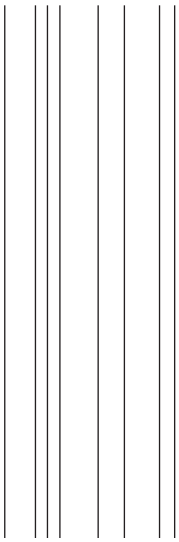

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2017 | Faculty of Medicine and Life Sciences

DOCTORAL DISSERTATION

Optimizing clinical management in donor insemination programs

Doctoral dissertation is submitted to obtain the degree of
Doctor of Biomedical Science, to be defended by

Annelies Thijssen

Promoter: Prof. Dr Willem Ombelet | UHasselt / tUL
Co-promoters: Prof. Dr Lena De Ryck | UHasselt / tUL
Dr Nathalie Dhont | Ziekenhuis Oost-Limburg

D/2017/2451/4

Promotor

W. Ombelet, MD PhD

Co-promotors

L. De Ryck, MSc PhD

N. Dhont, MD PhD

Jury

G. Pennings, MSc PhD

E. Klerkx, MSc PhD

E. Bosmans, MSc

B. Cohlen, MD PhD

A. Cantineau, MD PhD

H. Tournaye, MD PhD

Proefschrift voorgelegd tot het behalen van de graad van
Doctor in de Biomedische Wetenschappen

Met dank aan het 'Limburg Clinical Research Program (LCRP) UHasselt-ZOL-Jessa', met ondersteuning van de stichting Limburg Sterk Merk, de provincie Limburg, de Vlaamse overheid, de Universiteit Hasselt, het Ziekenhuis Oost-Limburg en het Jessa Ziekenhuis.

"If you ever feel like giving up,

just look back on how far you've come already"

Voor mama, papa en Davy

Table of Contents

List of abbreviations

7-AAD	7-aminoactinomycin
AID	artificial insemination with donor sperm
APC	allophycocyanin
ART	assisted reproductive technologies
ASRM	American society for reproductive medicine
BAS	British andrology society
BELRAP	Belgian register for assisted procreation
BMI	body mass index
CBRC	cross-border reproductive care
CC	clomiphene citrate
CF	cystic fibrosis
CMV	cytomegalovirus
CPA	cryoprotective agent
CPR	clinical pregnancy rate
CRF	case report form
DGC	density gradient centrifugation
DMSO	dimethylsulfoxide
EBSS	Earle's balanced salt solution
EG	ethylene glycerol
EUG	extrauterine gestation
FACS	fluorescence-activated cell sorting
FITC	fluorescein isothiocyanate
FSH	follicle stimulating hormone
GEE	generalized estimating equations
hCG	human chorionic gonadotropin
HFEA	human fertilisation and embryology authority
HIV	human immunodeficiency virus
hMG	human menopausal gonadotropin
HSA	human serum albumin
ICSI	intracytoplasmic sperm injection
IMC	inseminating motile count
IU	international units

IUI	intrauterine insemination
IVF	in vitro fertilization
LH	luteinizing hormone
LN_2	liquid nitrogen
NC	natural cycle
NGDT	national gamete donation trust
recFSH	recombinant follicle stimulating hormone
RT	room temperature
SD	standard deviation
TMSC	total motile sperm count
TYB	test yolk buffer
WHO	world health organization

Samenvatting

In deze thesis hebben we ons toegespitst op drie belangrijke domeinen voor het optimaliseren van het klinisch management in donorinseminatie-programma's. In het eerste deel wilden we een helder beeld geven van de situatie waarin de Belgische spermabanken zich momenteel bevinden. Daarnaast bekeken we ook de motivatie en attitudes van de huidige kandidaat spermadonoren in België. De resultaten toonden aan dat er een grote variatie is in de gebruikte methodes bij Belgische spermabanken. Strategieën voor donor rekrutering, screening van donoren, aantal geaccepteerde donoren, limieten voor spermakwaliteit, methodes voor sperma opwerking, methodes voor invriezen van sperma en zelfs de financiële vergoeding voor donatie verschillen sterk tussen de centra onderling. Bovendien zijn, door een tekort aan Belgische donoren, 15/17 (88%) van de centra afhankelijk van de import van buitenlands donorsperma. Omdat er in België momenteel een discussie plaatsvindt om de anonimiteit van de spermadonor af te schaffen, vrezen de spermabanken voor de beschikbaarheid van hun donoren. Onze resultaten toonden aan dat slechts 26% van de huidige kandidaat donoren bereid is om niet-anoniem te doneren. In het volgende deel hebben we de spermakwaliteit en -overleving getest bij verschillende bewaarmethodes. We konden aantonen dat de spermakwaliteit beter bewaard blijft wanneer stalen voor 24u bewaard worden bij kamertemperatuur in plaats van op 35°C. Ook hebben we, met het invriezen van sperma in kleine druppeltjes, een techniek ontwikkeld die gemakkelijker en sneller toe te passen is en een hoger aantal beweeglijke zaadcellen overhoudt na invriezen en ontdooien in vergelijking met de 'slow freezing' techniek. Tenslotte hebben we onderzocht in welke mate bepaalde parameters, geassocieerd met het inseminatieproces, een invloed hebben op de kans op zwangerschap bij donorinseminatie. Op basis van dit onderzoek hebben we een model kunnen bouwen waarmee we de kans op zwangerschap voor een specifieke patiënt kunnen voorspellen, gebaseerd op gegevens die ingevoerd worden voor de leeftijd van de vrouw, roken, primaire/secundaire infertiliteit, progesterone waarde op dag 0 van de cyclus en ovariële stimulatie. Deze resultaten kunnen bijdragen tot de optimalisatie van de methodiek van donorinseminaties en de kans op zwangerschap na donorinseminatie bevorderen.

Summary

In this PhD thesis, we have focused on three main domains for the optimization of clinical management in donor insemination programs. In the first part, we aimed to provide a clear overview of the current situation on sperm banking in Belgium. In addition, we surveyed the motivation and attitudes of the current candidate sperm donor population in Belgium. The results showed that a wide variation in methods associated with sperm banking could be observed between Belgian centers. Donor recruitment strategies, screening of donors, acceptance rates, thresholds for acceptable sperm quality, sperm preparation techniques, freezing methods and even the financial reimbursement per sperm sample differed substantially between the centers. Moreover, we were able to demonstrate that, due to a shortage in Belgian sperm donors, 15/17 (88%) of the Belgian centers have to rely on the import of foreign donor sperm. Since the discussion is currently ongoing to abolish donor anonymity in Belgium, sperm banks are fearing the possible consequences on the availability of donors. Results from our study indicated that only 26% of our current candidate donor population would be willing to continue donating non-anonymously. In the second part, we have looked further into the impact of different sperm storage methods on sperm quality and survival. We showed a significantly better and longer preservation of sperm quality when samples were incubated for 24h at room temperature compared with 35°C. Also, with the freezing of spermatozoa in small droplets, we were able to develop a technique that is easier and faster to apply and yields higher numbers of post-thaw progressive sperm motility compared with the conventional slow freezing process. In the final part, we aimed to evaluate the extent to which different parameters associated with the insemination process, can influence pregnancy rate in a donor insemination program. With this study, we were able to build a model which predicts the probability for a specific patient to achieve pregnancy based on values that are entered for female age, smoking, primary/secondary infertility, progesterone levels on day 0 of the cycle and the use of ovarian stimulation. These results can aid in the optimization of the methods used for donor insemination programs and help to increase pregnancy rates following donor insemination.

Chapter 1

General introduction

1.1 The history of donor insemination

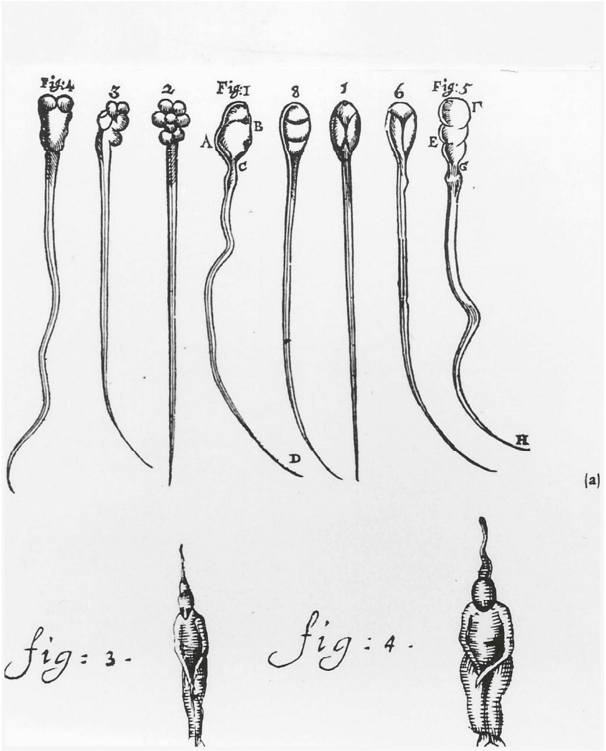

Figure 1.1-1: The 17th century conception of spermatozoa (A van Leeuwenhoek) [1]

Although artificial insemination with donor sperm (AID) is often seen as a modern technology, for its origination, we have to go back to 1678, when Antoni van Leeuwenhoek (1632-1723) was the first to visualize spermatozoa as 'zaaddiertjes' or living animalcules in human semen. More than 100 years later, in 1784, an Italian priest and physiologist named Lazzaro Spallanzani (1729-1799) reported the first successful artificial insemination in a dog. He was also the first to realize that for an embryo to develop, there had to be actual physical contact between the oocyte and spermatozoa. Only 16 years later, in 1790, dr. John Hunter (1728-1793) recorded the first successful intrauterine insemination (IUI) of a woman with her husband's sperm.

Then, for over a century, nothing was heard on the subject. Until in 1909, a letter appeared in the American journal, Medical World, claiming the first human

donor insemination had been performed 25 years earlier, in 1884, at the Jefferson Medical College in Philadelphia by dr. William Pancoast (1835-1897). When a 31-year-old woman came to see him about her inability to conceive, he discovered, after numerous exams, that the issue was her husband's low sperm count. When he discussed the case with his medical students, one suggested that semen could be collected from the 'best looking' member of the class to inseminate the woman. Dr. Pancoast agreed, but instead of disclosing this information to the patient, he called her in for another examination. After the woman was anesthetized with chloroform, dr. Pancoast inseminated her with semen collected from one of his students. After it became apparent that the woman had conceived, dr. Pancoast informed her husband. Although the man was pleased with the result, he asked for his wife to be kept in ignorance. So the story remained a secret until 1909, after the death of dr. Pancoast, when Addison Davis Hard (1853-1930), one of the students present at the day of the insemination, published his letter.

After the publication of Hard's letter, the moral and social implications of AID were highly debated and the Catholic Church objected to all forms of artificial insemination, stating donor insemination was a form of adultery, and the practice faded into oblivion. If doctors were treating infertility with donor insemination, they were doing it in secrecy.

In 1953, the debate concerning donor insemination was heated again when dr. Jerome K. Sherman introduced a simple method for sperm freezing. Using glycerol as a cryoprotectant and carbon dioxide as a refrigerant, combined with a slow cooling rate, he was the first to demonstrate that frozen sperm, when thawed, was able to regain its function and fertilize an oocyte. His research led to the first successful human pregnancy with frozen sperm. Only about two decades later, in the 1970s, the sperm bank industry became popular and commercialized.

1.2 Current status on sperm banking

At the beginning, sperm donation was performed in utmost secrecy, not only to protect the identity of the sperm donor, but also for the woman receiving treatment, her partner and the doctor performing the insemination. In the early

1900s, donor insemination was considered adultery on the mother's part and the child conceived was considered illegitimate. Records on the identity of the sperm donor were destroyed to avoid paternity claims and doctors tried their best to match physical characteristics of the sperm donor to those of the infertile husband in order to avert questions.

In more recent years, this anonymity status of the sperm donor has been changing. In 1985, Sweden was the first country to abolish donor anonymity [2]. Nowadays, even more countries have changed their legislation (e.g. Austria 1992, Switzerland 2001, the Netherlands 2004, Australia 2005, Norway 2005, United Kingdom 2005, …), allowing the donor conceived child to inquire about his/her genetic origin at a mature age.

As sperm donation has gained popularity over the years, an increasing number of women are relying on the procedure to fulfill their wish for a child. Where in the past the use of donor sperm was restricted to hetero couples with an azoospermic husband or when the male partner carried an inheritable genetic disease, nowadays more lesbian couples and single women call for donor insemination [3-5]. Since legislations concerning donor anonymity and which women, i.e. heterosexual, lesbian or single, are entitled to use donor insemination differ greatly between countries, reproductive tourism has developed [6-8]. Belgium is one of those countries experiencing reproductive tourism. Since our legislation allows for both anonymous and non-anonymous sperm donations to be performed on heterosexual, lesbian and single women, we observe a high inflow of patients from our neighboring countries, trying to evade the more restrictive laws in their home country. This has led to a shortage in Belgian donor sperm and an increased import of donor sperm from Denmark. However, since there is no central registration system for sperm donation in Belgium, it is difficult to support this statement with figures. In this PhD research, we have therefore aimed to obtain a detailed overview of the sperm banking facilities in Belgium by sending questionnaires to all Belgian centers for assisted reproduction with laboratory facilities. A summary of the Belgian legislation concerning donor insemination is provided in **Appendix C**.

As our neighboring countries have been changing their legislation towards a non-anonymous donor system, Belgian governments are currently also debating about adding other options. At the moment, Belgian patients can choose

between a completely anonymous sperm donor, or a donor which is known to them from the start of the treatment; no options exist in between. Debates are ongoing to add the option of an identifiable donor, i.e. a donor whose identity can be revealed to the donor-conceived child at a mature age, or to abolish donor anonymity completely and move to an identifiable donor program in which donor conceived children will be able to obtain non-identifiable information on their donor at the age of 12 and identifiable information at the age of 16 or 18 years old. Since other countries have suffered a downturn in the number of available sperm donors after removing donor anonymity [2, 9-12], Belgian sperm banking facilities are fearing the consequences on the availability of donor semen. In order to predict the possible impact of a changed legislation, we have examined the characteristics of the current candidate donor population and evaluated their view on sperm donation.

1.3 Optimal conditions for sperm storage

It is well known that the testis temperature is approximately 2-3°C below body temperature [13], as this is required for the production and maintenance of viable spermatozoa [14, 15]. Despite the numerous articles published on the harmful effects of long-term in-vitro sperm incubation at body temperature, it is still current practice in most IVF laboratories to store prepared sperm samples at this unfavorable temperature prior to their use in assisted reproduction treatment [16]. Therefore, our study aimed to examine the effects of long-term (24h) in-vitro sperm incubation at room temperature (RT; 23°C) versus testis temperature (35°C) on various sperm-quality parameters.

Since the introduction of sperm freezing in 1953, it has become standard practice in donor insemination programs to use frozen donor semen in order to allow time to screen the male donors for contaminations, such as human immunodeficiency virus (HIV), hepatitis B and C virus and other common infectious diseases, all before the cryopreserved sperm sample is used in clinical applications [17, 18]. Cryopreservation of donor semen and the use of cryoprotective agents, however, reduce post-thaw sperm survival, motility and pregnancy rates [19-21]. Although the freezing of oocytes and embryos has evolved from the slow freezing process towards the more successful vitrification

method, the vitrification of spermatozoa remains challenging and slow freezing continues to be the standard method of choice. In an attempt to increase the survival rate of spermatozoa after freezing and thawing and to reduce the costs and time spent on the slow freezing procedure, we have compared two methods of cryoprotectant-free vitrification for spermatozoa, i.e. large volume vitrification and small volume vitrification, with conventional slow freezing and evaluated the effects on post-thaw sperm quality.

1.4 Factors predicting AID success

There is an ever-increasing demand for more cost-effectiveness in health care. However, due to the shortage of good-quality prospective cohort studies and the persistent lack of standardization in study protocols, controversy remains about the effectiveness of IUI, particularly in relation to the more refined techniques such as in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI), and the question is increasingly asked if we should continue performing IUI in the future [22]. Although the World Health Organization (WHO) [23] provides a manual for the examination and processing of human semen, not all centers follow these guidelines. Moreover, terms like total motile sperm count (TMSC) and inseminating motile count (IMC) are very inconsistently used, making it difficult to compare results amongst different studies. Most frequently, studies do not describe when and how TMSC was calculated. Some studies report TMSC values pre-wash, while others report post-wash TMSC, the latter often representing IMC reported in other studies. Also, some studies count TMSC using the percentage of progressively motile sperm, whilst others use total motile counts. In addition, most of the studies performed in the past are retrospective and statistical analysis does not account for the multivariate nature of the dataset and the fact that the same patients are coming back for treatment after previous failed attempts. Therefore, in this PhD-research, we aimed to prospectively determine the factors influencing pregnancy rates after IUI with frozen donor semen in order to increase success rates and decrease costs per treatment cycle. A novel form of multivariate logistic regression analysis, i.e. Generalized Estimating Equations (GEE), was used on the data to take into account the correlation between observations from the same patient.

1.5 Research objectives

This PhD thesis focusses on three domains, in which we will further explore five main objectives:

1. **Current status on sperm banking.**
    - Obtain a detailed overview of the sperm banking facilities in Belgium.
    - Study the motivation and attitudes of a candidate sperm donor population in Belgium.
2. **Optimal conditions for sperm storage**
    - Examine the effects of long-term (24h) in-vitro sperm incubation at room temperature (RT; 23°C) versus testis temperature (35°C) on various sperm-quality parameters.
    - Compare two methods of cryoprotectant-free vitrification for spermatozoa, i.e. large volume vitrification and small volume vitrification, with conventional slow freezing and evaluate the effect on post-thaw sperm quality.
3. **Factors predicting AID success**
    - Evaluate the extent to which different parameters, such as age, smoking, inseminating motile count (IMC), etc., can influence pregnancy rate in a donor insemination program.

Chapter 2

Current status on sperm banking

2.1 Sperm banking in Belgium

Artificial insemination with donor sperm (AID): heterogeneity in sperm banking facilities in a single country (Belgium)

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original paper

Annelies THIJSSEN, Nathalie DHONT, Eva VANDORMAEL, Annemie COX, Elke KLERKX, Eva CREEMERS, Willem OMBELET

Facts Views Vis Obgyn, 2014, 6 (2): 57-67

2.1.1 Abstract

Due to the high inflow of foreign patients seeking cross-border reproductive care in Belgium and the increased number of lesbian couples and single women who call for artificial insemination with donor sperm (AID), Belgian sperm banks nowadays face a shortage in donor sperm. However, since there is no central registration system for sperm donors in Belgium, no figures are currently available supporting this statement. Therefore, a study was performed to obtain a detailed overview of the sperm banking facilities in Belgium. Questionnaires were sent to all Belgian centres for assisted reproduction with laboratory facilities (n=18) to report on their sperm banking methods. The results showed that 82% of the centres rely partially or completely on foreign donor sperm. Moreover, four of the thirteen centres that have their own sperm bank use imported donor sperm in >95% AID cycles. Our results show that in 63% of the Belgian AID cycles imported Danish donor sperm is being used. Donor recruitment is mainly performed through the centre's website (61%) or by distributing flyers in the centre (46%) and 9 to 180 potential donors have been recruited per centre in 2013. Eventually, 15 to 50% of these candidate donors were accepted. Different criteria for donor acceptance are handled by the centres: donor age limits range from 18-25 to 36-46 years old, and thresholds for sperm normality differ considerably. We can conclude that a wide variation in methods associated with sperm banking is observed in Belgian centres.

KEYWORDS: artificial insemination, donor sperm, Belgium, questionnaire, sperm banking

2.1.2 Introduction

According to the Belgian Register for Assisted Procreation (BELRAP), the number of initiated artificial insemination cycles with donor sperm (AID) in Belgium has increased from 8766 AID cycles in 2008-2009 [4] to 13048 in 2010-2011 [5]. Although this increase of AID cycles may partially be attributed to additional centres reporting to the BELRAP in recent years, Belgian centres also reported a substantial increase in the number of patients relying on sperm donation (personal communications).

Where in the past the use of donor sperm was restricted to hetero couples with an azoospermic husband or when the male partner carried an inheritable genetic disease, nowadays more lesbian couples and single women call for donor insemination [3-5].

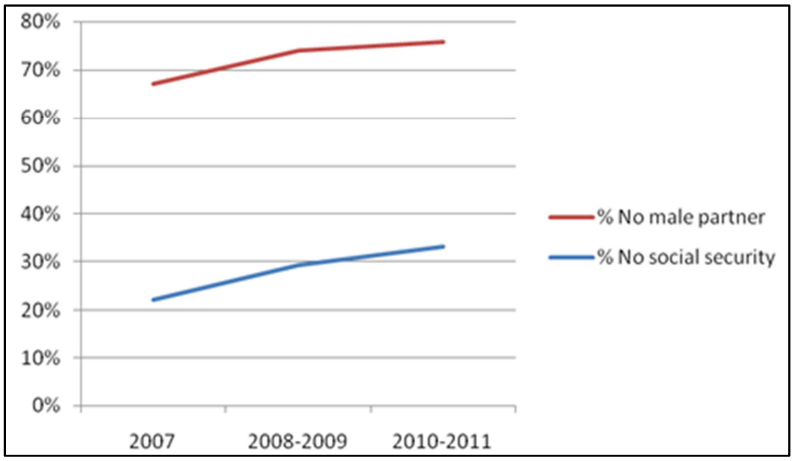

Figure 2.1-1: Rise in the percentage of patients with no male partner (lesbian couples and single women) and no social security (foreign patients), applying for AID in Belgium.

AID: Artificial Insemination with Donor sperm. No social security indicates the percentage of patients treated for AID in Belgium that are not connected to a Belgian social security fund, mostly indicating foreign patients. (Based on results adapted from reports of the Belgian Register for Assisted Procreation [3-5]).

Secondly, Belgian centres for Reproductive Medicine are overwhelmed with patients seeking cross-border reproductive care (CBRC) [3-5, 7], trying to avoid restrictive laws in their home country (**Fig. 2.1-1**) [6, 8]. For example, in the

Netherlands and the United Kingdom, anonymous sperm donation is abolished since 2004 and 2005 respectively [24, 25]. Furthermore, the treatment of lesbian couples and single women with donor sperm is restricted in France and Germany [26, 27]. On the contrary, Belgian legislation allows for anonymous as well as non-anonymous sperm donation to be performed on hetero and lesbian couples as well as single women (**Figure 2.1-2**) [28]. However, in contrast to countries like Spain and Denmark where the government supports men in becoming a sperm donor, the Belgian government does not allow any form of advertisement for the recruitment of sperm donors [29]. Moreover, according to the Belgian legislation only six women can become pregnant from a single sperm donor [28].

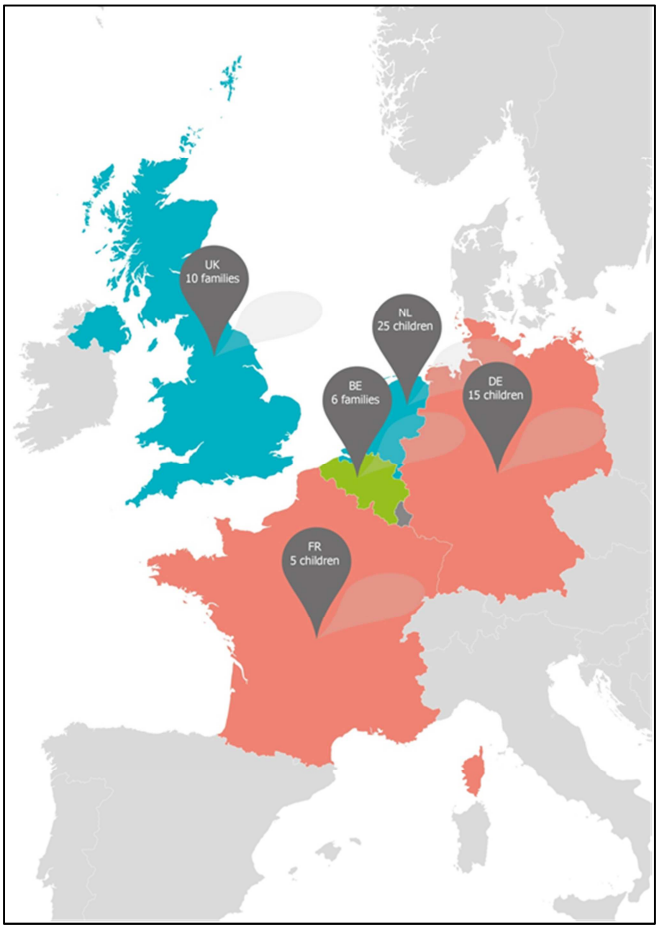

Figure 2.1-2: Legislation in Belgium and neighbouring countries

Legislation allows for hetero couples as well as lesbian couples and single women to be treated with donor insemination + sperm donation may be anonymous as well as non-anonymous, Legislation only allows hetero couples to be treated with donor insemination, Legislation only allows non-anonymous sperm donation.
BE: Belgium; DE: Germany; FR: France; NL: The Netherlands; UK: United Kingdom.

Nowadays, Belgian sperm banks face a shortage in donor sperm [30]. However, since there is no central registration system for sperm donors in Belgium, there are currently no figures supporting this statement. In line of the setup of our own sperm bank, we performed a study to obtain a detailed overview of the sperm banking facilities in Belgium. To our knowledge no such study has been performed in Belgium or in any other country before. Questionnaires (see **Appendix B**) were sent to all Belgian centres for assisted reproduction with laboratory facilities for intra-uterine insemination (IUI) and in vitro fertilisation (IVF) (B-centres) to report on their sperm banking methods, with special attention to the methods and criteria used for recruitment, screening and selection of potential sperm donors, procedures for sperm washing and freezing and costs associated with sperm donation.

2.1.3 Materials and Methods

Questionnaires involved a mix of open and closed questions (i.e. multiple choice), with the possibility of making additional remarks (see **Appendix B**).

At a first stage, a short questionnaire was sent by e-mail to all Belgian centres for assisted reproduction with laboratory facilities for IUI and IVF, the so-called B-centres (n=18). Only B-centres are allowed to have a sperm donor bank. This short questionnaire was designed to obtain basic information on the use of AID in Belgium; i.e. the number of AID cycles performed by the centre, the origin of the donor sperm (Belgian or imported donor sperm), to what ratio the centres use Belgian versus imported donor sperm, for which patients (hetero/lesbian couples or single women) donor insemination is performed, whether the centre performs anonymous or non-anonymous sperm donation and, in case the centre has its own sperm bank, methods for the recruitment of potential donors. The short questionnaire was distributed in English to all Belgian centres.

Based on the results of the first questionnaire, the centres having their own sperm bank were selected (n=13). A more detailed questionnaire was sent by e-mail to these centres to collect more information on their procedures for sperm banking. Additional questions included specific donor recruitment strategies and numbers, screening and selection criteria for candidate donors, the freezing and

washing procedures for donor semen and the payment of donors. The detailed questionnaires were sent in Dutch to the Flemish speaking centres and in French to the French speaking centres and they were distributed between December 2013 and April 2014.

All data were stored and analysed in Excel spreadsheets (Microsoft Excel 2010).

2.1.4 Results

Response rate and geographical distribution of the participating centres

Belgium counts eighteen B-centres for Reproductive Medicine (n=18). For both questionnaires, a response rate of 100% was accomplished. The different centres for Reproductive Medicine are distributed between the three Belgian regions as follows: eight centres in Flanders, four centres in Brussels and six centres in Wallonia.

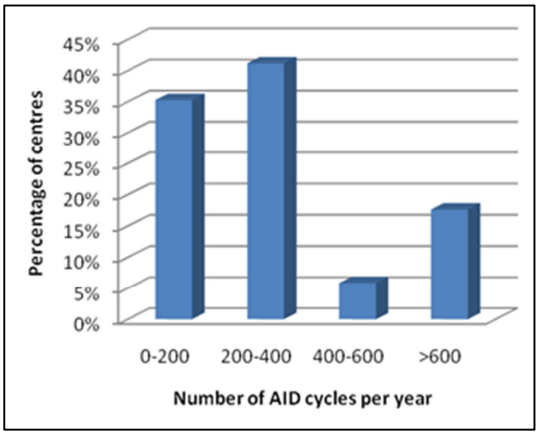

Figure 2.1-3: Number of AID cycles per year.

Overview of het total number of AID cycles performed by the centres each year.

Size of the participating centres

The results from the first questionnaire showed that all but one (17/18 or 94,4%) of the B-centres for Reproductive Medicine perform AID. The size of the centres can be determined by the number of AID cycles they perform each year. These are clearly shown in **Figure 2.1-3**.

Patients treated with AID

All three patient groups, i.e. hetero and lesbian couples and single women, can apply for AID in thirteen out of the seventeen (76%) centres. The remaining centres (n=4) do not offer AID to single women and only perform AID for hetero and lesbian couples (**Figure 2.1-4**).

Anonymous or non-anonymous donor insemination

About half of the centres (8/17 or 47%) always perform anonymous donor inseminations. The other centres (9/17 or 53%) are open to both anonymous and non-anonymous sperm donation, although they often state that the request for non-anonymous donor insemination is extremely rare (**Figure 2.1-5**).

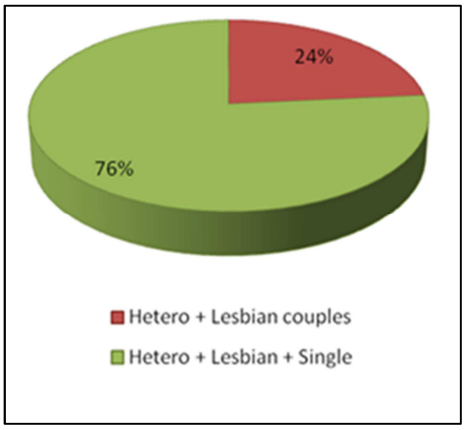

Figure 2.1-4: The percentage of centres treating hetero and lesbian couples and (no) single women.

Figure 2.1-5: The percentage of centres performing only anonymous or anonymous + non-anonymous donor inseminations.

The origin and use of donor sperm

Only two out of the seventeen centres (12%) rely completely on their own sperm bank. The other fifteen centres (15/17 or 88%) rely partially (11/17 or 64%) or entirely (4/17 or 24%) on the import of foreign donor sperm (**Figure 2.1-6**). Of the 11 centres using imported donor sperm as well as Belgian donor sperm from their own sperm bank, three centres use Belgian donor sperm in >95% of the AID cycles they perform, in four centres the ratio for Belgian and imported donor sperm used for AID is fifty/fifty and another four centres use imported donor sperm in >95% of their AID cycles (**Figure 2.1-6**).

Imported donor sperm in Belgium is exclusively obtained from Denmark, i.e. Nordic Cryobank (Nordic Cryobank ApS, Frederiksberg, Denmark) or Cryos International sperm bank (Cryos International ApS, Aarhus, Denmark), and none of the centres imports donor sperm from other Belgian sperm banks. Our results indicate that 63% of the donor inseminations performed in Belgium are accomplished with imported donor sperm from Denmark.

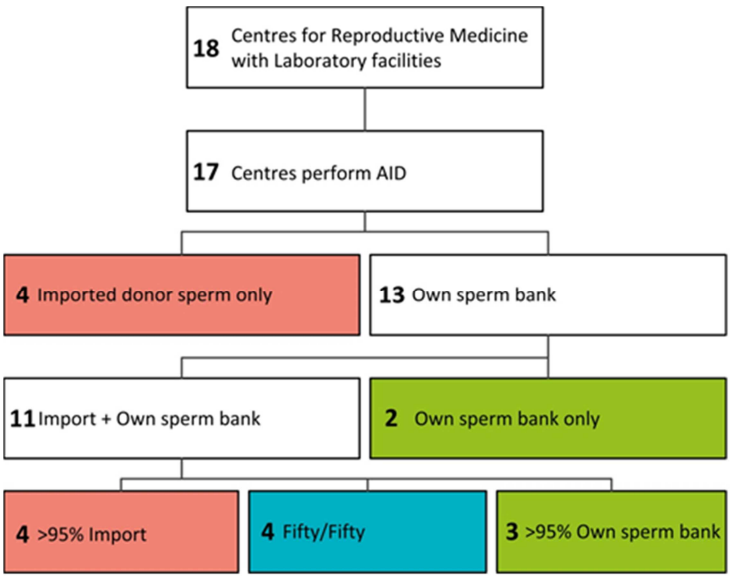

Figure 2.1-6: Origin and use of donor sperm

Donor recruitment

Donor recruitment is mostly done by providing information on donor insemination and the possibility of becoming a sperm donor on the centres website (8/13 or 61%) or by distributing flyers in the centre (6/13 or 46%). Other ways to recruit new potential donors are oral advertisement (3/13 or 23%) or asking partners of women who became pregnant after infertility treatment and with no male factor infertility involved (normal semen sample) to become a donor (solidarity principle) (4/13 or 31%). Most centres use a combination of different donor recruitment strategies.

In 2013 the total number of candidate donors applying to the Belgian centres for a first semen analysis was 602, with one centre reporting 9 candidate donors and another centre reporting 180 candidate donors. The average acceptance rate of the Belgian candidate donors is 32% (range 15% to 50%). In 2013 a total number of about 260 candidate donors were accepted in Belgium (**Table 2.1-I**).

To make sure a donor will only donate in one specific centre, five out of the thirteen sperm banks (38%) make use of a contract with the donor. This implicates that eight sperm banks (62%) do not make use of such a written contract.

Criteria for sperm donors

The minimum age for a candidate sperm donor was reported at 18 years for 11 out of the 13 sperm banks (85%). The other two centres reported a minimum age of 21 and 25 years old respectively. The maximum donor age differs substantially between the centres, with a minimum of 36 and a maximum of 46 years old (**Figure 2.1-7**, **Table 2.1-I**).

All sperm banks perform the statutory infection screenings: hepatitis B and C, human immunodeficiency virus (HIV)1,2, syphilis and chlamydia. Furthermore, all centres but one (12/13 or 92%) also screen for cytomegalovirus (CMV) infections. Of the centres who screen for CMV infection, the majority (9/12 or 75%) accepts CMV+ donors, with 8 of them also matching CMV+ donors with CMV+ recipients. In addition, one centre also reported to screen for Neisseria gonorrhoea. Next to the infection screening, all centres also perform a genetic screening for cystic fibrosis (CF) and determine the karyotype

of the candidate sperm donor. Additionally, one centre reported to test for Y chromosome microdeletions and two other centres reported to test for hemoglobinopathy. A psychological screening of the candidate donors is only performed in three out of the 13 Belgian sperm banks (23%).

The minimum criteria for sperm quality parameters differ substantially between the different sperm banks. The lower limit for native sperm concentration ranges from 15 up to 60 million spermatozoa per ml. Additionally, the threshold for good forward progressive motility has to be at least 30% in one centre and up to 70% in another centre. Minimum criteria for sperm morphology range from 4% to 25% morphologically normal spermatozoa. Minimum criteria for inseminating motile counts (IMC) after sperm freezing and thawing, range from 1 up to 5 million (**Table 2.1-I**).

Before donor acceptance, the majority of the sperm banks (10/13 or 77%) also perform a test procedure for sperm washing. Two centres perform the washing procedure prior to sperm freezing, seven centres after sperm freezing and one centre performs a washing procedure of the sperm sample both before and after sperm freezing.

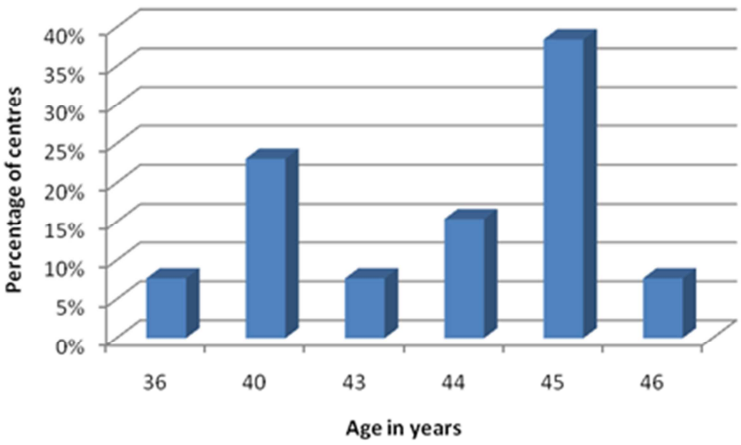

Figure 2.1-7: Maximum donor age limits used by the centres

Handling of donor sperm

The majority of the sperm banks (9/13 or 69%) always freeze donor semen in its fresh state. One centre (1/13 or 8%) indicated to perform both freezing of

fresh and concentrated semen and another centre always concentrates the semen sample prior to freezing. The remaining two centres freeze the sperm sample after capacitation. In addition, all centres reported to make use of straws as a carrier material for sperm freezing (**Table 2.1-I**).

The majority of the sperm banks (11/13 or 85%) store semen samples from donors that were not yet serologically tested in a separate quarantine container. Of the centres that accept CMV+ sperm donors, only one (1/9 or 11%) stores these samples separately.

Donor payment

A Belgian donor is paid €66 per donation on average, with a minimum of €50 and a maximum of €100 per donation (**Table 2.1-I**).

2.1.5 Discussion

Belgium is a small country, with only 13 sperm banking facilities. It is well known that country regulations concerning all different aspects of gamete donation vary a lot because of political, ethical, socio-cultural and religious differences. The results we obtained only describe the Belgian situation.

Belgian sperm banks nowadays face a shortage in donor sperm. Therefore, this study aimed to present an overview of the sperm banking facilities in Belgium with special attention to the methods and criteria used for recruitment, screening and selection of potential sperm donors, procedures for sperm washing and freezing and costs associated with sperm donation.

Since three quarter of the Belgian centres offer donor insemination to lesbian couples and single women as well as hetero couples, and anonymous donor insemination is available in all Belgian centres, foreign patients frequently come to Belgium seeking CBRC. According to Pennings et al. [7], most of these patients are coming from France (38%), the Netherlands (29%), Italy (12%) and Germany (10%). Patients coming from the UK constitute only 2% of all foreign patients treated in Belgium, since they are more likely to go to other European countries like Spain [31]. More specifically for sperm donation, Pennings et al. [7] reported that 80% of the treatment cycles were performed for French women, 13% for Dutch women and 3% for Italian women.

Table 2.1-I: Overview of the results

SPERM BANK	A	B	C	D	E	F	G	H	I	J	K	L	M
DONOR RECRUITMENT (2013)													
Number of candidate donors	53	20	21	20	100	80	10	19	180	9	30	10	50
Percentage accepted donors	17	33	50	30	15	50	20	46	NA	NA	25	30	40
DONOR AGE (years)													
Minimum	21	18	18	25	18	18	18	18	18	18	18	18	18
Maximum	44	46	43	45	40	44	45	45	45	40	40	45	36
DONOR SPERM QUALITY													
Fresh													
Concentration (million/ml)	15	40	50	60	15	40	30	20	20	20	20	20	20
Motility (%A+B)	32	50	50	60	42	50	70	40	30	50	40	50	50
Morphology (%)	4	4	10	10	4	4	10	4	10	4	4	25	7
Thawed													
IMC	2	2	2	1,5	5	1	1	1	NA	5	NA	1	3
SPERM FREEZING													
Status	fresh	fresh	fresh	conc	fresh	fresh	fresh	fresh	fresh	capac	capac	fresh/conc	fresh
Carrier	straws	straws	straws	straws	straws	straws	straws	straws	straws	straws	straws	straws	straws
DONOR PAYMENT													
€/sample	60	50	50	50	85	75	50	75	50	70	75	100	80

IMC: inseminating motile count (i.e. the total number of good progressive motile spermatozoa after sperm washing procedure); NA = not available.

Since 2004, there has been a steady increase in the number of French patients coming for donor insemination [7, 32]. For Dutch patients, the number for donor insemination almost doubled between 2004 and 2005, following the abolishment of donor anonymity in the Netherlands [7, 24].

Our results showed that in 2013 about 63% of the total number of donor inseminations performed in Belgium were accomplished with imported donor sperm from Denmark. This figure seems to be alarming, but one has to keep in mind that in the period 2010-2011 63.3% of AIDs in Belgium were performed in foreign patients (8262/13048 AID cycles) [5].

Out of the 13 centres having their own sperm bank, 4 centres still use imported donor sperm in over 95% of the AID cycles. Some of them stated they are keeping their own sperm bank for 'times of need', in case the Belgian government would decide to prohibit the import of foreign donor sperm.

In addition to the shortage in donor sperm, Belgian legislation only allows pregnancies with sperm from a single donor to a maximum of six different women [28]. Recently, a comment was added to this law, stating that a lesbian couple applying for donor insemination accounts for one women [33]. The maximum of six women is rather low in comparison to most of our neighbouring countries, allowing a maximum of 25 children per sperm donor in the Netherlands, 15 children in Germany and a maximum of 10 families in the United Kingdom (**Figure 2.1-2**). Only France has, with a maximum of 5 children, a lower rate for the number of children that are allowed to be born from a single donor. Because there is no central registration system for sperm donors in Belgium, it is questionable if this law is actually implemented in practice. Some centres ask their sperm donors to donate sperm exclusively in their centre by signing a contract in order to avoid that the same donor is going to different centres.

Legislation in Belgium also makes it difficult to recruit sufficient sperm donors, mainly because any form of public advertisement for the recruitment of sperm donors is prohibited by law [29]. Therefore, the centres mainly use their website or the distribution flyers in their centre to inform potential donors on the possibility of sperm donation. According to many AID-centres, a rise in the number of sperm donor applicants is observed whenever sperm donation is mentioned in the media, although this has never been properly investigated

and/or documented. If so, this would be a good argument for asking the government to start with awareness campaigns. The National Gamete Donation Trust (NGDT) in the United Kingdom is a very good example of how to organise recruitment for gamete donation. The NGDT is the national body running the National Gamete Donation Services. They work with potential recipients, UK licensed fertility clinics, the media and support organisations to raise awareness of the need for gamete donors. They realized to have an increase of sperm donors, although anonymous donation is forbidden in the UK (National Gamete Donation Trust, http://www.ngdt.co.uk).

Belgian donors are paid between €50 and €100 per donation. This payment is only meant to cover the transportation costs or loss of salary for the donor when he comes to donate his sperm [28]. The reason why some centres pay much more than other centres cannot be found.

Apart from the low number of candidate donors applying to the centres, the average acceptance rate for sperm donors in Belgium is also rather low. On average between 15 and 50% of the sperm donor applicants meet all criteria to become a sperm donor. We couldn't find a good reason why the acceptance rate differed so much between centres.

The threshold values for sperm quality differ strongly between centres. Different cut-off values for normality are used mostly due to different methodology for sperm analysis and different criteria used, especially for sperm morphology [34]. We observed that in all centres the criteria for defining normal sperm quality are substantially higher than the criteria posed by the World Health Organization indicating a normal semen sample [23]. However, the sometimes higher threshold for sperm normality of a certain centre does not always correspond to a lower acceptance rate in that specific centre (Table I). On the other hand, higher values of sperm parameters do not necessarily result in better pregnancy rates [35, 36].

The Belgian law implies that a sperm donor tests negatively for hepatitis B and C, HIV1,2, syphilis and chlamydia. In addition, all but one of the Belgian centres also screen for CMV infection. With CMV being the leading cause of congenital viral infection, potentially leading to foetal death, severe birth disease or subsequent development of neurological or sensory impairment, it is indeed an important parameter to take into account [37, 38]. Different strategies for

handling CMV+ sperm donors have been proposed by different agencies. According to the American Society for Reproductive Medicine (ASRM) guidelines, gametes from CMV seropositive donors should only be used for seropositive recipients [39]. On the other hand, the British Andrology Society (BAS) recommends that only CMV seronegative men should be allowed to donate sperm [40]. This recommendation would however lead to an even greater shortage in sperm donors since about 50-95% of all people at reproductive age are CMV positive [41, 42]. Although we found that most Belgian sperm banks screen potential donors for CMV infection and either reject or accept the CMV+ donor if he can be matched to a CMV+ acceptor, one centre does not match their CMV+ samples and another centre doesn't screen for CMV at all. The Belgian law is not clear on whether a candidate sperm donor should even be tested for CMV since it only states that: "In certain situations, further tests are needed, depending on the history of the donor and the characteristics of the donated human body material (e.g. CMV)." [43]. Literature makes it even more confusing, because there is no consensus on whether CMV is only present in the seminal plasma [44] and thus could be eliminated by sperm washing or also in the spermatozoa themselves [45]. Additionally, research by Doerr et al. [46] showed that the sero-immunoglobulin status (i.e. IgG or IgM positive) is not indicative for whether or not CMV is shed in the semen. Other researchers therefore recommend to test each sperm sample for presence of CMV via PCR, rather than testing the donor [47]. Furthermore, CMV seropositive women are only partially protected against a new CMV infection [48, 49].

According to the results of our study we can conclude that a wide variation in methods associated with sperm banking is observed in Belgian centres. Donor recruitment strategies, screening of donors, acceptance rates, thresholds for acceptable sperm quality, sperm preparation techniques, freezing methods and even the financial reimbursement per sperm sample differ substantially between the centres. Furthermore, we have shown that there is indeed a shortage of non-imported donor sperm by demonstrating that about two-third of the donor inseminations in Belgium are performed with Danish donor sperm. In order to increase the number of candidate donors, the Belgian government should be more supportive, for example by allowing advertisement for sperm donor

recruitment or by organizing awareness campaigns themselves as they do in the United Kingdom (National Gamete Donation Trust).

2.2 Attitudes of candidate sperm donors

Motivations and attitudes of candidate sperm donors

original paper

Annelies THIJSSEN, Veerle PROVOOST, Eva VANDORMAEL, Nathalie DHONT, Guido PENNINGS, Willem OMBELET

Submitted to Fertility and Sterility

2.2.1 Abstract

Objective: Study the motivation and attitudes of a candidate sperm donor population in Belgium.

Design: Anonymous survey.

Setting: Tertiary referral infertility center.

Patients: 100 candidate sperm donors applying to the center for a first semen analysis between April 2013 and March 2016.

Intervention(s): All candidate sperm donors applying to the center for a first semen analysis were invited to fill in a questionnaire. The questionnaire was filled in anonymously and candidate donors were informed that their participation in the study would not influence the selection process.

Main Outcome Measure(s): Demographic characteristics, recruitment methods, motivations and attitudes towards payment, donor anonymity, disclosure to offspring, donation to lesbian couples and single women, views on the donor children and social aspects of sperm donation.

Results: The media was the most reported information source for candidate donors (78%). The most important motivations for donation were altruistic (90%). The financial compensation related with sperm donation was only an important motivational factor in 31% of the candidate sperm donors. The majority of our candidate sperm donors did not have a problem with donating to lesbian couples or single women (93% and 97% respectively). Eighty-two percent of men would be willing to reveal non-identifying information on themselves to donor offspring, but only 26% would be willing to donate if anonymity was abolished completely.

Conclusion: The majority of our current candidate donor population were older men, with a partner and children, who are donating sperm for altruistic reasons. They showed little interest in the outcome of their donation and most of them were not willing to donate non-anonymously.

KEYWORDS: attitude, demography, gamete donor, semen, motivation

2.2.2 Introduction

Sperm banking is very differently regulated between countries [50], with differing legislations concerning donor anonymity, donation to lesbian couples and single women, payment to donors, etc., and can even differ greatly between sperm banks in a single country [51]. Since the abolishment of donor anonymity in Sweden in 1985 [2], other countries have followed in their footsteps and changed legislation towards an identity-release system in which donor conceived children have the possibility to inquire about their genetic origin at a mature age. The impact of this change on donor recruitment is still unclear; some countries removing donor anonymity were confronted with a significant drop in the number of available donors, leading to long waiting lists for patients, increased cross-border health care and more import of foreign donor sperm [2, 9-12]. Other countries seem to have recovered and have, through intensive campaigning, reached an equal or higher number of donors [52, 53].

Belgian sperm banks nowadays are facing a shortage in donor sperm due to the increased number of women relying on donor insemination, i.e. lesbian couples and single women, and the high inflow of patients from neighboring countries seeking cross-border reproductive care [7, 51]. Current Belgian law allows both donation from anonymous sperm donors as well as from a donor known to the recipient couple or woman from the start, e.g. a family member or friend. In addition, donation is allowed for heterosexual as well as lesbian couples and single women. Recently, several new law proposals have been submitted. The proposals contain different suggestions regarding the system of anonymity/ identifiability of donors. In one proposal, they plea for the addition of a third possibility next to the two existing options of an anonymous and a known donor, i.e. the identifiable donor. The parents are then free to decide what they think is best for their family. With this identifiable donor system, the donor child will have the possibility to know the donor's identity at the age of 18. Furthermore, they want to introduce a system by which previously anonymous sperm donors will have the opportunity to make themselves identifiable to their offspring. For this, they will give two complementary 'keys', one to the donor and one to the recipient couple. If both 'keys' are returned to the organization controlling the donor information, identifiable data can be exchanged. On the

opposite side, other political parties want to abolish donor anonymity completely and move to an identifiable donor program in which donor conceived children will be able to obtain non-identifiable information on their donor at the age of 12 and identifiable information at the age of 16 or 18.

At the moment, very little information is available on the donor population in Belgium. In anticipation of a possible abolishment of donor anonymity, Ide et al. [54] performed an opinion poll amongst potential sperm donors to evaluate whether they would still be willing to donate if donor anonymity was abolished. About 71% of the men replied that they would not. However, this is the only study on sperm donors in Belgium. Belgian fertility centers fear the possible drop in the availability of sperm donors if donor anonymity would be abolished completely. Some political parties however reject this argument and state that abolishing donor anonymity does not attract fewer donors but only another type of donors. According to Daniels et al. and Jadva et al. [55, 56] identity-release donation systems attract men with different demographic characteristics and different motivations for donation compared with anonymous systems. It is stated that donors in an identity-release system are more likely to be older, to have children of their own and to donate for altruistic rather than financial reasons. Other published data however contradict these statements. Results from the Human Fertilisation and Embryology Authority (HFEA) [53] in the UK indicated that the proportion of sperm donors in the youngest age group (aged 25 years and under) increased from 15% in the year 2011 to 22% in 2013 and the proportion in the older age groups has decreased. In addition, the percentage of sperm donors with children has decreased over the years, with 41% in 2004, 33% in 2008 and 25% in 2013. More studies are needed to find out how rules and regulations affect donor characteristics.

As a first step, it is essential to examine the characteristics of the current candidate donor population and evaluate their view on sperm donation. Therefore, we surveyed the motivations and attitudes of a total of 100 candidate donors by means of a questionnaire. The results of this study may be of great importance for clinicians as well as law makers.

2.2.3 Materials and Methods

This survey was conducted at the sperm bank of the Genk Institute for Fertility Technology (Ziekenhuis Oost-Limburg, Genk, Belgium). Between April 2013 and March 2016, a total of 100 candidate sperm donors, who applied to the center for a first semen analysis, were invited to complete a questionnaire. The questionnaire was filled in anonymously and candidate donors were informed that their participation in the study would not influence the selection process. All candidate donors effectively provided a semen sample for analysis after filling in the questionnaire (response rate 100%).

The questionnaire consisted of three main sections. First, a list of statements on sperm donation was scored on a 5-point Likert scale ranging from totally disagree to totally agree. Statements included topics such as payment for donors, attitudes toward donor anonymity, disclosure to offspring and donation to lesbian couples and single women, views on the donor children and social aspects of sperm donation. Second, a list of statements regarding the motivation for donation was presented, which was answered on a 5-point Likert scale ranging from very unimportant to very important. Third, socio-demographic characteristics including age, religion, educational level, relationship status and children were collected. In addition, three questions were posed on their familiarity with fertility problems (self or partner), donor-conceived children and men who donated or wanted to donate (i.e. 'Did you or your partner have fertility problems?', 'Do you know people whose children were conceived with donor sperm?' and 'Do you know other men who have donated sperm or wanted to donate?'). Finally, information sources were reviewed by asking how candidate donors came into contact with our center for sperm donation by means of multiple choice questions and the option 'other'. Multiple answers were possible for this question.

Statistical analysis

For the opinion statements, responses were combined into three categories (totally agree/agree, neutral, disagree/totally disagree. Analysis was conducted using Fisher's Exact (Cytel Studio StatXact-8) to compare the distribution of categorical variables and the independent t-test (Statistical Package of the

Social Sciences, SPSS version 23) to compare the mean age between two categories of a particular attitude (totally agree and agree versus neutral, disagree and totally disagree) or motivation to donate (very important and important versus neutral, unimportant and very unimportant).

Ethical approval

Ethical approval of the study was granted by the ethics committee of Ziekenhuis Oost-Limburg and Hasselt University (reference 13/032U, approved 5 April 2013).

2.2.4 Results

Socio-demographic characteristics

Socio-demographic characteristics of the candidate donor population are presented in **Table 2.2-I**. The mean age of the candidate donors was 31.5 years (min 19, max 45, SD 6.6). Different age groups were represented as follows: 24.5% of men ranging 19-25 years, 46.9% of men ranging 26-35 years and 28.6% of men ranging 36-45 years. Thirty-nine percent of the candidate donors had a denominative religion (i.e. Roman Catholic, Protestant, Muslim or Eastern Orthodox). Furthermore, 11% stated they were religious but had no specific religion and half of the men were non-religious. About half (49.5%) of the candidate donors had been to college or university and the other half (49.5%) had finished high school. One candidate donor only finished elementary school. Fifty-eight percent of the candidate donors had a partner and 31% had children of their own, whereas 3% of men indicated their partner has children. Thirty-three percent of the candidate sperm donors were also blood donor and/or stem cell or bone marrow donor. Finally, only 3% of candidate donors had experienced fertility problems themselves or with their partner, 22% knew donor-conceived children and 18% knew other men who donated or wanted to donate sperm.

Table 2.2-I: Socio-demographic characteristics of the candidate sperm donors (N=100)[a]

	n	%
Partner		
Yes	58	58.0
No	42	42.0
Children		
Yes, of my own	31	31.0
Yes, of my partner	3	3.0
No	66	66.0
Donor of other body material		
Blood donor	19	19.0
Stem cell or bone marrow donor	4	4.0
Both	10	10.0
No donor	67	67.0
Religion		
Roman Catholic	35	35.0
Protestant	1	1.0
Muslim	2	2.0
Jewish	0	0.0
Religious, but no specific religion	11	11.0
Non-religious	50	50.0
Other (i.e. 'Eastern Orthodox')	1	1.0
Education		
Elementary school	1	1.0
High school	49	49.5
College/University	49	49.5
Familiarity with fertility problems (self or partner)		
Yes	3	3.0
No	97	97.0
Familiarity with donor-conceived children		
Yes	22	22.0
No	78	78.0
Familiarity with men who donated or wanted to donate		
Yes	18	18.0
No	82	82.0
Age		
19-25 years	24	24.5
26-35 years	46	46.9
36-45 years	28	28.6
Mean (years)	31.5	
(SD, range)	(±6.6, 19-45)	

[a] Number of missing cases vary between 0-2 per variable.

Information source

The media was by far the most reported information source with 78%, followed by the center's website (29%) and being told by an acquaintance or friend (9%). Some men indicated more than one source (results not shown).

Attitudes towards sperm donation

Table 2.2-II provides an overview of the attitudes towards sperm donation, with questions grouped according to different topics concerning sperm donation and results given for the total group of respondents and subdivided according to different characteristics of the candidate donor.

Views on the donor children

Although about half of the candidate donors (46.5%) would like to know how many children were conceived with their sperm, only about a quarter would like to have information about the family where the child would grow up or on the children who were conceived with their sperm (21% and 27% respectively). Men with children of their own were significantly less interested to know how many children were conceived with their sperm (29.0% versus 54.4%; p=0.029) and showed a tendency towards less interest in information about the family in which the child would grow up (9.7% versus 26.1%; p=0.069) compared with men who had no children of their own. Also, men with a partner showed significantly less interest in obtaining information about the children conceived with their sperm (17.2% versus 40.5%; p=0.012) compared with men without a partner.

Views on single women and lesbian couples

The majority of the candidate sperm donors did not have a problem with donating sperm to lesbian couples or single women (93% and 97% respectively). Men who were financially motivated were significantly more reluctant to donate to lesbian couples than men who were not financially motivated (9.7% versus 0.0%; p=0.028) (results not shown).

Disclosure to offspring

Concerning disclosure to the offspring, the majority of the candidate donors (62.6%) agreed that the parents should be able to decide for themselves whether or not to inform the child about his or her genetic origin, and about half of them believe that parents should be honest with their children (49.5%).

Donor anonymity

Although 82% of men indicated that they would be willing to provide information about themselves, such as physical characteristics and interests, to the children born from their donation, only 26% of the candidate donors would also be willing to donate if their name would be passed on to the children born from their donation. On the other hand, 43% of the candidate donors believed that children conceived with donor sperm should have the right to know their genetic origin and 32% of men indicated they would be willing to meet the children who were conceived with their sperm in the future. Men with children of their own seemed less prepared to meet their donor-conceived children in the future (19.4% versus 37.7%; p=0.104), but this result was not significant. Men with a partner were significantly less prepared to meet the children conceived with their sperm in the future (20.7% versus 47.6%; p=0.005) compared with men without a partner. On the other hand, men who were also blood donor and/or stem cell or bone marrow donor seemed more prepared to donate non-anonymously (36.4 versus 20.9; p=0.145) and to meet their donor-conceived children (42.4 versus 26.9; p=0.171) compared with men who were not donors of other body materials. Additionally, men who had experienced fertility problems themselves or with their partner seemed less prepared to provide non-identifying information about themselves (66.7% versus 82.5%; p=0.452) or to meet their donor-conceived children in the future (0.0% versus 33.0%: p=0.549) (**Appendix D Supplementary Table A**). Also, men who knew donor conceived children were significantly less prepared to meet their own donor-conceived children in the future (4.5% versus 39.7%; p=0.001; **Appendix D Supplementary Table A**). Finally, men who were prepared to donate without the financial reimbursement were also significantly more prepared to meet their donor conceived children (45.8% versus 19.2%; p=0.005) and agreed significantly more with the statement that children conceived with donor sperm should have the right to know their genetic origin (55.3% versus 32.7%; p=0.027) (results not shown).

Willingness to donate/to use donor sperm

Only 22% of men thought that many men are prepared to donate sperm. Furthermore, 47% of the candidate donors would be prepared to use donor

sperm if they would experience fertility problems themselves. Men with a university/college degree showed significantly more willing to use donor sperm if they would experience fertility problems themselves (59.2% versus 35.4%; p=0.025; **Appendix D Supplementary Table A**) compared with men without a university/college degree.

Social aspects of sperm donation

Seventy-three percent of the candidate donors thought that their (future) partner has the right to know that they are (were) a donor and that important people in their life would accept their decision to donate sperm. Still, 29% of candidate donors indicated that they plan to keep their donor status a secret from everyone. Men without a partner agreed significantly less with the statement that their future partner has the right to know they were a donor (61.9% versus 81.0%; p=0.0414) compared with men with a partner. Men stating that they planned to keep their donor status a secret from everyone were significantly older compared with men not agreeing with this statement (34.54 versus 30.20 years; p=0.003; **Appendix D Supplementary Table A**). Additionally, men who were familiar with donor conceived children also agreed more with this statement (40.9% versus 25.6%; p=0.188; **Appendix D Supplementary Table A**). However, men who were familiar with other men who donated or wanted to donate sperm were less planning to keep their donor status a secret (11.1% versus 32.9%; p=0.086; **Appendix D Supplementary Table A**). In addition, these men also agreed more to the statement that the important people in their life would support their decision to donate sperm (94.4% versus 68.3%; p=0.037), as was also the case for men with a university/college degree (81.6% versus 64.0%; p=0.070) and men with a denominative religion (82.1% versus 67.2%; p=0.113) (**Appendix D Supplementary Table A**)

Motivation for donation

An overview of the motivations to donate is presented in **Table 2.2-IV**. Results are given for the total group of respondents and subdivided according to different characteristics of the candidate donor.

The main motivation for donation among our group of candidate sperm

donors was to help people to fulfil their child wish or sympathy with people having difficulties to conceive (96% and 90% respectively), followed by personal satisfaction (64%) and to test their semen quality (57.6%). The financial compensation related with sperm donation was an important motivational factor in 31% of the candidate sperm donors. Moreover, 48% of men would also be prepared to donate sperm if their expenses would not be reimbursed. Candidate donors who already had children of their own showed significantly less interest in knowing their semen quality (19.4% versus 75.0%; $p<0.001$) and the financial compensation (12.9% versus 39.1%; $p=0.010$) compared with men without children. This was also the case for men who were familiar with fertility problems of themselves or their partner (0.0% versus 59.4%; $p=0.073$ and 0.0% versus 32.0%; $p=0.550$ respectively; Supplementary Table B). Men who were familiar with men who donated or wanted to donate sperm seemed more interested in knowing their semen quality (77.8% versus 53.1%; $p=0.068$). Also, the men interested to know their semen quality were significantly younger compared with men indicating this motivation as neutral or (very) unimportant (29.45 versus 33.98 years; $p=0.001$; **Appendix D Supplementary Table B**). Moreover, wanting to know their semen quality was significantly related to the financial motivation: those with a financial motivation were much more interested in knowing their semen quality (76.6% versus 49.3%; $p=0.008$) (results not shown).

Table 2.2-II: Attitudes towards sperm donation (according to characteristics of the candidate donor)

	All respondents (N=100)[a]						Blood donor and/or stem cell or bone marrow donor		Children [b]		Relationship status	
	(Totally) agree		Neutral		(Totally) disagree		Yes	No	Yes	No	Yes	No
	n	%	n	%	n	%	%[c]	%[c]	%[c]	%[c]	%[c]	%[c]
Views on donor children												
I would like to know how many children were conceived with my sperm.	46	46.5	22	22.2	31	31.3	53.1	43.3	**29.0***	**54.4***	38.6	57.1
I would like information about the family in which the child would grow up.	21	21.0	28	28.0	51	51.0	24.2	19.4	9.7	26.1	15.5	28.6
I would like information about the children conceived with my sperm, without receiving their names.	27	27.0	20	20.0	53	53.0	33.3	23.9	16.1	31.9	**17.2***	**40.5***
Views on single women and lesbian couples												
I would be reluctant to donate to a lesbian couple.	3	3.0	4	4.0	93	93.0	0.0	4.5	0.0	4.3	3.4	2.4
I would be reluctant to donate to a single mother.	1	1.0	2	2.0	97	97.0	0.0	1.5	3.2	0.0	0.0	2.4
Disclosure to offspring												
Parents should be honest to their children about their genetic origin.	49	49.5	43	43.4	7	7.1	45.5	51.5	51.6	48.5	51.7	46.3
The parents should (be able to) decide whether or not they want to inform their child about his or her genetic origin.	62	62.6	28	28.3	9	9.1	60.6	63.6	51.6	67.6	61.4	64.3
Donor anonymity												
I would be prepared to give information about myself (e.g. physical appearance) to the children born from my donation, without giving them my name.	82	82.0	11	11.0	7	7.0	90.9	77.6	77.4	84.1	87.9	73.8

I would be prepared to donate if my name would be revealed to the children resulting from my donation.	26	26.0	19	19.0	55	55.0	36.4	20.9	**19.4**	**29.0**	20.7	33.3
I would be prepared to meet the children conceived with my sperm if they want that.	32	32.0	22	22.0	46	46.0	42.4	26.9	19.4	37.7	**20.7****	**47.6****
Children conceived with donated sperm should have the right to know their genetic origin.	43	43.4	28	28.3	28	28.3	48.5	40.9	35.5	47.1	**39.7**	**48.8**
Willingness to donate / to use donor sperm												
I think that many men are prepared to donate sperm.	22	22.0	41	41.0	37	37.0	15.2	25.4	19.4	23.2	25.9	16.7
If I would have fertility problems, I would be prepared to use donor sperm.	47	47.9	42	42.9	9	9.2	56.3	43.9	41.4	50.7	44.6	52.4
I would be prepared to donate even if my expenses would not be reimbursed.	48	48.0	23	23.0	29	29.0	57.6	43.3	58.1	43.5	48.3	47.6
Social aspects of sperm donation												
I think my (future) partner has the right to know that I am (was) a donor.	73	73.0	17	17.0	10	10.0	81.8	68.7	77.4	71.0	**81.0***	**61.9***
I am planning to keep my donor status a secret from everyone.	29	29.0	24	24.0	47	47.0	27.3	29.9	38.7	24.6	31.0	26.2
I think that the important people in my life, when they would be aware, would support my decision to donate sperm.	73	73.0	21	21.0	6	6.0	78.8	70.1	71.0	73.9	72.4	73.8

[a] Number of missing cases vary between 0-2 per variable.
[b] Only respondents who answered 'Yes, I have children of my own' on the question 'Do you have children?' (excluding those who answered 'My partner has children').
[c] Percentage of respondents answering 'agree' or 'totally agree' within each category (for instance those who answered 'yes' and those who answered 'no' on the question: 'Are you in a partner relationship?').
* Percentages expressing significant differences are printed in bold: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Table 2.2-III: Motivation to donate (according to characteristics of the candidate donor)

	All respondents (N=100)[a]						Blood donor and/or stem cell or bone marrow donor		Children [b]		Relationship status	
	(Very) important		Neutral		(Very) unimportant		Yes	No	Yes	No	Yes	No
	n	%	n	%	n	%	%[c]	%[c]	%[c]	%[c]	%[c]	%[c]
To reproduce	18	18.2	30	30.3	51	51.5	21.2	16.7	16.1	19.1	21.1	14.3
To know my semen quality	57	57.6	23	23.2	19	19.2	57.6	57.6	**19.4*** **	**75.0*** **	50.9	66.7
Because I sympathize with people who are having difficulties to conceive	90	90.0	7	7.0	3	3.0	87.9	91.0	96.8	87.0	89.7	90.5
Because I think it would give me a certain degree of satisfaction	64	64.0	25	25.0	11	11.0	66.7	62.7	58.1	66.7	56.9	73.8
Because I believe I have high quality genetic material	22	22.2	61	61.6	16	16.2	15.2	25.8	22.6	22.1	22.4	22.0
Because of the financial compensation	31	31.0	32	32.0	37	37.0	24.2	34.3	**12.9***	**39.1***	31.0	31.0
To help people to fulfil their child wish	96	96.0	3	3.0	1	1.0	97.0	95.5	100.0	94.2	93.1	100.0
Because I want to give something back for the fact that I used to be a patient in this center	1	1.1	20	21.1	74	77.9	0.0	1.5	0.0	1.5	0.0	2.5
Because I consider it my duty	16	16.3	43	43.9	39	39.8	21.9	13.6	12.9	17.9	20.7	10.0
Other [d]	9	100.0	0	0.0	0	0.0						

[a] Number of missing cases vary between 0-5 per variable.
[b] Only respondents who answered 'Yes, I have children of my own' on the question 'Do you have children?' (excluding those who answered 'My partner has children').
[c] Percentage of respondents answering 'agree' or 'totally agree' within each category (for instance those who answered 'yes' and those who answered 'no' on the question: 'Are you in a partner relationship?').
[d] Other: 'Because I know people with similar problems (X2); 'Why not: blood, plasma, semen,…'; 'Blood check'; 'Nature instinct'; 'My girlfriend does not want children'; 'Because I know what it means to be a 'parent''; 'Happiness for childless parents with a high child wish'; 'To help the LGBT community'.
* Percentages expressing significant differences are printed in bold: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

2.2.5 Discussion

This study aimed to give an overview of the motivations and attitudes of the current candidate sperm donor population. A total of 100 candidate donors applying to our center for a first semen analysis received a questionnaire that was filled in anonymously. Donors in our center received a sum of €60 per donated semen sample as compensation for their effort and time.

It has been claimed that with the abolition of donor anonymity, donor characteristics changed from students mainly motivated by financial gain towards older men with children, mainly motivated by altruism [55-57]. However, although all sperm donors in our center donated anonymously, the demographic characteristics of our candidate donor population did not match those of a mainly student based population. The mean age in our study group was 31.5 years, which fits better with the mean age of sperm donors in the non-anonymous (32.7 years) compared with the anonymous (27.5 years) donor group reported by Bay et al. [58]. Also, only about a quarter (24.5%) of our candidate donor population was aged ≤25 years. Furthermore, 58% of our candidate donors had a partner and 31% already had children of their own, which was also in accordance with previously published results from countries offering non-anonymous sperm donation [53, 58]. Candidate donors in our center were recruited in a non-university setting, which could also contribute to the lower proportion of students.

While this tendency towards an increasing group of older, married donors who already have children of their own is seen as a positive evolution, the attitudes regarding anonymity and possible contact with donor offspring in this group of men seems to contradict the aim of moving towards a system of open-identity gamete donation. While most men felt comfortable with disclosing non-identifying information, our results showed that married men or men with children of their own were less prepared to donate if their name would be revealed or to meet their donor conceived children in the future compared with single men without children. In addition, they were less interested in the number of children conceived with their sperm and did not want to receive information about the donor conceived child or the family it would grow up in. Also, men who were familiar with donor conceived children were less prepared to meet

their own donor conceived children in the future, possibly indicating they have experienced the impact such a meeting could have on the family.

Overall, only 26% of our candidate donor population was willing to continue donating non-anonymously. These results are in accordance with Ide et al. [54], who reported that 71.2% of Belgian candidate donors would not be willing to donate if anonymity could not be guaranteed. These results indicate that a change in law could have a huge impact on the recruitment of new sperm donors. However, as has been pointed out before [54, 55, 57], our results on attitudes towards anonymity could be biased since candidate donors in Belgium are currently being recruited in an anonymous system, evidently they would want to remain anonymous. In addition, Ide et al. [54] also asked if sperm donors would be willing to donate if a neutral institution would control the identity of sperm donors, being able to contact the sperm donor on request by the donor conceived child (e.g. for medical information) and whereby the donor would be free to respond or not. Surprisingly, 89% of the candidate donors would be willing to apply under those conditions. These results show that there is a possibility for creating more openness, without compromising on the availability of new sperm donors.

A systematic review by Van den Broeck et al. [57] revealed that media advertising is the most important way to recruit potential donors. Unfortunately, Belgian law does not allow advertisement for the recruitment of sperm donors. However, occasionally the media report on the shortage of donor sperm, which always leads to a sudden increase in applications from candidate donors (personal communications). Results from our study showed, in accordance with previous reports [9, 55, 59, 60], that the media play an important role in the recruitment of new sperm donors since the majority of our candidate donors reported this as their source of information. We therefore believe that it is important, prior to a change of law, to scrutinize the current population of potential sperm donors, for example through the use of media campaigns, and adapt current recruitment methods for sperm donors.

Motivations for donation in our candidate donor population were mainly altruistic in nature, in accordance with previous studies [9, 58]. This strong desire to help other people was also demonstrated by the fact that our candidate sperm donor group counted 33% of men who were also blood donor and/or

stem cell or bone marrow donor, while generally only 3% of the Belgian population regularly donates blood. Payment for donation was only an important motivational factor in 31% of the candidate donors and about half of them would also be willing to donate if their expenses would not be reimbursed. These results contradict findings by Bay et al. [58], who reported economic compensation as a motivational factor for 71% of their active donors in 2012. Moreover, only 14% of their donors were willing to continue donating without economic compensation [58]. Also in contrast with previous findings [55, 57, 59], we did not find any significant differences in age indicating that older potential donors (>25 years) were mainly driven by altruistic motives whereas younger donors (<25 years) were mainly interested in the financial compensation. Still, men with children showed significantly less interest in the financial compensation. Men interested in knowing their semen quality were significantly younger or had no children of their own, which seems plausible since their fertility had not yet been confirmed.

As this is the first study being performed on the motivation and attitudes of Belgian candidate sperm donors, results of this study could be of great importance to the clinics recruiting potential donors and to the law makers. We have shown that the majority of our current candidate donor population were older men, with a partner and children, who are donating sperm for altruistic reasons. They showed little interest in the outcome of their donation and most of them were not willing to donate non-anonymously. These results contradict previous reports on the characteristics of sperm donors recruited in an anonymous donor system. In order to recruit more candidate sperm donors in the future, it is important to be able to predict the response to certain rules and to adapt the recruitment methods accordingly.

Chapter 3

Optimal conditions for sperm storage

3.1 Temperature and sperm preparation

Influence of temperature and sperm preparation on the quality of spermatozoa

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original paper

Annelies THIJSSEN, Elke KLERKX, Carin HUYSER, Eugene BOSMANS, Rudi CAMPO, Willem OMBELET

Reprod Biomed Online, 2014, 28: 436-442

3.1.1 Abstract

This study investigated the effects of long term (24h) in-vitro sperm incubation at room temperature (RT; 23°C) versus testis temperature (35°C) on various sperm-quality parameters. Semen samples (n=41) were prepared both by density-gradient centrifugation (DGC) and the swim-up technique in order to compare the influence of sperm preparation on sperm quality after incubation. Progressive motility and morphology were significantly higher after incubation at RT compared with 35°C ($p<0.001$ and $p<0.01$, respectively). The proportions of acrosome-reacted, apoptotic and dead spermatozoa were significantly lower in samples incubated for 24h at RT compared with 35°C ($p<0.001$, $p=0.01$ and $p<0.001$, respectively). The number of motile, morphologically normal, non-acrosome-reacted and non-apoptotic spermatozoa recovered after sperm preparation was significantly higher in DGC compared with swim-up samples ($p<0.001$). However, spermatozoa prepared by swim-up showed better survival after incubation compared with DGC-prepared spermatozoa, especially when incubated at 35°C. In conclusion, this study indicates a significantly better and longer preservation of sperm quality when incubation is performed at RT. These findings may convince laboratories to change the routinely used sperm storage conditions in order to maximize the quality of the prepared sperm sample.

KEYWORDS: acrosome reaction, apoptosis, flow cytometry, sperm preparation, sperm quality, temperature

3.1.2 Introduction

Sperm quality is a very important factor in the IVF laboratory since male infertility accounts for 20-30% of the infertility cases [61] and treatment options are mainly based on sperm-quality improvement techniques [13]. The sperm quality parameters routinely used in the IVF laboratory are concentration, motility, viability and morphology [23, 62]. However, sperm-function assessments could additionally provide a valuable indication of sperm quality. Fertilization of an oocyte with an apoptotic spermatozoon has been shown to have detrimental effects on fertilization rate, implantation rate and embryo survival in assisted reproduction treatment [63]. Furthermore, occurrence of the acrosome reaction is essential to achieve fertilization in intrauterine insemination [64]. Both of these sperm-function parameters can be easily determined by use of flow cytometry; however this technique is not routinely available in the IVF laboratory.

The quality of the sperm sample is influenced by various laboratory factors, including: (i) use of different sperm preparation techniques [65-67]; (ii) temperature during sperm preparation [68, 69]; (iii) time interval from sperm preparation to IUI [70]; and (iv) temperature during long-term in-vitro incubation of prepared sperm samples [16, 71-73]. It is well known that the testis temperature is approximately 2-3°C below body temperature [13], as this is required for the production and maintenance of viable spermatozoa [14, 15]. Despite the numerous articles published on the harmful effects of long-term in-vitro sperm incubation at body temperature, it is still current practice in most IVF laboratories to store prepared sperm samples at this unfavorable temperature prior to their use in assisted reproduction treatment [72].

Therefore, this study aimed to examine the effects of long-term (24h) in-vitro sperm incubation at room temperature (RT; 23°C) versus testis temperature (35°C) on various sperm-quality parameters. In order to compare the influence of sperm preparation on sperm quality, native semen samples were split and prepared either by density-gradient centrifugation (DGC) or the swim-up technique. Conventional sperm-quality parameters such as concentration, progressive motility, normal morphology and viability [23] were taken into account. Additionally, sperm function was analyzed by determining

the proportions of spontaneously acrosome-reacted and apoptotic spermatozoa in the sample via flow cytometry.

3.1.3 Materials and methods

Collection of semen samples

Following a 2-7-day abstinence period, semen samples (n=41) were obtained through masturbation from patients presenting at the fertility center for an initial diagnostic semen analysis. Inclusion criteria were a sperm concentration of ≥15 million/ml and a motility of ≥32% progressively motile spermatozoa, according to World Health Organization (WHO) reference limits for normal semen samples [23]. Routine sperm analysis and sperm preparation were initiated after liquefaction at RT (23°C) and within 1h of production. Ethical approval of the study was granted by the ethics committee of Ziekenhuis Oost-Limburg (reference 13/055U, approved 31 May 2013).

Experimental design

Figure 3.1-1 gives a schematic overview of the experimental design. The native semen sample was split and one half of the sample was prepared by DGC while the other half was prepared by the swim-up technique. Aliquots of the DGC and swim-up prepared sperm samples were then incubated for 24h at RT or 35°C. Prior to incubation, samples were gassed with a gas mixture (6% CO_2, 5% O_2 and 89% N_2) for 90s in order to maintain the pH of the incubation medium. Samples incubated at 35±0.5°C were placed in a Labotect transport incubator (Cell-Trans 4016, Labor-Technik, Göttingen, Germany); while the RT samples were incubated on a bench in the laboratory (23±1°C). Although RT samples were exposed to light during incubation, in contrast to the samples incubated at 35°C, this should not have an influence on sperm quality [74]. For the final hour of the incubation period, the samples that were stored at RT were placed in the Labotect transport incubator in order to restore sample temperature to 35°C and obtain an equivalent motility count for both samples [75]. Analysis of sperm quality parameters, routine as well as flow cytometry measurements, was performed in duplicate on the native sample, after sperm preparation and after incubation.

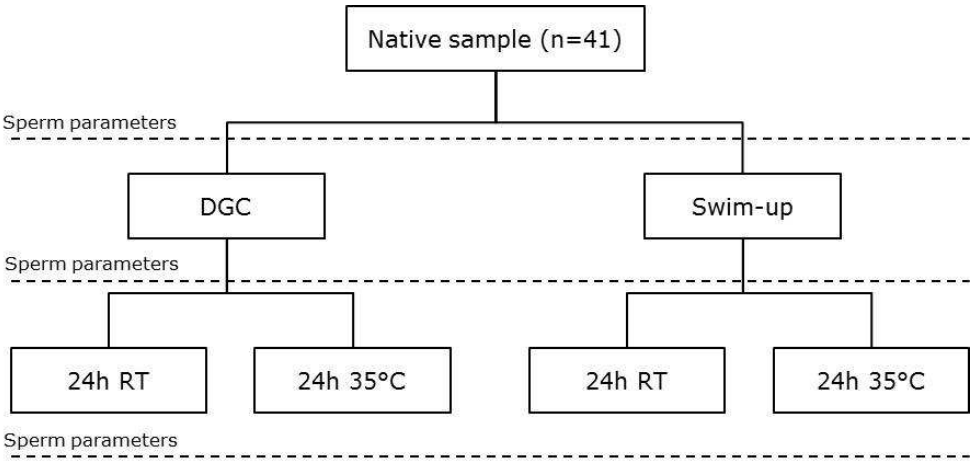

Figure 3.1-1: Schematic overview of the experimental design. DGC = density gradient centrifugation; RT = room temperature (23°C).

Sperm preparation

Earle's balanced salt solution (EBSS; E3024, Sigma; Origio, The Netherlands) was supplemented with sodium pyruvate (S8636; Sigma; Origio), penicillin–streptomycin 100x solution (Life Technologies, Invitrogen, Belgium) and 5% human serum albumin (HSA; Red Cross, Belgium) for use in sperm preparation [76]. A three-layer gradient (90%, 70%, 40%) was prepared by diluting PureSperm® 100 (Nidacon International; Origio) with supplemented EBSS [66]. The 90% gradient layer of 1.5 ml was layered with 1 ml of 70%, followed by 1 ml of 40% and a maximum of 1 ml of liquefied semen on top. This was centrifuged for 20 minutes at 310g. Spermatozoa were removed from the base of the 90% gradient layer and washed twice with 10 ml supplemented EBSS by centrifuging for 10 minutes at 350g. The final pellet (300µl) was resuspended in 0.7 ml supplemented EBSS medium.

For sperm swim-up, semen samples (0.5 ml) were placed under a 2-ml layer of supplemented EBSS medium [76]. The sample was incubated for 1h at 35±0.5°C at a 45° angle [69]. After 1h of incubation, the uppermost 1.5 ml of medium, containing highly motile sperm cells, was collected [76].

Routine sperm analysis

The laboratory has an accreditation according to the ISO15189 standards. Routine sperm analysis was performed by a single person and included concentration, motility, viability and morphology assessments, according to WHO guidelines [23, 77]. In order to evaluate sperm motility, the progressive movement of spermatozoa was graded A, B, C or D, with A+B motility representing the progressively motile sperm population [77]. Viability of the spermatozoa was estimated by assessing the membrane integrity of the cells using eosin dye exclusion [23]. An eosin B (Merck, Belgium) 0.5% (w/v) solution was added to a semen aliquot in a 1:1 ratio, mixed and left to stabilize for 30 s. The number of stained and unstained spermatozoa was counted under a microscope equipped with negative phase-contrast optics. Sperm morphology was scored by classifying spermatozoa as normal or abnormal according to strict criteria [78] after Papanicolaou staining [23, 34, 76, 79]. According to the World Health Organization (23) guidelines, 200 spermatozoa were counted per slide and in duplicate if a sufficient amount of spermatozoa was present. In total, 41 patients were evaluated. Therefore, group averages represented a count of approximately 16,400 spermatozoa per group (200 x 2 x 41).

Flow Cytometry

The general staining protocol from BD Biosciences was adapted in order to stain human spermatozoa for flow cytometry assessment of apoptosis (annexin V), acrosome reaction (CD46) [64] and viability status (7-aminoactinomycin; 7-AAD). A total of 100,000 spermatozoa were washed with 500 µl Dulbecco's Ca^{2+} Mg^{2+}-free phosphate-buffered saline (BioWhittaker, Lonza, Belgium). Cells were pelleted at 380g for 10 min, supernatant was then removed, and cells were resuspended in 100 µl 1x annexin-V Binding Buffer (BD Biosciences, Belgium). The annexin-V allophycocyanin (APC; BD Biosciences, Belgium) conjugate was 1:3 diluted with 1 x annexin-V-binding buffer. Subsequently, 5 µl of the 1:3 diluted annexin-V-allophycocyanin, 5 µl mouse anti-human CD46 fluorescein isothiocyanate (FITC; BD Biosciences), and 5 µl 7-AAD (BD Biosciences) were added for detection of apoptotic, acrosome-reacted and dead spermatozoa, respectively. Cells were then incubated for 15 min at RT in the dark. In a final step, cells were pelleted at 380g for 10 minutes, the supernatant was discarded

and cells were resuspended in 400 µl 1 x annexin-V-binding buffer prior to flow cytometry analysis (BD FACS Canto II).

The spermatozoa population was gated on the forward versus side scatter dot plot, in order to exclude the interference of debris and clumps during analysis. Fluorescence data were recorded for a minimum of 10,000 sperm cells using a high flow rate. The APC signal was obtained via the 633 nm excitation laser and the red fluorescence channel (650-670 nm filter range), whereas the FITC and 7-AAD signals were obtained via the 488 nm excitation laser and the green (515-545 nm filter range) and red (≥670 nm filter range) fluorescence channels, respectively. Compensation settings for the fluorochromes were performed by labelling cells with each fluorochrome separately and measuring the spectral overlap. Flow cytometry results were analyzed using BD FACS Diva software (version 6.3.1).

Statistics

For each parameter, normality of distribution was tested using the D'Agostino and Pearson omnibus normality test. Statistical analysis was performed using the paired sample t-test for normally distributed samples and the Wilcoxon signed rank test for matched pairs was used in case of an non-Gaussian distribution of the values. Statistical significance was established at $p<0.05$ and power calculations revealed an overall power of >85%. Statistical analysis was performed using Predictive Analytics SoftWare (PASW version 17.0 for Windows). Data were represented as box and whisker plots, whereby boxes depict the 25^{th} and 75^{th} percentiles with indication of the median value, and whiskers depict the 10^{th} and 90^{th} percentiles.

3.1.4 Results

Sperm-quality parameters after incubation at RT compared with 35°C

The motility of spermatozoa was significantly higher in DGC-prepared samples incubated at RT compared to 35°C ($p<0.001$, **Figure 3.1-2A**). Furthermore, in both DGC and swim-up samples, the proportion morphologically normal spermatozoa was significantly higher in samples incubated at RT compared with

35°C ($p<0.001$ and $p=0.004$, respectively, **Figure 3.1-2B**). For the DGC-prepared samples, the proportion of $CD46^+$ spermatozoa was significantly lower in samples incubated at RT compared with 35°C ($p<0.001$, **Figure 3.1-2C**). However, swim-up samples incubated at RT showed a significantly lower proportion of total annexin-V^+ spermatozoa compared with samples incubated at 35°C ($p=0.01$, **Figure 3.1-2D**). Finally, in both DGC and swim-up samples, the proportion of $eosin^+$ and 7-AAD^+ spermatozoa was significantly lower after incubation at RT compared with 35°C ($p<0.001$, **Figures 3.1-2E** and **F**).

DGC and swim-up sperm preparations for selection of improved-quality spermatozoa

Both DGC and swim-up yielded significantly higher proportions of motile and morphologically normal spermatozoa when compared with the native sample ($p<0.001$, **Figures 3.1-2A** and **B**). Furthermore, both techniques significantly decreased the proportion of $CD46^+$, annexin-V^+, $eosin^+$ and 7-AAD^+ spermatozoa in the sample ($p<0.001$, **Figures 3.1-2C**-**F**). DGC and swim-up selected equally for progressively motile spermatozoa. Additionally, there was no significant difference for the proportion of total $CD46^+$ cells between both sperm preparations. Swim-up samples contained significantly less total annexin-V^+ spermatozoa compared to the DGC ($p<0.001$, **Figure 3.1-2D**). Total $CD46^+$ and total annexin-V^+ spermatozoa represent the sum of the $CD46^+$/7-AAD^- and $CD46^+$/7-AAD^+, and annexin-V^+/7-AAD^- and annexin-V^+/7-AAD^+, subpopulations of spermatozoa, respectively. In addition, the proportions of $eosin^+$ and 7-AAD^+ spermatozoa were also significantly lower in swim-up samples compared with DGC ($p<0.001$, **Figures 3.1-2E** and **F**). On the other hand, DGC selected better for normal morphology than swim-up ($p=0.01$, **Figure 3.1-2B**).

Number of spermatozoa after incubation at RT compared with 35°C

Due to the significant difference in concentration between DGC and swim-up samples ($p<0.0001$, results not shown), data were also corrected for concentration to indicate the actual number of spermatozoa (in 10^6/ml) present in the samples.

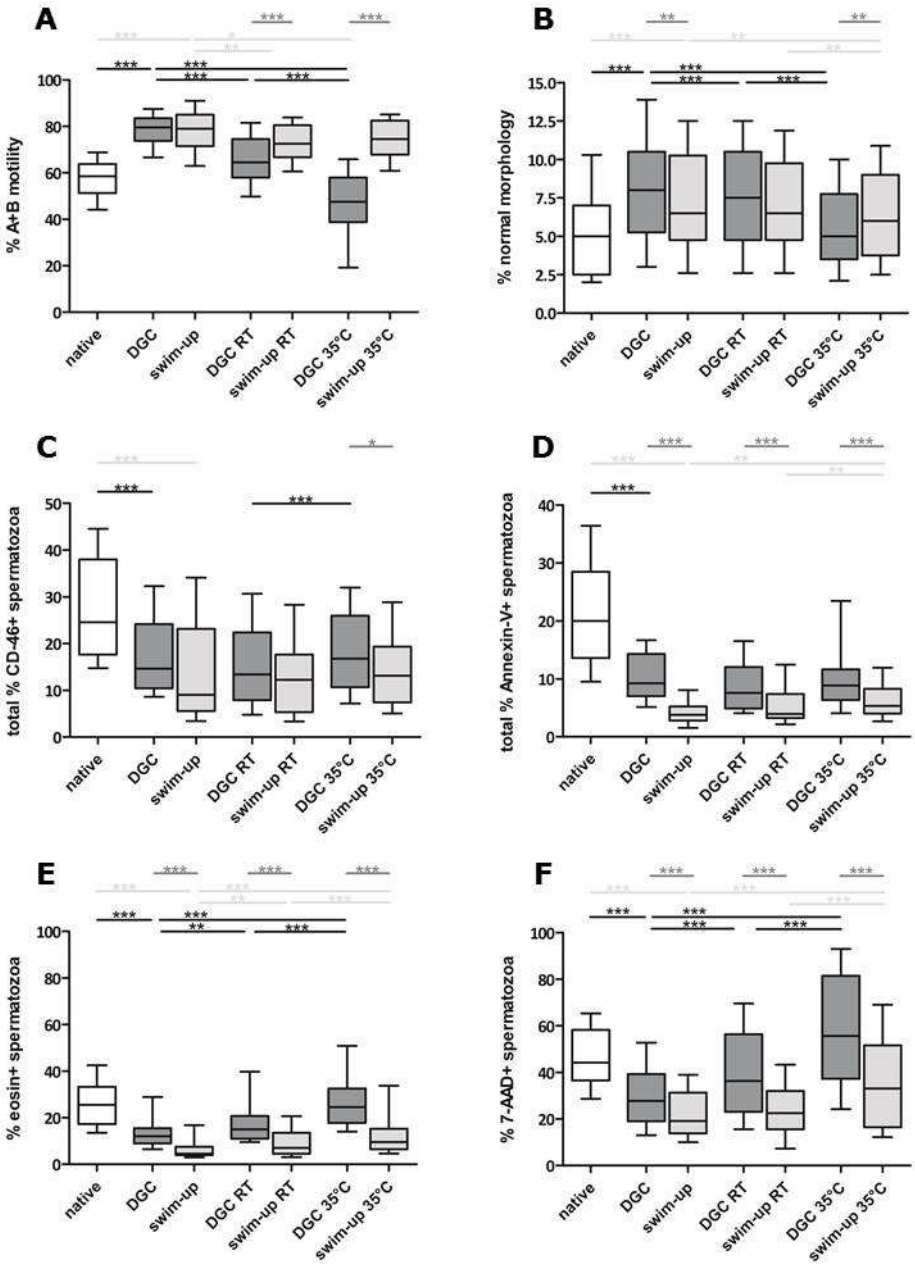

Figure 3.1-2: Sperm-quality parameters for DGC and swim-up samples after incubation at RT compared with 35°C: (A) Progressively motile spermatozoa (A + B motility); (B) normal morphology (strict criteria); (C) acrosome-reacted spermatozoa ($CD46^+$); (D) apoptotic spermatozoa (annexin-V^+); (E) dead spermatozoa ($eosin^+$); (F) dead spermatozoa (7-aminoactinomycin+; 7-AAD^+). Boxes depict the 25^{th} and 75^{th} percentiles

with indication of the median, and whiskers depict the 10th and 90th percentiles. White = native semen samples; light grey = swim-up samples; dark grey = DGC samples. *p<0.05, **p<0.01, ***p<0.001. DGC = density-gradient centrifugation; RT = room temperature (23°C).

Therefore, the number (No) of motile, morphologically normal and CD-46$^-$/Annexin-V$^-$ spermatozoa was significantly higher in DGC compared to SU prepared samples (p<0.001, **Figure 3.1-3**). A calculation was performed by multiplying the concentration of the sperm sample with the proportion of motile spermatozoa, proportion of normal morphology, proportion of non-acrosome-reacted spermatozoa and the proportion of non-apoptotic spermatozoa in the samples in order to become an overview of the 'ideal' spermatozoa population. This showed that the number of motile, morphologically normal, CD46$^-$/annexin-V$^-$ spermatozoa was significantly higher in DGC compared with swim-up samples (p<0.001, **Figure 3.1-3**). Furthermore, the number of motile, morphologically normal CD-46$^-$/annexin-V$^-$ spermatozoa in DGC-prepared samples showed such a strong decline that the significant difference between DGC and swim-up samples still seen after incubation at RT (p<0.001) was completely lost when samples were incubated at 35°C (p=0.304, **Figure 3.1-3**).

3.1.5 Discussion

The purpose of this study was to investigate the changes in sperm-quality parameters after long-term (24h) in-vitro sperm incubation at RT (23°C) versus testis temperature (35°C). Parameters that were taken into account to define a high-quality sample were concentration, progressive motility, normal morphology, viability, acrosome reaction and the presence of apoptotic spermatozoa in the sample. A total of 41 semen samples, with a sperm concentration of ≥15 million/ml and progressive motility of ≥32% [23], were analyzed.

The results presented in this study showed a significantly better and longer preservation of sperm quality when samples were incubated at RT compared with 35°C. This was in accordance with results proposed by Schuffner et al. [80], who reported a significant loss of motility and an increased

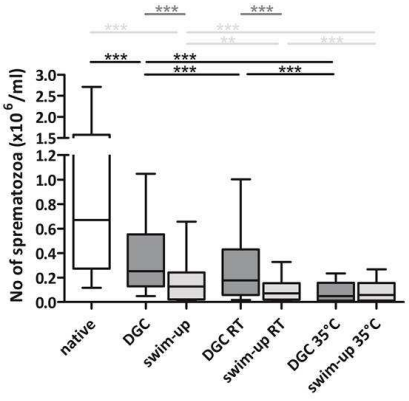

Figure 3.1-3: Number of motile, morphologically normal, CD46$^-$/annexin-V$^-$ spermatozoa in DGC and swim-up samples after incubation at RT compared with 35°C. Boxes depict the 25th and 75th percentiles with indication of the median, and whiskers depict the 10th and 90th percentiles. White = native semen samples; light grey = swim-up samples; dark grey = DGC samples. **p<0.01, ***p<0.001. DGC = density-gradient centrifugation; RT = room temperature (23°C).

incidence of apoptosis after 24h incubation at 37°C. Furthermore, Aitken et al. [16] showed a significant decline in the motility of spermatozoa after incubation at ambient temperatures (i.e. 22°C), although incubation had no effect on the viability of the spermatozoa or their potential to undergo the acrosome reaction. It is assumed that when spermatozoa are incubated at lower temperatures, they adopt a resting state, which allows them to preserve their energy. In 2009, Gallup proposed 'the activation hypothesis' as a mechanism of spermatozoa capacitation *in vivo*. He postulated that the rise in temperature when spermatozoa enter the female reproductive tract could act as a trigger for the activation of sperm, making them hyperactive [81]. This hypothesis possibly explains the diminished survival of sperm at 37°C compared with lower temperatures. Finally, in addition to the previous sperm parameters, morphology of the spermatozoa also decreased significantly after incubation and especially after incubation at 35°C. The change in morphological characteristics has not been recorded during this study. However, an indication for this was found in a study by Peer et al. [82], who reported that in-vitro incubation of sperm samples for ≥2h at 37°C caused the appearance of large nuclear

vacuoles, an effect which was not seen after incubation at 21°C.

Comparison between DGC and swim-up samples after incubation showed a superior preservation of sperm quality in swim-up samples. A possible explanation could be that the centrifugation steps performed during DGC sperm preparation rendered the spermatozoa more vulnerable to certain incubation conditions compared with the more natural selection of spermatozoa by the swim-up technique. Additionally, the higher concentration of spermatozoa, and therefore the higher number of dead sperm cells, in DGC-prepared samples compared with swim-up could have resulted in greater amounts of reactive oxygen species present in DGC-prepared samples. High reactive oxygen species production may induce peroxidative damage and a loss of sperm function [23, 83]. DNA damage in both the nuclear and mitochondrial genomes may also be generated, leading to a more rapid decline in sperm viability. Therefore, when sperm samples are to be incubated in-vitro for an extended period of time (i.e. 24h) it is recommended to use the swim-up technique for preparation of the sample.

The results indicated that both preparation techniques provided a significantly better sperm population compared to the native sample. However, no solid conclusion could be drawn whether one technique provided a qualitatively better sperm population compared to the other. Firstly, both techniques selected equally for progressively motile spermatozoa and DGC-prepared samples showed higher proportions of morphologically normal spermatozoa compared with swim-up. This was in contrast to results presented by Evliyaoglu et al. [84] and Ng et al. [85], who demonstrated a higher proportion of progressively motile spermatozoa in Percoll-selected samples and more morphologically normal spermatozoa in swim-up compared with Percoll-selected samples. Secondly, in accordance with the results presented by Evliyaoglu et al. [84] and Ng et al. [85], DGC yielded a significantly higher amount of spermatozoa compared with swim-up, whereby swim-up-selected samples showed a tendency towards a higher proportion of intact acrosomes. Thirdly, swim-up preparations also resulted in a significantly higher proportion of non-apoptotic and viable spermatozoa in the sample compared with DGC. The swim-up technique is a low-cost procedure and is less time consuming in comparison to DGC. However, selection of spermatozoa by swim-up is based on

motility and consequently dependent on a sufficient number of motile spermatozoa in the sample. Therefore, in assisted reproduction treatment it is usually recommended to use DGC as it can also be modulated to prepare severe quality sperm samples [86].

Various reports [73, 87, 88] showed no significant changes in sperm parameters evaluated after 4-6h of incubation at both room and body temperatures. However, sperm parameters did change significantly after 24h of storage in all three investigations. Furthermore, long-term in-vitro incubation of spermatozoa could serve multiple purposes. Firstly, according to Eskandar [89], zero 24h sperm motility is related to a lower fertilization rate and a higher incidence of failed fertilization. Secondly, preservation of sperm quality for up to 24h could be used for in-vitro maturation of immature metaphase I oocytes for intracytoplasmic sperm injection to be performed the following day [90]. Thirdly, Aitken et al. [16] suggested 24h storage of sperm samples at ambient temperature (i.e. 22°C) to allow transportation to a centralized, accredited, diagnostic laboratory in order to standardize laboratory assessments of semen quality in the context of multicenter clinical trials.

In conclusion, results of this study show a significantly better preservation of sperm quality when samples are incubated for 24h at RT compared with 35°C. However, further investigation is needed to confirm whether these results would also translate into an improvement in pregnancy rates if prepared sperm samples were to be stored at RT before their use in assisted reproduction treatment. Currently, a study is being performed in this study center whereby prepared sperm samples are alternately incubated at RT or 37±0.5°C before their use in IUI. Finally, the findings presented in this study may convince laboratories to change the routinely used sperm storage conditions in order to maximize the quality of the prepared sperm sample.

3.2 Sperm freezing

Cryopreservation of spermatozoa by means of small and large volume vitrification compared with conventional slow freezing: the effects on post-thaw sperm quality

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original paper

Annelies THIJSSEN, Thomas NELISSEN, Eva VANDORMAEL, Eugene BOSMANS, Ingrid VAN DER AUWERA, Willem OMBELET

Submitted to J Assist Reprod Genet

3.2.1 Abstract

This study aimed to compare two methods of cryoprotectant-free vitrification, i.e. large volume vitrification and small volume vitrification, with conventional slow freezing. Forty-one semen samples were prepared by density gradient centrifugation (DGC) with PureSperm®, divided into three aliquots and cryopreserved by conventional slow freezing, large volume (400µl) vitrification and small volume (40µl) vitrification respectively. Inclusion criteria were based on WHO reference limits for a normal semen sample: a concentration of ≥15 million/ml and a good forward progressive motility of ≥32%. Sperm quality parameters were analyzed after DGC prior to freezing and after thawing of the spermatozoa. The routine parameters sperm motility and morphology were taken into account, as well as acrosome reaction, apoptosis and dead spermatozoa analyzed by flow cytometry. Compared with conventional slow freezing, small volume vitrification resulted in a significantly higher percentage of post-thaw progressive sperm motility ($p<0,05$). Additionally, the percentages of living non-apoptotic or non-acrosome reacted spermatozoa were not significantly different in small volume vitrification compared with slow freezing ($p>0,05$). With further research, small volume vitrification of spermatozoa could provide interesting options for future IVF lab practices, as it has previously been described as a simpler, faster and more cost-effective technique than conventional slow freezing.

KEYWORDS: cryopreservation, cyroprotectant-free, slow freezing, sperm, vitrification

3.2.2 Introduction

Cryopreservation is a widely used method for preserving a wide range of cell types and tissues, including male gametes [91]. This technique aims to preserve the physiological and reproductive functions of the spermatozoa, thereby making long-term storage without the loss of viability possible [92]. It has become standard practice in assisted reproductive technologies (ART) in order to preserve male fertility before radiotherapy and/or chemotherapy, e.g. childhood cancer [17, 91, 93]. Furthermore, cryopreservation also finds its use in surgical sperm retrieval in cases of azoospermia, thereby avoiding the need for a repeat biopsy or aspiration [17]. In other applications, like donor insemination programs, cryopreservation is used to allow time to screen the male donors for contaminations, such as human immunodeficiency virus (HIV), hepatitis B and C virus and other common infectious diseases, all before the cryopreserved sperm sample is used in clinical applications [17, 18]. In this study, we will focus on the freezing of donor sperm in a donor insemination program.

Despite extensive research on selecting the optimal methods for sperm freezing, sperm motility is still significantly reduced after thawing and shows a wide inter-individual variability [91]. The technique that is most commonly used for the cryopreservation of spermatozoa is the conventional slow freezing method. This technique uses a progressive cooling procedure prior to preserving the cells. However, the slow solidification process of living cells is accompanied by the formation of intracellular and extracellular ice crystals during cooling. In order to protect the spermatozoa from the damaging ice formation, permeable cryoprotective agents (CPAs) are used, most commonly dimethylsulfoxide (DMSO), ethylene glycerol (EG), propylene glycerol and glycerol. These mixtures partially penetrate and solubilize the plasma membrane so the cells are less prone to get punctured, while also interrupting the lattice of ice and consequently suppressing the formation of crystals [94]. Although they provide cryoprotection, spermatozoa have shown low tolerance levels for high concentrations of CPA's [17, 18, 91, 93].

In order to shorten the freezing procedure and eliminate the cost of expensive programmable freezing equipment, vitrification was introduced [94]. With the vitrification technique, also known as “snap freezing”, cryopreservation

is achieved by directly freezing the cells in liquid nitrogen, causing a rapid cooling rate and thus avoiding the formation of damaging ice crystals [91, 95]. In contrast to conventional slow freezing, this method does not rely as much on the usage of CPAs, which in vitrification can be replaced by a protein and carbohydrate solution, typically a mixture of human serum albumin (HSA) and sucrose [92, 95, 96]. These non-permeating cryoprotective solutions mainly act as dehydration promotors prior to freezing, while also creating a stable osmotic gradient over the cell membrane and keeping the pH of the semen sample constant.

A variation of methods has been proposed for the vitrification of spermatozoa, differing in freezing volume, cooling procedure, use of recipients and thawing temperature. At first, only small volumes of spermatozoa (1-40µl) could be vitrified [97]. Later however, larger volumes of up to 500µl also proved possible [92, 98]. Vitrification typically involves directly plunging the spermatozoa suspension in liquid nitrogen, with or without the use of copper loops or plastic capillaries [91, 96]. Others make use of solid surface vitrification, using a precooled aluminum block [17, 99]. After freezing, spermatozoa may be stored in either cryovials or straws [92, 93]. Another important step in the vitrification protocol is the thawing temperature of the cryopreserved spermatozoa, since a rapid freezing process needs to be accompanied by a sufficient rapid thawing. Many thawing temperatures have been proposed for vitrification, ranging from 37°C to 42°C [91, 92]. Mansilla et al. [100], assessed the viability of spermatozoa thawed at distinct temperatures. The study concluded that a thawing temperature of 42°C was needed to optimally preserve the sperm's physiological parameters.

The present study aimed to individually compare two different methods of sperm vitrification, i.e. large volume vitrification and small volume vitrification, with the conventional slow freezing method. Both vitrification techniques varied in freezing volume, sperm concentration, cooling procedure and thawing temperature. Sperm quality parameters analyzed were sperm motility and morphology as well as additional flow cytometry measurements for viability, acrosome reaction and apoptotic rate.

3.2.3 Materials and Methods

Institutional review board approval for the study was granted by the ethics committee of Ziekenhuis Oost-Limburg and Hasselt University (reference 15/017U, approved 17 April 2015). Informed consent was received from all participants.

Collection of semen samples

Semen samples (n=41) were collected from male patients visiting the fertility center for an initial diagnostic semen analysis. Samples were obtained through masturbation following a 2-5-day abstinence period. Routine sperm analysis and sperm preparation were initiated within 1 hour of production and after liquefaction at room temperature (RT; 23°C). Inclusion criteria for the study were based on WHO reference limits for a normal semen sample: a concentration of ≥15 million/ml and a good forward progressive motility of ≥32% [23].

Sperm preparation

Native semen samples were capacitated via density gradient centrifugation (DGC) with PureSperm® (PureSperm® 100, Nidacon). A two layer gradient of 80% and 40% was used, according to the manufacturer's instructions, by diluting PureSperm® 100 with Earle's balanced salt solution (EBSS; E3024, Sigma), supplemented with sodium pyruvate (S8636; Sigma), penicillin–streptomycin 100 x solution (Life Technologies, Invitrogen) and 5% human serum albumin (HSA; Red Cross) [76, 101]. Briefly, 2 ml of 80% PureSperm® was layered with 2 ml of 40% PureSperm® and a maximum of 1,5 ml of liquefied semen on top. The gradient solution was centrifuged for 20 minutes at 300g. Afterwards, the spermatozoa were aspirated from the pellet (lowest 0.5 ml base of the tube) and washed twice with 10 ml supplemented EBSS (E3024, Sigma) by centrifuging for 10 minutes at 500g. The resulting pellet of spermatozoa was resuspended in supplemented EBSS to a final volume of 1 ml.

Experimental design

After routine sperm analysis and sperm preparation, aliquots of the DGC prepared sperm samples were split and cryopreserved for a minimum of 24 hours according to three different cryopreservation protocols: (i) conventional slow freezing, (ii) large volume vitrification and (iii) small volume vitrification. Sperm quality parameters were determined on the DGC prepared sperm sample before freezing and after thawing and included the routine parameters sperm motility and morphology as well as additional flow cytometry measurements for viability, acrosome reaction and apoptotic rate.

Conventional slow freezing is the standard cryopreservation protocol used in our laboratory. Sperm CryoProtec II™ (Nidacon) is used as a CPA and cryopreservation is performed according to the manufacturer's instructions. The alternative cryopreservation technique of large volume vitrification varied from conventional slow freezing in cooling rate, thawing rate and usage of CPA. The protocol was based on the research by Slabbert et al. [92] and Satirapod et al. [99]. Identical to the conventional slow freezing method, cryostraws of 400 µl volume (CBS^{TM} High security sperm straw 0.4 ml, Cryo Bio System) were used. The protocol by Slabbert et al. [92] suggested to plunge the straws into the liquid nitrogen. However, preliminary results after applying this technique in our laboratory concluded that nearly 100% of the post thaw spermatozoa died in the process, probably due to the described freezing step. We decided to focus on an alternative cooling step, the solid surface vitrification technique, as explored in the research done by Satirapod et al. [99], while still maintaining the same sample volume, freezing medium and thawing temperature from the protocol by Slabbert et al. [92]. The third cryopreservation method of small volume vitrification differed from conventional slow freezing in cooling rate, thawing rate, the freezing medium used and the freezing volume of the sample. The protocol for this cryopreservation technique was based on the research by Slabbert et al. [92] and Isachenko et al. [97]. Small droplets of 40µl sperm sample were vitrified on a precooled aluminum surface in liquid nitrogen. The freezing medium used in this technique was the same as used in Slabbert et al. [92].

Sperm cryopreservation techniques

Conventional slow freezing

The cryopreservation suspension was made by gently adding 1 part of Sperm CryoProtec II™ (Nidacon) to 3 parts of the DGC prepared sperm sample. Via a syringe, the prepared suspension was aspirated into a 400 µl straw (CBS™ High security sperm straw 0.4 ml, Cryo Bio System), with a noticeable air bubble present to prevent rupturing when immersed into LN_2. Both ends of the straw were sealed using a heat sealer (Cryo Bio System). The straw was equilibrated and cooled in the fridge for 60 minutes at 4-5°C. Afterwards, the straw was horizontally placed ±2 cm above the liquid nitrogen (LN_2), in the LN_2 vapor, and incubated for another 30 minutes. Finally, the straw was quickly transferred to LN_2 and stored for a minimum of 24 hours in a LN_2 reservoir. After the incubation period, the straw was placed in a warm water bath at 37°C for 30 seconds. Next, the straw was whipped, cut open at two ends and the semen sample content collected in a falcon tube. The sample was resuspended in 5mL PureSperm® Wash (PureSperm® Wash, Nidacon) and centrifuged at 500g for 10 minutes. After centrifugation, the obtained pellet was again resuspended in 1ml PureSperm® Wash.

Large volume vitrification

As with the conventional slow freezing protocol, large volume vitrified samples were frozen in 400 µl straws (CBS™ High security sperm straw 0.4 ml, Cryo Bio System). The capacitated sperm sample was diluted in a 1:1 ratio with a solution of distilled water, supplemented with 0.5M sucrose and 1% HSA (Red Cross), as described by Slabbert et al. [92]. The prepared suspension was aspirated into a 400 µl straw (CBS™ High security sperm straw 0.4 ml, Cryo Bio System) and both ends were sealed using a heat sealer (Cryo Bio System). The straw was left at RT for 10 to 15 minutes in order for the sperm cells to partially dehydrate to prevent bursting of the cells during cooling. In a following step, the straw was frozen via solid surface vitrification by placing the straw horizontally on a metal plate, precooled at -196°C, with the surface sticking out of the LN_2. After 5-10 minutes, the fully frozen straws were transferred to LN_2 and stored for a minimum of 24 hours in the LN_2 reservoir [99]. After the incubation period,

individual straws were quickly thawed in a warm water bath at 42°C for approximately 15 to 20 seconds [92]. Similarly to the conventional slow freezing thawing procedure, each straw was whipped, cut open at two ends and the sperm sample content collected. The sample was resuspended in 5mL PureSperm® Wash (Nidacon) and centrifuged at 500g for 10 min, supernatant was removed and the pellet was resuspended in 1ml PureSperm® Wash.

Small volume vitrification

Small volume vitrification involved freezing of spermatozoa in 40 µl drops. Preliminary testing results showed a good post-thaw survival of the spermatozoa in semen samples with an initial 2:1 dilution. Therefore, samples were prepared by diluting 300 µl of the capacitated semen sample with 150 µl of distilled water, supplemented with 0.5M sucrose and 1% HSA (Red Cross) [92]. Consequently, the sample had a volume of 450 µl. In order to keep the prepared sample volumes consistent along the three cryopreservation methods, only 400µl was aspirated and used for vitrification. The prepared samples were again left at RT for 10 to 15 minutes. In a following step, each 400 µl sample was frozen via solid surface vitrification by pipetting 10 separate droplets of 40 µl on an aluminum container (bowl aluminum 133 ml, Sterisets®), precooled at -196°C and floating on the LN_2 surface [97]. Each small volume droplet froze instantly upon touching the aluminum surface. Afterwards, all droplets were transferred to cryovials (1,8 ml Nunc® Cryotubes®) and stored for a minimum of 24 hours in the LN_2 reservoir. For thawing, the frozen sample droplets were directly transferred to an individual falcon tube, filled with 5 ml PureSperm® Wash (Nidacon) at 37°C [97]. The falcon tube was gently shaken to maintain a fast and steady thawing process. Since all droplets had a small volume of 40 µl, the thawing occurred instantaneous. Afterwards, all sperm samples were centrifuged (fixed angled rotor, EBA20, Hettich Zentrifugen) at 500g for 10 min, supernatant was removed and the pellet resuspended in 1 ml PureSperm® Wash.

Sperm quality analysis

Sperm motility was evaluated according to WHO (2010) [23] guidelines and involved counting the percentages of progressively motile, non-progressively motile and immotile spermatozoa in the sample. Sperm morphology was scored

after Papanicolaou staining [23, 34, 79] by classifying spermatozoa as normal or abnormal according to strict criteria [78]. Flow cytometry analysis of sperm viability, acrosome reaction and apoptotic rate was performed by staining the cells with 7-aminoactinomycin (7-AAD), CD46 and Annexin V respectively and measuring fluorescence on the BD FACS Canto II with BD FACS Diva software (version 6.3.1), according to the protocol described by Thijssen et al. [101].

Statistical analysis

Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS Statistics version 22.0 for Windows). Each individual parameter was tested for normality of distribution using the D'Agostino and Pearson omnibus normality test. Paired sample t-test was used for Gaussian distributed samples and the Wilcoxon signed rank test for matched pairs was used in case of a non-Gaussian distribution of the values. The level of statistical significance was set at $p<0.05$. Data were represented as box and whisker plots, whereby boxes depict the 25^{th} and 75^{th} percentiles with indication of the median value, and whiskers depict the 10^{th} and 90^{th} percentiles.

3.2.4 Results

Cryopreservation of spermatozoa significantly reduces sperm quality

The percentage progressive motility and normal morphology of the spermatozoa was significantly reduced after sperm freezing and thawing in all three cryopreservation techniques compared with the original DGC prepared sperm sample ($p<0,0001$ and $p<0,05$ respectively). In addition, the percentages acrosome reacted ($CD46^+$), apoptotic (Annexin-V^+) and dead (7-AAD^+) spermatozoa in the samples all significantly increased as a result of sperm cryopreservation ($p<0,0001$). Results are presented in **Figure 3.2-1**.

Small volume vitrification leads to a higher preservation of sperm motility after thawing

Progressive motility of the spermatozoa was significantly lower after large volume vitrification of the spermatozoa compared with slow freezing ($p<0,0001$; **Figure 3.2-1A**). However, small volume vitrification did preserve sperm motility

significantly better than the conventional slow freezing technique ($p<0,05$; **Figure 3.2-1A**). Furthermore, none of the three cryopreservation techniques differed significantly in the percentage of normal morphology spermatozoa ($p>0,05$; **Figure 3.2-1B**).

As for the flow cytometry parameters, both vitrification techniques resulted in a significantly higher percentage of acrosome reacted spermatozoa compared with slow freezing ($p<0,0001$; **Figure 3.2-1C**). On the other hand, there was no significant difference between slow freezing and vitrification in terms of the percentage apoptotic spermatozoa in the samples. However, small volume vitrification showed to result in a significantly lower percentage of apoptotic spermatozoa compared with large volume vitrification ($p<0,05$; **Figure 3.2-1D**). The percentage dead spermatozoa in the samples was significantly increased when large volume vitrification was used compared with slow freezing ($p<0,01$; **Figure 3.2-1E**), but not with small volume vitrification. In addition, small volume vitrification resulted in a significantly lower percentage of dead spermatozoa compared with large volume vitrification ($p<0,05$; **Figure 3.2-1E**).

When looking at the population of good quality spermatozoa, i.e. $CD46^-$/7-AAD^- and Annexin-V^-/7-AAD^- spermatozoa, large volume vitrification significantly reduced sperm quality compared with slow freezing ($p<0,0001$; **Figure 3.2-1F-G**) but results with small volume vitrification were not significantly different.

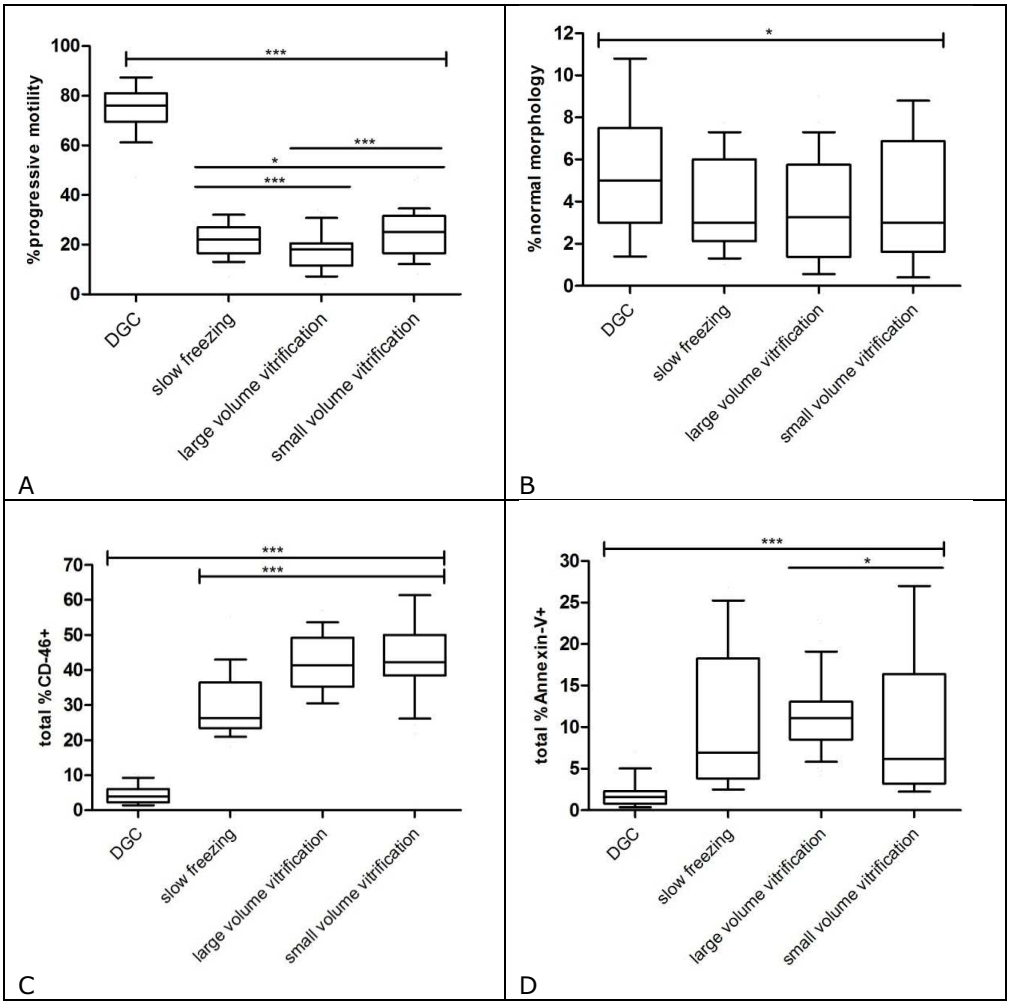
3.2.5 Discussion

The study aimed to compare two different methods of vitrification with the conventional slow freezing method currently used in our laboratory on their ability to preserve sperm quality after freezing and thawing. Both vitrification techniques were based on preliminary research done by Isachenko et al. [97], Slabbert et al. [92] and Satirapod et al. [99], and could be largely classified according to cryopreservation volume. Sperm quality before and after freezing was assessed via routine sperm quality parameters progressive motility and morphology and flow cytometry measurements of acrosome reaction, apoptosis and cell death.

All cryopreservation methods showed a significant decrease in post-thaw progressive sperm motility. Earlier conclusions on motility by Watson [102],

Donnelly et al. [103] and Simon and Lewis [104] stated this parameter to be the most heavily affected by freezing, while also being a strong indicator of a sample to achieve fertilization. At present, however, it is still unclear exactly how the mechanism of motility is affected by freezing, as the decrease might be mechanical or due to a physical-chemical nature [91].

Previous study results on sperm vitrification indicated that the cryopreserved volume in combination with the applied CPA played an important role in preserving post-thaw progressive sperm motility [91, 92, 94, 96]. Our protocol for large volume vitrification was largely based on the research done by Slabbert et al. [92]. But although they concluded no significant differences could be found between post-thaw progressive motility after freezing by means of vitrification compared with conventional slow freezing, our study results contradicted these findings and observed a significantly higher decrease of progressive motility after large volume vitrification compared with conventional slow freezing. It is important to note that although both studies had similar cryopreservation protocols, they were not identical in freezing volume, freezing step and conventional slow freezing protocol. Firstly, the study by Slabbert et al. [92] was performed using a 300 µl sperm suspension, while our large volume vitrification protocol used a freezing volume of 400 µl. This was done for practical implications and general convenience in order to fully compare it to the routinely performed conventional slow freezing method in our laboratory. However, preliminary vitrification results from Slabbert et al. [92] stated that no significant differences in sperm quality parameters were observed between 300 µl and 500 µl sperm suspensions, suggesting that a larger volume of 400 µl should not have influenced the post-thaw sperm quality parameters. Secondly, we compared the large volume vitrification technique with the conventional slow freezing technique currently used in our laboratory. The protocol described by Slabbert et al. [92] used TYB (Test yolk buffer; Irvine Scientific®, Santa Ana, CA, USA) as a permeable CPA. The study added TYB in a 1:1 ratio to the washed spermatozoa, while our study opted for a 1:3 ratio of Sperm CryoProtec II™ (Sperm CryoProtec II™, Nidacon) as prescribed by the manufacturer. Lastly, solid surface vitrification was used as an alternative cooling step in our large volume vitrification protocol. This was in contrast to the cooling step described in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DGC
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large volume vitrification
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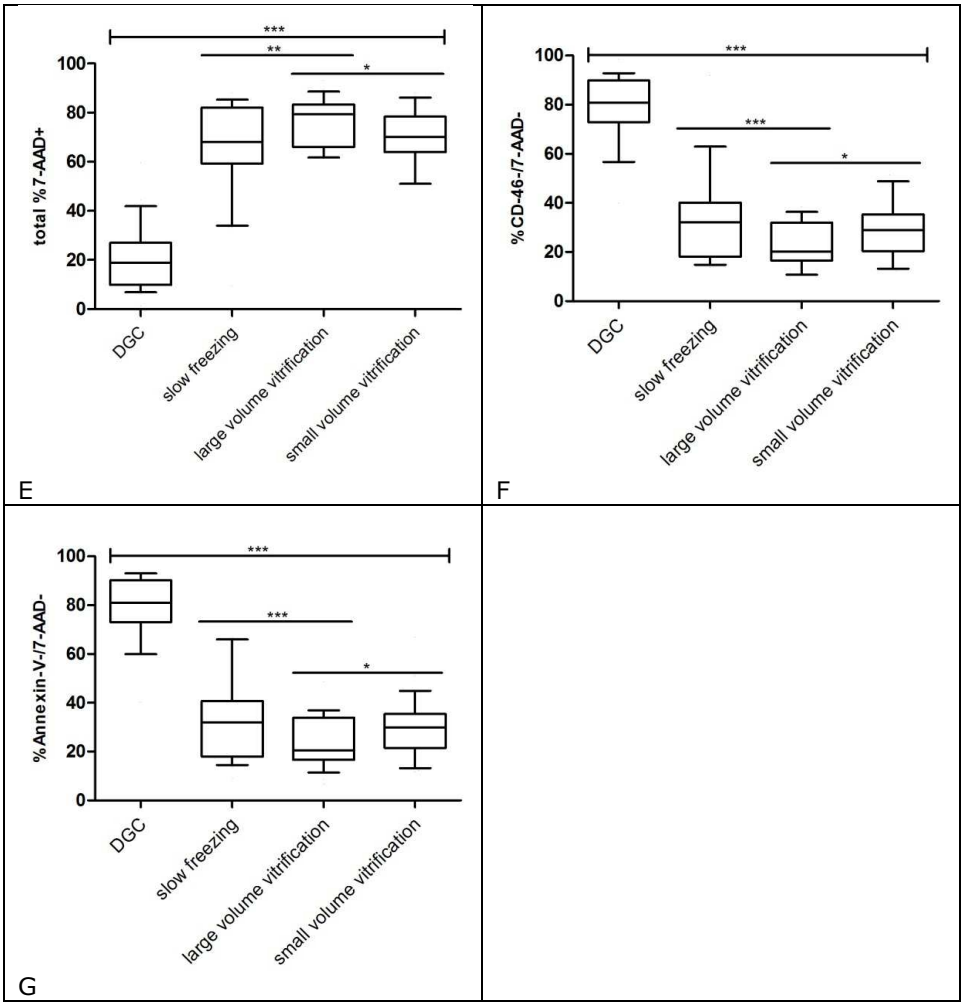

Figure 3.2-1: Sperm quality parameters before and after freezing/thawing of spermatozoa

|—| significant difference between the first condition compared with each of the following conditions

—— significant difference between two indicated conditions

Boxes depict the 25th and 75th percentiles with indication of the median, and whiskers depict the 10th and 90th percentiles. * $p < 0,05$; ** $p < 0,01$; *** $p < 0,0001$. 7-AAD: marker for cell death; Annexin-V: marker for apoptosis; CD-46: marker for acrosome reaction; DGC: density gradient centrifugation.

the study by Slabbert et al. [92], where straws were vitrified by directly plunging them into LN_2. However, after multiple attempts, our preliminary research concluded that due to a boiling effect on LN_2 contact, the suspension was unable to get to a glassy vitrified state and instantly turned white, demonstrating ice crystal formation. Subsequently, nearly 100% of the post-thaw spermatozoa died during the cooling process. We therefore based the cooling step on the research done by Satirapod et al. [99] and Vutyavanich et al. [17] on solid surface vitrification. This technique had a higher percentage of post-thaw spermatozoa survival, because a precooled metal surface is a more efficient method of heat transfer [99, 105]. As explained in the study by Dinnyes et al. [105], the LN_2 liquid boils when it comes in contact with a warm object, temporarily creating an isolating LN_2 vapor around the object that lowers the cooling rate. A precooled metal surface will eliminate the insulation effect.

Although this solid surface vitrification technique can be considered a more successful approach, the post-thaw progressive motility of the large volume vitrified samples was still significantly lower than the conventional slow freezing samples. A possible explanation for this difference was the usage of a larger freezing volume. Vitrification depends on three important factors to consistently achieve a glass-like solidification: high cooling rates, high viscosity of the cryopreservation medium and a minimal freezing volume [106]. The vitrification solution should be kept at a minimum such that the duration of the solidification of the liquid phase is reduced and most of the specimen will be immersed in LN_2, leading to higher cooling rates [91, 94]. Our study results suggested that a large sperm suspension of 400 µl complicated the vitrification procedure by possibly altering this initial cooling rate. Additionally, this also allowed the opportunity for a damaging recrystallization to occur due to a slower thawing rate [94, 100].

Our second small volume vitrification protocol allowed us to further explore the effects of freezing volume on the post-thaw progressive motility. The small volume vitrification protocol varied from the large volume vitrification protocol in cooling step, CPA dilution and more importantly freezing volume. The small volume sperm suspensions were vitrified by dropping small droplets of 40 µl on a precooled aluminum tray floating on the LN_2. The technique was based on the research by Isachenko et al. [97] and had a great resemblance to the previously mentioned solid surface vitrification technique. The droplets instantly solidified

on contact with the metal surface due to the efficient way of heat transfer [105]. When compared with the large volume vitrification, this direct liquid on cooled surface method was probably even more efficient, as it eliminated the interference of the cryostraw with the cooling/thawing rate. Moreover, the thawing of the droplets in the 37°C wash medium was also almost instantaneous, providing a fast thawing rate with a minimal ice recrystallization. As stated by Isachenko et al. [94], the successful survival rate of spermatozoa in a small specimen size is just as well due to a low probability of recrystallization during thawing and dissolving this specimen in a large volume of agitated warm medium will provide this sufficient high speed and short time of warming. Furthermore, the direct transfer to a warm washing medium made the thawing procedure on itself less elaborate. Additionally, the small volume vitrification sperm suspension was diluted at a 2:1 rate with the CPA, while the large volume vitrified samples were prepared at a 1:1 dilution rate as prescribed by Slabbert et al. [92]. So compared with the large volume vitrification technique, the small volume vitrification technique had the advantage to cryopreserve approximately twice the concentration spermatozoa for an identical suspension volume of 400µl. Moreover, with small volume vitrification there is no need to thaw an entire sperm sample when needed, as the desired concentration of spermatozoa can be calculated just by the amount of droplets needed. This shows a great advantage over any cryopreservation technique using straws when dealing with refreezing samples for later usage as this can have detrimental effects to the spermatozoa quality. The research by Thomson et al. [107] on repeated freezing concluded a significant loss of motility and viability with severe DNA fragmentation, worsening after each additional freeze/thaw cycle.

In summary, results on post-thaw sperm motility concluded that the small volume vitrification technique was significantly better at preserving the post-thaw progressive motility compared with the large volume vitrification technique and the conventional slow freezing procedure. Since both vitrification techniques mainly differed in freezing volume, study results supported the notion that a small sperm suspension volume played an important role in permeable CPA free vitrification. Cryopreservation studies, such as Nawroth et al. [108] and Isachenko et al. [91, 96, 97], compared permeable cryoprotectant free

vitrification of small freezing volumes (ranging from 10 µl to 40 µl) versus the conventional technique of slow freezing and also concluded vitrification to be a superior technique at preserving motility.

Furthermore, it is well understood that morphology, just as motility, viability and mitochondrial activities, is susceptible to damage induced by cryopreservation [18, 109]. Alike various research on the subject, our study results on morphology also showed and reinstated the significant decrease of normal morphology after cryopreservation [18, 108, 109]. As described previously, cryodamage to morphology is most probably a combinatory effect of cellular sensitivity to excessive cell swelling or shrinking during cooling and thawing procedures, as well as lipid peroxidation due to ROS formation [18, 91, 109-111]. Furthermore, there were no significant differences between both cryopreservation techniques. In addition, these findings were in accordance with previous research by Nawroth et al. [108], Isachenko et al. [91, 110] and Vutyavanich et al. [17].

Post-thaw spermatozoa cryopreserved with vitrification showed a significantly higher percentage of acrosome loss compared with slow freezing samples in our study. Potentially, this loss could be caused by the difference in thawing temperature between the vitrification and the slow freezing technique [81, 100]. Large volume vitrification samples were thawed at 42°C as opposed to slow freezing samples which were thawed at 37°C, which could have induced acrosome reaction. Furthermore, to assure a fast thawing process, the small volume vitrification droplets were submerged in a relatively large volume (5 ml) of sperm wash at 37°C. This quick dilution of the relatively high sucrose concentration in the vitrification medium might induce an osmotic shock, leading to water rushing into the cells and the acrosome to burst [112]. Also, most $CD46^+$ labeled spermatozoa were simultaneously labelled with an 7-AAD probe, indicating that the $CD46^+$ labelling process may also be due to a loss of membrane integrity and not an acrosome reaction. It is also important to note, it was possible these spermatozoa genuinely experienced an acrosome reaction in the first place and at a later stage lost their viability. Therefore, total percentage results did not specify which event occurred first and consequently did not show a distinction between an initial acrosomal loss and a general loss of viability. Most importantly, the fraction of $CD46^-$/7-AAD^- and Annexin-V^-/7-AAD^-

spermatozoa showed no significant differences between slow freezing and small volume vitrification.

In conclusion, small volume vitrification could be considered as an alternative method for sperm freezing since it resulted in a significantly higher percentage of post-thaw progressive sperm motility compared with slow freezing. Additionally, the percentages of living non-apoptotic and non-acrosome reacted spermatozoa were not significantly different. Furthermore, the technique is easier and faster to apply and the ability to thaw individual droplets of sperm will reduce the need for recurrent freezing. Furthermore, this technique does not require any expensive cryopreservation medium nor specialized or extra freezing equipment. Further research is however needed to evaluate the influence of small volume vitrification on chromatin structure and long term survival of spermatozoa.

Chapter 4

Factors predicting AID success

4.1 Factors predicting AID success

Predictive factors influencing pregnancy rates after intrauterine insemination with frozen donor semen: a prospective cohort study of 1264 cycles

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Original paper

Annelies THIJSSEN, An CREEMERS, Wim VAN DER ELST, Eva CREEMERS, Eva VANDORMAEL, Nathalie DHONT, Willem OMBELET

Accepted for publication in Reprod Biomed Online

4.1.1 Abstract

This study aimed to prospectively examine to which extent certain parameters can influence pregnancy rates after IUI with frozen donor semen. During the period of July 2011 until September 2015, 402 women received a total of 1264 IUI cycles with frozen donor semen in a tertiary referral infertility center. A case report form (CRF), filled in by the midwife together with the patient, was used to prospectively collect the data. The primary outcome measure was clinical pregnancy rate (CPR), confirmed by detection of a gestational sac and fetal heartbeat using ultrasonography at 7-8 weeks of gestation. Statistical analysis was done using Generalized Estimating Equations (GEE) to take into account the correlation between observations from the same patient. Overall CPR per cycle was 17,2%. Multivariate GEE analysis revealed the following parameters as predictive for a successful pregnancy outcome: female age (p=0,0003), non-smoking or smoking less than 15 cigarettes a day (p=0,0470 and p=0,0235 respectively), secondary infertility (p=0,0062), low progesterone levels at D0 of the cycle (p=0,0164) and use of ovarian stimulation with hMG/recFSH compared with clomiphene citrate and natural cycle (p=0,0006 and p=0,0004 respectively). We observed that these parameters are the most important factors influencing the success rate in a sperm donation program.

KEYWORDS: Donor sperm, IUI, prognostic factor, prospective cohort study, success rate

4.1.2 Introduction

Intrauterine insemination (IUI) with frozen donor semen is indicated for couples suffering from severe male factor infertility including azoospermia, men with sexually transmitted infectious diseases, in males with Y chromosome linked genetic disorders that might be transmissible to the progeny, as well as lesbian women and women without a male partner [113]. In recent years, the request for IUI with frozen donor semen has increased due to an increased number of lesbian couples and single women relying on donor insemination and a high inflow of patients seeking cross-border reproductive care in order to avoid restrictive laws in their home country [6, 7, 51].

Furthermore, there is an ever-increasing demand for more cost-effectiveness in health care. Therefore, it is important to determine the factors influencing pregnancy rates after IUI with frozen donor semen in order to increase success rates and decrease costs per treatment cycle.

For donor inseminations, only frozen sperm can be used. Cryopreserved donor semen, quarantined for a minimum of 6 months, is inseminated to prevent the transmission of sexually transmitted diseases such as human immunodeficiency virus (HIV) and Hepatitis B and C [114, 115]. Cryopreservation of donor semen and the use of cryoprotective agents, however, reduce post-thaw sperm survival, motility and pregnancy rates [19-21].

The influence of different parameters such as female age, ovarian stimulation protocols, sperm parameters, etc. on pregnancy rates after IUI with frozen donor semen has been described thoroughly in the past, however with diverging results. Mostly, female age has been indicated as one of the most important prognostic factors for predicting pregnancy rate after donor insemination [21, 113, 116-123]. Recently a study by Koh et al. [124] also revealed sperm donor age as a factor influencing clinical pregnancy rate (CPR) in donor insemination cycles. Matorras et al. [125] and Zuzuarregui et al. [113] reported that the use of follicle stimulating hormone (FSH) for ovarian stimulation results in significantly higher pregnancy rates compared with clomiphene citrate (CC) or natural cycle (NC). However, Ferrara et al. [117] and De Brucker et al. [123] concluded that ovarian stimulation did not significantly

improve pregnancy rates and delivery rates. Numerous sperm parameters have also been suggested as possible predicting factors, going from pre-freezing sperm motility [126], to post-thaw sperm motility [127], forward progression [128, 129], inseminating motile count (IMC) [113, 118, 120] and total motile sperm counts (TMSC) [129, 130]. However, many studies were unable to detect such relationships [117, 118, 126-128, 131].

These widely conflicting data are mainly the result of differences in study design and methodology [36]. Most of the studies performed in the past are retrospective and statistical analysis does not account for the multivariate nature of the dataset and the fact that the same patients are coming back for treatment after previous failed attempts.

This study aimed to prospectively evaluate the extent to which different parameters can influence the success rate in a donor insemination program. Although this was a cohort study, the nature of the study was prospective since patient specific data were recorded by means of a case report form (CRF) at the time of insemination. Furthermore, since patients are coming back after previously failed attempts, the data cannot be analyzed as independent, which is mostly assumed in classical statistical analysis like t-tests, linear regression models and logistic regression models. Therefore, a novel form of multivariate logistic regression analysis, i.e. Generalized Estimating Equations (GEE), was used on the data to take into account the correlation between observations from the same patient.

4.1.3 Materials and Methods

Patients

Between 1 July 2011 and 30 September 2015, data from 402 women with a total of 1264 IUI cycles with frozen donor semen were collected prospectively in a tertiary referral infertility center. A CRF was used to review all possible contributing factors to IUI outcome. This was filled in by a midwife together with the patient during the 20 minutes of mandatory bedrest following insemination [132, 133]. The results of the CRFs were examined by a third person for possible lack of data on a monthly basis.

The average age of the patient population was 33±5 years (range 20 – 46

years). All women were either single or lesbian or hetero couples with an azoospermic partner or a partner with a y-linked chromosome genetic disorder. In all patients a complete infertility work-up was done, including a medical history, physical examination, pelvic ultrasound, serum hormone assays between day 2 and 4 of the menstrual cycle and mid luteal serum progesterone in women with regular menstrual cycles. If an implantation abnormality or uterine abnormality was suspected on ultrasound, a hysteroscopy and/or laparoscopy was performed. Tubal patency was assessed either by hysterosalpingography and/or laparoscopy. Biochemical and ultrasound screening was performed in all IUI cycles.

Institutional Review Board approval was obtained for this study.

Parameters studied

Data analyzed included female age (years), smoking (non-smoking, 1-14 cigarettes a day, ≥15 cigarettes a day), BMI (kg/m^2), primary/secondary infertility, cycle number, ovarian stimulation method (NC, CC, human menopausal gonadotropin (hMG)/recombinant FSH (recFSH)), day (D) 0 estradiol (ng/l) and progesterone (µg/l) levels, human chorionic gonadotropin (hCG)-insemination time interval (hours), easy or difficult insemination with difficult being defined as multiple trials needed to get into the uterus (easy-clamp (C), easy+C, difficult+C, difficult+C+dilator, intracervically), occurrence of obvious uterine bleeding during or after insemination (yes, no), post-thaw sperm quality parameters (i.e. concentration (million/ml), motility grade A (%), motility grade A+B (%), TMSC grade A (million), TMSC grade A+B (million), and IMC (million)) and sperm washing procedure (wash, density gradient centrifugation (DGC)).

The primary outcome measure was the clinical pregnancy rate (CPR), defined as a pregnancy with ultrasound visualization of a gestational sac and an embryonic pole with heartbeat at 7-8 weeks of gestation.

Ovarian stimulation and IUI

Women were inseminated in a natural cycle when cycles were regular and ovulatory. After a minimum of three failed attempts in the natural cycle or in case of anovulation, ovarian stimulation was used. Ovarian stimulation was

conducted by either administering CC or hMG/recFSH. According to the Belgian law, hMG/recFSH ovarian stimulation can only be reimbursed if at least 3 cycles with CC were performed without success or when patients are CC resistant or have a thin endometrium (<6 mm). When using CC, patients received 50 mg (100 mg in case of a BMI≥30) daily from day 3 to day 7. hMG and/or recFSH were administered in a minimal dose step-up regimen, starting off with 50 IU or 75 IU on day 3 of the cycle. The ovarian response was monitored by performing regular ultrasonography and biochemical monitoring of LH, estradiol and progesterone level measurements, for patients in a natural cycle as well as patients using ovarian stimulation with CC or hMG/recFSH. Ovulation was induced with a single hCG injection of 5000 IU when the average diameter of the dominant follicle was 18 mm or more. IUI was performed at 20-24 hours post-hCG on average. A fraction of the washed motile spermatozoa (0,3 ml) was inserted up to the uterine fundus and expelled into the uterine cavity. No reflux of the inseminate was observed upon insemination. A supine position was maintained by the women for 20 minutes post-IUI. Cycles were cancelled if three or more follicles of at least 15 mm were present. Serum β-hCG level measurement was carried out 2 weeks after IUI and, if positive, clinical pregnancy was confirmed by ultrasound at 7-8 weeks of gestation. If pregnancy was not achieved within a minimum of 6 IUI cycles, the physician would, in consultation with the patient, decide whether to continue IUI or move to IVF/ICSI. If pregnancy was achieved within a minimum of 6 cycles and patients come back for another attempt counting started again from cycle number 0 up to 6.

Sperm preparation

Frozen donor sperm was imported from Denmark, both from Nordic Cryobank (87,2%) (Nordic Cryobank ApS, Frederiksberg, Denmark) and Cryos International sperm bank (12,8%) (Cryos International ApS, Aarhus, Denmark). Semen samples from Cryos International sperm bank had been frozen in their native state and were therefore, after thawing in our laboratory, prepared by DGC with PureSperm® (PureSperm® 40/80, Nidacon) according to the manufacturer's instructions. On the other hand, frozen donor sperm derived from Nordic Cryobank had been frozen in a purified state and was therefore,

after thawing in our laboratory, prepared by washing twice in 5 ml of PureSperm® Wash (Nidacon). The final pellet was resuspended in 1 ml of medium. For insemination, the washed sperm sample was concentrated to a volume of 0,3 ml. Thawed and purified sperm samples were evaluated for concentration and progressive motility. Sperm concentration was determined by pipetting 10 µl of purified sperm in a Makler counting chamber and counting the number of spermatozoa in the grids (10 grids counted: total number of spermatozoa x 10^6/ml; all grids counted: total number of spermatozoa x 10^5/ml). Sperm motility was determined by pipetting 10 µl of purified sperm in a Makler counting chamber and assessing sperm motility of a minimum of 100 spermatozoa in at least 5 microscopic fields. The motility of spermatozoa was graded, rapid progressive (A; >25µm/second), slow progressive (B; 5-25 µm/second), non-progressive (C; <5µm/second) or immotile (D; 0 µm/second) according to World Health Organization (WHO) [77] guidelines. Microscopes were equipped with phase contrast optics and a heat tray for counting sperm motility at 37°C±0,5°C. TMSC was determined by multiplying grade A or grade A+B progressive sperm motility percentages with sperm volume (0,3 ml) and concentration. IMC was determined by multiplying the percentage grade A motility spermatozoa with sperm volume (1 ml) and concentration. Native semen parameters were unavailable for the imported semen samples.

Statistical analysis

One of the most important assumptions of all classical statistical analyses (like t-tests, linear regression models, logistic regression models, etc.) is the assumption of independency. This means they all assume independent observations for their inferences to be valid.

However, for the dataset considered here, this assumption is not fulfilled. When a first attempt to become pregnant failed, the patient probably will come back for a second, a third, … attempt. Even if they became pregnant, they will come back for a second, third, … child. This shows that not all observations are independent: some observations come from the same patient, while other observations come from different patients. To take into account this dependency, several statistical models are possible.

In this paper, we will model the probability to become pregnant. To take into

account the previous described dependency, not an ordinary logistic model, but Generalized Estimating Equations (GEE) were used [134]. This can be seen as an extension of ordinary logistic regression, where the correlation between observations from the same person are taken into account. In this paper, the correlation structure is assumed to be of an 'exchangeable' type. Although it might not be the correct one, GEE is known to be robust against misspecification of the working correlation structure. Since the amount of missing data was low, cycles that contained missing data were not included in the GEE analysis. All GEE analyses were done in the software package SAS 9.4. For full details of GEE analysis, we refer to Molenberghs and Verbeke [135].

4.1.4 Results

There were 402 women who received a total of 1264 IUI cycles with frozen donor semen. Outcome results were not available for 11 cycles (0,9%) since patients were followed abroad. Outcomes out of the 1253 cycles were distributed as follows: 980 β-hCG negative (78,2%), 25 biochemical pregnancies (2,0%), 7 extrauterine gestations (EUG) (0,6%), 25 early miscarriages (i.e. presence of a gestational sac without heartbeat; 2,0%) and 216 (17,2%) clinical pregnancies, of which 13 (6%) were twin pregnancies and 1 (0,5%) triplet pregnancy. Of the twin pregnancies, 1 occurred in a NC (1,2%; n=1/83), 5 resulted from stimulation with CC (8,8%; n=5/57) and 7 resulted from stimulation with hMG/recFSH (9,3%; n=7/75). The triplet pregnancy occurred in a hMG/recFSH stimulated cycle (1,3%; n=1/75). On average, 2,4 cycles (range 1-8) were needed to achieve pregnancy in a NC, 3 cycles (range 1-11) with CC stimulation and 4,8 cycles (range 1-11) with hMG/recFSH stimulation.

Univariate analysis

Table 4.1-I provides an overview of the main characteristics of the study population for the total patient group and for the pregnant and not pregnant groups separately. The univariate relationship between the different covariates and the outcome is shown in **Tables 4.1-II to 4.1-IV** (**Appendix E** shows the same results in graphs instead of tables). Continuous variables were categorized for graphical representation only, not for actual statistical analysis. Keep in mind

that **Tables 4.1-I to 4.1-IV** only show the effect of one covariate at a time. This can be misleading since it is, for example, possible that average age in the stimulation group hMG/recFSH is lower compared with the NC or CC groups, which may implicate an effect of stimulation while the graph is actually showing an effect of age.

Table 4.1-I: Main characteristics of the study population

	Total	**Pregnant**	**Not pregnant**	**p-value**
No. cycles	1264	216	1037	
Patient characteristics				
Age patient (years)	33,4±5,0 (20,1-45,9)	32,7±4,2 (20,7-43,6)	33,5±5,1 (20,1-45,9)	**0,01**
BMI patient (kg/m^2)	25,5±5,0 (16,4-46,6)	25,4±4,5 (16,6-46,6)	25,5±5,1 (16,4-43,8)	0,91
IUI procedure characteristics				
hCG-insemination interval (hours)	20,9±4,4 (1,0-39,5)	21,3±4,1 (12,0-28,1)	20,9±4,4 (1,0-39,5)	0,18
Estradiol D0 (ng/l)	332,7±213,5 (1,7-1976,0)	342,1±204,4 (11,8-1302,0)	330,8±214,7 (1,7-1976,0)	0,47
Progesteron D0 (µg/l)	0,7±1,0 (0,1-25,4)	0,6±0,3 (0,1-2,1)	0,7±1,1 (0,1-25,4)	**<0,01**
Post-thaw sperm characteristics				
IMC (million)	8,4±5,4 (0,0-39,9)	8,6±5,3 (0,1-30,7)	8,3±5,4 (0,0-39,9)	0,44
Concentration (million/ml)	78,4±29,7 (0,1-340,0)	80,6±27,3 (19,0-165,0)	78,0±30,2 (0,1-340,0)	0,20
Grade A motility (%)	24,6±11,5 (0,0-93,0)	25,8±11,3 (0,0-56,0)	24,4±11,5 (0,0-93,0)	0,11
Grade A+B motility (%)	53,3±11,8 (16,0-85,0)	53,8±11,8 (25,0-83,0)	53,2±11,9 (16,0-85,0)	0,49
TMSC-A (million)	6,0±3,6 (0,0-23,1)	6,3±3,3 (0,0-18,2)	5,9±3,6 (0,0-23,1)	0,21
TMSC-AB (million)	12,9±5,3 (0,0-40,1)	13,2±4,9 (3,8-28,4)	12,9±5,4 (0,0-40,1)	0,32

BMI: body mass index; D0: day 0; hCG: human chorionic gonadotropin; IMC: inseminating motile count; SD: standard deviation; TMSC: total motile sperm count. Data are presented as mean±SD (min-max).

Patient related factors

Univariate statistical analysis showed that CPR per cycle significantly decreased with advancing patient age (p=0,0226). Also, patients presenting with primary infertility showed a significantly lower pregnancy rate compared with patients suffering from secondary infertility (p=0,0054). Additionally, in women smoking

15 cigarettes or more daily, CPR was significantly lower compared with women smoking 1-14 cigarettes a day (p=0,0140) and there was a tendency towards a significantly lower CPR compared with non-smoking women (p=0,0520). The covariate BMI showed a tendency towards a lower CPR when BMI was <20 kg/m^2 and ≥30 kg/m^2, but this was not statistically significant (p=0,8344). When looking further into the severe obese (≥35 kg/m^2) and morbidly obese (≥40 kg/m^2) categories, pregnancy rates were even lower, with a CPR of 0,0962 (5/52) and 0,0909 (1/11) respectively, but not significantly different. Results from the univariate analysis on patient related factors are shown in **Table 4.1-II**.

Table 4.1-II: Univariate analysis of patient related factors

	CP	Total	CPR	SE	p-value
Age (years)					
<30	56	312	0,179	±0,022	**0,0226**
30-34,99	97	492	0,197	±0,018	
35-39,99	57	320	0,178	±0,021	
≥40	6	128	0,047	±0,019	
Infertility					
primary	138	903	0,153	±0,012	**0,0054**
secondary	78	348	0,224	±0,022	
Smoking (n/day)					
0 cig.	188	1117	0,168	±0,011	0,0877[a]
1-14 cig.	23	94	0,245	±0,045	0,0520[b]
≥15 cig.	2	36	0,056	±0,039	**0,0140**[c]
BMI (kg/m^2)					
<20	13	118	0,110	±0,029	0,8344
20-24,99	105	565	0,186	±0,016	
25-29,99	59	327	0,180	±0,021	
≥30	35	237	0,148	±0,023	

BMI: body mass index; cig.: cigarettes; CP: number of clinical pregnancies; CPR: clinical pregnancy rate; SE: standard error.
Age and BMI were continuous variables, therefore p-values represent overall significance levels. Smoking and infertility were categorical variables and p-values represent if CPR in one of the groups significantly differed from another. [a] 0 cig. versus 1-14 cig., [b] 0 cig. versus ≥15 cig., [c] 1-14 cig. versus ≥15 cig.

IUI procedure specific factors

Ovarian stimulation showed to be an important factor influencing pregnancy rate. Ovarian stimulation with hMG/recFSH protocols resulted in significantly higher CPR compared with cycles stimulated with CC or NC (p=0,0001).

Although shorter time intervals between hCG administration and insemination resulted in a lower CPR, this result was not significant (p=0,1620). Hormone levels of estradiol on D0 of the cycle did not reflect a significant influence on pregnancy rate (p=0,3561), while higher progesterone levels on D0 resulted in a significantly lower CPR (p=0,0061). The insemination procedure itself, i.e. easy or difficult, with or without use of clamp or dilator or intracervical insemination, the occurrence of an obvious uterine bleeding during or after insemination and cycle number did not significantly influence CPR per cycle. Results are shown in **Table 4.1-III**.

Table 4.1-III: Univariate analysis of IUI procedure specific factors

	CP	Total	CPR	SE	p-value
Stimulation					
NC	83	550	0,151	±0,015	0,7998[a]
CC	57	402	0,142	±0,017	**0,0001**[b]
hMG/recFSH	75	298	0,252	±0,025	**0,0001**[c]
hCG-insemination interval (hours)					
<15	6	51	0,118	±0,046	0,1620
15-22,99	117	732	0,160	±0,014	
≥23	89	439	0,203	±0,019	
Estradiol D0 (ng/l)					
1,69-195	41	300	0,137	±0,020	0,3561
195-268	61	307	0,199	±0,023	
268-415	49	295	0,166	±0,022	
415-1976	57	301	0,189	±0,023	
Progesteron D0 (μg/l)					
<0,5	72	355	0,203	±0,021	**0,0061**
0,5-0,99	110	637	0,173	±0,015	
1-1,49	21	137	0,153	±0,031	
≥1,5	3	42	0,071	±0,040	

CC: clomiphene citrate; CP: number of clinical pregnancies; CPR: clinical pregnancy rate; D0: day 0; hCG: human chorionic gonadotropin; hMG: human menopausal gonadotropin; NC: natural cycle; recFSH: recombinant follicle stimulating hormone SE: standard error.
Levels of estradiol and progesterone at D0 and the hCG-insemination time interval were continuous variables, therefore p-values represent overall significance levels. Stimulation was a categorical variable and the p-value represents a significant difference in CPR between NC or CC and hMG/recFSH stimulated groups: [a] NC versus CC, [b] NC versus hMG/recFSH, [c] CC versus hMG/recFSH.

Post-thaw sperm quality factors

None of the post-thaw sperm quality parameters showed a significant influence on CPR (**Table 4.1-IV**). There was no difference in CPR when semen samples

had lower sperm concentrations or IMC (p=0,2031 and p=0,3991 respectively). Low grade A and grade A+B sperm motility parameters resulted in a slight decrease in pregnancy rates, however this was not statistically significant (p=0,0842 and p=0,5129 respectively). A similar tendency could be observed with TMSC-A and TMSC-AB (p=0,1927 and p=0,3158 respectively). Furthermore, there was no significant difference in CPR when sperm samples were prepared for IUI with either DGC or wash (p=0,8745).

Multivariate GEE analysis

The final multivariate GEE model contained the covariates age, smoking, infertility, progesterone D0 and stimulation as significant factors influencing CPR. As shown in **Table 4.1-V**, age and progesterone D0 were both continuous variables with a negative parameter estimation, indicating decreasing pregnancy rates with increasing values for age and progesterone D0. Smoking 15 or more cigarettes a day significantly decreased CPR compared with smoking 1-14 cigarettes a day or non-smoking. No significant difference in CPR was observed between women smoking 1-14 cigarettes a day and non-smokers (p=0,2870). Women suffering from primary infertility showed a significantly lower CPR compared with women presenting with secondary infertility problems. Using no ovarian stimulation (NC) or ovarian stimulation with CC, resulted in significantly lower CPR compared with ovarian stimulation using hMG/recFSH protocols. No significant difference in CPR was observed between women inseminated in their NC compared with women stimulated with CC (p=0,9896).

Table 4.1-IV: Univariate analysis of post-thaw sperm quality factors

	CP	Total	CPR	SE	p-value
IMC (million)					
<1	8	50	0,160	±0,052	0,3991
1-1,99	10	60	0,167	±0,049	
2-4,99	36	255	0,141	±0,022	
5-9,99	94	484	0,194	±0,018	
≥10	68	400	0,170	±0,019	
Concentration (million/ml)					
0,1-50	22	150	0,147	±0,029	0,2031
50-100	147	872	0,169	±0,013	
100-150	42	200	0,210	±0,029	
150-340	5	30	0,167	±0,070	
Grade A motility (%)					
0-10	14	115	0,122	±0,031	0,0842
10-20	53	315	0,168	±0,021	
20-30	73	405	0,180	±0,019	
30-40	55	290	0,190	±0,023	
40-93	21	127	0,165	±0,033	
Grade A+B motility (%)					
16-40	27	166	0,163	±0,029	0,5129
40-55	80	475	0,168	±0,017	
55-70	94	516	0,182	±0,017	
70-85	15	95	0,158	±0,038	
TMSC-A (million)					
0-3	31	261	0,119	±0,020	0,1927
3-5	51	287	0,178	±0,023	
5-10	103	551	0,187	±0,017	
10-23,1	31	153	0,203	±0,033	
TMSC-AB (million)					
0,0171-10	57	388	0,147	±0,018	0,3158
10-15	86	479	0,180	±0,018	
15-20	51	263	0,194	±0,024	
20-40,1	22	122	0,180	±0,035	
Wash					
DGC	29	161	0,180	±0,030	0,8745
Wash	187	1092	0,171	±0,011	

CP: number of clinical pregnancies; CPR: clinical pregnancy rate; DGC: density gradient centrifugation; IMC: inseminating motile count; SE: standard error; TMSC: total motile sperm count.

All sperm parameters were continuous variables, therefore the p-values represent the overall significance level.

Table 4.1-V: Results from the multivariate GEE analysis

Covariate	Parameter estimation (SE)	p-value
Intercept	0,4407 (0,9177)	0,6311
Age	-0,0620 (0,0172)	**0,0003**
Smoking (0 cig.)	1,3461 (0,6777)	**0,0470**
Smoking (1-14 cig.)	1,6308 (0,7197)	**0,0235**
Smoking (≥15 cig.)	reference	
Infertility (primary)	-0,5150 (0,1880)	**0,0062**
Infertility (secondary)	reference	
Progesterone D0	-0,5691 (0,2372)	**0,0164**
Stimulation (NC)	-0,7146 (0,2020)	**0,0004**
Stimulation (CC)	-0,7173 (0,2095)	**0,0006**
Stimulation (hMG/recFSH)	reference	

Cig.: cigarettes, IMC: inseminating motile count, SE: standard error.

4.1.5 Discussion

Because of the increased request for donor insemination [6, 7, 51], it has become important to be able to accurately predict the likelihood of success after IUI treatment with frozen donor semen. In this study, the relationship between certain covariates such as female age, smoking habits, BMI, use or non-use of ovarian stimulation, post-thaw IMC etc., and pregnancy outcome, was explored through a prospective analysis of 1264 cycles in a total of 402 women presenting at a tertiary referral infertility center between July 2011 and September 2015. Data were registered trough CRFs filled in by the midwife together with the patient. Moreover, statistical analysis was performed with GEE to account for the correlation between observations from the same patient.

According to the literature, pregnancy rates per insemination cycle with donor semen range from 6,4% to 16,5% [21]. Our results showed an overall CPR per cycle of 17,2%, which was also notably higher than the average CPR of 7,5% reported in the latest report of the Belgian Register for Assisted Procreation (BELRAP) [136]. Although overall CPR in our study group was generally high, the occurrence of multiple pregnancies was lower in comparison with other studies, probably because, according to our study protocol, cycles with three or more mature follicles were cancelled. Matorras et al. [125] reported a multiple pregnancy rate of 12,5% in CC stimulated cycles and 20% in FSH stimulated cycles, of which 6,7% were triplets or more. Overall multiple pregnancy rate in our study was 6,5%, with 10,7% in cycles stimulated with

hMG/recFSH, 8,8% in CC stimulated cycles and 1,2% in NC. Nevertheless, care should be taken in avoiding multiple pregnancies when ovarian stimulation is used.

Results from our multivariate GEE analysis indicated that cycles stimulated with hMG/recFSH resulted in significantly higher pregnancy rates compared with cycles stimulated with CC or NC. These results are largely in accordance with the results proposed by Zuzuarregui et al. [113], who showed significantly higher pregnancy rates following donor IUI treatment using FSH compared with hMG, and the latter higher than CC stimulated cycles ($p<0,01$). Additionally, also Matorras et al. [125] reported significantly higher per-started cycle pregnancy rates in FSH compared with CC stimulated cycles ($p=0,02$). On the other hand, Ferrara et al. [117], Botchan et al. [21] and De Brucker et al. [123] were not able to show a significant influence of using different ovarian stimulation protocols on pregnancy and delivery rates resulting from IUI with donor semen. hCG triggering was used in our study protocol, instead of spontaneous LH monitoring as proposed by Kyrou et al. [137], for practical reasons and uniformity. Since our study had already started at the time the paper by Kyrou et al. [137] was published, we were unable to take spontaneous LH monitoring into account. However, this might be interesting to evaluate in a following study.

The most predictive factor for a positive pregnancy outcome was the patient's age, as reported before in many studies [21, 113, 116-123]. Our results showed a significantly decreasing CPR with advancing age, with the strongest decline in patients aged 40 years or older. This age related decline in CPR can be attributed to decreasing ovarian reserves with advancing age [123]. Furthermore, women presenting with secondary infertility were more likely to achieve pregnancy than primary infertile women.

No previous studies have reported on the influence of progesterone levels at D0 on pregnancy rates after IUI treatment. Our results showed that the CPR significantly decreased when high levels of progesterone were measured at D0 of the cycle, especially at levels of 1,5 µg/l or more. However, no cut-off value could be established since progesterone levels were used as a continuous variable.

Post-thaw IMC and other sperm factors did not show a significant influence on CPR in our study. The highest pregnancy rates were obtained with an IMC of

5-9,99 million, although this result was not statistically significant. However, Kang and Wu [118] and Williams and Alderman [120], among others, have demonstrated significantly higher pregnancy rates in IUI with donor semen when more than 20 million motile spermatozoa were inseminated. It is possible that sperm factors did not yield significant results in our study because we routinely use a simplified method of counting sperm concentration and motility before IUI since robust diagnostic semen analysis according to WHO guidelines [23, 77] has already been done at an earlier stage. This could therefore be a significant limitation of our study.

Smoking only affected CPR in our study when women were smoking 15 or more cigarettes a day. Augood et al. [138] reviewed the association between smoking and the risk of infertility in women of reproductive age and described a 60% increased risk of infertility among cigarette smokers. However, Farhi and Orvieto [139] were unable to detect a significant difference in pregnancy rates between groups of smoking and non-smoking women in a study on the influence of smoking on the outcome of homologous IUI with controlled ovarian hyperstimulation.

BMI was not found to be a significant factor influencing CPR in our study, although a slight decrease in CPR could be observed in women with a BMI of less than 20 kg/m^2 and 30 kg/m^2 or more. Results found in the literature concerning BMI as a factor influencing pregnancy rate after homologous IUI treatment differ greatly, varying from no influence [140-142], to decreased pregnancy rates in women with high BMI [143] and even demonstrating positive effects of being overweight on pregnancy rates [144].

In conclusion, results from our study show that the CPR resulting from IUI treatment with frozen donor semen is significantly higher in young women, non-smoking or smoking less than 15 cigarettes a day, presenting with secondary infertility, having lower progesterone levels at D0 of the cycle and using low dose protocols of hMG or recFSH for ovarian stimulation. This study provides some important strengths compared with previous studies on this topic. First of all, collection of the data was prospective, since different patient and treatment specific factors were recorded by means of a CRF at the time of insemination. The results of the CRFs were examined by a third person for possible lack of data on a monthly basis. Secondly, the multivariate GEE analysis used in this

study has a major advantage over previously used ordinary logistic regression models since it takes into account the correlation between observations from the same patient when patients are coming back for treatment after previous failed attempts.

Chapter 5

General discussion

This PhD thesis aimed to shed a light on some currently underinvestigated topics in the (Belgian) sperm banking industry. In the first part we aimed to provide a clear overview of the current situation on sperm banking in Belgium, with special attention to the methods and criteria used for recruitment, screening and selection of potential sperm donors, procedures for sperm washing and freezing and costs associated with sperm donation. In addition, we surveyed the motivation and attitudes towards payment, donor anonymity, disclosure to offspring, donation to lesbian couples and single women, views on the donor children and social aspects of sperm donation of the current candidate sperm donor population in Belgium. Secondly, we have examined the impact of different sperm storage methods on sperm quality and survival by investigating sperm incubation for 24h at room temperature (23°C) versus testis temperature (35°C) and comparing two methods of cryoprotectant-free vitrification, i.e. large volume vitrification and small volume vitrification, with conventional slow freezing. Lastly, we aimed to evaluate the extent to which different parameters, such as age, smoking, inseminating motile count (IMC), etc., can influence pregnancy rate in a donor insemination program.

Current status on sperm banking

It is well known that regulations concerning all different aspects of gamete donation vary a lot between countries because of political, ethical, socio-cultural and religious differences. However, we wanted to show the variation in methods associated with sperm banking used within a single country. Therefore, we have questioned all Belgian centers for assisted reproduction with laboratory facilities (n=18). The results showed that a wide variation in methods associated with sperm banking could be observed in Belgian centers. Donor recruitment strategies, screening of donors, acceptance rates, thresholds for acceptable sperm quality, sperm preparation techniques, freezing methods and even the financial reimbursement per sperm sample differed substantially between the centers. In addition, since the request for donor semen has increased, Belgian sperm banks face a shortage in donor sperm. However, since there is no central registration system for sperm donors in Belgium, this statement cannot be supported with figures. We were able to demonstrate that, in 2013, about 63%

of the total number of donor inseminations performed in Belgium were accomplished with imported donor sperm from Denmark. Moreover, only two out of the seventeen centers (12%) were able to rely completely on their own sperm bank. The other fifteen centers (15/17 or 88%) had to rely partially (11/17 or 64%) or entirely (4/17 or 24%) on the import of foreign donor sperm. Also, Belgian legislation only allows pregnancies with sperm from a single donor to a maximum of six different women. However, because of the lack of a central registration system for sperm donors in Belgium, it is questionable if this law is actually implemented in practice. Some centers ask their sperm donors to donate sperm exclusively in their center by signing a contract in order to avoid that the same donor is going to different centers, but there is no way to control if donors comply to this, since donor information is not exchanged between centers.

After our neighboring countries have abolished donor anonymity, the discussion started also in Belgium to add the option of identifiable donors to the current system or even to abolish donor anonymity completely, aiming to attract more older donors, who are potentially married and already have children of their own. However, little is known about the motivations and attitudes towards donation of the current candidate donor population, which makes it difficult to predict the impact of such a change in law on the availability of new donors. We have shown that our current candidate donor population is not a highly student based population, donating for the financial compensation, as is mostly assumed in anonymous donor systems. However, these older, married donors indicated they were more reluctant towards meeting potential donor children in the future, which seems to contradict with the aim of moving to an open-identity donor system. Also, only 26% of our candidate donor population indicated they were willing to continue donating non-anonymously. These results indicate that a sudden change in law could have a huge impact on the recruitment of new sperm donors. However, 32% indicated they would be prepared to meet the children conceived from their donation and moreover, 43.4% thought the child had the right to inquire about his/her genetic origin and 49.5% believed that parents should be honest with their children. It is possible that the candidate donors may feel that the child has the right to know that he/she was donor conceived, however, as long as they are anonymous donors, the decision to

inform the child has no impact on their life, so this question is rather hypothetical. Surprisingly however was the fact that 32% of the candidate donors indicated they would be prepared to meet the children conceived from their donation, while only 26% was willing to donate non-anonymously. We do not really have an explanation for this discrepancy. This clearly shows that people answer certain questions in a different way while the outcome of these two statements is rather the same; i.e. their donation would not be anonymous. Finally, Belgian law doesn't allow advertisement for the recruitment of gamete donors. However, the majority of candidate donors indicated the media as their most important information source. Therefore, we believe that it is important, prior to a change of law, to scrutinize the current population of potential sperm donors towards identity-release donation, for example through the use of media campaigns, and adapt current recruitment methods for sperm donors.

To summarize, the Belgian government should set up an organization controlling donor information for all Belgian centers. Through this central registration system, centers will be able to control when the maximum number of families using sperm from a single donor is reached. This organization could also be responsible for organizing national awareness campaigns for gamete donation through the media for the recruitment of new candidate sperm donors and develop standards for donor screening, thresholds for acceptable sperm quality and the financial reimbursement of the donor.

Optimal conditions for sperm storage

Since sperm banking requires the preservation of sperm samples for an extended period of time, in order to screen the male donors for contaminations, conditions for sperm storage have to be optimized to preserve sperm function. However, sperm storage and cryopreservation still significantly reduce post-thaw sperm survival, motility and pregnancy rates. The results presented in this study showed a significantly better and longer preservation of sperm quality when samples were incubated for 24h at RT compared with 35°C. It is assumed that when spermatozoa are incubated at lower temperatures, they adopt a resting state, which allows them to preserve their energy. Comparison between density gradient centrifugation (DGC) and swim-up samples after incubation showed a

superior preservation of sperm quality in swim-up samples. Furthermore, by comparing cryoprotectant-free vitrification with conventional slow freezing, we showed that small volume vitrification was superior in preserving post-thaw progressive sperm motility. Furthermore, the technique is easier and faster to apply and the ability to thaw individual droplets of sperm will reduce the need for recurrent freezing. In addition, this technique does not require any expensive cryopreservation medium nor specialized or extra freezing equipment.

Factors predicting AID success

There is an ever-increasing demand for more cost-effectiveness and personalized medicine in health care. Therefore, it is important to determine the factors influencing pregnancy rates after IUI with frozen donor semen in order to strive for a better patient selection and prediction of success rates following treatment. Results from our study showed that the clinical pregnancy rate (CPR) per cycle resulting from IUI treatment with frozen donor semen was significantly higher in young women, non-smoking or smoking less than 15 cigarettes a day, presenting with secondary infertility, having lower progesterone levels at day 0 of the cycle and using low dose protocols of hMG or recFSH for ovarian stimulation. Based on the results of the final multivariate model, we were able to build a calculation tool for the prediction of CPR for any given patient.

No previous studies have reported on the influence of high progesterone levels at D0 of the cycle (i.e. day of hCG injection or spontaneous LH surge) on pregnancy rates after IUI treatment with frozen donor semen. The rationale behind this effect is not entirely clear. Also in IVF cycles, the clinical influence of a rise in progesterone levels in the early follicular phase has been controversial, with studies reporting negative effects on pregnancy rate [145, 146], while others have not been able to demonstrate an association between increased progesterone levels and outcome [147, 148]. It was shown before in IVF that an elevation of preovulatory progesterone levels can negatively affect endometrial receptivity, therefore we believe that this is the most plausible reason to explain our finding [149, 150]. Since we have been the first do demonstrate a negative effect of high progesterone levels on CPR in a donor insemination cohort, it would be valuable if these results could be repeated by a different research

group.

In addition to the study determining factors influencing pregnancy rates after IUI with frozen donor semen, we have performed the same analysis on patients receiving homologous IUI treatment (**Appendix A**). Since this study does not relate to donor insemination, it was not included in the main part of this thesis. However, there were some interesting discrepancies between the parameters we found significantly influencing CPR in the donor insemination group versus homologous insemination. Female age and primary/secondary infertility significantly influenced CPR per cycle in both study groups. However, while female smoking was a significant factor in the donor insemination group, it was partner smoking in the homologous insemination group which significantly affected the outcome. Also, the homologous group showed a significant influence of the inseminating motile count (IMC), while common sperm parameters were not important in the donor insemination group. These differences could be largely explained by the fact that the female population in both study groups is completely different. The donor insemination patients have a better prognosis in relation to the homologous insemination patients, since these women never had the chance to become pregnant before. Patients for homologous insemination are on average probably less fertile since if they were normally fertile, they would have been pregnant before, even when the semen quality of their partner is slightly impaired.

Future prospects

An organization controlling donor information for all Belgian centers should be set up by the government. Through this central registration system, centers will be able to control when the maximum number of families using sperm from a single donor is reached.

Standards should be developed for donor screening, thresholds for acceptable sperm quality and financial reimbursement of the donor.

The Belgian government should also invest in awareness campaigns for the recruitment of new sperm donors, not only to supplement the current donor population, but also to scrutinize the potential sperm donors towards open-identity donation.

In addition, it is important to extend the research we did on candidate sperm donors by examining the attitudes of the entire potential sperm donor group. Also, it would be interesting to follow sperm donors in time in order to evaluate changes in their attitudes towards donation.

Further investigation is needed to confirm whether the improved preservation of sperm quality at room temperature (RT) can translate into increased pregnancy rates if prepared sperm samples were to be stored at RT instead of 37°C before their use in assisted reproduction treatment.

In order for cryoprotectant-free vitrification of spermatozoa to become a standard method for sperm freezing, more research is needed to assess the effects of vitrification on sperm DNA damage. Also, the impact of the thawing temperature on acrosomal loss should be investigated further.

Appendix A

Factors predicting IUI success

6.1 Review of the literature

Semen quality and prediction of IUI success in male subfertility: a systematic review

Mini-review

Willem OMBELET, Nathalie DHONT, **Annelies THIJSSEN**, Eugene BOSMANS, Thinus KRUGER

Reprod Biomed Online, 2014, 28: 300-309

6.1.1 Abstract

Many variables may influence success rates after intrauterine insemination (IUI), including sperm quality in the native and washed semen sample. A literature search was performed to investigate the threshold levels of sperm parameters above which IUI pregnancy outcome is significantly improved and/or the cut-off values reaching substantial discriminative performance in an IUI program. A search of MEDLINE, EMBASE and Cochrane Library revealed a total of 983 papers. Only 55 studies (5.6%) fulfilled the inclusion criteria and these papers were analyzed. Sperm parameters most frequently examined were: (i) inseminating motile count after washing: cut-off value between 0.8 and 5 million; (ii) sperm morphology using strict criteria: cut-off value 5% normal morphology; (iii) total motile sperm count in the native sperm sample: cut-off value of 5–10 million; and (iv) total motility in the native sperm sample: threshold value of 30%. The results indicate a lack of prospective studies, a lack of standardization in semen testing methodology and a huge heterogeneity of patient groups and IUI treatment strategies. More prospective cohort trials and prospective randomized trials investigating the predictive value of semen parameters on IUI outcome are urgently needed.

KEYWORDS: assisted reproduction, intrauterine insemination, predictive value, pregnancy rate, semen, sperm quality

6.1.2 Introduction

Intrauterine insemination (IUI) is a simple and noninvasive technique which can be performed without expensive infrastructure with a reasonable cumulative live birth rate within three or four cycles [22]. The rationale behind artificial IUI is increasing the gamete density at the site of fertilization. IUI has been proven to be easier to perform, less invasive and less expensive than other more complex methods of assisted reproduction [151]. Risks are minimal provided that the multiple gestation incidence can be reduced to an acceptable level and efforts are made to decrease horizontal transmission of sexually transmitted infections, including HIV.

Increasing interest in IUI is undoubtedly associated with the refinement of techniques for the preparation of washed motile spermatozoa [65, 152]. Semen washing procedures can remove prostaglandins, infectious agents, antigenic proteins, non-motile spermatozoa, leukocytes and immature germ cells. This may enhance sperm quality by decreasing the formation of free oxygen radicals after sperm preparation. The final result is an improved fertilizing capacity of the spermatozoa *in vitro* and *in vivo*.

Despite the extensive literature on IUI and due to a lack of good-quality prospective cohort trials, controversy remains about the effectiveness of this treatment procedure, particularly in relation to IVF and intracytoplasmic sperm injection (ICSI) [22, 153-156]. This may be explained by the fact that most studies are retrospective and not only vary in the comparison of the study group (different groups of male subfertility) but also in the use or non-use of different ovulation induction regimens, the number of inseminations per treatment cycle, methods of timing ovulation, sites of insemination, methods of sperm preparation and use of additives such as kallikrein, platelet-activating factor and antioxidants, as shown in **Figure 6.1-1**.

According to the new and updated recommendations published in the NICE clinical guidelines, IUI is not recommended anymore for unexplained and mild male factor infertility [157]: ‘For people with unexplained infertility, mild endometriosis or ‘‘mild male factor infertility’’, who are having regular unprotected sexual intercourse, it is advised not to offer IUI routinely, either with or without ovarian stimulation, but advise them to try to conceive for a

total of 2 years before IVF will be considered'.

Despite the NICE recommendations, it can be expected that artificial insemination with husband's semen remains a widely used treatment option for many couples with unexplained infertility, cervical factor subfertility, physiological or psychological sexual dysfunction and mild-to-moderate male subfertility.

To find out which couples can benefit from IUI in the case of male subfertility, the power of different semen parameters in predicting success after IUI need to be investigated. Although the World Health Organization (WHO) tried to standardize the performances of semen analysis and related procedures in order to reduce variation in the results obtained, a literature search on this topic is frustrating due to the ongoing lack of standardization of interpretation of semen results [158].

This structured review aimed to investigate the accuracy of sperm parameters in predicting IUI success. Therefore, threshold levels of sperm quality above which IUI pregnancy outcome is significantly improved were examined. Secondly, the cut-off values reaching substantial discriminative performance considering IUI outcome were investigated. For the current systematic review, no written protocol was registered.

6.1.3 Materials and Methods

Search strategy

By means of a computerized MEDLINE search, the literature for a 31-year period, from January 1982 until December 2012, was reviewed. The following search terms were used: (success OR outcome OR pregnancy OR predictive value) AND (semen OR spermatozoa) AND (IUI OR intrauterine insemination OR artificial insemination). Other relevant studies were identified by searching EMBASE using the same search terms and Cochrane Controlled Trial register published until December 2012. The reference lists of all selected articles were examined to identify papers that were not captured by the electronic search and the 'related articles' function of PubMed was also used. There was no language

restriction and the identification of relevant studies was performed independently by two authors (WO and ND).

Study inclusion criteria and data extraction

Studies were only included if the authors reported on the value of sperm parameters on the prediction of IUI success in couples with male subfertility. Only studies with a minimum of 200 IUI cycles using homologous spermatozoa were included.

Male subfertility was defined as semen quality below the standards of WHO during that specific period. Studies in a population with unexplained infertility and studies reporting on results in a sperm donor program were excluded.

The outcome most frequently used was clinical pregnancy defined as a pregnancy confirmed by a gestational sac and/or fetal heart activity on ultrasound. Nowadays, results should be expressed as live birth rates (or at least ongoing pregnancy rates) per couple applying intention to treat analysis, but these outcome parameters were not used in the selected studies.

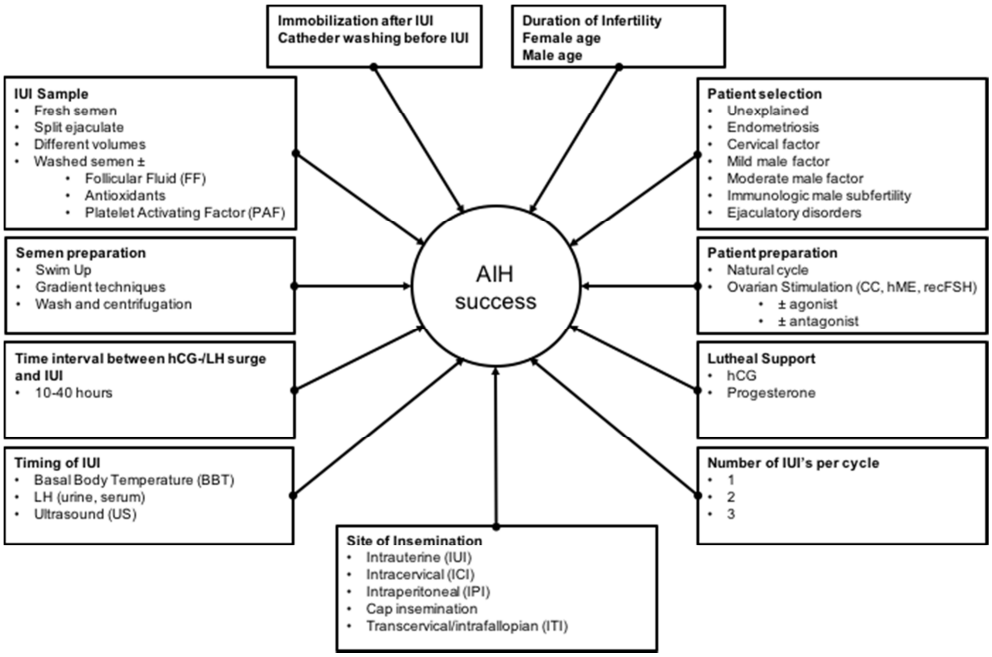

Figure 6.1-1: Factors influencing the success rate of artificial intrauterine insemination with homologous spermatozoa. CC = clomiphpene citrate; HCG = human chorionic gonadotrophin; HMG = human menopausal gonadotrophin; IUI = intrauterine insemination; rec FSH = recombinant FSH.

6.1.4 Results

In the Cochrane Library, 10 reviews could be selected, but none of these evaluated the predictive value of semen parameters on IUI outcome. The EMBASE and MEDLINE search revealed a total of 983 papers. Only 55 studies (5.6%) fulfilled the inclusion criteria and these papers were analyzed (**Table 6.1-I** and **Figure 6.1-2**). In the majority of IUI studies, the predictive value of sperm parameters was not investigated at all; in many other studies, quality assurance associated with semen analysis and a successful service, as indicated by acceptable pregnancy rates, was clearly not available.

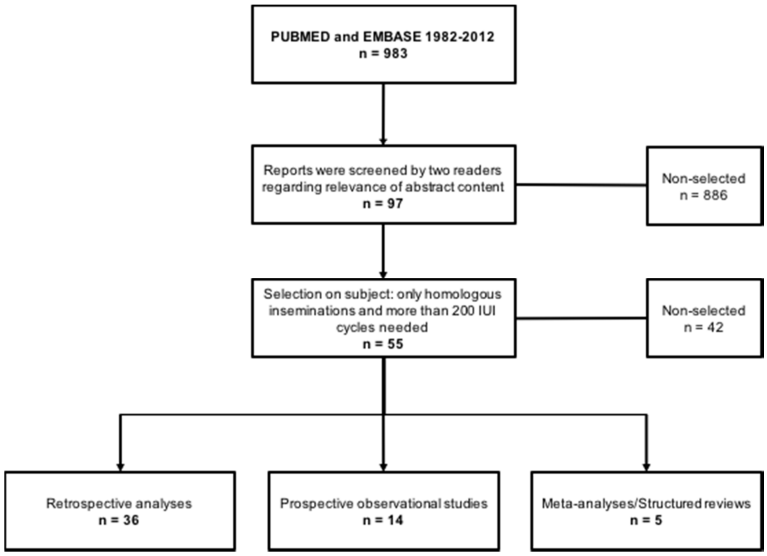

Figure 6.1-2: Overview of the systematic literature search concerning the prognostic value of sperm quality parameters in an intrauterine insemination program.

Out of the 55 selected studies, 36 papers performed a retrospective analysis and 14 articles described the results of a prospective observational study. Five structured reviews and/or meta-analyses were obtained.

In the meta-analysis of Van Waart et al. [159], six studies yielded a risk difference between the pregnancy rates achieved in the patients below and above the 4% strict sperm morphology criteria threshold of -0.07 (95% CI -0.11 to 4.03; $P < 0.001$). In the meta-analysis of 16 studies by Van Weert et al. [160], receiver operating characteristics (ROC) curves indicated a reasonable predictive performance towards IUI outcome for the inseminating motile count (IMC). At cut-off levels between 0.8 and 5 million, the specificity of the IMC, defined as the ability to predict failure to become pregnant, was as high as 100%; the sensitivity of the test, defined as the ability to predict pregnancy, was limited.

According to Ombelet et al. [161, 162], an IMC of 1 million can be used as a reasonable threshold level above which IUI can be performed with acceptable pregnancy rates. Overall, sperm morphology and IMC, as an individual parameter, were of no prognostic value using ROC curve analysis. Sperm morphology turned out to be a valuable prognostic parameter in predicting IUI

success if the IMC was <1 million (area under ROC curve 77.6%). The cumulative live birth rate (CLBR) after three IUI cycles was 13.6% if the IMC was <1 million, significantly different from the group with an IMC >1 million (22.4%, P < 0.05). Considering only patients with IMC <1 million and sperm morphology >4%, the CLBR was 21.9%, comparable with the CLBR of all cycles with an IMC of >1 million [161].

In the systematic review of Castilla et al. [163], investigating the clinical value of the sperm chromatin structure assay (SCSA) and classical semen parameters, it was shown that in couples treated with IUI the clinical validity was higher for SCSA compared with sperm morphology, with a positive likelihood ratio (LR+) of 6.1 (95% CI 2.6–14.6) and 1.9 (95% CI 1.1–3.0) for SCSA and sperm morphology, respectively. They also concluded that, despite this finding, the clinical value of SCSA was not enough to introduce this parameter as a routine test in male infertility work up.

The four sperm parameters that were most frequently examined and cited were the following: (i) IMC; (ii) sperm morphology using strict criteria; (iii) total motile sperm count in the native sperm sample (TMSC); and (iv) total motility in the native sperm sample (TM) (**Table 6.1-I**).

In 24 articles, the IMC was cited as an important predictive parameter, in seven out of 20 studies, a cut-off value of 1 million was mentioned, in four studies a cut-off values of between 1 and 2 million was used and in four studies, the authors calculated a threshold value of 5 million.

Sperm morphology using strict criteria was the second most cited sperm parameter. In 11 out of 16 studies, 5% normal forms was reported as the best cut-off value to predict IUI outcome. When utilizing these cut-off values of sperm morphology and IMC, there is poor sensitivity for predicting who will conceive but a high specificity for predicting failure to conceive with IUI.

TMSC was also reported to be an important predictive parameter in 12 papers with a cut-off value of 5 million in three papers and 10 million in six papers. A TM threshold value of 30% was found in three out of six articles in which TM was found to be a good predictor of success.

Other semen parameters less frequently cited were the initial concentration of the native sperm sample, SCSA, the DNA-fragmentation index, computer-assisted sperm analysis parameters and the Hemizona index.

Table 6.1-I: Overview of papers examining and reporting on the influence of sperm quality on IUI outcome (1982–2011).

Publication	Country	Couples (n)	Cycles (n)	Sperm parameter	Threshold	Type of study
Berker et al. (2012) [164]	Turkey	338		Motility grade A/TMSC	>10 million if motility grade A=0	RA
Sun et al. (2012) [165]	China	412	908	Morphology SC	≥5%	RA
Demir et al. (2011) [166]	Turkey	212	253	TMSC Morphology SC	>10 million >4%	RA
Dorjpurev et al. (2011) [167]	Japan	283	1177	TM TMSC	>30 % >10 million	RA
Nikbakht and Saharkhiz (2011) [168]	Iran	445	820	TMSC IMC Morphology SC	5-10 million >10 million ≥5%	POS
Yang et al. (2011) [169]	China	482		SCSA-DFI	<25%	POS
Youn et al. (2011) [170]	China		383	CASA conc. CASA motility grade AB CASA motility grade A	111 million 51,40% 30,10%	RA
Castilla et al. (2010) [163]	Spain			SCSA-DFI		Structured review
Merviel et al. (2010) [171]	France	353	1038	TMSC	>5 million	RA
Tijani and Bhattacharya (2010) [172]	UK			TMSC	>10 million	Structured review
Badawy et al. (2009) [173]	Egypt	393	714	IMC Morphology WHO	>5 million >30%	POS
Haim et al. (2009) [174]	France		248	Motility grade A	>10%	POS
De La Cuesta Benjumea et al. (2008) [175]	Spain	183	500	IMC	>1,5 million	RA
Guven et al. (2008) [176]	Turkey	232	255	Morphology SC	>4%	RA
Bungum et al. (2007) [177]	Denmark		387	SCSA-DFI	≤30%	RA
Kdous et al. (2007) [178]	Tunisia	138	206	IMC	>1,1 million	RA
Tay et al. (2007) [179]	Malaysia	317	507	IMC/TMSC	>20 million	RA
Arslan et al. (2006) [180]	USA	82	313	HZI	<30%	POS
Mehrannia (2006) [181]	Iran	824	824	IMC	>10 million	RA
Grigoriou et al. (2005) [182]	Greece	615	1641	Morphology SC	>10%	RA
De La Cuesta et al. (2004) [183]	Spain	168	430	IMC	0,8-5 million	RA
Shibahara et al. (2004) [184]	Japan	160	682	Morphology SC CASA-RASP	>15,5% ≥25,5%	POS
Van Weert et al. (2004) [160]	The Netherlands			IMC	0,8-5 million	Meta-analysis
Wainer et al. (2004) [185]	France	889	2564	IMC Morphology WHO	>5 million >30%	RA
Yalti et al. (2004) [186]	Turkey	190	268	TM	>30%	RA
Zhao et al. (2004) [187]	USA	431	1007	TM	>80%	RA
Makkar et al. (2003) [188]	Hong Kong	292	600	IC Morphology SC IMC	>20 million/ml ≥7% >1 million	RA

Ombelet et al. (2003) [162]	Belgium			Morphology SC IMC	>4% >1 million	Structured review
Saucedo de la Llata et al. (2003) [189]	Spain		787	Morphology WHO	>20 million	RA
Lee et al. (2002) [190]	China	209	244	Morphology SC	>4%	POS
Lee et al. (2002) [191]	Singapore	1479	2846	IMC TM	>1 million >30%	RA
Miller et al. (2002) [192]	USA	438	1114	IMC	>10 million	POS
Hauser et al. (2001) [193]	Isreal	108	264	Morphology SC	>4%	POS
Khalil et al (2001) [194]	Denmark	893	2473	IMC	>5 million	RA
Montanaro Gauci et al. (2001) [195]	South Africa		495	Morphology SC	>4%	RA
Van Voorhis et al. (2001) [196]	USA	1039	3479	TMSC TM	>10 million >50%	RA
Van Waart et al. (2001) [159]	South Africa			Morphology SC	>4%	Structured review
Branigan et al. (1999) [197]	USA	414	1100	IMC Sperm survival 24h	≥10 million ≥70%	POS
Dickey et al. (1999) [198]	USA	1841	4056	Motility grade AB TC TMSC	≥30% ≥10 million ≥5 million	RA
Stone et al. (1999) [199]	USA		9963	TMSC TM	≥4 million ≥60%	RA
Cohlen et al. (1998) [200]	The Netherlands	74	308	TMSC	>10 million	POS/RCoT
Shulman et al. (1998) [201]	Isreal	160	544	Semen parameters	Not useful	RA
Van der Westerlaken et al. (1998) [202]	The Netherlands	566	1763	IMC	>10 million	RA
Berg et al. (1997) [203]	Germany	902	3037	IMC	>0,8 million	RA
Karabinus and Gelety (1997) [204]	USA	193	538	Morphology SC	Not useful	RA
Ombelet et al. (1997) [161]	Belgium	373	792	IMC Morphology SC	>1 million >4%	RA
Burr et al. (1996) [205]	Astralia	163	330	Morphology SC IMC	>10% Not useful	RA
Camapana et al. (1996) [206]	Switzerland	332	1115	IMC	>1 million	POS
Huang et al. (1996) [207]	China	939	1375	IMC	>5 million	POS
Ombelet et al. (1996) [208]	Belgium	412	1100	Morphology SC	≥4%	RA
Matorras et al. (1995) [209]	Spain	74	271	Morphology SC	Not useful	POS
Toner et al. (1995) [210]	USA	126	395	IMC Morphology SC	>2 million >4%	RA
Brasch et al. (1994) [211]	USA	546	1205	IMC	>20 million	RA
Francavilla et al. (1990) [212]	Italy	86	411	Morphology WHO TMSC	>50% >5 million	RA
Horvath et al. (1989) [213]	USA	232	451	IMC	>1 million	RA

CASA = computer-assisted sperm analysis; DFI = DNA fragmentation index; HZI = Hemizona index; IC = initial concentration in native sperm sample; IMC = inseminating motile count or post-wash total motile sperm count; POS = prospective observational study; RA = retrospective analysis; RCoT = randomized crossover trial; SC = strict criteria; SCSA = sperm chromatin structure assay; TM = total motility in native sperm sample; TMSC = total motile sperm count in native sperm sample; WHO = World Health Organization criteria.

6.1.5 Discussion

Most selected studies in this search are retrospective and not only vary in the comparison of the study group (different groups of male subfertility) but also in the use or non-use of different ovulation induction regimens, the number of inseminations per treatment cycle, methods of timing ovulation, methods of sperm preparation and use of additives such as platelet-activating factor, pentoxifylline and antioxidants. All these factors may influence the impact of sperm quality on IUI success [65, 214-217]. The most important determinant regarding IUI outcome is undoubtedly the use of ovarian stimulation protocols and, in particular, multifollicular development. According to the meta-analysis of van Rumste et al. [218], multifollicular growth is associated with increased pregnancy rates in IUI with ovarian stimulation, but at the expense of an increased multiple pregnancy rate. The authors also stated that the presence of three or four follicles was associated with an increased multiple pregnancy rate without substantial gain in overall pregnancy rate. They concluded that IUI with ovarian stimulation should not aim for more than two follicles. One stimulated follicle should be the goal if safety is the primary concern, whereas two follicles may be accepted after careful patient counselling.

Also, the duration of subfertility and the female age differed tremendously between studies or were not mentioned at all, although these factors are well recognized to be associated with IUI success, indirectly influencing the impact of semen quality as a predictor of IUI outcome.

Another confounding factor when interpreting these data is the wide and complex variation in methods of sperm preparation and semen testing methodology. A uniform approach in the interpretation of seminal parameters is mandatory, the best example being the persistent variance in sperm morphology scoring between and even within laboratories [34, 79, 158]. This ongoing error associated with inconsistent semen-testing methodologies means that many men at the margins of treatment decision making can wrongly be excluded, or conversely men are sometimes included who perhaps should not be.

As a result of this literature search, the calculations are based on evidence levels 2 or 3. Nevertheless, it seems that the following cut-off values can be used when talking about semen parameters with an important and substantial

discriminative performance in an IUI program: IMC >1 million, sperm morphology using strict criteria >4%, TMCS of 5–10 million and TM of >30%. When using these cut-off levels, the ability to predict pregnancy was limited (poor sensitivity) for all parameters, but the specificity defined as the ability to predict failure to become pregnant was much better.

The results also do not mean that below these cut-off levels IUI can't be used as a good and effective first-line treatment in male subfertility cases, they only indicate that above these threshold levels the success rate after IUI seems to be significantly improved. When reviewing the literature, it is also clear that prewash semen parameters do not always reflect post-wash semen characteristics. Selecting a couple for IUI in male factor infertility cases includes the study of both pre- and post-wash semen characteristics before starting the IUI treatment.

The lack of large prospective cohort studies is easy to understand. Because natural-cycle IUI and clomiphene citrate stimulation are frequently used in IUI programs, the budget for IUI studies is almost negligible when compared with the budget spent on other methods of assisted reproduction such as IVF and ICSI. Studies on the predictive value of sperm quality on IUI success supported and organized by the pharmaceutical industry are not available. It is obvious that the pharmaceutical industry is not really interested in performing good-quality studies at the moment. The lack of valuable studies can also partially be explained by the fact that a lot of IUI procedures are performed as a first-line therapy in non-IVF centers. The experience to perform scientific studies is mostly lower in these centers compared with university-based IVF centers. The results also give the impression that the majority of IVF centers are not really interested in performing prospective high-quality studies in the field of IUI. It should be investigated whether this finding can be explained by a conflict of interest amongst authors who advocate the use of IVF.

A nice example showing the discrepancy between theory (evidence-based medicine) and clinical practice is a study performed in 2002 by Miskry and Chapman [219]. A postal survey was sent to 37 well-known fertility centers within Australia and New Zealand to establish current clinical IUI practice. Although 80% of centers recognized considerable advantages to the patient in terms of risk/benefit ratio and financial cost associated with IUI compared with

IVF in moderate male infertility cases, nearly a third of centers promoted IVF as first-line treatment even in the presence of patent tubes and normal semen. When semen parameters were reduced, IUI was rarely considered. According to the authors, it appears that evidence-based medicine is not yet translated into clinical practice in many units.

Nevertheless, from a societal point of view, considering the economical impact due to the indirect costs associated with IUI because of high rates of multiple pregnancies, well-organized randomized studies are urgently needed to define usable cut-off values for selecting couples for IUI in male subfertility cases, taking into account the cost-effectiveness of the different methods of assisted reproduction [220].

Looking to the future, a marked increase in pregnancy rates with IVF compared with IUI can be expected in general. A recent study modelling outcomes and costs showed that moving directly to IVF might be more cost-effective than starting with gonadotrophin-stimulated IUI for unexplained and mild male factor infertility [221], but in this study only the short-term costs were included in the analysis. The costs of complications arising from multiple births were not included, although these costs are of crucial when talking about cost-effectiveness.

IUI is a simple and noninvasive technique with minimal monitoring and risks, at least if multiple pregnancy rates can be avoided. It can be performed without expensive infrastructure with a reasonable success rate within three or four cycles in most centers. IUI is undoubtedly a more patient-friendly strategy compared with IVF/ICSI [222] and it has been shown that a substantial number of subfertile couples prefer to be treated in a patient-centered clinic rather than going to centers only focusing on success rates. A lack of patient-centeredness was the most cited nonmedical reason for changing fertility clinics [223].

Belgian data on IUI clearly show that, although the pregnancy rate per cycle is significantly higher in IVF/ICSI versus IUI, the price per delivery is significantly lower for IUI [224, 225]. For IUI, the live birth rate per insemination with husband's semen was 7.7%, taking into account that gonadotrophins were only used in 20% of IUI. In almost 30% of cases, IUI was performed in a natural cycle. The multiple pregnancy rate was 5.8% (twins 5.7%, triplet 0.1%). In the same year (2009), the live birth rate per oocyte

recovery for IVF/ICSI was 19.8%, with a multiple pregnancy rate of 11.9% (twins 11.7%, triplet 0.2%). The calculated price per delivery, even not taking into account the higher multiple pregnancy rate for IVF/ICSI, was significantly lower for IUI compared with IVF/ICSI (Ombelet, unpublished data).

Nevertheless, when the difference in cumulative live birth rate per couple between IUI and IVF continues to increase, it will be very difficult to argue that IUI clinics are acting in the best interest of their patients. It's time for action: clinicians are obliged to increase pregnancy rates in IUI programs by making use of different evidence-based strategies improving success rates. It has been proven that at least 10–15 min of immobilization should be applied after every IUI [132], which can result in significantly higher pregnancy rates in higher pregnancy rates compared with single IUI in couples with male factor subfertility [226, 227]. Oral antioxidants given to infertile men with high semen oxidative stress result in significant reduction in semen ROS (reactive oxygen species) and serum Inhibin B levels, significant increase in the sperm linear velocity and per cycle IUI pregnancy rates [228]. Novel sperm selection methods have recently been developed, these methods aim at isolating mature, structurally intact and non-apoptotic spermatozoa with high DNA integrity [217], and their value needs to be investigated in IUI as well.

Until today, the balance of published studies still favors to start with IUI before moving to IVF in the treatment of mild and moderate male subfertility. It is time to realize that a better selection of those couples who benefit most from IUI as a first-line treatment is needed and therefore a better understanding of the effect of sperm quality on IUI success is mandatory. The prevalence of multiple births will become one of the most important determinants in deciding which treatment strategy has to be used, taking into account the economical restraints in most countries.

In conclusion, the literature did not reveal level 1 evidence on the relationship between sperm quality and IUI success. Although more prospective observational cohort studies and well-organized retrospective analyses are urgently needed, this structured review indicates that IMC >1 million with IUI is probably the best cost-effective treatment before starting IVF, irrespective of sperm morphology. More answers to the question as to when to perform IUI in male factor infertility cases will never be obtained until more multicenter

prospective trials according to standard protocols are organized. Despite the current ongoing debate concerning cost-effectiveness of IUI versus IVF in moderate male factor infertility, other factors might be important, such as the well-known differences between both strategies in risk profile and patient satisfaction.

6.2 Factors predicting IUI success

Predictive value of different covariates influencing pregnancy rate following intrauterine insemination with homologous semen: a prospective cohort study

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Original paper

Annelies THIJSSEN, An CREEMERS, Wim VAN DER ELST, Eva CREEMERS, Eva VANDORMAEL, Nathalie DHONT, Willem OMBELET

Accepted for publication in Reprod Biomed Online

6.2.1 Abstract

The aim of this study was to examine the value of different covariates in the prediction of IUI success. During the period of July 2011 until September 2015, data from 1401 IUI cycles with homologous semen in 556 couples were collected prospectively, by means of a questionnaire, in a tertiary referral infertility center. Statistical analysis was performed using Generalized Estimating Equations (GEE). GEE was used instead of an ordinary logistic regression model to take into account the correlation between observations from the same person. The primary outcome parameter was clinical pregnancy rate (CPR), confirmed with a gestational sac and fetal heartbeat on ultrasonography at 7-8 weeks of gestation. An overall CPR of 9,5% per cycle was observed. Univariate statistical analysis revealed female and male age (p=0,0007 and p=0,0127 respectively), male smoking (p=0,0165), female BMI (p=0,0319), ovarian stimulation (p=0,0102) and inseminating motile count (IMC; p=0,0273) as covariates significantly influencing CPR per cycle. Multivariate GEE analysis revealed that the only valuable prognostic covariates included female age (p=0,0005), male smoking (p=0,0079) and infertility status (i.e. primary/secondary infertility; p=0,0403). IMC showed a significant curvilinear relationship (p=0,0062), with first an increase and then a decrease of the pregnancy rate.

KEYWORDS: homologous semen, IUI, prognostic factor, prospective cohort study, success rate

6.2.2 Introduction

Homologous intrauterine insemination (IUI) is generally used as a first-line treatment for couples with subfertility due to ejaculatory disorders, cervical factor infertility, moderate male factor and unexplained infertility.

Throughout the years, there has always been a search for predictive factors, influencing successful ongoing pregnancy rates after IUI. Multiple studies have reported on the impact of factors such as female age [167, 173, 179, 194, 195, 199, 204, 206, 229-231], duration of infertility [232, 233], type of infertility [179, 194, 195, 204], hormone levels (i.e. follicle stimulating hormone (FSH), estradiol, progesterone) [234], use of different ovarian stimulation protocols [194, 200, 204, 230, 235-238], timing/induction of ovulation [194, 239, 240], number of preovulatory follicles [194, 195, 199, 232, 234], endometrial thickness by the time of ovulation [194, 232], frequency of insemination (single or double) [241-243], sperm parameters (i.e. concentration [195], progressive motility [187, 195, 199, 229, 232, 234], morphology [161, 187, 195, 204, 209, 210, 244], total motile sperm count (TMSC) [179, 202, 206, 245], inseminating motile count (IMC) [161, 194, 199, 230]) and sperm washing procedures [199, 204, 246, 247]. Also the influence of body mass index (BMI) [234] and smoking habits [139] on IUI outcome have been studied before. However, results on the predictive value of these parameters remain highly contradicting.

The vast majority of studies performed in the past are retrospective cohort studies, which has led to a great variety in outcome results because of differences in patient selection criteria, presence of various infertility factors, use or non-use of different ovarian stimulation protocols, number of inseminations per treatment cycle, number of cycles performed, methods of timing ovulation, sites of insemination, sperm parameters and sperm preparation techniques. Furthermore, since most studies are based on small patient populations and because clinical pregnancy rates (CPRs) per cycle with IUI are generally low, interpretation of study results is complicated and results vary. Therefore, controversy remains about the effectiveness of IUI, especially in relation to more refined techniques such as in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI).

This study aimed to prospectively evaluate the extent to which the

pregnancy outcome after homologous IUI is influenced by certain covariates such as age, smoking habits, BMI, infertility status, ovarian stimulation method, easy or difficult insemination, sperm quality parameters, etc. Covariates taken into account for our study were based on a thorough review of the literature [36] and our personal experience.

6.2.3 Materials and Methods

Patients

During the period between 1 July 2011 and 30 September 2015, the medical records of 556 subfertile couples, who received a total of 1401 homologous IUI cycles, were reviewed prospectively in a tertiary referral infertility center. During the 20 minutes of mandatory bed rest following IUI [132, 133], a midwife sat next to the patient to review all possible contributing factors together by means of a questionnaire. On a monthly basis, the results of the questionnaires were examined for possible lack of data. Institutional Review Board approval was obtained for this study.

All couples had been trying unsuccessfully to conceive for at least one year. Prior to IUI treatment, female patients were subjected to an infertility work-up, including medical history, physical examination, pelvic ultrasound, serum hormone assays between day 2 and 4 of the menstrual cycle, ultrasound monitoring of folliculogenesis, ovulation assessment by mid-lutheal phase concentrations of progesterone in women with regular cycles, and a mid-cycle post-coital test. Hysterosalpingography (HSG) and/or laparoscopy was used to assess the uterine cavity and presence of at least one patent tube. In case of a suspected tubal or uterine abnormality, a hysteroscopy and/or laparoscopy was performed. In all men at least two sperm examinations, microbiological tests and analysis of anti-sperm antibodies (ASA) were performed. All couples were tested for hepatitis B and hepatitis C virus before receiving any treatment. Couples suffering from unexplained infertility, including mild endometriosis, oligo-/anovulation and moderate male factor infertility, with at least one patent fallopian tube and an IMC of >1 million were considered eligible for IUI treatment. IUI cycles with use of frozen semen or escape IUI cycles, i.e. couples

allocated to IVF/ICSI treatment who received escape IUI treatment because of low response to ovarian stimulation, were excluded from the study.

Covariates

Covariates taken into account included female and male age (years), smoking (non-smoking, 1-14 cigarettes a day, ≥15 cigarettes a day), BMI (kg/m^2), primary/secondary infertility, cycle number, ovarian stimulation method (natural cycle (NC), clomiphene citrate (CC), human menopausal gonadotropin (hMG)/recombinant FSH (recFSH)), day (D) 0 estradiol (ng/l) and progesterone (µg/l) levels, abstinence period (days), human chorionic gonadotropin (hCG)-insemination time interval (hours), easy or difficult insemination (easy-clamp (C), easy+C, difficult+C, difficult+C+dilator, intracervically), occurrence of blood loss after insemination and sperm quality parameters (i.e. volume (ml), concentration (million/ml), total count (million), motility grade A (%), motility grade A+B (%), TMSC grade A (million), TMSC grade A+B (million), morphology (%), ASA (no factor, 10-50%, >50%) and IMC (million)).

Ovarian stimulation

Patients were treated in a natural cycle in case of regular cycles. Ovarian stimulation with CC or hMG/recFSH protocols was used in case of unexplained infertility or oligo-/anovulation. With the CC protocol, a single dose of clomiphene (50 mg or 100 mg ; Clomid®, Sanofi, Belgium) was administered on days 3, 4, 5, 6 and 7 of the cycle. hMG and/or recFSH (Menopur®, Ferring, Belgium; Puregon®, MSD, Belgium) were administered in a minimal dose step-up regimen, starting off with 50 IU or 75 IU on day 3 of the cycle. Follicular ultrasonography and serum estradiol determination were carried out on day 8-9 of the cycle and thereafter every other day. hCG 5000 IU injection (Pregnyl®, MSD, Belgium) was given to induce ovulation when the average diameter of the dominant follicle was 18 mm or more, except when spontaneous LH rise had already occurred on the day of hCG injection (1,8%; 25/1401). LH was measured on a serum sample via electrochemiluminescence immunoassay (ECLIA; Cobas®, Cobas e602, Roche Diagnostics, Belgium). If 3 or more follicles of at least 15 mm were present, the cycle was cancelled and protected intercourse was advised.

Sperm examination and preparation

On the day of insemination, the semen sample was obtained through masturbation after a 2-5 day abstinence period and collected in a sterile cup. Within one hour of production and after liquefaction at room temperature, the specimen was examined for initial volume, concentration and progressive motility according to World Health Organization (WHO) guidelines [23, 77]. TMSC was determined by multiplying grade A or grade A+B sperm motility percentages with sperm volume and concentration. Sperm capacitation was performed using density gradient centrifugation (DGC) with PureSperm® (PureSperm® 40/80, Nidacon), according to the manufacturer's instructions, in order to remove seminal fluids and enhance sperm quality for IUI. IMC was determined after sperm preparation by multiplying the percentage hyperactive and grade A motility spermatozoa with sperm volume (1 ml) and concentration. Morphology and ASA scores were adapted from the first semen examination since these parameters are not routinely examined on IUI samples in our laboratory. Sperm morphology was scored according to strict criteria [78] after Papanicolaou staining [23, 34, 79, 248]. ASA detection on spermatozoa was performed using SperMar IgA/IgG tests (FertiPro) according to the manufacturer's instructions.

Intrauterine insemination

IUI was performed at 24-36h post-hCG. A fraction of the washed motile spermatozoa was inserted up to the uterine fundus and expelled into the uterine cavity. The women remained supine for 20 min after IUI. Serum β-hCG was determined 14-16 days after IUI. Clinical pregnancy was confirmed when presence of a gestational sac and fetal heartbeats showed on ultrasonography 7-8 weeks after IUI.

Statistical analysis

Since most patients received multiple treatments, either because of failed previous attempts or in order to have a second, third, … child, observations could not be analyzed as independent. Classical statistical analysis, like t-tests, linear regression models, logistic regression models, etc., all assume independency of the dataset and do not take into account the correlation

between observations from the same person. Therefore, type 3 Generalized Estimating Equations (GEE) [134] were used instead of an ordinary logistic regression model to model the probability to become pregnant. The correlation structure was assumed to be of an 'exchangeable' type and statistical significance was established at $p<0.05$. All GEE analyses were done in the software package SAS® version 9.4 for Windows (Belgium). For more detailed information about GEE analysis, we refer to Molenberghs and Verbeke [135].

Tables 6.2-I to 6.2-IV represent the univariate relationships between the different covariates. Care should be taken with the interpretation of these results since the effect of only one covariate at a time is shown, which could be influenced by other factors. Continuous variables were categorized for presentation only, not for actual statistical analysis. Results from **Tables 6.2-II to 6.2-IV** are presented as graphs in **Appendix F**.

6.2.4 Results

A total of 1401 IUI treatments were given to 556 subfertile couples. The pregnancy outcome was unknown in a total of 8 IUI cycles (0,6%) because these were foreign patients who were followed abroad. CPR outcome was considered negative in case of negative β-hCG measurement (86,9%; 1210/1393), biochemical pregnancy (1,4%; 20/1393), extrauterine gestation (0,2%; 3/1393) and early miscarriage, i.e. presence of a gestational sac without heartbeat (2,0%; 28/1393). Positive CPR outcome was considered when singleton or twin pregnancies with fetal heartbeat (9,5%; 132/1393) were visualized on ultrasound at 7-8 weeks of gestation. Per pregnancy multiple pregnancy rate in our study was 6,1% (8/131), with 10,9% (6/55) resulting from ovarian stimulation with hMG/recFSH, 3,8% (2/53) resulting from stimulation with CC and none (0/24) resulting from IUI in a natural cycle.

An overview of the main characteristics of the study population is provided in **Table 6.2-I**. Results are given for the total patient group and for the pregnant and not pregnant groups separately.

Table 6.2-I: Main characteristics of the study population

	Total	Pregnant	Not pregnant	p-value
No. cycles	1401[a]	132	1261	
No. couples	556[b]	132	420	
Patient characteristics				
Age patient (years)	32,1±5,0 (19,1-46,9)	30,6±5,0 (19,1-43,3)	32,3±5,0 (20,6-46,9)	**<0,01**
Age partner (years)	34,6±6,0 (22,3-62,5)	33,2±5,9 (23,0-52,8)	34,7±6,0 (22,3-62,5)	**0,01**
BMI patient (kg/m^2)	23,9±4,5 (16,3-43,4)	24,6±4,7 (16,9-42,8)	23,8±4,5 (16,3-43,4)	**0,04**
BMI partner (kg/m^2)	25,8±3,5 (16,7-46,1)	26,1±3,3 (19,4-35,4)	25,8±3,5 (16,7-46,1)	NS
IUI procedure characteristics				
hCG-insemination interval (hours)	20,8±4,2 (1,0-31,0)	20,9±4,4 (6,0-30,3)	20,8±4,2 (1,0-31,0)	NS
Estradiol D0 (ng/l)	417,0±242,9 (12,0-1700,0)	409,4±222,7 (101,0-1182,0)	418,5±245,5 (12,0-1700,0)	NS
Progesteron D0 (µg/l)	0,7±0,9 (0,1-20,1)	0,7±1,0 (0,1-12,0)	0,7±0,9 (0,1-20,1)	NS
Sperm characteristics				
IMC (million)	17,1±20,1 (0,0-180,4)	14,0±13,3 (0,0-68,4)	17,3±20,5 (0,0-180,4)	**0,01**
Concentration (million/ml)	60,8±47,2 (0,4-408,0)	57,8±38,8 (7,9-228,0)	61,0±47,2 (0,4-408,0)	NS
Total concentration (million)	182,9±172,4 (0,85-2622,0)	171,7±120,7 (11,7-660,0)	182,3±163,3 (0,85- 1625,0)	NS
Grade A motility (%)	17,4±12,0 (0,0-67,0)	17,2±12,4 (0,0-48,0)	17,4±12,0 (0,0-67,0)	NS
Grade A+B motility (%)	48,4±14,7 (0,0-86,0)	47,6±13,5 (10,0-77,0)	48,5±14,9 (0,0-86,0)	NS
TMSC-A (million)	38,2±59,7 (0,0-1075,0)	32,9±44,9 (0,0-273,6)	37,9±53,6 (0,0-565,9)	NS
TMSC-AB (million)	93,0±100,9 (0,0-1704,3)	83,9±74,7 (3,3-434,7)	9,7±92,9 (0,0-843,4)	NS
Morphology (%)	5,1±3,5 (0,0-55,0)	5,0±3,2 (0,0-14,0)	5,1±3,5 (0,0-55,0)	NS

[a] Outcome unknown in eight cycles. [b] Outcome unknown in four couples. BMI: body mass index; D0: day 0; hCG: human chorionic gonadotropin; IMC: inseminating motile count; SD: standard deviation; TMSC: total motile sperm count. Data are presented as mean±SD (min-max).

Univariate analysis

Covariates related to patient characteristics

CPR per cycle decreased significantly with both advancing patient and partner age (p=0,0007 and p=0,0127 respectively). A higher patient BMI resulted in a significantly higher CPR (p=0,0319) up until a BMI of 30 kg/m^2, whereas partner BMI did not significantly influence CPR per cycle (p=0,4184). On the other hand, smoking significantly negatively affected CPR in cases where the male partner smoked 1-14 cig. a day compared with non-smokers (p=0,0165), however patient smoking did not significantly affect outcome results. Furthermore, the univariate statistical analysis did not show a significant difference between patients presenting with primary or secondary infertility (results not shown). Results are shown in **Table 6.2-II**.

Covariates related to the IUI procedure

Ovarian stimulation showed to be the only IUI procedure related factor significantly influencing CPR per cycle. Cycles stimulated with CC resulted in a significantly lower CPR compared with cycles stimulated with hMG/recFSH (p=0,0102) (**Table 6.2-III**). Hormone levels of estradiol and progesterone on day 0 and time interval between hCG injection and insemination had no significant influence on CPR per cycle (**Table 6.2-III**). Also, easy or difficult insemination, occurrence of blood loss during or after insemination, number of days abstinence before delivery of the semen sample for IUI and number of IUI attempts did not significantly influence CPR per cycle (results not shown).

Covariates related to sperm quality

CPR showed a steady increase with increasing IMC values up until an IMC of 9,99 million, after which it dropped significantly (p=0,0273). CPRs were the highest when sperm concentration was 15-19,99 million/ml, total sperm count was 73,8-143 million, grade A sperm motility was 8-15%, grade A+B sperm motility was 32-49,99%, TMSC grade A was 3-4,99 million or TMSC grade A+B was 31-64 million, however these results were not statistically significant. Furthermore, although ASA scores of more than 50% resulted in slightly lower CPRs per cycle, this result was not significantly different from cycles with no ASA

factor or 10-50% ASA (data not shown). Also sperm morphology scores did not significantly influence CPR per cycle. Results from the univariate statistical analysis on covariates related to sperm quality are shown in **Table 6.2-IV**.

Table 6.2-II: Univariate analysis on covariates related to patient characteristics

	CP	Total	CPR	SE	p-value
Age patient (years)					
<30	69	533	0,129	±0,014	**0,0007**
30-34,99	36	483	0,075	±0,012	
35-39,99	21	263	0,080	±0,017	
≥40	7	113	0,062	±0,023	
Age partner (years)					
<30	42	340	0,124	±0,018	**0,0127**
30-34,99	48	492	0,098	±0,013	
35-39,99	25	311	0,080	±0,015	
≥40	17	249	0,068	±0,016	
Smoking patient (n/day)					
0 cig.	115	1183	0,097	±0,009	NS[a]
1-14 cig.	14	178	0,079	±0,020	NS[b]
≥15 cig.	3	32	0,094	±0,052	NS[c]
Smoking partner (n/day)					
0 cig.	106	976	0,109	±0,010	**0,0165**[a]
1-14 cig.	16	270	0,059	±0,014	NS[b]
≥15 cig.	10	147	0,068	±0,021	NS[c]
BMI patient (kg/m^2)					
<20	16	245	0,065	±0,016	**0,0319**
20-24,99	58	728	0,080	±0,010	
25-29,99	43	264	0,163	±0,023	
≥30	14	149	0,094	±0,024	
BMI partner (kg/m^2)					
<20	3	30	0,100	±0,056	NS
20-24,99	55	595	0,092	±0,012	
25-29,99	55	606	0,091	±0,012	
≥30	19	154	0,123	±0,027	

BMI: body mass index; cig.: cigarettes; CP: number of clinical pregnancies; CPR: clinical pregnancy rate; SE: standard error.

Age and BMI were continuous variables, therefore p-values represent overall significance levels. Smoking was a categorical variable and p-values represent if CPR in one of the groups significantly differed from another. [a] 0 cig. versus 1-14 cig., [b] 0 cig. versus ≥15 cig., [c] 1-14 cig. versus ≥15 cig.

Table 6.2-III: Univariate analysis on covariates related to the IUI procedure

	CP	Total	CPR	SE	p-value
Stimulation					
NC	24	223	0,108	±0,021	NS[a]
CC	53	707	0,075	±0,01	NS[b]
hMG/recFSH	55	461	0,119	±0,015	**0,0102**[c]
hCG-insemination interval (hours)					
<15	5	41	0,122	±0,052	NS
15-22,99	79	863	0,092	±0,010	
≥23	48	463	0,104	±0,014	
Estradiol D0 (ng/l)					
12-240	40	342	0,117	±0,017	NS
241-368	30	342	0,088	±0,015	
369-533	23	340	0,068	±0,014	
534-1700	37	344	0,108	±0,017	
Progesteron D0 (μg/l)					
<0,5	41	457	0,090	±0,013	NS
0,5-0,99	71	741	0,096	±0,011	
1-1,49	13	123	0,106	±0,028	
≥1,5	5	38	0,132	±0,056	

CC: clomiphene citrate; CP: number of clinical pregnancies; CPR: clinical pregnancy rate; D0: day 0; hCG: human chorionic gonadotropin; hMG: human menopausal gonadotropin; NC: natural cycle; recFSH: recombinant follicle stimulating hormone SE: standard error.

Levels of estradiol and progesterone at D0 and the hCG-insemination time interval were continuous variables, therefore p-values represent overall significance levels. Stimulation was a categorical variable and the p-value represents a significant difference in CPR between NC or CC and hMG/recFSH stimulated groups: [a] NC versus CC, [b] NC versus hMG/recFSH, [c] CC versus hMG/recFSH.

Table 6.2-IV: Univariate analysis of covariates related to sperm quality

	CP	Total	CPR	SE	p-value
IMC (million)					
<1	9	161	0,056	±0,018	
1-1,99	4	74	0,054	±0,026	
2-4,99	18	188	0,096	±0,022	**0,0273**
5-9,99	39	270	0,144	±0,021	
≥10	62	697	0,089	±0,011	
Concentration (million/ml)					
0-4,99	0	36	0,000	±0,000	
5-9,99	1	45	0,022	±0,022	
10-14,99	6	70	0,086	±0,034	NS
15-19,99	8	62	0,129	±0,043	
20-408	116	1177	0,099	±0,009	
Total count (million)					
0,85-73,7	26	345	0,075	±0,014	
73,8-143	40	343	0,117	±0,017	NS
144-243	34	345	0,099	±0,016	
244-2620	31	343	0,090	±0,016	
Grade A motility (%)					
0-7	35	347	0,101	±0,016	
8-15	35	333	0,105	±0,017	NS
16-24	27	346	0,078	±0,014	
25-67	35	366	0,096	±0,015	
Grade A+B motility (%)					
<20	2	47	0,043	±0,030	
20-31,99	11	145	0,076	±0,022	NS
32-49,99	61	519	0,118	±0,014	
≥50	58	681	0,085	±0,011	
TMSC-A (million)					
0-2,99	11	218	0,050	±0,015	
3-4,99	11	91	0,121	±0,034	NS
5-9,99	17	154	0,110	±0,025	
10-86	92	913	0,101	±0,010	
TMSC-AB (million)					
0,0-30	27	346	0,078	±0,014	
31-64	46	339	0,136	±0,019	NS
65-125	31	345	0,090	±0,015	
126-1704,3	27	345	0,078	±0,014	
Morphology (%)					
<4	54	535	0,101	±0,013	
4-5,99	26	308	0,084	±0,016	NS
≥6	51	535	0,095	±0,013	

CP: number of clinical pregnancies; CPR: clinical pregnancy rate; IMC: inseminating motile count; SE: standard error; TMSC: total motile sperm count.
All sperm parameters were continuous variables, therefore the p-values represent the overall significance level.

Multivariate GEE analysis

According to the final multivariate GEE model (**Table 6.2-V**), the covariates patients' age, smoking partner, primary/secondary infertility and IMC showed to significantly influence CPRs. Female age was a continuous variable with a negative parameter estimation. This indicates that with increasing values for patients' age, CPRs will decrease when corrected for the other significant covariates (p=0,0005). The categorical parameters, i.e. smoking partner and primary/secondary infertility, were compared with their respective reference category. Cycles in which partners smoked 1-14 cigarettes a day showed a significantly lower CPR compared with cycles with a non-smoking partner (p=0,0079). However, there was no significant difference in CPR when partners smoked more than 15 cigarettes a day compared with non-smoking partners, but the p-value was rather borderline, indicating a possible effect of partner smoking on CPR (p=0,0888). When CPRs were compared in groups with partners smoking 1-14 cigarettes a day versus partners smoking more than 15 cigarettes a day, outcome results were not significantly different. Couples suffering from primary infertility showed a significantly lower CPR compared with couples presenting with secondary infertility (p=0,0403).

For the parameter IMC, we had to take into account the curvilinear relationship, showing first an increase followed by a decrease in CPR with increasing values for IMC, which was seen in the univariate analysis. This was done by modeling the parameter of IMC squared. This resulted in a non-significant influence of IMC, but the curvilinear relationship of IMC^2 resulted in a significant p-value of 0,0062, indicating the existence of an optimum at IMC 5-9,99 million.

Based on the results of the final multivariate GEE model, we were able to build a calculation tool for the prediction of CPR for a given patient. Values can be entered for female age (years) and IMC (million), whereas smoking of the partner and primary/secondary infertility can be added by entering value '0' for 'untrue' and '1' for 'true'. For the CPR calculation tool see the supplementary table available on the website.

Table 6.2-V: Results from the multivariate GEE analysis

Covariate	Parameter estimation (SE)	p-value
Intercept	-0,0612 (0,7582)	0,9357
Age patient	-0,0739 (0,0216)	**0,0005**
Smoking partner (≥15 cig.)	0,6388 (0,3352)	0,0888
Smoking partner (1-14 cig.)	0,6825 (0,2879)	**0,0079**
Smoking partner (0 cig.)	reference	
Infertility (primary)	-0,4053 (0,1912)	**0,0403**
Infertility (secondary)	reference	
IMC	-0,0125 (0,0109)	0,2391
IMC^2	-0,0007 (0,0003)	**0,0062**

Cig.: cigarettes, IMC: inseminating motile count, SE: standard error.

6.2.5 Discussion

Due to the shortage of good-quality prospective cohort studies and the persistent lack of standardization in study protocols, controversy remains about the effectiveness of IUI and the question is increasingly asked if we should continue performing IUI in the future. This study aimed to elucidate the factors significantly influencing IUI outcome in order to strive for a better patient selection and prediction of success rates following IUI treatment.

Overall success rates per IUI cycle are rather low in comparison with other techniques such as IVF/ICSI, with a generally accepted range of 10-20% CPR per cycle for all etiologies [249]. Results from our study showed an overall CPR per cycle of 9,5% and a multiple pregnancy rate of 6,1%, which was in accordance with the results proposed by the latest report of the Belgian Register for Assisted Procreation (BELRAP; 9,9% and 5,7% respectively) [136]. In order to increase the success rate, IUI treatment is often performed in conjunction with ovarian stimulation. The Belgian law implies the use of CC for ovarian stimulation before moving on to the use of gonadotropins. Unfortunately, CC does not seem to have a significant influence on the success rate of IUI [194, 230, 235], as was also demonstrated by the results reported in this study. Ovarian stimulation with use of hMG/recFSH protocols on the other hand did result in significantly higher pregnancy rates according to some studies [236, 238], however others described no significant influence of the type of stimulation on CPR after IUI [200, 204, 231]. Our results showed a significantly higher CPR in IUI cycles stimulated with hMG/recFSH compared with CC

stimulated cycles in the univariate analysis, but this was not statistically significant in the multivariate analysis.

Women presenting with secondary infertility appeared to have higher CPRs compared with primary infertile women, which is in accordance with the results proposed by Dinelli et al. [231]. This could be explained by the fact that secondary infertile women present with a proven fertility, since they have been pregnant before (either normal or pathological) with the same or a different partner.

Female age has been linked with IUI success before by multiple studies [167, 173, 179, 194, 195, 199, 204, 206, 229-231]. An overall consensus in assisted reproductive technology (ART) is that female age represents an indirect indicator of oocyte quality [249]. Therefore, CPRs decrease significantly with increasing female age, as was also indicated by the results in our study.

A systematic review by Ombelet et al. [36] on semen quality and the prediction of IUI success revealed sperm morphology using strict criteria, TMSC and IMC amongst the sperm parameters most frequently examined and cited in relation to IUI success. In contrast with other studies, most frequently showing increased pregnancy rates with a sperm morphology score of more than 4% [159, 162, 166, 176, 190, 193, 195, 210], our results did not show a significant influence of sperm morphology on CPR. Furthermore, TMSC is a parameter that is very inconsistently used amongst studies. Most frequently, studies do not describe when and how TMSC was calculated [167, 198]. Some studies report TMSC values pre-wash [168, 171], while others report post-wash TMSC, the latter often representing IMC reported in other studies [206, 250]. Also, some studies count TMSC using the percentage of progressively motile sperm [168], whilst others use total motile sperm [179]. This discrepancy makes it very difficult to compare results for TMSC amongst different studies. According to the systematic review by Ombelet et al. [36], a TMSC of more than 10 million was most frequently indicated as a threshold above which pregnancy rates after IUI increase significantly. In our study, TMSC did not show to have significant influence on CPR, although TMSC-A indicated higher CPRs starting from 3-4,99 million. Finally, results for IMC values in our study showed a steady increase in CPRs with increasing IMC starting from an IMC of 2 million up until 9,99 million, after which CPRs decreased again. In accordance with a recent study by

Lemmens et al. [251], our multivariate analysis showed the existence of an optimum, with the highest CPRs obtained at an IMC of 5-9,99 million spermatozoa. It can be hypothesized that couples with a good male fertility do not benefit from IUI treatment [252]. Apart from IMC, a slight decrease in CPR was also noted with high values of A+B motility and TMSC-AB in our study. It is possible that with these patients, other unknown female factors contributed to the decreased CPR and more elaborate female fertility checkups should be performed, for example a diagnostic laparoscopy, as was also recently proposed by Lemmens et al. [251]. Another possible explanation for the decreased CPR at high IMC levels could be the extensive generation of reactive oxygen species (ROS) by spermatozoa undergoing sperm washing and centrifugation. The higher the concentration of spermatozoa, the more ROS is being produced, possibly affecting sperm motility and fertilizing ability [253, 254].

Female BMI showed to be of significant influence in the univariate statistical analysis, since CPRs increased significantly with increasing BMI up until a BMI of 30 kg/m^2. However, this result was no longer significant in the multivariate model. Our results are largely in accordance with the results proposed by Wang et al. [144], who also demonstrated a significant increase in fecundity from underweight to obese women. However, other studies reported no significant differences in pregnancy rates amongst different BMI groups [140-142] or demonstrated a negative effect of high BMI on pregnancy rates [143]. It has also been pointed out that when ovarian stimulation is adjusted to overcome the weight effect, CPRs in obese women are comparable to women with a normal BMI [140].

Finally, studies relating male smoking and fertility are mostly reporting on significantly reduced sperm concentrations, motility and morphology [255]. However, these results do not conclusively indicate an impact of smoking on male fertility as such. Results presented in this study showed that couples with a male smoking partner had significantly reduced CPRs. No significant influence on CPR could be detected for women smoking in our study, however there were only 32 cycles reported in which women smoked more than 15 cigarettes a day. Nonetheless, a study by Farhi and Orvieto [139] was also unable to detect a significant difference in pregnancy rate between groups of smoking and non-smoking women receiving ovarian stimulation and IUI treatment. However, they

concluded that smoking women required a significantly higher gonadotropin dosage than non-smoking women to achieve comparable pregnancy rates.

In conclusion, although this was a cohort study and patients were not prospectively randomized into groups, there are some strengths associated with this study. Firstly, data were recorded prospectively by means of a questionnaire that was completed by a midwife sitting next to the patient during the 20 minutes of mandatory bed rest after insemination. Secondly, GEE analysis instead of an ordinary logistic regression model was used to statistically analyze the data to take into account the correlation between observations from the same patient. The final multivariate GEE model revealed that the only valuable prognostic covariates for the prediction of CPR after homologous insemination included female age, male smoking and infertility status (i.e. primary/secondary infertility). IMC showed a significant curvilinear relationship, with first an increase and then a decrease of the pregnancy rate.

Appendix B

Questionnaire Belgian centers

Questionnaire: Artificial insemination with donor sperm (AID) in Belgium

General information

Name of the clinic: ..... City.....

1. **Does your centre perform AID?**
   ☐ No ☐ Yes
2. **If yes, how many cycles per year?**
   ☐ 0-200 ☐ 200-400 ☐ 400-600 ☐ More than 600 ☐ Unknown
3. **Where do you obtain your donor sperm from?** *Multiple answers are possible*
   ☐ Own sperm bank
   ☐ Belgian sperm bank
   Which centre? .....
   ☐ International sperm bank
   Which centre?
   ☐ Cryos
   ☐ Nordic
   ☐ Other:.....
4. **In your centre, what ratio is used for AID of:** *Total = 100%*
   ☐ Donor sperm from own centre: .....%
   ☐ Imported Belgian donor sperm: .....%
   ☐ Imported international donor sperm: .....%
5. **Which patients can be treated with AID in your centre?** ***Multiple answers are possible***
   ☐ Hetero couples ☐ Lesbian couples ☐ Singles
6. **In your centre AID is**
   ☐ Always anonymous ☐ Never anonymous ☐ Both anonymous and non-anonymous
7. **This question is only for centers with their own sperm bank :**
   Can you indicate how you recruit the sperm donors?
   ☐ Orally (e.g. during lectures or couples coming to or previously treated in the IVF centre)
   If yes, describe how (for example: solidarity principle)
   .....
   ☐ Through website
   ☐ Advertisement in papers or magazines
   If yes, where and how? .....
   ☐ Flyers in centre
   ☐ Other (specify):.....

Detailed information

1. **Number of cycles**

- **How many cycles with donor sperm do you perform in your centre each year?**

2011:	☐	0-200	2012:	☐	0-200
	☐	200-400		☐	200-400
	☐	400-600		☐	400-600
	☐	more than 600		☐	more than 600

2. **Donor Recruitment**

- **How many candidate donors apply to your centre each year for a first semen analysis?**

[.....] candidate donors a year

- **How many of the recruited candidate donors are accepted?**

[.....] %

- **Do you work with a contract which binds the sperm donor to donate in your centre only?**

☐ No ☐ Yes

3. **Screening/selection criteria**

- **What are the inclusion-/exclusion criteria for sperm donors?**

☐ Age
≥ [.....] yr. ≤ [.....] yr.

☐ Sperm quality

Fresh semen:

Conc: ≥ [.....] m/ml Mot: ≥ [.....] % Morph: ≥ [.....] % Vol: ≥ [.....] ml

After thawing:

IMC ≥ [.....]

- **Are candidate sperm donors psychologically screened before acceptance as a donor?**

  ☐ No ☐ Yes

- **Which serological tests are performed on the (candidate) sperm donor? (Multiple answers are possible)**

  ☐ HCV ☐ Syphilis ☐ HIV1,2 ☐ Other (specify):.....

  ☐ HBV ☐ Chlamydia ☐ CMV

- **If a candidate sperm donor tests positive for CMV, the candidate donor is:**

  ☐ Refused

  ☐ Accepted, but matched with a CMV+ acceptor

  ☐ Other (specify):.....

- **Is there a quarantine nitrogen vessel for donor samples that are not yet serologically screened?**

  ☐ No ☐ Yes

- **If your centre accepts CMV+ sperm donors, is there a separate quarantine nitrogen vessel for CMV+ sample storage?**

  ☐ No ☐ Yes

- **Is there a chromosomal test performed on the (candidate) sperm donor? (Multiple answers are possible)**

  ☐ No ☐ Yes

  ☐ CF-screening ☐ Karyotype ☐ Other (specify):.....

- **Is there a test procedure for sperm washing performed on the semen sample?**

  ☐ No ☐ Yes

  ☐ Before freezing ☐ After freezing ☐ Before and after freezing

4. Freezing and washing procedures

- **How is the sperm sample frozen?**

  ☐ Fresh ☐ Washed ☐ Concentrated ☐ Capacitated

  ☐ Gradient centrifugation

  ☐ Swim-up

  ☐ Other (specify):.....

- **Which carrier material is used for sperm freezing?**

  ☐ Straws ☐ Vials ☐ Other (specify):.....

5. Cost

- **Is the sperm donor reimbursed?**

  ☐ No

  ☐ Yes

  [.....] € per donation

- **If yes, when is the sperm donor reimbursed?**

  ☐ After every donation

  ☐ At the end of the donation series

  ☐ Other (specify):.....

Appendix C

Summary of the Belgian legislation concerning donor insemination

Who can apply for donor insemination in Belgium?

A request for the implantation of embryos or insemination with gametes may be submitted by adult women up to the age of 45 years. However, the implantation of embryos or insemination with gametes cannot be performed in women older than 47 years.

Reimbursement of the donor

The donation of gametes is free of charge. However, the King may determine a fee to cover for the donor's travel expenses or loss of wages.

Data of the donor

The following donor data are recorded by the fertility center: (i) medical information related to the gamete donor that can be important for the healthy development of the unborn child; (ii) the physical characteristics of the gamete donor; (iii) the information which is necessary for the application of this law. The King lays down a system for the exchange of information between the fertility centers. The medical information related to the gamete donor may be communicated by the fertility center (i) to the recipient of the gametes or the pair that receives the gametes, when they ask for it at the time that they make a decision or (ii) to the extent that the health of the person which is conceived by the insemination with gametes requires this, to the latter's general practitioner or to that of the recipient of the gametes or the pair that receives the gametes.

Lineage

From insemination of the donated gametes, the lineage is determined to the advantage of the candidate parent(s) who received the gametes. Donors of gametes cannot set a legal claim on the lineage or the resulting financial consequences. The recipient(s) of gametes and the child born through gamete insemination cannot bring an action concerning the lineage or the resulting financial consequences to the donor(s) of gametes.

When gametes are used for a donation program, the fertility center should

make any information that could lead to the identification of the donor inaccessible. Non-anonymous donation is permitted resting on the consent of the donor and the recipient(s).

Selection criteria and biological tests

The donors are selected based on their age, health and medical history using a questionnaire and an interview with a qualified and trained practitioner of a health professional. This assessment must include all relevant factors that may assist in identifying and screening out persons whose donation could be dangerous to the health of others, such as the possibility of transmitting diseases (such as sexually transmitted infections), or health risks to themselves (e.g. superovulation, sedation, the risks from the egg collection procedure or the psychological consequences of being a donor).

The donors must be negative on testing for HIV 1 and 2, HCV and HBV and syphilis on a serum or plasma sample. Moreover, sperm donors must respond negatively to a test for Chlamydia on a urine sample tested by the nucleic acid amplification technique (NAT). There are tests for HTLV-I antibodies performed in donors living in or coming from an area with a high incidence of the infection or with sexual partners or parents in such an area. In certain situations, further tests are needed, depending on the case history of the donor and the characteristics of the donated human body material (e.g. RhD, malaria, CMV, Trypanosoma cruzi).

After permission has been granted, a genetic screening is performed for autosomal recessive genes which, through international scientific data, are known to occur in the ethnic background of the donor and an assessment is made for the risk of transmission of inherited disorders which are known to occur in the family.

For donations other than by partners blood samples should be collected at the time of each donation.

Donations of gametes by others than they are kept in quarantine for at least 180 days, after which the tests are repeated. If the blood sample from the donor at the time of the donation is also tested using the nucleic acid amplification

technique (NAT) for HIV, HBV and HCV, the investigating of a second blood sample, as well as the quarantine period referred to above, can be canceled. The repeat test is also not required if the processing includes an inactivation step that has been validated for the viruses concerned.

What is forbidden by law?

Trade in human gametes is prohibited. Also, it is forbidden to donate gametes for eugenic selection and for the purpose of sex selection, with the exception of the selection for the prevention of sex-linked disorders. Matching donor(s) and receiver(s) is not regarded as a eugenic practice. It is not allowed to simultaneously inseminate gametes from different donors. Gametes from the same donor may not be used to give birth to one or more children in more than six different women. Two prospective parents of the female sex, who declare that they have a common desire to have children, are hereby considered as a single woman. Every advertisement for carrying out the removal of or actions with human body materials is prohibited, with the exception of cases concerning a public campaign to raise awareness for allogeneic donation of human body material, only in interest of the public health.

Appendix D

Supplemental figures attitudes of candidate donors

Supplementary Table A: Attitudes towards sperm donation (according to characteristics of the candidate donor)[a]

	Familiarity with fertility problems (self or partner)		Familiarity with donor conceived children		Familiarity with men who donated or want to donate		Education: University/college degree		Religion [b]		Age		
	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No	Mean age (years)		
	%[c]	%[c]	%[c]	%[c]	%[c]	%[c]	%[c]	%[c]	%[c]	%[c]	Agree	Others	p-value
Views on donor children													
I would like to know how many children were conceived with my sperm.	66.7	45.8	47.6	46.2	50.0	45.7	50.0	42.0	35.9	53.3	30.87	31.92	0.434
I would like information about the family in which the child would grow up.	0.0	21.6	13.6	23.1	22.2	20.7	20.4	22.0	20.5	21.3	29.71	31.91	0.175
I would like information about the children conceived with my sperm, without receiving their names.	0.0	27.8	27.3	26.9	16.7	29.3	28.6	24.0	25.6	27.9	30.12	31.92	0.232
Views on single women and lesbian couples													
I would be reluctant to donate to a lesbian couple.	0.0	3.1	4.5	2.6	11.1	1.2	4.1	2.0	5.1	1.6	29.00	31.52	0.516
I would be reluctant to donate to a single mother.	0.0	1.0	0.0	1.3	0.0	1.2	0.0	2.0	2.6	0.0	37.00	31.38	0.397
Disclosure to offspring													
Parents should be honest to their children about their genetic origin.	66.7	49.0	59.1	46.8	33.3	53.1	46.9	51.0	43.6	53.3	31.40	31.46	0.967
The parents should (be able to) decide whether or not they want to inform their child about his or her genetic origin.	66.7	62.5	59.1	63.6	64.7	62.2	69.4	55.1	64.1	61.7	31.33	31.75	0.762
Donor anonymity													
I would be prepared to give information about myself (e.g. physical appearance) to the children born from my donation, without giving them my name.	66.7	82.5	86.4	80.8	94.4	79.3	87.8	76.0	82.1	82.0	31.68	30.39	0.455

I would be prepared to donate if my name would be revealed to the children resulting from my donation.	33.3	25.8	18.2	28.2	22.2	26.8	22.4	30.0	20.5	29.5	31.50	31.42	9.56
I would be prepared to meet the children conceived with my sperm if they want that.	0.0	33.0	**4.5****	**39.7****	27.8	32.9	36.7	26.0	30.8	32.8	31.53	31.40	0.925
Children conceived with donated sperm should have the right to know their genetic origin.	66.7	42.7	50.0	41.6	33.3	45.7	43.8	42.0	39.5	45.9	30.86	32.11	0.348
Willingness to donate / to use donor sperm													
I think that many men are prepared to donate sperm.	33.3	21.6	27.3	20.5	33.3	19.5	20.4	22.0	23.1	21.3	30.33	31.74	0.386
If I would have fertility problems, I would be prepared to use donor sperm.	66.7	47.4	57.1	45.5	66.7	43.8	**59.2***	**35.4***	43.6	50.8	31.6	31.47	0.933
I would be prepared to donate even if my expenses would not be reimbursed.	100.0	46.4	40.9	50.0	50.0	47.6	49.0	48.0	43.6	50.8	32.02	30.88	0.392
Social aspects of sperm donation													
I think my (future) partner has the right to know that I am (was) a donor.	100.0	72.2	72.7	73.1	72.2	73.2	71.4	74.0	74.4	72.1	31.18	32.15	0.519
I am planning to keep my donor status a secret from everyone.	33.3	28.9	40.9	25.6	11.1	32.9	30.6	28.0	25.6	31.1	**34.54**	**30.20**	**0.003**
I think that the important people in my life, when they would be aware, would support my decision to donate sperm.	100.0	72.2	77.3	71.8	**94.4***	**68.3***	81.6	64.0	82.1	67.2	31.34	31.70	0.807

[a] N=100; Number of missing cases vary between 0-4 per variable.
[b] Religion: Yes = all candidate donors who have a denominative religion (e.g. Christian, Roman Catholic, Protestant, Islamic, Eastern Orthodox); No = candidate donors who are not religious or who do not have a specific religion or denomination.
[c] Percentage of respondents answering 'agree' or 'totally agree' within each category (for instance those who answered 'yes' and those who answered 'no' on the question: 'Are you familiar with donor-conceived children?').
* Percentages expressing significant differences are printed in bold: $p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$.

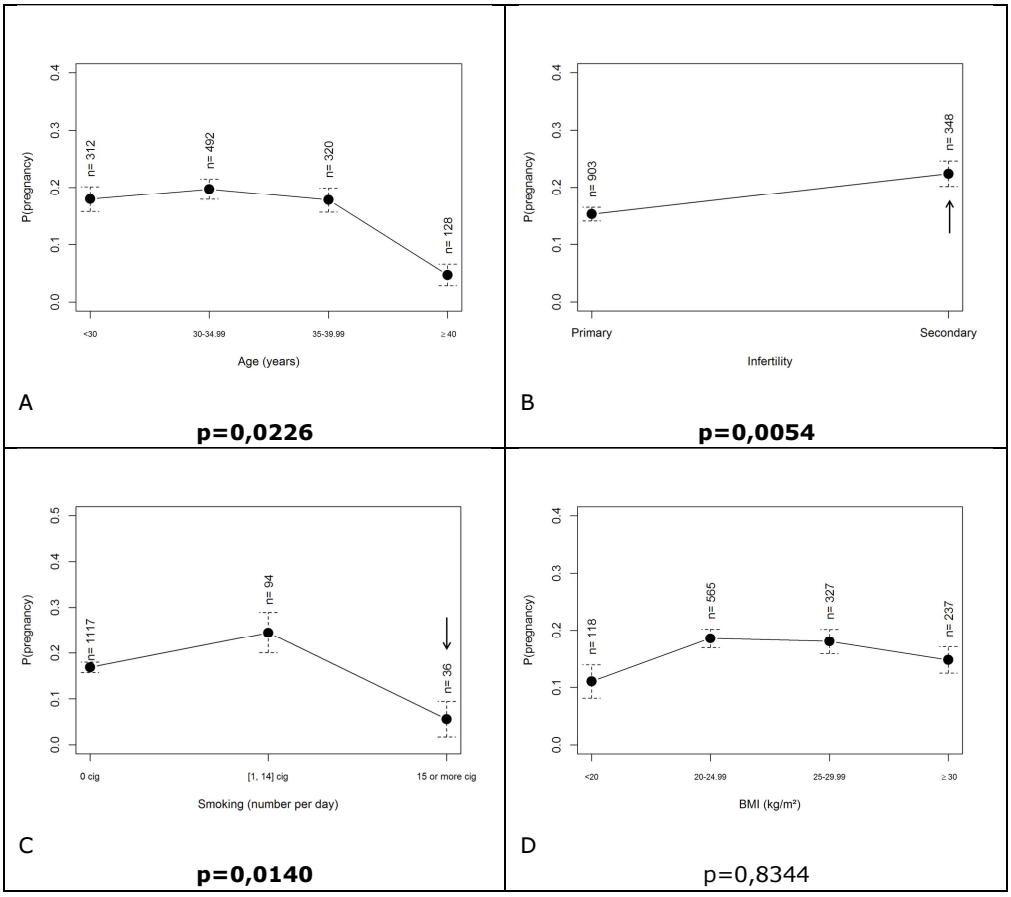
Supplementary Table B: Motivation to donate (according to characteristics of the candidate donor)[a]

	Familiarity with fertility problems (self or partner)		Familiarity with donor conceived children		Familiarity with men who donated or want to donate		Education: University/college degree		Religion [b]		Age		
	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No	Mean age (years)		
	%[c]	%[c]	%[c]	%[c]	%[c]	%[c]	%[c]	%[c]	%[c]	%[c]	Agree	Others	p-value
To reproduce	33.3	17.7	22.7	16.9	11.1	19.8	18.8	18.0	20.5	16.7	29.50	31.85	0.174
To know my semen quality	0.0	59.4	63.6	55.8	77.8	53.1	60.4	54.0	64.1	53.3	**29.45**	**33.98**	**0.001**
Because I sympathize with people who are having difficulties to conceive	100.0	89.7	95.5	88.5	94.4	89.0	85.7	94.0	94.9	86.9	31.85	27.80	0.064
Because I think it would give me a certain degree of satisfaction	33.3	64.9	68.2	62.8	72.2	62.2	59.2	68.0	74.4	57.4	31.74	30.92	0.551
Because I believe I have high quality genetic material	33.3	21.9	18.2	23.4	11.1	24.7	22.4	22.4	17.9	25.0	31.18	31.61	0.787
Because of the financial compensation	0.0	32.0	18.2	34.6	27.8	31.7	36.7	26.0	25.6	34.4	30.47	31.87	0.332
To help people to fulfil their child wish	100.0	95.9	100.0	94.9	100.0	95.1	93.9	98.00	97.4	95.1	31.64	26.75	0.145
Because I want to give something back for the fact that I used to be a patient in this center	0.0	1.1	0.0	1.4	0.0	1.3	0.0	2.0	2.8	0.0	31.00	31.43	0.949
Because I consider it my duty	33.3	15.8	27.3	13.2	16.7	16.3	23.4	10.0	13.2	18.3	31.50	31.40	0.956

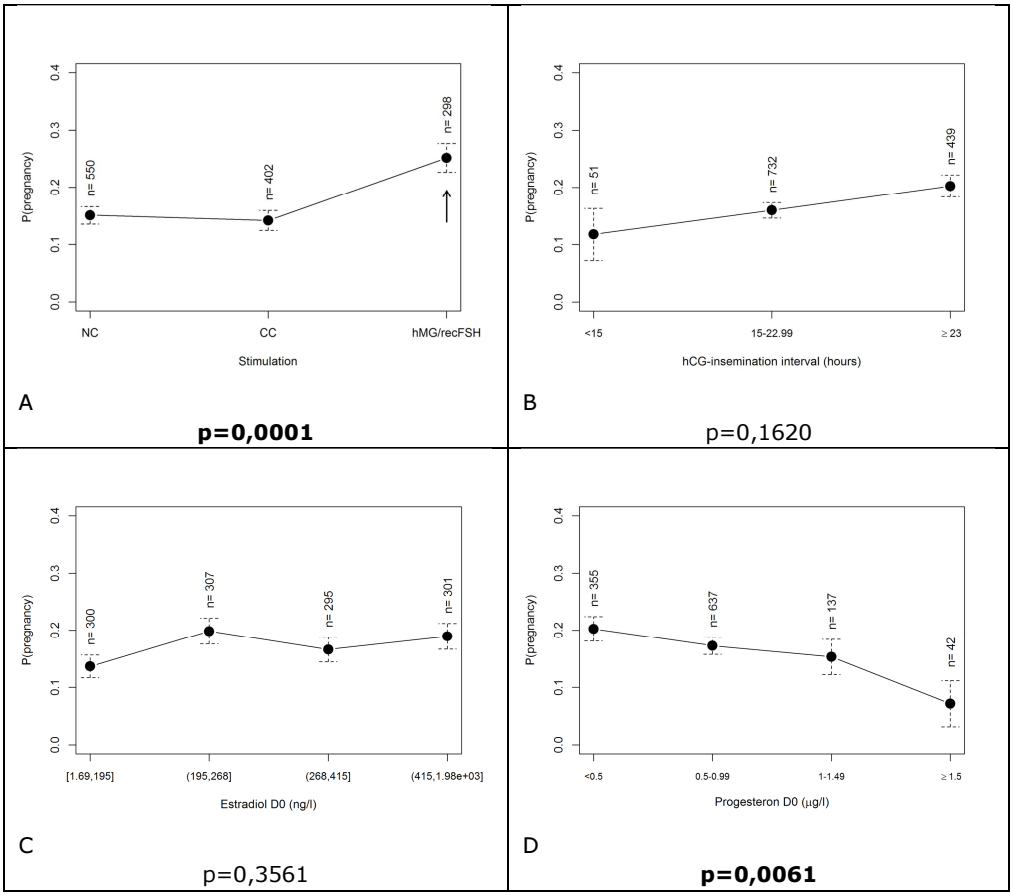
[a] N=100; Number of missing cases vary between 0-7 per variable.
[b] Religion: Yes = all candidate donors who have a denominative religion (e.g. Christian, Roman Catholic, Protestant, Islamic, Eastern Orthodox); No = candidate donors who are not religious or who do not have a specific religion or denomination.
[c] Percentage of respondents answering 'agree' or 'totally agree' within each category (for instance those who answered 'yes' and those who answered 'no' on the question: 'Are you familiar with donor-conceived children?').
* Percentages expressing significant differences are printed in bold: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

Appendix E

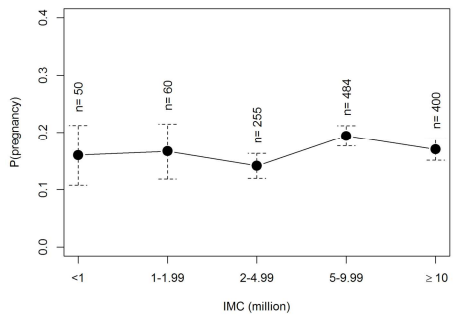
Supplemental figures factors predicting AID succes



Supplementary Figure 4.1-2: Univariate analysis of patient related factors
BMI: body mass index; cig.: cigarettes;
Age and BMI were continuous variables, therefore p-values represent overall significance levels. Smoking and infertility were categorical variables and the p-value for smoking represents a significant difference in CPR between smoking 1-14 cig./day and ≥15 cig./day.

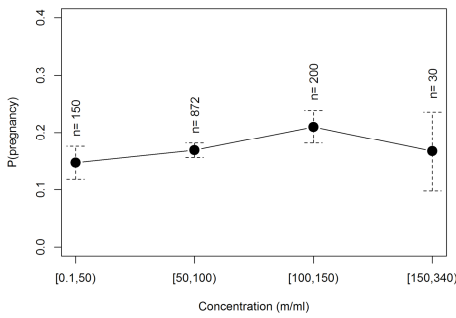


Supplementary Figure 4.1-3: Univariate analysis of IUI procedure specific factors
CC: clomiphene citrate; D0: day 0; hCG: human chorionic gonadotropin; hMG: human menopausal gonadotropin; NC: natural cycle; recFSH: recombinant follicle stimulating hormone.
Levels of estradiol and progesterone at D0 and the hCG-insemination time interval were continuous variables, therefore p-values represent overall significance levels. Stimulation was a categorical variable and the p-value represents a significant difference in CPR between NC or CC and hMG/recFSH stimulated groups



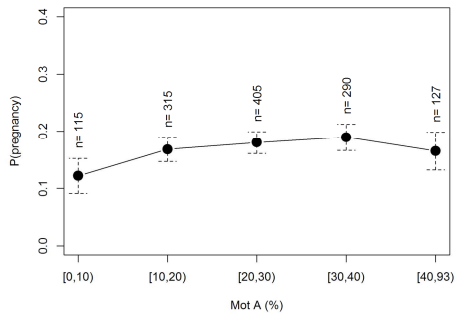
A

p=0,3991



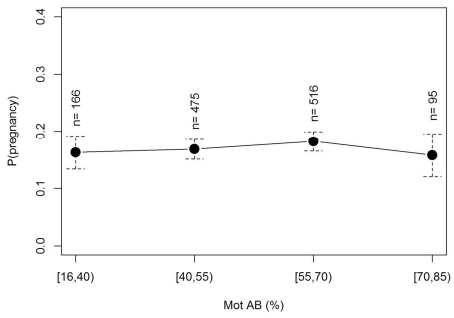
B

p=0,2031



C

p=0,0842



D

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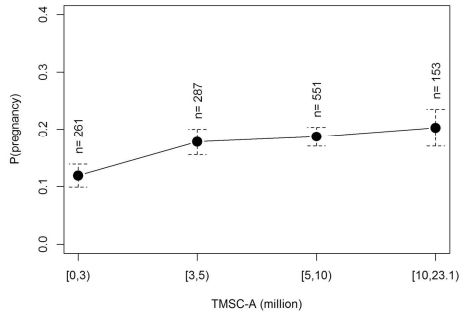

E

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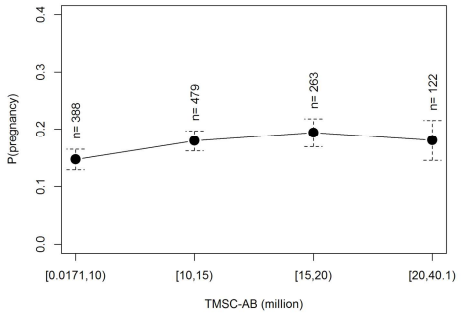
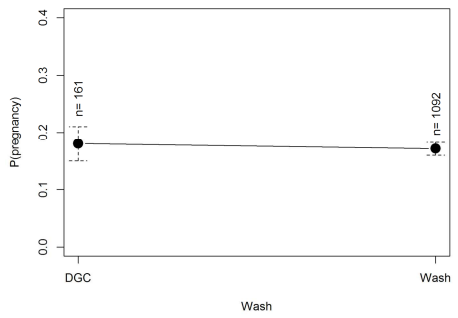


F

p=0,3158



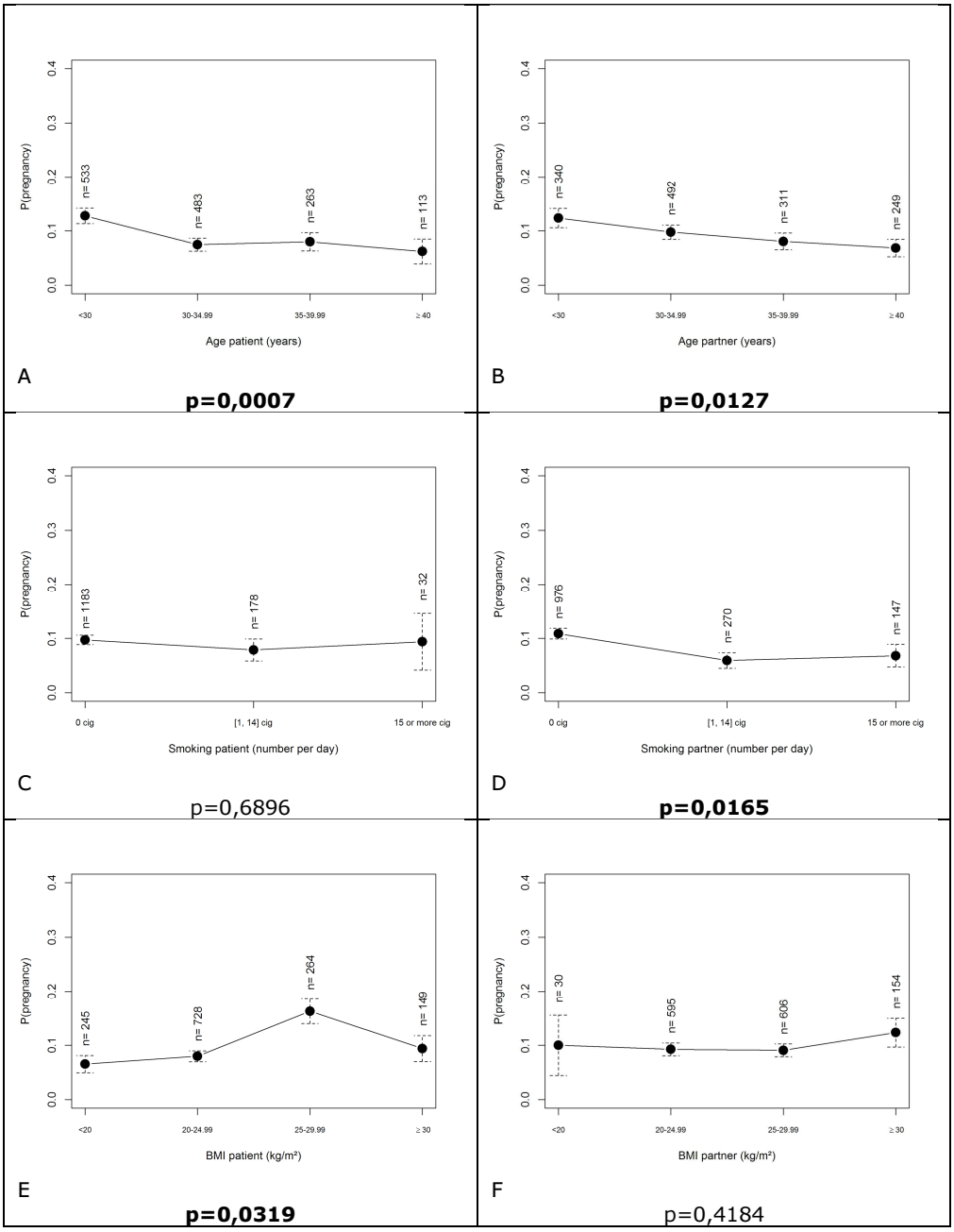
G

p=0,8745

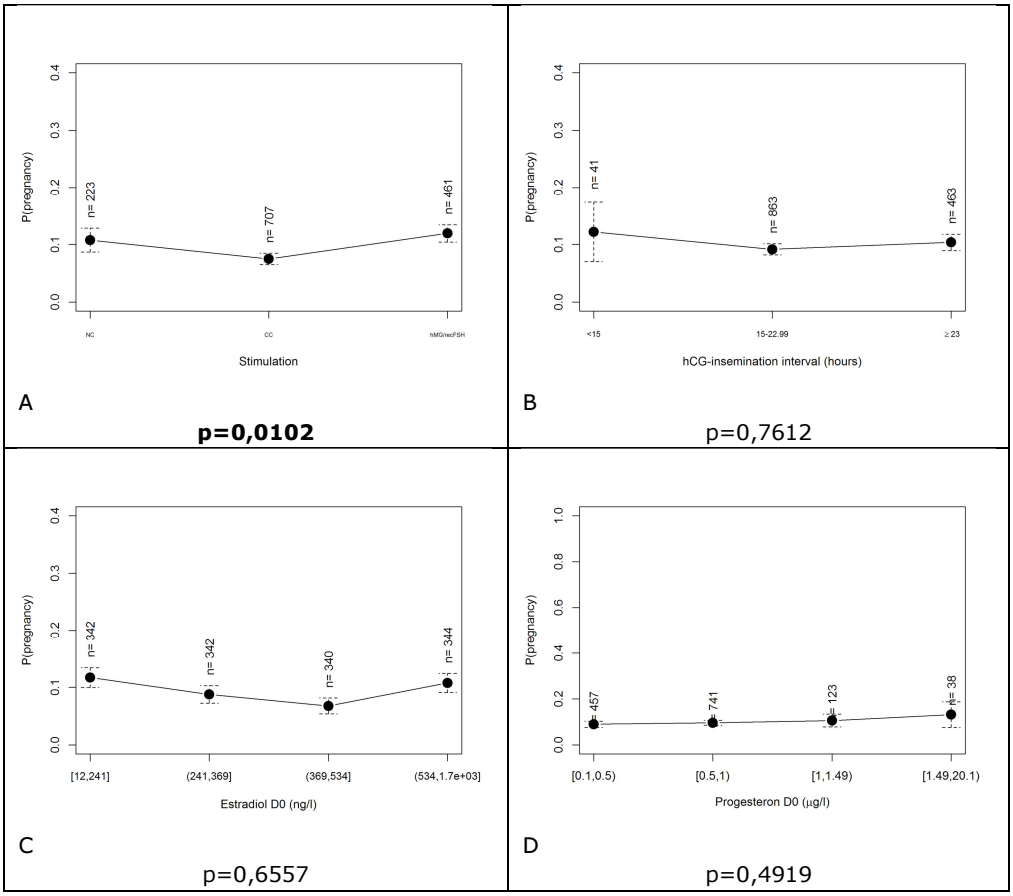
Supplementary Figure 4.1-4: Univariate analysis of post-thaw sperm quality factors
DGC: density gradient centrifugation; IMC: inseminating motile count; TMSC: total motile sperm count.
All sperm parameters were continuous variables, therefore the p-values represent the overall significance level.

Appendix F

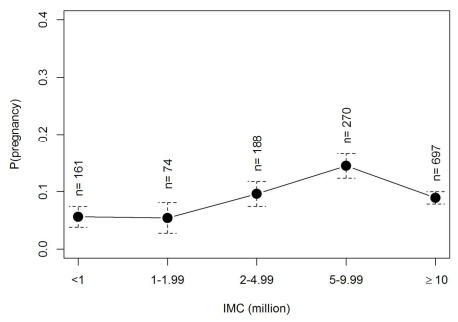
Supplemental figures factors predicting IUI success



Suppl. Figure 6.2-2: Univariate analysis on covariates related to patient characteristics BMI: body mass index; cig.: cigarettes.
Age and BMI were continuous variables, therefore p-values represent overall significance levels. Smoking was a categorical variable and p-values represent if CPR in one of the groups significantly differed from another. The p-value for partner smoking represents a significant difference in CPR between smoking 0 cig./day and 1-14 cig./day.

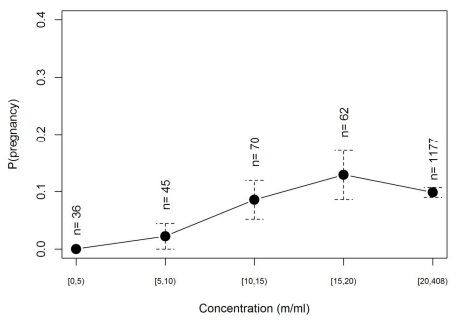


Suppl. Figure 6.2-3: Univariate analysis on covariates related to the IUI procedure
CC: clomiphene citrate; D0: day 0; hCG: human chorionic gonadotropin; hMG: human menopausal gonadotropin; NC: natural cycle; recFSH: recombinant follicle stimulating hormone.
Levels of estradiol and progesterone at D0 and the hCG-insemination time interval were continuous variables, therefore p-values represent overall significance levels. Stimulation was a categorical variable and the p-value represents a significant difference in CPR between CC and hMG/recFSH stimulated groups.



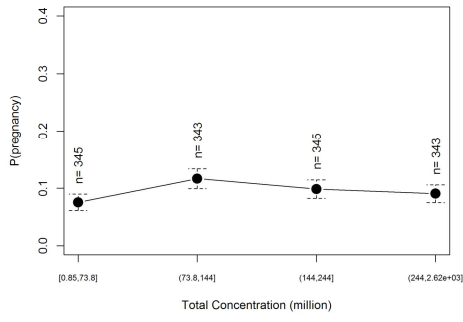
A

p=0,0273



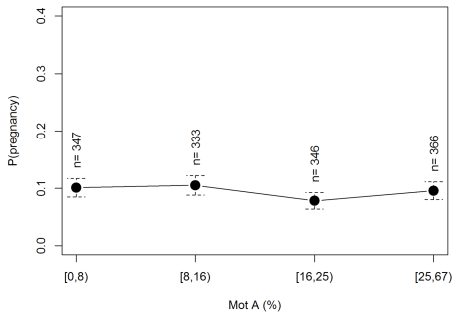
B

p=0,4017



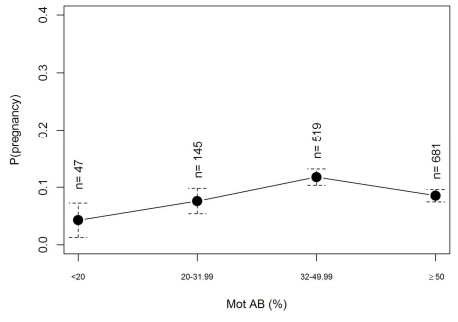
C

p=0,3931



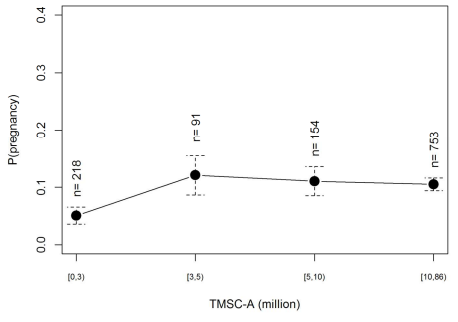
D

p=0,9137



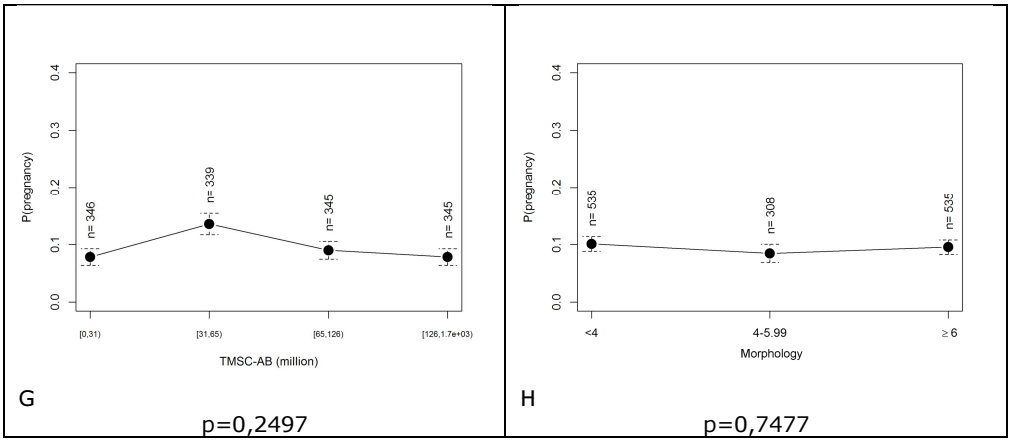
E

p=0,4621



F

p=0,2974



Suppl. Figure 6.2-4: Univariate analysis of covariates related to sperm quality
IMC: inseminating motile count; TMSC: total motile sperm count.
All sperm parameters were continuous variables, therefore the p-values represent the overall significance level.

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Thijssen A., Nelissen T., Vandormael E., Van Der Auwera I., Ombelet W. *Cryopreservation of spermatozoa by means of small and large volume vitrification compared with conventional slow freezing and the effects on post-thaw sperm quality.* J Assist Reprod Genet.

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Thijssen A., Klerkx E., De Ryck L., Bosmans E., Campo R., Ombelet W. *Influence of Temperature and Preparation Techniques on Sperm Quality.* 29th Annual Meeting, ESHRE 2013 London, United Kingdom. 7th to 10th of July 2013.

Thijssen A. *'Help mijn kind is een Viking!': nood aan Belgische spermadonoren.* Symposium georganiseerd door de PhD studenten van het Limburg Clinical Research Program (LCRP): Medisch-wetenschappelijk onderzoek in de Limburgse Ziekenhuizen, Hasselt, Belgium. 9th of November 2013.

Thijssen A. *De geboorte van een Limburgse spermabank.* Symposium georganiseerd door het Genks Instituut voor Fertiliteitstechnologie (GIFT): 25 jaar IVF in Limburg: toekomst van de geassisteerde reproductie, Hasselt, Belgium. 7th of December 2013.

Thijssen A., Dhont N. *Uitbouw van een spermabank: medische, ethische en economische aspecten.* Samenkomst van de Limburgse centra voor reproductieve geneeskunde: Intra-Uterine Insemination: Evidence-based guidelines for daily practice, Genk, Belgium. 27th of February 2014.

Thijssen A., Klerkx E., Cox A., Vandormael E., Dhont N., Ombelet W. *Artificial insemination with donor sperm (AID): heterogeneity in sperm banking facilities in a single country.* 30th Annual Meeting, ESHRE 2014 Munich, Germany. 30th of June to 2nd of July 2014.

Thijssen A., Meier A., Panis K., Ombelet W. *Fertility Awareness Based Methods for the subfertile couple: a systematic review.* Sensiplan Meeting, NFP-Vlaanderen, Leuven, Belgium. 28th of March 2015

Thijssen A., Vankerrebroeck H., Van der Elst W., Mestdagh G., Dhont N., Campo R., Ombelet W. *Predictive factors influencing pregnancy rates after artificial insemination with donor semen: a prospective observational study of 935 cycles.* 18th World IVF Congress of In Vitro Fertilization, ISIVF 2015 Copenhagen, Denmark. 27th to 30th of September 2015.

Thijssen A., Vankerrebroeck H., Van der Elst W., Mestdagh G., Dhont N., Campo R., Ombelet W. *Predictive value of different factors influencing pregnancy rate following intrauterine insemination with homologous semen: results of a prospective observational study of 1041 inseminations.* 18th World IVF Congress

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Thijssen A. *Recruteren van spermadonoren in België: is er een tekort?* Samenkomst A&B centra, VVOG 2015 Boom, Belgium. 27th of November 2015.

Thijssen A. *Factors influencing pregnancy outcome in a sperm donor programme.* Donor sperm banking: medical, socio-cultural, ethical and legal considerations, SIG Andrology Campus Workshop ESHRE 2015 Leuven, Belgium. 10th to 11th of December 2015.

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Dankwoord

Na meer dan 5 jaar te hebben doorgebracht op de dienst Fertiliteit van het Ziekenhuis-Oost Limburg komt dit hoofdstuk in mijn leven stilaan op zijn einde. Het was een periode met hoogtepunten en dieptepunten, zoals elk doctoraat dit kent, maar ik kan nu met een goed gevoel terugkijken op de mooie tijd die ik hier beleefd heb.

De voltooiing van dit werk was echter niet mogelijk geweest zonder de hulp en steun van velen. Daarom een speciaal woordje van dank voor ieder van jullie.

Bedankt aan de **juryvoorzitter** prof. dr. Sven Hendrix en de **juryleden** dr. Ben Cohlen, dr. Astrid Cantineau en dr. Herman Tournaye voor uw tijd en interesse om mijn thesis te lezen en uw bijdrage te leveren aan dit werk. Het is voor mij een hele eer om zulke grote namen op het gebied van fertiliteitsonderzoek als juryleden te mogen ontvangen.

Dr. Ombelet, bedankt om in mij te geloven en mij de kans te geven om na mijn masterstage dit doctoraatsproject onder uw deskundige begeleiding te mogen verderzetten. U wist me steeds opnieuw te motiveren bij het schrijven van de artikels wanneer er (in mijn ogen) soms moeilijk te beantwoorden commentaren terugkwamen van de reviewers. Ook uw drijfveer en onuitputtelijke bron aan nieuwe ideeën voor onderzoeken en artikels zullen mij altijd blijven inspireren. Naast de momenten van intense arbeid, had u ook aandacht voor het creëren van een hechte groep op de dienst. Ik denk dan ook met veel plezier terug aan de momenten waarop u als enige haan in het hoenderhok op pad ging met uw ±20 hennen op teambuilding. Ik wens u nog veel succes bij de verderzetting van het Walking Egg project en uw droom om IVF ook toegankelijk en betaalbaar te maken voor de mensen in derdewereldlanden.

Ook een speciaal woordje van dank aan **dr. Dhont** om mij bij te staan bij het schrijven van de artikels. Steeds stond u klaar om mijn artikels grondig na te lezen, te verbeteren en me te wijzen om eventuele leemtes en deed me zaken vanuit een andere invalshoek bekijken. Ik kon altijd bij u terecht met mijn

vragen (al dan niet werkgerelateerd) en uw hulp wordt dan ook zeer gewaardeerd.

Bedankt ook **prof. dr. De Ryck** om mij op te nemen in uw onderwijsteam. Het begeleiden van de labo's en werkzittingen bij de studenten Biomedische Wetenschappen en Geneeskunde waren voor mij een zeer leerrijke ervaring. U bent als docent een groot voorbeeld voor mij en onder uw begeleiding heb ik dan ook de leerkracht in mezelf ontdekt en verder kunnen ontwikkelen.

Prof. dr. Pennings, bedankt om uw expertise met ons te willen delen voor het opstellen van de vragenlijst voor de kandidaat spermadonoren en voor het schrijven van het artikel.

Voor een deel van mijn onderzoek kon ik ook steeds terecht in het labo van **dhr. Eugene Bosmans**, waar ik gebruik mocht maken van de flow cytometer. Ook mijn masterstudent heeft tijdens zijn stage van dezelfde diensten gebruik mogen maken. Bedankt Eugene om ons uw labo ter beschikking te stellen en om ons steeds bij te staan met uw grenzeloze kennis en briljante ideeën.

Dr. Elke Klerkx, je was een superbegeleidster bij mijn eerste stappen in de wereld van de fertiliteit tijdens mijn masterstage. Ook bij aanvang van mijn doctoraat wist je me keer op keer met de juiste insteek op te peppen in moeilijke momenten en met volle overgave me te helpen waar nodig. Toen je onze dienst verliet wist ik even niet hoe ik de komende 2 jaar van mijn doctoraat zou 'overleven' zonder jouw steun. Maar we zijn contact blijven houden en ondertussen kan ik zeggen dat we vriendinnen voor het leven zijn geworden. Bedankt voor je geduld, niet aflatende interesse in mijn doctoraat maar ook in mij als persoon.

Verder wil ik ook graag **dr. Wim Van der Elst**, **dr. An Creemers** en **dr. Wilfried Cools** van de UHasselt en **dr. Veerle Provoost** van de UGent bedanken voor hun statistische ondersteuning bij dit werk.

Een groot woord van dank gaat uit naar alle medewerkers van de **dienst fertiliteit**. Groot of klein, iedereen van jullie heeft háár (want bemerk goed, naast dr. Ombelet en dr. Campo werken er geen mannen op deze dienst)

steentje bijgedragen aan dit werk. Ook al was ik niet dagelijks bij jullie op de werkvloer aanwezig, ik heb me altijd zeer welkom gevoeld en een deel van het team.

De **secretaresses**, Ingrid, Noelle, Ellen en Emily, jullie waren er altijd om mij te berichten wanneer dr. Ombelet aanwezig was. Ingrid en Emily, bedankt ook om steeds zo nauwgezet de database met de resultaten van de inseminaties aan te vullen.

De **consulenten**, Petra, Hilde, Sarah, Cathérine, Véronique, Véronique en Ann, dank jullie wel voor alle moeite die jullie gedaan hebben (en nog steeds doen) om bij elke inseminatie de papieren in te vullen voor onze database.

Aan de vrouwen op het **IVF labo**, Mia, Annemie, Elke, Eva C. en Eva V., bedankt om mij op te nemen in jullie groep en mij met veel geduld de kunst van het spermatellen bij te brengen. Jullie stonden altijd voor me klaar bij mijn werk in het labo en ik amuseerde me steevast met de soms hilarische gesprekken tijdens de lunches. Ingrid, met jouw komst als hoofd van het labo waaide er een nieuwe kritische wind door de dienst, waar ik met veel plezier gebruik van heb kunnen maken. Bedankt ook om mij bij de staan bij de begeleiding van de studenten. Kristien, ik reken jou voor het gemak ook even bij de mensen van het labo, bedankt voor je hulp bij het opstarten van nieuwe studies. Marina, dankjewel voor de leuke attenties en de fleurige aankleding van de stafvergadering bij Kerstmis. Anja, dit rijtje zou gewoon niet compleet zijn zonder jou.

Dr. Campo en **dr. Mestdagh**, ook jullie toonden steeds interesse in mijn werk tijdens presentaties op de wekelijkse stafvergaderingen, waarvoor dank.

Bedankt ook aan alle **studenten** Biomedische Wetenschappen en Geneeskunde die hun jaarwerkstuk of stage kwamen doen op onze dienst. Ook hun werk resulteerde in enkele publicaties, waarvoor dank.

Lieve **collega-doctoraatstudenten**: Kathleen, Ingrid, Amber, Frederik, Anneleen, Cornelia, Sharona, Christophe, Joren, Petra, Philippe, Pieter, Lieselotte, Ward, Rob, Dorien, Hélène, Thijs, Kristof en Özgür. 5 jaar lang

deelden we lief en leed op de bureau. Ons aantal breidde exponentieel uit, waardoor we elk jaar noodgedwongen een nieuw onderkomen moesten zoeken. Na mijn eerste jaar als doctoraatsstudent nog te hebben doorgebracht in het 'studentenkotje' mocht ik eindelijk mijn bureau verhuizen naar 'de visbokaal' en maakte ik deel uit van de groep. Een jaar later stond er echter alweer een grote verhuis op de planning en gingen we naar de kelder, waar de aanwezigheid van muizen en padden soms een welkome afleiding vormden tussen het data verzamelen, SPSS'en en schrijven van artikels door. Weer een jaar later barstte ons kantoor echter al opnieuw uit zijn voegen en zijn we verhuisd naar onze aparte 'kottekes'. Ook al zaten we sindsdien niet meer allemaal bij elkaar, de sfeer is er in elk geval niet minder op geworden. We konden nog steeds bij elkaar terecht voor een babbel wanneer het nodig was en er werd steevast 's middags op elke deur geklopt om iedereen mee te vragen voor de lunch. **Sharona** ('Shiwi'), mijn kotgenootje, bedankt voor je gezelschap en de bemoedigende gesprekken. Jij bent de volgende in de rij om ons kotje te verlaten. Veel succes bij het analyseren van je 1000 patiënten! **Cornelia** ('Corrie'), partner-in-crime bij het indienen van onze thesis, de vele mailtjes over de juiste interlinie, lettergrootte, marges en indeling van onze thesis vormden een welkome afleiding tussen het schrijven door. Bedankt ook voor de leuke intermezzo's wanneer ik een dag alleen in mijn kotje zat. Lieve collega's, ik ga jullie missen. Ik wens jullie allemaal nog veel succes met de verderzetting van jullie onderzoek!

Ook bedankt aan **Lars** en **Inge** als begeleiders van onze alsmaar groter wordende groep doctoraatsstudenten. Jullie leveren fantastisch werk.

Aan de vrienden- en meidengroep van de **Koninklijke Harmonie Ons Verlangen**, de dames van klassiek gevorderd bij **Cadans**, en de dansparen en trainers bij **dansschool Maex**, bedankt om mijn vrije tijd op te vullen met leuke en (inspannende) ontspannende activiteiten. Van vrijgezellenfeestjes, tot concerten, balletoptredens en danswedstrijden in binnen- en buitenland, bij jullie kon ik altijd even mijn hoofd leegmaken en de batterijen terug opladen.

Mijn **schoonouders en zussen** Stephanie, Lisa en Ilona wil ik bedanken voor de ontspannende vrijdagavonden, die uitgegroeid zijn tot onze vaste

spelletjesavond. Stephanie, nog eens extra bedankt om de Franse vertaling te maken van de vragenlijst voor de Belgische spermabanken.

Mama en papa, dank jullie wel voor alle kansen die jullie mij gegeven hebben in het leven. Bedankt voor mijn onbezorgde jeugd, om mij te laten studeren. Jullie hebben mij altijd gesteund en staan altijd voor mij klaar. Zonder jullie was dit nooit mogelijk geweest. **Ward**, bedankt om gewoon mijn broer te zijn en mij met **Indra** nog een extra zusje te bezorgen.

Davy, liefde van mijn leven, beste vriend, danspartner, steun en toeverlaat. Bedankt voor de schouder om op uit te huilen als ik het even niet meer zag zitten en voor het duwtje in de rug wanneer ik het nodig had om mij terug op gang te trekken. Ondanks je eigen drukke agenda nam je zelfs huishoudelijke taken van mij over als ik moest doorwerken om een deadline te halen. Zonder jouw steun had ik dit nooit bereikt. Ik heb het gevoel dat we samen alles aankunnen en kijk dan ook vol verwachting uit naar onze verdere toekomst samen.