Graduate School for Health Sciences
University of Bern

UNIVERSITÄT BERN

Obstetric and perinatal outcomes of women treated for subfertility and children born after in vitro fertilisation

PhD Thesis submitted by

Vera Ruth Mitter

from Täuffelen, BE

for the Degree of

PhD in Health Sciences (Epidemiology)

Thesis advisor

Prof. Dr. med. Michael von Wolff

Division of Gynecological Endocrinology and Reproductive Medicine, University Women's Hospital, Faculty of Medicine, University of Bern

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Accepted by the Faculty of Medicine and the Faculty of Human Sciences of the University of Bern

Bern, Dean of the Faculty of Medicine

Bern, Dean of the Faculty of Human Sciences

For Malin, Lenny & Mike

There is no better time for epidemiology

Thesis in times of COVID-19

“We have therefore made the assessment that COVID-19 can be characterized as a pandemic”

Tedros Ghebreyesus, March 2020

Content

SUMMARY

The use of assisted reproductive technologies has increased over the past decades. To date, 8 million children have been born worldwide following assisted reproductive technologies. In Switzerland, this refers to around 2-2.5% of newborn's every year, this were 2'188 children in 2017.

The introduction of this thesis gives an overview of the reasons for increasing global infertility, and of the use of medically assisted reproduction. It also presents the treatment options available today, and the risks associated with them for mothers and offspring. It goes on to describe the methods and data used in the research projects conducted for this thesis.

In the following section, the four publications included in this thesis are presented. The first article on children within the Bern IVF Cohort compares the birthweights and birthweight percentiles of children born after natural cycle in vitro fertilisation, to those born after conventional in vitro fertilisation. This shows that the increased risk of small-for-gestational age infants being born may be associated with hyperstimulation of oocyte growth in conventional in vitro fertilisation, especially when high estradiol levels are reached on the day of ovulation induction.

The second article uses follow-up data on breastfeeding collected within the Bern IVF Cohort. Breastfeeding prevalence and duration is compared to data from the Swiss Infant Feeding Study, which served as a control population. The findings demonstrate that women after fertility treatment breastfeed their children as much and for as long as mothers in the control population. This suggests that fertility treatment does not affect the potential and ability to breastfeed in Switzerland.

The third article relates to the endometrium in natural cycle in vitro fertilisation, where the endometrium is not affected by hormonal stimulation. Data on endometrial thickness was collected from women during their first treatment cycle and the outcome measured, was successful pregnancy. It was shown that both very thin but also thick endometrium is associated with adverse pregnancy outcomes in natural cycle in vitro fertilisation.

The fourth article analyses data from a historical cohort of women under treatment due to repeated implantation failure and recurrent pregnancy loss from 2014-2018. The two conditions seem to be associated with chronic endometritis, and their discussion is controversial: so far, no agreement on a standardised diagnosis or treatment has been reached. The aim was to assess the effect of endometrial diagnostic biopsy on subsequent treatment in cases of chronic endometritis, which was introduced in 2016, compared to hysteroscopic assessment alone. This demonstrated that diagnostic endometrial biopsy and subsequent treatment of chronic endometritis shortens time-to-pregnancy and live birth.

ABBREVIATIONS

aOR	Adjusted odds ratio
AMH	Anti-Müllerian hormone
APS	Antiphospholipid syndrome
ART	Assisted reproductive technology
BMI	Body mass index
CC	Clomiphene citrate
CE	Chronic endometritis
CI	Confidence interval
cIVF	Conventional gonadotropin stimulated in vitro fertilisation
DNA	Deoxyribonucleic acid
DOHaD	Developmental origins of health and disease
EC	Ethic commission
ESHRE	European Society of Human Reproduction and Embryology
ET	Embryo transfer
E2	Estradiol
FET	Frozen embryo transfer
FIVNAT-CH	National registry on data from all ART treatment cycles in Switzerland
FOPH	Federal Office of Public Health (Bundesamt für Gesundheit)
FSH	Follicle stimulating hormone
FSO	Federal Statistical Office (Bundesamt für Statistik)
GnRH	Gonadotropin-releasing hormone
hCG	human chorionic gonadotropin
HIV	Human immunodeficiency virus
HMG	Human menopausal gonadotropins (includes FSH and LH)
HR	Hazard ratio
ICMART	International Committee for Monitoring Assisted Reproductive Technologies
ICSI	Intracytoplasmic sperm injection
ISMAAR	International Society for Mild Approaches in Assisted Reproduction
IU	International unit
IUI	Intrauterine insemination
IVF	In vitro fertilization

LBR	Live birth rate
LBW	Low birthweight (< 2’500g)
LH	Luteinising hormone
MAR	Medically assisted reproduction
mIVF	Minimal stimulation in vitro fertilisation
NSAID	Non-steroidal anti-inflammatory drugs
NC-IVF	Natural cycle in vitro fertilisation treatment
OHSS	Ovarian hyperstimulation syndrome
OPU	Oocyte pick up
OR	Odds ratio
PCOS	Polycystic ovary syndrome
PTB	Preterm birth (< 37 gestational weeks)
RIF	Repeated implantation failure
RMA	Federal act on medically assisted reproduction (Reproductive Medicine Act)
RPL	Recurrent pregnancy loss
SET	Single embryo transfer
SGA	Small-for-gestational-age
SGRM	Swiss Society for Reproductive Medicine (Schweizerische Gesellschaft für Reproduktionsmedizin)
SWIFS	Swiss infant feeding study
SNC	Swiss National Cohort
TESE	Testicular sperm extraction
TSH	Thyroid-stimulating hormone (thyrotropin)
WHO	World Health Organization

1. Introduction

The World Health Organization (WHO) and the International Committee for Monitoring Assisted Reproductive Technology (ICMART) define infertility as "a disease of the reproductive system defined by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse, or due to impairment of a woman's capacity to reproduce".[1,2]

Some couples remaining childless is quite normal, as is couples having abundant offspring. Couples affected by infertility can seek medical help, at least in most countries of the developed world. However, infertility remains an ongoing problem worldwide, impacting gender equity and health equality. Unfortunately, the highest rates of infertility are often in countries with the most limited access to assisted reproductive technologies (ART).[3]

It is important to respect infertility or subfertility as a disease, because affected couples often feel that their bodies are not working properly and healthily. They are not achieving what normal healthy couples can naturally achieve – namely, a pregnancy and a live birth of a child.[4] According to the latest definition by the WHO, infertility is a disease, which constitutes a disability and an impairment. Sometimes infertility is the result of a disorder, which itself needs treatment and therefore should be diagnosed.[4]

1.1.Infertility – epidemiology, aetiology and consequences

Infertility is a highly prevalent condition and affects 8-12% of couples of reproductive age.[5] Worldwide, an estimated 97 – 186 million people are suffering from some form of infertility, infecundity or childlessness, mainly in developing countries.[3,6,7] The inability to conceive following a prior pregnancy – secondary infertility – is the most common form of infertility worldwide. 25-30% of women of sub-Saharan African countries suffer from secondary infertility.[6] A high prevalence of sexually transmitted infections may lead to female and male infertility. Additionally, poor maternity care, leading to post-abortive and postpartum infections, causes secondary infertility in females, especially in African countries.[3,8] Despite the fact that in half of the infertile couples male factors contribute, it is often seen as a female failure, and the social burden falls on the woman, possibly leading to stigmatisation.[8] The treatment and diagnosis of infertility is not only important for gender equity, but also in identifying possibly severe underlying health conditions generally reducing health-related quality of life.[4,8]

Major factors affecting the likelihood of spontaneous conception are:

- Time of unwanted non-conception (the longer a couple tries, the lower the chances become)
- Age of the woman
- Disease-related infertility

Time of unwanted non-conception

The time of unwanted non-conception is an important determinant of the severity of subfertility. Usually, 80% of pregnancies occur within six months of regular unprotected intercourse. After six more months, half of the remaining 20% of couples achieve a pregnancy. After 12 months of trying, the remaining 10% can be diagnosed with subfertility. However, even among this group, nearly 55% will achieve a spontaneous pregnancy within the next 36 months. 5% will remain infertile, with an extremely low chance of becoming pregnant.[9]

Female age-related fertility decline & social trends

In the 1960's, the first hormonal contraceptive method, the anti-baby pill, came on the market. It allowed women to control their fertility and to become more sexually independent. Today, 73% of women aged 15-49 in Switzerland use some form of contraceptive method.[10] At the same time, expectations for parenthood increased. Women aimed for better and longer educations, and focused on their professional careers. As a consequence of these societal changes, couples delayed starting their families, and the mean childbearing age in many countries increased.[11,12] In Switzerland, the mean age of mothers at birth was 27.9 in the 1980s, 29.8 in 2000 and reached 32.2 in 2019.[13] The total current fertility rate is below the replacement level of 2.1 in all European countries (average EU: 1.6, World: 2.43).[10] Delaying childbearing until less fertile ages is increasing involuntary childlessness, and is an important contributor to the decrease in births and fertility rates.[14] Women are most fertile between the ages of 20-29. From 32 onwards, female fertility decreases gradually, and from 37 on, the decrease becomes more rapid. Women over 35 years do not only have more difficulties in becoming pregnant, but are also at a higher risk of loss of pregnancy.[15] This is due to the continuous decrease of the ovarian reserve in both ovaries, and the hormonal change induced by the perimenopause. Additionally, deterioration of oocyte quality increases the risk of genetic aberrations and spontaneous abortions.[16] Some women are at risk of entering menopause much earlier due to premature ovarian failure.[17] With increasing age, the risk of health problems causing infertility, and the consequences of an unhealthy lifestyle become more important. Sadly, many men and women of younger ages are not aware of age-related declining fertility, and many believe ART can mitigate this problem.[18] However, the response to ovarian stimulation in ART is lower if the ovarian reserve is reduced. This reduces levels of treatment success in older women. Either having already had a live

birth, or having donated eggs, increases the chance of a live birth through ART for women above the age of 40 years, but after 45 the chances are close to zero.[19]

Disease related infertilities

Many diseases, dysfunctions or malformations are associated with lower fertility or subfertility. Pathologies affecting the sexual functioning of the body or sexual organs, as well as systemic diseases, may influence fertility. It may affect both genders or be specific to one gender only.

1.1.1. Female infertility

Ovulatory dysfunction

Ovulatory dysfunction means the non- or malfunction of the ovaries, and is usually characterized by fewer menstruations (oligomenorrhea) or no menstruation at all (amenorrhea). It is mostly caused by endocrine disorders, meaning the hormones regulating the female cycle are not produced in the necessary amount, or do not function normally. The most common endocrine disorder in women of reproductive age is polycystic ovary syndrome (PCOS), with a prevalence of 5-15%. The follicles fail to release the oocyte, which leads to numerous small persisting follicles in the ovaries which can be detected by ultrasound.[20,21] In rare cases, ovulatory dysfunction is caused by genetic conditions such as Turner syndrome.[22]

Tubal factor

The fallopian tubes collect the oocyte after ovulation and transport it towards the cavum. Transport relies on effective ciliary activity, which can be impaired by inflammations or pathogens. The most common infectious diseases causing tubal problems are *Chlamydia trachomatis* or *Neisseria gonorrhoea*. Systematic diseases, such as primary ciliary dyskinesia or cystic fibrosis, can also impair ciliary structure and function. Further reasons may be tubal endometriosis, peritubal adhesions or salpingitis isthimica nodosa.[5,23]

Uterine factor

The uterine physiology and anatomy is important in the successful implantation of a fertilized egg. Uterine myomas are the most common benign tumours of the female reproductive system, and are present in 5-10% of infertile women, but are only the sole cause of infertility in 2-3% of infertile women. Their localisation may interfere with the implantation of the embryo.[24] Other uterine abnormalities such as fibroid, polyps, or adenomyosis can also reduce the chance of achieving a pregnancy.[25] Congenital uterine malformations such as uterus septus or subseptus, or more complex malformations such as uterus unicornus, bicornus or didelphys, may in rare instances lead to infertility or recurrent pregnancy loss.[26] Surgical treatment is sometimes needed.

Endometriosis

Endometriosis is characterised by the presence of endometrial mucosa (the lining of the uterus) in other locations within the female pelvic area. It is a pathological and chronic inflammatory process. Some women have cyclical abdominal pain and/or pelvic pain during or after sexual intercourse. The severity and localisation of endometriosis can vary highly. The association of endometriosis with infertility ranges from anatomical distortions due to adhesions and fibrosis, to endocrine abnormalities and immunological disturbances.[27] Endometriosis decreases the embryonic implantation potential, which reduces ART success. It also leads to a higher risk of miscarriages independent of the origin of the pregnancy.[28] Endometriosis often remains undiagnosed. Its prevalence in women of reproductive age is unclear, and estimates range from 0.8% to 10%. In subfertile women it ranges from 20-50%.[29,30]

1.1.2. Male factor infertility

Male infertility contributes to 40-50% of cases where a couple is infertile, and in 20-30% it is the sole cause. Male infertility is mostly due to testicular deficiency or post-testicular impairment. Both can be congenital, acquired or idiopathic. Congenital problems may be the malformation or lack of testicles, the absence of vas deferens in men with cystic fibrosis or genetic abnormalities. Genetic abnormalities are Klinefelter (47 XXY) syndrome and Y chromosome microdeletions, causing issues from a light disturbance of spermatogenesis up to a complete azoospermia.[5,25] Trauma, testicular torsion or inflammation of the testes can lead to testicular failure, as can medication or varicocele (varicose veins in the scrotum). Varicocele are present in 14.8 - 25.4% of men with abnormal semen.[31] Ejaculatory dysfunction (retrograde ejaculation) or obstruction in the epididymis, vas deferens or ejaculatory duct can be the result of infections, vasectomy or hernia repair. About 6% of men produce antibodies against their own sperm.[5,31]

Another important issue in male fertility is the worldwide decrease in sperm quality seen over the last decades.[32] In 2010 the WHO lowered their reference limits for human semen characteristics based on men whose female partner got pregnant within 12 months (volume: 1.5ml; total sperm count: 39 million; sperm concentration: 15 million/ml; vitality: 58%, progressive mobility: 32%; motility: 40%; and 4% morphologically normal forms). In a study on the semen quality of young men recruited for the Swiss army, only 38% reached the sperm concentration, motility and morphology values of the WHO semen reference criteria. The median sperm concentration was among the lowest in Europe.[33] The reasons for this trend are manifold, but environmental and lifestyle factors seem to play important roles. The consumption of caffeine, nicotine, alcohol and fatty acids, and a diet rich in full fat diary, meat and sugar, as well as obesity, potentially reduce fertility in men.[32,34] Chemicals with a hormonal influence on the body (endocrine disrupting compounds) can interfere with female

and male fertility. Other pollutants such as organohalogens, air pollutants or heavy metals such as mercury could influence semen quality, as could pesticides and herbicides used in agriculture.[35,36]

1.1.3. Idiopathic infertility

In around 20-30% of infertile couples, infertility remains unexplained. It may be due to subtle, undetectable defects in the reproductive process, or just due to incidentally reduced fecundity. Many chronic or systematic diseases may influence fecundity. This is true of unstable diabetes, uncontrolled celiac disease and active autoimmune conditions, which may also be subclinical.[37,38] Psychological stress, lifestyle factors, use of caffeine or nicotine, too much or too little body weight and extensive sport training may be associated with lower fertility in men and women.[36,39,40]

Repeated implantation failure and recurrent pregnancy loss

Among the main challenges in fertility treatment remain repeated implantation failure (RIF) and recurrent pregnancy loss (RPL) as stated by the European Society of Human Reproduction and Embryology (ESHRE). RIF means the failure to reach a stage of clinical pregnancy after several ART cycles with subsequent embryo transfer, but no distinct definition exists.[41] RPL is defined as three or more consecutive miscarriages by the WHO and 1-5% of women are affected.[42–44] Risk factors associated with RIF and RPL are parental age, obesity, environmental exposure (including smoking and alcohol), genetic factors, uterine malformations and thyroid autoimmunity. Antiphospholipid syndrome is a distinct risk factor for RPL.[44] Additional factors related to IVF therapy might play a role in RIF: gamete/embryo factors such as oocyte quality, sperm quality (e.g. deoxyribonucleic acid (DNA)-fragmentation), ovulation induction protocol, hydrosalpinges and assumed immunological and pathophysiological factors. Other physiological and immunological factors have recently been discussed in relationship to RIF and RPL (e.g. natural killer cells, T-helper 1/T-helper 2 ratio and tumour necrosis factor α levels, autoantibodies, altered expression of associated molecules, implantation window), but more research is needed to provide evidence of any association.[45,46] Imbalances of the uterine immunological cell pattern can lead to persistent inflammatory processes, and to chronic endometritis (CE). CE often remains undiagnosed, as it can be subtle and asymptomatic, or present with untypical symptoms such as pelvic pain, vaginal discharge, abnormal bleeding, dyspareunia and leucorrhoea.[47,48] ART cannot successfully bypass CE, as the problem lies in the implantation of the embryo. There is little agreement on standardized diagnosis and treatments for CE.[49] The formation of the first connection to the uterine endometrium, and the maintenance and support of this connection to establish a secure pregnancy, is a very complex process.

1.2.Assisted reproduction

1.2.1. Definitions

The following section clarifies and defines terms often used in medically assisted reproduction (MAR) and assisted reproductive technologies (ART).

Medically assisted reproduction includes "various interventions, procedures, surgeries and technologies to treat different forms of fertility impairment and infertility. These are ovulation induction, ovarian stimulation, ovulation triggering, all ART procedures, uterine transplantation and intra-uterine, intracervical and intravaginal insemination with semen of husband/partner or donor".[1]

Assisted reproductive technologies "includes all interventions with the in vitro handling of both human oocytes and sperm or embryos for the purpose of reproduction".[1] This includes in vitro fertilisation (IVF), embryo transfer (ET), intracytoplasmic sperm injection (ICSI) and many other treatments which are not relevant for this thesis. Importantly, ART does not include assisted intrauterine insemination (IUI).[1] In this thesis, the term ART is used when referring to children born after IVF or ICSI.

The term subfertility is interchangeable with infertility. Infertility and subfertility are defined by a specified time period; this is one year of unwanted non-conception, per the WHO definition. Sterility is a permanent state of infertility.[1]

A further distinction can be made between primary and secondary infertility. A woman with primary infertility has never been diagnosed with a clinical pregnancy. Secondary infertility means that a woman has previously been diagnosed with a clinical pregnancy, but is not able to establish a further clinical pregnancy.[1]

1.2.2. Development and global perspective

In England in 1978, the first baby was born through IVF. This success was among the major medical breakthroughs of the 20th century. IVF was in this case developed to overcome the mother's blocked tubes.[50,51] Since Steptoe, Edwards and Purdy succeeded with IVF, the use of ART has increased worldwide.[51] Approximately 8 million children have been born following ART since 1978, mainly in high-income countries.[3] In developing countries, access to adequate fertility treatment is still difficult and unaffordable for many, although access is improving every year.[3] Even in developed countries, health insurance does not always cover ART, compromising reproductive autonomy.[52,53] Many infertile couples benefit from improvements in MAR. In only 40 years, many technologies have been developed to address different causes of infertility.[50]

1.2.3. Fertility treatment

Fertility treatment usually starts with the investigation of the couple for the cause of subfertility. Firstly, a comprehensive assessment of the medical, gynaecological and urological histories, the function of sexual organs, clinical investigation and specific diagnostic tests are conducted. These tests usually include a transvaginal ultrasound examination including the antral follicle count to assess ovarian reserve. At the beginning of the cycle a measurement of serum hormones (follicle stimulating hormone (FSH), luteinising hormone (LH), estradiol (E2), thyroid stimulating hormone (TSH), prolactin and total testosterone) are performed. Anti-Müllerian hormone (AMH) is measured for additional information on the ovarian reserve. Preovulatory a hysterosalpingography is performed to assess uterine cavity and the patency of the tubes. Male fertility is assessed by the analysis of ejaculate including concentration, motility, and the morphology of the sperm.[22,25]

Today, many causes of infertility are treatable with different techniques developed in MAR. IUI and monofollicular ovarian stimulation may be sufficient for couples with less severe fertility problems, where a spontaneous conception is possible. Both the stimulation and the inhibition of ovulation are important factors in synchronising ovulation within the cycle. If supported spontaneous conception is not successful or possible, the fertilisation can be done in culture in a laboratory. Sperm is collected to inseminate retrieved oocytes outside the human body.

Hormonal ovarian stimulation supports the growth of more than one oocyte and impedes the natural selection of just one oocyte. Ejaculate is provided by the man himself, and then worked-up in the laboratory to select high quality sperm. The oocyte is fertilized in vitro either by adding an amount of sperm to the oocyte (IVF), or by the injection of just one selected sperm directly into it (ICSI). ICSI was originally developed to overcome severe male infertility, more than 25 years ago. In 2015, ICSI was used in 71.2% of treatments in Europe, avoiding natural sperm selection.[54]

The fertilized oocytes are cultured for several days before they are transferred to the uterus of the mother. This is a fresh embryo transfer. They are either transferred at the cleavage stage (day 2 or 3) or at the blastocyst stage (day 5) of their development. The extension of culture duration allows a better assessment of embryo quality, as their morphological structure, their kinetics and their longer survival are good indicators of viability. Freezing techniques have been developed to store supernumerous embryos. Current vitrification techniques allow storage and the thawing of embryos to be transferred in later cycles. This is called a frozen embryo transfer (FET). The advantage of a FET is that the uterus, especially the endometrium, can be specifically prepared. The disadvantage is that the embryo can get damaged by the vitrification technique. Today, it is possible to diagnose embryos for genetic defects before transfer to the uterus. This is called preimplantation genetic testing. If a

couple is a known carrier of a hereditary disease, a few cells of the embryo can be genetically analysed for this mutation. This allows the selection of a healthy embryo for transfer. It is even possible to screen an embryo's potential to develop unknown chromosomal or structural aberrations.

Some versions of MAR are not legal in Switzerland, such as the donation of oocytes or surrogacy motherhood. Sperm donation, on the other hand, is allowed. All these technologies and many more developed in the last 40 years.[55] Reproductive medicine is a relatively young medical field. Cumulative pregnancy rates in ART can under optimal conditions reach natural fertility rates.[56] However, even with ART support, a proportion of couples will remain childless. Unsuccessful fertility treatment can lead to psychological distress, or to problems in the couple's relationship.[57,58] Reproductive medicine is also an area of conflict in many societies. It is caught between ethical, religious and legal factors which shape the options and attitudes of the couples affected, and the way they access and experience support.[57] Health insurance coverage differs between countries.[52] Different legal restrictions, costs and access to treatment options increase cross-border fertility tourism between countries and continents.[59]

1.3. Treatment schemes used at the University Women's Hospital in Bern

At the University Women's Hospital in Bern, individual and patient-oriented IVF therapy is important. Therefore, natural cycle IVF (NC-IVF) is offered as an alternative alongside conventional gonadotropin stimulated IVF therapies (cIVF). In Switzerland, this is only the case at Bern, Baden, St. Gallen and Lausanne, but Bern has the longest experience with this treatment. Centres are organized within the IVF-Naturelle (www.ivf-naturelle.ch) network. This chapter gives an overview of the IVF treatments conducted at the Bern IVF center. The focus is on treatments with IVF/ICSI fertilization followed by a fresh embryo transfer.

Natural cycle IVF (Schema A)

In natural cycle IVF, gonadotropins are avoided for the stimulation of oocyte growth. The focus is on natural follicle recruitment and selection. The women undergoing NC-IVF have a regular menstrual cycle (26-32 days), and at least one ovary accessible transvaginally for the oocyte pick-up (OPU) needle. The treating physicians monitor the cycle by ultrasound, and measure levels of E2 and LH once before the ovulation is expected. When the follicle reaches a diameter of at least 16 mm and the estimated E2 level reaches > 700 pmol/L, a single injection of 5'000 international units (IU) of human chorionic gonadotropin (hCG) is given, to trigger the ovulation. Within 36 hours, follicular aspiration is conducted using a 19G single lumen needle, without anaesthesia. Follicular flushing up

to five times increases the chance of collecting the oocyte.[60] In case of the LH surge (>10 IU/L) on the day of trigger administration, 400 mg ibuprofen is given every 8 hours until the day of follicular aspiration.[61,62] Non-steroidal anti-inflammatory drugs (NSAID) such as Ibuprofen or indomethacin can postpone the ovulation by several hours if started before or at the onset of the LH surge.[63,64] If the LH surge is already high, the OPU is planned within 24 hours in order not to miss the oocyte. In NC-IVF there is always a risk that ovulation has already taken place, and the oocyte is lost. Besides NSAID, there is also the possibility of giving a single injection of a gonadotropin-releasing hormone (GnRH) antagonist if the LH surge has not yet started. This can be used to postpone follicular aspiration by one day for organisational reasons (e.g. not on a Sunday).

NC-IVF using clomiphene citrate (Schema C)

Low-dose clomiphene citrate can reduce the risk of premature ovulation by delaying the premature LH surge. The woman takes 25 mg every morning from cycle day 6 or 7, depending on the cycle length, until the day when ovulation is triggered by 5'000 IU of hCG. The subsequent procedure remains similar to the NC-IVF protocol described above.[61,62] Clomiphene citrate can reduce premature ovulation by 21%, and increase subsequent ET rate by 14.6%.[62] However, higher doses of clomiphene citrate should be avoided, as they can reduce endometrial thickness.[25]

Minimal stimulation IVF (Schema E)

If a woman under IVF treatment has a thin endometrium and is at high risk for preterm ovulation, a minimal hormonal stimulation can be used (mIVF). The woman injects Merional® 75 IE from cycle day 3-5, until one day before the administration of HCG for ovulation trigger. Merional® is human menopausal gonadotropin, which is a mix of human FSH and LH. Menopausal gonadotropin is gained from the urine of postmenopausal women. When the diameter of the leading follicle is 18-20 mm, and E2 concentration is estimated to be ≥ 700-800 pmol/l, 5'000 IU of hCG is injected and oocyte retrieval is conducted 36 hours later as described above. If many follicles have developed, OPU is conducted with anaesthesia. This stimulation scheme is similar to a cIVF protocol using antagonists, with much lower doses of gonadotropins.[25,65] Low-dose gonadotropins are sometimes combined with clomiphene citrate if a better response to the stimulation is desired.[25,66,67]

Conventional gonadotropin stimulated IVF

The vast majority of ART treatments worldwide are performed with cIVF. For multifollicular stimulation, HMG was primarily used, and is still used, but because of HMG's urinary origin, the use of recombinant FSH is more common nowadays.[68]

Generally in cIVF, multifollicular growth is stimulated by FSH and a GnRH agonist or antagonist is added in combination to inhibit the gonadotropin production of the pituitary gland. This suppresses

LH release from the pituitary gland and prevents spontaneous ovulation. As LH surge therefore does not naturally occur, the ovulation has to be induced by hCG or GnRH agonists, artificially creating an LH surge. Without the LH surge, the first meiotic division and morphological changes of the oocytes would not occur.

In Bern, different cIVF treatment protocols are used, with gonadotropin agonists or antagonists. In the short or long agonist protocol, the downregulation with Decapeptyl® (GnRH agonist) starts from day 1 of the menstrual cycle (short), or in the mid-luteal phase of the preceding cycle (long). The stimulation with 150-300 IU of gonadotropins (either HMG, e.g. Merional®, Menopur® or FSH, e.g. Gonal-F® or Pergoveris®) starts from cycle day 2-3.[69,70] The physician individually decides the dose and the exact product based on the age, body mass index (BMI), AMH level, and antral follicle count and medical history of the woman.

For the antagonist protocol, 150 – 300 IU HMG are administered for follicular stimulation once a day. From day 6 to 8 onwards, the GnRH antagonist (e.g. Cetrotide® or Orgalutran® 0.25 mg) is added to avoid preterm ovulation. In all cIVF protocols, cycles are monitored by ultrasound and serum E2 level is measured. Once the leading follicles reach a diameter of at least 18 mm, and the concentration of E2 corresponds to the number of follicles, ovulation is triggered by urinary hCG (e.g. 6500 IU Ovitrelle®). The transvaginal oocyte retrieval takes place 36 hours after hCG administration under conscious sedation.[71,72]

Due to the downregulation of the pituitary gland and to the reach of supraphysiological E2 levels, the luteal phase after gonadotropin stimulation is insufficient. Progesterone is vaginally administrated to prepare the endometrium for implantation after embryo transfer up to 12 weeks of gestation.[73]

Definition of different treatments

NC-IVF is defined by the International Society for Mild Approaches in Assisted Reproduction (ISMAAR) as IVF without the use of any medication. However, it is also used as an umbrella term for all IVF treatments not using gonadotropins or other stimulating hormones for follicular growth. Von Wolff suggests naming these treatments “monofollicular IVF”, even if certain medications are used to support steps within the cycle to improve the outcome. He suggests further distinguishing between minimal stimulation IVF, and conventional IVF. In minimal stimulation IVF, a lower dose of stimulating hormones is used to stimulate the growth of some follicles. This could be named “oligofollicular IVF”. In conventional IVF, GnRH antagonists or agonists are used to suppress the LH surge and to stimulate the growth of many follicles, which should be named “polyfollicular IVF”.[61]

1.4. Perinatal health of children born after ART

The first successes in ART soon met with religious and ethical concerns. In ART, the treatment not only affects the parental couple, but also the offspring. Concerns followed about complications in pregnancy and birth, and over the health of children conceived using ART.[74] Perinatal outcomes for ART children have since been comprehensively studied. There are many risks, which are particularly associated with multiple births, but are also present in single births after ART.[75] The first systematic reviews on singletons with data from the 80's and 90's showed a twofold higher risk of preterm birth (PTB), low birthweight (LBW) and small-for-gestational age (SGA), in comparison to spontaneously conceived singletons.[75,76] Later systematic reviews and meta-analyses (after the year 2000) still found an odds ratio (OR) of 1.5 for preterm birth.[77,78] Anja Pinborg et al. found increased risks of preterm birth in spontaneously conceived children when time-to-pregnancy was more than one year (adjusted OR 1.35).[78] Furthermore, they found higher risks in ART singletons as compared to their spontaneously conceived siblings (aOR 1.27, 95% CI 1.08 – 1.49), suggesting that factors related to ART methods per se may still play a role.

Epigenetic modifications control the activity of genes, but do not change the DNA structure. They are either associated with the health and fertility levels of the parents, or are a consequence of the early environment of the embryo. The first epigenetic programming occurs during gametogenesis in either parent. A second wave of epigenetic reprogramming happens at fertilization with the demethylation of the paternal and maternal genomes.[78,79] Several studies show differences in DNA methylation levels in children born after ART.[80–82] However, a recent study demonstrates that this epigenetic changes seem not to persist into adult life.[83]

Researchers have identified various factors, which may increase the risk of ART causing epigenetic changes during conception, pregnancy and early life. These are:

- Hormonal stimulation
- Fertilisation
- In vitro culture
- Cryopreservation

The following section offers some insight in the possible associations.

Hormonal stimulation

Hormonal stimulation may interfere with oocyte selection, follicular fluid and early pregnancy.[84–86] Epigenetic changes may occur during gametogenesis, and in genes critical to endometrial remodelling during early implantation.[87]

Different stimulation protocols used within ART have been comprehensively researched and discussed, with a focus on the type and total dose of hormones used.[88,89] Research suggests that the birthweight and gestational age of newborns is lower after gonadotropin stimulation.[90–93] This is especially true when women show a high response to the stimulation, which leads to supraphysiological estrogen levels around ovulation, and so to superovulation.[94] Low birthweight and small-for-gestational-age, as well as the risk of preterm birth, seem to be associated with high estrogen levels and a high number of oocytes.[95–97] However, the risk of low birth weight also seems to be influenced by parental characteristics and pregnancy conditions.[98]

Fertilisation

In IVF, several sperms compete for fertilisation. In ICSI an embryologists arbitrary selects a single sperm to inject it into the oocyte, which is quite invasive. However, compared to IVF, most large studies found similar or lower risks for PTB and LBW in singletons born after ICSI. A large cohort study found an association between ICSI and a higher risk of birth defects, but this was not confirmed by a meta-analysis.[99,100] Male infertility is inherited from the father by male offspring born after ICSI.[101,102]

In vitro culture

Culture procedures in the laboratory seem to introduce epigenetic changes in the sensitive phase of foetal programming.[103,104] It has been shown that two culture media, from different manufacturers, lead to differences in birthweight. Although the components in the culture media are known, the manufacturers do not disclose the exact concentrations.[105–108]

Changes in Swiss law, and advances in culture techniques, have permitted embryo culture up to day five, until the embryo reaches the blastocyst stage. This enables the self-selection of viable embryos, and a better synchronisation with the maternal cycle. A Cochrane review showed increased live birth rates after fresh blastocyst transfer by OR 1.48 (95% confidence interval (CI) 1.20 – 1.82), as compared to cleavage stage transfer.[109] Blastocyst transfer may also lead to higher birthweight.[110] However, blastocyst transfer was also associated with a higher risk of preterm and very preterm delivery, although the risk of intrauterine growth restriction was lower.[111]

Cryopreservation

To minimise the risks of hormonal stimulation to the onset of pregnancy, freeze-all strategies are becoming increasingly proposed. A single thawed embryo is transferred during a normal menstrual cycle, without gonadotropin stimulation of oocyte growth but only hormonal preparation of the endometrium. Babies born from the transfer of thawed embryos have a higher risk of increased birthweight and large baby syndrome.[112–114] A meta-analysis concluded there was a lower risk of

preterm delivery, low birth weight and small-for-gestational age. However, a systematic review and meta-analysis found the risks of preeclampsia and of hypertensive disorders during pregnancy, were found to be higher.[115] This result was confirmed by a large randomised controlled trial conducted in China, in which they also found a higher pregnancy rate after single frozen blastocyst transfer.

Multiple pregnancies

Multiple pregnancies are increased in ART due to the transfer of more than one embryo. Often multiple embryos are available after hormonal stimulation. Despite the fact that multiple pregnancies are associated with higher risks, the transfer of multiple embryos increases pregnancy and live birth rates.[116] Many experts support the elective single embryo transfer (SET) strategy. Therefore, in some countries, laws or insurance policies encourage or require the use of SET.[117] Even when a singleton is born after the transfer of more than one embryo, there are increased risks. If one embryo does not develop further, this is called a vanishing twin. The remaining singleton is physiologically still seen as a twin during the pregnancy. This can lead to intrauterine growth restrictions, with subsequent lower birth weight and a higher risk of being SGA.[118,119]

Risks for the mother

Hormonal stimulation likewise increases risks for the mother-to-be. The use of hormones is always associated with a higher risk for thrombosis in women. Furthermore, ovarian hyperstimulation syndrome (OHSS) is clearly associated with high-dose gonadotropin or clomiphene stimulation. The risks are generally not foreseeable, as they are dependent on the individual's reaction on the stimulation. OHSS is mainly characterised by increased vascular permeability and ovarian enlargement. OHSS can range from mild to severe, and in rare cases is even fatal. Moderate to severe cases may occur in 3-10% of cases.[120]

In extensive meta-analyses or cohort studies, the following risks have been found to significantly increase during pregnancy after ART: pregnancy-induced hypertension, gestational diabetes, placenta praevia, placental abruption, ante- or postpartum haemorrhage, and, in multiple pregnancies, the premature rupture of membranes. This puts the health of child and mother at risk of complications and preterm birth.[121,122] ART children are more frequently delivered by caesarean section. It is not clear whether this is due to parental wishes, whether complications occur more frequently or simply whether obstetricians tend to be especially careful.[77,112,122] However, an Italian study comparing the delivery of uncomplicated singleton term pregnancies still found higher odds of operative delivery for ART children (adjusted OR 1.40; 95% CI 1.01-1.95), and this is also true within the Bern IVF cohort.[123]

Research on NC-IVF

NC-IVF allows research into the influence of gonadotropin stimulation, as outcomes can be compared to those of cIVF. As SET is standard, multiple pregnancies can easily be avoided in NC-IVF. Research at the University Women's Hospital in Bern has proven NC-IVF to be less stressful for infertile patients.[124] It is less expensive but more time consuming per achieved pregnancy when compared to cIVF.[66,125] In NC-IVF, the quality of the embryo seems to be better, due to natural follicle selection and fewer alterations of the follicular fluid.[126–128] Implantation and placentation seem to be improved due to increased endometrium receptivity.[85,86,129,130] NC-IVF and minimal-stimulation IVF are valuable, cost-effective alternatives to cIVF, and it is important to investigate the possible reduction of further risks associated with ART by promoting NC-IVF.[66,131]

1.5.Long-term health of children born after ART

In David Barker's investigations into infant mortality and stroke, he found a relationship between conditions in early life and later health outcomes. At that time, low birthweight was the most frequent cause of infant mortality.[132] He developed the theory of developmental origins of health and disease (DOHaD).[133] In a Finnish cohort, in which fetal growth was measured, he identified fetal growth restriction, undernutrition and compensatory growth in childhood as the key risk factors associated with cardiovascular risk and type 2 diabetes. He postulated that the circumstances of conception, and conditions during pregnancy, may influence later health, even in adult life. Hunger and growth restriction in the womb may especially affect cardiometabolic outcomes.[134] Some concerning results have recently been published regarding the cardiovascular health of children born after ART. A Spanish research group found cardiovascular remodelling even during pregnancy in foetuses conceived through ART, which persisted until age 3. [135,136] A cohort study conducted in Switzerland found increased intima-media thickness, increased flow-mediated dilation, higher pulse wave velocity and higher blood pressure in children at age seven to nine born after ART.[137] These findings were later found to persist into adolescence (16-18 years).[138] A study of an ART cohort at the Netherlands found elevated blood pressure at age four, although this was not found to persist at age nine.[139–141] An Australian study of 193 ART and 86 non-ART adults did not find elevated vascular or cardiometabolic risks, when adjusted for important perinatal factors and quality of life.[142] A recent systematic review and meta-analysis including 19 studies with 2'112 ART and 4'096 non-ART children, found small but significantly higher blood pressure levels in ART children. [143]

Singletons born after IVF perform well in neurological and mental development, and show normal intelligence, although Zhu et al. found a modest delay in psychomotor developments in a large Danish birth cohort.[144,145] However, the duration of infertility seems to be associated with lower cognition and behavioural performance.[146] The incidence of autism spectrum disorders seems to be inconclusive, but rather not to be elevated in singletons, but is in multiples following ART.[147,148] Initial results in adolescents show no persistence of autism spectrum disorders.[149] A tendency toward an increased risk for cerebral palsy has been observed, mainly associated with preterm birth or multiples.[144,145] No difference has been identified regarding growth (weight & height) in ART children in comparison to spontaneously conceived children.[150–152]

Cancer is very rare during childhood; it is therefore difficult to identify differences between ART and non-ART children. Two systematic reviews and meta-analyses summarized several large cohort- and linkage studies, and found a small but significantly increased risk for all types of childhood cancer in children born following ART.[153,154] The more recent review from 2019 found that risks were mainly

increased for leukaemia and retinoblastoma.[154] Two recent studies from the USA and Denmark, not yet included in the latest reviews, found a significant association between childhood cancer and frozen embryo transfer.[155,156]

The question of whether children conceived through ART are at an increased risk of infections, especially severe infections requiring hospitalisation, has mainly been studied by looking at general health and morbidity or hospital admissions.[157–163] A somewhat increased risk was mainly associated with multiple births and lower birthweight.[160,163] For infectious and parasitic diseases, Kettner et al. found in their review eight studies presenting odds ratios (OR) between 0.37 – 5.7.[164] For the burden of otitis media, acute tonsillitis, urinary infections or pneumonia, Kallen et al. found a higher odds (OR 1.08 – 1.33) among ART children, in a population based study,[161] whereas Pinborg et al. did not find differences in a questionnaire-based study in which they compared ART twins to ART singletons and SC twins.[160]

Only a few immune-mediated diseases have been studied in ART children. Of these, asthma is probably the most studied.[165–170] Many of these studies show an association between ART and increased odds ratios from 1.9 to 6.0.[157,159,161,167,168] Those studies which have information on parental subfertility (time to conception), all found a higher risk of asthma among the spontaneously conceived children of subfertile parents, as compared to the children of parents without fertility problems.[168,169,171,172] Kuiper et al. found the higher use of asthma medication in ART children to be associated with hormonal-stimulated IVF, as compared to modified natural cycle IVF.[173] Two studies on adolescents and young adults describe a higher prevalence of breathing problems and asthma in ART children; however, this difference was no longer observed in adolescence.[142,166]

Another important disease to look at is type 1 diabetes. Only two studies have been published on the topic, both very recently.[174,175] Norrman et al. found increased HR for diabetes type 1 in an unadjusted analysis, but no longer once adjusted. They also observed an association between type 1 diabetes with FET.[175] Kettner et al. found no association between type 1 diabetes and fertility treatment in a primary analysis, but found some increased risk associated with follicle-stimulating hormones in a secondary analysis.[174] However, while these two studies remained somewhat inconclusive, there is evidence that glucose metabolism might be altered by ART.[143,176] This may increase the risk of the development of type 2 diabetes later in life. The DOHaD theory also supports this hypothesis. As low birthweight is associated with impaired metabolic functions, a higher susceptibility to type 2 diabetes might be expected.[177,178]

2. Methods and data

In the following section, the study designs and data sets used in the different projects included in this thesis will be presented.

2.1.The Bern IVF Cohort

The main data source for this thesis is the Bern IVF Cohort. I coordinated the cohort's establishment by helping secure ethical approval, setting up the RedCap database and recruiting the participants. One of the main aims of my thesis was the set-up of a control group of spontaneously conceived children. I performed the matching of cohort members with control group members, and coordinated the participant recruitment and data collection.

The aim of the Bern IVF Cohort is to compare different fertility treatments, mainly NC-IVF and cIVF. NC-IVF avoids gonadotropins for stimulation of oocyte growth. cIVF uses ≥150 IU of gonadotropins per day for the stimulation. The Bern IVF Cohort allows comparison of outcomes between the two treatments. This allows estimating influence of hormonal stimulation on pregnancy and health of children. In Bern, NC-IVF and cIVF are offered, and sufficient numbers of both treatments are conducted to allow comparison. The cohort is therefore unique, both in Switzerland and even at an international level.

2.1.1. Bern IVF Cohort - description

The Bern IVF cohort was established in 2016-2017, retrospectively including woman treated from 2010 to 2015, and with the aim of including women treated in 2016 and 2017. The registration of women treated in 2017 is not yet complete. Data is collected on parental characteristics, medical and fertility history, ART treatment, pregnancy complications and outcome, the characteristics of births and perinatal health. Follow-up data on breastfeeding duration and child growth until age four is also collected. All participants are contacted for informed consent, and receive a newsletter every year. The newsletter contains information about current and planned projects, and helps in keeping addresses updated. The Bern IVF cohort has ethical approval, issued by the cantonal ethics committee of Bern (No. 2012-00235).

Inclusion criteria: All couples (mothers and fathers) where the woman was 18-42 years at the time of treatment, and all children born after successful IVF treatment and fresh embryo transfer performed at the Bern University Hospital from 2010-2017.

Exclusion criteria: Children born after FET, and women whose treatment cycles did not lead to a pregnancy. Pregnancy as defined as having had one positive pregnancy test for hCG.

In 2017, the law governing reproductive medicine in Switzerland was changed, allowing for a longer duration of embryo culture. This change was adopted from October 2017 onwards at the Bern University Hospital. Women treated after this change are not recruited into the cohort, as the duration of embryo culture is considered an important factor affecting perinatal outcomes.[109]

The Bern IVF Cohort currently includes n=319 women, with a total of n=488 pregnancies. Of the pregnancies n=190 ended in a miscarriage and n=298 in a delivery (272 singletons, 25 twins, 1 triplet), with 269 singletons born alive (Table 1). (Article 1 and Article 2)

Table 1: Live births per treatment in the Bern IVF Cohort (per achieved pregnancy)

		Adverse pregnancy outcomes			**Livebirths**	
	Pregna-ncies	**Biochem. only**	**Miscarriages**	**Perinatal death***	**Singletons (LBR)**	**Multiples (twins/triplets)**
NC-IVF (A)	110	14	26		70 (63.6%)	0
NC-IVF (C) with CC	124	21	25	1	74 (62.1%)	3
mIVF (D) with CC	25	4	2		17 (76.0%)	2
mIVF (E)	47	10	9	1	23 (57.4%)	4
cIVF	182	44	35	1	85 (56.0%)	16 /1
Total	**488**	**93**	**97**	**3**	**269**	**26 (53)**

LBR: Live birth rate including multiples; NC-IVF: Natural cycle IVF, mIVF: minimal stimulation IVF, CC: Clomiphene citrate, cIVF: conventional stimulated IVF

**Perinatal death is defined as death from 28 gestational weeks until 7 days after birth*

Table 2: Singletons born alive by treatment scheme and year of OPU in the Bern IVF Cohort

Treatment	**2010**	**2011**	**2012**	**2013**	**2014**	**2015**	**2016**	**Total**
NC-IVF (A)	10	1	14	3	11	12	19	70
NC-IVF (C) with CC	5	5	9	14	9	18	14	74
mIVF (D) with CC	0	0	0	5	0	6	6	17
mIVF (E)	2	0	2	5	5	2	7	23
cIVF	15	17	7	7	6	12	21	85
Total	**32**	**23**	**32**	**34**	**31**	**50**	**67**	**269**

NC-IVF: Natural cycle IVF, mIVF: minimal stimulation IVF, CC: Clomiphene citrate, cIVF: conventional stimulated IVF

2.1.2. The Bern IVF Cohort: establishment of control group

In 2018, the decision was made to establish a control group of spontaneously conceived children for comparison to the IVF children of the Bern IVF Cohort Study. The recruitment of possible controls was conducted through the obstetrics department of the University Women's Hospital in Bern. Women coming for ultrasound in the first trimester of their pregnancy were matched with the mothers of singletons from the Bern IVF cohort. It was decided to only match women treated with NC-IVF or cIVF. A total of n=155 women were matched in a 1:3 ratio. The matching criteria were the age of the mother (+/- 1 year), the parity (primipara vs multipara) and the (planned) due date (+/- 6 months).

Exclusion criteria were the same as for ART treatment at the Bern University Women's hospital: Mothers with problems of drug abuse or parents infected by human immunodeficiency virus (HIV) were excluded. Additionally couples, which used more than 12 months to become pregnant were declared as subfertile. Couples who used ART treatment to achieve the pregnancy were excluded as well. To identify the couples to exclude, a questionnaire was sent with the patient information and the informed consent form. The plan was to match 465 controls. 398 women were eligible for contact. A first reminder letter was sent to 255 (65.5%) women and 227 got a phone call as a second reminder (58.4%) Figure 1 shows the procedure for the contact of the controls. Figure 2 shows the population of the control group. Finally, complete data on 268 women, their pregnancies and births, was available.

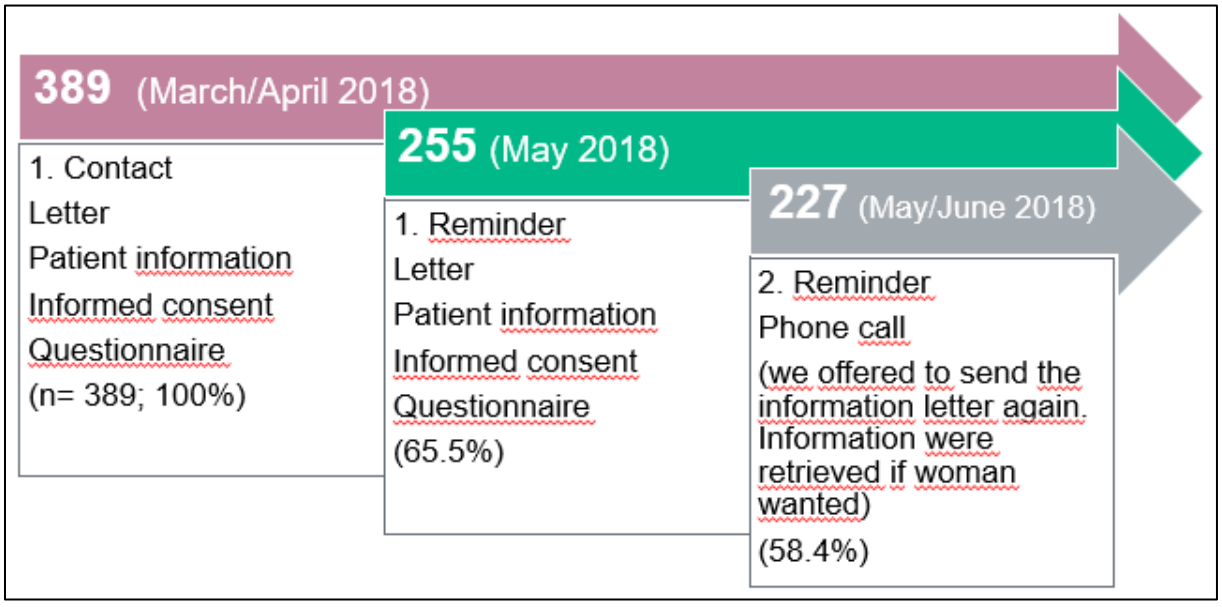

Figure 1: Contact of controls and reminders, process to retrieve informed consent

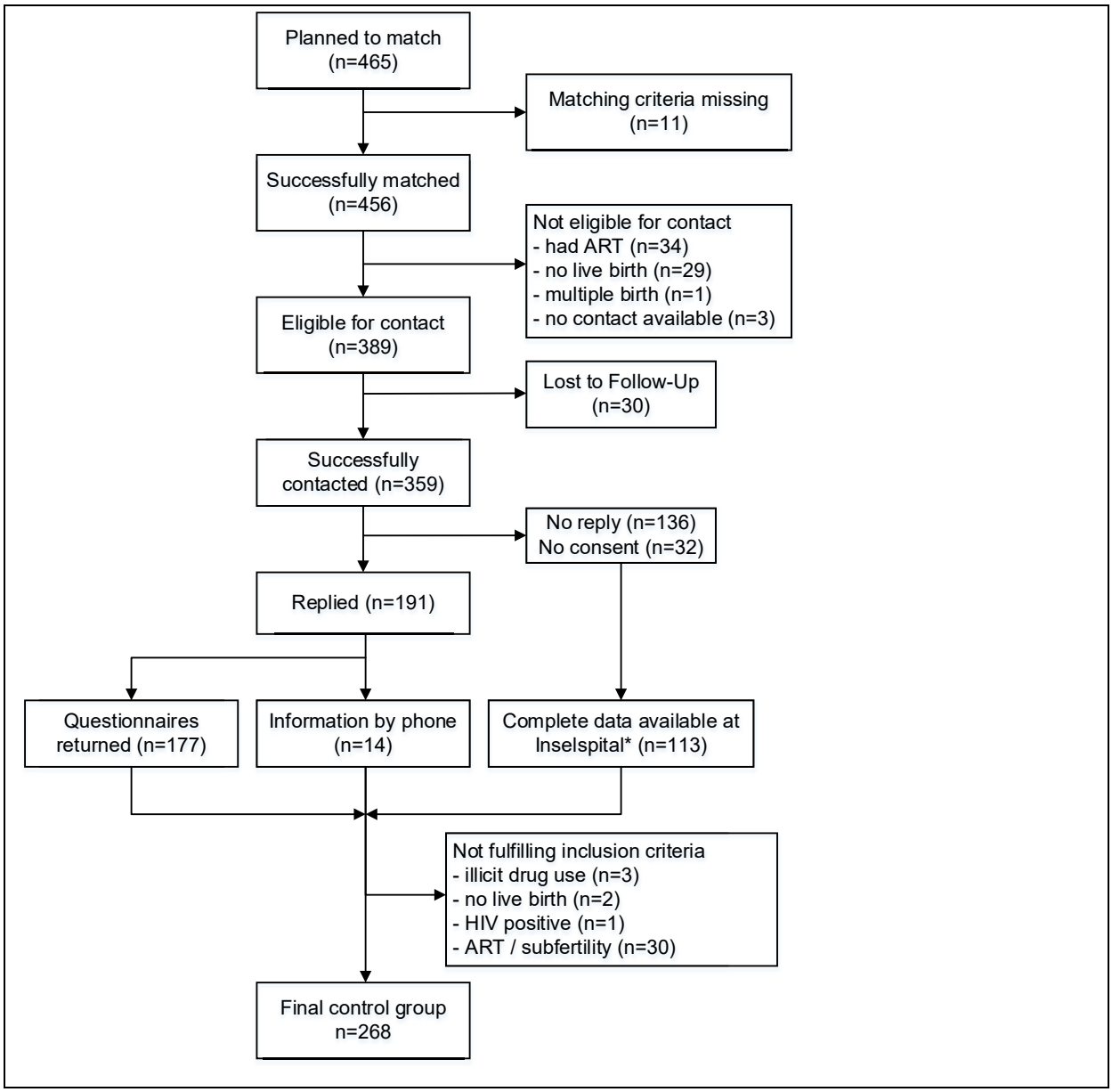

*Figure 2: Final population of the control group * either general consent or no documented refusal available at Inselspital. ART: Assisted reproductive technologies, HIV: Human immunodeficiency virus*

2.2.The Swiss Infant Feeding Study

The Swiss Infant Feeding Study (SWIFS) was the third study on the prevalence and duration of breastfeeding and infant nutrition in Switzerland. It was conducted by the Swiss Tropical and Public Health Institute at the University of Basel. It is a cross-sectional study using written postal questionnaires. Data was collected on social-economic status, nationality and lifestyle, alongside information on pregnancy and birth. Additionally, in 2014, information on employment conditions, child and maternal health, health-related behaviour and social networks was collected. The questionnaire also contained a 24-hour nutrition protocol. The Swiss Parent Counselling Services (Mütter- und Väterberatungsstellen) randomly recruited mothers from the German, French and Italian speaking regions of Switzerland. They sent a reminder two weeks after the first questionnaire. 40% (n=1650) of the questionnaires were returned.[179]

The Swiss Tropical and Public Health Institute provided the data they used to analyse breastfeeding duration in Switzerland during the first year of life (n=1421). It served as reference group to the breastfeeding data from the Bern IVF Cohort. (Article 2)

2.3.Endometrial thickness in women after NC-IVF

This was a retrospective cohort in which 225 women undergoing their first NC-IVF cycle were identified. 111 did not have subsequent embryo transfer, and nine were excluded for other reasons (endometriosis and TESE). In this sample of 105 women who met the inclusion criteria, the influence of endometrial thickness on pregnancy rates is analysed. In NC-IVF, the endometrium is not influenced by the hormonal stimulation of follicular growth. Research on the association between endometrial thickness and pregnancy rates has mostly been done on stimulated IVF cycles. (Article 3)

2.4.Women with hysteroscopy assessment

A cohort of 217 women who had a planned hysteroscopy assessment for different reasons between 2014-2019 was set up at the Bern University Women's Hospital. Women diagnosed with RIF and RPL usually have a hysteroscopy assessment in order to identify possible causes. 127 women did fulfil the definitions of repeated implantation failure and recurrent pregnancy loss. This group of women was used for our retrospective analysis on time-to-pregnancy and time to live birth after the diagnosis and treatment of chronic endometritis. (Article 4)

3. Objectives of the thesis

The aim of this thesis is to contribute to the understanding of factors influencing pregnancy and perinatal outcomes after different ART treatments for infertility.

Specific objectives included:

1) To further develop the Bern IVF cohort by including patients treated in 2016 and setting up a control group of spontaneously conceived children
2) To evaluate perinatal outcomes within the Bern IVF cohort: birthweight, gestational age and birthweight percentile (Article 1)
3) To assess breastfeeding behaviour and duration of mothers with children born within the Bern IVF cohort in comparison to a random sample of mothers in Switzerland (Article 2)
4) To support the understanding of CE in women with RIF and RPL and to analyse time-to-pregnancy and live birth depending on the biopsy and subsequent treatment in cases of CE (Article 4)
5) To support the research on fertility and fertility treatments and their outcomes in the division of gynaecological endocrinology (Article 3)

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5. Manuscripts

5.1 Article 1: Small-for-gestational-age

The greater incidence of small-for gestational-age newborns after gonadotropin-stimulated in vitro fertilization with a supraphysiological estradiol level on ovulation trigger day

Contributions

This project was the first planned publication out of the Bern IVF Cohort. As project coordinator, I was involved in this project from the very beginning, supporting all the steps mentioned in the manuscript. Furthermore, I was responsible for the data management, including data extraction, cleaning, data quality and preparation for analysis.

I contributed to the conception and design of this manuscript, to the collection of the data and to the drafting of the text, including a literature review and citing of references.

I planned and conducted the analysis and interpreted the data.

Alexandra Kohl-Schwartz and I wrote the first draft.

I contributed substantially to the finalisation of the manuscript.

I created Figure 1, Figure 2, Figure 3 and Figure 4, and Tables 1, 2 and 3.

I was heavily involved in revising the analysis and rewriting the manuscript.

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ORIGINAL RESEARCH ARTICLE

The greater incidence of small-for-gestational-age newborns after gonadotropin-stimulated in vitro fertilization with a supraphysiological estradiol level on ovulation trigger day

Alexandra S. Kohl Schwartz[1] | Vera R. Mitter[1] | Sofia Amylidi-Mohr[2] | Pascale Fasel[1] | Mirja A. Minger[1] | Costanzo Limoni[3] | Marcel Zwahlen[4] | Michael von Wolff[1]

[1]Division of Gynecological Endocrinology and Reproductive Medicine, Bern University Hospital, University of Bern, Inselspital, Bern, Switzerland

[2]Department of Feto-maternal Medicine and Obstetrics, University Women's Hospital, University Hospital, University of Bern, Inselspital, Bern, Switzerland

[3]University of Applied Sciences and Arts of Southern Switzerland, Lugano, Switzerland

[4]Institute of Social and Preventive Medicine, University of Bern, Bern, Switzerland

Correspondence
Alexandra Sabrina Kohl Schwartz, Division of Gynecological Endocrinology and Reproductive Medicine, Inselspital, Bern University Hospital, Friedbuehlstrasse 19, 3010 Bern, Switzerland.
Email: alexandra.kohl-schwartz@insel.ch

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Abstract

Introduction: Reproductive scientists have postulated various risk factors for lower birthweight following conventional gonadotropin-stimulated in vitro fertilization compared with spontaneously conceived children: parental factors (age, health, duration of subfertility and smoking habits); ovarian stimulation; laboratory procedures; the number of oocytes retrieved and the number of embryos transferred. Our aim was to investigate the impact of gonadotropin stimulation and serum estradiol level on the risk of a newborn being small-for-gestational-age.

Material and methods: We conducted a cohort study (2010-2016) of singletons (n = 155) born either after conventional gonadotropin-stimulated in vitro fertilization (using ≥150 IU/d human gonadotropin for stimulation) or after natural cycle in vitro fertilization without any stimulation. We analyzed perinatal outcomes using birthweight percentiles, adjusted for gestational age and sex.

Results: The proportion of small-for-gestational-age was 11.8% following conventional gonadotropin-stimulated in vitro fertilization and 2.9% after natural cycle in vitro fertilization ($P = 0.058$). The odds of small-for-gestational-age were significantly higher with supraphysiological estradiol levels in maternal serum on ovulation trigger day (unadjusted odds ratio 4.58; 95% confidence interval 1.35-15.55; $P = 0.015$). It remained significant after adjusting for maternal height, age and body mass index (adjusted odds ratio 3.83; 95% confidence interval 1.06-13.82; $P = 0.041$).

Conclusions: We found an associated risk of children being born small-for-gestational-age after conventional gonadotropin-stimulated in vitro fertilization compared with natural cycle in vitro fertilization. This higher risk is significantly associated with supraphysiological estradiol levels. We propose a reduction in the dosage of

Abbreviations: BMI, body mass index; CI, confidence interval; c-IVF, conventional gonadotropin-stimulated IVF; E2, estradiol; FA, follicle aspiration; IVF, in vitro fertilization; LBW, low birthweight; NC-IVF, natural cycle in vitro fertilization; OR, odds ratio; SGA, small-for-gestational-age.

Alexandra S. Kohl Schwartz and Vera R. Mitter are joint first authors.

gonadotropin to minimize the risk of small-for-gestational-age and future health consequences.

KEYWORDS
gonadotropin, high-risk pregnancy, in vitro fertilization, infertility, pregnancy, reproductive endocrinology

1 | INTRODUCTION

Birthweights in children born following conventional gonadotropin-stimulated in vitro fertilization (c-IVF) and fresh embryo transfer are shown in several systematic reviews and meta-analyses[1,2] to be lower than birthweights of spontaneously conceived singletons. Various risk factors were postulated:[3] parental factors (mainly pre-existing diseases, duration of subfertility[4] and parental smoking); in vitro fertilization (IVF) laboratory procedures;[5] the number of oocytes retrieved;[6] and the number of embryos transferred. After the transfer of more than one embryo, fetal intrauterine growth restriction may be a consequence of vanishing twins.[2] Two studies addressing E2 levels in maternal serum at ovulation trigger day postulated a dosage-dependent impact of gonadotropins on birthweight and birthweight percentile.[7,8]

Comparing c-IVF with natural cycle IVF (NC-IVF) with natural follicle selection and without the use of gonadotropins offers an excellent way to study the influence of hormones on obstetric and perinatal outcomes. A recent systematic review found a significantly higher risk of low birthweight (LBW) after c-IVF (risk ratio 1.95; 95% confidence interval [CI] 1.03-3.67) compared with modified IVF/NC-IVF therapy.[9] Studies included in the review focused on birthweight as the main outcome and defined LBW as <2500 g, without taking into consideration exact gestational age.[6,10,11] Using birthweight percentiles adjusts for gestational age and sex of the neonate.

Our study compares birthweight and birthweight percentiles of c-IVF with those of NC-IVF newborns.

Key message

Supraphysiological serum estradiol levels under gonadotropin stimulation in in vitro fertilization are associated with a higher incidence of children born small-for-gestational-age. We propose a reduction in gonadotropin dosage to minimize potential negative health consequences.

2 | MATERIAL AND METHODS

2.1 | Study population

The cohort of this study includes women treated for infertility at the Bern University Hospital, Division of Gynecological Endocrinology and Reproductive Medicine, Switzerland, from 2010 to 2016. We retrieved prospectively collected data and prepared it for analysis using REDCap electronic data (REDCap 8.5.19 Vaderbilt University, Nashville, USA) capture tools hosted at the Clinical Trials Unit, University of Bern.

We included data on all women with a singleton live birth after a fresh cleavage-stage embryo transfer who underwent either c-IVF or NC-IVF. Women with a regular menstrual cycle chose the treatment (NC-IVF or c-IVF) as per their preference. We did not include women receiving thawing cycles and cycles with modified IVF treatment (eg, the use of clomiphene citrate [CC]). At our clinic, we offer treatments with modifying drugs such as CC and low-dose human menopausal gonadotropin only as second-line options after several unsuccessful NC-IVF treatments. Women receiving those treatments had previously several unsuccessful treatment cycles. Our aim was to compare two clearly distinct approaches, namely, NC-IVF without the use of any stimulating drugs with c-IVF, both used as first-line treatments.

2.2 | Data sources

We collected data on patient characteristics and reproductive treatment from medical records. Chronic disease included asthma, Hashimoto's thyroiditis and arterial hypertension. From the treating gynecologist or the maternity hospital we received results of later pregnancies, delivery reports and perinatal information on mother and child. We considered the following conditions to be pregnancy complications: gestational hypertension, gestational diabetes, placental pathologies (eg, abnormal placentation such as placenta previa or placenta accreta/increta), antepartum hemorrhage, preterm contractions, preterm rupture of membranes and fetal growth restriction. We calculated fetal weight during pregnancy using the Hadlock formula. From the available fetal growth charts, we evaluated uteroplacental dysfunction (decrease in growth rate) and characteristic fetal perfusion changes if Doppler ultrasound was available. We defined according to the TRUFFLE study for fetal growth restriction[12] an abnormal umbilical artery Doppler with a pulsatility index above the 95th percentile of the Doppler reference chart[13] with or without reversed or absent end-diastolic flow as pathologic. We calculated gestational age as the time from fertilization at the day of follicular aspiration (FA) to delivery, plus 14 days. We used growths charts from Voigt & Fenton[14] to determine the birthweight percentile. We defined a priori small-for-gestational-age (SGA) as a birthweight

≤5th percentile. This reflects a degree of smallness that is more likely to be pathologic rather than constitutional.[15,16]

2.3 | Conventional gonadotropin-stimulated IVF

At our center, we perform c-IVF as either a short agonist protocol or an antagonist protocol. For the short agonist protocol, we use triptoreline (gonadotropin-releasing hormone agonist, eg, Decapeptyl®) and 150-300 units of follicle-stimulating hormone (eg, Fostimon®) or human menopausal gonadotropin (Menopur® or Merional®) for follicular stimulation, depending on age, Anti-Müllerian hormone level and antral follicle count. For the antagonist protocol, we use human menopausal gonadotropin (150-300 units a day) for follicular stimulation and 0.25 mg cetrorelix (gonadotropin-releasing hormone antagonist, eg, Cetrotide®) once a day, beginning at stimulation day 6 or 7. We monitor cycles using ultrasound, check serum E2 levels, and trigger ovulation with human choriogonadotropin (eg, Ovitrelle®) when the size of the leading follicles is >17 mm. We perform FA 36 hours later under conscious sedation.

2.4 | Natural cycle IVF

We perform NC-IVF in accordance with best practice.[17] We perform follicle monitoring by ultrasound and analysis of E2 and luteinizing hormone (LH) concentrations, once preovulatory. When the follicular diameter reaches at least 16 mm and the E2 concentration is expected to be ≥800 pmol/L, we administer a single dose of 5000 IU human choriogonadotropin as a trigger and FA is performed 36 hours later without anesthesia using a 19G single lumen needle. In the event of luteinizing hormone-surge (>10 IU/L) on the day of human choriogonadotropin administration, we give 400 mg ibuprofen every 8 hours starting a maximum of 48 hours before FA until the day of FA. If information on the E2 concentration on ovulation trigger day is not available, we calculate it based on follicular phase measurements.

For both types of treatment, we fertilize the mature oocytes by intracytoplasmic sperm injection, and we culture a cleavage stage embryo. For the assessment of embryo quality, we use ASEBIR classification[18] based on the number of cells, the heterogeneity of the cells, and the fragmentation rate of the embryo. We transfer the embryo at cleavage stage with a soft transfer catheter under ultrasound surveillance. Women receive luteal phase support twice a day after c-IVF with intravaginal micronized progesterone (eg, Utrogestan® 200 mg or Crinone®), beginning the evening of the day of FA until the 12th week of pregnancy.

2.5 | Statistical analyses

We performed statistical analysis using Fisher's exact test (SGA and LBW) or chi-square test and Mantel-Haenszel statistics for categorical outcomes and linear regression for continuous outcomes. As seven women with two IVF children each were included in the dataset, we used

TABLE 1 Patient characteristics

	NC-IVF		c-IVF		
	n = 70	%/SD	n = 85	%/SD	*P* value
Maternal age (y)	34.23	3.76	34.57	4.15	0.598[a]
Maternal height (cm)	166.99	6.04	167.01	7.98	0.982[a]
Maternal weight (kg)	62.18	10.57	62.28	11.17	0.956[a]
Maternal BMI (kg/m^2)	22.32	3.76	22.33	3.72	0.982[a]
Parity (nulliparous)	52	74.29	72	84.71	0.107[b]
Smoking before pregnancy (y/n)	7	10	15	17.65	0.248[c]
Smoking during pregnancy (y/n)	0	0	4	4.71	0.141[c]
Chronic disease mother (y/n)	12	17.14	19	22.40	0.420[b]
AMH mother	21.52	22.30	24.79	21.32	0.358[a]
Indication for IVF					
Male factor	45	64.30	42	49.41	0.381[c]
Endometriosis	11	15.70	18	21.18	
Tube factor	3	4.30	5	5.88	
PCO-S	0	0.00	3	3.53	
Idiopathic	10	14.29	15	17.65	
Other	1	1.43	2	2.35	

AMH, Anti-Müllerian hormone; BMI, body mass index; c-IVF, conventional IVF; cm, centimeter; IVF, in vitro fertilization; kg, kilogram; m^2, square meter NC-IVF, natural cycle IVF; PCO-S, polycystic ovary syndrome; SD, standard deviation; y, years; y/n, yes/no.
[a]Linear regression.
[b]Chi-square test.
[c]Fisher's exact test.

TABLE 2 Stimulation and pregnancy outcome characteristics

	NC-IVF		c-IVF		
	N = 70	%/SD	N = 85	%/SD	*P* value
Stimulation					
Nb previous transfers (n)	1.94	1.74	1.62	0.88	0.138[a]
Day of retrieval (d)	12.65	1.87	12.19	2.09	0.207[a]
Nb oocytes retrieved (n)	1.01	0.12	8.20	4.73	**<0.001**[a]
Nb of embryos transferred (n)	1.01	0.12	2.01	0.36	**<0.001**[a]
Day of embryo transfer (d)	2.69	0.71	2.61	0.74	0.531[a]
Total gonadotropin dosage (IU)	—	—	2322.77	758.41	—
Estradiol at trigger day (pmol/L)	1028.94	330.39	10 459.78	4552.12	**<0.001**[a]
Estradiol at trigger day (>10 000 pmol/L)	0	0	40	48.19	**<0.001**[b]
Endometrium thickness (mm)	8.59	1.75	9.86	2.37	**<0.001**[a]
Pregnancy					
Duration of pregnancy (wk.d)	39.2	1.52	38.94	2.51	0.451[a]
Pregnancy hypertension (n)	1	1.43	1	1.18	0.890[c]
Pregnancy complication (n)	16	22.56	26	30.59	0.309[b]
Induction of labor (n)	22	35.48	28	34.57	0.909[b]
Very preterm birth (<31 wk)	0	0	2	2.35	0.418[c]
Preterm birth (31-36 wk)	5	7.14	5	5.88	
Term birth (≥37 wk)	65	92.85	78	91.76	
Infant gender female	36	51.43	34	40	0.155[b]
Infant gender male	34	48.57	51	60	
Birthweight (g)	3310.34	475.08	3218.25	704.38	0.352[a]
VLBW (<1500 g)	0	0	3	3.53	0.231[b]
LBW (1500-2500 g)	2	2.86	4	4.71	
Birthweight ≥2500 g (n)	68	65.9	78	91.76	
Birthweight ≥4000 g (n)	6	8.57	9	10.59	0.673[b]
Percentile (mean)	43.14	26.74	38.58	27.91	0.304[a]
SGA (≤5th percentile)	2	2.86	10	11.76	**0.039**[b]
Birthweight ≤10th percentile (n)	10	14.49	18	21.18	0.285[b]
Vanishing twin (n)	1	1.43	6	7.32	0.088[c]

Values are presented as means with standard deviations or n with proportions (%).
c-IVF, conventional IVF; d, day; g, gram; IU, international units; IVF, in vitro fertilization; LBW, low birthweight; mm, millimeter; NC-IVF, natural cycle IVF; n, number; pmol/L, pico mol per liter; SD, standard deviation; SGA, small-for-gestational-age; VLBW, very low birthweight; wk, week of gestation; wk.d, week.day.
[a]Linear regression.
[b]Chi-square test.
[c]Fisher's exact test.
Bold values are statistically significant.

robust standard errors controlling for clusters in the regressions. For E2 and birth percentile, we performed multivariate linear regression, and for binary outcomes, multivariate logistic regression. We adjusted for the known risk factors: maternal age, maternal height and body mass index (BMI) according the large US multicenter study from Gardosi & Francis[19] and controlled for multicollinearity between BMI and height. For parity (nulliparous/parous), vanishing twins and smoking during pregnancy (yes/no), we conducted a sensitivity analysis using exact logistic regression. We also used supraphysiological E2 levels (>10 000 pmol/L) as a binary exposure. The E2 cut-off levels in previous studies ranged between 9178 and 12 665 pmol/L.[7,8,20] We considered E2 >10 000 pmol/L to be supraphysiological, "because this E2 level is higher than that of 10 mature follicles and lower than that of high-risk ovarian hyperstimulation syndrome"[8] and it comprises median and mean E2 levels of our c-IVF patients (9590 pmol/L; 10 459 pmol/L). We present odds ratios (OR) and 95% CIs; a *P* value <0.05 is considered statistically significant.

For statistical analysis, we used STATA statistical software Version 15 (Stata Corporation, College Station, Texas, USA).

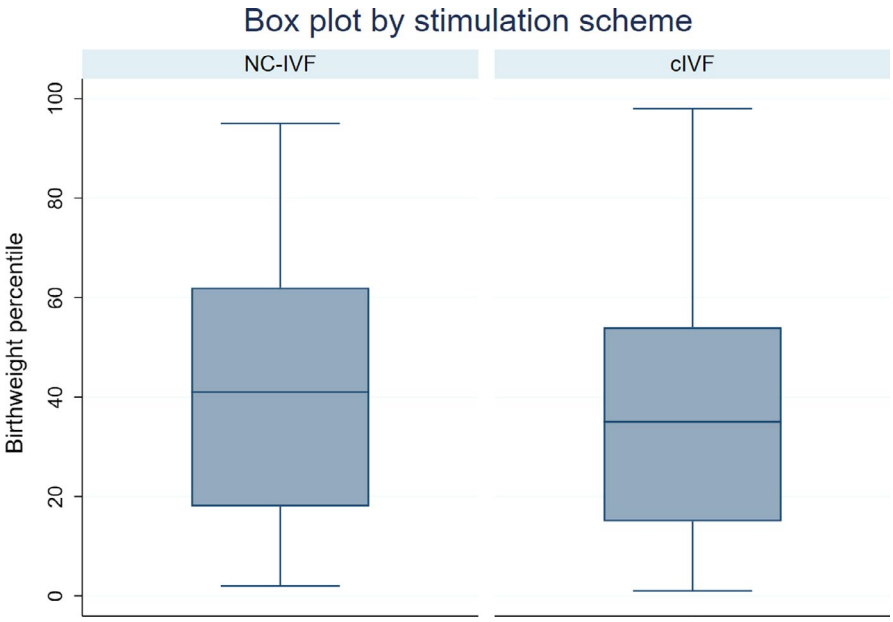

FIGURE 1 Boxplot of birthweight percentiles of conventional gonadotropin-stimulated in vitro fertilization (c-IVF) vs natural cycle in vitro fertilization (NC-IVF) [Color figure can be viewed at wileyonlinelibrary.com]

2.6 | Ethics approval

The cantonal ethical committee of Bern (KEK Bern, 397/15), Switzerland, approved the study on 26 January 2016.

3 | RESULTS

We identified 643 pregnancies from the 2010-2016 period. We excluded 159 births after frozen embryo transfer. Of 484 women who underwent a fresh embryo transfer, one woman was lost to follow up, 188 had a miscarriage and 26 had a multiple pregnancy. We excluded 114 singleton deliveries because of modifications (see Study population 2.1.). These resulted in 155 singleton deliveries in the analysis: 85 conceived after c-IVF and 70 after a NC-IVF treatment. Twelve women did not consent to further data collection associated with their child.

3.1 | Study population characteristics

Within the c-IVF group, 21 had an agonist protocol and 64 had an antagonist protocol. See Table 1 for patient and treatment characteristics. We could not find a significant difference in age, BMI, parity or smoking between the two groups. Prevalence of chronic diseases was comparable between c-IVF and NC-IVF (22.40% vs 17.14%; $P = 0.420$).

We found that stimulation characteristics were significantly higher in c-IVF but the number of previous embryo transfers was similar (Table 2).

3.2 | Pregnancy characteristics

We note that there were no differences in pregnancy complications, eg, gestational hypertension, between the two groups (Table 2). The rate of preterm births (<37 gestational weeks) was 8.24% (n = 7) in the c-IVF group and 7.14% (n = 5) in the NC-IVF ($P = 1.000$) group.

The overall mean birthweight and percentile were 3218 g (±704 g), 38.6th percentile for c-IVF and 3310 g (±475), 43.1st percentile for NC-IVF, respectively, $P = 0.352$. The proportion of LBW was 8.24% (n = 7) in c-IVF and 2.90% (n = 2) in NC-IVF, $P = 0.188$, whereas the incidence of birthweight >4000 g was 10.59% (n = 9) in the c-IVF group and 8.57% (n = 6) in the NC-IVF group, $P = 0.673$.

3.3 | Small-for-gestational-age

More children were born as SGA following c-IVF (11.76%, n = 10; 4 male, 6 female) than following NC-IVF (2.86%, n = 2; both male), with an odds ratio of 4.53 for an SGA child in a logistic regression (95% CI 0.95-21.61, $P = 0.058$, Figure 1). After we adjusted for confounding factors such as maternal age, height and BMI, the odds ratio was somewhat attenuated (OR 4.23; 95% CI 0.87-20.41; $P = 0.073$, Model I in Table 3). If we additionally adjust for the E2 level, the odds ratio is further reduced to 1.01 (95% CI 0.87-1.19, $P = 0.971$, Model III in Table 3).

3.4 | Supraphysiological estradiol level

The influence on birthweight percentile of an E2 level of >10 000 pmol/L on ovulation trigger day was significant in the crude analysis (unadjusted OR 4.58; 95% CI 1.35-15.55; $P = 0.015$) and remained significant when adjusted (adjusted OR 3.83; 95% CI 1.06-13.82; $P = 0.041$) (Model II in Table 3; Figure 2). The sensitivity analysis regarding parity, smoking and vanishing twins showed very similar odds ratios for stimulation schemes in crude and adjusted analyses (OR 1.45 vs 1.41, 1.48 and 1.41, respectively). In the linear regression, the association between E2 level and birthweight and birthweight percentile is modest. Birthweights and birthweight

TABLE 3 Multilevel logistic regression for small-for-gestational-age

N = 155	Unadjusted OR (95% CI) (for each determinant individually)	*P* value	Model I Adjusted OR (95% CI)	*P* value	Model II Adjusted OR (95% CI)	*P* value	Model III Adjusted OR (95% CI)	*P* value
Stimulation (c-IVF)	4.53 (0.95-21.61)	0.058	4.23 (0.87-20.41)	0.073			1.05 (0.87-1.17)	0.971
Mother age (continuous)	1.01 (0.89-1.17)	0.820	0.99 (0.88-1.13)	0.952	1.01 (0.87-1.19)	0.811	1.01 (0.87-1.17)	0.901
Mother age (>36 y)	0.98 (0.28-3.48)	0.987						
Mother height (continuous)	0.88 (0.79-0.99)	0.026	0.89 (0.81-0.98)	0.019	0.90 (0.81-0.99)	0.042	0.90 (0.81-.99)	0.032
Mother height (<160 cm)	5.46 (1.42-20.92)	0.013						
Mother BMI (continuous)	0.99 (0.83-1.18)	0.944	0.96 (0.78-1.17)	0.668	0.96 (0.74-1.24)	0.753	0.98 (0.77-1.24)	0.847
Mother BMI (<20 m^2)	1.24 (0.35-4.40)	0.735						
E2 level (continuous)	1 (1.00-1.00)	0.003					1.00 (1.00-1.00)	0.127
E2 level on trigger day (<10 000 vs ≥10 000)	4.58 (1.35-15.55)	0.015			3.83 (1.06-13.82)	0.041		
Nulliparous (0 vs ≥1)	3.81[a,b]	0.188						
Smoking during pregnancy (y/n)	0.99 (0-1.04) [b]	0.984						
Vanishing twin (y/n)	2.25 (0.24-20.73)	0.474						
Pregnancy complication (y/n)	2.88 (0.87-9.6)	0.083						
Pregnancy hypertension (y/n)	12.90 (0.75-222.94)	0.078						
Induction of labor (y/n)	0.92 (0.26-3.26)	0.902						
LBW (<2500 vs ≥2500 g)	24.64 (5.4-113.98)	<0.001						

Model I: adjusted multivariate logistic regressions with SGA as outcome and stimulation as exposure adjusted for age, height and BMI as continuous variables.
Model II: adjusted multivariate logistic regression with SGA as outcome and supraphysiological E2 level as exposure adjusted for age, height and BMI as continuous variables.
Model III: adjusted multivariate logistic regression with SGA as outcome and stimulation as exposure adjusted for E2, age, height and BMI as continuous variables.
c-IVF, conventional IVF; cm, centimeters; g, gram; IVF, in vitro fertilization; NC-IVF, natural cycle IVF; y, years; n, number; m^2, square meter; OR, odds ratio; pmol/L, picomol per liter; y/n, yes/no; 95% CI, 95% confidence interval.
[a]Median unbiased estimate.
[b]Exact logistic regression.

percentiles are lower with higher E2 levels on ovulation trigger day. The adjusted linear regression of NC-IVF children shows a significant decrease of three percentiles by E2 increase of 100 pmol/L at ovulation trigger day (CI −4.61 to −1.39; *P* <0.001, Figure 3). For c-IVF this is a decrease of 0.05 percentiles with an E2 increase of 100 pmol/L (CI −0.02 to 0.18; *P* = 0.451, Figure 4). The effect of the stimulation scheme was completely leveled out when we controlled for the E2 level.

3.5 | Doppler analysis

We obtained complete pregnancy records for 8 of 12 children born as SGA. The evaluation of their fetal growth charts showed four (42%; 1 NC-IVF, 3 c-IVF) cases with utero-placental dysfunction (late flattening growth, pathological Doppler analysis) and one case with a placental infarction (c-IVF) and pathological Doppler analysis. We did not have comparable information for the other four cases because the treating gynecologist did not perform a Doppler measurement.

4 | DISCUSSION

This cohort study of singletons conceived after fresh IVF therapy focuses on the effect of ovarian stimulation on birth outcomes. Overall, gonadotropins seem to reduce birthweight and birthweight

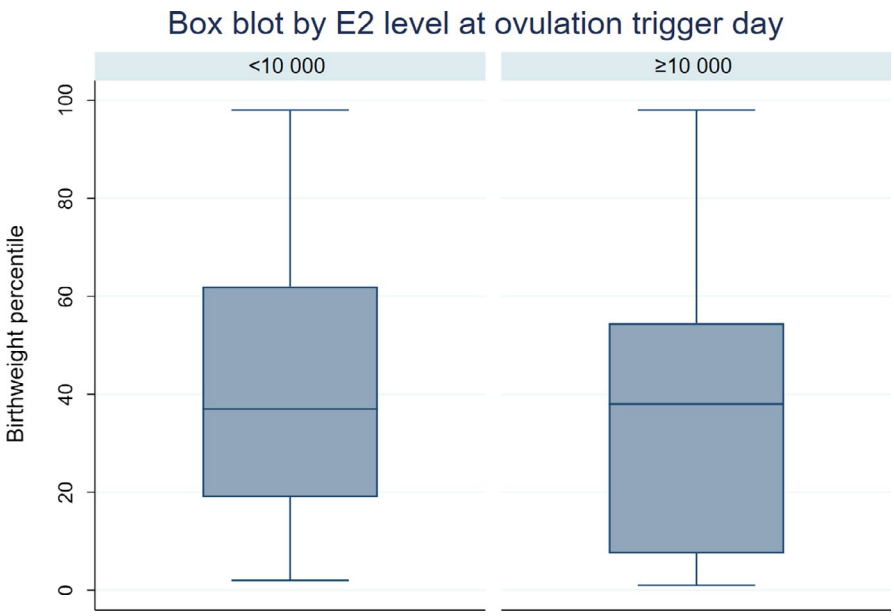

FIGURE 2 Boxplot of birthweight percentiles estradiol (E2) level <10 000 pmol/L vs ≥10 000 pmol/L E2 level [Color figure can be viewed at wileyonlinelibrary.com]

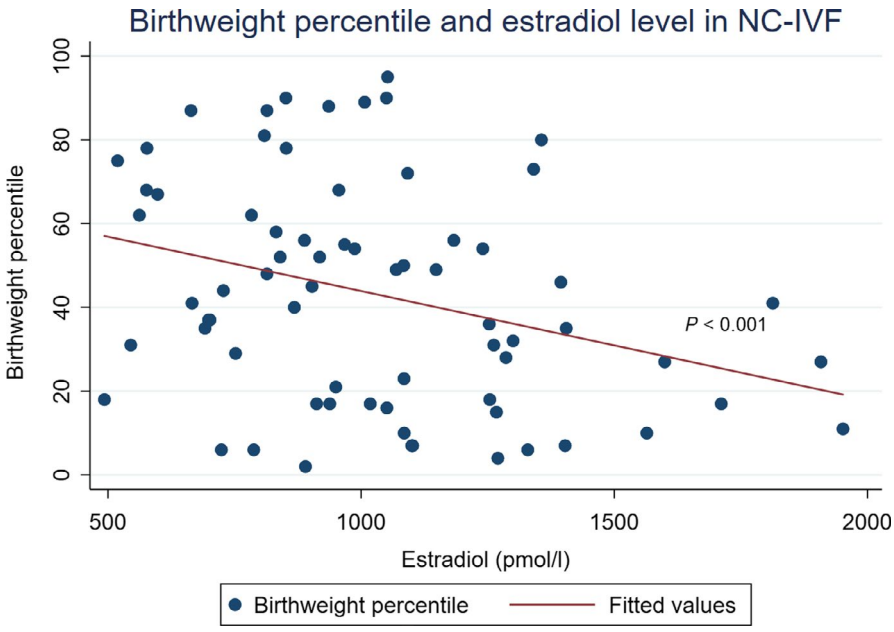

FIGURE 3 Birthweight percentile with higher estradiol (E2) level (in maternal serum, pmol/L) on ovulation trigger day in natural cycle in vitro fertilization [Color figure can be viewed at wileyonlinelibrary.com]

percentiles, especially when used for ovarian stimulation reaching supraphysiological E2 levels on ovulation trigger day. The two main findings of our study are: (1) the risk associated of children being born SGA after c-IVF compared with NC-IVF; and (2) the association of SGA children with supraphysiological E2 levels on ovulation trigger day. After adjusting for established risk factors, the supraphysiological E2 level in particular was determined to be a relevant risk factor for SGA; this even levels out completely the effect of the stimulation scheme.

These results contribute to the important debate on whether ovarian stimulation poses a risk for SGA or LBW and to the health of the IVF children later in life and what could be the determining factors.

For children conceived by NC-IVF, the incidence of SGA was analyzed only once, by Mak et al.[10] They compared 190 singletons after NC-IVF with 174 after c-IVF. In both groups, the percentage of preterm births was exceptionally high (31.5% vs 42%) but the difference in birthweights was significant (NC-IVF 3426 ± 420 g vs c-IVF 3273 ± 574 g, $P = 0.01$). Because of the high rate of preterm neonates (36.3%), this population is not completely comparable to ours (7.74% preterm only).

Our results suggest that the stimulation is detrimental if a supraphysiological estradiol level is reached. Measures of intensive ovarian hyperstimulation, such as supraphysiological E2 levels on ovulation trigger day[7,20] and a high number of oocytes retrieved,[6] have been identified previously as independent risk factors for lower

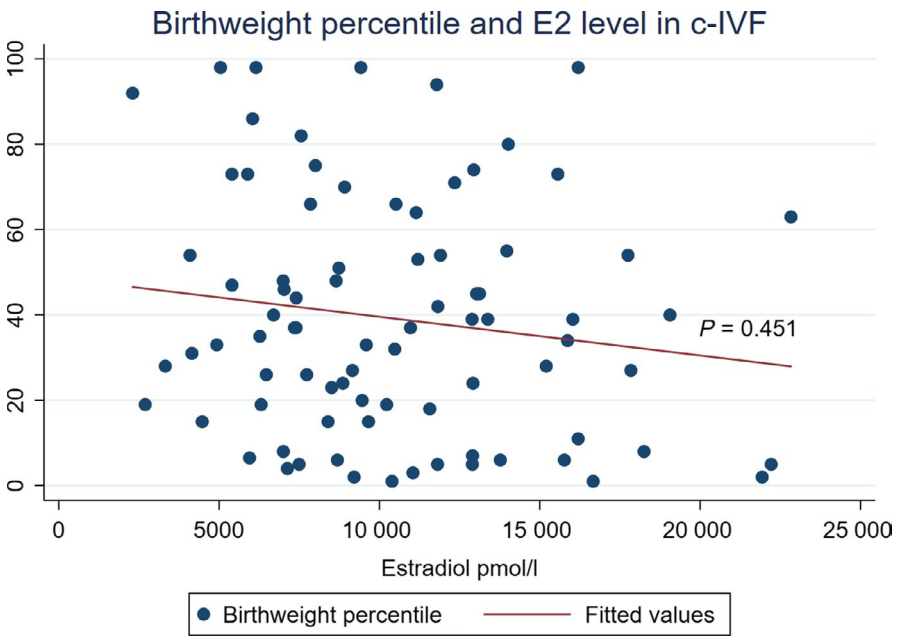

FIGURE 4 Birthweight percentile with higher estradiol (E2) level (in maternal serum, pmol/L) on ovulation trigger day in conventional gonadotropin-stimulated in vitro fertilization [Color figure can be viewed at wileyonlinelibrary.com]

birthweight in IVF therapy. Supraphysiological E2 levels are also associated with a higher risk of preeclampsia (18). Our results even suggest that the effect of E2 level on ovulation trigger day outweighs the choice of stimulation scheme and the amount of gonadotropins used. It would be necessary to predict more accurately individual stimulation responses and the E2 levels.

The strength of our study is the equal access of all couples to both treatments in our center, which reduces (but does not eliminate) selection bias. The only difference between the groups is the use of gonadotropins for stimulation, resulting in higher serum E2 levels, a higher number of oocytes retrieved, and a higher number of embryos transferred within the c-IVF group, also resulting in more vanishing twins. All other factors, such as laboratory conditions and the staff providing the treatment, were similar for the two groups. With regard to parental health and underlying subfertility as well as manipulation and culture of the embryos, we would not expect any differences between the two groups. Additionally, the use of birthweight percentiles increases the prognostic value of this particular parameter compared with birthweight alone.

A limitation of our study is the small sample size. We recruited within our center to increase comparability between both groups and we were able to have access to complete treatment data for all cases. A prospective design with random allocation of fertility treatment is not possible in Switzerland because the couple completely pays for fertility treatment themselves.

Further factors may affect our results; when two or more embryos are transferred and one gestation vanishes, the remaining fetus is physiologically seen as a twin. Pinborg showed its association with LBW and SGA (2). In our population, we had six vanishing twins in the c-IVF group and one in the NC-IVF group; this did not affect the incidence of SGA.

Animal models showed that high serum E2 levels suppress the extra villous trophoblast vascular endothelial growth factor and hinder uterine spiral artery invasion into the placenta.[21] A supraphysiological E2 environment, such as in gonadotropin-stimulated cycles, may result in an edematous endometrium impairing trophoblast differentiation and abnormal placentation, compared with the physiologic endometrium conditions in NC-IVF.[22] We hypothesize that these mechanisms might be one reason for the increased incidence of SGA children after gonadotropin-stimulated IVF therapies compared with spontaneously conceived children.

In 1998, Barker linked maternal nutrition during the preimplantation period to intrauterine growth; his work showed that intrauterine-affected individuals are at greater risk of developing coronary heart disease, hypertension and diabetes; this is known as the "Barker's hypothesis" or the "developmental origins of health and disease theory".[23]

IVF offspring have not yet reached late adult life. Multiple studies suggest that c-IVF children, especially with an associated fetal growth restriction,[24] may face health issues later in life, such as reduced insulin sensitivity, cardiovascular dysfunction and higher blood pressure at school age.[25,26] Larger prospective cohort studies should investigate further the effects of gonadotropins and supraphysiological E2 levels on intrauterine growth and the health of IVF children.

The extent to which gonadotropin stimulation is associated with LBW and SGA, and consequently their possible negative health conditions, is still not clear. However, while we cannot alter many factors in IVF therapy to achieve acceptable pregnancy outcomes, we can reduce the use of gonadotropin and the dosage when it is used. Furthermore, the risk of SGA can be reduced by frozen embryo transfer cycles, but there instead a higher risk of large-for-gestational-age (LGA),[27] and preeclampsia[28] has been described recently. In NC-IVF, the serum E2 milieu remains within physiologic limits,[17,29] whereas gonadotropin stimulation in c-IVF alters it. NC-IVF as well as low-dose

stimulation-IVF may be options to reduce the risk of supraphysiological E2 levels and consequently LBW and SGA. Several studies that looked at mild stimulation found similar pregnancy and live birth rates, better quality oocytes and less adverse effects of the stimulation. Although Baart et al.[30] found fewer aneuploidy and mosaic embryos following mild stimulation, there is still not much known about perinatal outcome including the effect on LBW and SGA.

5 | CONCLUSION

Lower stimulation dosages are associated with lower E2 levels at ovulation trigger day. There is consecutively a lower risk of ovarian hyperstimulation syndrome and we assume lower associated perinatal health risks. Even if the effect of gonadotropins on the risk for SGA is not yet fully proven, we advise reproductive specialists to consider natural cycle or low-dose ovarian stimulation.

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CONFLICT OF INTEREST

The authors have stated explicitly that there are no conflicts of interest in connection with this article.

ORCID

Alexandra S. Kohl Schwartz https://orcid.org/0000-0002-7558-1327
Vera R. Mitter https://orcid.org/0000-0002-1483-5020
Sofia Amylidi-Mohr https://orcid.org/0000-0001-5996-4064
Mirja A. Minger https://orcid.org/0000-0002-0022-9580
Costanzo Limoni https://orcid.org/0000-0002-2549-9050
Marcel Zwahlen https://orcid.org/0000-0002-6772-6346
Michael Wolff https://orcid.org/0000-0003-4303-2734

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5.2 Article 2: Breastfeeding following in vitro fertilization

Breastfeeding following in vitro fertilization in Switzerland - does mode of conception affect breastfeeding behaviour?

Contributions

This project uses follow up data on breastfeeding from the Bern IVF Cohort and compares them to a random sample of mothers recruited within the Swiss Infant Feeding Study.

For this manuscript, I contributed to the conception and design of the study and to the collection of data on breastfeeding. I established the contact with the team of the Swiss Infant Feeding Study, negotiated the contract on the use of SWIFS data with the Federal Food Safety and Veterinary Office and coordinated data transfer.

I was responsible for data management and the preparation of data for analysis.

I planned and conducted the analysis and interpreted the data.

I supported writing and finalising the manuscript; I produced Figure 1, Figure 2 and Figure 3, and contributed to Table 1 and Table 2. I also contributed the revision of the manuscript.

Submitted

Breastfeeding following in vitro fertilization in Switzerland – does mode of conception affect breastfeeding behavior?

Short title: In vitro fertilization has no impact on breastfeeding

Livia Amanda Purtschert MMed[a,b,c,*], Vera Ruth Mitter MSc[a], Jarmila Anna Zdanowicz MD[d], Mirja Amadea Minger MMed[a,b,e], Anna Spaeth MPH[f, g] , Michael von Wolff MD[a], Alexandra Sabrina Kohl Schwartz MD[a]

[a] Division of Gynecological Endocrinology and Reproductive Medicine, Department of Obstetrics and Gynecology, Bern University Hospital, University of Bern, Inselspital, Friedbuehlstrasse 19, 3010 Bern, Switzerland

[b] Medical Faculty, University of Bern, Murtenstrasse 11, 3008 Bern, Switzerland

[c] Department of Internal Medicine, Cantonal Hospital of Nidwalden, Ennetmooserstrasse 19, 6370 Stans, Switzerland

[d] Department of Obstetrics and Gynecology, Bern University Hospital, University of Bern, Inselspital, Friedbuehlstrasse 19, 3010 Bern, Switzerland

[e] Department of Pediatric Surgery, Bern University Hospital, University of Bern, Inselspital, Freiburgstrasse 15, 3010 Bern, Switzerland

[f] Swiss Tropical and Public Health Institute, Socinstrasse 57, 4002 Basel, Switzerland

[g] University of Basel, Basel, Switzerland

***Corresponding author:**

Alexandra Sabrina Kohl Schwartz

Division of Gynecological Endocrinology and Reproductive Medicine, Department of Obstetrics and Gynecology, Bern University Hospital, University of Bern, Inselspital, Friedbuehlstrasse 19, 3010 Bern, Switzerland

alexandra.kohl-schwartz@insel.ch

Tel.: +41 31 632 13 03

Abstract

Aim: To investigate whether breastfeeding initiation and duration differ between women with pregnancies conceived through in vitro fertilization and women who conceived spontaneously.

Methods: Breastfeeding behavior was studied using a comparative cross-sectional study design. The in vitro fertilization group included mothers who had a singleton live birth between 2010 and 2016 conceived through in vitro fertilization (N=198), either with or without gonadotropin stimulation. The population-based control population (N=1421) consisted of a randomly selected sample of mothers living in Switzerland, who gave birth to a singleton in 2014.

Results: A total of 1619 women were included in this analysis. Breastfeeding initiation rate was similar between the in vitro fertilization group (93.4%) and control group (94.8%). No increased risk of earlier breastfeeding cessation after in vitro fertilization treatment could be found compared to the control group over the whole observational period of 12 months (HR=1.00, 95% CI 0.83-1.20, p=0.984). There was no difference in breastfeeding initiation or duration after gonadotropin stimulated in vitro fertilization compared to in vitro fertilization performed without gonadotropin stimulation.

Conclusions: In Switzerland, in vitro fertilization treatments are not associated with earlier breastfeeding cessation. This result is reassuring for mothers undergoing in vitro fertilization.

Keywords

breastfeeding, lactation, breastfeeding duration, fertility, gonadotropins

Key Notes

- Our cross-sectional study found that breastfeeding initiation and duration are not affected by in vitro fertilization.
- Mothers and health care professionals who might be concerned about an adverse impact of in vitro fertilization on maternal breastfeeding ability can be reassured.
- High doses of gonadotropin do not influence breastfeeding ability.

Introduction

The World Health Organization (WHO) recommends exclusive breastfeeding for a minimum period of six months after delivery, and to continue breastfeeding for two years or more after the introduction of complementary food.(1) Numerous advantages of breastfeeding have been shown, not only for the health and wellbeing of the infant, but also for the mother and mother-infant relationship.(2,3) These include a decreased risk of respiratory infections, as well as as a lower mean blood pressure, lower prevalence of diabetes or a decreased risk of becoming overweight in adulthood.(2,3) To support breastfeeding identification of possible influencing factors associated with unfavorable breastfeeding outcomes is crucial.

Previous studies have shown a decreased probability of breastfeeding initiation and a shorter mean breastfeeding duration with infants born preterm, of low birth weight, by caesarean section, of male sex or with health issues. Maternal factors associated with breastfeeding outcomes include: maternal age (showing the longest breastfeeding duration between 30-39 years of age), smoking, obesity and maternal level of education.(2,4-6)

The effect of fertility treatment on breastfeeding initiation and duration has only been sparsely assessed. Subfertility and infertility treatments include in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI), and are referred to as assisted reproductive technologies (ART).(7) ART impose substantial distress on parents.(8) Previous studies showed that ART may affect breastfeeding behavior.(6,9–13) Moreover, pregnancies after ART are associated with higher risks of small for gestational age, low birthweight, caesarean sections and preterm births.(14,15) These risk factors, especially when provoked by gonadotropin hyperstimulation, may possibly multiply the risks for disadvantageous breastfeeding behavior.(15,16)

This paper aims to answer the following two questions:

First, what effectively influences breastfeeding behavior: infertility and ART treatment, or its association with adverse obstetric and perinatal outcomes, and second, what is the effect of high-dose gonadotropin stimulation on breastfeeding behavior?

An American cross-sectional study, the largest to date (N=15.615), showed lower odds of breastfeeding at eight weeks postpartum among women who conceived after fertility treatment, but this difference was no longer significant after adjustment for multiples and preterm deliveries.(13) Studies with a longer observation period showed an increased risk for earlier breastfeeding cessation after fertility treatments at four (11), six (10) or eight months.(12) In adjusted analysis often other factors additionally to ART were as well influencing breastfeeding behavior such as maternal age, mode of delivery or adverse perinatal outcomes (10-13,17). On the other hand, a small Canadian study assessing breastfeeding difficulties including 76 infants conceived by ART did not show any difference to spontaneously conceived children.(18) For Switzerland, no research on breastfeeding after ART exists so far as well as on the impact of gonadotropin stimulation on breastfeeding outcomes. Given the great importance of breastfeeding for mother and child, further research in different cultural settings is needed.

The aim of our study was to investigate whether breastfeeding initiation and duration in women with pregnancies after ART differs from the breastfeeding behavior of a randomly selected sample of mothers. Additionally, we assessed the potential effect of gonadotropin stimulation.

Methods

Setting

This is a comparison of two cross-sectional surveys about the initiation and duration of breastfeeding, with a total number of 1619 mothers of singletons included. They either had fertility treatment at the Division of Endocrinology and Reproductive Medicine at the Department of Obstetrics and Gynecology in University Hospital Bern (IVF population), or participated in the Swiss Infant Feeding Study (SWIFS population) (see Figure 1).

Population

The IVF population included 198 women who had singleton live births after fertility treatment with or without hormonal stimulation followed by a fresh embryo transfer from 2010 to 2016. Fertilization was achieved either by IVF or by ICSI. Conventional IVF treatment (cIVF) was conducted according to standard agonist or antagonist protocols using ≥ 150 international units (IU) of human menopausal gonadotropin (HMG) for hormonal stimulation and urinary human chorionic gonadotropin (hCG) for ovulation induction.(15) Natural cycle in vitro fertilization (NC-IVF) avoids gonadotropin stimulation to enable spontaneous follicular recruitment.(19) In NC-IVF, a trigger of 5000 IU urinary hCG for ovulation induction was applied, when the follicular maturity was achieved as described elsewhere.(15,19) Women with a regular menstrual cycle chose either NC-IVF or cIVF according to their preference.(15,19)

Pregnancies originating from cryopreserved embryo transfers were excluded, as were pregnancies resulting in miscarriages, perinatal deaths and multiple births.

The SWIFS population (N = 1421) served as the control population. It was the third national cross-sectional study about early infant feeding within Switzerland. The survey includes mothers with infants born in 2014, randomly selected by their local parent counselling service.(2) All women with missing information on either breastfeeding initiation or breastfeeding duration were excluded for this analysis.

Data collection IVF population

Data on breastfeeding initiation and duration were collected using postal questionnaires. Questions were “Have you ever breastfed your baby?” (yes/no), “If ‘yes’, for how long?” and possible answers were < 1 month, 1 month, 2 months, 3 months etc. up to 12 months or > 12 months. Mothers who did not initially respond were additionally contacted by phone.

Demographic data, data on medical and obstetric history, information on ART treatment, pregnancy and delivery outcomes was either collected from medical records, or from the delivery

clinic. Data was collected using REDCap electronic data capture tools (REDCap 8.5.19 Vaderbilt University, Nashville, USA) hosted at the Clinical Trials Unit, University of Bern.

Outcomes

The primary outcomes were defined as 1) breastfeeding initiation rate and 2) duration of breastfeeding. Primary outcomes were compared between the IVF- and the SWIFS population and between mothers after IVF treatment with and without gonadotropin stimulation (NC-IVF vs. cIVF).

Breastfeeding initiation was defined as receiving any breast milk after delivery (breastfed or pumped). Breastfeeding duration was defined as the period of time infants received any breast milk in addition to food or other liquids.

Further factors were assessed: maternal age, level of education, smoking during pregnancy, body mass index (BMI), parity, mode of delivery, gestational age, birthweight and infants' sex.(2,6) For the IVF population, we also assessed duration of preceding subfertility (≤24 months and >24 months) and cause of subfertility (male, female or idiopathic).

A high level of education was defined as graduation from an university, moderate level of education as an apprenticeship, vocational school or high school diploma and low level of education as elementary school level or below. BMI categories were defined as underweight < 18.5, overweight 25.1-29.9 or obese ≥ 30 kg/m^2. For delivery data, low birth weight was defined as below 2 500g, and preterm delivery as delivery before 37 completed gestational weeks.

Statistical analysis

Statistical analysis was performed using Stata 16.0 (StataCorp LLC, Texas, USA). To compare groups, the Chi Square test was used for categorical variables, and univariable linear regression for continuous variables. Time-to-event analysis, using Kaplan-Meier survival estimates, was

159 performed to compare duration of breastfeeding between different study groups. Follow-up time
160 started at delivery and the event of interest was the complete cessation of breastfeeding. In the
161 SWIFS, breastfeeding duration was recorded in weeks and for the IVF population in months.
162 This was kept for the analysis as to not lose any information. For women who did not initiate
163 breastfeeding at all, the duration was set to 0.01. This allows the survival probabilities to reflect
164 the true proportion of women breastfeeding at each stage. Risk of breastfeeding cessation was
165 evaluated using multivariable Cox regression to adjust for confounding factors. Results were
166 considered significant if $p < 0.05$.
167 To analyze the effect of hormonal stimulation and perinatal outcomes, three statistical models
168 were built. The first model included important maternal characteristics (age, BMI, smoking,
169 education) as covariate, the second focused on delivery characteristics (parity, delivery mode,
170 gestational age) and the third included perinatal characteristics (birthweight, infant's sex). All
171 significant covariate from each model were introduced in the final model (smoking, BMI,
172 cesarean section, gestational age, birthweight, infant sex).
173
174 *Ethics*
175 The study "Obstetric and perinatal outcome of children born after different IVF therapies (Bern
176 IVF cohort) compared to spontaneously conceived children" was approved by the local Ethics
177 Committee (EC Bern, 397/15) on 26 January 2016 and again on 29 August 2019 (3rd
178 Amendment). SWIF Study was approved by the ethic commission of Basel (EC 259/13) on
179 October 17th 2013.
180

Results

Characteristics of study populations

The population characteristics are summarized in Table 1. Mothers in the IVF population were on average 2.6 years older than SWIFS mothers smoked less often during pregnancy and had on average a lower BMI. The maternal level of education was higher in the SWIFS population, as was parity. Regarding delivery, the proportion of caesarean section was higher after IVF, but preterm deliveries did not differ.

Breastfeeding initiation

In both groups, a very high proportion of mothers started breastfeeding, with 93.4% among the IVF population and 94.8% among the control group (p=0.427). Overall, maternal smoking during pregnancy and increased maternal BMI were both strongly associated with lower breastfeeding initiation. 11.0% of mothers who smoked during pregnancy did not initiate breastfeeding versus only 4.7% of non-smokers (p=0.001). Mean BMI of breastfeeding mothers was 22.4 versus 23.5 in mothers not breastfeeding (p=0.008). Only 4.0% of mothers with a higher level of education did not start breastfeeding compared to 7.2% of mothers with low or middle education (p=0.015). Furthermore, primiparous women were more likely to initiate breastfeeding than multiparous women (95.6% vs. 93.3%; p=0.042). Mothers after caesarean section had a higher risk of not initiating breastfeeding when compared to mothers after a vaginal delivery (92.4% vs 95.7%; p=0.006). Preterm versus term birth (89.7% vs 95.2%; p=0.014) and low versus regular birthweight (89.6% vs 94.9%; p=0.044) were important factors with a negative influence on breastfeeding initiation. Mothers with cIVF (92.6%) started breastfeeding as often as mothers with NC-IVF (93.8%).

206 *Breastfeeding duration*

207 The duration of breastfeeding did not differ between the IVF population and the SWIFS
208 population in the first year after delivery (Figure 2). At four weeks postpartum, 92.4% of the
209 women conceived by IVF and 88.5% of the women in the control group were still breastfeeding.
210 Similar results were seen at 12 weeks (IVF: 81.3% vs. control: 79.7%), 24 weeks (IVF: 65.2%
211 vs. control: 62.9%) or 32 weeks (IVF: 48.5 vs. control: 46.5). After the tenth month, 35.4% of the
212 IVF population was still breastfeeding and 18.2% continued breastfeeding for more than a year.
213 There was no difference between cIVF and NC-IVF for the duration of breastfeeding. Four
214 weeks postpartum, 88.89% of the mothers after cIVF treatment were breastfeeding their infants
215 versus 93.75% after NC-IVF.
216 Factors significantly associated with a shorter duration of breastfeeding were maternal smoking
217 during pregnancy, increased BMI and caesarean section. No difference could be found in
218 breastfeeding duration between male and female infants (see Table 2).
219 The higher the gestational age and the birthweight, the longer the babies were breastfed.
220 Preterm birth in general was not significantly associated with shorter breastfeeding duration, but
221 low birthweight was.
222 In the final model after adjustment for the important influencing factors, the HR regarding IVF
223 was not changed (see Table 2).
224 In the subanalysis within the IVF population, the cause of infertility did not influence
225 breastfeeding initiation or duration. If male infertility was the reason for IVF treatment, mothers
226 were breastfeeding in 94.02% compared to 90.57% with female subfertility (p=0.553). The
227 duration of preceding infertility ≤ 24 months had no influence on breastfeeding outcomes
228 compared to longer duration of infertility (≤ 24 months: 93.42% vs. >24 months: 93.44%,
229 p=0.995).
230

Discussion

This study aimed to analyze the influence of IVF treatment on breastfeeding behavior in Switzerland by comparing a random population sample from the Swiss Infant Feeding Study to the population of the Bern IVF cohort. In Switzerland, the proportion of mothers starting breastfeeding is 95%, which is very high in comparison to other countries such as France (60.2%) or Germany (90%).(2,6,20) In our sample, breastfeeding initiation was high and at a similar level in both the IVF group and the SWIFS population sample.

Our results allow us to conclude that the mode of conception does not affect breastfeeding duration – a reassuring finding for mothers and health care professionals concerned about IVF adversely affecting maternal breastfeeding ability.

These findings are in line with previous studies, presenting similar breastfeeding initiation rates and breastfeeding durations when comparing mothers who conceived through any form of fertility treatments to mothers with spontaneous conceptions.(18,21–23)

Of the studies cited above, only the Canadian study of O'Quinn et al. focused on breastfeeding rates, difficulties and duration as outcomes. The other three studies collected information on breastfeeding as covariates, but their focus was motherhood or aspects of the development of the child.

O'Quinn et al. found that 54.1% of the infants who were conceived through intrauterine insemination, fertility enhancing drugs, IVF or ICSI (n=76), and 59.7% of the infants conceived spontaneously (n=150), were breastfed at four months postpartum and no differences existed regarding breastfeeding initiation, breastfeeding duration or breastfeeding difficulties.(18) From the studies collecting breastfeeding as covariates, an Australian study from 1997 showed no differences regarding breastfeeding behavior focusing on the psychological adjustment to early motherhood after conception by IVF (n=65, 68.8%) versus spontaneous (n=62, 74.6%) at four months postpartum.(21) A prospective cohort study from Belgium (ART: n=118, non-ART: n=59) (23), and a cross-sectional study from France (ART: n=66, non-ART: n=33) (22), both

investigating psychomotor development in infants, found no differences in breastfeeding behavior between the two groups.

A particular strength of our study is the focus on breastfeeding behavior as an outcome in a country like Switzerland. Another strength is the confirmation of previous risk factors for impaired breastfeeding. A recent meta-analysis showed a relative risk (RR) of 1.23 for caesarean section to be associated with shorter breastfeeding duration, and our study confirmed this finding (HR 1.22).(17,24) Furthermore, our data confirms that the higher the gestational age (HR 0.99) and the birthweight (HR 0.99), the longer babies were breastfed, as described in previous research.(4) Women within our IVF group had a higher rate of caesarean sections and mean gestational age was lower. Both are important risk factors for shorter breastfeeding duration, especially after fertility treatment.(10,13,25) Despite the higher incidence of these risk factors in the IVF population, breastfeeding initiation and duration are not affected. This could be due to overcompensation on the part of the mother, which might be due to the strong internalized pressure of perception of failing further at motherhood. This is often reported by mothers after fertility treatments.(18,25,26)

Our study further confirms influence of lifestyle and demographic factors on breastfeeding duration.(2,4,5,27) Smoking during pregnancy was associated with earlier breastfeeding cessation (HR 1.71), similar to findings of Cohen et al. (RR 1.91).(24) Our HR of 1.04 for shorter breastfeeding duration in obese women confirms what is known from a large systematic review with HR's ranging from 1.24 to 2.54.(28) However, it is difficult to disentangle the different risk factors within the IVF group (more caesarean sections/lower gestational age), as they smoke less and have a lower BMI – both factors that positively influence breastfeeding.(24,28) As presented in the meta-analysis from Cohen et al. (RR 1.68) our results further confirm that women with a lower level of education breastfeed for a shorter time period (HR 1.48).(24)

The critical factors affecting breastfeeding behavior are the same in women after IVF as after spontaneous conception, and include the perinatal health of the infant, maternal factors and the

support of the health care system. Support in early motherhood is an important factor in promoting breastfeeding.(29) In Switzerland, one third of all obstetric clinics are certified by the United Nations Children's Fund (UNICEF) and the WHO as baby-friendly hospitals, which accounts for the high breastfeeding initiation rate.(30,31) Breastfeeding support in Switzerland further includes: discussions about breastfeeding during routine pregnancy consultations, support in breastfeeding management postnatally through nursing staff at the delivery clinic and breastfeeding support through a midwife after discharge.(2,32) These services are covered by the mandatory health insurance, which all persons have.(2,32) The mode of conception itself does not seem to influence breastfeeding behavior.

However, there is also controversial literature on the effect of mode of conception.(9–13,17,18,21–23) Five studies on breastfeeding following fertility treatment found differences at certain time points during the first year postpartum.(9–13) First, a large Chinese prospective cohort study (N=935) found lower breastfeeding rates at 6 months postpartum in the fertility treatment group compared to a spontaneous conception group; however, similar breastfeeding rates were found at 12 months postpartum.(10) Similarly to our study, they accounted for confounding factors such as maternal BMI or infant's birthweight.(10) Second, a smaller Italian retrospective case-control study (N=188) found that a lower percentage of women after fertility treatment were breastfeeding at 6 weeks postpartum, compared to women who gave birth after spontaneous conception, but at 6 months postpartum breastfeeding rates no longer differed.(9) Third, similar results are reported in an Australian prospective cohort study (N=183) showing lower percentages of women breastfeeding at 6 weeks and 8 months in the ART group.(12) However, in both latter studies, small sample sizes and a single center study design limit their evidence.(9,12) Fourth, Michels et al. showed that mothers after fertility treatment were more likely to cease breastfeeding before the 12th month after birth, even after adjustment for confounders such as preterm birth.(11) In a US cohort study including 1361 women after ART, they accounted for different factors, but not for twins.(11) Contrary to the results of Michels et al.

(11), the fifth, a recent cross-sectional surveillance from the US reported lower odds of breastfeeding at 8 weeks postpartum among women who conceived through fertility treatments (n=1056), but this difference was no longer significant after adjustment for multiples and preterm birth.(13) However, breastfeeding duration was only analyzed at 8 weeks postpartum.(13) Furthermore, both US studies were unable to conclude whether a specific treatment or a combination of treatments may impact breastfeeding, as different fertility treatments were grouped together (e.g. intrauterine insemination, ovulation induction only, IVF).(11,13) Unlike previous studies, we were able to focus on breastfeeding outcomes specifically after IVF and ICSI in combination with fresh embryo transfer, by excluding fertility enhancing methods or intrauterine insemination. Furthermore, by comparing gonadotropin stimulated IVF to non-gonadotropin stimulated IVF, it is possible to assess the effect of high-dose hormonal stimulation on breastfeeding outcomes. Since this comparison showed no significant impact on breastfeeding initiation and duration in our study, it is reassuring that the use of high doses of hormones does not seem to influence breastfeeding ability.

The following two aspects are limitations of this study: First, the randomized sample of mothers in the SWIFS could possibly include infants conceived through fertility treatments, as the SWIFS did not collect information on mode of conception. Because only 2.5% of children born in Switzerland are generated by fertility treatments, the potential effect on our data analysis is very small.(33) Second, a selection bias cannot be ruled out, meaning that well-educated women are possibly more interested in research, and that this has subsequently led to higher participation rate in both study groups.

Conclusion

Three conclusions can be drawn from this study. First, in Switzerland, in vitro fertilization treatments are not associated with earlier breastfeeding cessation. Second, our results confirm

findings from other studies: infants with low gestational age, low birthweight or infants born by caesarean section are less frequently breastfed, and maternal obesity, smoking and low level of education negatively influence breastfeeding behavior. Third, in our study there is no difference in breastfeeding behavior between mothers undergoing NC-IVF compared to mothers undergoing hormonally stimulated cIVF therapy.

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List of abbreviations

- ART: Assisted reproductive technology
- BMI: Body mass index
- cIVF: Conventional in vitro fertilization
- EC: Ethic commission
- hCG: Human chorionic gonadotropin
- HMG: Human menopausal gonadotropin
- HR: Hazard ratio
- ICSI: Intracytoplasmic sperm injection
- IU: International units
- IVF: In vitro fertilization
- NC-IVF: Natural cycle in vitro fertilization
- SWIFS: Swiss Infant Feeding Study
- UNICEF: United Nations Children’s Fund
- WHO: World Health Organization
- 95% CI: 95% Confidence interval

Conflict of interest

The authors declare no potential conflicts of interest with respect to research, authorship, and/or publication of this article.

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Table legends

Table 1: Characteristics of IVF population and SWIFS (Swiss Infant Feeding Study) population.
Note. Values are presented as means with standard deviations or n with proportions (%).

a) in vitro fertilization, b) mean, c) standard deviation, d) body mass index kg/m^2, e) maternal level of education was defined as follows: high (graduation from a university), moderate (apprenticeship or high school degree), low (elementary school level or below), f) preterm delivery ≤ 37 weeks, g) vacuum extraction or forceps.

Table 2: Multivariable Cox regression models for the duration of breastfeeding.

Note. HR >1 means a higher risk for breastfeeding cessation, so shorter breastfeeding time

Model I: adjusted multivariable Cox regression model with breastfeeding duration as outcome and IVF treatment as exposure adjusted for maternal characteristics

Model II: adjusted multivariable Cox regression model with breastfeeding duration as outcome and IVF treatment as exposure adjusted for delivery characteristics

Model III: adjusted multivariable Cox regression model with breastfeeding duration as outcome and IVF treatment as exposure adjusted for perinatal characteristics

Final Model: adjusted multivariable Cox regression model with breastfeeding duration as outcome and IVF treatment as exposure adjusted for smoking, BMI, cesarean section, gestational age, birthweight and infant sex

a) hazard ratio, b) confidence interval, c) maternal level of education was defined as follows: high (graduation from a university), moderate (apprenticeship or high school degree), low (elementary school level or below), d) body mass index kg/m2, e) vacuum extraction or forceps, f) preterm delivery ≤ 37 weeks

Figure legends

Figure 1: Study population flow-chart.

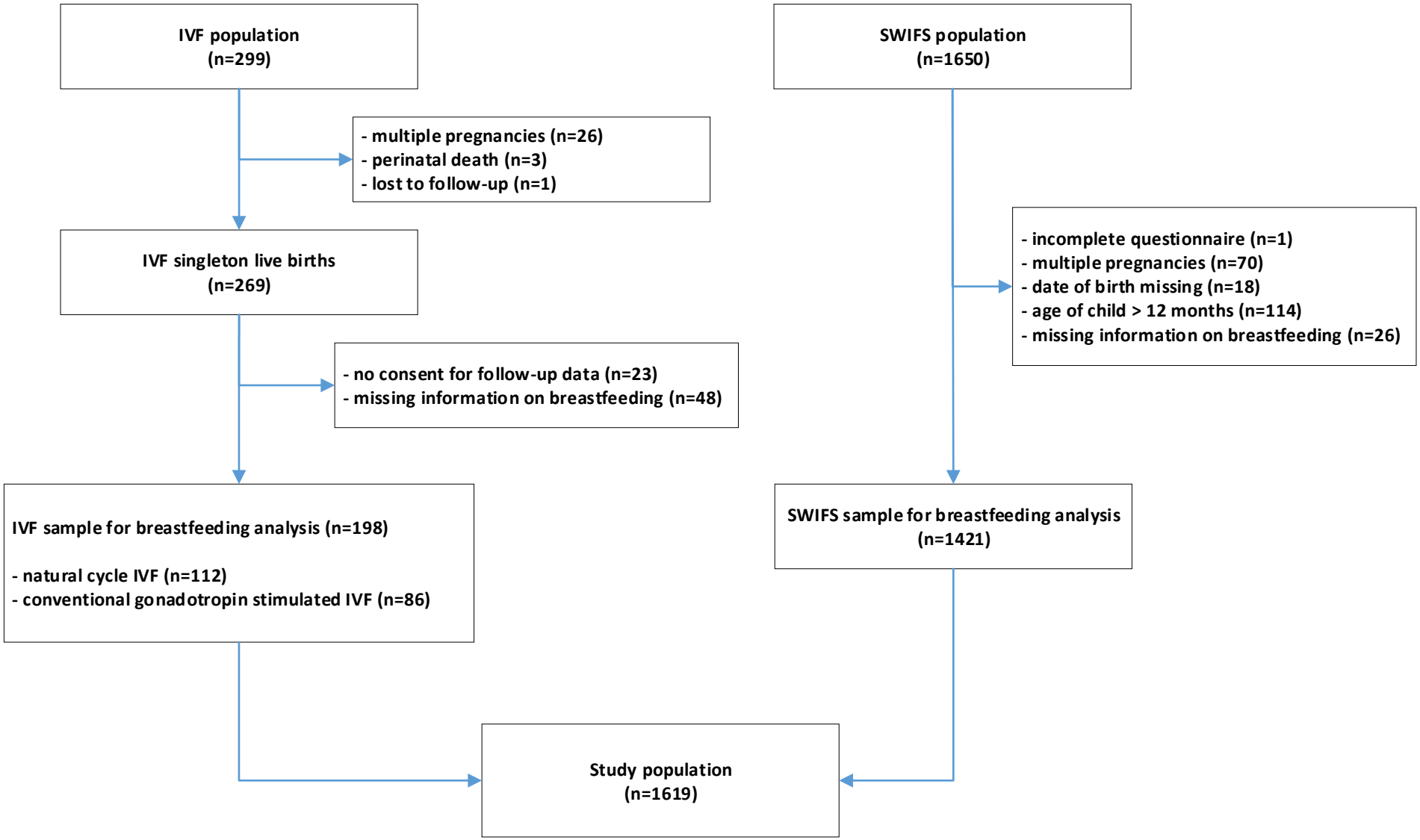
Figure 2: Kaplan-Meier estimates displaying the proportion of women breastfeeding over time after delivery.
Up: IVF population vs SWIFS population; below: Subanalysis within IVF population

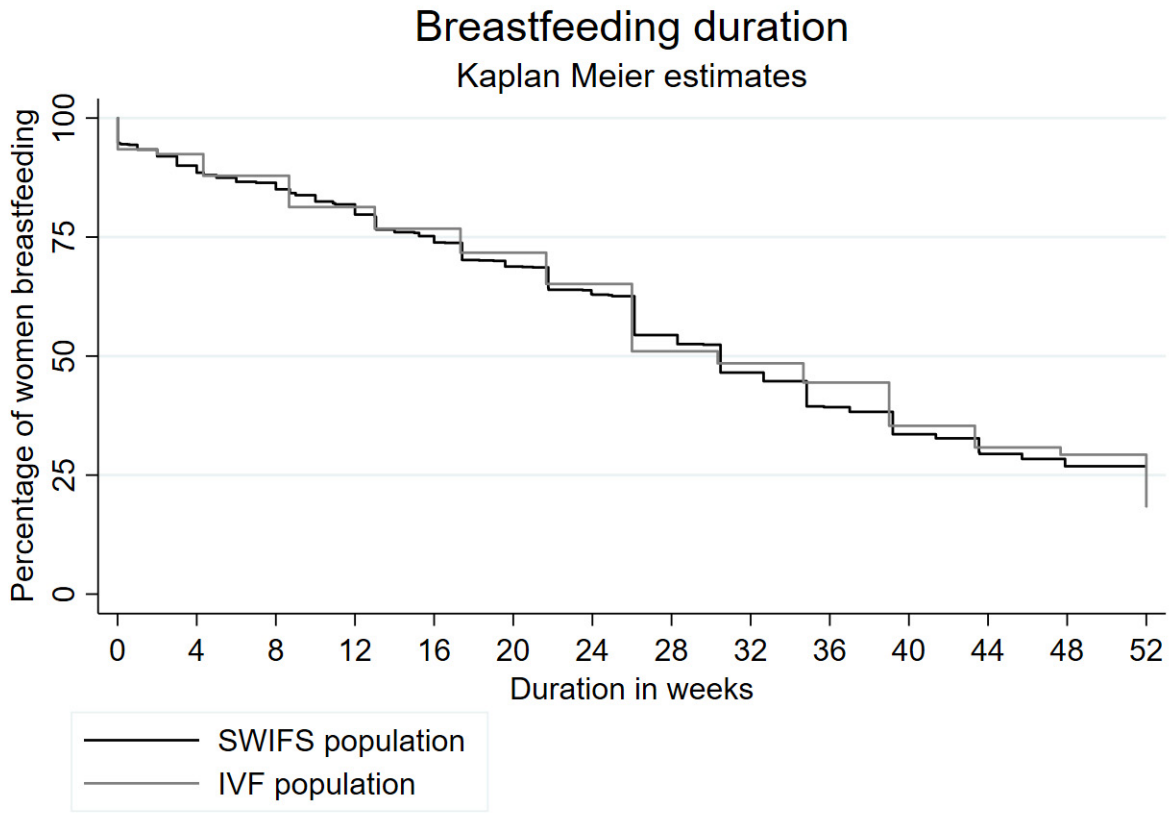
Table 1: Characteristics of study population

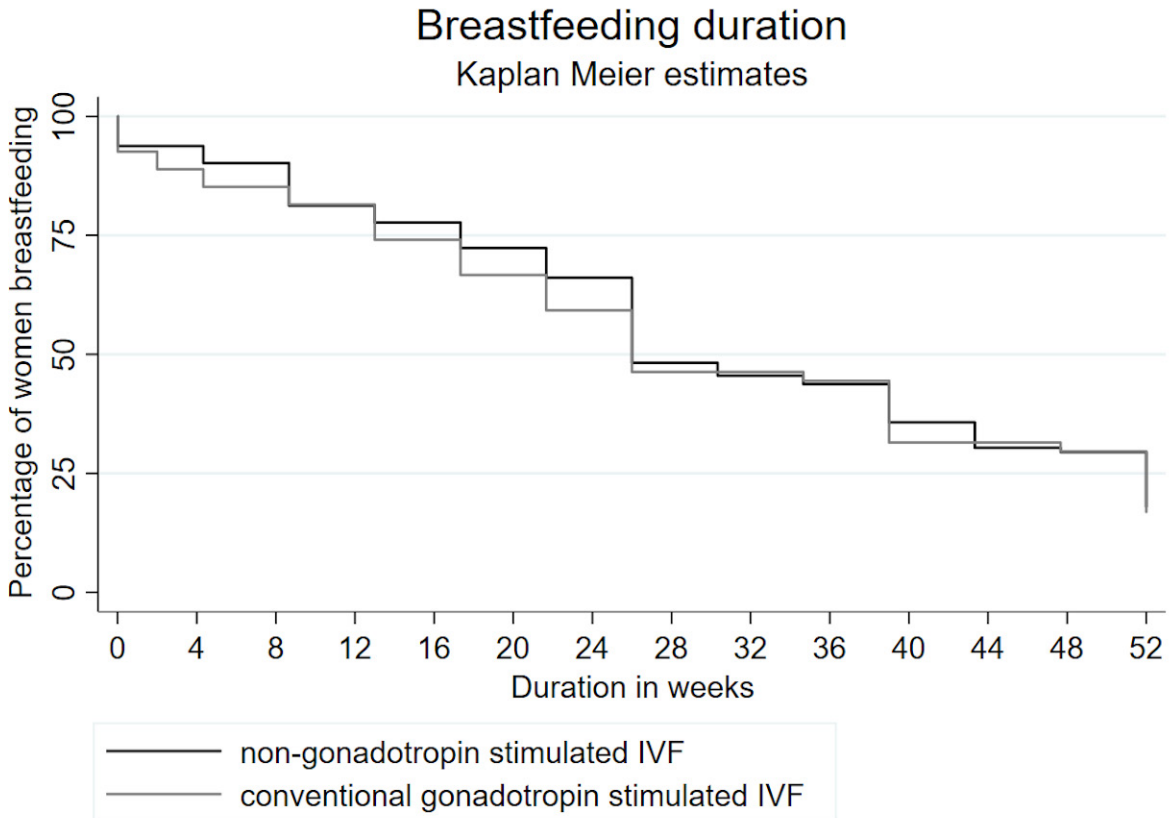
	IVF[a] Group n = 198 n/μ[b]	%/SD[c]	SWIFS Group n = 1421 n/μ[b]	%/SD[c]	P-value
Demographics					
Maternal age (years) (n=1615)	34.79	3.76	32.23	4.30	**<0.001**
Maternal smoking during pregnancy	5	2.53	168	11.84	**<0.001**
Maternal BMI (kg/m^2)[d] (n=1563)	21.97	3.29	22.51	3.64	**0.047**
Maternal education in categories[d] (n=1569)					
High	71	44.10	757	53.76	
Moderate	89	55.28	600	42.61	**0.003**
Low	1	0.62	51	3.62	
Fertility status					
Parity (n=1607)					
Primiarous	157	79.29	751	53.30	**<0.001**
Multiparous	41	20.71	658	46.70	
Delivery details					
Gestational age (days) (n=1533)	274.59	13.02	276.23	12.37	**0.085**
Gestational age in categories					
≥ 37 gestational weeks	187	94.44	1239	92.81	
33-37 gestational weeks	9	4.55	87	6.52	**0.047**
29-32 gestational weeks	1	0.51	9	0.67	
24-28 gestational weeks	1	0.51	0	0.00	
Preterm birth[f] (n=1542)	11	5.56	96	7.14	**0.412**
Delivery mode (n=1619)					
Spontaneous vaginal delivery	80	40.40	843	59.32	
Instrumental vaginal delivery[g]	28	14.14	159	11.19	**<0.001**
Caesarean Section	90	45.45	419	29.70	
Infant					
Birthweight (grams) (n=1588)	3274.99	580.12	3321.16	505.99	**0.239**
Birthweight in categories					
Normal	186	93.94	65	4.68	**0.396**
Low	12	6.06	1325	95.32	
Infant sex					
Female	89	44.95	704	49.61	**0.219**
Male	109	55.05	715	50.39	

Table 2. **Multivariable Cox regression models for the duration of breastfeeding**

	Unadjusted HR[a] (95% CI[b]) (for each covariate individually)	P value	Model I Adjusted for maternal characteristics HR[a] (95% CI[b])	P value	Model II Adjusted for delivery characteristics HR[a] (95% CI[b])	P value	Model III Adjusted for perinatal characteristics HR[a] (95% CI[b])	P value	Final model Adjusted HR[a] (95% CIb)	P value
In vitro fertilization	1.00 (0.83 – 1.20)	0.984	1.04 (0.85 – 1.28)	0.698	0.96 (0.80 – 1.16)	0.695	1.00 (0.83 – 1.21)	0.968	1.05 (0.87 – 1.27)	0.615
Maternal demographics										
Maternal age (years)	0.98 (0.97 – 1.00)	0.054	0.99 (0.98 – 1.01)	0.438						
Maternal education[c]										
High	1.00		1.00							
Moderate	1.41 (1.23 – 1.62)	<0.001	1.31 (1.13 – 1.52)	<0.001						
Low	1.83 (1.25 – 2.67)	0.002	1.48 (0.97 – 2.25)	0.067						
Maternal factors										
Smoking during pregnancy (yes/no)	1.68 (1.37 – 2.06)	<0.001	1.60 (1.30 – 1.99)	<0.001					1.71 (1.38 – 2.13)	<0.001
BMI (kg/m²)[d]	1.04 (1.02 – 1.06)	<0.001	1.05 (1.03 – 1.07)	<0.001					1.04 (1.02 – 1.03)	<0.001
Fertility status										
Parity (continuous)	0.93 (0.84 – 1.02)	0.108								
Primiparous	1.03 (0.90 – 1.19)	0.601			1.02 (0.88 – 1.17)	0.814				
Multiparous	1.00				1.00					
Delivery										
Mode of delivery										
Spontaneous vaginal delivery	1.00				1.00					
Instrumental vaginal delivery[e]	1.12 (0.91 – 1.39)	0.276			1.13 (0.91 – 1.40)	0.287				
Caesarean section	1.29 (1.11 – 1.50)	0.001			1.30 (1.12 – 1.51)	0.001				
Caesarean section (yes/no)	1.27 (1.10 – 1.46)	0.001							1.22 (1.04 – 1.41)	0.012
Perinatal										
Gestational age (days)	0.99 (0.98 – 0.99)	0.001					0.93 (0.99 – 1.00)	0.093	1.00 (0.99 – 1.00)	0.299
Gestational age in categories										
≥ 37 gestational weeks	1.00									
33-37 gestational weeks	1.06 (0.79 – 1.43)	0.680								
29-32 gestational weeks	1.24 (0.55 – 2.76)	0.606								
24-28 gestational weeks	2.05 (0.29 – 14.56)	0.474								
Preterm birth[f]	1.09 (0.83 – 1.44)	0.536								
Birthweight (grams)	1.00 (1.00 – 1.00)	0.003					1.00 (1.00 – 1.00)	0.166	1.00 (1.00 – 1.00)	0.086
Birthweigth in categories										
Normal	1.00									
Low (< 2'500g)	1.37 (1.02 – 1.84)	0.034								
Infant sex										
Female	1.00						1.00		1.00	
Male	1.04 (0.91 – 1.19)	0.585					1.07 (0.93 – 1.23)	0.362	1.09 (0.94 – 1.26)	0.256


IVF population
(n=299)
- multiple pregnancies (n=26)
- perinatal death (n=3)
- lost to follow-up (n=1)
IVF singleton live births
(n=269)
- no consent for follow-up data (n=23)
- missing information on breastfeeding (n=48)
IVF sample for breastfeeding analysis (n=198)
- natural cycle IVF (n=112)
- conventional gonadotropin stimulated IVF (n=86)
SWIFS population
(n=1650)
- incomplete questionnaire (n=1)
- multiple pregnancies (n=70)
- date of birth missing (n=18)
- age of child > 12 months (n=114)
- missing information on breastfeeding (n=26)
SWIFS sample for breastfeeding analysis
(n=1421)
Study population
(n=1619)


Breastfeeding duration
Kaplan Meier estimates
Percentage of women breastfeeding
0
25
50
75
100
0
4
8
12
16
20
24
28
32
36
40
44
48
52
Duration in weeks
SWIFS population
IVF population


Breastfeeding duration
Kaplan Meier estimates
Percentage of women breastfeeding
0
25
50
75
100
0
4
8
12
16
20
24
28
32
36
40
44
48
52
Duration in weeks
non-gonadotropin stimulated IVF
conventional gonadotropin stimulated IVF

5.3 Article 3: Endometrial thickness in NC-IVF

Thin Endometrium Is Also Associated With lower clinical pregnancy rate in unstimulated menstrual cycles: a study based on natural cycle IVF

Contributions

For this project, I supported data collection and completion, and in the interpretation of the results.

I contributed to the writing of the final manuscript.

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Thin Endometrium Is Also Associated With Lower Clinical Pregnancy Rate in Unstimulated Menstrual Cycles: A Study Based on Natural Cycle IVF

Michael von Wolff[1], Monika Fäh[1], Marie Roumet[2], Vera Mitter[1], Petra Stute[1], Georg Griesinger[3] and Alexandra Kohl Schwartz[1]*

[1] Division of Gynaecological Endocrinology and Reproductive Medicine, University Women's Hospital, Inselspital University Hospital, Bern, Switzerland, [2] CTU Bern, Institute of Social and Preventive Medicine, University of Bern, Bern, Switzerland, [3] Division of Gynaecological Endocrinology and Reproductive Medicine, University Women's Hospital, University Hospital Schleswig-Holstein, Lübeck, Germany

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***Correspondence:**
Michael von Wolff
michael.vonwolff@insel.ch

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Introduction: Does the endometrial thickness (EMT) at the time of follicle aspiration correlate with the pregnancy rate in unstimulated menstrual cycles?

Materials and Methods: This is a retrospective, observational single center study.105 women with regular menstrual cycles undergoing their first NC-IVF cycle with an embryo transfer were analyzed. Clinical pregnancy and live birth rates were calculated and data were adjusted for women's age, cycle day of follicle aspiration and body mass index (BMI).

Results: Age of participants was 35.0 y [32.0; 37.0]. Follicle aspiration was performed on day 14.0 [12.0; 15.0] of the cycle. Total clinical pregnancy rate was 24.8% and live birth rate 15.2% per transfer. Pregnancy rate in women with endometrial thickness $\leq$7 mm (n = 27) was 7.4 and 30.8% in women >7 mm (n = 78) (OR 5.56, 1.22–25.36) (P = 0.03). Live birth rates were not significantly different. Quadratic regression analysis revealed lower pregnancy rates in women with thin (around <8 mm) as well as with thick (around >11 mm) endometria. P-value after crude quadratic analysis was 0.028 and after adjustment for age, day of aspiration and BMI was 0.039. Significance was not reached for live birth rates.

Conclusion: Thin endometrium should also be considered as an independent negative prognostic factor for achieving pregnancy in women without ovarian stimulation.

Keywords: endometrium, pregnancy rate, live birth rate, natural cycle IVF, spontaneous cylce

INTRODUCTION

The importance of the endometrium for the development and maintenance of pregnancy is clearly proven. However, it is unclear which endometrial factors are of relevance (1). Histological examination in couples who wish to conceive makes little sense as a biopsy would be necessary. In transvaginal ultrasound evaluation endometrial thickness, the echo pattern and endometrial perfusion are evaluated (2). Ultrasound analysis of endometrial thickness (EMT) is most commonly performed, as this is the easiest and best reproducible technique.

The significance of the endometrial thickness has been investigated in numerous studies and in meta-analyses. The investigations are essentially limited to *in vitro* fertilization treatments (IVF) with high-dose stimulation therapy and intrauterine insemination treatment (IUI) with different ovarian stimulation regimes.

In IVF treatments, a thin endometrium is associated with lower pregnancy rates. The clinical pregnancy rate is related to a lower chance of pregnancy if endometrial thickness is ≤7 mm (OR 0.42, 95% CI: 0.27, 0.67) (3). The data regarding a thick endometrium is not so clear. A previous study described reduced pregnancy rates in women with endometrium >14 mm (4), whereas other studies did not find decreased or found even increased pregnancy rates (5–7).

In women undergoing IUI with low dose stimulation, such a relationship does not appear to exist. In a recent meta-analysis with IUI treatments combined with a gonadotrophin, clomifene citrate, or aromatase inhibitor stimulation, there was no evidence of a difference in EMT between women who conceived and women who did not conceive (MDrandom: 0.51, 95% CI: −0.05, 1.07) (8).

Because ovarian stimulation treatments were used, the results of these studies cannot be transferred to the unstimulated situation. As a result, the study findings have only limited use in a fertility work-up for infertility to assess the relevance of the endometrium as a cause of sterility.

Based on this, we investigated the pregnancy rate as a function of the endometrial thickness using the Natural Cycle IVF (NC-IVF) model, in which no ovarian stimulation except ovulation induction with human chorionic gonadotropin, hCG, and luteal phase progesterone supplementation was administered.

Since strict inclusion and exclusion criteria such as the transfer of only one embryo were defined and thus numerous confounders on the pregnancy rate could be excluded, this may be the first study which also allows a cautious estimate of the importance of endometrial thickness for a pregnancy event in a spontaneous cycle.

METHODS

Study Population and Participants

The retrospective, observational single center study was performed between 2011 and 2016. A total of 225 women, 18–42 years of age with regular menstrual cycles (24–32 days) and basal FSH concentrations <10 IU/L undergoing their first IVF cycle treatment with transfer of a single embryo were screened. Women had been offered both, NC-IVF and conventional IVF but decided themselves which therapy they preferred. Women without a transfer, with endometriosis >rAFS II° (revised American Fertility Society) (as diagnosed by laparoscopy or clinical and ultrasound analysis), with fibroids as diagnosed by ultrasound or in case ultrasound was not conclusive by hysteroscopy and with sperm collection by testicular sperm extraction (TESE) were excluded.

NC-IVF patients were monitored using ultrasound and analysis of luteinizing hormone (LH) and E_2 concentrations. When the follicle diameter reached at least 18 mm and the E_2-concentration was expected to be ≥800 pmol/L, 5000 IU of hCG (Pregnyl®, MSD Merck Sharp & Dohme GmbH, Lucerne, Switzerland) were administered and patients were scheduled 36 h later for oocyte retrieval. EMT was measured at the time of oocyte aspiration by different physicians and different ultrasound machines. Endometrium thickness was measured in mm without decimal numbers in our clinical routine as intraindividual and interindividual variations did not justify a more precise measurement. Follicles were aspirated without anesthesia and without analgesia using 19G single lumen needles (220 mmHg) as described elsewhere (9). After aspiration, follicles were flushed and aspirated 3 times each using 2–5ml flushing medium with heparin (SynVitro® Flush, Origio, Berlin, Germany). The flushing volume was adapted according to the size of the follicles. Fertilization was achieved by standard ICSI in all cases. Embryos were transferred on day 2 or 3 after aspiration as long term culture was not required with only one embryo. Women received luteal phase support with vaginal micronized progesterone. EMT at the time of follicle aspiration as well as biochemical and clinical (defined as ultrasound detection of an amniotic sac) pregnancy rates and live birth rate were analyzed per embryo transfer.

The study was carried out in accordance with the recommendations of the local ethical committee of the IRB Internal Review Board, Inselspital Bern on October 12th 2012 (IRB 12–223). All subjects gave informed written consent in accordance with the Declaration of Helsinki.

Statistical Analysis

Endometrial thickness was first considered as a categorical variable and therefore women were divided into two endometrial thickness groups (≤7 mm vs. >7 mm). Patients' baseline characteristics were compared for quantitative variables by using the *t*-test, or if normality assumption was not satisfied, by non-parametric Wilcoxon-test. For qualitative variables (cause of infertility), the Chi-square test was used or by Fisher's exact test when the sample size was small.

Clinical pregnancy and life birth rate where compared using a logistic regression. For each outcome, we first assessed the crude (unadjusted) association between EMT categories and the outcome. We then adjusted the model for potential confounders by including women's age, day of follicle aspiration and BMI in the model. The cause of the infertility was not considered as we had been shown in another, not yet published, study, that the cause of infertility is not a prognostic factor in NC-IVF. Endometrial thickness was then considered as a continuous variable and its effect on pregnancy and on live birth was further analyzed using logistic regression. For each outcome, we first assessed the crude (unadjusted) and adjusted association between EMT and the outcome, using endometrial thickness as a linear term. Then, we examined the linearity of the effect of EMT on the outcomes by fitting a crude and adjusted quadratic regression model and by testing whether the addition of a quadratic term significantly increased the fit of the model. Models were compared using likelihood ratio tests. *P*-value and the confidence interval of models parameters were estimated using the normal approximation.

RESULTS

Two hundred and twenty five women undergoing a NC-IVF cycle were identified. One hundred and eleven women (49.3%) were excluded due to missing transfer (premature ovulation 14%, aspirations without oocyte 15%, oocytes without fertilization or arrested embryo growth 16%), 5 women (2.2%) due to endometriosis, and 4 women (1.8%) due to TESE, resulting in 105 women to be included in the analysis. Cycles and transfers were not canceled due to thin endometrium. The basic characteristics of these women are shown in **Table 1**. Age of participants was 35.0 y [32.0; 37.0], cycle day of follicle aspiration was 14.0 [12.0; 15.0] and duration of infertility was 3.00 y [2.00; 4.00]. Infertility factors were severe male factor (sperm < 5 Mill/ml) ($n = 26$, 24.8%), moderate and mild male factor (sperm 5- < 15 Mill/ml or total motility <40%) ($n = 24$, 22.9%), tubal factor (peritubal adhesions, blockage of one or both fallopian tubes), endometriosis rAFS I-II° and mixed factors ($n = 22$, 21.0%), and idiopathic infertility ($n = 33$, 31.4%).

Overall AMH concentrations were 12.0 pmol/l [6.0; 22.0]. Clinical pregnancy rate and live birth rate as a function of endometrial thickness are shown in **Figures 1**, **2**. Endometrial thickness was 6 mm in 6 women, 7 mm in 21, 8 mm in 31, 9 mm in 17, 10 mm in 15, 11 mm in 9, 12 mm in 5, and 16 mm in 1 woman.

The pregnancy rate was firstly compared in women with endometrial thickness ≤7 mm ($n = 27$) vs. >7 mm ($n = 78$). The groups did not differ regarding women's age, cycle day of aspiration, duration of infertility, BMI and cause of infertility (**Table 1**). Clinical pregnancy rates in women with endometrial thickness ≤7 mm ($n = 27$) vs. >7 mm ($n = 78$) were 7.4 and 30.8%, respectively.

Crude and adjusted logistic regression, adjusted for women's age, day of follicle aspiration and BMI, showed an increased odds of pregnancy in patients with increased endometrial thickness >7 mm (crude OR 5.56, 95% CI: 1.22–25.36, $p = 0.027$; adjusted OR 5.50, 95% CI: 1.14–26.62, $p = 0.034$). Likewise, the odds of live birth was also increased in patients with increased endometrial thickness, however, associations were not significant (crude OR 6.19, 95% CI: 0.78–49.27, $p = 0.085$; adjusted OR 5.98, 95% CI: 0.74–48.56, $p = 0.094$).

We also used endometrial thickness as a continuous variable in logistic models. There is a not significant trend of a linear relationship between continuous endometrial thickness and pregnancy [crude OR (per log (mm)): 6.63, 95% CI: 0.61–71.99, $P = 0.12$]; adjusted OR [per log (mm)): 4.52, 95% CI: 0.38–53.14, $P = 0.23$]. Likewise, there is a non-significant trend of a linear, non-significant relationship between continuous endometrial thickness and live birth [crude OR (per log (mm)): 8.99, 95% CI: 0.54–150.06, $P = 0.13$]; adjusted OR [per log (mm)): 8.02, 95% CI: 0.41–157.41, $P = 0.17$]. However, the model fit is better when modeling a quadratic relationship instead of a linear relationship between endometrial thickness and clinical pregnancy (p-value from a model comparison = 0.03). The crude and adjusted quadratic models indicate a decreased odds of pregnancy for thinner (around <8 mm, $n = 27$) but also for very thick (around >11 mm, $n = 5$) endometrium (p-value for quadratic relationship: crude $p = 0.028$; adjusted $p = 0.039$).

Likewise, there was also a trend of a better model fit when modeling a quadratic relationship between endometrial thickness and live birth (p-value from a model comparison = 0.08). The crude and adjusted quadratic models indicate a decreased odds of pregnancy for thinner but also for very thick endometrium (p-value for quadratic relationship: crude $p = 0.066$; adjusted $p = 0.093$).

DISCUSSION

Main Findings

This study described for the first time the association of pregnancy rates with endometrial thickness in unstimulated menstrual cycles with fresh embryo transfers. The evaluation was adjusted for the main factors that could influence the chance of pregnancy (age) (10) and the EMT (day of aspiration and BMI) (11).

Strengths and Limitations

To minimize the influence of possible influencing variables, the investigation was carried out using 105 NC-IVF cycles, in which–as in almost all NC-IVF cycles–only one embryo was transferred. Different numbers of embryos would not have allowed a comparison of pregnancy rates. However, it needs to be noted that first we performed a retrospective analysis and second that the strict inclusion and exclusion criteria resulted in a limited number of participants. This might be a reason why significance was only reached for pregnancy but not for live birth rate.

Endometrium thickness was analyzed by several physicians using different ultrasound machines. Therefore, and due to the intra und interindividual variations of endometrial measurements, EMT was analyzed without decimal numbers, which could have affected the precision of the analysis.

We defined the ultrasound detection of an amniotic sac as a clinical pregnancy, which might explain the high miscarriage rate. However, the miscarriage rate could not be allocated to a specific endometrial thickness.

Interpretation

In all studies published to date, the association of endometrial thickness with pregnancy rate was performed only with high-dose IVF stimulations, cryopreserved embryos (12) or low-dose IUI stimulations. The IVF studies suggested an increase in the pregnancy rate with an endometrium >7 mm (3), whereas the IUI studies failed to demonstrate such a relationship (8). Our study confirmed the reduced pregnancy rate in gonadotrophin-stimulated IVF therapies with an endometrial thickness of ≤7 mm. However, an increase in the pregnancy rate in a particularly thick endometrium of >11 mm (6), >13 mm (5), or >14 mm (7), as demonstrated with gonadotrophin-stimulated IVF therapies, could not be confirmed. In contrast, we even observed a tendency to lower pregnancy rates in women with particularly thick endometrium. In IUI treatments with low dose

TABLE 1 | Baseline characteristics of all analyzed patients ($n = 105$) and of women with endometrial thickness ≤7 mm ($n = 27$) vs. >7 mm ($n = 78$) (data are shown as median and upper and lower quartile ranges).

	Total ($n = 105$)	EMT ≤7 mm ($n = 27$)	EMT >7 mm ($n = 78$)	*P*-value
Age at time of aspiration (years)	35.0 (32.0; 37.0)	37.0 (33.5; 39.5)	34.0 (32.0; 36.0)	0.06
Cycle day of follicle aspiration	14.0 (12.0; 15.0)	14.0 (13.0; 15.0)	14.0 (12.0; 15.0)	0.46
Infertility since (years)	3.00 (2.0; 4.0)	3.0 (2.0; 3.0)	3.0 (2.0; 4.0)	0.41
Body mass index, BMI	21.3 (20.0; 23.4)	20.8 (19.2; 22.3)	21.6 (20.2; 23.5)	0.13
Cause of infertility (n, %)				0.98
Severe male factor	26(24.8%)	7(25.9%)	19(24.4%)	
moderate/mild male factor	24(22.9%)	6(22.2%)	18(23.1%)	
Tubal factor, endometriosis rAFS I-II° and mixed factors	22(21.0%)	5(18.5%)	17(21.8%)	
Idiopathic	33(31.4%)	9(33.3%)	24(30.8%)	
Thickness of the endometrium(mm)	8.0 (7.0; 10.0)	7.0 (7.0; 7.0)	9.0 (8.0; 10.0)	Not applicable

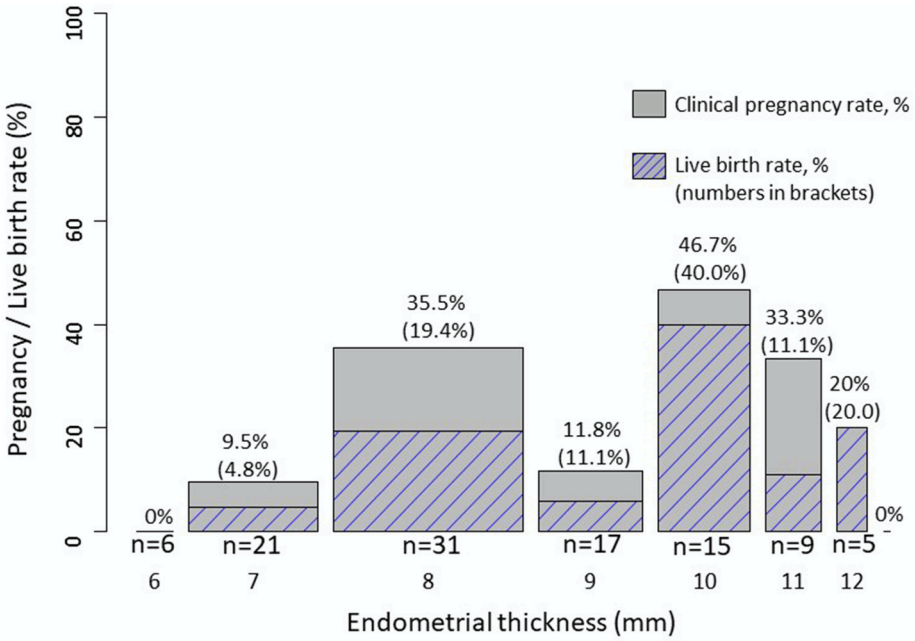

FIGURE 1 | Clinical pregnancy (not hatched) and live birth (hatched) rates as a function of endometrial thickness. The bar width is proportional to the number of women in each category.

gonadotrophin stimulation, neither an increased nor a reduced pregnancy rate (8) was found.

The reason for the reduced pregnancy rates in patients undergoing gonadotrophin-stimulated IVF therapies with an endometrial thickness ≤7 mm compared to an endometrial thickness >7 mm is unclear. It has been speculated that basal layer endometrial oxygen concentrations are increased in patients with a thin endometrium, which might be detrimental for embryo implantation (13). It was further speculated that embryos developing *in vitro* are especially susceptible to this higher oxygen exposure (8).

In hormone-stimulated IUI treatments, there is no significant correlation between the endometrial thickness and the pregnancy rate (8). As a possible reason for this, it was discussed that embryos develop more robustly *in vivo* and are less susceptible to high oxygen exposure (8). However, this explanation is purely hypothetical. Therefore, it could be speculated that in hormone-stimulated IUI treatments a thin endometrium is associated with lower pregnancy rates, but that this association could not be

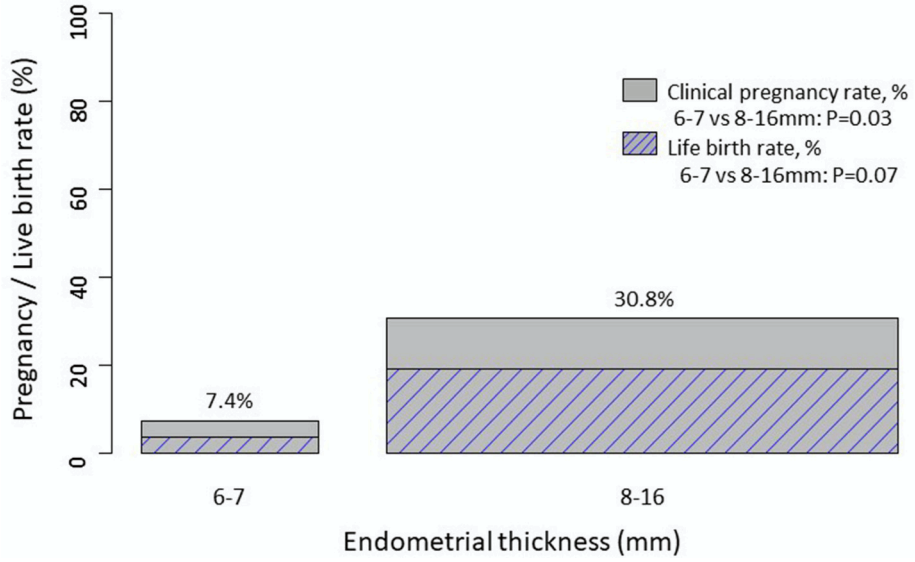

FIGURE 2 | Clinical pregnancy rate (not hatched) and live birth (hatched) rates in women with endometrial thickness ≤7 mm vs. >7 mm. The bar width is proportional to the number of women in each category.

detected. In the meta-analysis by Weiss et al. (8), the primary analysis showed a significantly thinner endometrium in women who did not conceive (MD: 0.48, 95% CI: 0.18, 0.77). The significance was only lost when the calculation was performed using a random effects model (MD random: 0.51, 95% CI: −0.05, 1.07) which was chosen due to the heterogeneity of studies. This raises the question of whether a thin endometrium is also associated with lower pregnancy rates in stimulated IUI treatments; however, this association could not be detected due to the heterogeneity across the studies.

Pregnancy rates have also been studied in modified natural cycles with frozen-thawed embryo transfers (12). Mean endometrial thickness did not differ between patients achieving ongoing pregnancy and those who did not. However, the pregnancy rates in women with endometrium of <7 mm (n = 41) was only 9.8% whereas in women with an endometrium of ≥7 mm it was 21.0% (12). Even though the differences were not statistically different, these data support the hypothesis, that pregnancy rates are lower in women with thin endometrium, even in unstimulated cycles.

Lower pregnancy rates with a thin endometrium in unstimulated cycles are unlikely to be biologically plausible. It is unlikely that a tendency toward a thin endometrium could be inherited if it significantly affected fertility. There are, of course, numerous factors that lead to a thin endometrium or which are associated with a thin endometrium and lower the chances of pregnancy. The most relevant factors are multiple curettages (14) and exposure of the uterus to radiation (15). However, these factors are either iatrogenic or due to an acquired pathology and therefore cannot explain the reduced pregnancy rates with a thin endometrium as described in other studies. In our study only 1/6 (16.7%) of the women with an endometrial thickness of 6 mm and 4/21 (19.0%) with an endometrial thickness of 7 mm had undergone a curettage and none an uterine radiation. Accordingly a curettage could be a reason for a thin endometrium is a few women but not in the majority.

On the other hand, the question arises of how relevant a reduced pregnancy rate with a thin endometrium really is. Of the 6 non-pregnant women in our study with an endometrial thickness of 6 mm, 3 women became pregnant later on. Thus, the clinical relevance of a thin endometrium without a recognizable cause such as multiple curettages etc., is questionable.

If a patient's endometrium is very thin, and if this may be a possible cause of infertility, the question of possible therapeutic options arises. Stimulation with estrogens can hardly be carried out in a spontaneous cycle, since high estrogen concentrations reduce FSH release and inhibit folliculogenesis and also impair endometrial function (16). Santamaria et al. (17), developed a treatment with bone marrow-derived stem cells, which seems to increase the chances of pregnancy in refractory Asherman's syndrome and endometrial atrophy. Whether such a complex and still experimental therapy is useful in cases with physiologically thin endometrium is questionable, since in such cases the endometrium is thinner but presumably functionally intact.

The differences in studies regarding the effect of a thick endometrium on the pregnancy rate are contradictory. In the gonadotrophin-stimulated IVF studies, a thick endometrium appears to be associated with a higher pregnancy rate (5–7). However, such a dependence could not be demonstrated in hormone-stimulated IUI therapies (8). We even found a tendency toward a reduced pregnancy rate. However, it needs to be noted that this finding is based on a statistical model which only provides a very vague tendency toward a lower pregnancy rate with very thick endometrium. Furthermore, the thickness which leads to decreased pregnancy rates cannot be defined.

The differences of the studies are barely explainable. It possibly concerns physiologically different endometrial function and IVF activity states, which do not allow a comparison of the different treatments as the endometrium is likely to be more proliferated and oedematous with gonadotrophin stimulation. It is also possible that the differences are due to the low number of patients in our study which can be considered a weakness of our study. Since the inclusion and exclusion criteria were very strict to be able to examine a patient population as homogeneously as possible, the patient numbers are limited.

In conclusion, the study confirmed that thin endometrium is also associated with lower pregnancy rates in unstimulated cycles. Therefore, thin endometrium should be regarded as an independent prognostic factor for achieving a pregnancy. However, as the pregnancy rate in women with thin endometrium is not zero but only reduced, thin endometrium should not be regarded as an infertility but rather as a fertility-reducing factor.

AUTHOR CONTRIBUTIONS

MvW designed the study, analyzed the data, and prepared the manuscript. MvW, MF, VM, PS, and AK collected data. MF prepared the data. MR and GG performed the statistics. All authors contributed to the data collecting, interpretation of the results, and the revision of the final manuscript.

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Conflict of Interest Statement: GG has received consultant fees from MSD, Merck Serono, Glycotope, Ferring, IBSA, VitroLife, Finox, ReprodWissen GmBH, and TEVA GmBH, ZIVA, Abbott, NMC Healthcare; has received speaker fees from Merck Serono, MSD, IBSA, VitroLife, ReprodWissen GmBH and Abbott.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

5.4 Article 4: Time to live birth in subfertile women

Chronic endometritis - Shortened time to live birth in subfertile women undergoing hysteroscopy and endometrial diagnostic biopsy

Contributions

I was involved in the planning and conceptualisation of the study. I supported data collection, and conducted an in-depth literature review.

SG and I wrote the first draft. I contributed significantly to redrafting of the manuscript, introduced most of the references and prepared the final draft.

I prepared the data for analysis, conducted the analysis and interpreted the data.

I produced Figure 1, Figure 3, Table 1 and Table 2.

I also undertook the revision of the analysis and manuscript.

Submitted

Chronic endometritis - Shortened time to live birth in subfertile women undergoing hysteroscopy and endometrial diagnostic biopsy

Vera R. Mitter, MSc [1]

Sheila Meier, MM [1]

Tilman T. Rau, MD[2]

Tessa Gillon, MD [3]

Michael D. Mueller, MD [3]

Marcel Zwahlen, PhD, Prof.[4]

Michael von Wolff, MD, Prof.[1]

and Alexandra S. Kohl Schwartz, MD [1,*]

[1] Division of Gynecological Endocrinology and Reproductive Medicine, Bern University Hospital and University of Bern, Bern, Switzerland

[2] Institute for Pathology, Bern University Hospital and University of Bern, Bern, Switzerland

[3] Department of Gynecology and Gynecological Oncology, University Hospital of Bern and University of Bern, Bern, Switzerland.

[4] Institute of Social and Preventive Medicine, University of Bern, Bern, Switzerland

[*] Correspondence address:
Dr. Alexandra S. Kohl Schwartz
Division of Gynecological Endocrinology and Reproductive Medicine, Bern University Hospital, Inselspital, Friedbuehlstrasse 19, Bern 3010, Switzerland
Tel: +41 31 632 18 33, E-mail: alexandra.kohl-schwartz@insel.ch

Conflict of interest

The authors have stated explicitly that there are no conflicts of interest in connection with this article.

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Abstract

Introduction

Research has suggested that chronic endometritis is associated with repeated implantation failure and recurrent pregnancy loss. However, standardized diagnostic procedures to detect chronic endometritis and its treatment are still controversially discussed. We therefore aimed to analyse the effect of endometrial diagnostic biopsy and subsequent antibiotic treatment in case of chronic endometritis on time to live birth.

Materials and Methods

We conducted a historical cohort study (January 2014 – December 2018) at our University based infertility center including subfertile women (n=108) with repeated implantation failure and recurrent pregnancy loss as indications. We excluded women with relevant pathologies for mentioned indications according to international guidelines. 67 women (62%) had an endometrial diagnostic biopsy with immunohistochemically staining for CD138 to detect plasma cells from 2016-2018 (biopsy group), whereas the 41 women treated from 2014-2016 only had an office hysteroscopy (reference group). If ≥ 1 plasma cell was detected, women were diagnosed with chronic endometritis and treated with doxycycline 100 mg twice a day orally for two weeks. We performed survival analysis (Kaplan-Meier) and Cox regression stratified for indication.

Results

Biopsy group and reference group did not differ regarding patient`s characteristics. The indications, repeated implantation failure and recurrent pregnancy loss, did not substantially impact the outcome in sensitivity analysis. Women in the biopsy group had higher chances for a pregnancy (hazard ratio 2.28; 95% confidence interval 1.23-4.24; $P = 0.009$) and for a live birth (hazard ratio 2.76; 95% confidence interval 1.30-5.87; $P = 0.008$) compared to women of reference group. Within the biopsy group, overall live birth rate tended to be slightly higher in the 42 (63.0%) women diagnosed and treated for chronic endometritis compared to 25 (37%) women without chronic endometritis (live birth: hazard ratio 1.75; 95% confidence interval 0.57-5.38; $P = 0.327$).

Conclusions

Diagnostic intervention such as endometrial biopsy with subsequent antibiotic treatment in case of chronic endometritis in women with repeated implantation failure and recurrent pregnancy loss shortens time-to- live birth, suggesting the procedure to be beneficial.

Keywords

Endometrial diagnostic biopsy/ chronic endometritis / recurrent pregnancy loss / repeated implantation failure / plasma cell / time-to-pregnancy/ time to live birth

Abbreviations

CE, Chronic endometritis

CEneg, group of women with negative diagnosis of chronic endometritis

CEpos, group of women with positive diagnosis of chronic endometritis

CI, 95% Confidence interval

HR, Hazard ratio

IVF, In vitro fertilisation

RPL, Recurrent pregnancy loss

RIF, Repeated implantation failure

HSC, Hysteroscopy

Key message

Diagnostic endometrial biopsy with subsequent antibiotic treatment in case of chronic endometritis (≥1 plasma cell) is associated with a shorter time-to-pregnancy and time to live birth compared to hysteroscopy assessment in women with recurrent pregnancy loss or repeated implantation failure.

Introduction

Repeated implantation failure (RIF) and recurrent pregnancy loss (RPL) impose a heavy burden on women desiring children, especially when etiology remains unclear. Implantation failure has been identified by the European society of human reproduction and embryology as one of the main unresolved issues in reproductive medicine.

Known risk factors for RIF and RPL are parental age, obesity, environmental exposure (including smoking and alcohol), genetic factors, uterine malformations or pathologies and thyroid autoimmunity. (1–3) Additionally, antiphospholipid syndrome is a risk factor for RPL. (3) Work-up according to guidelines includes not only ultrasound examination, but also diagnostic hysteroscopy.

In RIF the transfer of an embryo following in-vitro fertilization (IVF) treatment fails repeatedly to reach a stage of clinical pregnancy (more than three good quality blastocysts/ six good quality cleavage stage embryos). (4) The World Health Organization defines RPL as three or more consecutive miscarriages; it affects from 1% to 5% of women. (2,5,6)

Chronic endometritis (CE) is often subtle and asymptomatic, or it presents with atypical symptoms such as pelvic pain, vaginal discharge, abnormal bleeding, dyspareunia, and leucorrhea. While there is no doubt that CE is of high importance clinically, there is still no universally accepted definition, standardized diagnostic procedure, or treatment confirmed by randomized clinical trials.(7–9) The prevalence of CE in women with RIF reported in published studies ranges from 14.0% up to 67.5%; the prevalence of CE in women with RPL from 9.3% up to 67.6%.(7) It is unclear if this differences in prevalence result from differences in study populations, prevalence of different pathogens or diagnostic assessment techniques. (10)

By hysteroscopy (HSC) CE can be suspected by the presence of mucosal edema, focal or diffuse endometrial hyperemia, and micro polyps (<1mm). (11,12) The diagnosis should be confirmed by an endometrial biopsy stained immunohistochemically with Syndecan-1 for plasma (CD138) cells.(10,13,14) A meta-analysis showed higher pregnancy and live birth rates after antibiotic treatment for plasma cell positive chronic endometritis in women with repeated implantation failure.(15) This was also found in a meta-analysis of trials on endometrial scratch injury in women who had two or more implantation failures.(16)

Overall, the impact of chronic endometritis and its treatment on pathologies like RIF and RPL, which are both affected by endometritis is still not clear. As chronic endometritis seems to affect implantation and the early pregnancy, the most relevant outcome parameter would be live birth rate, or even better time to live birth.

Accordingly, the aim of our study was to focus on the effect of endometrial diagnostics and treatments on time-to-live birth by using the method of survival analysis (Kaplan-Meier) n women undergoing hysteroscopy for both, RIF or RPL

Materials and Methods

Population

We screened retrospectively all women treated at our center for RIF or RPL between January 2014 and December 2018. We defined RIF as a failure to achieve a pregnancy after the transfer of six or more cleavage-stage embryos.(4) For RPL we used the WHO definition of ≥3 recurrent pregnancy losses. (2,5)

Screening work-up consisted of: thyroid function and antibody testing, exclusion of antiphospholipid syndrome and assessment of the uterine cavity by ultrasound. Consecutively we defined the following inclusion criteria for our study: women undergoing HSC or HSC and biopsy, age≤42 years at HSC, body mass index (BMI) between 18 and 35 kg/m^2, and a regular uterine anatomy, assessed with transvaginal ultrasound or hysterosalpingosonography. As exclusion criteria, we defined according to guidelines, the following conditions known to be associated with RIF and RPL: parental chromosomal abnormalities, sperm retrieved by testicular sperm extraction, or severe thyroid dysfunction. We did not exclude other thrombophilic conditions with the exception of antiphospholipid syndrome in women with RPL (Figure 1).(17,18)

Diagnosis and histological assessment of chronic endometritis

Before we conducted HSC, we ruled out chlamydia (*Chlamydia trachomatis*) and gonorrhea (*Neisseria gonorrhoeae*) with PCR from cervical-vaginal swabs. We performed the HSC and biopsy during late follicular phase, in an outpatient setting without anesthesia. For HSC we used a rigid 30° view 2·9 mm diameter hysteroscope with an atraumatic tip (TROPHYscope; Karl Storz, Tuttlingen Germany). We did not provide preoperative analgesia, sedation, or antibiotics, but cervical preparation with dequalinium chloride (Fluomizin®). We documented the appearance of the uterine cavity and the endometrium with photos (Figure 2). At the end of the exam, we performed an endometrial biopsy using a flexible biopsy device (Pipelle) and we conserved endometrial tissue in 10% formaldehyde solution. We immunohistochemically stained the sections of the paraformaldehyde-fixed paraffin-embedded endometrial biopsy samples with a monoclonal antibody against CD138, a specific marker for plasma cells according to routine protocols (Figure 2). Formalin-fixed paraffin-embedded slides with a

thickness of 3µm were pretreated with heat (95°C, 20 minutes) and then incubated with the CD138 antibody (Serotec, Cologne, Germany) in EDTA buffer at pH 9 for 15 minutes at a dilution of 1:1600, with diacetylbenzene serving as the chromogenic agent. We counterstained with hemalaun-eosin (HE) to compare the two diagnostic methods. For the diagnosis of CE as defined within this study, we relied on immunohistochemistry, considering n≥1 plasma cell per whole-slide tissue section (one plasma cell in the hotspot) as sufficient for the diagnosis of CE.(11) Before 2016 HSC without biopsy was the standard diagnostic intervention in women with RPL or RIF, these women served as historical controls.

Treatment of chronic endometritis

Women diagnosed with CE according to immunohistochemistry were treated with doxycycline 100 mg twice daily for two weeks. Women with an intolerance to doxycycline received ciprofloxacin 400 mg daily for two weeks. We did not perform a test-of-cure. In case of recurrent pregnancy loss after treatment, the women were re-biopsied and retreated with ciprofloxacin as a second-line treatment, as described above.

Fertility treatment

For women with RIF, IVF treatment was started again after biopsy, one month after termination of antibiotic therapy, or when the couple felt ready. The subsequent IVF cycles were conducted either as natural IVF cycle (19) or as conventional gonadotropin-stimulated IVF, either in an agonist or antagonist protocol. We provided luteal phase support using progesterone for up to 12 weeks of gestation.(20) For women with RPL we provided vaginal micronized progesterone 200 mg per day in the subsequent pregnancy.(21,22)

Statistical analysis

We compared the women from the biopsy group to the women who had HSC only, the reference group. For subgroup analysis we subdivided the women with biopsy according to the immunohistochemically diagnosis of plasma cells with CE; biopsy without diagnosis of CE (CEneg) and biopsy with diagnosis of CE (CEpos). We compared baseline characteristics and outcomes (pregnancy rates, live birth rates, time-to-pregnancy, and time to live birth among the biopsy and the reference group and between RIF and RPL. We used chi-squared test for categorical variables and univariable linear regression for continuous outcomes. For the time-to-event analyses, follow-up time started at the date of HSC or biopsy (biopsy is performed during HSC). The follow-up time ended at the event of interest or at last contact

date recorded for each woman; the event of interest is either the date of clinical pregnancy confirmed by ultrasound display of an amniotic cavity (time-to-pregnancy) or the delivery date (time to live birth). We used survival analysis (Kaplan-Meier) to compare time-to-pregnancy and live birth or expected live birth between the groups. In further comparisons, we used multivariable Cox regression models including the following information: age of the women (continuous) and parity (parous vs nulliparous). We stratified Cox regression models for RIF and RPL women allowing for different baseline hazards via separate risk set definitions in the RIF and RPL group. In a sensitivity analysis, we conducted separate Cox regressions for women with RIF and women with RPL and among the two subgroups, CEneg, and CEpos, compared to reference group. For the survival curves in Figure 3 we used inverse probability weighting to account for different proportions of RIF or RPL women and different age structure of mothers within the treatment groups.(23) For statistical analysis, we used STATA version 16.0, (STATA Corporation LLC, Texas, USA). We considered a p-value < 0.05 as significant.

Ethical approval

The local ethical committee approved this study (BASEC & Kantonale Ethik Kommission Bern: 2017-01739), allowing for the use of encoded clinical data after patient information or the further use of biomaterials after informed consent retrieval.

Results

Population and diagnosis of chronic endometritis

The flow-chart of the study population is presented in Figure 1. In total, 108 women fulfilled the inclusion criteria of whom 47 (43.5%) had RIF and 61 (56.5%) had RPL. From 2014-2016 we performed only HSC in 41 (38.0 %) women (reference group); from 2016- 2018 we performed HSC with subsequent biopsy in 67 women. 24 (35.8%) women showed suspicion of CE at hysteroscopy with strawberry aspects, endometrial edema, irregular endometrium, and hyperemic areas with prominent leukoplakia (Figure 2). Among the women with biopsy, a total of 42 women were diagnosed positive for CE (CEpos) which refers to a prevalence of 62.7%, and 25 (37.3%) women did not have any plasma cells according to histology and immunohistochemically CD138 staining (CEneg).

Among the women with a biopsy, with HSC we could correctly identify 18 women as CE positive out of 42 (sensitivity of 42.8%) and 19 as CE negative out of 25 (specificity of 76.0%) when comparing to immunohistochemically stained biopsy samples ($P = 0.119$). HE

staining alone was not sufficient to identify plasma cells as well, with only 11 women correctly diagnosed as positive for CE (sensitivity of 26.2%) and 21 as negative for CE (specificity of 84.0%) in comparison to immunohistochemistry ($P = 0.333$). False positive cells in HE staining were due mainly to pseudo-decidua of endometrial stromal cells, which could display a crescent-like cytoplasmic rim and represent a mimicker of plasma cells (Figure 2). The 41 patients diagnosed with CE (CEpos) took a first course of doxycycline and one women took ciprofloxacin.

Patient characteristics

Patient characteristics are displayed in Table 1. The reasons for infertility are different between RIF and RPL patients ($P < 0.005$) but not among the three studied groups. Nine patients with RPL suffered from endometriosis.

Observation time

The mean observation time until live birth or last follow-up date was the longest for the control group, with 1.86 years (SD 1.34). For CEneg the mean time was 0.88 years (SD 0.61), and for CEpos it was 0.75 years (SD 0.47).

Fertility outcomes

The chance for a clinical pregnancy and for a live birth was significantly higher for women with biopsy and subsequent management of endometrial pathology compared to the reference group (Table 2). The hazard ratio (HR), stratified for indication is 2.28 (CI 95% 1.23 – 4.24, $P = 0.009$) for a clinical pregnancy and 2.76 for a live birth (CI 95% 1.30 – 5.86, $P = 0.008$). Survival analysis shows a shorter time-to-pregnancy and to live birth for the biopsied women (Figure 3).

In a subgroup analysis the chance for clinical pregnancy is highest for CEpos women (HR 2.39, 95% CI 1.20-4.36, $P = 0.010$) and only somewhat higher for CEneg women (HR 2.04; 95% CI 0.85-4.86, $P = 0.110$). This trend is also confirmed by subgroup analysis, independent if the women had RIF or RPL. Adjustment for maternal age and parity does not substantially affect the outcomes. All detailed results of the Cox regression are presented in Table 2. Detailed fertility outcome are presented in supplemental Table 1.

Discussion

This is a historical cohort study on the effect of biopsy with subsequent management of endometrial pathology on time to live birth in women with RIF and RPL. Our results show that a diagnostic endometrial biopsy with subsequent diagnosis and treatment in case of CE is associated with reduced time-to-pregnancy and reduced time to live birth in both conditions, RIF and RPL. Women who had a biopsy and were treated for CE (CEpos) had the same chances compared to those without CE and a better chance compared to women with HSC only (reference group).

This leads us to two hypotheses: first, diagnostic endometrial biopsy and CD138 staining with subsequent treatment of CE is important in the high-risk population of women with RIF and RPL; and second, even women not diagnosed with CE may achieve a better reproductive outcome in a shorter time after a diagnostic endometrial biopsy.

Our study has two strengths: a differentiated diagnostics with HSC, endometrial biopsy, and immunohistochemically staining for CD138; an outcome that was not yet investigated in combination with CE, namely time-to-pregnancy and live birth. The sensitivity of our HSC investigation correlates well with a study published recently. (12)

Our study also has several limitations. First it is an observational historical cohort and the sample size is limited. We analyzed patients with RIF and RPL as one group. Even though we did this on purpose, it might be questionable. We therefore focused on time to live birth as the main outcome and addressed this problem by a stratification of the Cox regression models for RIF and RPL. For the survival curves in Figure 3 we used inverse probability weighting to account for different proportions of RIF and RPL and different age structure of mothers.(23)

Live birth as outcome is valid for both conditions and subgroup analysis confirmed that the procedure is mainly beneficial for women diagnosed and treated for CE (CEpos) independent if they had RIF and RPL. The inclusion of women with ongoing pregnancy might result in slightly overestimating the results presented for live births. However, we were generally interested in the long-term follow-up of women after the intervention independent of the course between intervention and the recorded outcome. Compared to many other studies looking at one subsequent embryo transfer or the next subsequent pregnancy, we did not restrict the observation time under the assumption that processes may use some time and influence of interventions might be beneficial later.

Our results is based on the suggestion that hysteroscopy alone or HE staining is no longer considered sufficient to clearly diagnose CE or to identify plasma cells (13).

Immunohistochemically staining of CD138 cells is considered to be best practice, but there

are various methods regarding quantification and what threshold should lead to the confirmation of a diagnosis of CE (10). We chose a more conservative approach of diagnosing with the threshold of one plasma cell per high power field in the hot spot, which may have led to an overestimation of CE prevalence.

CE is often associated with the presence of bacterial pathogens. This leads to an unbalanced resident microbiota of the uterus and abnormal pattern of lymphocyte subset in the endometrium which may influence reproductive immunology (24). The immunological changes may be associated with poor endometrial receptivity (25). We assume that treatment of CE helps to reconstitute the uterine microenvironment and patterns of lymphocytes. This might strengthen the endometrial receptivity and improve reproductive outcome.

A prospective study analyzing immunologic cells within the endometrium of 178 RIF and 155 RPL women revealed a significantly higher prevalence of uterine Natural killer cells (NKs) (53.2 vs 45.2 & 42.9%, $P < 0.001$) in women with RIF compared to women with RPL. However, all sub-fertile populations had increased percentage of peripheral type NKs ($P = 0.001$), and exhibited increased of CD69+ activation ($P = 0.005$), higher levels of B cells ($P < 0.001$), an elevated ratio of CD4:CD8 ($P < 0.001$) and a higher proportion of Th1+ CD4s ($P = 0.001$) (26). Furthermore, defective endometrial prostaglandin synthesis has been observed in women with RIF (27). Based on the presence of uterine NK cells and defective prostaglandin synthesis, different immunological responses on the implantation process seems to be additionally unfavourable in women with RIF compared to women with RPL.

Our results show a difference in time-to-pregnancy and, more important, time to live birth between the biopsy group and the reference group. It is questionable if the biopsy also has an impact on women without CE (CEneg) as their reproductive outcome also seem so benefit from the procedure. A scratch induces an inflammatory response and may trigger immunological reconstitution. (28,29) It might be particularly relevant in a more vulnerable RIF and RPL population. A large randomized controlled trial has recently shown that endometrial scratching following IVF or ICSI does not increase the implantation rate in a subsequent transfer cycle, but its effect in women with RIF or RPL or the long-term effect were not addressed in this trial.(30) Endometrial scratch injury was shown to be beneficial in a meta-analysis in women who had two or more implantation failures, but not for women with one failed embryo transfer only. The greatest effect was associated with double luteal endometrial scratch injury with pipelle.(16) It is not well researched what reactions are caused by endometrial scratch injury and by endometrial diagnostic biopsy and to what extent they can be considered as comparable.(29)

Our results support the increasing evidence that in women with either RIF or RPL, CE needs to be diagnosed and treated. Our results particularly show a shorter time to live birth. However, prospective studies with larger number of participants are needed.

Conclusion

Diagnostic endometrial biopsy with subsequent antibiotic treatment in case of chronic endometritis shortens time to live birth and time-to-pregnancy, compared to women undergoing hysteroscopy only. Randomized clinical trials are needed to establish standards for diagnosis and treatment of CE and to cure implantation failure in the future.

Authors' roles

ASK, VRM, SM, TG and TTR conceptualized and designed the study. AKS, VRM, SM, MvW, MDM and TTR collected the epidemiological, clinical and pathological data. VRM and MZ conceptualized and performed the statistical analysis. All authors contributed substantially to the interpretation of the findings. SM and VRM wrote the first draft of the manuscript. All authors contributed to redrafting of the manuscript. AKS, VMI, MvW, MDM and MZ revised the article critically for important intellectual content. All authors approved the final submitted version.

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Details of ethical approval

Kantonale Ethikkommission Bern (KEK): 2017-01739 (Project ID)

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Table and Figure legends

Table 1 Patient characteristics and fertility characteristics

Patient characteristics of the biopsy group and the reference group

AMH: Anti-Müllerian Hormone, BMI: Body Mass Index, CE: Chronic endometritis, cm: centimeter, HSC: Hysteroscopy, m: mean, na: not applicable, n: number, PCOS: Polycystic ovary syndrome, RIF: Repeated implantation failure, RPL: Recurrent pregnancy loss, SD: Standard deviation

[a] linear regression, [b] Pearson's chi-squared test

Table 2 Chances for clinical pregnancy and live birth (Cox models)

CE: Chronic endometritis, CEneg: women diagnosed negative for chronic endometritis, CEpos: women diagnosed positive for chronic endometritis, 95% CI: 95% Confidence Interval, HR: Hazard ratio, n: no, y: yes, RIF: Repeated implantation failure, RPL: Recurrent pregnancy loss

° Cox regression models adjusted for maternal age (continuous) and parity (nullipar vs par)
Stratified Cox regression models all stratified for repeated implantation failure or recurrent pregnancy loss

Figure 1: Study population

RPL: Recurrent pregnancy loss, RIF: Repeated implantation failure, TESE: Testicular sperm extraction, HSC: Hysteroscopy, CE: Chronic endometritis, CEneg: women diagnosed negative for chronic endometritis, CEpos: women diagnosed positive for chronic endometritis

Figure 2: Images of patient with and without CE in hysteroscopy, hemalaun-eosin staining and immunohistochemically CD138 staining

Comparison of a patient with bland endometrium (A,C,E) versus chronic endometritis (B,D,F) in hysteroscopy (A,B), conventional histology (C,D) and immunohistochemistry for CD138 (E,F). Note the reddish inflamed mucosal surface in chronic endometritis (B) and the intermingled plasma cells in endometrial stroma in immunohistochemistry (F, central region). These differences in plasma cell densities can't be distinguished in conventional histology (C,D) of the same patients. Of note, regular endometrial glands serve as a positive internal control as CD138 also known as syndecan-1 is positive in epithelial cells.

HSC: Hysteroscopy, CE: Chronic endometritis; HE: Hemalaun-eosin staining

Figure 3: Time-to-pregnancy and time to live birth

Kaplan-Meier failure estimates. Observation time: from hysteroscopy or biopsy date to clinical pregnancy or live birth (expected live birth); with applied inverse probability weighting to account for the proportion of women with repeated implantation failure and recurrent pregnancy loss respectively as well as the maternal age structure within each of the groups compared (according to Cole SR & Hernan MA).

P-values for the two groups compared:

a) $P = 0.009$ b) $P = 0.008$ c) $P = 0.327$ d) $P = 0.004$

Supplemental Table 1: Patient characteristics and fertility characteristics

Patient characteristics of the women diagnosed with chronic endometritis (CEpos group), the women without chronic endometritis (CEneg group), both from biopsy group, compared to women with hysteroscopy only (reference group).

AMH: Anti-Müllerian Hormone, BMI: Body Mass Index, CE: Chronic endometritis, cm: centimeter, HSC: Hysteroscopy, m: mean, na: not applicable, n: number, PCOS: Polycystic ovary syndrome, RIF: Repeated implantation failure, RPL: Recurrent pregnancy loss, SD: Standard deviation

[a] linear regression, [b] Pearson's chi-squared test

Supplemental Table 2: Fertility outcomes

Fertility outcomes of the women diagnosed with chronic endometritis (CEpos group), the women without chronic endometritis (CEneg group) both from biopsy group, compared to women with hysteroscopy only (reference group).

HSC: Hysteroscopy, CE: Chronic endometritis, Tx: Therapy, CP: Clinical pregnancy, n: number of events or persons, y: year, RIF: Recurrent implantation failure, RPL: Recurrent pregnancy loss, na: not applicable

** Fisher's exact test

° Cox regression

+ Cox regression stratified for repeated implantation failure and recurrent pregnancy loss

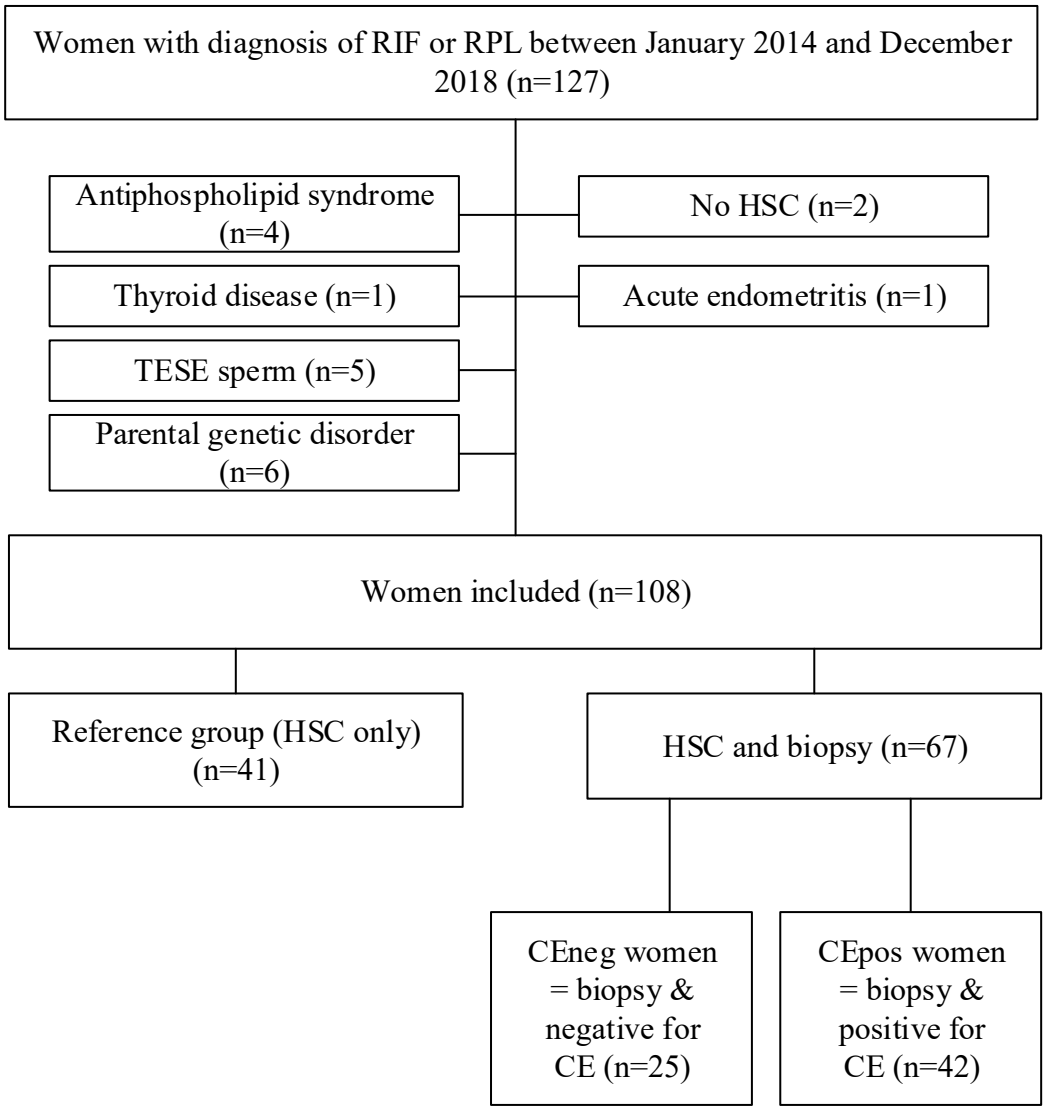
Table 1 - Patient characteristics and fertility characteristics

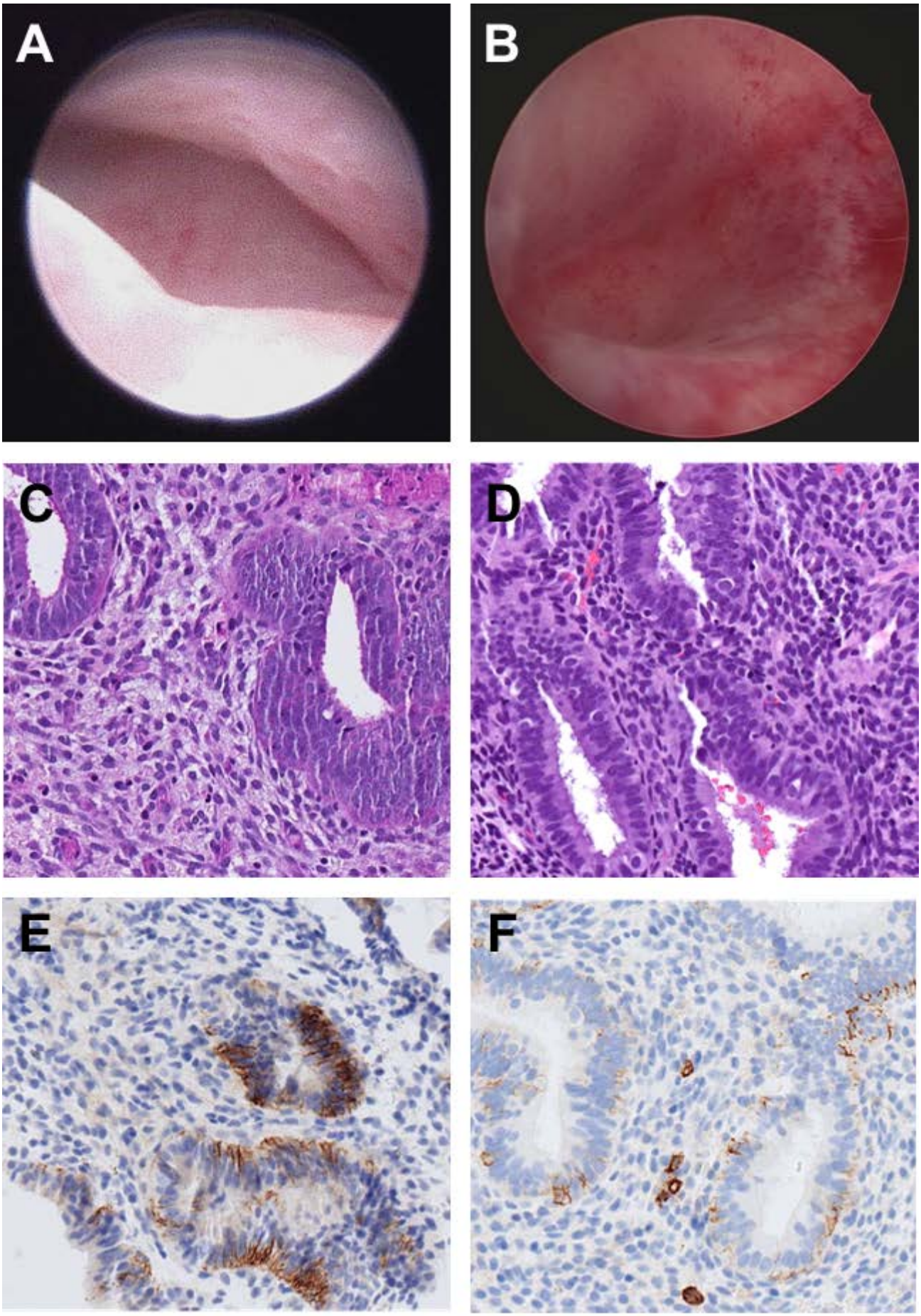
	Biopsy group							Reference group							Biopsy group vs reference group	RIF vs RPL
	RIF		RPL		Total		RIF vs RPL	RIF		RPL		Total		RIF vs RPL		
	m/n	SD/%	m/n	SD/%	m/n	SD/%	*P* value	m/n	SD/%	m/n	SD/%	m/n	SD/%	*P* value	*P* value	*P* value
n	28	42	39	58	67	62		19	46	22	54	41	38		108	61 vs 47
Mean age (years)[a]	37.8	3.1	36.9	5.2	37.3	4.4	0.422	37.6	4.0	35.3	3.5	36.4	3.8	0.061	0.294	0.094
Median age (years)	37.9	na	37.4	na	37.7	na	na	37.8	na	35.2	na	37.0	na	na	na	na
Mean weight (kg)[a]	65.6	11.1	66.4	10.3	66.1	10.5	0.777	64.3	10.1	63.5	9.8	63.9	9.8	0.806	0.301	0.883
Mean height (cm)[a]	166.6	4.8	166.1	7.6	166.3	6.6	0.782	166.5	5.6	165.2	6.6	165.8	6.1	0.529	0.731	0.561
Mean BMI (kg/m^2)[a]	23.7	4.2	24.1	3.8	23.9	3.9	0.689	23.2	3.5	23.3	3.8	23.2	3.5	0.924	0.396	0.661
Current smoker (n)[b]	3	10.7	8	20.5	11	16.4	0.286	3	15.8	0.0	0	3	7.3	0.053	0.172	0.957
AMH (pmol/l)[a]	34.1	38.6	26.0	31.1	29.7	34.7	0.375	23.1	18.5	23.4	32.3	23.2	26.5	0.971	0.334	0.459
Time from intention to conceive until HSC (years)[a]	5.3	3.6	4.0	2.7	4.5	3.1	0.080	4.6	3.3	3.2	1.9	3.9	2.7	0.104	0.257	5.0 vs 3.7 (*P*=0.02)
Deliveries before treatment (n)[b]																
0	23	82.1	23.0	59.0	46.0	68.6		14.0	73.7	11.0	50.0	25.0	0.6			
1	5	17.9	13.0	33.3	18.0	26.7	0.087	5.0	26.3	10.0	45.5	15.0	36.6	0.244	0.522	0.027
2	0	0.0	3.0	7.7	3.0	4.5		0.0	0.0	1.0	4.5	1.0	2.4			
No. of embryos transferred before treatment (RIF)[a]	11	7.4					na	10.3	3.5					na	0.645	na
Reason for infertility (n)[b]																
male factor	10	35.7						7	3.7							
tubal factor	2	7.1						0	0.0							
endometriosis	3	10.7					na	4	21.0					na	0.492	na
idiopathic	12	42.9						6	31.6							
anovulation/PCOS	1	3.6						2	10.5							

Table 2: Chances for clinical pregnancy and live birth (Cox models)

	Outcome: Clinlical pregnancy						Outcome: Live birth					
	unadjusted stratified Cox models			adjusted stratified Cox models°			unadjusted stratified Cox models			adjusted stratified Cox models°		
	HR	95% CI	*P* value	HR	95% CI	*P* value	HR	95% CI	*P* value	HR	95% CI	*P* value
Reference group (baseline)	1.00			1.00			1.00			1.00		
Biopsy group	2.28	1.23-4.24	0.009	2.62	1.39-4.49	0.003	2.76	1.30-5.86	0.008	2.88	1.35-6.16	0.006
Maternal age (continuous)	0.94	0.88-1.01	0.083	0.92	0.86-0.99	0.018	0.97	0.89-1.06	0.547	0.96	0.87-1.05	0.364
Duration of subfertility (continuous)	1.05	0.96-1.14	0.301				0.95	0.81-1.12	0.572			
Parity (y/n)	0.84	0.47-1.51	0.570	0.84	0.47-1.52	0.568	0.98	0.47-2.05	0.958	0.86	0.40-1.85	0.697
Smoking (y/n)	1.05	0.44-2.51	0.904				1.04	0.40-2.74	0.924			
Subgroup analyses (stratified for RIF/RPL)												
Reference group (baseline)	1.00			1.00			1.00			1.00		
Diagnosed positive for CE	2.39	1.20-4.36	0.010	2.86	1.46-5.63	0.002	3.53	1.48-8.40	0.004	3.91	1.62-9.41	0.002
Diagnosed negative for CE	2.04	0.85-4.86	0.110	2.11	0.88-5.08	0.095	1.95	0.70-5.42	0.203	1.92	0.69-5.35	0.213
Subgroup analyses (not stratified for RIF/RPL)												
RIF and biopsy	2.18	0.73-6.50	0.162	2.12	0.71-6.35	0.179	2.95	0.91-9.62	0.073	2.9	0.89-9.46	0.077
RIF and CEpos	3.03	0.85-10.90	0.089	3.23	0.88-11.89	0.078	5.88	1.42 - 24.28	0.014	6.78	1.63-28.10	0.008
RIF and CEneg	1.73	0.49-6.04	0.390	1.61	0.46-5.67	0.454	2.04	0.52-8.02	0.309	1.88	0.48-7.42	0.367
RPL and biopsy	2.33	1.10-4.96	0.027	2.98	1.36-6.52	0.006	2.63	0.99-7.00	0.052	2.76	1.04-7.35	0.042
RPL and CEpos	2.28	1.06-4.92	0.036	3.06	1.37-6.85	0.006	2.74	0.97-7.70	0.056	3.34	1.13-9.83	0.029
RPL and CEneg	2.73	0.81-9.20	0.107	2.61	0.73-9.35	0.138	2.31	0.75-11.15	0.298	1.62	0.30-8.69	0.573

Figure 1: Study population

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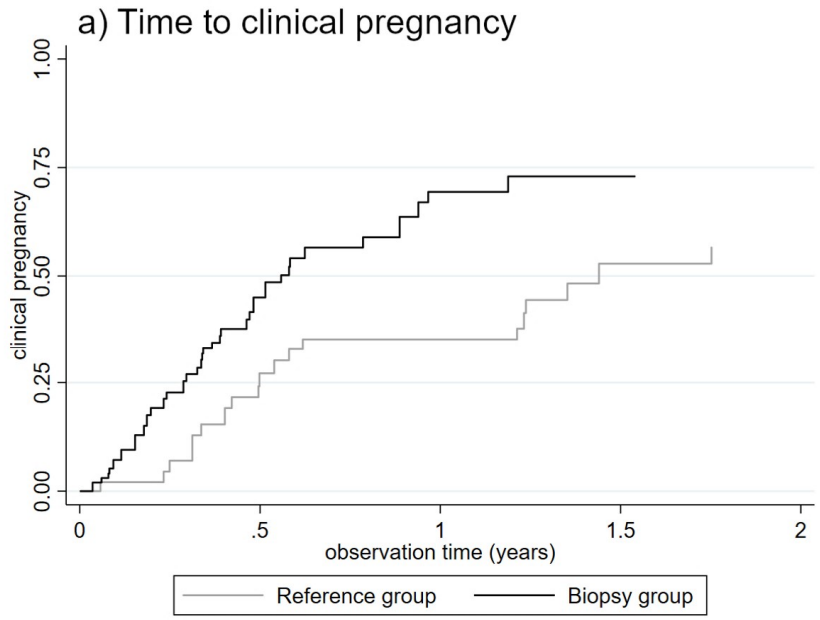
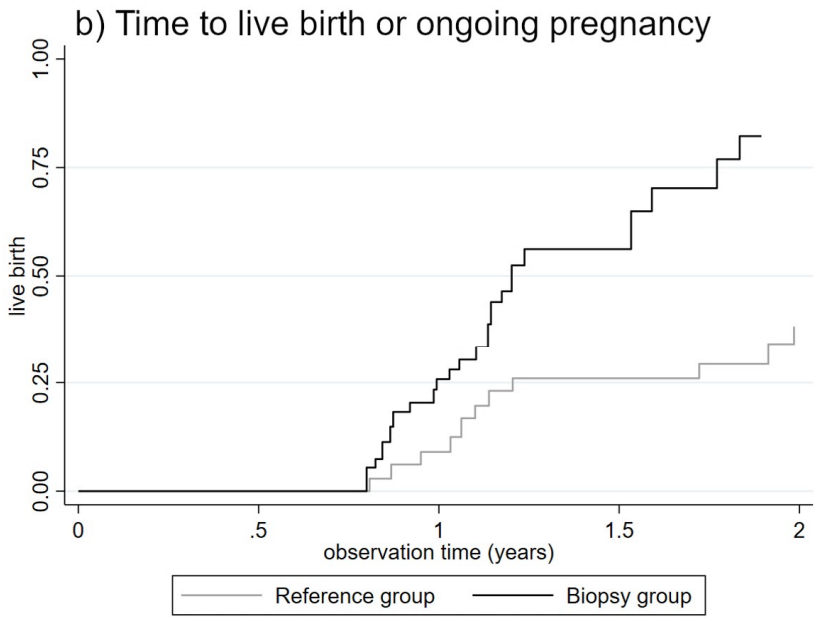
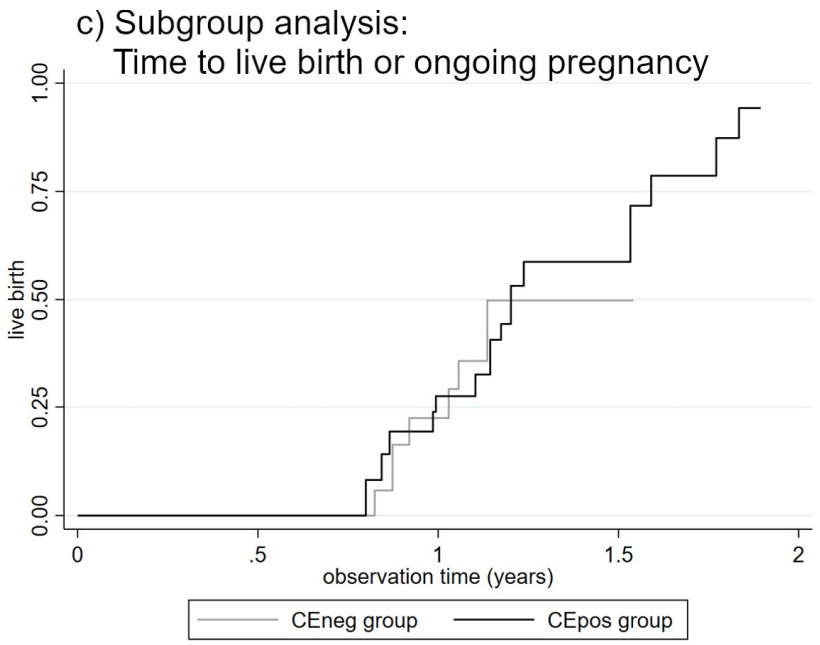
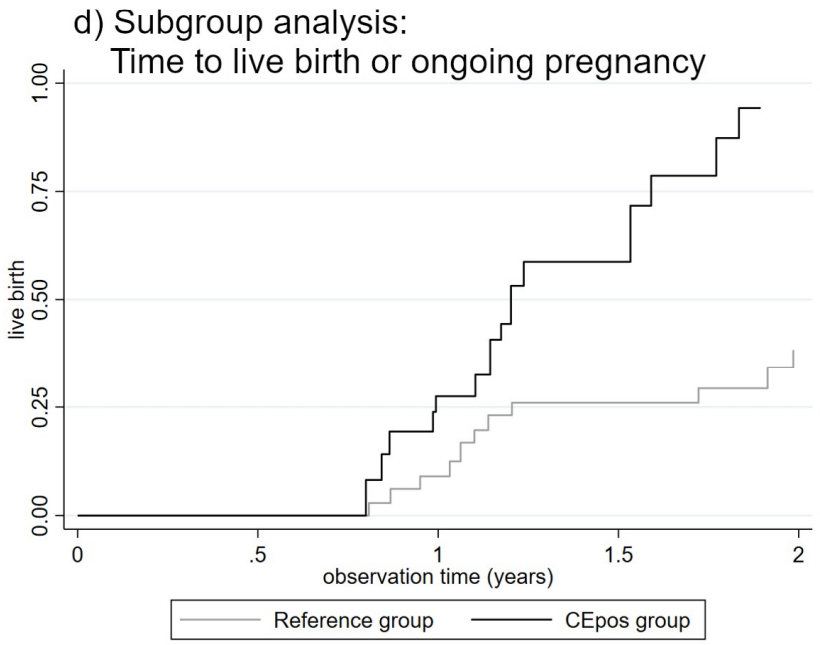

a) Time to clinical pregnancy
clinical pregnancy
0.00
0.25
0.50
0.75
1.00
0
.5
1
1.5
2
observation time (years)
Reference group
Biopsy group


b) Time to live birth or ongoing pregnancy
live birth
0.00
0.25
0.50
0.75
1.00
0
.5
1
1.5
2
observation time (years)
Reference group
Biopsy group


c) Subgroup analysis:
Time to live birth or ongoing pregnancy
live birth
0.00
0.25
0.50
0.75
1.00
0
.5
1
1.5
2
observation time (years)
CEneg group
CEpos group


d) Subgroup analysis:
Time to live birth or ongoing pregnancy
live birth
0.00
0.25
0.50
0.75
1.00
0
.5
1
1.5
2
observation time (years)
Reference group
CEpos group

Supplemental table 1 - Patient characteristics & fertility characteristics

	Biopsy group (biopsy)													**Reference group (ref)**							*P* value	*P* value
	not diagnosed with CE (CEneg)						*P* value	diagnosed with CE (CEpos)					*P* value							*P* value	over three groups	over all
	RIF		RPL		Total		RPL vs RIF	RIF		RPL		Total	RPL vs RIF	RIF		RPL		Total		RPL vs RIF	ref/CEneg/CEpos	RIF vs RPL
	m/n	SD/%	m/n	SD/%	m/n	SD/%		m/n	SD/%	m/n	SD/%	m/n	SD/%		m/n	SD/%	m/n	SD/%	m/n	SD/%			
n	15	60	10	40	25	23		13	31	29	69	42	39		19	46.3	22	53.7	41	38		108	61 vs 47
Mean age (years) [a]	38.3	2.5	34.5	6.4	36.8	4.7	0.046	37.2	3.7	37.7	4.5	37.6	4.2	0.709	37.6	4.0	35.3	3.5	36.4	3.8	0.061	0.431	0.094
Median age (years)	38.1	na	36.0	na	37.7	na	na	36.3	na	38.7	na	38.1	na	na	37.8	na	35.2	na	37.0	na	na	na	na
Mean weight (kg) [a]	66.5	7.8	69.4	9.5	67.7	8.4	0.440	64.6	14.1	65.4	10.5	65.2	11.5	0.841	64.3	10.1	63.5	9.8	63.9	9.8	0.806	0.376	0.883
Mean height (cm) [a]	166.8	4.2	169.2	10.6	167.8	7.4	0.458	166.3	5.6	165.1	6.3	165.45	6.1	0.554	166.5	5.6	165.2	6.6	165.8	6.1	0.529	0.372	0.561
Mean BMI (kg/m^2) [a]	24	3.3	24.3	2.7	24.1	3	0.847	23.4	5	24.04	4.1	23.8	4.4	0.651	23.2	3.5	23.3	3.8	23.2	3.5	0.924	0.676	0.661
Current smoker (n) [b]	2.0	13.3	4	40.0	6.0	24	0.126	1	7.7	4	13.7	5	11.9	0.572	3	15.8	0	0	3	7.3	0.053	0.142	0.957
AMH (pmol/l) [a]	27.1	30.9	29.9	28.3	28.12	29.3	0.834	41.6	45.6	24.7	32.5	30.63	37.9	0.197	23.1	18.5	23.4	32.3	23.2	26.5	0.971	0.603	0.459
Time from intention to conceive until HSC (years) [a]	6.1	4.5	3.34	1.3	5	3.8	0.078	4.5	2	4.2	3	4.29	2.7	0.735	4.61	3.3	3.23	1.9	3.9	2.7	0.104	0.351	5.0 vs 3.7 (p=0.02)
Deliveries before treatment (n) [b]																							
0	13	86.7	7	70.0	20	80		10	76.9	16	55.2	26	61.9		14	73.7	11	50	25	61			
1	2	13.3	2	20.0	4	16	0.392	3	21.1	11	37.9	14	33.3	0.337	5	26.3	10	45.5	15	36.6	0.244	0.456	0.027
2	0	0	1	10.0	1	4		0	0	2	6.9	2	4.8		0	0	1	4.5	1	2.4			
No. of embryos transferred before treatment (RIF) [a]	10.1	4.8					na	12.31	9.59					na	10.3	3.5					na	0.567	na
Reason for infertility (n) [b]		8																					
male factor	3	20						7	53.8						7	3.7							
tubal factor	2	13.3						0	0						0	0							
endometriosis	2	13.3					na	1	7.7					na	4	21					na	0.069	na
idiopathic	8	53.3						4	30.8						6	31.6							
anovulation/PCOS	0	0						1	7.7						2	10.5							

Supplemental table 2: Fertility outcomes

	Biopsy group								Reference group (ref)				*P* value over ref	*P* value over all
	CEneg group			*P* value	Cepos group			*P* value	Reference group			*P* value	over ref	over all
	RIF	RPL	Total	RIF vs RPL	RIF	RPL	Total	RIF vs RPL	RIF	RPL	Total	RIF vs RPL	CEneg/CEpos	RIF vs RPL
n (%)	15 (60%)	10 (40%)	25 (23%)		13 (31%)	29 (69%)	42 (39%)		19 (46.3%)	22 (53.7%)	41 (38%)		108	
Women with a first ClinP after Tx	5 (33.3%)	4 (40%)	9 (36.0%)	1.00**	5 (38.5%)	21 (72.4%)	26 (61.9%)	0.047**	5 (26.3%)	14 (63.6%)	19 (46.3%)	0.028**	0.102	0.001
First ClinP ended in miscarriage	2 (40.0%)	2 (50.0%)	4 (44.4%)	1.00**	1 (20%)	12 (57.1%)	13 (50.0%)	0.322**	0 (0.0%)	6 (42.9%)	6 (31.6%)	0.128**	0.147	
First ClinP ended in live birth	3 (60.0%)	2 (50.0%)	5 (55.6%)		4 (80.0%)	9 (42.9%)	13 (50.0%)		5 (100%)	8 (57.1%)	13 (68.4%)			0.001
Women with a second ClinP after Tx	1 (6.7%)	1 (10%)	2 (8.0%)	1.00**	0 (0%)	5 (17.2%)	5 (11.9%)	0.302**	0 (0.0%)	4 (18.2%)	4 (9.8%)	0.111 **	0.872	0.015
Second ClinP ended in miscarriage	0 (0.0%)	1 (100.0%)	1 (50.0%)	1.00**	0 (0.0%)	3 (60.0%)	3 (60.0%)	na	0 (0.0%)	1 (25.0%)	1 (25.0%)		0.572	0.338
Second ClinP ended in live birth	1 (100.0%)	0 (0.0%)	1 (50.0%)		0. (0.0%)	2 (40.0%)	2 (40.0%)		0 (0.0%)	3 (75.0)%	3 (75.0%)	na		
Total number of ClinP (first and second)	6 (40.0%)	6 (60.0%)	12 (80.0%)	na	5 (38.5%)	26 (89.7%)	30 (71.4%)	na	5 (26.3%)	18 (81.8%)	23 (56.1%)	na	na	na
Total number of miscarriages after ClinP (n)	2 (13.3%)	3 (30.0%)	5 (20.0%)	0.567**	1 (7.7%)	15 (51.7%)	16 (38.1%)	0.172**	0 (3.2%)	7 (31.8%)	7 (17.1%)	0.095	0.339	0.011
Total number of live births (n)	4 (26.7%)	2 (20.0%)	6 (24.0%)		4 (30.8%)	11 (37.9%)	15 (35.7%)		5 (26.3%)	11 (50.0%)	16 (39.0%)		0.445	0.205
median time to ClinP (years)	na	0.48	na	0.237°	0.89	0.47	0.56	0.263°	na	0.62	1.44	0.014°	**0.023**$^{+}$	<0.001°
median time to live birth (years)	na	1.14	na	0.472	1.14	1.24	1.21	0.223	na	2.08	2.27	0.182	**0.013**$^{+}$	0.197°
Women with an embryo transfer after Tx (RIF)	12 (80.0%)				9 (69.2%)				14 (73.7%)				0.804	na
Total nb embryos transferred after Tx (n)	43				35				49				0.617	na

6. Discussion

6.1. Main findings

Small-for-gestational age

This project investigated birthweight and birthweight percentiles after cIVF and NC-IVF. Birthweight percentile is a better outcome measure than birthweight alone, as it is adjusted for gestational age and sex of the infant. After cIVF, 11.8% of singletons were small-for-gestational-age compared to 2.9% after NC-IVF in the Bern IVF Cohort. The odds were significantly increased when supraphysiological estradiol levels (>10'000pmol/l) in the maternal serum were detected at trigger day (unadjusted OR 4.58 95% CI 1.35 – 15.55; p=0.015). After adjustment for maternal height, age and body mass index it remained significant (aOR 3.83; 95% CI 1.06-18.82; p=0.041)[1]. These findings are in line with the work by Pereira et al. and are also supported by the findings from Sunkara et al. They both found higher risks associated with high estradiol level or a high amount of oocytes collected.[2–4]

Breastfeeding after ART treatment

It is reassuring that in Switzerland ART treatment does not affect the initiation or duration of breastfeeding in the first year after delivery. Mothers after IVF reach the same high breastfeeding prevalence (93.4 %) compared to mothers from the control group (94.8%).[5] Additionally, hormonal stimulation is not associated with lower breastfeeding prevalence or earlier breastfeeding cessation as no differences between cIVF or NC-IVF were found. Factors associated with lower breastfeeding prevalence or earlier cessation in the whole study population were factors already familiar from the literature: maternal smoking during pregnancy, high BMI, low-birthweight, primiparity, preterm-birth and lower education. Mothers with IVF treatment smoked less and had a lower BMI, but had a slightly higher proportion of low-birthweight, preterm birth and were more often primiparous.

Endometrial thickness in NC-IVF women

The optimal endometrial thickness for achieving a pregnancy in a NC-IVF treatment is between 8-11mm. The pregnancy rate in women with endometrial thickness ≤7mm was 7.4% and in women with >7mm it was 30.8%, but live birth rate did not differ. Quadratic regression analysis showed that thick endometrium is also associated with lower pregnancy success. Thin or very thick endometrium should be considered as a negative predicting factors for achieving a pregnancy in women treated by NC-IVF.[6]

Time to pregnancy in women with RPL and RIF

In a historical cohort of 108 women fulfilling the inclusion criteria, 47 were diagnosed with RIF and 61 with RPL. From 2014 – 2016, 41 women had a hysteroscopic assessment only (reference group). Of 67 women who had a biopsy from 2016-2018, 42 were diagnosed positive for chronic endometritis and 25 were negative in immunohistochemically staining of plasma cells (CD138). Women who had an endometrial diagnostic biopsy with subsequent diagnosis and treatment in case of chronic endometritis had a significantly higher chance for a clinical pregnancy (Hazard ratio (HR) = 2.28, CI 95% 1.23 – 4.24, p=.009) and for a live birth (HR = 2.76, CI 95% 1.30 – 5.87, p=.008) compared to the reference group. In subgroup analysis it was found that women diagnosed positive for CE also had a high chance for clinical pregnancy (HR=2.39; 95% CI 1.20 – 4.36; p=.010) and live birth (HR=3.53; 95% CI 1.48 – 8.40; p=.004) compared to the women of the reference group. The indication of RIF or RPL did not substantially affect the outcome of the analysis. This finding demonstrates the importance of endometrial diagnostic biopsy and the subsequent treatment in case of a positive result for CE, in women suffering from both, RIF and RPL. However, it also shows that women without CE might benefit from an endometrial biopsy, which is similar to an endometrial scratch injury caused by the endometrial diagnostic biopsy. This was also found by Vitagliano et al.[7] It has been shown that endometrial scratch injury influences immunological processes in the endometrium of the uterus.[8] It would be important to confirm these findings through a randomized controlled trial.

6.2. Broader interpretation of the findings

Hormonal stimulation IVF

Gonadotropin stimulation seems to be especially risky for women with a strong response to stimulation. However, it is difficult to predict a woman's response to the gonadotropin stimulation. Generally, it can be said that women of younger ages, with a good ovarian reserve and a normal body weight, are at higher risk of generating a high response.[9] High-responders are at higher risk of developing OHSS, which may interfere with early pregnancy and may potentially affect the intellect in the offspring.[10] In Natural cycle IVF there is no risk for multiple pregnancies, as usually only one embryo is generated. Natural cycle IVF allows the research of the influence of the hormonal stimulation in singletons born after ART per se, as the only difference between cIVF and NC-IVF is the use of gonadotropins. In both treatments, other underlying factors remain similar. Parents of both treatments suffer from subfertility, and laboratory technologies and embryo culture are comparable. NC-IVF is therefore a suitable model to research the effects of the hormonal stimulation on the health of children.[11,12] On the other hand, NC-IVF is not offered by many centres providing fertility treatment. This makes it difficult to achieve numbers high enough to get sufficient power for

statistical analysis. It would be necessary to collaborate with other centres, even at an international level, to set up cohorts large enough. However, even a reduction in the doses of hormones in minimal stimulation IVF is beneficial for the mother and the child, and should be considered by more treating centres.[13,14]

Outcomes of ART research

One of the most important outcomes in reproductive medicine has long been and remains the pregnancy rate. Clinical pregnancy is usually confirmed by the detection of an amniotic sac by ultrasound. It is not even agreed, if confirmation also involves the heartbeat of a viable embryo. This means that an amniotic sac absent a viable embryo would count as a successful outcome, were the endpoint is defined as such. For some years now, a shift towards live birth rate as a more suitable outcome has been in progress. The aim of the fertility treatment is a healthy newborn, and not a clinical pregnancy with unsure outcome.[15] However, according to the DOHaD theory perinatal health matters, and may matter throughout an entire human life.[16,17] This raises the question – is live birth the correct desired outcome? Should perinatal health, at least, if not long-term health, be assessed? Quite a lot of research exists on childhood health after ART. For adulthood, many of the cohorts are still too young. ART technologies develop so quickly that the health of adults researched today may not be relevant for children born after ART today. Therefore, perinatal health is often the most feasible proxy measure for long term health outcomes. Nevertheless, it remains important to establish cohorts, or to access informative data from existing cohorts and registries and to follow-up existing cohorts until adult life.[18]

Unexplained infertility

In fertility treatment, there are still a high proportion of couples with so-called unexplained infertility. It is possible that some problems leading to unexplained infertility have not yet been identified, and research is scarce. For many suspected causes of unexplained infertility, no randomized controlled trials have been conducted and no treatment guidelines exist.[19,20] This is especially true for infertility due to endometriosis, and for women with RIF and RPL. It seems that immunological problems may play a role in some cases of unexplained infertility. Immunological aspects are also suspected to be associated with RIF,RPL and chronic endometritis.[21]

Women's health matters

Many female health problems are not well researched. A report from Public Health England revealed that 31 % of women suffered from a reproductive or gynaecological problem within a 12 month period, but only a very small proportion of funding is allocated to this area.[22,23] Underfunding may lead to many small observational single-centre analyses. But this will not help to find evidence on

pending questions. What is really lacking for many unresolved issues in female fertility are well-conducted, randomized controlled trials with a sufficient power to detect possible differences among the groups compared. This becomes even more difficult with rare conditions, which would require collaborative efforts. RIF and RPL is a well-documented example of such a condition. Many published studies exist with women suffering from RIF and RPL but most of them are small and of a retrospective study design. Because diagnosis of CE is not standardised, comparability of the results is not possible.[24] Recently, a first RCT on diagnosing CE though fluid hysteroscopy was published.[25] On www.clinicaltrials.gov one RCT on antibiotic therapy in CE conducted in China is recorded.[26] Not one single RCT on the treatment of CE has been published so far.

Problems in current ART research

The influence of ART on the health of children is a very complex subject, with many influencing factors, mediators and confounders. It remains difficult to identify the true origin of adverse outcomes in children born after ART. Several factors are involved when a couple seeks fertility treatment. Besides the ART technologies and the hormonal stimulation, other particularly important factors include underlying parental health, parental age and the particular causes of subfertility.[27] As nowadays childbearing is often postponed for many reasons, the average maternal age is above 30 in most European countries, including Switzerland. Parents seeking fertility treatment are usually older; a high proportion is between 35-40 years. Pregnancy at age 35 – 40 is associated per se with higher risks of pregnancy complications and adverse perinatal outcomes.[28,29] One of the pending questions is how to separate the parental influence from any epigenetic changes induced through ART by applying sensible research methodologies. In current ART research, this separation remains especially crucial. Much published research cannot answer these questions. The main problems are: I) Small single-centre cohort studies; II) The selection of an appropriate control group; III) The comparison of diverse reproductive technologies making the aggregation of results difficult for systematic reviews and meta-analyses; and IV) The frequent lack of details on the ART treatment in large population-based studies.[30] The problem of selecting an appropriate group for comparison is discussed by Berntsen et al. Either spontaneously conceived children of subfertile couples or discordant siblings would be valuable controls.[27] To identify spontaneously conceived children born to subfertile couples, pregnancies caused by IUI could be examined. In couples not using any medical support for conception, the time until conception is either not documented, or relies on self-reported information provided by the couple. Discordant sibling couples are rare and difficult to identify. Only a few studies have used discordant siblings as controls.[31–34] One study used sibling couples where one was born after a fresh embryo transfer and the other after FET to explore the cause for LGA.[35] The differences in perinatal outcomes between pairs of siblings were lower

compared to existing research[31,34], or not present.[33] This supports the hypothesis that parental factors and subfertility are the primary influences on health outcomes in children.

6.3.Strengths and limitations

The strengths and limitations of each study are discussed in detail in each of the presented manuscripts.

The Bern IVF Cohort is a unique cohort of IVF children, with data on the parents, the details of the treatments and pregnancies, as well as birth outcome. The proportion of parents consenting is high, and the children are followed-up on until today. This cohort is a great opportunity for future research, including research on long-term health outcomes. The possibility of connecting perinatal outcomes to detailed treatment information and detailed laboratory data is a strength of the projects. Bern is a specialist centre for natural cycle IVF. NC-IVF is offered to all women with regular menstrual cycles, and the decision on treatment is made together with the physician. This is the reason why nearly half of the live births came following an NC-IVF treatment. This allows the study of the influence of gonadotropins. Comparable cohorts are rare, even on an international level.

Generally, all projects face the typical problems of research into ART, as discussed in the introduction. All are using data of one single centre, have a limited sample size and are retrospectively observational. Only one study included a control group for comparison. As patients treated by IVF or ICSI are highly selected already, it is difficult to find a good group for comparison. If, as in the manuscripts presented, data of a sample of the population is used, information on conception is often lacking. Children conceived with the support of ART methods are therefore present in the samples. The proportion of children born after ART in Switzerland is about 2-2.5%.[36] The contamination caused by this proportion can be considered to be low, and will not affect the medians of the groups for comparison.

This thesis demonstrates the possibilities and difficulties of ART research very well. When the goal is to investigate children's outcomes, it is not easy to find enough children born after the different treatment methods. This necessitates the collection of the data over several years, or collaboration with other ART centres. In multicentre studies, there are issues around the comparability of treatment schemes, as well as laboratory procedures, which might be different at each centre. It therefore increases the sample size by introducing more heterogeneity. As ART develops rapidly and the law changes, similar issues arise with collecting data over many years. For example, in 2017 the reproductive medicine act (RMA) was changed and allowed the extension of cultural duration until

day 5. Since then, pregnancy rates have increased in Switzerland.[37] But it also introduced an additional confounder when comparing children created before or after the change.

It is important to understand that ART is quite a special domain within medicine. Firstly, there are strict laws in place, clearly defining what is allowed and what is forbidden. Many ethical aspects are linked to ART. Society and politics may influence the scope of such a law. The RMA of Switzerland is, in comparison to other European countries, rather strict. It does not allow surrogate motherhood or egg donation. Furthermore, it does not allow the treatment of homosexual couples. However, it does allow sperm donation, which raises questions as to gender equity regarding accessing fertility treatment.

Fertility treatment is not covered by the mandatory health insurance, which all persons have in Switzerland. Only diagnosis, and up to three IUI and/or the stimulation of ovulation by hormones up to one year, is reimbursed. All IVF or ICSI treatments have to be paid for by the couples themselves. This leads to a selection bias, as more couples who are able to afford treatment will go for fertility treatment. For less privileged couples, it may lead to a further delay of treatment onset, as money may have to be saved first.

All these aspects may limit the transferability of the results of ART research to other countries.

6.4. Implications for future research

Access to data

The Swiss Society for Reproductive Medicine (SGRM) collects data on fertility treatment within Switzerland via its national database on ART (FIVNAT-CH). FIVNAT-CH was founded in 1993 and remains active today. It reports key figures on a yearly basis to the Federal Statistical Office (FSO). The RMA requires the cantons to collect the data, and to monitor the quality of fertility treatment.

In the FIVNAT-CH, data on fertility treatment on a national level is available for monitoring and quality control.[38] It would be desirable to allow access to this data for research. This would require certain changes in the way the SGRM and the FIVNAT-CH are organised. First, the FIVNAT-CH would need ethical approval to collect the data for the purpose of research. Only recently, Swissethics introduced a basic ethical approval for registries collecting health-related personal data.[39] Several medical associations have published guidelines on how to establish a medical registry.[40] This would of course require the centres to adequately inform their patients, and to collect informed consent. Second, the SGRM would need to clearly regulate access to the data for the purposes of research. A process of requesting data, and of the validation of research projects by the steering board of SGRM

or FIVNAT-CH would need to be put in place. It would allow research with larger dataset on a national level.[40] Standardized and high-quality data, including pregnancy and birth outcomes, should be collected systematically. Variable collection could be extended to allow the investigation of important research questions. Follow-up research on families, where informed consent is granted accordingly, would be possible. This would allow the contacting of families for future research on the long-term health consequences of ART. ART data collection must also adequately follow changes in treatment options. Today, with the possibility of freezing oocytes for social or medical reasons, or the vitrification of embryos, the fertility treatment of the same individual can last over decades.[41,42]

Transparency

Data collected within FIVNAT-CH is used to monitor the number of cycles conducted in fertility treatment and the number of children born following ART in Switzerland.[38,43] It allows certain conclusions to be drawn about the characteristics of the couples in treatment, treatment success and the children born. However, it is not yet used to assess the quality and outcome of the centres in a standardized way. Instead, each centre is asked to provide its own key performance indicators. Even if they are defined, differences in assessing them may exist. Each centre also determines how transparently they report their figures. Calculating the key performance indicators should be done centrally and independently by using the numbers of FIVNAT-CH. FIVNAT-CH should include all variables necessary to be able to independently calculate the performance of the centres.

Furthermore, FIVNAT-CH and the FSO only collect data on couples seeking treatment in Swiss fertility clinics. Compared to other European countries, RMA in Switzerland is still rather restrictive, and costs for fertility treatment are high. This leads to reproductive tourism, with Swiss couples seeking fertility treatment abroad. In Switzerland, it is impossible to estimate the number of couples seeking help abroad, or to know how many children are born in Switzerland after ART treatment abroad. FIVNAT-CH, on the other hand, documents foreign couples seeking treatment within Switzerland. A European or international collaboration of national registries would be necessary to enable the extent of reproductive tourism to be estimated. It would also require standardized data collection on an international level to better aggregate and exchange data. ISMAARC and ESHRE are currently improving their data collection.[44] This will possibly help in future to estimate the extent of fertility tourism by Swiss couples.[18,41]

Health of children should be the focus

Research on perinatal outcomes in children born after ART in Switzerland has so far been limited to qualitative aspects analysed within the FIVNAT-CH data.[38,43]

The Bern IVF Cohort is the first Swiss cohort to have been set up with the aim of also investigating the long-term health of children born after different ART treatments.

Previously, research conducted by fertility specialists on the long-term health consequences in children born after ART in Switzerland did not exist. It was a bit of a surprise when cardiologists around Urs Scherrer communicated the higher cardiovascular health risks they discovered in children born after ART. It led to a media response throughout Switzerland, and even at international level. Many fertility specialists in Switzerland found themselves confronted with patients asking critical questions. This would be the moment for specialists engaged in fertility treatment to wake up and to create a supportive atmosphere to facilitate research. It is the moment to seek collaborations. It is our responsibility towards our patients to become more engaged in research, or at least to facilitate it with better data and improved access to it.[45]

Future research projects

As a first step, it would be important to investigate the perinatal health of children born after ART in comparison to that of children born to fertile couples within Switzerland. FIVNAT-CH and the Bern IVF Cohort could be a good basis on which to establish research focused on long-term follow-up in Switzerland.

Long-term cardiovascular consequences should be investigated in collaboration with cardiologists. Children from the Bern IVF Cohort could be asked to participate in follow-up investigations.

Linkage studies with data from FIVNAT-CH would allow the identification of risks of other diseases. Many registries exist in Switzerland, some on typical childhood diseases. A linkage with the registry for childhood cancer, for example, would be an option.

A linkage with the Swiss NeoNet registry would allow the identification of children born at very low gestational age, to further research and follow up the risks of very preterm birth after ART.

A randomized, blinded multicentre study should be set up on the diagnosis and treatment of women with RIF and RPL. It would be important to define clear inclusion criteria. As number of women with these conditions are not many, an international approach could be considered. Women diagnosed with CE would get a blinded course of antibiotics or placebo. One months later, a test for cure should be performed by endometrial biopsy. A crossover design could be considered to give the chance for treatment to each women participating in the trial. The women should then be followed up for at least one year.

6.5.Implications for future policy

Society and fertility

The trend towards the postponement of childbearing has been observed for decades across all European countries. Many explanations highlight changes in women's position and opportunities within society. However, women's actual reasons for the postponement of childbearing are not well researched nor understood. Especially in Switzerland, not much research exists on topics such as fertility awareness, parity progression, child wish or aimed family size. This leads to the fact that it is not well known how women could be motivated to move towards having children at younger ages. Which political measures would have to be taken to encourage younger couples to build a family? To sociologically and demographically research these questions might allow politics to improve circumstances for couples to start families earlier. The positive effect would be to lower the use of ART due to ageing.

Access to fertility treatment

Unequal access to fertility treatment may be increasing the risks for offspring. In Switzerland, only very basic fertility treatment is paid for by the mandatory health insurance. Neither IVF nor ICSI are covered, denying access to couples with lower financial resources. It also leads to couples delaying treatment during their more fertile years increasing risks for the mothers and the children. Access to fertility treatment should be equal for all genders and couples independent of their sexual orientation.[46]

Towards safer ART in Switzerland

Research, alongside ever-increasing experience and knowledge, leads to a reduction of some of the risks associated with birth following ART. For example, many countries have now moved towards a single embryo transfer policy. Low-dose stimulation schemes and variations, and the further development of cIVF have also reduced certain risks associated with gonadotropin stimulation as well as the number of embryos generated. Laboratory procedures have developed rapidly, and freeze-all strategies avoid the interference of hormonal stimulation with the onset of pregnancy. However, while clear answers are missing for some of the new technologies, the causes of any poorer perinatal outcomes often seem to be a combination of parental factors and ART itself. The one thing that is crystal clear is that multiple pregnancies bear the highest risk of adverse perinatal outcomes. Despite this clear evidence, the transfer of multiple embryos is still common in many countries, leading to twin and higher-order pregnancies. It would be advisable for Switzerland to employ a policy of single embryo transfer in both fresh and frozen cycles.

7. Conclusion

The following findings can be drawn from the different research projects described in this thesis:

I) There is a need to further investigate the effects of gonadotropin stimulation; and NC-IVF provides an opportunity to do this;

II) Child health and long-term outcomes should be the focus of reproductive medicine, rather than the current focuses of pregnancy rates and live birth rates;

III) Many reasons for infertility and women's health are still unresolved and require further, more focused and well-designed research.

The main conclusion is that fertility treatment matters and better research on fertility treatment should be encouraged, especially in Switzerland. Numbers are often too low in any one centre so collaboration would be key to expanding and improving research.

It remains difficult to disentangle all the influencing factors on child health. Gaining more knowledge on these aspects is crucial. Especially in a country, where maternal age at child birth, infertility and the use of ART will increase further, it is of tremendous importance to further improve the safety of ART treatment and to follow-up their future children.

Personal conclusion

This thesis was an excellent chance for me to increase my experience in research. It gave me insight in a topic, ART, where I had not previously had any knowledge. It also allowed me to combine my main interests: women's health, reproduction, demography and child health. I am very happy for this experience.

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CURRICULUM VITAE

Personal Information

Name	Vera Ruth Mitter-Iseli
Date of birth	04.01.1980
Place of birth	Bern, Switzerland
Marital status	Married
Children	Lenny, 03.12.2011 & Malin, 02.07.2014

Education

09.2018 – present	PhD in Health Sciences, **Division of Gynecological Endocrinology and Reproductive Medicine,** University Women's Hospital, Insel Gruppe AG, & Graduate School for Health Science, University of Bern, Switzerland & Swiss School of Public Health (SSPH+)
09.2008 – 10.2009	MSc Demography & Health, **London School of Hygiene and Tropical Medicine**, London, UK
09.2003 – 11.2005	Federally graduated Pharmacist, **University of Basel,** Switzerland
08.2002 – 08.2003	cand. pharm, **École Polytechnique Fédéral de Lausanne (EPFL),** Lausanne; **Pharmacy du Croset**, Ecublens, Switzerland
08.2000 – 08.2002	Pharmacy, University of Bern
08.1995 – 05.2000	**Gymnasium Bern-Kirchenfeld,** High school with focus on economics and law

Work Experience

11.2014 – 01.2018	Pharmacist, Clinical Trial Coordinator, **Institute of Hospital Pharmacy**, Insel Gruppe AG, Bern, Switzerland
08.2016 – 08.2017	Research Assistant (20%), **Division of Gynecological Endocrinology and Reproductive Medicine**, University Women's Hospital, Insel Gruppe AG, Bern, Switzerland
10.2009 –11.2014	Project coordinator of the Swiss Childhood Cancer Registry, **Institute of Social and Preventive Medicine**, University of Bern, Switzerland
01.2010 – 03.2012	Pharmacist, emergency service, **Notfall Apotheke**, Basel, Switzerland (20%)
05.2008 – 09.2008	Pharmacist, **St. Jakobsapotheke,** Basel, Switzerland
03.2008 – 09.2008	Trainer/Coach for professionals on health products and sale, **Health Invest**
03.2007 – 01.2008	Hospital pharmacist, **St. Claraspital,** Basel, Switzerland
07.2006 – 08.2006	IAESTE Traineeship, sales and marketing, **Supor Pharmaceuticals,** Hangzhou, China
01.2006 –05.2006	Pharmacist, **Apotheke zur Waage,** Basel, Switzerlan

Teaching activity

03.2018	**Good clinical Practice und Ethics** – Ethische Fragen im klinischen Forschungsalltag, Lecturer, Bachelor Pharmacy and Biology, University of Basel, Switzerland (1 lesson per year 2018 – 2020)
2015 – 2017	**The Role of the Hospital Pharmacy in Clinical Trials**, Lecture, Clinical Investigators II: Advanced GCP and clinical research training course, CTU Bern, Switzerland (2 lessons per year in 2015, 2016; 3 lessons 2017)
2016	**The Role of the Hospital Pharmacy in Clinical Trials**, Lecture, CAS Clinical Research Coordinator, Bern University of applied Sciences, Switzerland (1 afternoon in 2016)
06.2011	**Das Schweizer Kinderkrebsregister – ein Beispiel für ein Diagnoseregister,** Gastlesende in Gesundheitspsychologie, University Wuppertal, Germany
11.2009	**Risks associated with fertility and fertility control**, ISPM Bern, Research Meeting, University of Bern, Switzerland

Supervision of students for master theses

Main Supervisor

2014	Diana Moser, cand. MSc of Science in Pharmacy, “Cardiac late effects after treatment of cancer in childhood”
2014	Sandra Blau, cand. MSc of Science in Pharmacy, “Secondary tumours as late effects after treatment of cancer in childhood”

Co-Supervision of medical master students, not official supervisor, supervision and support for coordination, data management, patient contact and consent as well as statistical analysis.

- Marlen Berchtold, Master 2017
- Daniela Steiner, Master 2017
- Mirja Minger, Master 2017
- Livia Purtschert, Master 2017
- Pascale Fasel, Master 2017
- Maria Paulsson, Master 2018-2020
- Flavia Grädel, Master 2018
- Ursina Küng, Master 2018
- Lena Bommer, Master 2018
- Caroline Imhoof, Master 2020

PUBLICATIONS

Peer reviewed Journals

Kohl Schwartz AS*, **Mitter VR*,** Amylidi-Mohr S, Fasel P., Minger MA, Limoni C, Zwahlen M, von Wolff M. The greater incidence of small-for-gestational-age newborns after gonadotropin-stimulated in vitro fertilisation with a supraphysiological estradiol level on ovulation trigger day. *Acta Obstetricia et Gynecologica Scandinavica. 2019; 98:1575-1584*

*** shared first authorship**

von Wolff M, Fäh M, Roumet M, **Mitter V,** Stute P, Griesinger G, Kohl Schwartz A.Thin Endometrium is also Associated with Lower Clinical Pregnancy Rate in Unstimulated Menstrual Cycles: A Study Based on Natural Cycle IVF. *Frontiers in Endocrinology*, 2018 Dec 20;9:776

Singer S, **Mitter V**, Porsch U. No Evidence for Benefits of the Depression Coach. *Psychotherapy and Psychosomatics*, 2018 Sep 21:1 *[letter to the editor]*

Purtschert LA, **Mitter VR**, Mosimann B, Zdanowicz JA, Minger MA, Fasel P, von Wolff M, Kohl Schwartz AS. Breastfeeding following in vitro fertilization in Switzerland – does mode of conception affect breastfeeding behaviour? [submitted]

Mitter VR, Meier S, Gillon T, Rau TT, Mueller MD, Zwahlen M, von Wolff M, Kohl Schwartz AS. Chronic endometritis- shortened time to live birth in subfertile women undergoing hysteroscopy and endometrial diagnostic biopsy. [submitted]

Non peer reviewed journals

Kohl Schwartz AS, **Mitter V**, von Wolff M. Eizellen für die Zukunft. Gynäkologie, Juli 2017

CONFERENCE PRESENTATIONS

Mitter V, Kohl Schwarz A, Fäh M, Griesinger G, von Wolff M. Endometrial thickness is associated with the clinical pregnancy rate in unstimulated menstrual cycles – a study based on Natural Cycle IVF. Poster presentation at the Conference of *European Society of Human Reproduction and Embryology* 2017, Geneva, Switzerland – Abstract published: Human Reproduction, Vol 32, Supp 1, 2017 Abstract book; P-473; p i351.

Mitter VR, Kohl Schwartz AS, Amylidi-Mohr S, Fasel P, Zwahlen M, Von Wolff M. The risk for «small for gestational age» after gonadotropin-stimulated in-vitro fertilisation (IVF) compared to natural cycle IVF: a cohort study. Poster presentation at the *Symposium of the Graduate School for Health Sciences* 2018, University of Bern, Switzerland

Mitter VR, Fasel P, Minger M, Purtschert L, Zwahlen M, von Wolff M, Kohl Schwartz AS. The Bern IVF Cohort: Perinatal outcomes compared to births to the general population of Switzerland. Talk at the Swiss Public Health Conference 2019, Winterthur, Switzerland

Mitter VR, Science Flash Talk, Finalist, Swiss Public Health Conference 2019, Winterthur, Switzerland

Mitter VR, Meier S, Kohl Schwartz AS. Time to live birth in subfertile couples and couples with recurrent pregnancy loss, Talk at the *Symposium of the Graduate School for Health Sciences 2019*, Gunten, Switzerland

Mitter VR, Krebs D, Paulsson M, Mosimann B, von Wolff M, Kohl Schwartz AS. Getting consent – challenges and difficulties for conducting research using health-related patient data. Talk at the 21.Jahrestagung of the Evidence based Medicine- Network, February 2020, University of Basel, Switzerland

Mitter VR, Meier S, Rau TT, von Wolff M, Zwahlen M, Kohl Schwartz AS. Shortened time to live birth in subfertile women undergoing endometrial diagnostic biopsy. Accepted as ePoster for the Conference of *European Society of Human Reproduction and Embryology* 2020, Copenhagen, Denmark; (virtual instead because of COVID-19 pandemic)

Declaration of Originality

Last name, first name: Mitter Vera

Matriculation number: 00-104-828

I hereby declare that this thesis represents my original work and that I have used no other sources except as noted by citations.

All data, tables, figures and text citations which have been reproduced from any other source, including the internet, have been explicitly acknowledged as such.

I am aware that in case of non-compliance, the Senate is entitled to withdraw the doctorate degree awarded to me on the basis of the present thesis, in accordance with the "Statut der Universität Bern (Universitätsstatut; UniSt)", Art. 69, of 7 June 2011.

Place, date

Muri b. Bern, 27.04.2020

Signature