcoxon sum rank

Obstetrical outcome

The chemical pregnancy rate and LBR per warming cycle were significantly higher in the intact embryo group versus the cell loss group (585/1953, 30%, versus 91/374, 24.3%, $p = 0.028$, and 267/1953, 13.7%, versus 35/374, 9.4%, $p = 0.023$). However, LBR per positive hCG was equivalent between group A and B (45.6% vs 38.5%, $p = 0.2$). Bio- chemical pregnancy rate per frozen embryo transfer (4.2% vs 2.7%, $p = 0.115$) and miscarriage rate (5.3% vs 6.9%, $p = 0.252$) were similar between groups with and without blastomere loss, respectively **(Table 5)**. Newborn measurements (length, weight, and head circumference at birth) showed no statistical difference between the two groups (50.1±2.7 cm vs 50.1±2.8 cm, p=0.918; 3334.7±643.7 g vs 3362.4±517.7 g, p=0.774; 38.2±37.2 cm vs 34.5 ± 35.2 cm, $p = 0.169$, respectively, for intact and dam- aged embryos) **(Table 4)**.

Table 4 Obstetrical outcomes between the two groups: intact embryos and embryos with blastomere loss after warming

	Grouping		
	Group A ($n=1953$)	Group B ($n=374$)	p values
Chemical pregnancy, no. (%)	585 (30)	91(24.3)	0.028
Biochemical pregnancy (per frozen embryo transfer)	53 (2.7)	16 (4.2)	0.115
Miscarriage, no. (%)	134 (6.9)	20 (5.3)	0.252
Live birth (per ET cycle), no. (%)	267 (13.7)	35 (9.4)	0.023
Live birth rates (per positive hCG) (%)	45.6	38.5	0.2
Gestational age (including all positive hCG)	35.9 ± 23.5	34.8 ± 10.7	0.619
Neonatal length (cm)	50.1 ± 2.7	50.1 ± 2.8	0.918
Neonatal weight (g)	3334.7 ± 643.7	3362.4 ± 517.7	0.774
Neonatal head circumference (cm)	38.2 ± 37.2	34.5 ± 35.2	0.169

Data are expressed as mean ± SD or number (no.) and percentage (%)
ET embryo transfer, *n* number, *hCG* human chorionic gonadotropin

Multivariable logistic regression analysis

Multivariable logistic regression analysis showed no association between transfer of intact or damaged embryos and LB (adjusted OR = 1.4, 95% CI = 0.86–2.2, $p = 0.18$), when adjusting for the potential confounder such as patient age at cryopreservation **(Table 5)**.

Table 5 Multivariable logistic regression. Outcome LB, predictors: intact vs cell loss (CL) embryos, maternal age at cryopreservation

	OR	p value	95% C.I	
Intact vs CL	1.4	0.18	0.86	2.2
Age at cryo	0.98	0.25	0.95	1.01

LB live birth, *OR* odds ratio, *C.I.* confidence interval, *Intact vs CL* intact embryos versus cell loss embryos after thawing, *Age at cryo* maternal age at cryopreservation

Discussion

The results of this large retrospective cohort study indicated that transfer of embryos with blastomere loss derived from V/W was associated with lower live birth and chemical pregnancy rates when compared with those of fully intact

embryos. However, when adjusting for patient age at cryopreservation, the negative effect of cell damage on LB was not observed. Moreover, we noted that LBR per positive hCG was equivalent between the intact and cell loss groups indicating that if implantation occurs, LB is not affected by cell loss. Furthermore, cell loss after warming of cleavage- stage embryos had no impact on newborn measurements (neonatal length, weight, and head circumference at birth). These findings confirm the results of our previous study (Van Landuyt et al 2013), in which an association between cell loss and diminished developmental potential was demonstrated, even in case such a loss was limited. However, the latter study showed that if the embryo resumed cleavage after warming, there was no effect of the number of cells lost on its implantation potential (although analyzed on small numbers). Our findings are in line with other studies (El-Toukhy et al., 2003; Guerif et al., 2002; Zhang et al., 2009), which report that dam- aged embryos with blastomere loss after cryopreservation may diminish embryo developmental potential and pregnancy rate, when only considering blastomere loss per se. FET cycles performed with completely intact embryos after warming achieved superior reproductive outcomes than those performed with partially damaged embryos. Indeed, a fully intact embryo represents a marker of better embryonic developmental potential, irrespective of its morphological quality (El-Toukhy et al., 2003; Yu et al., 2017). In this context, several studies (Van Den Abbeel and Van Steirteghem, 2000; Van der Elst et al., 1997) reported that the capacity of the frozen–thawed embryo to further cleave in vitro is impaired in embryos showing blastomere loss after warming compared with intact embryos. Consistent with this notion,

blastomere loss after thawing accounts for 30% of implantation potential reduction and approximately for 40% of clinical pregnancy rate decrement (Edgar et al., 2000). In addition, necrotic blastomeres may produce a toxic effect on the remaining cells and affect embryo viability (Elliott et al., 2007). Considering the proportion of cell loss after cryopreservation, embryos with less than 50% of the original number of blastomeres are considered unsuitable for transfer (El-Toukhy et al., 2003), while scientific evidence suggested that embryo competency is not impacted below the limit of 25% of blastomere loss (Capodanno *et al.*, 2016; Wu *et al.*, 2018). According to this, a double embryo transfer should not be performed, even to compensate for those with blasto- mere loss, to avoid the risk for multiple pregnancy. However, after vitrification, extensive cell loss is less frequent than after slow freezing (Van Landuyt *et al*. 2013). Our results are partly in agreement with those reported by Wu et al. (Wu *et al.,* 2018),who found that the transfer of embryos with blastomere loss following vitrification is associated with lower rates of implantation, clinical pregnancy, and live birth, compared to transfers of fully intact embryos. However, the same authors reported several adverse outcomes in newborns resulting from the implantation of embryos with blastomere loss, such as a higher rate of small for gestational age babies or transient tachypnea at birth. Nevertheless, the study conducted by Wu et al. (Wu *et al.,* 2018) included both single and double embryo transfers. Moreover, although it aimed to collect data about major birth defects, a long-term follow-up on neonatal growth and development was not performed. In addition, our results are in line with those recently described by Jiang et al. (Jiang et al., 2022) who showed that transfer of

vitrified/warmed day 3 embryos with blastomere loss is related to impaired embryo developmental potentials in terms of live birth rates when compared to transfer of fully intact embryos. However, the neonatal outcomes of embryos derived from blastomere loss embryos and intact embryos were similar, concluding that blastomere loss was not associated with increased risk of any adverse neonatal condition.

Finally, it has to be mentioned that HRT protocol for FET, per se, seems to be associated with increased risk of adverse maternal and neonatal outcomes such as preeclampsia (Von Versen-Hoÿnck et al., 2019; Zaat et al., 2021), preterm birth, and low birth weight when compared to NC-FET (Zong et al., 2020).

The strength of the present study relies on its design, including a large sample size of cleavage-stage vitrified day 3 embryos. Moreover, only single embryo transfers were included in the analysis. Study limitations, however, exist and should be taken into consideration when interpreting the results. The retrospective nature of our study is inherent to risk of bias. Therefore, although a significant effort was made to eliminate all known sources of systematic error through multivariate analysis, unknown sources of bias may exist and have an impact on measured out- comes. Finally, neonatal outcomes might only be analyzed for a specific subgroup of patients (those who delivered); however, demographic characteristics were comparable between the two groups included in the initial analysis (Bradburn et al., 2020).

In conclusion, the transfer of embryos with blastomere loss caused by V/W is associated with lower chemical pregnancy and live birth rates when compared

with trans- fer of fully intact embryos. However, the negative effect of blastomere loss is not observed when adjusting for patient age at cryopreservation. Moreover, if implantation occurs, LBR is not impacted by cell loss. Finally, blastomere loss is not associated with neonatal length, weight, and head circumference at birth. Larger cohort studies are warranted to validate these findings.

Impact of GnRH antagonist pretreatment on oocyte yield after ovarian stimulation: a retrospective analysis

Introduction

The use of GnRH antagonists has been progressively increased in Assisted Reproductive Technique (ART) clinics worldwide, GnRH antagonists act by suppressing immediately and irreversibly the gonadotropin secretion, which results in a shorter duration of treatment with less patient distress (Al-Inany et al., 2006; Devroey et al., 2009; Van Hooren et al., 2001). Moreover, the use of GnRH antagonist protocol is associated with lower risk of hospital admission due to OHSS (Kolibianakis et al., 2006). On the other hand, several ART centers use the GnRH agonist protocol as first line option due to several reasons. First, the GnRH antagonist protocol has been associated with uncoordinated antral follicle growth under certain condition (Fanchin et al., 2003); second, the start of ovarian stimulation in a long GnRH antagonist protocol relies on the occurrence of spontaneous menses (Guivarc'h-Levêque et al., 2010; Levy et al., 2013; Tremellen and Lane, 2010) whereas the GnRH agonist protocol is more flexible allowing a more controlled scheduling of oocyte retrievals which means also the reduction or even the avoidance of oocyte retrievals during the weekend. In the clinical practice, pretreatment with Oral Contraceptive Pill (OCP) is used in antagonist protocols to obtain a more flexible scheduling of the start of ovarian stimulation. However, this practice is associated a decrease of ongoing pregnancy rate (OPR) (Farquhar et al., 2017; Griesinger et al., 2010) as well as fresh and cumulative live birth rates (LBRs) (Lu et al., 2020).

Furthermore, an increase of duration of ovarian stimulation with higher gonadotropin consumption has been reported (Griesinger et al., 2008).

In addition, higher serum gonadotropin concentrations as well as higher E2 concentration are found at the onset of ovarium stimulation in GnRH antagonist protocol when compared with a pituitary down regulation protocol. As a result, the unsuppressed FSH level at the start of a GnRH antagonist cycles allows the initial growth of a few leading follicles before the addition of exogenous rFSH (Åbyholm et al., 2000; Albano et al., 2000; Van Hooren et al., 2001). Menstrual administration of an antagonist before starting ovarian stimulation might reduce size and improve homogeneity of antral follicles (Fanchin et al., 2003). It has already been shown that elevated progesterone at the onset of ART cycles, and reduced fertility outcome, can be solved by the administration of GnRH antagonists for 3 consecutive days before the start of OS (Blockeel et al., 2011a). Furthermore, a pilot study conducted in women under 36 years old, found that GnRH antagonist pretreatment during 3 consecutive days before the initiation of ovarian stimulation had a trend towards a higher number of retrieved cumulus-oocyte complexes (COCs) with improved pregnancy outcome (Blockeel et al., 2011b). Using a similar protocol, improved maturation and fertilization rates of retrieved oocytes was showed (Younis et al., 2010). The current study aims to investigate whether a 3-day pretreatment course with a GnRH antagonist in the early follicular phase may increase the number of oocytes retrieved in a GnRH antagonist stimulation protocol using a large data set.

Material and methods

Study design

This was a retrospective, single-centre cohort study (crossover, match – control design) at a tertiary referral university hospital including all consecutive women undergoing ovarian stimulation for IVF/ICSI at Brussels IVF, the University Hospital of Brussels in Belgium from January 2011 to December 2020. The study was approved by the institutional Review Board of Universitair Ziekenhuis Brussel (approval B.U.N. 143201838385).

Study population

Eligible patients were those who did not get pregnant after one standard GnRH antagonist stimulation cycle ("standard cycle") and proceeded with one GnRH antagonist stimulation cycle preceded by early administration of GnRH antagonist for 3 days ("pretreatment cycle") with fresh embryo transfer or frozen embryo transfer. All women may have used the same or a lower initial dose of gonadotropins in their first IVF cycle (standard cycle), both cycles needed to be performed in a time interval of <12 months.

The age of included patients ranged from 20 to 44 years. Patients were excluded from the study if they had planned to undergo ovarian stimulation for preimplantation genetic diagnosis or screening, oocyte donation, social or medical egg freezing and in vitro maturation (IVM) of oocytes. All women that had basal progesterone levels >1.5ng/ml, were deemed non-eligible. All cycles were divided into two groups: group 1 (standard cycles) and group 2 (pretreatment cycles).

Treatment protocol

In standard cycles ovarian stimulation was started on day 2 or 3 of the menstrual cycle with daily injections of gonadotrophins, followed by a daily dose of 0.25 mg of GnRH antagonist in a fixed protocol, starting 6 days later. In pretreatment cycles ovarian stimulation was preceded by the early administration of GnRH antagonist started on day 2 or 3 of the menstrual cycle for 3 consecutive days. Gonadotropins used were recombinant FSH (rFSH) Gonal-F®, Merck Pharmaceuticals, Darmstadt, Germany; Ovaleap®, Theramex, Ireland Limited; Puregon®, Organon, Whitehouse Station, NJ, USA; or highly purified HMG (hpHMG) Menopur®, Ferring Pharmaceuticals, St. Prex, Switzerland. Cycle monitoring was performed through serum E2, P, FSH and LH assessments, and serial transvaginal ultrasound examinations (Popovic-Todorovic et al., 2018). Ovulation triggering was performed with the administration of hCG as soon as three follicles of 17 mm diameter were observed (Humaidan et al., 2013). Oocyte retrieval took place 36 hours later. Collected oocytes were inseminated either via conventional IVF or intracytoplasmic sperm injection (ICSI) or via IVF/ICSI. Embryos were cultured up to Day 3 or Day 5 following oocyte retrieval and the embryo transfer (ET) was performed under ultrasound guidance. Luteal phase support consisted in vaginal progesterone tablets of 200 mg three times daily, administered from the day after oocyte retrieval onwards until 7 weeks of pregnancy (Andersen et al., 2002; Liu et al., 2012). In case of frozen ET of embryos obtained from the same cycle, hormonal replacement therapy (HRT), natural cycle (NC) and NC with triggered ovulation protocols were used to prepare the endometrium.

Main outcome measures

The primary outcome parameter was the total number of retrieved COCs after ovarian stimulation. The secondary outcomes were consumption (IU) of gonadotrophins and duration (days) of ovarian stimulation.

Statistical analysis

Continuous data are presented as mean ± standard deviation (SD) and median with interquartile range (IQR). Categorical data are described by number of cases, including the numerator and denominator, and percentages. Differences in continuous variables (including the primary endpoint: total number of retrieved COCs after ovarian stimulation) between patients' 2nd IVF cycle (with GnRH antagonist pretreatment) and their preceding cycle were calculated via dependent-sample t-tests or Wilcoxon signed-rank tests, as appropriate. Categorical variables were analyzed via Mc Nemar test, as appropriate. Continuous variables were analyzed by regression models with estimation by generalized estimating equations (GEE) to assess the effect of antagonist pretreatment in the number of oocytes and embryo utilization rate, after accounting for several confounders such as dose of gonadotropin used, type of gonadotropin used, age, cause of infertility and duration of ovarian stimulation. GEE was used to account for the within subject correlation in outcomes for repeated treatments. Results are presented with adjusted odds ratios (ORs) and 95% confidence intervals (CIs). All statistical tests used a two-tailed α of 0.05.

Analyses were performed using STATA 13.0. A p-value <0.05 was considered as statistically significant.

Results

Baseline patient baseline characteristics in the general population

In total, 430 patients undergoing 860 cycles were included. The average female age was 34.4 ± 4.8 years. Indications for fertility treatment included unexplained infertility (34.3%), male-factor infertility (33.3%), age (16.9%), PCOS (8.2%), Tubal-factor infertility (4.7%) and endometriosis (2.6%). All cycles were divided into two groups: group 1 (standard, 430 cycles) and group 2 (pretreatment, 430 cycles). The average cohort AMH value was 2.61 ± 2.52. Basal progesterone (assessed on day 2 or 3 of the menstrual cycle) was significantly higher in group 2 (0.66 ± 0.72 vs 0.51 ± 0.3, p <0.005) **(Table 1)**.

Table 1. Baseline patient characteristics

Parameter	n 430
Age **(mean ± SD)** **median (IQR)**	 34.4 ± 4.8 35 (31-38)
AMH **(mean ± SD)** **median (IQR)**	 2.61 ± 2.52 2.04 (1.01-3.43)
Indication n (%) **Male factor** **Endometriosis** **Age/ovarian insufficiency** **Idiopathic** **PCOS** **Tubal**	 143 (33.3) 11 (2.6) 73 (16.9) 148 (34.3) 35 (8.2) 20 (4.7)

Stimulation characteristics in the two groups: standard treatment and antagonist pre-treatment

Prior-triggering hormonal assessment revealed that E2, P, LH and FSH levels were significantly higher in Group 2 than in Group 1 (2289.7 ± 1355.6 vs 1628.4 ± 971.3, p<0.001; 1.02 ± 0.65 vs 0.88 ± 0.53, p<0.001; 3.9 ± 4.65 vs 2.5 ± 3.17, p<0.001; 18.08 ± 7.2 vs 15.8 ± 6.9, p<0.001, respectively) **(Table 2)**.The mean duration of stimulation was similar in both groups (10.3 ± 1.6 vs 10.3 ± 2.2; p=0.28) **(Table 3)**. The starting dose of gonadotropin and the total amount of gonadotropins used were significantly higher in group 2 than in group 1 (234 ± 60.9 vs 196.7 ± 54.4 p<0.001; 2419 ± 758.4 vs 2020 ± 674.9, p<0.001) **(Table 3)**. In both groups, rFSH, was more used than hMG [389/531(73.3) vs 142/531(26.7); 284/531 (53.5) vs 247/53 (46.5), p<0.001] **(Table 3)**.

Table 2. Basal and prior-triggering hormonal assessment in the two groups: standard treatment and antagonist pre-treatment

Hormonal assessment	Standard treatment (430)	Antagonist pre-treatment (430)	P-value
	Basal hormonal assessment		
E2 (ng/ml) (mean ± SD) median (IQR)	39.8 ± 24.1 38 (27-50)	42.5 ± 19.9 39 (28-56)	0.006
P (ng/ml) (mean ± SD) median (IQR)	0.51 ± 0.3 0.45 (0.29-0.7)	0.66 ± 0.72 0.47 (0.3-0.8)	<0.005
LH (IU/L)			0.41

(mean ± SD) **median (IQR)**	6.03 ± 2.71 5.75 (4.4-7.4)	6.18 ± 2.77 5.8 (4.2-7.7)	
FSH (IU/L) **(mean ± SD)** **median (IQR)**	7.84 ± 2.97 7.6 (6-9.4)	7.68 ± 3 7.2 (5.8-8.9)	0.18
	Hormonal assessment at trigger		
E2 (ng/ml) **(mean ± SD)** **median (IQR)**	1628.4 ± 971.3 1461 (990-2094)	2289.7 ± 1355.6 1977.5 (1337-2972)	<0.001
P (ng/ml) **(mean ± SD)** **median (IQR)**	0.88 ± 0.53 0.8 (0.5-1.12)	1.02 ± 0.65 0.89 (0.6-1.31)	<0.001
LH (IU/L) **(mean ± SD)** **median (IQR)**	2.5 ± 3.17 1.6 (0.8-3.1)	3.9 ± 4.65 2.55 (1.2-4.69)	<0.001
FSH (IU/L) **(mean ± SD)** **median (IQR)**	15.8 ± 6.9 14.7 (10.8-20)	18.08 ± 7.2 17 (12.6-22.9)	<0.001

Table 3. Stimulation characteristics in the two groups: standard treatment and antagonist pre-treatment

	Standard treatment (430)	**Antagonist pre-treatment (430)**	**P-value**
Gonadotrophin Starting Dose (IU) **(mean ± SD)** **median (IQR)**	196.7 ± 54.4 200 (150-225)	234 ± 60.9 225 (200-300)	<0.001
Total Gonadotrophin consumption (IU) **(mean ± SD)** **median (IQR)**	2020 ± 674.9 2000 (1500-2400)	2419 ± 758.4 2250 (1800-3000)	<0.001
Stimulation Length (days)	10.3 ± 1.6 10 (9-11)	10.3 ± 2.2 10 (9-11)	0.28

(mean ± SD) **median (IQR)**			
Type of gonadotropins ***n* (%)** **rFSH** **hMG**	389/531(73.3) 142/531(26.7)	284/531 (53.5) 247/53 (46.5)	<0.001

Stimulation and cycle outcomes in the two groups: standard treatment and antagonist pre-treatment

The total number of obtained COCs and the number of mature oocytes were significantly higher in group 2 than in group 1 (10 ± 6.6 vs 7.8 ± 5.5, p<0.001; 8 ± 5 vs 5.8 ± 4, p<0.001, respectively).

Fertilization rate, number of cryopreserved D3 Embryos, embryo utilization rate and the incidence and severity of OHSS were similar between the two groups [68 ± 27 vs 70 ± 25, p=0.27; 0.3 ± 0.8 vs 0.47 ± 1.1, p=0.08; 52 ± 36 vs 51 ± 33, p=0.32; No OHSS: 531/531 (100) vs 529/531(99.6), Mild OHSS: 0/531 (0) vs1/531 (2), Moderate OHSS: 0/531 (0) vs 1/531 (2), p= 0.36]. The number of cryopreserved blastocysts was significantly higher in group 2 than in group 1 (1.09 ± 2.2 vs 0.28 ± 0.7, p <0.001) **(Table 4)**.

Table 4. Stimulation and cycle outcomes in the two groups: standard treatment and antagonist pre-treatment

	Standard treatment (430)	**Antagonist pre-treatment (430)**	**P-value**
COC **(mean ± SD)** **Median (IQR)**	7.8 ± 5.5 7 (4-10)	10 ± 6.6 9 (6-14)	<0.001

MII Oocyte (mean ± SD) Median (IQR)	5.8 ± 4 5 (3-7)	8 ± 5 7 (4-11)	<0.001
Fertilization rate[a] (mean ± SD) Median (IQR)	68 ± 27 71 (50-100)	70 ± 25 75 (60-90)	0.27
Cryo Embryos D3 (mean ± SD) Median (IQR)	0.3 ± 0.8 0 (0-0)	0.47 ± 1.1 0 (0-0)	0.08
Cryo Embryos D5 (mean ± SD) Median (IQR)	0.28 ± 0.7 0 (0-0)	1.09 ± 2.2 0 (0-1)	<0.001
Embryo utilization rate[b] (mean ± SD) Median (IQR)	52 ± 36 50 (25-100)	51 ± 33 50 (25-75)	0.32
Incidence and severity of OHSS n (%) **No OHSS** **Mild OHSS** **Moderate OHSS**	 531/531 (100) 0/531 (0) 0/531 (0)	 529/531(99.6) 1/531 (2) 1/531 (2)	 0.36

[a]*calculated as number of oocytes fertilized divided by number of COC, multiplied by 100*
[b]*calculated as number the number of embryos utilized (transferred or cryopreserved) per number of 2PN zygotes*

Multivariate regression analysis

The generalized estimating equation (GEE) multivariate regression analysis showed that the pretreatment strategy had a significant positive effect on the number of COCs (coefficient 2.4, 95 % C.I. 3.15 to 1.76, p <0.001), after

adjusting for the confounders (age, indication of infertility, stimulation dose, type and duration of stimulation). On the other hand, the older age had a significant negative effect on the number of COCs (coefficient -.28, 95 % C.I. -.38 to -.18, p<0.001) **(Table 5)**.

Table 5. Multivariable logistic regression. Outcome: number of COCs, predictors: maternal age, indication, type and dose of gonadotropins, duration of stimulation

	Coefficient	95 % C.I.	P value
Pretreatment	2.4	3.15 to 1.76	<0.001
Age	-.28	-.38 to -.18	<0.001
Indication of infertility			
Male factor	-	-	
Endometriosis	-1.59	-4.66 to 1.48	0.31
Age/ovarian Insufficiency	0.79	-.67 to 2.26	0.28
Idiopathic	3.07	0.29 to 5.85	0.3
PCOS	1.48	-.33 to 3.31	0.11
Tubal	0.64	-1.56 to 2.86	0.56
Dose of gonadotropins (IU)	-.007	-.01 to 0.00	0.06
Type of gonadotropins	-.59	-.1.41 to 0.21	0.15
Duration of stimulation (days)	-.14	-.34 to 0.05	0.15

Note. C.I. (confidence interval)

Discussion

The result of this retrospective study indicated that a 3-day course of GnRH antagonist pretreatment increases the number of COCs and MII oocytes obtained after ovarian stimulation compared to conventional antagonist protocol. However, it has to be mentioned that a higher starting dose and consumption of gonadotropins were observed in patients who received GnRH antagonist pretreatment, while stimulation length was equivalent between the two groups. As expected, we noted that patients' older age had a significant negative impact on the number of retrieved COCs. These findings confirm the results of an older study from our group in which an association between early follicular phase GnRH antagonist pretreatment and a trend toward a higher number of retrieved oocytes was demonstrated in women aged <36 years who underwent fixed GnRH antagonist protocol. However, in spite of the promising findings, caution needed to be applied when interpreting the results, as the study was a small pilot trial (Blockeel et al., 2011b).

The same topic was recently investigated by a RCT including 136 normal ovulatory women undergoing IVF/ICSI with r-FSH in a flexible GnRH antagonist protocol. The patients were randomized into two equal groups with or without GnRH antagonist administration from day 2 of the menstrual cycle for 3 days before stimulation. In contrast with our results, this study findings showed that the number of retrieved oocytes did not significantly vary between

the two groups. Limitations were underlined by the authors who auspicated for a larger future multicentre trial to confirm their conclusions (Zhang et al., 2021).

Similarly, a case-control study by Viardot-Foucault et al. (2015) reported no difference in terms of number of collected oocytes between 70 patients undergoing GnRH antagonist pretreatment before ovarian stimulation with flexible GnRH antagonist protocol and the control group (Viardot-Foucault et al., 2015) In disagreement with our findings, this study results described that in the pretreatment antagonist group a significant lower total dose of rFSH was used for ovarian stimulation compared to the control group. However, these findings were flawed by the semi-retrospective design of the study which represented a potential bias.

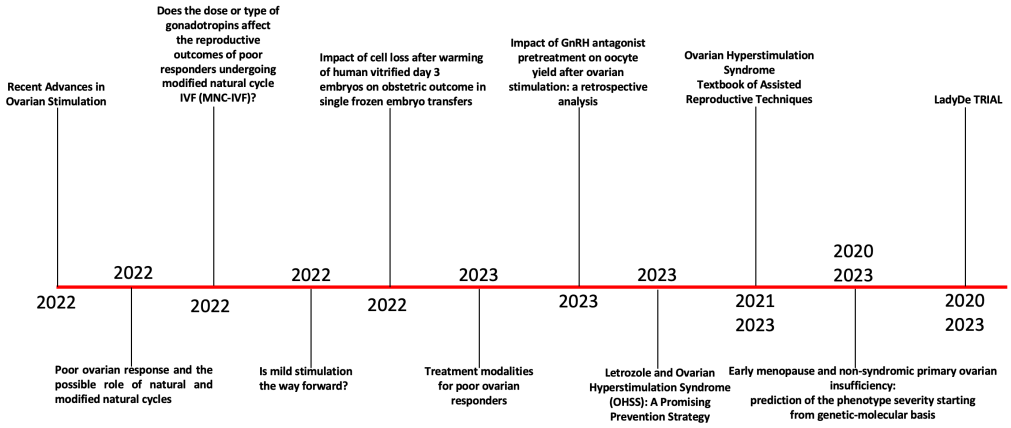
Furthermore, the use of GnRH antagonist pretreatment has been also investigated in specific groups of subfertile patients such as poor ovarian responders (PORs). A multicenter RCT including 160 PORs patients selected according to Bologna Criteria evaluated reproductive outcomes between two equal groups obtained after randomization (Maged et al., 2015). Group I received standard ovarian stimulation in a flexible GnRH antagonist protocol, Group II underwent flexible GnRH antagonist protocol preceded by GnRH antagonist pretreatment administered from day 2 to day 8 of the menstrual cycle. Conclusions showed that delayed start protocol significantly improved CPR and IVF cycle parameters in PORs. Indeed, a statistically significant higher number of fertilized and metaphase II oocytes as well as grade I embryos were reported in Group II compared to Group I. Finally, a small RCT by Aflatoonian et al.

(2017) compared reproductive outcomes obtained in 60 POR selected according to Bologna criteria. Patients were randomly assigned to two groups: case group (*n*=30) in which delayed start GnRH antagonist protocol was initiated from day 2 to 8 of the menstrual cycle immediately after estrogen priming treatment administered from day 21 of the previous cycle for 10 days onwards (double suppression) and control group (*n*=30) treated with only estrogen priming treatment and antagonist protocol. Results showed no statistically significant difference between the two groups in terms of oocyte maturation and embryo formation rates. On the other hand, a trend toward higher implantation, chemical, clinical and ongoing pregnancy rates was described in delayed start cycles, although it was not statistically significant (Aflatoonian et al., 2017). A major strength of the presented study relies on its large sample size. Nonetheless, limitations should be considered when interpreting the results. The retrospective nature of the study is inherent to risk of bias. Although a significant effort has been made to eliminate all known sources of systematic error through multivariable analysis, there might still exist non apparent sources of bias. Moreover, it is important to mention that our study population included patients who did not get pregnant after one standard GnRH antagonist stimulation cycle and proceeded with another GnRH antagonist stimulation cycle preceded by early administration of GnRH antagonist pretreatment. This category may represent patients with a potential suboptimal prognosis. In addition, the inclusion of potential PORs among our cohort cannot be excluded, as patients' age range for inclusion in the study was from 20 to 44 years. Besides, the impact

of GnRH antagonist pretreatment on pregnancy rate cannot be assessed because of the study crossover design.

In conclusion, a 3-day pretreatment course with a GnRH antagonist administered in the early follicular phase increases the number of oocytes retrieved in a GnRH antagonist stimulation protocol. Furthermore, as the initiation of ovarian stimulation in a GnRH antagonist protocol relies on the unpredictable occurrence of spontaneous menses, addition of three days of GnRH antagonist pretreatment may enhance scheduling flexibility without reducing efficacy.

Chapter 5.
Timeline



A summary of the studies displayed in the timeline:

Recent advances in ovarian stimulation

This study comprises current evidence on ovarian stimulation indicating innovative approaches to achieve successful reproductive outcomes: supplementation with LH, progestin-primed ovarian stimulation protocol and adequate supplementation with serum folate, Ca and Mg before ovarian stimulation in normogonadotropic women, was associated with an increment of IVF results.

Poor ovarian response and the possible role of natural and modified natural cycles

This study highlights the role of natural cycle/MNC-IVF in PORs, offering a milder and patient-friendly approach that represent a valuable alternative to conventional/high-dose ovarian stimulation in this group of patients, especially after failure of stimulated cycles and/or if they do not wish to undergo egg donation.

Is mild stimulation the way forward?

This study clarifies the aim of mild stimulation IVF (MS-IVF) indicating a "procedure in which the ovaries are stimulated with gonadotropins and/or other pharmacological compounds, with an intention of limiting the number of oocytes obtained for IVF to fewer than 8". Recent evidence show that the use of MS-IVF in patients with uncompromised ovarian reserve could optimize fresh and cumulative LBR, respectively. However, our study offers a critical point of view regarding this argument.

Treatment modalities for poor ovarian responders (PORs)

This study analyses therapeutic approaches aiming to enhance fertility of PORs. More in deep, discusses argument such as: gonadotropin dose and type, adjuvant treatments as well as oocyte rejuvenation. However, most of the strategies mentioned in the study, have not yet shown any significant effect, while they were limited by small sample size and heterogeneous populations. In this view, well designed, RCTs performed in homogeneous subgroups of low-prognosis women are warranted.

Letrozole and Ovarian Hyperstimulation Syndrome (OHSS): A Promising Prevention Strategy

This study discusses the use of letrozole for OHSS prevention. Although this pharmacological compound has not yet gained "official" acceptance, promising findings seem to support its administration as an effective therapeutical option to reduce OHSS incidence. The oral administration of 7.5 mg letrozole daily for 5 consecutive days beginning on the day of oocyte retrieval seems to be the best option to prevent OHSS in high-risk women. In the future, large prospective randomized trials are required to evaluate the effect of letrozole and its endocrine impact on the development of OHSS.

Chapter in Textbook of Assisted Reproductive Technique:

Ovarian Hyperstimulation Syndrome (OHSS)

Ovarian Hyperstimulation Syndrome is a rare as well as challenging complication of ovarian stimulation. This chapter reports the current evidence regarding OHSS, discussing approaches for its prevention and treatment.

A summary on the ongoing projects:

Early menopause and non-syndromic primary ovarian insufficiency: prediction of the phenotype severity starting from genetic-molecular basis

This project aims to predict the severity of clinical and biological manifestation of POI. The primary goal is to individuate the possible age of cessation of ovarian function to treat patients (IVF or oocytes cryopreservation) before this happens. Preliminary results show a cohort of women with POI having an average age of 33 and AMH <1 ng/ml. We are still far to predict the age of exhausting of ovarian function.

Lady-De trial

This pivotal trial aims to investigate a more compliant protocol for ovarian stimulation in oocytes donors. The combination of follitropin delta, as gonadotrophin, and dydrogesterone, as oral ovulation suppressor, should encourage patients to donate their oocytes experiencing less stimulation complication and minimal discomfort.

Conclusions

COS is undeniably a pivotal phase in IVF procedures, aiming to stimulate the development of multiple follicles and subsequently to retrieve multiple oocytes. Although, numerous scientific endeavors have focused to optimize the effectiveness of COS, it's essential to note that, the presence of established IVF protocols are not sufficient to overcome infertility issue.

In the era of customized therapeutic strategies, it is of paramount importance that IVF protocols are tailored to the individual characteristics of the patients, including woman's clinical history, age, and ovarian reserve.

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