EMJ EUROPEAN MEDICAL JOURNAL

# REPRODUCTIVE HEALTH

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# CONTENTS


EDITORIAL BOARD..... 4

CONGRESS REVIEW..... 12

- Review of the 33rd Annual Meeting of the European Society of Human Reproduction and Embryology (ESHRE), held in Geneva, Switzerland, 2nd–5th July 2017

INTERVIEWS WITH *EMJ REPRODUCTIVE HEALTH* EDITORIAL BOARD..... 24

ABSTRACT AWARDS..... 32

ABSTRACT REVIEWS..... 36

ARTICLES

- EDITOR'S PICK: SELECTION OF APPROPRIATE TOOLS FOR EVALUATING OBESITY IN POLYCYSTIC OVARY SYNDROME PATIENTS..... 48

  Yang Xu

- TORSION OF NORMAL ADNEXA IN THE THIRD TRIMESTER OF PREGNANCY MIMICKING ACUTE APPENDICITIS..... 54

  Mariam Mathew et al.

# REPRODUCTIVE HEALTH


- REGENERATIVE MEDICINE, DISEASE MODELLING, AND DRUG DISCOVERY IN HUMAN PLURIPOTENT STEM CELL-DERIVED KIDNEY TISSUE..... 57

  Navin Gupta et al.

- OOCYTE DONATION: AN OVERVIEW..... 68

  Nalini Mahajan

- TARGETING MAST CELLS AS A VIABLE THERAPEUTIC OPTION IN ENDOMETRIOSIS..... 76

  David A. Hart

- PARENTAL PROTEIN MALNUTRITION PROGRAMMES OF OFFSPRING GROWTH AND VASCULATURE TO INCREASE RISK OF CARDIOVASCULAR, PANCREATIC, AND METABOLIC DISEASE. LESSONS LEARNED FROM ANIMAL STUDIES..... 84

  Maria Dolores Ruiz-Diaz et al.

WHAT'S NEW..... 90

EVENTS..... 96

BUYER'S GUIDE..... 98

# EDITORIAL BOARD




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# Welcome

From all of us here at the European Medical Journal, I would like to welcome you to this latest edition of *EMJ Reproductive Health*, the go-to place for the very latest advances in this ever-evolving field. In this edition, we present an independent review of the 33rd Annual Meeting of the European Society of Human Reproduction and Embryology (ESHRE), including abstract summaries composed by the researchers themselves. You can also look forward to a selection of interviews from the *EMJ Reproductive Health* Editorial Board, followed by a range of high-quality peer-reviewed papers.

Switzerland's capital city of Geneva provided the perfect backdrop for this year's ESHRE Congress, and we have detailed the unmissable highlights from the 4-day programme. The research shown at the congress includes the use of artificial intelligence in assessing embryo quality as well as assessing the correlation of male partner age with *in vitro* fertilisation (IVF) delivery rates. Concluding the Congress Review section is a variety of comprehensive abstract reviews chosen from the >1,000 abstracts carefully selected for oral and poster presentation at ESHRE.

“Switzerland's capital city of Geneva provided the perfect backdrop for this year's ESHRE Congress, and we have detailed the unmissable highlights from the 4-day programme.”

We are also pleased to present contributions from *EMJ Reproductive Health's* prestigious Editorial Board in this eJournal, who discuss topics ranging from assisted reproductive technology, to the factors responsible for oocyte activation. This section is not to be missed if you want to gain a valuable insight into the interests, achievements, and future aspirations of leading specialists in the field of reproductive health.

Lastly, the much-anticipated final section of *EMJ Reproductive Health 3.1* consists of a selection of high-quality peer-reviewed articles. The Editor's Pick for this edition is a paper penned by Xu entitled 'Selection of Appropriate Tools for Evaluating Obesity in Polycystic Ovary Syndrome Patients', summarising the important potential of tools used as alternatives to BMI in these often mis-diagnosed patients. In addition, Mathew et al. deliver a fascinating review of maternal ovarian torsion, with particular emphasis on the third trimester of pregnancy and Gupta et al. analyse the roles of human induced pluripotent stem cells and bioengineering techniques in the development of novel renal replacement therapies. These are just a few of the top-quality papers this eJournal has to offer.

Whether you are an industry professional or medical specialist, the range of content within this latest edition of *EMJ Reproductive Health* means there is something to interest everyone and we hope you enjoy reading this issue. We would also like to thank the Editorial Board and authors for their insightful contributions, and we look forward to seeing you all at next year's ESHRE Congress in Barcelona, Spain!

Spencer Gore

*Director, European Medical Journal*

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# Foreword

**Prof Joep Geraedts**

*Maastricht University, Maastricht, Netherlands.*

Dear Colleagues,

This year, *EMJ Reproductive Health* offers you a number of very interesting manuscripts that have undergone peer review. The list of papers ranges from basic science to applied laboratory science, clinical diagnostics, and therapeutic applications.

Such a wide coverage reminds me of the 33rd Annual Meeting of the European Society of Human Reproduction and Embryology (ESHRE) in Geneva, Switzerland. It had more attendees than ever before. As always, there were many interesting free communications, both orally and as posters. The start was overwhelming with two very interesting invited presentations at the plenary session on the first morning. Carlos Simon (Valencia, Spain), showed that autologous cell therapy with CD133+ bone marrow-derived stem cells is an efficient therapy for patients with refractory Asherman's syndrome and endometrial atrophy who wish to conceive. Two months after stem cell therapy, all but one patient exhibited an improved uterine cavity, resulting in pregnancies, both spontaneously as well as after embryo transfer. However, the effect was only temporary, with a return to the initial levels 6 months after treatment.

“This year, *EMJ Reproductive Health* offers you a number of very interesting manuscripts that have undergone peer review. The list of papers ranges from basic science to applied laboratory science, clinical diagnostics, and therapeutic applications.”

The second presentation was a state of the art lecture by Dennis Lo (Hong Kong), the true pioneer of non-invasive prenatal testing. His group is now able to detect fetal *de novo* mutations on a genome-wide level. Furthermore, they have shown that there are recurrent DNA sites that plasma DNA molecules tend to preferentially end on. Such positions are called 'preferred DNA ends'. Interestingly, circulating DNA fragments derived from the fetus and those derived from the pregnant woman have sets of different preferred ends. This development allows one to predict the likelihood that a plasma DNA fragment is of fetal or maternal origin without using DNA polymorphisms. This will certainly increase the possibilities of non-invasive prenatal testing.

Kind regards,

**Joep Geraedts**

Emeritus Professor and Former Head of the Department of Genetics and Cell Biology, Maastricht University, Maastricht, Netherlands.

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# ESHRE ANNUAL CONGRESS 2017

THE PALEXPO,
GENEVA, SWITZERLAND
2ND–5TH JULY 2017

## Welcome to the European Medical Journal review of the 33rd Annual Meeting of the European Society of Human Reproduction and Embryology

**Citation:** EMJ Repro Health. 2017;3[1]:12-23. Congress Review.

The diverse city of Geneva, Switzerland was the location for the 33rd Annual Meeting of The European Society of Human Reproduction and Embryology (ESHRE), which warmly welcomed senior scientists and clinicians from around Europe to attend this much anticipated meeting from 2nd–5th July. Known as both the 'city of peace' and Europe's most international city, with >40% of its population originating from outside of Switzerland, Geneva also boasts spectacular views of Lake Geneva and the breath-taking Alps mountain range, as well as offering a rich and vibrant history, making it the perfect backdrop for this prestigious congress.

The 4-day meeting involved an open programme selected from >1,700 abstracts, of which the highest number came from China, for the first time in the congress' history. Breaking yet another record, the number of abstracts submitted by females exceeded that by males, totalling 985 and 740, respectively. ESHRE Past Chairman, Dr Kersti Lundin, in her pre-congress statement in 'Focus on Reproduction', was proud to announce: "No other meeting in reproductive medicine can now command this sort of support year after year." In our Congress Review section, we will bring you unmissable details and highlights from the 235 abstracts that were selected for oral presentation and a further 800 for poster presentation. As part of the social programme, attendees took a well-deserved break from congress by participating in ESHRE's charity run on Monday 3rd July, which helped raise funds for patient groups throughout Europe including Fertility Europe, ESHRE's partner patient organisation.

The high-quality scientific programme commenced with two keynote lectures. The first winning study was presented by world renowned clinical investigator, Prof Carlos Símon, and was based on his paper 'Autologous cell therapy with CD133+ bone-marrow derived stem cells for refractory Asherman's syndrome and endometrial atrophy: a pilot cohort study'. Presenting to one of the largest audiences in reproductive medicine, Prof Símon was followed by chemical pathologist Prof Dennis Lo for the second keynote presentation, providing the spectators with an update on non-invasive prenatal testing, which he argued will

play a vital role in obstetric care. The meeting programme was packed full of topics of great importance to the discipline, including ovarian rejuvenation, germline gene editing, endocrine disruptors, cryopreservation, artificial gametes, and pregnancy failure. Of particular interest was the revelation of the much anticipated first results from ESHRE's ESTEEM trial, a preimplantation genetic testing study of polar body analysis by array comparative genomic hybridisation (aCGH).

> "No other meeting in reproductive medicine can now command this sort of support year after year."

To bring this magnificent meeting to an end, newly appointed ESHRE Chairman Dr Roy Farquharson presented the awards and closing ceremony. João Pedro Alves Lopes (Sweden) was awarded the Basic Science Award for oral presentation; Heleen Zandstra (Netherlands) was awarded the Clinical Science Award for oral presentation; Ellen Casser (Germany) received the Basic Science Award for poster presentation; Paula Piomboni (Italy) was awarded the Clinical Science Award for poster presentation; Mina Popovic (Belgium) was given an educational travel grant to present her oral presentation at the annual meeting of the Fertility Society of Australia; Sarah Bailey (UK) was awarded the best oral presentation by a nurse; and Sofie Ellegiers (Belgium) was awarded the best oral or poster presentation made by a laboratory technician. In addition, Marc Germond (Switzerland) and Rob Norman (Australia) were appointed as the new ESHRE honorary members.

In the following review highlights, we will explore the very best of the pioneering research presented at this year's ESHRE meeting, providing a convenient overview for those of you who were unable to attend, or a welcome refresher for those who would like to re-visit the fascinating meeting. We hope you will enjoy reading our highlights and we look forward to seeing many of you at next year's ESHRE meeting, 1st–4th July, in the home of Gaudí architecture, Barcelona, Spain.

## Artificial Intelligence Improves Assessment of Embryo Quality

ERRORS in the morphological assessment of embryo quality could be improved by advances in artificial intelligence, according to a ESHRE press release dated 4th July 2017.

Embryo quality is considered the main determinant of implantation and pregnancy in *in vitro* fertilisation (IVF). Morphology is used to measure embryo quality; however, a visual morphological grading can lead to variation. Recently, morphological grading has become more accurate with the introduction of time-lapse imaging. Unfortunately, many embryos graded as 'good quality' still fail to implant in the uterus and lead to pregnancy. It is thought that this is due to chromosomal abnormalities, which are not detected by morphological assessment.

A recent study from São Paulo State University, São Paulo, Brazil, has analysed images taken from the development of 482 7-day-old bovine embryos, which were used to 'train' an artificial intelligence system. Thirty-six assessment variables were identified during the analysis, 24 of which formed the input of the artificial network architecture. By using mathematical variables derived from time-lapse images of embryo development, an algorithm can classify images of an embryo's development automatically, removing the human variable from morphological assessment. During the initial set-up phase, 'serious errors' occurred in only 6% of the assessments and, overall, the artificial intelligence system had a 76% accuracy.

By increasing the objectivity and repeatability in embryo assessment, accuracy of diagnosing embryo viability can be improved. Clinicians can use this artificial intelligence to customise their treatment strategies and better predict a patient's chance of pregnancy.

Investigator Prof Jose Celso Rocha, São Paulo State University, suggested that embryo assessment led by artificial intelligence could be ready for routine clinical use within the next 2 years, at least as a controlled and test version; however, the extent to which it will improve embryo grading, and thus outcome in IVF, will depend on how thoroughly the system is 'trained' and how wide the sampling of embryo images is in that training.

> "By increasing the objectivity and repeatability in embryo assessment, accuracy of diagnosing embryo viability can be improved."



## Quantity and Quality Are Both Important for IVF Success

A HIGHER number of eggs retrieved during an *in vitro* fertilisation (IVF) treatment cycle is associated with more chromosomally normal embryos available for transfer. Results from the Australian study, conducted by Dr Christos Venetis, University of New South Wales, Sydney, New South Wales, Australia, and IVF Australia, were presented at this year's ESHRE conference and summarised in a ESHRE press release dated 3rd July 2017.

The study assessed the number of chromosomally normal (euploid) embryos identified during preimplantation genetic screening in 724 cycles at three IVF centres. The euploid embryos were then correlated with the number of oocytes received during that treatment cycle. Results showed that the number of euploid embryos identified per cycle was negatively associated with female age. For one euploid embryo to be produced, a 34-year-old woman would need to produce five oocytes, whilst a 38-year-old would need 10; as for two euploid embryos, 14 and 24 would be needed, respectively. It was also shown that the number of oocytes received was positively associated with the number of euploid embryos generated; the higher the number of oocytes, the higher the number of euploid embryos produced. Previous research had indicated live birth rates were also correlated to the number of oocytes received.

> "...a higher number of oocytes leads to a higher number of euploid embryos available for transfer..."

Euploid embryos are known to have a high probability of implantation (50–60%) and low probability of miscarriage (6%). This study suggests, according to Dr Venetis, University of New South Wales, that a higher number of oocytes leads to a higher number of euploid embryos available for transfer which "clearly correlated with the cumulative chance of a pregnancy after a single stimulated cycle." Simply, the more oocytes collected during a single round of IVF treatment, the higher the chance of euploid embryos developing, leading to an increased chance of healthy live births.

There is no universal oocyte number to collect, as other parameters need to be taken into account, including specific patient's wishes. Dr Venetis mentioned that aiming for a high number of eggs (>15) can increase the risk of ovarian hyperstimulation syndrome (OHSS) and potential complications. New stimulation protocols, however, greatly reduce the risk of OHSS, which may allow the yield from a single stimulation to be much greater.

## Mosaic Embryos Can Lead to a Healthy Pregnancy

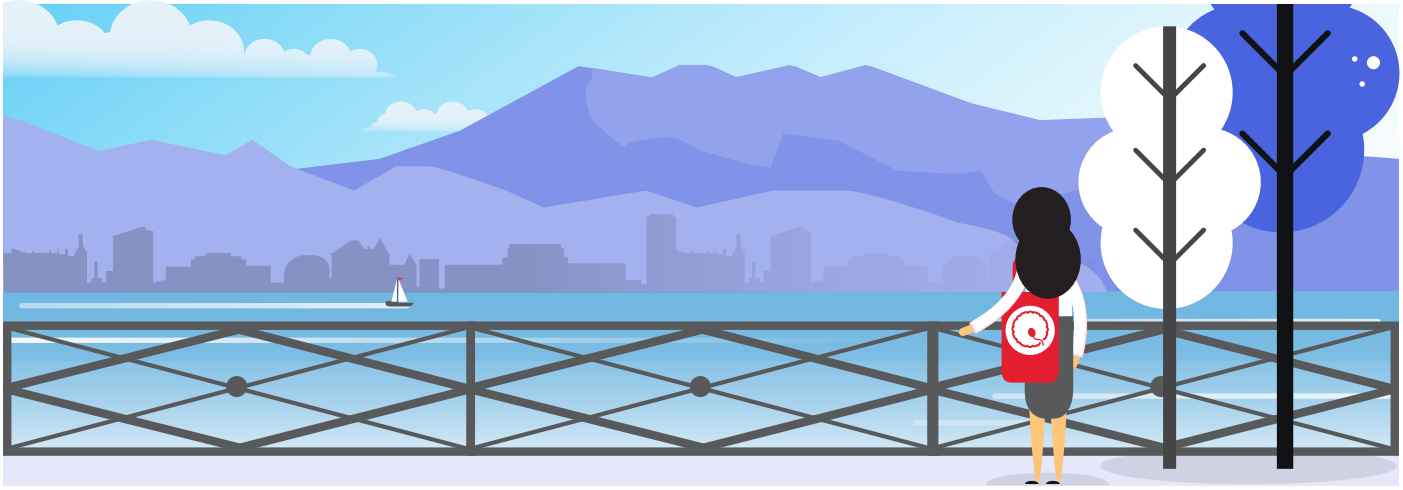
IMPLANTING mosaic embryos during *in vitro* fertilisation (IVF) can result in a healthy pregnancy. This is according to the results of a new study by Dr Francesco Fiorentino, GENOMA Laboratory, Rome, Italy, and colleagues, reported in a ESHRE press release dated 4th July 2017. Mosaic embryos are those whose cells have ≥2 cell lineages that are genetically distinct. Generally, one cell lineage will have a normal chromosome composition and the other will have a chromosome abnormality.

> “...we suggest that mosaic embryos should only be transferred in women with no euploid embryos available.”

During IVF, preimplantation genetic testing for aneuploidy is typically used to discern euploid (chromosomally normal) embryos and thereby boost the likelihood of a successful IVF outcome. Until recently, mosaic embryos were not commonly utilised in IVF, as it was believed their use would not result in a successful procedure. However, a previous study by Dr Fiorentino’s group had suggested that mosaic embryos could lead to a healthy pregnancy.

Building on this finding, the study involved 73 women who did not have any euploid embryos for transfer but did have mosaic embryos. Researchers classified the mosaic embryos into two categories: high aneuploidy (≥50%) and low aneuploidy (<50%). It was found that transfers of embryos in the low aneuploidy category led to a live birth rate of 39.5% and a miscarriage rate of 7.0%. This was in comparison to a live birth rate of 16.7% and a miscarriage rate of 10.0% in the high aneuploidy category. This finding led Dr Fiorentino to suggest that: “Priority for transfer should be given to mosaic embryos with low levels of aneuploidy.”

Furthermore, the researchers concluded that their results demonstrated mosaic embryos should now be considered a distinct category in regard to likelihood of a successful IVF procedure. Dr Fiorentino explained: “Euploid embryos have a higher implantation potential than mosaic embryos, and because of this we suggest that mosaic embryos should only be transferred in women with no euploid embryos available.”



## Male Partner Age Affects IVF Delivery Rates

INCREASING male age was the subject of a recent study which investigated the correlation between the age of a male partner and live birth rates in couples using *in vitro* fertilisation (IVF) to conceive. Aside from a handful of studies into natural conception and the impact of male partner age, little was known about this subject until recently, because female age is such a dominant factor in successful conception.

In a ESHRE press release dated 3rd July 2017, Dr Laura Dodge, Harvard Medical School, Boston, Massachusetts, USA, explained: "Generally, we saw no significant decline in cumulative live birth when women had a male partner the same age or younger. However, women aged 35–40 did significantly benefit from having a male partner who is under age 30, in that they see a nearly 30% relative improvement in cumulative incidence of live birth when compared to women whose partner is 30–35, from 54% to 70%."

The team analysed the IVF cycles that had been completed between 2000 and 2014 at a large IVF centre in Boston. This amounted to nearly 19,000 cycles for 7,753 couples, with female partners grouped according to the following age ranges: <30, 30–35, 35–40, and 40–42 years of age. Male partners were also grouped into these age bands, but there was an additional group for >42 years. They found that couples where the female was in the 40–42-year age range had the lowest cumulative live birth rate, and in these instances the age of the male partner had no impact on the success of the cycle. This confirms the impact of female age on success rates of IVF. Increasing male age, however, was shown to have a negative effect on the success rates when the female was in other age groups; for example, if the female partner was aged <30 years, a male partner aged 40–42 years was associated with a 46% lower cumulative birth rate than if the male partner was aged 30–35 years. If female partners were aged 35–40 years, a younger male partner was more likely to produce a higher live birth rate.

> "...women aged 35–40 did significantly benefit from having a male partner who is under age 30, in that they see a nearly 30% relative improvement in cumulative incidence of live birth when compared to women whose partner is 30–35, from 54% to 70%."

## Clomiphene Citrate to Become the Drug of Choice for Ovarian Stimulation Before Intrauterine Insemination

A NON-HORMONAL fertility drug, in addition to being much cheaper, has been shown to be just as effective as hormones for ovarian stimulation before intrauterine insemination (IUI) in a study reported on in a ESHRE press release dated 3rd July 2017. According to the researchers from the AMC Centre for Reproductive Medicine, Amsterdam, Netherlands, these results will make this the drug of choice for IUI in couples with unexplained and mild male infertility.

> "We showed that IUI stimulated with FSH is not superior to IUI and clomiphene in terms of ongoing pregnancies, live births, and time to pregnancy..."

The ovarian stimulation prior to IUI method is associated with an increased risk of multiple pregnancy, and therefore there has been some debate as to whether it should be performed with a course of routinely used injections of follicle stimulating hormone (FSH) or with a shorter course of the non-hormonal drug clomiphene citrate (CC).

This study compared the two options in couples with unexplained or mild male infertility in 24 fertility centres in the Netherlands, randomising a cohort of women (N=738), to IUI with FSH (n=369) and to IUI with clomiphene (n=369). Of the patients studied, 113 (31%) and 97 (26%) had an ongoing pregnancy following IUI-FSH and IUI-CC, respectively. There were five (1%) cases of multiple pregnancies in the IUI-FSH cohort, and eight (2%) in the IUI-CC group. These differences were statistically insignificant.

Additionally, ovarian stimulation with CC was much cheaper financially than with FSH, with the latter estimated as costing just €5 per patient after 5 days of daily tablets. This compared to an estimated €200 per patient for those who received FSH injections for a mean duration of 8 days.

"We showed that IUI stimulated with FSH is not superior to IUI and clomiphene in terms of ongoing pregnancies, live births, and time to pregnancy," commented investigator Dr Noor Danhof, AMC Centre for Reproductive Medicine. "We also found a comparable low multiple pregnancy rate between IUI-FSH and IUI-CC, and these are now the reasons why we recommend using the least expensive stimulation agent."

## Pregnancy in Cancer Survivors Quantified for the First Time

FEMALE survivors of cancer are 38% less likely to achieve a pregnancy than women who have never been subject to the disease, suggests a study performed by the Medical Research Council Centre for Reproductive Health, Queen's Medical Research Institute, University of Edinburgh, Edinburgh, UK, presented at the ESHRE annual meeting, 2017, and described in a ESHRE press release dated 3rd July 2017.

The study noted the incidents of pregnancy in 23,201 cancer survivors ≤39 years of age, who were diagnosed in Scotland between 1981 and 2012, and found that only 20.6% of them achieved pregnancy, compared to 38.7% of the control group. This chance of pregnancy was reduced in all age groups and was particularly low in those women with cervical cancer, breast cancer, and leukaemia. "The major impact of pregnancy after some common cancers highlights the need for enhanced strategies to preserve fertility in girls and young women," said Prof Richard Anderson, University of Edinburgh.

Another notable discovery was that those diagnosed with cancer later in the study (2005–2012) had higher rates of pregnancy than those diagnosed earlier (1981–88), indicating that the impact of some cancer treatments on fertility rates has reduced.

Prof Anderson was quick to point out the limitations of the study, not least that some women may have simply chosen not to have a pregnancy, explaining: "While these results do show an expected reduction in the chance of pregnancy after chemotherapy and radiotherapy, having a pregnancy after cancer does involve a range of complex issues that we cannot address in this study."

> "The major impact of pregnancy after some common cancers highlights the need for enhanced strategies to preserve fertility in girls and young women..."

Researchers are hopeful that these data will help clinicians to advise their patients more accurately concerning their future chances of pregnancy, particularly with respect to fertility preservation methods, such as oocyte and embryo freezing. "Even for patients considered at low risk of infertility as a result of treatment, a fertility discussion is recommended before treatment begins," concluded Prof Anderson.

## Single-Mother-By-Choice Children Do Just as Well

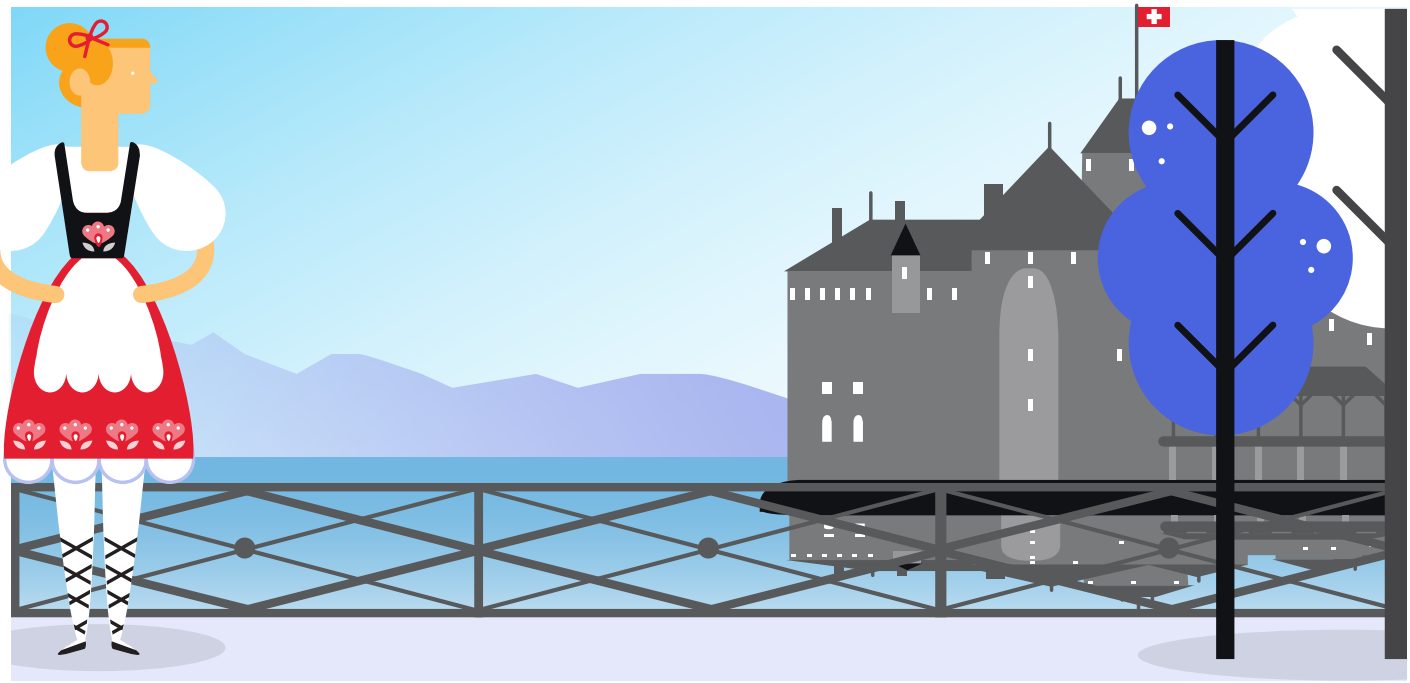
CHILDREN in single-mother-by-choice families do as well as those in heterosexual two-parent families, according to a ESHRE press release

dated 5th July 2017. Fertility treatment for single women is now an increasingly popular choice for women who wish to become pregnant without a partner, but some specialists have raised concerns about the wellbeing and development of the resulting children. A study from the Centre of Expertise on Gender Dysphoria, VU University Medical Centre, Amsterdam, Netherlands, found no differences in terms of parent-child relationship or child development, and discovered that single-mothers-by-choice had a greater social support network.

> “...any negative influence on child development depends more on a troubled parent-child relationship and not on the absence of a father.”

The study compared 69 single-mothers-by-choice (who had knowingly chosen to raise their children alone) and 59 mothers from heterosexual two-parent families with a child between 1.5 and 6 years of age. Using three validated questionnaires, parent-child relationships, mothers’ social support networks, and children’s wellbeing were compared. The study found that, for parent stress or emotional involvement there were no significant differences between family types; single-mothers-by-choice showed significantly higher scores on the social support they received; and there were no significant differences in the children’s wellbeing between both family types. The study reported that children growing up with single-mothers-by-choice seemed to enjoy a similar parent-child relationship to those in heterosexual two-parent families.

Ms Mathilde Brewaeys, study investigator from the Centre of Expertise on Gender Dysphoria of the VU University Medical Centre, said: “The assumption that growing up in a family without a father is not good for the child is based mainly on research into children whose parents are divorced and who thus have experienced parental conflict. However, it seems likely that any negative influence on child development depends more on a troubled parent-child relationship and not on the absence of a father.”



The study also found that single-mothers-by-choice and their children benefit from a good social support network and that this should be emphasised in the counselling of women who want to have a child without a partner.

## Use of Assisted Reproductive Technology in Europe

GLOBAL need for advanced fertility treatments are not being met by the majority of European countries, according to a presentation displayed in a ESHRE press release dated 4th July 2017. This study covered 707,171 treatment cycles performed in 2014 and the subsequent 146,232 babies born, making this the largest, most accurate snapshot of assisted reproductive technology (ART) in Europe. Spain was found to be leading in ART activity, performing 109,275 treatment cycles in 2014, followed by Russia (94,985) and, former leaders, France (90,434); the UK performed 61,000 treatments in 2013. The total number of treatment cycles included *in vitro* fertilisation (IVF), intracytoplasmic sperm injection (ICSI), egg donation, and intrauterine insemination.

Numerous findings were presented, with ICSI being continually favoured over IVF in clinics, with 336,123 and 123,809 procedures performed, respectively, despite pregnancy rates, per embryo transfer, being higher with IVF (34.6%) than ICSI (33.1%). ICSI was first developed in the 1990s to allow infertile men to conceive, but now the procedure is predominantly used for occasions with no male partners.

Throughout Europe, pregnancy rates have stabilised for both IVF and ICSI, 35% and 33%, respectively, but pregnancies resulting from egg donation have continued to rise, currently at ˜50%. However, these statistics do fluctuate between countries. It was also acknowledged that pregnancy rates from blastocyst transfers are continuously higher than Day 3 transfers, across all processes. Rates from frozen donor eggs (49%) were found to be very similar to fresh donor eggs (51%), but higher than frozen embryos. Twin pregnancy rates, once common in IVF, are continuing to fall, reaching ˜17% in 2014. Dr Calhaz-Jorge, Chairman, European IVF Monitoring Consortium for ESHRE summarised: "The rate of multiple pregnancy continues its slow but steady decline. Success rates seem to have stabilised."

Unfortunately, availability of ART remains very uneven across Europe; a study calculated a global need for ART at ˜1,500 cycles per million population per year. Dr Calhaz-Jorge commented: "Only a minority of European countries meet this need."

> "The rate of multiple pregnancy continues its slow but steady decline. Success rates seem to have stabilised."

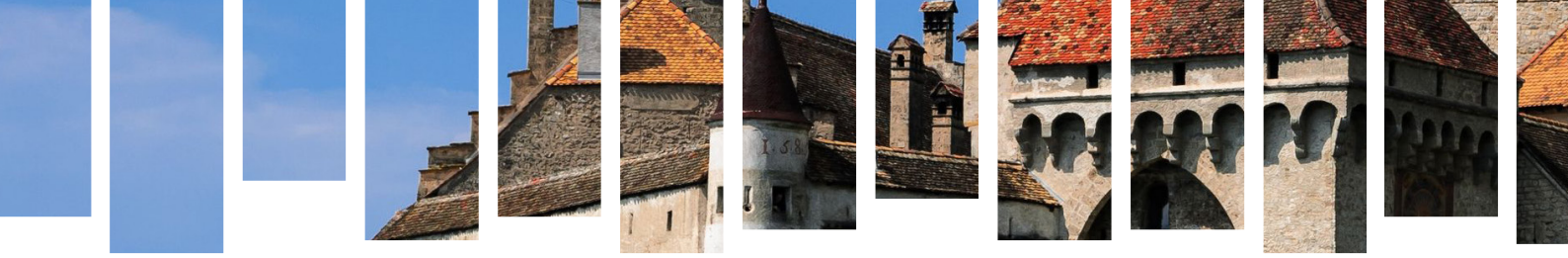
## Positive Trials Towards Personalised Medicine in Fertility Patients

ANALYSES of two Phase III clinical trials (ESTHER-1 and ESTHER-2) have demonstrated that treatment with Rekovelle® (follitropin delta) produces similar live birth rates to conventional follitropin alfa treatment. The analysis, performed by Ferring Pharmaceuticals, Saint-Prex, Switzerland and presented at the ESHRE Annual Meeting 2017, showed the cumulative live birth rate following *in vitro* fertilisation to be 43.9% with follitropin delta; just 0.6% lower than with follitropin alfa. Ongoing pregnancy rates were equally similar (45.1% and 45.7%, respectively). "These new Rekovelle analyses add further evidence for a personalised approach to fertility treatment for patients," said Per Falk, Executive Vice President and Chief Scientific Officer, Ferring Pharmaceuticals.

A separate analysis of the safety profile of the drug was also performed. Using data from the ESTHER-1 trial, researchers evaluated ongoing pregnancy rates, early ovarian hyperstimulation syndrome (OHSS), and preventative interventions for early OHSS in women with varying anti-Müllerian hormone (AMH) levels. In women with high AMH (≥35 pmol/L) results showed a lower incidence of early OHSS for follitropin delta in comparison to conventional follitropin alfa dosing (4.7% versus 11.9%, respectively). The analysis also revealed a significant improvement in the amount of patients requiring preventative interventions for early OHSS; 4.7% for follitropin delta treatment versus 23.8% for follitropin alfa. It was notable that patients' ongoing pregnancy rate was sustained.

Researchers were quick to note that the ESTHER trials were not powered for this analysis, thus further study will be required before the efficacy of the drug can be validated. Nonetheless, researchers are confident that these analyses represent a promising step towards personalised fertility treatment. "Rekovelle's individualised dosing regimen, based on a patient's AMH level and body weight, provides clinicians with a consistent, evidence-based approach to personalising treatment for their patients," concluded Per Falk.

> "These new Rekovelle analyses add further evidence for a personalised approach to fertility treatment for patients..."



## Monica Dăscălescu

Senior Embryologist, Columna Medical Center, Bucharest, Romania.

**Q:** Could you please describe your day-to-day roles and responsibilities as a senior embryologist at Columna Medical Center, Bucharest, Romania?

**A:** The Columna Clinic is a clinic with about 500 cycles a year. My duties as a senior embryologist are multiple. I perform all types of laboratory procedures, from sperm preparation, checking of the ova, *in vitro* fertilisation (IVF), and intracytoplasmic sperm injection, to vitrification and thawing. I oversee the daily check of environmental conditions.

**Q:** What initially piqued your interest in embryology; was there something or someone in particular that inspired you to specialise in this area?

**A:** IVF came into my life suddenly, but I felt I had found my vocation. This changed the direction of my life by 180 degrees.

**Q:** What are the biggest breakthroughs that you have witnessed throughout your career? What positive impacts have they had on patients and clinical practice?

**A:** Fertility treatment has come a long way since the first IVF baby, Louise Brown, was born in 1978. A medical first that I think will change patients' perspectives is the birth of the first healthy baby from a transplanted womb.

**Q:** How would you like the see the reproductive health field develop over the next 10 years? Are there any innovative technologies that you believe will help make these goals achievable?

**A:** Development will be extremely accelerated in the next 10 years. As for an innovative technique that will change the course of reproductive health, I think it will be the one that greatly succeeds in rejuvenating and improving the quality of oocytes.

**Q:** What does your role as Romanian Representative at the European Society of Human Reproduction and Embryology (ESHRE) entail? Can you detail some of the goals of the society over the coming years and how you plan to achieve them?

**A:** The role of the Romanian Representative at ESHRE is, on the one hand, to correctly communicate the different information about the activity in Romania but, on the other hand, is also to try to apply the knowledge gained from international collaboration and implement it through those who perform medically assisted reproduction in Romania.

The main aim of ESHRE is to promote interest in, and understanding of, reproductive biology and medicine. It does this through facilitating research into human reproduction and embryology and disseminating the results of that research to the general public, scientists, clinicians, and patient associations. It also works to collaborate with politicians and policy makers throughout Europe.

**Q:** You are an active member of many different societies. Do you believe that this is an important role for healthcare professionals to undertake, and why?

**A:** Effective teamwork in healthcare and scientific organisations can have an immediate and positive impact on patient safety. The importance of this is increasing, due to factors such as the increasing complexity and specialisation of care and the increasing number of infertile patients. Patient safety, in the context of a complex medical system, recognises that it is essential to be involved in organisations that promote not only the research but also the continuous propagation of medical knowledge.

> “The main aim of ESHRE is to promote interest in, and understanding of, reproductive biology and medicine.”

> My proudest moments may not have looked spectacular from the outside, but in fact they are the moments when a child that came from my lab is born.

**Q:** Could you inform us of some of the benefits of using blastocyst cultivation? Do you believe this is the way forward for couples that have problems conceiving?

**A:** The point of growing embryos to the blastocyst stage in the laboratory is to deliberately weed out the embryos that do not have the genetic potential for continued growth. Of course, there is always the 'risk' that no embryos make it to the blastocyst stage in the laboratory, but (because the problem is related to the genetics of the embryo, not to the culture conditions in the laboratory) they would not have been viable in the uterus either. Only the embryos that make it to the blastocyst stage can generate a successful IVF pregnancy. In my lab, and many others, all embryos are grown to the blastocyst stage and only well-developed blastocyst stage embryos are transferred to the uterus on Day 5 or 6. Extra embryos are cryopreserved at the blastocyst stage. However, growing the embryos to the blastocyst stage prior to transfer does not automatically result in a pregnancy. Because the embryos have reached the blastocyst stage prior to transfer, it is reasonable to assume that the embryos are capable of successful implantation. However, once the embryos are transferred to the uterine cavity, they must still attach to the endometrium and continue the implantation process for 10 days before a pregnancy is established. The attachment and implantation processes are, currently, beyond our control.

**Q:** Could you share with us the importance and necessary stages of embryo evaluation?

**A:** Assessment of embryo quality, in order to choose the embryos that will most likely result in pregnancy, is the critical goal in assisted reproductive technologies. Assessment of morphological features, as a reliable non-invasive method that provides valuable information in the prediction of IVF or intracytoplasmic sperm injection outcome, is one of the critical steps in the embryology lab. Generally, quality and the rate of development in human embryos that are produced *in vitro* may vary widely. These differences may indicate the inherent diversity in the potential of gametes, as well as in details of the IVF method and culture medium status. Application of a proper embryo scoring system has many potential benefits, such as the accurate selection of embryos prior to transfer, the assessment of different culture media, and reduced risk of multiple pregnancies. There are several stages for the evaluation of an embryo's quality, but in the majority of European clinics the embryologists use the criteria that came from the ESHRE Istanbul consensus.

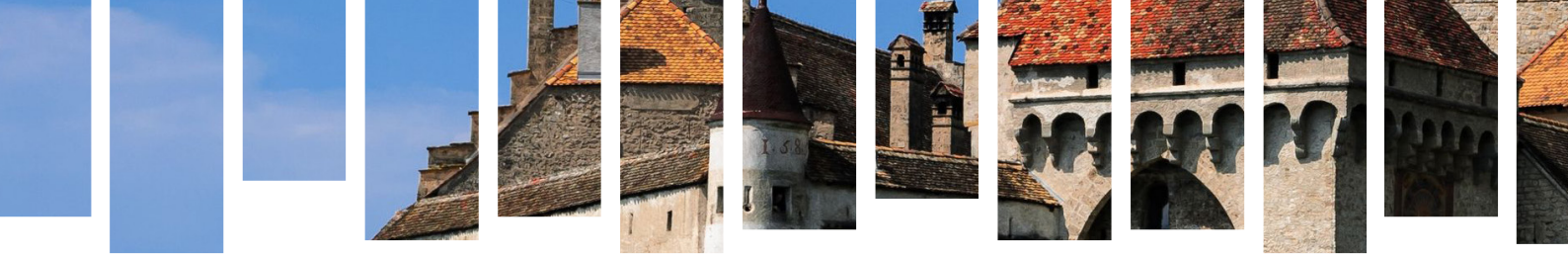
**Q:** Reflecting on your career, what stands out as your proudest professional achievement to date?

**A:** My proudest moments may not have looked spectacular from the outside, but in fact they are the moments when a child that came from my lab is born. From that point of view, there are quite a few!

**Q:** Finally, if you were to offer one piece of advice to aspiring medical students to help them in their future careers, what would it be?

**A:** Embryologists work closely with hopeful parents helping them in all aspects of reproduction, from fertilisation through pregnancy. Embryology can be tricky; it involves long hours in the lab, including at the weekend, emotional involvement, a lot of hard work, the constant need to learn new techniques, and good practical skills. But it is ultimately rewarding, so being an embryologist is not that bad.

> In my lab, and many others, all embryos are grown to the blastocyst stage and only well-developed blastocyst stage embryos are transferred to the uterus on Day 5 or 6.




## Ioana Rugescu

Safety and Quality Specialist, Bucharest, Romania; General Secretary, AER Embryologists Association; Elected Deputy, Safety and Quality Interest Group, European Society for Human Reproduction and Embryology (ESHRE); Romanian Data Collecting Representative, Data Consortium ESHRE-European *In Vitro* Fertilisation Monitoring (EIM).


**Q:** What was it that piqued your interest in the field of reproduction and from there, the issues of safety and quality in assisted reproductive technologies (ART)?

**A:** I strongly believe that who we are is unalterably linked to those whom we have known during our life, as well as our intrinsic nature. So, I focussed on biological science first, because of the interactions I had with my teachers during school and college. Faculty directed me to the beauty of research which was the start of my interest in the field of embryology and biology of reproduction and the clinical applications of it all. I learned that there are a lot of critical issues related with reproductive healthcare that affect patients. During my professional life, I was concerned about the stages that present ethical matters for patients, their families, practitioners, and not least the organisations that offer medical services. I believe that reproductive healthcare will continue to present critical concerns for practitioners who wish to provide the best care for their patients. All of this pointed me to the field of safety and quality, and my desire to grow the fulfilment of patient needs. In addition, advanced technologies that are currently available, or that will become available soon, will make the patient and practitioners decisions easier if, through protocols and quality management and operational procedures, we can prove that assisted human reproduction is not science fiction but a safe and reliable medical procedure.

> “I strongly believe that who we are is unalterably linked to those whom we have known during our life, as well as our intrinsic nature.”

**Q:** ART are developing rapidly and growing in popularity. Does this pose any challenges in your work as a safety and quality specialist?

**A:** Safety and quality professionals, in any organisation but especially in healthcare, is the hidden factor that saves lives. While many patients often do not receive the medical care necessary, others receive care that may be unnecessary, or even harmful. This is the point where the safety and quality professional steps in! Safety and quality of reproductive care is a dynamic and complex phenomenon, especially because of the rapid rate of development in new technologies. We discover new techniques and are tempted to use these new procedures in the clinic in order to help our patients, sometimes without proper caution, but this is not the biggest challenge in our work. The biggest challenge is diversity!

**Q:** How would you say safety and quality in ART differs across Europe and globally?

**A:** I mentioned that the most difficult point in safety and quality in reproductive healthcare is diversity. We have different laws, different medical procedures, even different points of view. Reproductive healthcare is challenged to standardise frameworks and language under which all care providers operate. There is no standard nomenclature for patient safety that is widely used and this must be changed. Standardisation provides consistency between interdisciplinary teams and can facilitate multisite patient care. My mission is to improve the lives of patients, not only their healthcare service, by building consensus for quality measurement and reporting in order to have good strategies to reduce any risks. Efforts to improve the safety and quality of care are resource intensive and take continued commitment.

**Q:** As an elected Deputy of the European Society for Human Reproduction and Embryology (ESHRE) Safety and Quality Interest Group, could you tell us about some of the responsibilities within this group? How important is it to undertake such work across a multinational perspective?

**A:** The Special Interest Group on Safety and Quality in Assisted Reproductive Technology (SIG SQART) was founded during the 18th Annual Meeting that took place in Vienna in 2002. I was elected deputy in this SIG for the past 2 years and my colleagues are deeply involved in all SIG SQART's projects.

Our SIG is active in several areas that are related to health economics, quality of treatment, psychosocial, sexual, and legal aspects and the impact on families and individuals confronted with ART treatments. We have also taken the task of co-ordinating the ESHRE's activities on the effects of viral diseases on reproduction. In addition to participating to the annual ESHRE conferences and organising campus courses, we provide written recommendations and guidelines of good medical practice on various aspects of ART. We are also creating a reference list of national guidelines which can serve as reference point and can facilitate clinical work and research across countries.

In order to answer your second question, I can tell you that it is really important to be involved in such work, but globalisation is a complex phenomenon which encompasses a great variety of trends and tendencies in the health economic, social, and cultural spheres. It has multidimensional characteristics and involves an intensified flow of ideas, information, and of course, efforts.

**Q:** What would you say are the biggest challenges facing the field of ART today, and how might these be overcome?

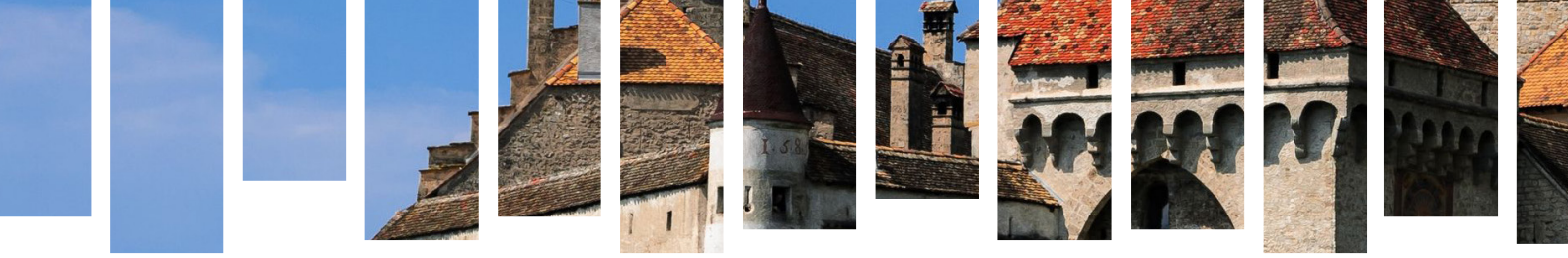
**A:** In the 1980s, the concerns surrounding ART were focussed on the safe administration of gonadotropins, transparency of pregnancy data from clinics, and addressing economic barriers to ART access; how we are more concerned about new techniques such as pronuclear transfer or preimplantation genetic screening and diagnosis; or gametes derived from stem cells or biomarkers. The biggest challenge remains that not all aspects can be measured so, if we cannot measure it, we cannot improve it!

**Q:** Could you provide us with any insight into the work you are currently undertaking or plan to undertake in the near future?

**A:** I am currently involved in a lot of projects and it is impossible to enumerate them all; in order to give you an insight I consider one of the priorities the involvement in the EURO GTP II Project. In this new European Union (EU)-funded project, the aim is to assess the technical requirements that are needed to determine the quality and safety of novel tissue and cells processes or treatments by demonstrating their efficacy based on key performance indicators and recipient outcomes. The goals of the project are to develop a tool that can categorise treatments, aid tissue establishments and ART centres to determine what is needed, and to implement a new process or procedure based on good risk assessment and follow-up.

**Q:** You are the Romanian Data Collecting Representative for the ESHRE-European *In Vitro* Fertilisation Monitoring (EIM) Consortium. Could you tell us more about what this role involves?

**A:** The EIM was established to collect, process, and finally publish regional data for Europe on direct clinical results and side effects, follow-up children's wellbeing, and the availability and structure of services in the different countries. I have been involved in working for Romanian data since 2008 and I can share that methods of data collection and analysis are well chosen and well implemented, which is essential for all types of evaluations. Since 2008 I have collected data based on EIM forms, from Romanian clinics and sent this data to ESHRE. Romania was a voluntary participant in the *In Vitro* Fertilisation Monitoring Consortium. This voluntary data collecting system allows us to aggregate and validate complex data, use the data until we are able to make scientific, evidence-based decisions. Data reporting brings fairness and benefits to



reporting clinics and clarity to patients. Daniel Keys Moran once said that: "You can have data without information, but you cannot have information without data" so, I like to believe in the sushi principle, that data is best when it is raw, fresh, and ready to consume!

**Q:** On the subject of data collection, what opportunities does collecting a large data set offer? Additionally, is there a need for caution when interpreting the results from a variety of locations?

**A:** Healthcare data will not get simpler in the future. Healthcare faces unique challenges and with that comes unique data challenges. The top five challenges in voluntary data collection are: multiple data locations, structured/unstructured data, inconsistent/variable definitions, the complexity of data, and changes in regulatory requirements. Having a large database that spans Europe allows different sources of information to integrate, the availability of novel technologies, and finally, the inclusion of geographical and environmental information that may further increase the capability of interpreting the data gathered with the ability to extract new knowledge from them. Quality can only be maintained if the outcome of the therapeutic endeavour is known and when occurring complications are recorded. Therefore, data reporting is an essential part of monitoring the effectiveness and safety of medical treatments. Under-reporting is the major flaw of data collection, which often leads to overestimation of treatment effectiveness and the underestimation of the safety of the offered treatments. There is a need for action when interpreting the results from the top five challenging points of view because we are trying to create order out of chaos.

**Q:** What advice would you give to medical students who are considering a career in the field of reproductive medicine?

**A:** If medical students enjoy a variety of challenges and an incredibly varied workload, then reproductive medicine could be the speciality that they are looking for. Multidisciplinary teamwork is at the core of this speciality. Excellent communication skills and an open-minded approach to patients is a must. An ability to empathise with patients is also vital. You will learn all your professional life but the results of your work will grow with you into the next generation!

**Q:** Finally, of the achievements across your career, which are you most proud of?

**A:** I am proud of a lot of things from my professional life. One of them is the Bucharest Embryology Symposium, the most important Romanian event in reproductive medicine.

## George Anifandis

Assistant Professor of Human Embryology, Clinical Embryologist, Department of Obstetrics and Gynaecology, Faculty of Medicine, University of Thessaly, Larissa, Greece.

**Q:** What first attracted you to specialise in obstetrics and gynaecology, and, more specifically, human embryology?

**A:** I studied biology and, after that, throughout my Master's and PhD, I specialised in human clinical embryology. Clinical embryology is the main part of human assisted reproduction that is an obstetrics and gynaecology specialty.

**Q:** Could you give us a brief overview of what your role within the Department of Obstetrics and Gynaecology entails? What are your main responsibilities?

**A:** At this moment, I am Assistant Professor of Embryology and Head of the Embryology and Spermatology Lab of the Human Assisted Reproduction Unit, Obstetrics and Gynaecology Clinic, University of Thessaly, Greece. Apart from the teaching responsibilities, I am a supervisor of the

Embryology Lab, with my main responsibility being to supervise the function of the entire lab.

**Q:** One of your more recent publications was focussed on the standards of care in infertility in Europe. Could you tell us more about this research? How important is the standardisation of care across different countries?

**A:** It was an overview of the standards of care in infertility around Europe. In this review, the standards that are essential for good practise during *in vitro* fertilisation (IVF) treatments were described. They were focussed only in Europe, but I think it is not far from what is happening in the USA, Australia, or even Asia and other developing (non-European) countries. I think that the review is most attractive for clinicians, in order for them to establish common ovarian stimulation protocols that will result in high IVF success rates.

**Q:** How important are events such as the European Society of Human Reproduction and Embryology (ESHRE) Annual Meeting to reproductive health professionals and embryologists?

**A:** It is by far the most important event for IVF specialists and clinical embryologists. New methodologies, new techniques, and innovative science at both a basic and clinical level are applied at this event, and every year the participation is increasing, gathering people from all over the world. For the last 10 years, at every ESHRE annual meeting there have been examinations for clinical embryologists, while for the last 3 years there have been examinations concerning obstetrician gynaecologists for laparoscopic surgery.

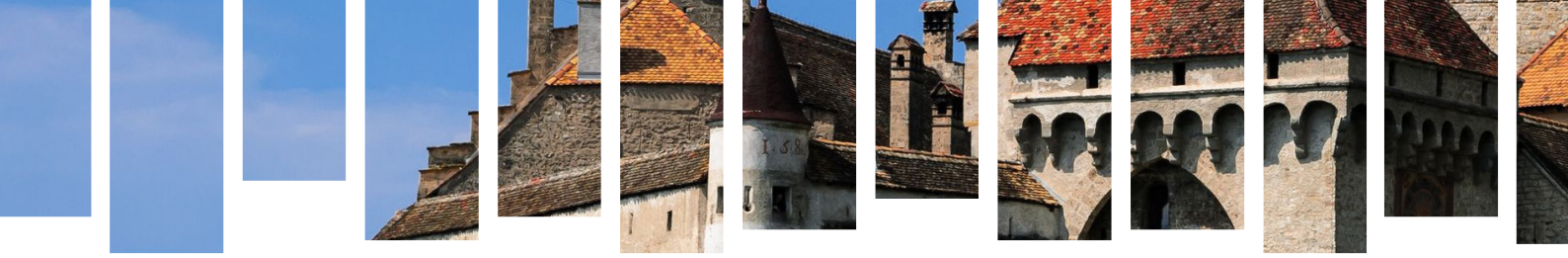
**Q:** Over the past few years, more reliable methods have been developed to assist in the management of infertility, including therapeutic and diagnostic procedures dependent on technologies. What are your thoughts on current advancements in technology within the field? Is it something we should be encouraging more of in the future?

**A:** Techniques on assisted reproduction have been developed very much indeed. For example, embryo evaluation has been changed dramatically following the usage of time lapse. Embryoscopes have been used widely to evaluate embryos in order patients to have bigger chances for pregnancy success. Any scientific advance in our field is welcomed, because our goal is to increase pregnancy rates. We should encourage basic research in this field to find out the molecular or the genetic pathways that are responsible for infertility and to discover new therapeutic tools.

**Q:** You have previously reported on the contributions sperm have in relation to oocyte activation, although this area is still relatively unknown. There is debate as to the feasibility of using phospholipase C (PLCζ) and post-acrosomal sheath WW domain-binding protein (PAWP) as diagnostic tools and hypotheses about their therapeutic potential. Could you provide some further explanation about this research? Are you planning any further investigation in this area?

**A:** Well, it was a comment about the factors that are responsible for oocyte activation, a process which is responsible for the formation of the two pronuclei, the formation of the zygote, and embryonic development. The candidates are PLCζ and PAWP. According to this mini review, it seems that PLCζ gained more ground over PAWP, indicating PLCζ as the only factor that can provoke oocyte activation. For that reason, couples that experience intracytoplasmic sperm injection fertilisation failure are potential candidates for assisted oocyte activation protocols. Human recombinant PLCζ protocols do not exist yet, as far as I know, but protocols with ionophores are plentiful and I am planning to investigate similar protocols in patients with total or partial fertilisation failure.

> “For the last 10 years, at every ESHRE annual meeting there have been examinations for clinical embryologists, while for the last 3 years there have been examinations concerning obstetrician gynaecologists for laparoscopic surgery.”



**Q:** Is your research focus likely to shift in the coming years? Are there any areas of research that you have not had a chance to explore that you would like to investigate in the near future?

**A:** My research interests are around clinical embryology and no, I am not planning to shift in the next few years. Nevertheless, I would like to investigate the genetic background of male or female infertility, something which I have already begun with a recent paper.

**Q:** What has been your proudest achievement so far during your medical career?

**A:** My proudest achievement was when I became Head of the Embryology and Spermatology Lab, supervising and helping the Unit and the Clinic to achieve their goals.

**Q:** Across the field of reproductive health in general, what are some of the most pressing issues that need to be addressed during 2017?

**A:** Some of the most pressing issues to be addressed include:

- Mild ovarian stimulation protocols and ovarian protocols for poor responders
- Protocols for women with polycystic ovary syndrome (PCOS)
- More accurate scoring systems for embryo morphology
- Human papilloma virus and IVF results, or even HIV and IVF results

**Q:** Finally, what advice would you give to ambitious medical students with an interest in a reproductive health specialism?

**A:** They should focus on IVF, because it is a field that develops more and more. They should also focus on the research in this field because current research is relatively poor. New blood will advance our field, something which can be seen taking place over the last 35 years within this speciality.

“My proudest achievement was when I became Head of the Embryology and Spermatology Lab, supervising and helping the Unit and the Clinic to achieve their goals.”

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# Award Winners at the 33rd ESHRE Annual Meeting 2017

The 33rd Annual Meeting of The European Society of Human Reproduction and Embryology (ESHRE) received a huge volume of abstract submissions. Over the 4 days the congress took place, a large number of these submissions were presented: 235 orally and 800 as posters. From amongst this selection, seven presentations were specifically highlighted. One presentation was nominated for the Fertility Society of Australia (FSA) Exchange Award and six received a cash prize of €2,000. The selections were made by special award committees comprising senior scientists and clinicians. In this Abstract Awards section, we place all the prize-winning presentations in the spotlight for your consideration.

## The Fertility Society of Australia Exchange Award

This award was won by Mina Popovic (Ghent, Belgium) for work that examined the extent trophectoderm biopsy could be used as a measure of the genomic status of the inner cell mass in human blastocysts. In this study, Popovic and colleagues found that an association between trophectoderm biopsy and inner cell mass was present in most of the samples they analysed. However, they commented that the nature of mosaicism could make it exceedingly difficult to use just one trophectoderm biopsy for making a diagnosis. In order to more completely understand mosaicism, in terms of occurrence and implications, as well as to obtain significant results, the authors are in the process of expanding their research to investigate a greater number of blastocysts. It is hoped that studies such as this will facilitate a more precise quantification of the diagnosis of mosaicism, which will assist selecting suitable embryos for *in vitro* fertilisation (IVF); this should improve the efficiency, safety, and outcomes of the procedure. The FSA Exchange Award is an educational travel grant that will enable Mina Popovic to travel to the FSA annual meeting and deliver her oral presentation there, allowing a wider audience to appreciate the important findings of this study.

## Basic Science Award for Oral Presentation

This award was given to João Pedro Alves Lopes (Stockholm, Sweden) as the first and presenting author of the best oral presentation on a basic science topic. This study sought to answer the question of whether germ and somatic testicular cells co-cultured in a three-dimensional gradient system (3DGS) could reorganise *in vitro* in a close to *in vivo* association.


While germ cell proliferation and differentiation have been extensively studied in regard to signalling pathways and cell-to-cell interactions, there is much that is not yet known, as the processes are exceedingly complex and influenced by a wide range of factors, many of which are interconnected. Previous research investigating the mechanisms that determine whether a germ cell proliferates or differentiates has made use of different approaches, including organ culture and the use of primary testicular cells to generate *de novo* seminiferous-like structures. The researchers sought to investigate whether the 3DGS provided a model that better captured the germ-to-somatic cell associations, in order to enable study of the germ cell niche *in vitro*.

It was found that rat germ and Sertoli cells co-cultured in the 3DGS reorganised in seminiferous-like structures, thus making it possible to study *in vitro* germ-to-somatic cell interactions. However, the authors cautioned that, currently, progression in spermatogenesis had not been seen to occur in the culture conditions.

## Clinical Science Award for Oral Presentation

This award was given to Heleen Zandstra (Maastricht, Netherlands) as the first and presenting author of the best oral presentation on a clinical science topic. Zandstra and her colleagues from the Maastricht University Medical Center carried out an observational cohort follow-up study to determine if the embryo culture medium used for IVF/intracytoplasmic sperm injection (ICSI) impacted the growth and body composition of singleton IVF children at 9 years of age.

Previous research has shown that the culture medium used in IVF/ICSI has an influence on birthweight and this affect persists up to 2 years old. That previous research alternated assignment to either GITM Version 3 (Vitrolife group) or K-SCICM (Cook group). Participants in this study were contacted after their child turned 9 years old and 46% agreed to participate in follow-up research. At 9-year follow-up, various anthropometric measurements were taken. After correcting for age, anthropometrics of the parents, sex, and other factors, it was found that the culture used for the Vitrolife group was responsible for an additional 1.7 kg of weight (p=0.047) and an additional 3.4 cm in waist circumference (61.4 cm versus 58.0 cm; p=0.01). As a result of these findings, the researchers commented that there was a need for further structured follow-up of IVF/ICSI to obtain more information about the potential long-term health impacts.

## Basic Science Award for Poster Presentation

Ellen Casser (Münster, Germany) scooped this award for the first and presenting author of the best poster presentation on a basic science topic. Casser and colleagues' research study focussed on whether identical (monozygotic) twin embryos cultured in different assisted reproductive technology (ART) media became functionally different.

Two-cell mouse embryos were mechanically bisected and then cultured in parallel in different media. There were two ART media (GM501, SAGE I-step) and one mouse medium utilised (KSOM[aa]). Twin blastocysts cultured in ART media were more likely to result in four epiblast cells, which represents the minimum threshold for further development, than in KSOM(aa) (GM501=5.33±0.46 cells; SAGE I-step=3.92±1.08 cells; KSOM[aa]=3.58±1.08 cells). Furthermore, it was found that a greater number of twins were delivered to term when ART media were utilised. Indeed, when both twins were developed in the same ART medium, 86 pairs were transferred to uterus, which resulted in 28 live births (6 monozygotic pairs). The corresponding figures for KSOM(aa) were 48, 10, and 1, respectively; when one twin was developed in KSOM(aa) and the other in either GM501 or SAGE 1-step, 30 pairs were transferred to uterus, which led to 9 live births (2 monozygotic pairs). The researchers explained, that if their results were produced in other species (including humans), they would demonstrate that embryo culture media have a substantial effect on embryo survival.

## Clinical Science Award for Poster Presentation

Paula Piomboni (Siena, Italy) took top honours in this award for the first and presenting author of the best poster presentation on a clinical science topic. Today, although many men are affected by infertility (around 7%), the causes are often unknown. As previous work had found that taste receptors were expressed in testis and sperm and varies among individuals, the researchers hypothesised that polymorphisms in taste receptors could affect sperm functionality and hence investigated potential associations between *TAS2Rs/TASIR* and *GNAT3* gene variability and fertility status in 452 men undergoing semen evaluation during an infertility diagnostic screening.

The findings implied that there was a correlation between *TAS2R3*-rs11763979 and sperm morphology; *TAS2R3*-rs11763979 and sperm concentration; and *TAS2R14*-rs3741843 and sperm motility. However, the researchers cautioned that, due to the low sample size, a larger study is needed to confirm the findings, especially because some taste receptor gene polymorphic variance have a low incidence in the population. Further genetic studies should be conducted in order to build a more comprehensive picture of the genetic components of infertility in males.

## The Nurses Award

The prize for the best oral presentation by a nurse was given to Sarah Bailey (Southampton, UK) for the presentation: 'Hope for the best… But prepare for the worst'. This qualitative study investigated women's experiences of the first 12 weeks of a new pregnancy following recurrent miscarriage. Participants for the study were recruited from recurrent miscarriage clinics at two major UK hospitals. All participants completed weekly questionnaires to assess their psychological condition and some participants took part in semi-structured interviews. This took place within the context of a randomised control trial feasibility study of a self-help intervention, which was intended to improve psychological well-being during this 12-week period.

A thematic analysis revealed six primary themes. These included women preparing for the worst and expecting another miscarriage would occur, social isolation and loneliness, a constant guilt about their 'failure' to have a successful pregnancy, and the positive impact of supportive care and understanding healthcare professionals. The study authors hope that these findings will highlight the care needs of this patient group, although they caution that the UK-based nature of the study may mean that findings cannot be extrapolated across different cultural and national contexts.

## The ART Laboratory Award

The award for the best oral or poster presentation by a laboratory technician was won by Sofie Ellegiers (Ghent, Belgium). The study authors endeavoured to answer the question of whether there was a difference in blastocyst quality and formation rate when using a time-lapse imaging system (TLIS) compared to a standard incubator. Although a previous review had found no significant difference in clinical outcome between TLIS and a standard incubator, currently, there is limited data on whether the undisturbed culture characteristic of TLIS could prove beneficial for embryo quality; thus, this was a timely study.

The researchers selected ICSI cycles with embryo transfer on Day 5 and then conducted sequential extended culture on either the TLIS Embryoscope™ (Vitrolife, Frölunda, Sweden) or a standard incubator (Binder CB210; Wolf Laboratories Limited, York, UK). A significant difference was discerned in the TLIS group compared to the standard incubator group in regard to category A quality (excellent) blastocysts, with 15.44% and 10.46% category A blastocysts, respectively. It was concluded that the study showed a possible benefit resulting from undisturbed extended blastocyst culture.

# CAN A SECOND DOSE OF KISSPEPTIN SAFELY IMPROVE OOCYTE MATURATION IN WOMEN AT HIGH RISK OF OVARIAN HYPERSTIMULATION SYNDROME?

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Keywords: Kisspeptin, ovarian hyperstimulation syndrome (OHSS), oocyte maturation trigger.

Kisspeptin is a novel neuropeptide that came to prominence in the field of fertility in 2003, when it was discovered that genetic mutations causing decreased kisspeptin signalling resulted in hypogonadotropic hypogonadism. Since then, several studies have established that kisspeptin is a key regulator of the hypothalamo-pituitary-gonadal axis, stimulating gonadotropin-releasing hormone (GnRH) secretion in the hypothalamus. Recent trials have demonstrated that kisspeptin can safely trigger oocyte maturation even in women at high risk of ovarian hyperstimulation syndrome (OHSS).[1] In 2017, a randomised controlled trial was presented at the European Society of Human Reproduction and Embryology (ESHRE) congress to investigate whether administering a second dose of kisspeptin can safely optimise oocyte maturation in women at high risk of OHSS (serum anti-Müllerian hormone [AMH]: ≥40 pmol/L or antral follicle count: ≥23).

In this trial, oocyte maturation was assessed using a patient-centric primary outcome. Whilst mean oocyte maturation provides a reasonable estimate of the efficacy of a trigger across a group, from an individual patient's perspective, it is more relevant to be advised of the likelihood of personally achieving a clinically effective oocyte yield that could increase the chance of progression to subsequent stages of *in vitro* fertilisation treatment. Thus, the primary outcome was the proportion of patients with retrieval of ≥60% of the oocytes that one would expect to be retrieved based on sonographic assessment on the day of trigger.

The results of this study showed that a second dose of kisspeptin significantly improved the proportion of patients achieving an oocyte yield ≥60%, increasing from 45% with one dose of kisspeptin to 71% with two doses of kisspeptin (p=0.042). Recently, there has been increasing focus to develop stimulation protocols that avoid ovarian under-response, but without induction of ovarian over-response. A second dose of kisspeptin eliminated the retrieval of <4 oocytes, but importantly this did not come at the expense of increasing the proportion of patients having ovarian over-response.

This finding may be due to a unique property of kisspeptin pharmacodynamics, which led to a variable rise in luteinising hormone (LH) following the second dose of kisspeptin. Those who had a lesser LH response following the first dose of kisspeptin had a greater subsequent rise following the second dose. Conversely, patients who already had a robust LH response following the first dose of kisspeptin had minimal further LH secretion

following the second dose. Thus, a second dose of kisspeptin triggered an 'individualised' response, providing further LH exposure only to those patients requiring it. Thus, a second dose of kisspeptin could optimise the chance of an intermediate ovarian response whilst minimising the chance of ovarian under or over-response. The population studied was at high risk of OHSS (mean AMH: 52 pmol/L; mean antral follicle count: 40) and had a median of 24 follicles ≥11 mm on the day of trigger. Thus, almost half of patients would not usually be offered a fresh embryo transfer due to high risk of OHSS with current triggers. Importantly, 98% of patients in this trial had a fresh embryo transfer, all of whom had no evidence of moderate-to-severe OHSS and a second dose of kisspeptin resulted in a live birth rate per protocol of 39%.

In summary, a second dose of kisspeptin was able to optimise oocyte maturation and safely enable fresh embryo transfer without increasing rates of OHSS, even in a high-risk population.

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# REPRODUCTIVE OUTCOMES OF TESTICULAR VERSUS EJACULATED SPERM FOR INTRACYTOPLASMIC SPERM INJECTION AMONG MEN WITH HIGH LEVELS OF DNA FRAGMENTATION IN SEMEN: SYSTEMATIC REVIEW AND META-ANALYSIS

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Keywords: Semen, male infertility, intracytoplasmic sperm injection (ICSI), sperm DNA fragmentation (SDF), testicular sperm.

Intracytoplasmic sperm injection (ICSI) has been widely used to overcome all forms of male factor infertility. Despite its overall acceptable success rates with the use of abnormal sperm, studies suggest that low sperm quality may adversely impact ICSI outcomes, likely due to altered sperm DNA content associated with impaired sperm characteristics.[1-3] The negative impact of damaged paternal chromatin is usually manifested by impaired embryo development and early pregnancy loss, thus decreasing assisted reproductive therapy success.[4]

Sperm DNA fragmentation (SDF) assays measure the proportion of sperm with damaged chromatin in the neat ejaculate.[4] Among couples undergoing ICSI, high SDF in the neat semen is found in ˜30% of men.[5] In recent studies, we found that the use of testicular sperm in preference over ejaculated sperm may offer better ICSI outcomes for men with high SDF in semen.[6] The biological plausibility seems to be related to increased SDF in ejaculated, compared to testicular sperm, most probably due to elevated levels of reactive oxygen species in the epididymis that can cause post-testicular harm.[7]

At the European Society of Human Reproduction and Embryology (ESHRE) congress 2017, hosted in Geneva, Switzerland, these findings were presented concerning a PRISMA systematic review and meta-analysis of ICSI outcomes for testicular (Testi-ICSI) and ejaculated (Ejac-ICSI) sperm among non-azoospermic infertile men with confirmed

post-testicular SDF. Our electronic search into major databases up to December 2016 identified seven studies, encompassing 507 ICSI cycles and 3,840 injected oocytes. Five studies provided paired data on SDF between ejaculated and testicular sperm, involving 143 patients who served as their own control. High SDF was defined according to the method and thresholds used in each study, most often terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) and 30%, respectively.

We found that SDF rates were lower in testicular rather than ejaculated sperm, with the mean difference by random-effect model (REM) being -24.58% (95% confidence interval [CI]: -32.53, -16.64; $I^2$=92%; p<0.001). The pooled odds ratio (OR) for fertilisation rates using REM was 0.81 (95% CI: 0.58–1.15; $I^2$=81%; p=0.24), with a trend to lower fertilisation rates in the Testi-ICSI group. Clinical pregnancy rates per fresh embryo transfer were higher with Testi-ICSI than Ejac-ICSI, with an OR using the fixed effects model (FEM) of 2.42 (95% CI: 1.57–3.73; $I^2$=34%; p<0.001). As for miscarriage rates, we found that results favoured Testi-ICSI as compared to Ejac-ICSI, with an OR by FEM of 0.28 (95% CI: 0.11–0.68; p=0.005; $I^2$=11%). Lastly, live birth rates per fresh embryo transfer were higher with Testi-ICSI as compared with Ejac-ICSI, with an OR by FEM of 2.58 (95% CI: 1.54–4.35; $I^2$=0%; p<0.001).

To our knowledge, this is the first systematic review and meta-analysis summarising the evidence currently available concerning Testi-ICSI in men with high SDF in neat semen. Our findings offer novel insights on SDF-related infertility and offer a possible therapeutic approach with the use of Testi-ICSI. Given the overall moderate quality of studies included in our meta-analysis, and the risks associated with sperm retrieval, the use of testicular over ejaculated sperm should be considered only in ICSI candidates with confirmed post-testicular SDF. In particular, couples with previous failed treatment cycles, where efforts to reduce SDF in the ejaculate have also failed, then injected sperm should be used.

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# MITOCHONDRIA TRANSFER: CAN IT IMPROVE OOCYTE QUALITY?


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Keywords: Cytoplasmic transfer, ooplasmic transplantation, mitochondrial DNA replication, repeated implantation failure, non-coding heteroplasmy, inbred mouse, nuclear transplantation, pronuclear transfer.


The exact causes of embryonic failure during assisted reproduction remain elusive. Aneuploidy can emerge during meiosis and early mitosis and

has been linked to reproductive senescence and failure of normal development. Cytoplasmic insufficiency due to mitochondrial replication errors and metabolic energy dysfunction has been suggested as another cause of reproductive failure. Pratt and Muggleton-Harris[1] were first to attempt to reverse cytoplasmic failure in a mouse two-cell block model nearly 30 years ago. They were able to overcome the two-cell block by injecting blocked blastomeres in embryos derived from an inbred strain, with cytoplasm extracted from non-blocked blastomeres of an F1-hybrid mouse. The success of these experiments led us to investigate the possibility of ooplasmic transplantation (or cytoplasmic transfer) in the mouse and human in order to investigate the impact of the procedure on embryos from patients with repeated implantation failure.[2] Variations on this basic theme have been introduced, investigated, and debated over the ensuing years. These pertain to intervention purpose (for disease prevention or infertility treatment), mode of cytoplasm delivery (nuclear transplantation, mitochondria, or cytoplasm injection), level of precision ('purified' mitochondria or whole cytoplasm usage), inter-cell stage synchrony (synchronous or asynchronous), and cytoplasm or mitochondria source (autologous or heterologous). Babies have now been born using all of these pathways. The focus has been mainly on three approaches:

a) Ooplasmic transplantation from donor egg to recipient:

The first children born following clinical application of this experimental approach are now 15–20 years old. A recent survey-based follow-up did not show any major concerns regarding the health and cognitive ability of the children.[3] The only exceptional finding was a low rate of disclosure of the intervention to offspring. Earlier concerns regarding non-coding (D-loop region) heteroplasmy and the potential developmental side effects of this condition in inbred mouse models appear not to be relevant in humans, based on this follow-up study.[4]

b) The use of egg precursor cells (EggPCSM, AUGMENT℠, OvaScience) to source mitochondria extracted from autologous ovarian tissue:

Pregnancies and births have been reported from this procedure.[5] Some findings, such as the resemblance of mitochondrial morphology in putative egg precursor cells (from the ovarian surface) with mitochondria in mature oocytes and a higher development rate to blastocysts following egg augmentation with these mitochondria[6] are intriguing. Both cytoplasmic transfer and AUGMENT data show that intervention is successful even when there has been a high number of previously failed *in vitro* fertilisation (IVF) cycles.

c) Pronuclear transfer in cases of repeated implantation failure:

Two babies were born earlier this year following treatment of 16 women, who had experienced previous failed IVF cycles, at the Nadiya clinic in Kiev, Ukraine[7] using pronuclear transplantation between donor and patient zygotes; another pregnancy is also ongoing. Zhang et al.[8] reported a twin pregnancy using this approach in 2003, but tragically, the babies died during a complicated delivery. The details of this case were reported only recently. Zhang et al.[9] were also the first to report the birth of a healthy baby boy to a carrier of a mitochondrial mutation that causes Leigh syndrome, a serious inheritable mitochondrial DNA disease. Women >40 years of age have not been treated using the cytoplasmic transfer approach, but these older women have been treated using the other two modalities. However, treatment has been unsuccessful in this age group. Mitochondrial and cytoplasmic transfers are subject to many ethical and legal limitations and data to support them remain sparse. Although a comparison of pregnancy rates in the younger groups with and without treatment suggests an increase in pregnancy rate with treatment, direct evidence of the efficacy and safety of mitochondrial or cytoplasmic augmentation is still lacking. Carefully conducted trials should therefore be seriously contemplated.

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# ANTIOXIDANT GENE EXPRESSION OF PEROXIREDOXIN DECREASES IN GRANULOSE CELLS FROM OOCYTES OF YOUNG WOMEN WITH LOW OVARIAN RESERVE

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Keywords: Oxidative stress, low ovarian reserve, peroxiredoxin (PRDX), granulose cells (GC).

## INTRODUCTION

Recent studies carried out by our team have shown that an increase in oxidative stress may have a negative impact on ovarian response.[1] Peroxiredoxins (PRDX), a family of peroxidases, are associated with various biological processes such as the detoxification of oxidants and cell apoptosis.[2] The latest studies have suggested that *PRDX2*, *PRDX3*, and *PRDX4* have the ability to protect against oxidative stress and apoptosis.[3] *PRDX1* may play an important role in the regulation of cell signalling pathways induced by reactive oxygen species, whereas *PRDX5* would act as a scavenger.[4] Nevertheless, little attention has been given to the role of *PRDX* in female infertility.

## OBJECTIVE

The aim of this work was to investigate gene expression of *PRDX* (1–6) in granulose cells (GC) and cumulus cells (CC) from the peri-ovulatory follicles of young women who experienced a low response in controlled ovarian stimulation cycles (COH), when compared with fertile donors from the same age cohort.

## MATERIALS AND METHODS

This prospective study compared the mRNA expression of *PRDX* (1–6) and caspase 3 in 56 oocyte-cumulus and 62 oocyte-granulose complexes retrieved from six healthy, fertile oocyte donors and five patients (≤5 oocytes retrieved) after gonadotropic stimulation from July–December 2016. All study participants were <35 years of age and stimulated with the same protocol (follicle-stimulating hormone receptor and triggering with gonadotropin-releasing hormone analogues). mRNA was extracted using the TaqMan® Gene Expression Cells-to-CT Kit (AM1729, Applied Biosystems, California, USA) and mRNA expression of *PRDX* genes and endogenous controls were measured by quantitative reverse transcription polymerase chain reaction (qRT-PCR) using TaqMan probes. No parametric tests were used to identify any significant differences between patients and healthy donors. Statistical significance was set at $p<0.05$.

## RESULTS

Following this study, we found that *PRDX1*, *PRDX2*, *PRDX3*, *PRDX4*, *PRDX5*, and *PRDX6* are expressed in GC and CC. The results obtained from comparative RT-PCR analysis revealed that the mean relative levels of mRNA coding for *PRDX2*, *PRDX3*, *PRDX4*, and *PRDX6* were significantly decreased in GC from women with low response in COH compared with healthy oocyte donors (*PRDX2* p=0.03; *PRDX3* p=0.022; *PRDX4* p=0.014; *PRDX6* p=0.014). mRNA expression of both *PRX1* and *PRX5* was much stronger in GC from the patient group compared with the expression observed in GC from the donor group (*PRDX1* p=0.05; *PRDX5* p=0.02). Also, an increase of caspase-3 expression in GC (p<0.001) was observed in the patient group, compared to the donor group. No significant differences were found in the levels of mRNA coding for *PRDX* (1–6) and caspase-3 in CC from young women with low response compared with oocyte donors.

## CONCLUSIONS

From our study, we can conclude that *PRDX* are differentially expressed in GC and CC. Our results suggest a lower antioxidant capacity and increased apoptosis level in GC of women with low response to COH compared with fertile donors, as well as a role for PRDX in human GC function and in the pathogenesis of low ovarian reserve in women undergoing infertility treatment with COH-*in vitro* fertilisation (IVF). These results suggest that antioxidant capabilities are diminished during low ovarian response, leading to an increase in oxidative damage in the ovary in a similar way to age-related oxidative damage. These findings could lead to the development of new therapeutic strategies for the treatment of low ovarian reserve.

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# WILL IT BE POSSIBLE (ONCE AND FOR ALL) TO ESTABLISH THE SINGLE EMBRYO TRANSFER (SET) AS THE GOLD (AND UNIQUE) STANDARD IN *IN VITRO* FERTILISATION?

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Keywords: Multiple pregnancy (MP), elective single embryo transfer (esET), preimplantation genetic screening (PGS).

The complications of multiple pregnancies (MP) should be well known by assisted reproduction specialists. Despite the general agreement that MP represent a serious and avoidable complication of assisted reproduction, in most procedures two, or even three, embryos are still transferred. Regrettably, the lack of legal limitation constitutes a 'pathway' to continue with this inappropriate and hazardous policy.

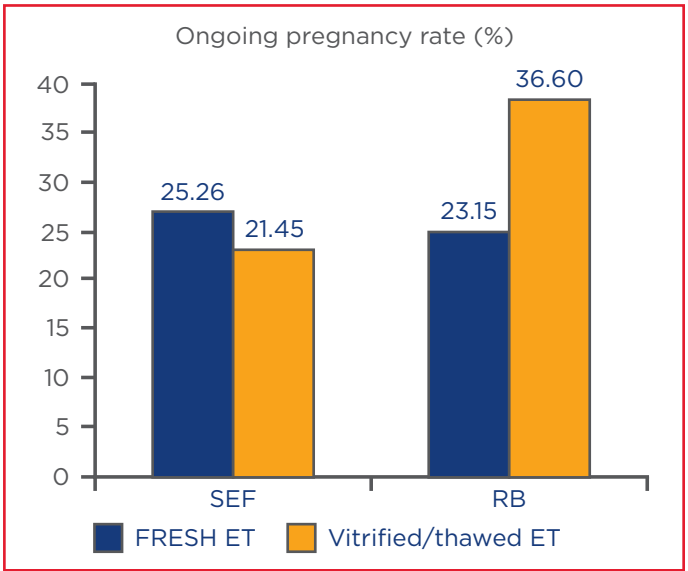


**Figure 1: Ongoing pregnancy rates per embryo transfer (Spanish Fertility Society and Reproducción Bilbao).**
ET: embryo transfer; RB: Reproducción Bilbao; SEF: Spanish Fertility Society.

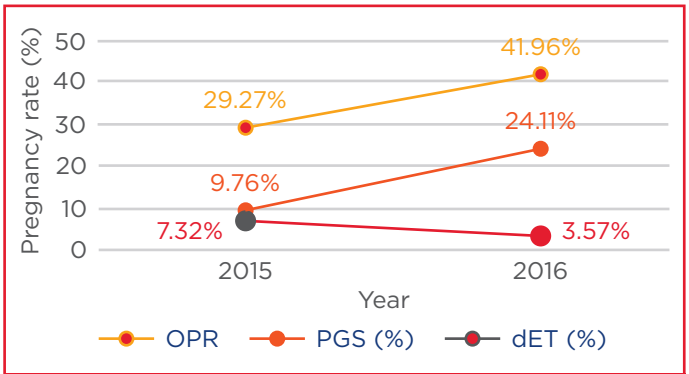


**Figure 2: Pregnancy rates (Reproducción Bilbao). Percentage of preimplantation genetic screening and double embryo transfer.**
dET: double embryo transfer; OPR: ongoing pregnancy rates; PGS: preimplantation genetic screening.

## MATERIALS AND METHODS

A retrospective cohort analysis, with a study group of *in vitro* fertilisation (IVF) cycles performed between June 2015 and May 2016, at a university-associated assisted reproductive technology centre, was assessed. The results were compared to those published by the Spanish Fertility Society (SEF),[1] corresponding to the activity of Spanish centres in 2014.

A total of 203 fresh and 194 vitrified-embryo transfers (ET) were compared to the 34,342 fresh and 19,549 thawed ET reported by SEF. Cycles in which donor eggs were used were not included. Ongoing pregnancy rates per transfer and differences in MP rates were compared. Pearson's Chi-squared test was used to analyse the differences.

## RESULTS

A single ET (sET) was performed in 184 out of 203 fresh ET (90.64%) and in 184 out of 194 frozen-thawed ET (94.85%). No triple embryo transfer was undertaken. Among the 51,591 ET performed in the control group, in 25.43%, 68.20%, and 6.37% of cases (among fresh ET) and in 37.64%, 57.47%, and 4.9% of cases (among thawed ET) one, two, and three embryos were transferred, respectively (p=0.000). The transfer of more than one embryo was not associated with a higher chance of pregnancy (Figure 1). Furthermore, the MP rate in our study group was 3.69%, whereas 18.30% and 0.19% of the pregnancies achieved in the control group were twins and triplets, respectively (p=0.000). The delivery of the double pregnancies of the control group was premature in 50.47% of twin and in 100% of triplet pregnancies (9.81% and 58.52% <32 weeks of amenorrhea).

Even though a sET might be associated with a lower chance of success per transfer, the use of strict selection (including morphokinetic and genetic) criteria overcome such a challenge. There was a linear relationship between pregnancy rates and the use of preimplantation genetic screening for embryo selection in our series, despite the rate of multiple embryo transfer being lower (Figure 2).

## CONCLUSION

MP is not an anecdotal issue but a side effect with potentially deleterious consequences. The ongoing pregnancy rate does not depend on the number of embryos transferred but on the selection of the most adequate embryo to be transferred.

The implementation of a strict sET policy does not limit IVF success and MP rates are reduced dramatically. As better selection criteria are used, pregnancy rates increase.


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# EVIDENCE BASE FOR INVESTIGATION AND MANAGEMENT FOR UNEXPLAINED RECURRENT IMPLANTATION FAILURE: A SYSTEMATIC REVIEW AND META-ANALYSIS


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Keywords: Recurrent implantation failure (RIF), *in vitro* fertilisation (IVF), immunological testing, antiphospholipid antibodies, thrombophilia, empirical treatment, endometrial injury, intravenous immunoglobulins, low molecular weight heparin, peripheral blood mononuclear cells.


Embryo implantation following transfer is considered a critical success-determining step of *in vitro* fertilisation (IVF); however, the probability that an embryo will successfully implant into the uterus after transfer is surprisingly low, standing at approximately 30%.[1] Implantation failure is devastating for the couple; it can have significant financial implications as well as the associated physical and psychological burden. In cases of recurrent implantation failure (RIF), these consequences are amplified. RIF arises after embryo transfer when implantation repeatedly fails to reach a stage identifiable by ultrasonography.[2]

At present, the management of RIF remains ambiguous among clinicians and investigations and treatments are often prescribed depending on the clinician. In response to this, we conducted a systematic review and meta-analysis aiming firstly to compare the prevalence of positive immunological test outcomes between women with a history of RIF and both subfertile and fertile women with no history of the condition; and secondly to evaluate the efficacy of a number of empirical treatments to improve IVF outcome in women experiencing unexplained RIF. We searched two scientific databases, MEDLINE and the Cochrane Library, from commencement to June 2017. To be eligible for inclusion, our first objective studies had to be observational in design, comparing the prevalence of positive immunological test outcomes between women with a history of RIF and fertile and subfertile women with no history of the condition. For the second objective, the studies had to be either observational or randomised controlled trials comparing IVF outcome between women with a history of unexplained RIF who were administered an empirical treatment prior to the cycle, and women with RIF who were administered a placebo or no treatment prior to the cycle.

Our search yielded a total of 10,034 citations and, after careful scrutiny, we identified 30 relevant citations for the first objective, and 17 relevant citations for the second. The quality of observational studies was assessed using the

Newcastle–Ottawa scale, whereas the quality of randomised controlled trials was evaluated using The Cochrane Risk of Bias Tool.

The prevalence of positive antiphospholipid antibody test outcomes was significantly higher in women with RIF than in controls (odds ratio [OR]: 3.35; 95% confidence interval [CI]: 2.05–6.05; p<0.001), more specifically lupus anticoagulant (OR: 5.03; 95% CI: 1.81–13.99; p=0.002). Similar results were seen for inherited thrombophilias, specifically Factor V Leiden (OR: 2.69; 95% CI: 1.28–5.63; p=0.009). While meta-analyses for the prevalence of high natural killer cells and Type 1 T helper:Type 2 T helper cell ratio were not possible, no difference was identified in the prevalence of anti-thyroid antibodies. The prevalence of certain human leukocyte antigen-G variants was found to be significantly higher in women with RIF; -14 base pair (bp) (bp/-14 bp and -14 bp/+14 bp genotype) as well as the 010101, 0106, 010106, and 0105 N alleles. Endometrial injury in the previous menstrual cycle was shown to benefit clinical pregnancy rate in women with RIF (OR: 3.38; 95% CI: 2.26–5.06; p<0.001). Similar results were seen for peripheral blood mononuclear cell administration to the uterus (OR: 3.68; 95% CI: 2.01–6.64; p<0.001).

Additionally, we showed a beneficial effect of intravenous immunoglobulin therapy on live birth rate in this cohort (OR: 10.51; 95% CI: 1.52–76.66; p=0.02). Whilst our results support testing for certain congenital and inherited thrombophilias in women with RIF, evidence to suggest a role of other immunological factors in the pathogenesis of this condition is limited. Furthermore, our results suggest that endometrial injury, intravenous immunoglobulin therapy, and peripheral blood mononuclear cell administration prior to embryo transfer show promise for the treatment of unexplained RIF in the future; however, this is not before optimisation studies are performed to establish appropriate clinical guidelines for their prescription.

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# LIFESTYLE FACTORS ASSOCIATED WITH INCREASED SPERM DNA FRAGMENTATION AND THEIR IMPACT ON INTRACYTOPLASMIC SPERM INJECTION

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Keywords: Sperm DNA fragmentation (SDF), BMI, intracytoplasmic sperm injection (ICSI) outcome, age, smoking.

Sperm DNA fragmentation (SDF) reduces fertilisation rate, embryo quality, and pregnancy rate,[1] and ~30–50% of infertility cases are due to sperm defects.[2] However, 20% of sperm abnormalities like SDF cannot be detected by light microscopy. Exposure to environmental pollutants, drugs, radiation, smoking, febrile illness, varicocele, advanced age, and obesity can increase SDF.[3] Intracytoplasmic sperm injection (ICSI) allows injection of sperm directly into the egg to induce fertilisation and embryo development, thus enabling a man with an extremely low sperm count to have a biological child. The purpose of this study was to investigate lifestyle factors associated with increased SDF and their impact on ICSI outcome.

The SDF data from 94 men undergoing ICSI from January 2015–June 2016 were analysed. The study

parameters were male age, BMI, smoking, standard semen parameters, and SDF. The semen samples were grouped into three categories based on percentage levels of SDF (Figure 1): <15%, 15–30%, and >30%. The SDF was determined by the Sperm Chromatin Dispersion Assay, using the Halosperm G2® kit (Halotech®, Madrid, Spain).

The results of this study are shown in Table 1. Male BMI and smoking were positively correlated with SDF. Age was also positively correlated with the 15–29% and >30% SDF categories. However, sperm concentration and progressive motility were negatively correlated with SDF, as well as the sperm morphology, which was negatively correlated with the <15% and 15–30% SDF categories. There was no difference in pregnancy rates after ICSI in low and moderate SDF categories; however, in the high SDF category, no men achieved pregnancy (data not shown).

The SDF is one of the male fertility predictors.[1] It is therefore vital for healthcare professionals to inform infertile male partners about the lifestyle factors that can result in high SDF and those that can have a negative impact on ICSI outcome.

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**Table 1: The correlations of SDF with lifestyle factors and semen parameters.**

| Parameter | <15% SDF | 15–30% SDF | >30% SDF |
|---|---|---|---|
| BMI | 0.210 | 0.420 | 0.561 |
| Smoking | 0.290 | 0.035 | 0.340 |
| Age | -0.273 | 0.603 | 0.548 |
| Sperm concentration | -0.020 | -0.133 | -0.009 |
| Progressive motility | -0.360 | -0.334 | -0.334 |
| Sperm morphology | -0.096 | -0.018 | 0.198 |

SDF: sperm DNA fragmentation.

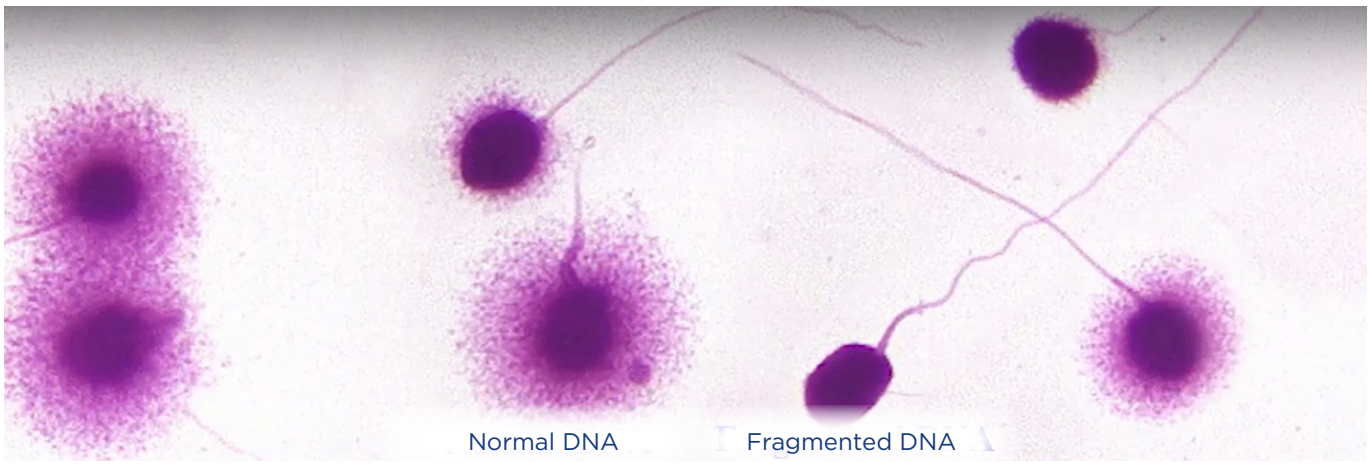


**Figure 1: A sperm chromatin dispersion analysis showing sperm with unfragmented DNA with a 'halo' and sperm with fragmented DNA without a 'halo'.**
*Source: Halotech®, Madrid, Spain.*

# METHYLENETRAHYDROFOLATE REDUCTASE (MTHFR) C677T ISOFORM EXPLAINS SOME HEAVY INFERTILITY. BACKGROUND AND SOLUTIONS: A PRELIMINARY REPORT

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Keywords: *MTHFR* isoform, sperm, methylation, one carbon cycle (1-CC), 5-methyltetrahydrofolate (5-MTHF).

In this first approach study, couples with a heavy infertility background, between 3 and 9 miscarriages and/or 3–5 failures of assisted reproductive technologies (ART), were tested for the presence of the *MTHFR* C677T isoform. *MTHFR* isoforms lead to a weakened methylation ability and a higher sensitivity to oxidative stress, leading to a decreased sperm quality.[1,2]

In male partners studied, no effect on sperm DNA fragmentation was observed, either for the heterozygous or homozygous patients. However, the isoform was responsible for nucleus decondensation (sperm decondensation index [SDI]) when compared to the control group (n=1,400). SDI was more important for the homozygous patients (HMZ, n=18) than for the heterozygous patients (HTZ, n=70; p<0.05) (Figure 1).

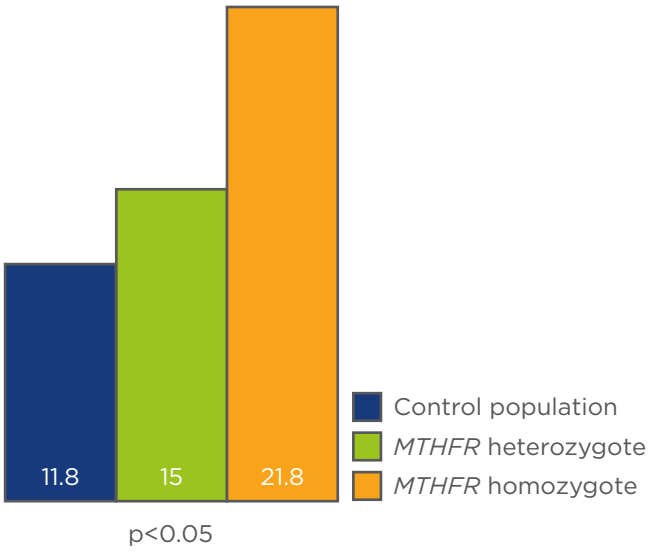


**Figure 1: *MTHFR* C677T isoform and sperm nucleus decondensation.**
The numbers represent mean decondensation indexes according to the genetic status.

Nucleus decondensation is a faulty compaction of the nucleus. It is known to induce early embryo developmental arrest,[3] as a result of the oocyte being poorly equipped to deal with nucleus tertiary structure anomalies.[4]

Of the 49 couples studied, 7 (14%) were wild type (WT) X WT and did not participate in the clinical study. Thirteen (26.5%) were HTZ X HTZ, and 12 (24.5%) were HTZ X HMZ. Two couples (4%) were HMZ X HMZ. Patients carrying this isoform have a weak capacity to metabolise folic acid, which impairs the one carbon cycle (1-CC, Figure 2), due to the active compound normally synthesised (5-methyltetrahydrofolate [5-MTHF]) being poorly formed. This leads to a strong decrease in early embryo quality.[5] This study proposed that supplementation with 5-MTHF, associated with vitamins B3, 6, and 12, and zinc (cofactors of the 1-CC), and N-acetylcysteine, for 4 months for *MTHFR* carriers, male or female, before starting another ART attempt.

A total of 31 patients fulfilled all of the 1-CC protocol requirements. Seventeen patients started a pregnancy, including one from a HMZ X HMZ couple, and eight originating from HMZ X HTZ couples. Of all the starting pregnancies, 11 were

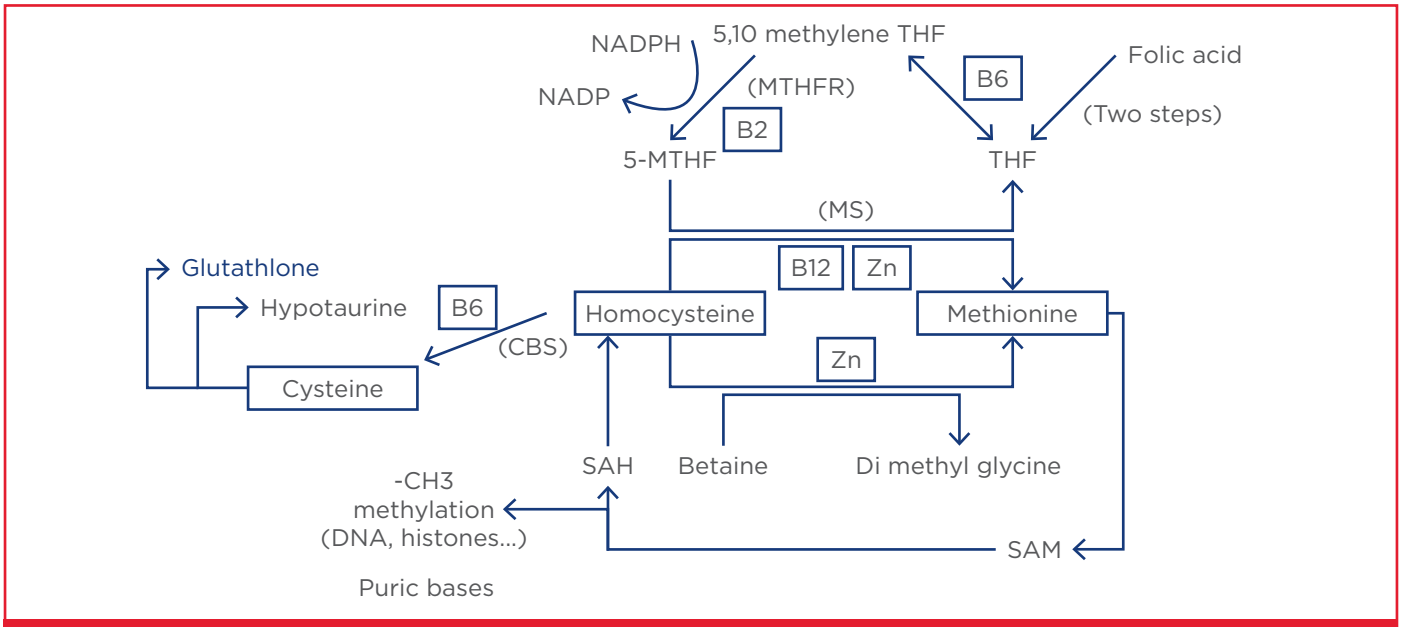


**Figure 2: The one carbon cycle (1-CC).**
THF: tetrahydrofolate; 5-MTHF: 5-methyltetrahydrofolate; NADP: nicotinamide adenine dinucleotide phosphate; NADPH: NADP reduced form; SAM: S-adenosylmethionine; SAH: S-adenosylhomocysteine.

spontaneous. However, four miscarriages were observed (7–9 weeks) from these 17 pregnancies.

In conclusion, the management of hypo-fertile patients must include a correct evaluation of the *MTHFR* background of both partners, not just the female partner as in current practice; especially if we consider the possibility that other composite homozygous mutations, the A1298C variation especially, have not been tested here. Nutritional supplementation with 5-MTHF, the metabolite downstream of MTHFR, associated with a support of the 1-CC, is a useful strategy for the isoform carriers. This also suggests that oocyte donation is not an inevitable path for patients with repeated ART failures. This clinical aspect is clinically relevant as current environmental pollution, especially related to the endocrine disruptor chemicals, increases the oxidative stress and exerts a negative pressure on methylation, imprinting, and epigenetics.[1]

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EDITOR'S PICK

In women of reproductive age, polycystic ovary syndrome (PCOS) is one of the most common abnormalities, and obesity is observed in about 80% of these patients. The relationship between PCOS and obesity is complex, and therefore the study "Selection of Appropriate Tools for Evaluating Obesity in Polycystic Ovary Syndrome Patients" is very welcome. The author concludes that using BMI to diagnose and classify obesity, a high fat content, or fat distribution of android type in PCOS patients with normal weight can be overlooked.

Prof Joep Geraedts

# SELECTION OF APPROPRIATE TOOLS FOR EVALUATING OBESITY IN POLYCYSTIC OVARY SYNDROME PATIENTS

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## ABSTRACT

Patients with polycystic ovary syndrome (PCOS) have unique endocrine and metabolic characteristics, whereby the incidence and potentiality of obesity, as well as the accompanying risk of metabolic and cardiovascular diseases, are significantly increased. Currently, BMI is widely used to diagnose and classify obesity. However, body fat is not accounted for in BMI calculations, and the missed diagnosis rate of obesity is nearly 50%. Since PCOS patients with normal weight are also characterised by a high content of fat or fat distribution of android type, some of these patients are often overlooked if an inappropriate diagnostic tool for obesity is selected, which affects the therapeutic effect. Herein, we have reviewed the mechanism and diagnostic methods of PCOS-related obesity and suggested that not only body weight and circumference alone, but also the body fat percentage and fat distribution, should be considered for the evaluation of obesity in PCOS patients.

Keywords: Polycystic ovary syndrome (PCOS), obesity, normal weight obesity, body fat percentage, body composition.

## INTRODUCTION

Polycystic ovary syndrome (PCOS) is a common endocrine disease in women of childbearing age. It is characterised by chronic anovulation and hyperandrogenism, and commonly manifests as irregular menstruation, infertility, hirsutism, and obesity. The pathophysiological changes of PCOS involve abnormalities in the neuroendocrine system, glucose metabolism, lipid metabolism, protein metabolism, and local ovarian regulatory factors. It affects a woman's reproductive function and causes long-term complications, such as Type 2 diabetes, cardiovascular disease (CVD), and endometrial cancer, which pose a serious threat to a woman's health.

Due to the heterogeneity of clinical manifestations of PCOS, its definition and diagnosis remain controversial. The clinical practice guidelines issued by the Endocrine Expert Working Group in the USA and Europe in 2013[1] recommended the use of criteria published in 2003 by the European

Society of Human Reproduction and Embryology and American Society for Reproductive Medicine (ESHRE/ASRM):[2] a patient can be diagnosed with PCOS if two of the following three criteria are met: oligoovulation or anovulation; clinical and/or biochemical signs of hyperandrogenism; polycystic ovaries and exclusion of other aetiologies, such as congenital adrenal hyperplasia, androgen-secreting tumours, and Cushing's syndrome.

Obesity is a common clinical manifestation of PCOS, with an incidence of 50–80%,[3] which differs with respect to race and geographical factors. Obesity refers to an increase in body weight due to an increase in the volume of body fat and/or an increase in the number of adipocytes, or an abnormal increase in the percentage of body fat (PBF). Additionally, in obesity, body fat is excessively accumulated in certain locations.[4] In the diagnosis and treatment guidelines for PCOS, weight loss and lifestyle changes are considered as the first-line treatment option for overweight or obese patients, but it is believed that PCOS patients with normal weight cannot benefit from weight loss.[1]

Obesity further increases the secretion of androgens, affecting the metabolic and reproductive functions. In PCOS, the levels of low-density lipoprotein, triglycerides, and cholesterol are increased, while the level of high-density lipoprotein is decreased, with an increased risk of arteriosclerosis and changes in vascular endothelial function.[5] In addition, PCOS also increases the risk of CVD as well as the related complications.[6] Obese PCOS patients have a higher incidence of menstrual disorders and long-term anovulation than patients with normal weight and, thus, show a poorer effect after receiving ovulation stimulation.

## MECHANISM OF POLYCYSTIC OVARY SYNDROME OBESITY

The pathogenesis of obesity is influenced by genetic and environmental factors, such as diet and lifestyle. Adipose tissue is not only an 'energy warehouse', but also an important endocrine organ. It can secrete numerous cytokines and hormones, and plays an important role in regulating body metabolism, inflammation, and immune response. Normal content and distribution of fat are essential for maintaining body functions and fertility potential in females. Gynoid fat distribution begins in adolescence, where an adequate fat content is conducive to triggering and maintaining a regular and ovulatory menstrual cycle. A significant decrease in adipose tissue in adulthood will lead to secondary amenorrhoea. Peripheral adipose tissue is an important site for the synthesis of oestrogen outside the ovary, where the androgen transforms into oestrogen through the aromatisation effect.[7]

There could be several mechanisms of obesity in PCOS. PCOS patients have a significant increase in intake of food rich in carbohydrates, high in glycaemic index, and high in saturated fat. They also have an excessively high intake of total energy and sedentary lifestyles,[8] which is particularly common in individuals with genetic and environmental susceptibility.[9] As compared to age and body weight matched controls, the basal metabolic rate[10] and postprandial calorigenesis[11] were found to be lower in PCOS patients. Due to the abnormal endocrine levels, PCOS patients have a retarded perception of satiety, which manifests as gluttony and food craving.[12] Mouse models have revealed that excessive androgen increases appetite and the frequency of eating,[11] as well as promoting the accumulation of abdominal fat.[13] At the onset of adolescence, obesity is associated with excessive androgen.[14] Thus, it is possible to control the progression of PCOS and even prevent it in adulthood by controlling the excessive androgen prior to adolescence. Veilleux et al.[15] showed us that adipocyte hyperplasia (the proliferation and differentiation of preadipocytes) is predominant in the subcutaneous adipose tissue, whereas adipocyte hypertrophy (the enlargement of existing adipocytes) is present both in subcutaneous and omental adipose tissue depots. The key point is that the hypertrophic adipocytes are associated with dyslipidaemia and insulin resistance.[15] Thus, obesity in PCOS not only exacerbates the existing characteristics but also affects the therapeutic results.[16]

## COMMON DIAGNOSTIC METHODS FOR OBESITY

- BMI: In 1842, the Belgian mathematician Quetelet[17] found that body weight was proportional to the square of height. Currently, BMI is often used for the diagnosis and classification of obesity, as well as for the prediction and assessment of disease risk in epidemiological studies due to its ease of use, safety, and low cost. However, BMI does not consider the body fat, which is very important, and is unable to distinguish fat, lean body weight, or bone. If BMI alone is used

to diagnose obesity, individuals with an increase in muscle mass will be mistakenly included as obese, while individuals with a high content of fat and low lean body weight will be considered to have normal BMI. In addition, with the same BMI, PBF may be different due to differences in sex, age, and race.[18]

- Waist circumference (WC), waist-to-hip ratio, and waist-to-height ratio: The waist-to-hip ratio can be used to indirectly measure the body fat distribution. The WC, alone or in combination with BMI, has a stronger correlation with an increased health risk than BMI alone.[19] The WC reflects the abdominal or visceral fat, and has no correspondence with BMI. Hip circumference reflects different body compositions at the gluteofemoral area, such as muscle, bone, and fat. A recent study indicated that women with central obesity with BMI <25 kg/m$^2$ are 2.03-times (95% confidence interval of adjusted odds ratio: 1.62–2.54) more likely to have at least one CVD factor when compared with normal weight women without central obesity.[20]
- Body composition analysis: It measures the body fat, fat-free body weight, overall body water, and basal metabolic rate, and calculates PBF. The main methods for measuring body fat include bioelectrical impedance, hydrostatics plethysmography, isotope dilution technique, dual-energy X-ray absorptiometry, and skin-fold thickness measurement.[21] A high PBF is associated with insulin resistance even in patients with normal body weight.[22] Thus, PBF is a good predictor of obesity,[23] and is increasingly used for its value in the diagnosis of obesity.[24]

Based on the different racial characteristics, the World Health Organization (WHO)[25] and the International Diabetes Federation (IDF)[26] have determined the cut-off values of BMI and WC for the diagnosis of obesity. For adult women in China, a BMI of 24 to <28 kg/m$^2$ is defined as overweight, and BMI ≥28 kg/m$^2$ is defined as obese;[4] a WC of ≥80 cm is defined as central obesity. Studies collected data from 13,601 adults to detect the accuracy of BMI in the diagnosis of obesity and found that if patients with a BMI >30 kg/m$^2$ were diagnosed as obese, bioelectrical impedance was used to calculate the body fat. BMI calculations showed a specificity of up to 97% but had a sensitivity of only 42% in detecting obesity, whereby 50% of the obese population diagnosed by PBF were missed by BMI.[27,28] Therefore, in the diagnosis and classification of obesity, it is necessary to refer to the content and distribution of body fat, rather than body weight and BMI alone. Nevertheless, there is a lack of consensus on the cut-off value of PBF for the diagnosis of obesity. Currently, it is recommended that the cut-off value of PBF for the diagnosis of obesity in women is 30–35%.[29] In addition, our previous study found that, at a cut-off point of 29%, PBF has a sensitivity of 88.2% and a specificity of 57.7% in diagnosis of PCOS, suggesting that PBF can be used to screen PCOS due to its increased sensitivity.[30]

Combining BMI, PBF, and various metabolic indicators, obesity can be divided into four phenotypes:[9] a) normal weight obese (NWO), b) metabolically obese normal weight (MONW), c) metabolically healthy obese, and d) metabolically unhealthy obese. Among them, body measurement data of NWO are described as BMI: 22.6±1.9, WC: 72.3±4.9, and PBF: 34.9±5.0, while those of MONW are described as: BMI: 22.5±2.0, WC: 77.5±0.3, and PBF: 31.8±5.9. A common feature of the two obese groups is that both BMI and WC are in the normal range. Kang et al.[31] found that the blood pressure and fasting blood glucose level are significantly increased in NWO individuals, along with dyslipidaemia. When compared to individuals with less body fat, the mortality due to CVD is increased by 2.2-times in NWO individuals.[32] MONW individuals also have a high risk of diabetes.[33] These two groups of high-risk patients are often excluded from the obese category if their diagnosis is solely based on body weight, BMI, or WC.

## NORMAL WEIGHT OBESITY

Ruderman et al.[34] found that normal weight individuals may suffer from Type 2 diabetes mellitus, premature coronary heart disease, hypertension, and hyperlipidaemia if they have insulin resistance. Occurrence of metabolic abnormalities may be associated with the fat distribution. For normal weight patients with metabolic abnormalities, their metabolic indicators can be improved after controlling their diet and energy as well as exercising.[35] In 2006, De Lorenzo et al.[36] proposed the concept of NWO in individuals with a normal BMI (<25 kg/m$^2$) but a high content of fat (PBF >30%). This type of obesity shows an incidence of 2–28% in women,[37] but there is a lack of data in PCOS patients. The NWO is highly correlated to subclinical vascular inflammation and cardiovascular metabolic disease,[38] and is also associated with mortality of coronary heart disease.[39]

There are significant differences in body composition and fat distribution between lean PCOS patients and lean controls. With matching body weights, the body fat is significantly increased and the lean body weight is significantly lower in lean PCOS patients as compared to the controls. The healthy controls show gynoid fat distribution, while majority of the lean PCOS patients (70%) show non-gynoid fat distribution, and 50% of them exhibit android fat distribution.[7] This distribution of fat is characterised by an increase in visceral fat and the degree of insulin resistance.[40] Lean PCOS women show the same metabolic characteristics as obese PCOS women.[3,41] As compared to patients with gynoid fat distribution in which the fat is peripherally distributed, the risk of cardiovascular metabolic disease is significantly increased in patients with abdominal fat or androgenic obesity.[42]

## CONCLUSION

In summary, PCOS patients with normal body weight or BMI may have adverse reproductive and health outcomes due to increased fat content or abnormal fat distribution. Presumably, if the lean PCOS patients are further classified by the diagnostic criteria of PBF, a considerable number of patients will be classified as NWO or MONW and may have been easily overlooked if inappropriate diagnostic tools are selected, which could affect their therapeutic options; for example, they would still benefit from controlling their body fat. Therefore, we propose that in addition to body weight and circumference, the total body fat and fat distribution should also be considered in evaluating obesity in PCOS patients.

In view of the issues mentioned, much work is still needed in the future. The methods and criteria for evaluating PCOS obesity need to be re-defined and incidences of NWO and MONW in PCOS patients need to be summarised using epidemiological statistics to facilitate early intervention and delay the progression and improve the outcome of PCOS and save medical resources.

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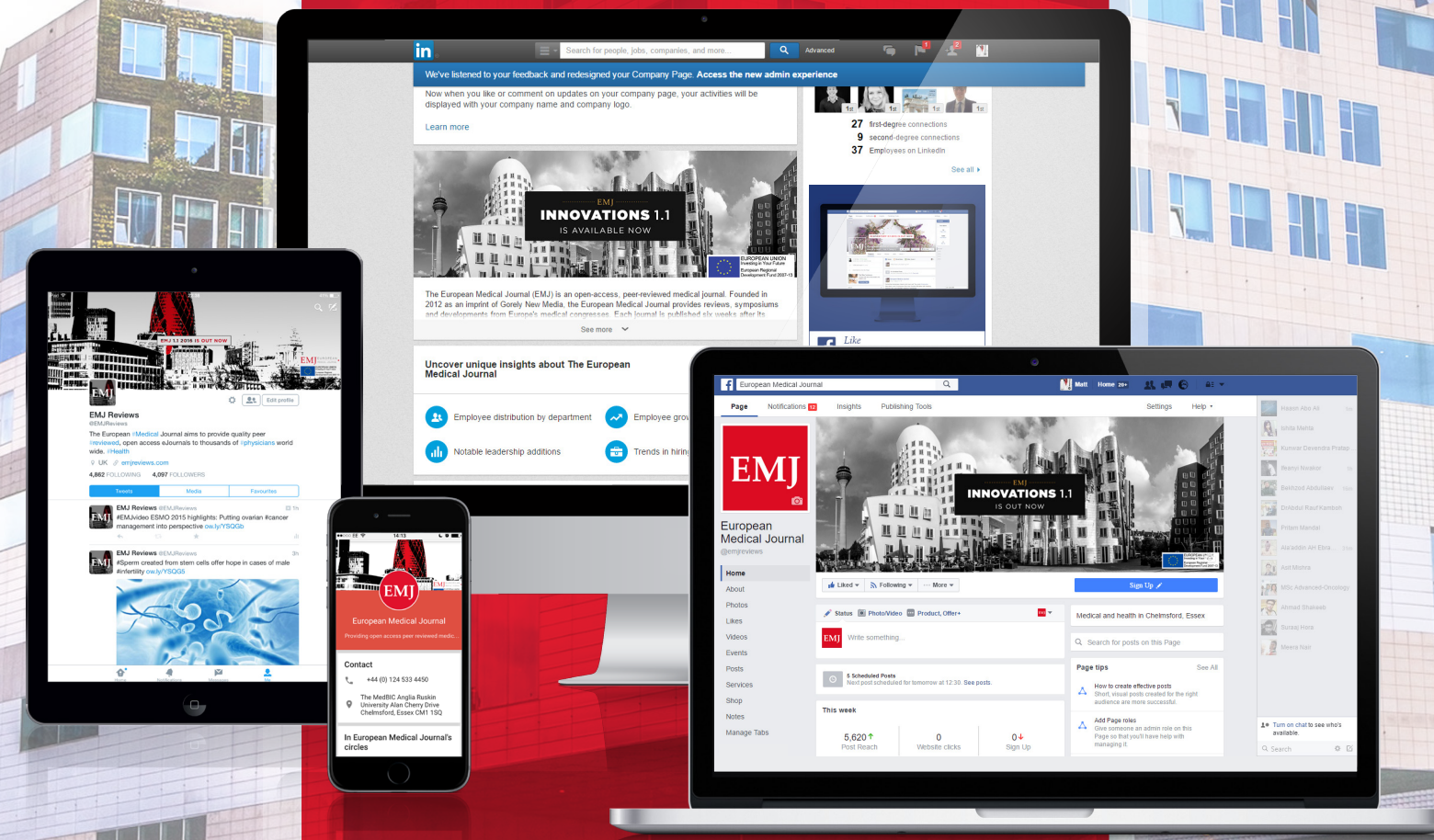


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# TORSION OF NORMAL ADNEXA IN THE THIRD TRIMESTER OF PREGNANCY MIMICKING ACUTE APPENDICITIS

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## ABSTRACT

Maternal ovarian torsion in pregnancy is very rare. Non-specific symptoms, signs, and imaging findings make the diagnosis difficult. A delay in diagnosis can lead to irreversible damage to adnexa and fetal compromise. Difficulties encountered in the diagnosis and management of adnexal torsion in the third trimester of pregnancy are highlighted in this article.

Keywords: Adnexal torsion, pregnancy, third trimester, salpingo-oophorectomy.

## INTRODUCTION

Adnexal torsion usually occurs in ovaries with functional cysts or tumours. Torsion of normal adnexa is rare during pregnancy, especially in the third trimester. The diagnosis of adnexal torsion is difficult to establish during pregnancy from symptoms, signs, and imaging techniques.[1] The patient usually presents with non-specific symptoms like abdominal pain, nausea, and vomiting, which can be easily mistaken for appendicitis, cholecystitis, pyelonephritis, or preterm labour. This can lead to delays in diagnosis and surgical management, resulting in loss of adnexa as well as fetal compromise.[2]

We report the case of a primigravida at 31 weeks' gestation, admitted to a tertiary care hospital with a presumptive diagnosis of acute appendicitis, which turned out to be torsion of a normal adnexa.

## CASE PRESENTATION

A 26-year-old primigravida at 31 weeks' gestation was admitted with a history of right lower abdominal pain, nausea, and vomiting of 1-day duration. She was seen in a secondary care hospital earlier in the day with a presumptive diagnosis of appendicitis. After receiving analgesics, she felt better, refused surgery, and went home against medical advice. After 6 hours, the pain got worse, and she became febrile and presented to our hospital. She was assessed by the obstetrician and the surgeon in the emergency room. The pain was severe, mainly in the right lumbar and lower abdomen with nausea, vomiting, and fever. There were no urinary symptoms or vaginal discharge, bleeding, or leaking. She felt normal fetal movements.

On examination, the patient was febrile and in distress. Her blood pressure was 120/80 mmHg, pulse 116/min, temperature 38°C, and oxygen saturation was 100% in room air. Abdominal examination revealed a relaxed gravid uterus corresponding to 30 weeks with tenderness, rigidity, and guarding in the right lower abdomen. A cardiotocogram showed reassuring fetal heart tracing with no uterine contractions. An abdominal ultrasound scan revealed fetal parameters corresponding to the gestation with normal amniotic fluid and fetal movements. The appendix was not visualised and no adnexal mass was detected.

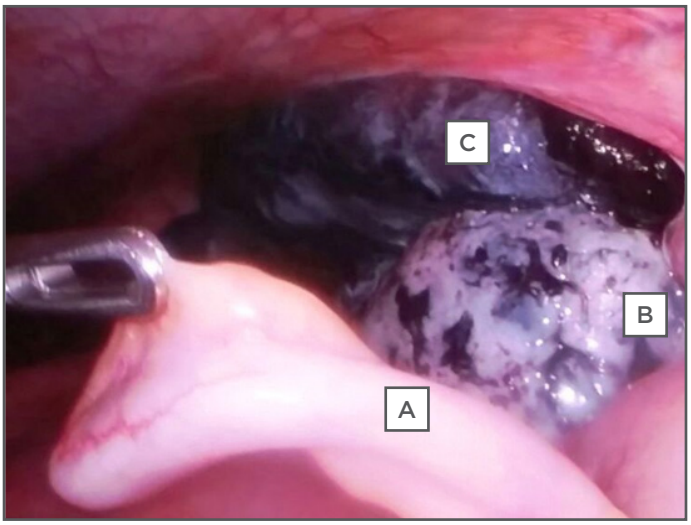


**Figure 1: A) Normal appendix; B) necrotic ovary; C) necrotic fallopian tube.**

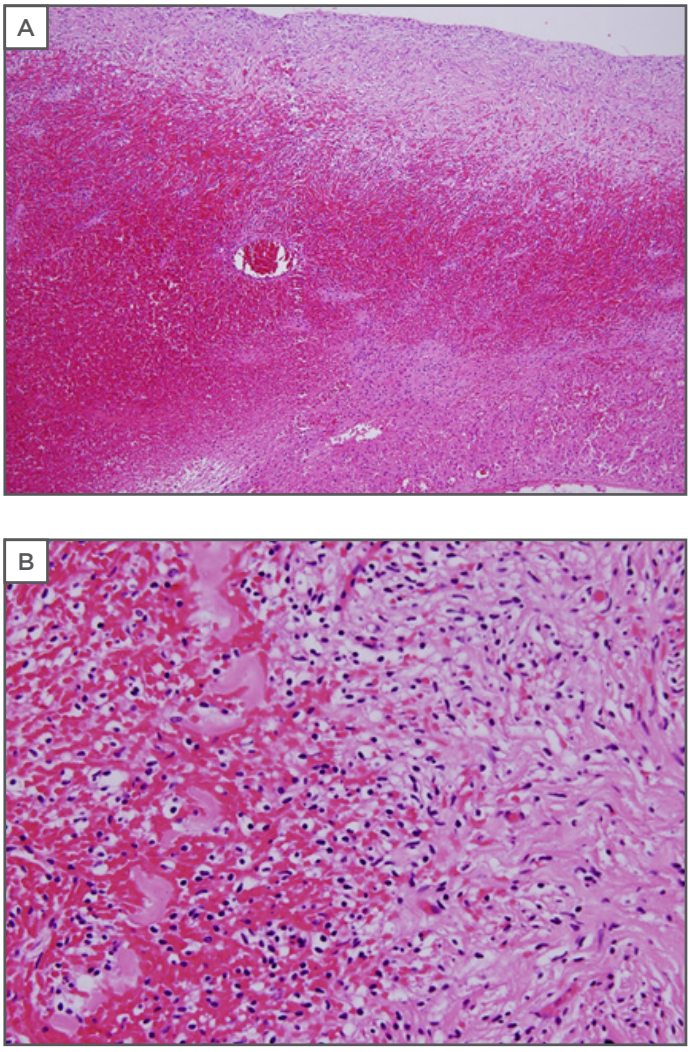


**Figure 2: A) Haemorrhage and necrosis of ovary H&E x 20; B) haemorrhage and necrosis of ovary. H&E x 40.**
H&E: haemotoxylin and eosin.

The patient's haemoglobin was 10.2 g/dL with a white cell count of 9,500/mm$^3$. C-reactive protein was 2 mg/L. Blood group was B Rh+. Urine analysis was normal. With a provisional diagnosis of acute appendicitis, the general surgeons took her for laparoscopy. Intraoperatively, the appendix was found to be normal in appearance with a twisted right ovary and tube (Figure 1) and the gynaecology team was called in.

As access to the adnexa was limited, and manipulation was technically difficult and risky due to the enlarged uterus, we proceeded with a subumbilical midline laparotomy. There was minimal haemoperitoneum; a sample was taken for cytology and culture. Both ovaries were found to be normal in size, the right ovary and tube were twisted three times, and the ovary and tube looked gangrenous and non-viable. As there was no evidence of functioning vascular supply on untwisting the ovary, a right salpingo-oophorectomy was performed. Intraoperative antibiotic (injection cefuroxime 1.5 mg at intubation) was given followed by oral cefuroxime 500 mg twice-daily for 5 days. As a prophylaxis against preterm labour, she was given an indomethacin suppository 100 mg preoperative, followed by an injection of hydroxyprogesterone hexanoate (Proluton Depot, 250 mg/mL x 1 mL) once-weekly for 4 weeks.

The patient had an uneventful stay in the hospital and was discharged on postoperative Day 4. Peritoneal fluid cytology was normal. High vaginal swab, urine, peritoneal fluid, and blood cultures were negative. Histopathology confirmed a gangrenous fallopian tube and ovary (Figure 2).

The pregnancy continued without problems until 38 weeks when she developed pre-eclampsia and underwent induction of labour with prostaglandin E2 gel. She delivered vaginally a baby girl weighing 2,480 g (<10$^{th}$ centile) with Apgar scores 9 and 10 at 1 and 5 minutes, respectively. Delivery was complicated with primary postpartum haemorrhage (blood loss 1.5 L) due to uterine atony, which was managed successfully with oxytocics.

## DISCUSSION

Torsion of adnexa in pregnancy is a rare cause of gynaecological emergencies where the adnexa rotates on its pedicle and compromises its blood supply, leading to stasis, venous congestion, haemorrhage, and necrosis.[3] The reported incidence is 1/1,000 during pregnancies, occurring mostly

during the first trimester, especially in women undergoing ovulation induction for infertility and in those diagnosed early to have an ovarian cyst.[4] Accurate diagnosis and early surgical intervention is essential as it facilitates conservative surgery, saving the adnexa, especially in young women where preservation of future fertility is of utmost importance.[5]

Diagnosis of ovarian torsion in advanced pregnancy is challenging as the symptoms, signs, and imaging findings are non-specific and can be confused with other acute abdominal conditions. Clinical examination is usually hampered by the anatomical displacement of the abdominal organs by the enlarged uterus. Ultrasound imaging of the ovaries is technically more difficult during pregnancy, especially in advanced gestation.[1,6] This leads to delay in surgical intervention, resulting in increased maternal and fetal morbidity. A combination of magnetic resonance imaging findings and lack of perfusion of ovarian parenchyma on colour Doppler sonography appears to be promising in the diagnosis of ovarian torsion in pregnancy.[1,7] However, the absence of Doppler signals may be a late sign and the presence of signals within the ovary does not exclude ovarian torsion.[8,9]

The type of surgical approach during pregnancy remains controversial and it depends on the availability of resources, the skill of the operator, and the patient's suitability. Several reports show that laparoscopy is technically feasible and as safe as laparotomy, even in the third trimester, with the additional advantages of minimally invasive surgery.[10-12] Simple detorsion is sufficient even in dark purple or black coloured ovary if vascular supply returns on untwisting the pedicle. Salpingo-oophorectomy may be necessary depending upon the degree of ischaemia and necrosis.[7] In our case, we proceeded with laparotomy and salpingo-oophorectomy, as laparoscopy was technically difficult and the tube and ovary looked gangrenous and non-viable even after detorting.

## CONCLUSION

Adnexal torsion may occur even in the absence of ovarian cysts. Torsion of normal adnexa should be considered as a differential diagnosis of acute abdominal pain in the third trimester of pregnancy, although rare. Prompt diagnosis and early surgical intervention might help in the conservation of adnexa, preservation of fertility, and reduction of maternal and fetal morbidity.

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# REGENERATIVE MEDICINE, DISEASE MODELLING, AND DRUG DISCOVERY IN HUMAN PLURIPOTENT STEM CELL-DERIVED KIDNEY TISSUE

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## ABSTRACT

The multitude of research clarifying critical factors in embryonic organ development has been instrumental in human stem cell research. Mammalian organogenesis serves as the archetype for directed differentiation protocols, subdividing the process into a series of distinct intermediate stages that can be chemically induced and monitored for the expression of stage-specific markers. Significant advances over the past few years include established directed differentiation protocols of human embryonic stem cells and human induced pluripotent stem cells (hiPSC) into human kidney organoids *in vitro*. Human kidney tissue *in vitro* simulates the *in vivo* response when subjected to nephrotoxins, providing a novel screening platform during drug discovery to facilitate identification of lead candidates, reduce developmental expenditures, and reduce future rates of drug-induced acute kidney injury. Patient-derived hiPSC, which bear naturally occurring DNA mutations, may allow for modelling of human genetic diseases to enable determination of pathological mechanisms and screening for novel therapeutics. In addition, recent advances in genome editing with clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 enable the generation of specific mutations to study genetic disease, with non-mutated lines serving as an ideal isogenic control. The growing population of patients with end-stage kidney disease is a worldwide healthcare problem, with high morbidity and mortality rates, that warrants the discovery of novel forms of renal replacement therapy. Coupling the outlined advances in hiPSC research with innovative bioengineering techniques, such as decellularised kidney and three-dimensional printed scaffolds, may contribute to the development of bioengineered transplantable human kidney tissue as a means of renal replacement therapy.

Keywords: Kidney, organoid, mini-organ, induced pluripotent stem cells (iPSC), pluripotent stem cell (PSC), directed differentiation, kidney development, glomeruli, tissue engineering.

## INTRODUCTION

Decades of developmental studies have elucidated the molecular pathways and genes necessary for normal kidney development. Historically, these studies enabled an understanding of human kidney developmental disease processes but largely have not translated into effective treatment, as human kidney development ceases prior to birth. There has been a renaissance in developmental biology with the advent of research involving human pluripotent stem cells (hPSC). By definition, hPSC have the ability to differentiate into cells of the three germ layers, namely the mesoderm, ectoderm, and endoderm.[1,2] Through directed differentiation, the sequential application of growth factors at specific concentrations for defined periods of time, hPSC can be transformed into particular organ tissues with a high degree of efficiency. Human induced pluripotent stem cells (hiPSC), a subset of hPSC, are generated from reprogramming adult cells through the activation of transcription factors characteristic of pluripotency.[2,3] Starting with any individual's terminally differentiated cells, an unlimited supply of isogenic hiPSC can be generated. Genomic retention permits modelling of genetic disease and provides an immunocompatible cell source for organ regeneration. The known physiology of vertebrate organogenesis serves as a guide towards the directed differentiation of hPSC into human tissue. Drawing from studies of vertebrate kidney development, researchers have discovered directed differentiation protocols that derive human kidney tissue *in vitro*.[4-10] Such human kidney tissue in a dish, potentially coupled with advances in biomedical engineering, may prove to revolutionise the fields of drug discovery, disease modelling, and kidney regenerative medicine (Figure 1).[11]

## VERTEBRATE KIDNEY DEVELOPMENT

The vertebrate kidney derives from the intermediate mesoderm (IM) of the mesodermal germ layer.

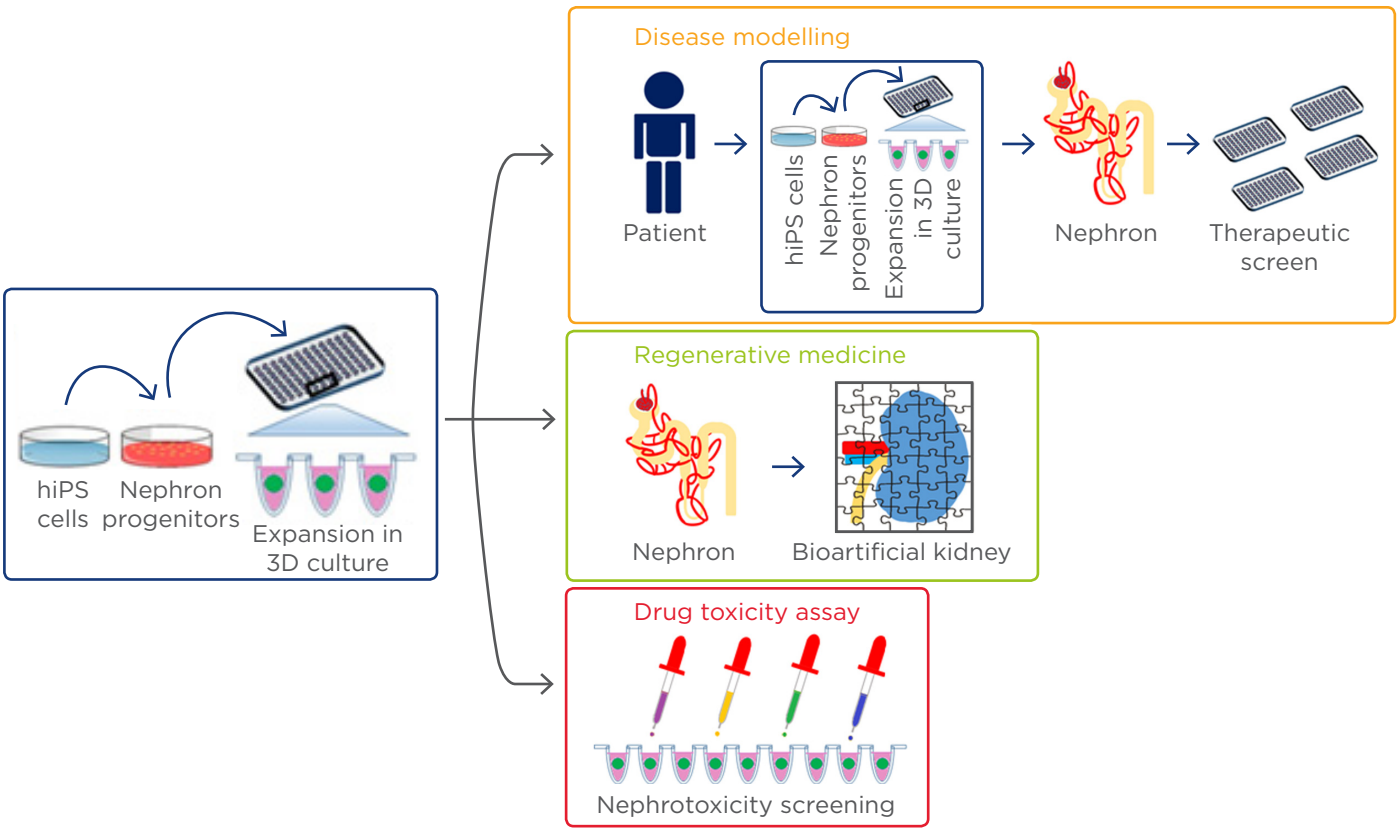


**Figure 1: Translational applications of hiPSC-derived kidney organoids.**
The discovery of directed differentiation protocols for the generation of three-dimensional kidney organoids from hiPSC may provide for numerous translational applications, including human genetic and congenital disease modelling, kidney regenerative medicine, and nephrotoxicity screening during drug development.
hiPSC: human induced pluripotent stem cells.

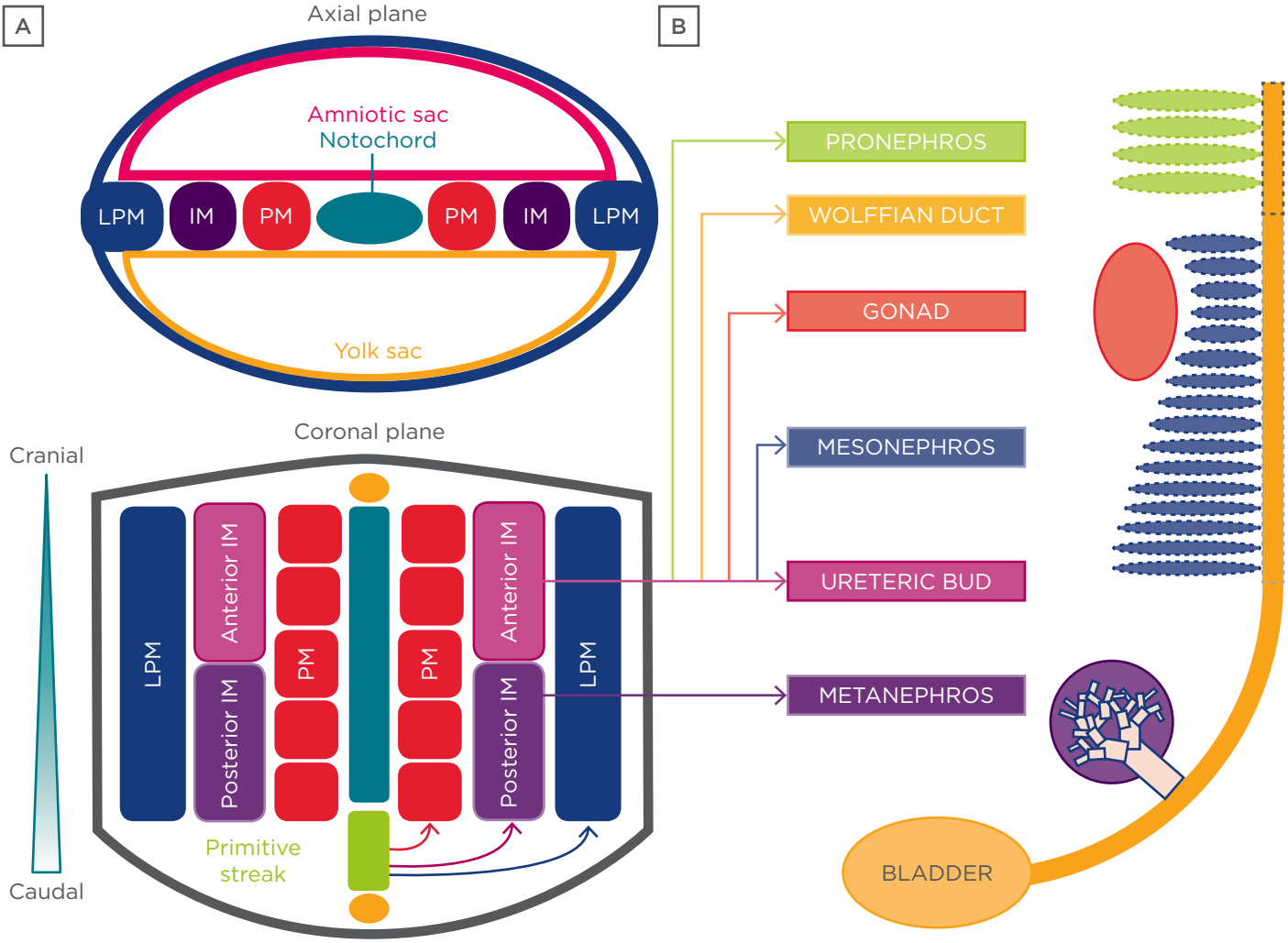


**Figure 2: Development of the vertebrate kidney.**

A) Vertebrate kidney development involves the serial progression of three distinct structures derived from the intermediate mesoderm, the pronephros, mesonephros, and metanephros. The pronephros is non-functional and regresses, the mesonephros provides primary exocrine functions until the eighth week of gestation, and the metanephros becomes the mature kidney in humans. Notably, the caudal portion of the mesonephric duct forms portions of the gonads in the male, while regressing in females. Each nephric stage associates with a collecting duct network derived from the Wolffian (or nephric) duct. B) The ureteric bud is an outpouching of the Wolffian duct, which derives from the anterior intermediate mesoderm. The metanephric mesenchyme contains the progenitor cells of all kidney epithelia, save the collecting duct, and derives from the posterior intermediate mesoderm.

LPM: lateral plate mesoderm; IM: intermediate mesoderm; PM: paraxial mesoderm.

During kidney organogenesis, the IM sequentially gives rise to the pronephros, mesonephros, and metanephros. In humans, the pronephros remains non-functional, regressing by the fourth week of gestation. The mesonephros forms just prior to degeneration of the pronephros in humans and serves as the primary excretory organ from the fourth to the eighth week of gestation. In females, the mesonephros degenerates, whereas in males it gives rise to portions of the gonads. The metanephros, which begins to form caudal to the mesonephros in the fifth week of gestation, becomes the definitive adult kidney in humans (Figure 2A).[12]

The metanephric kidney forms through the reciprocally inductive interactions between two distinct IM tissues, the metanephric mesenchyme (MM) and ureteric bud (UB).[4] The MM arises from the posterior IM and contains a population of multipotent nephron progenitor cells (NPC) that express the transcription factors Six2, Cited1, Pax2, Sall1, and Wt1.[4,13-15] The Six2$^+$ NPC cluster to form the

cap mesenchyme around each infiltrating UB tip.[15] UB-derived Wnt signals induce NPC to undergo mesenchymal-to-epithelial transition, giving rise to nearly all the epithelial cells of the nephron except for those of the collecting duct.[15,16] Meanwhile, the UB arises as an epithelial outpouching from the caudal Wolffian (or nephric) duct, which upon receiving inductive signals from the MM, undergoes iterative branching to form the collecting duct system of the kidney. Nephrogenesis in humans is completed between 32 and 36 weeks of gestation and results in the formation of approximately one million nephrons in each kidney. After birth, no new nephrons are formed, even under circumstances of kidney injury and repair.[17,18]

Recent work from Taguchi et al.[4] has provided important insight into the embryonic origins of NPC in the MM. Employing lineage tracing techniques in mice, the authors demonstrated that NPCs are derived from a population of T+ cells in the primitive streak that persists to give rise to $T^+Tbx6^+$ posterior nascent mesoderm followed by $Wt1^+Osr1^+$ posterior IM. In contrast, the UB originates from a $Pax2^+$ cell population known to be limited to the anterior IM[14,19,20] and is incapable of giving rise to MM (Figure 2B). Thus, careful consideration of these diverging developmental pathways is critical for the efficient differentiation of pluripotent stem cells (PSC) into cells of these two different lineages.

Current strategies to direct the differentiation of hPSC into cells of the kidney lineage have been based on vertebrate animal kidney development models.[4,7,8] Key transcription factors, growth factors, and membrane protein factors involved in kidney organogenesis have been identified using gene knockout and transgenic models with resultant phenotype demonstrating congenital anomalies of the kidney and urinary tract (Table 1).[21-38] These factors serve as critical markers of kidney induction during directed differentiation of hPSC.

## PLURIPOTENT STEM CELLS

PSC represent early embryonic progenitor cells, believed to correspond to the blastocyst or epiblast stage of mammalian embryos.[39] Cells at this stage arise 5–9 days post-conception in humans and are defined by two intrinsic properties: self-renewal and pluripotency. PSC have the ability to self-renew indefinitely in culture, without transformation or differentiation. Additionally, PSC are pluripotent, having the capacity to give rise to all cell types derived from the three embryonic germ layers, namely the mesoderm, endoderm, and ectoderm.[40]

PSC comprise embryonic stem cells (ESC) and induced pluripotent stem cells (iPSC). ESC are derived from the isolation and culture of cells from the inner cell mass of the embryonic blastocyst.[1] In contrast, iPSC are generated following the activation of four key transcription factors of pluripotency (Oct4, Sox2, Klf4, and c-Myc) in terminally differentiated cells, which directly reprograms them into cells that behave similarly to and appear morphologically identical to ESC.[2,3]

Although human ESC (hESC) remain the gold standard for stem cell research, hiPSC have a number of advantages. Unlike hESC, the derivation of iPSC does not involve the use of human embryos, a limitation that has led to ethical concerns over the use of hESC.[41] Protocols now exist to derive hiPSC from a variety of different terminally differentiated cell types, including peripheral blood mononuclear cells, keratinocytes, hepatocytes, urothelial cells, neural stem cells, and kidney mesangial and tubular epithelial cells, using both viral and non-viral reprogramming methods.[42-48] hiPSC can be generated from any human being, healthy or diseased, with retention of the host's individual genome. Therefore, hiPSC represent an isogenic substrate to generate theoretically immunocompatible tissue for organ regeneration. Additionally, hiPSC generated from patients with genetic diseases can be used to develop *in vitro* models to better study disease pathogenesis. For diseases that are particularly rare or do not have relevant animal models, iPSC offer a novel strategy to study disease mechanisms and develop new therapeutics.

In the absence of exogenous growth factors or chemicals, PSC undergo stochastic differentiation into embryoid bodies (EB) *in vitro* and teratomas *in vivo*.[1-3] Both EB and teratomas are heterogeneous tissues that contain cells of the three embryonic germ layers. The heterogeneity of the tissue confirms pluripotency, but imparts a low induction efficiency of any one specific cell type. Directed differentiation refers to the process by which PSC are sequentially treated with growth factors and chemicals to efficiently induce a particular cell or tissue type of interest. Directed differentiation protocols often employ a stepwise approach, through intermediate developmental stages, which mirror normal embryonic organogenesis.[49]

**Table 1: Genetic knockout and transgenic mouse models of kidney development.**

| GENE KNOCKOUT OR MUTATION | KIDNEY PHENOTYPE | RELATED HUMAN DISEASE |
|---|---|---|
| **Intermediate mesoderm** | | |
| *Eya1* | Renal agenesis, lack of UB branching[21] | Branchio-oto-renal syndrome[22] |
| *Lim1/LHX1* | Renal agenesis, UB branching defect, hydroureter[23] | Mayer-Rokitansky-Küster-Hauser syndrome[24] |
| *Osr1* | Renal agenesis[25] | |
| *Wt1* | Renal and gonadal agenesis[26] | Wilms tumor, Denys–Drash syndrome[27] |
| **Ureteric bud** | | |
| *Gata3* | Renal agenesis, ectopic ureteric budding[28] | N/A (Embryonic lethal in mice[29]) |
| *Pax2* | Renal agenesis[30] | CAKUT, renal-coloboma syndrome[31,32] |
| *Pax8* | Severe renal hypoplasia, reduced EB branching[33] | |
| **Metanephric mesenchyme** | | |
| *Sall1* | Severe renal hypoplasia[34] | Townes-Brock syndrome[35] |
| *Six2* | Renal hypoplasia, depletion of nephron progenitor cells[16] | |
| **MM-UB reciprocal induction** | | |
| *Gdnf* | Renal agenesis[36] | Stillbirth[37] |
| *Ret* | Renal agenesis[38] | MEN Type IIA and MEN Type IIB[38] |

UB: ureteric bud; MM: metanephric mesenchyme; EB: embryoid bodies; CAKUT: congenital anomalies of the kidney and the urinary tract; MEN: multiple endocrine neoplasia.

## Differentiation of Human Pluripotent Stem Cells to Kidney Lineage Cells

Chronologically, the earliest work to obtain IM from hESC involved the generation of WT1$^+$ and PAX2$^+$ kidney precursor cells.[50,51] Shortly thereafter, protocols to directly generate cells bearing markers of terminal kidney epithelia were published. Podocin$^+$Synaptopodin$^+$PAX2$^+$ podocyte-like cells were induced in EB from hiPSC using a combination of activin, bone morphogenetic protein (BMP)7, and retinoic acid.[52] Through a similar protocol, a monolayer culture of these podocyte-like cells was shown to integrate into WT1$^+$ glomerular structures, when combined with dissociated-reaggregated embryonic mouse kidneys. In renal epithelial growth medium, a combination of BMP2 and BMP7 induced the differentiation of hESC to aquaporin-1 (AQP1)$^+$ proximal tubule-like cells. Flow-sorted AQP1$^+$ cells integrated into tubular compartments of *ex vivo* newborn mouse kidneys and spontaneously formed cord-like structures when cultured on Matrigel®. AQP1$^+$ cells increased cyclic adenosine monophosphate production in response to exogenous parathyroid hormone, demonstrated functional γ-glutamyl transferase enzymatic activity, and produced ammonia.[53]

Recent studies have focussed on the efficient induction of kidney progenitor cells, particularly cells of the IM and MM. An OSR1-GFP hiPSC line was treated with the combination of the glycogen synthase kinase-3β inhibitor CHIR99021 (CHIR) and activin, followed by BMP7, to yield OSR1$^+$ cells with 90% efficiency within 11–18 days of differentiation. OSR1$^+$ cells differentiated into populations expressing markers of mature kidneys, adrenal glands, and gonads *in vitro* and integrated into dissociated-reaggregated embryonic mouse kidneys.[54] The sequential treatment of hPSC with CHIR, followed by fibroblast growth factor (FGF)2 and retinoic acid, generated PAX2$^+$LHX1$^+$ IM-like cells with >70% efficiency. PAX2$^+$LHX1$^+$ cells stochastically differentiated to form ciliated tubular structures expressing the proximal tubular markers *Lotus tetragonolobus* lectin (LTL), N-cadherin, and kidney-specific protein.[6] Meanwhile, directed differentiation of PAX2$^+$LHX1$^+$ cells, involving treatment with FGF9 and activin, generated cells co-expressing markers of MM including SIX2, SALL1, and WT1.[6] A similar protocol efficiently generated PAX2$^+$LHX1$^+$ IM cells from hESC within 6 days, using a combination of CHIR and FGF9.[5] On continued FGF9 treatment, these cells gave rise to SIX2$^+$ cells with 10–20% efficiency within 14 days.[5]

Mixing these cells with dissociated-reaggregated mouse embryonic kidneys resulted in three-dimensional (3D) aggregates of SIX2$^+$ cells containing tubular structures expressing kidney markers such as AQP1, AQP2, JAG1, E-cadherin, WT1, and PAX2.[5]

While considerable work has been done to differentiate hPSC into MM, efforts to differentiate hPSC into cells of the UB lineage have been limited. In a 4-day directed differentiation protocol involving initial treatment with BMP4 and FGF2, followed by retinoic acid, activin, and BMP2, hESC, and hiPSC formed PAX2$^+$OSR1$^+$WT1$^+$LHX1$^+$ IM-like cells.[55] These cells spontaneously upregulated transcripts of the UB markers HOXB7, RET, and GFRA1 within 2 days. Upon co-culture with dissociated-reaggregated embryonic mouse kidneys, these putative UB progenitor-like cells partially integrated into mouse UB tips and trunks.[55]

Two groups have demonstrated the ability to differentiate hPSC into 3D kidney organoids containing complex, multi-segmented nephron-like structures.[7,8] Treatment of hPSC with CHIR for 4 days, followed by FGF9 for 3 days, and transfer into 3D suspension culture ≤20 days generated kidney organoids consisting of nephron-like structures bearing markers of proximal and distal tubules, early loops of Henle, and podocyte-like cells.[7] To simulate UB-derived Wnt signalling, a transient 1-hour pulse of CHIR on transfer to suspension culture aided the induction of nephron-like structures. The organoids contained tubular structures expressing markers of collecting ducts, stromal cells expressing markers of the renal interstitium, and endothelial cells, suggesting the presence of a heterogenous mixture of the IM and lateral plate mesoderm.[7] More recently, a directed differentiation protocol was found to differentiate both hESC and hiPSC into SIX2$^+$SALL1$^+$PAX2$^+$WT1$^+$ NPC that could be induced to form nephron (kidney) organoids in both two-dimensional and 3D cultures.[8] In a stepwise approach that mirrored vertebrate kidney organogenesis, first T$^+$TBX6$^+$ primitive streak cells were induced with CHIR for 4 days, then WT1$^+$HOXD11$^+$ posterior IM cells were induced with activin, and then SIX2$^+$SALL1$^+$PAX2$^+$WT1$^+$ NPC were induced using low-dose FGF9 with ≤90% efficiency. Treatment of NPC with continued FGF9 and a transient CHIR pulse induced PAX8$^+$LHX1$^+$ renal vesicles that spontaneously formed nephron-like structures in two-dimensional culture. Transfer of NPC into 3D suspension culture resulted in the formation of kidney organoids containing multi-segmented nephron-like structures expressing markers of glomerular podocytes (NPHS1$^+$PODXL$^+$WT1$^+$), proximal tubules (LTL$^+$CDH2$^+$AQP1$^+$), loops of Henle (CDH1$^+$UMOD$^+$), and distal tubules (CHD1 UMOD$^-$) in a contiguous arrangement. Additionally, these kidney organoids demonstrated promise for applications involving studies of kidney development and drug toxicity.

The establishment of efficient protocols for directing the differentiation of hPSC into NPC and kidney organoids marks a significant advance in the ongoing effort to apply human stem cells to the regeneration of kidney tissue, modelling of human kidney disease, and drug testing for therapeutic efficacy and toxicity.[8,11] However, the development of definitive functional assays and the establishment of reliable genetic markers will be required to verify whether induced hPSC-derived kidney cells and tissues are sufficiently identical to their *in vivo* complements for translational applications.

## KIDNEY ORGANOIDS FOR NEPHROTOXICITY TESTING

Nephrotoxicity is a common manifestation of the toxic effects of drugs and their metabolites. The kidneys are highly vascularised, receiving 20–25% of the cardiac output, and may accumulate circulating toxins in the vascular, interstitial, tubular, or glomerular spaces. During drug development, 19% of failures in Phase III clinical trials are due to nephrotoxicity.[56] As the cost to bring a drug to market is currently ~2.6 billion dollars,[57] the availability of high-throughput nephrotoxicity screening systems during drug development may save considerable time and costs.

Recent reports have demonstrated that hPSC-derived kidney cell tissue may respond to nephrotoxic drugs in a manner that mimics *in vivo* kidney injury.[7,8] In the previously discussed protocols to generate hPSC-derived kidney organoids, the chemotherapeutic agent cisplatin was demonstrated to cause specific injury to the proximal tubular cells, consistent with known cisplatin toxicity *in vivo*. Cisplatin-induced proximal tubular injury was characterised by the upregulation of the DNA damage marker γH2AX, increased expression of kidney injury molecule-1 (KIM-1), and upregulation of cleaved caspase-3.[7,8,10] Additionally, treatment of kidney organoids with the antibiotic gentamicin upregulated KIM-1 in proximal tubules, without any discernible

effect on podocytes, consistent with the known nephrotoxicity of aminoglycosides.

## KIDNEY ORGANOIDS FOR MODELLING OF KIDNEY DISEASES

hiPSC-derived organ tissue represents a valuable platform for the study of human pathophysiology and the discovery of novel therapeutics. As hiPSC remain isogenic with the original host cell prior to reprogramming, they provide a means of modelling patient-specific genetic diseases. Importantly, the rarity of many genetic diseases precludes enrolment in clinical trials, which coupled with a lack of incentive for drug companies to develop treatments for rare diseases, fuels the hope that hiPSC-based assays can be a scalable and reliable option for preclinical studies at low cost. Once established, reliable human disease models may allow for clinical 'trials-in-a-dish'. Human stem cell-based systems may ultimately replace animal testing, known to be poorly predictive of the human response.[58] To date, hiPSC lines have been generated for autosomal dominant polycystic kidney disease (ADPKD),[59] autosomal recessive polycystic kidney disease (ARPKD),[43] and systemic lupus erythematosus.[60,61]

ADPKD is the most common potentially lethal monogenic disorder, affecting 1 in 600 to 1 in 1,000 live births. Approximately 50% of individuals with ADPKD develop end-stage kidney disease (ESKD) by 60 years of age.[62] The traditionally used mouse models are homozygous carriers for ADPKD mutations while afflicted humans are heterozygotes, calling into question the utility of ADPKD animal models.[63] hiPSC lines have been created from multiple patients with ADPKD and ARPKD.[59] A subsequent study from the same group used the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system to knockout *PKD1* or *PKD2*, the causative genes for ADPKD, in hESC lines.[64] Tubular organoids derived from these *PKD1* and *PKD2* knockout mutants developed cystic structures from $LTL^+$ kidney tubules, suggesting that this model could potentially serve as a novel means to study cystogenesis in ADPKD and screen for new therapeutics *in vitro*.

## PLURIPOTENT STEM CELLS FOR BIOENGINEERING KIDNEY TISSUE

The shortage of transplantable organs, coupled with the rising prevalence of ESKD, have led researchers to apply regenerative medicine techniques to kidney bioengineering.[65] Human iPSC serve as a theoretically immunocompatible and scalable cell source, with therapeutic applications for both chronic kidney disease and acute kidney injury. While the kidney is a complex organ consisting of >50 distinct cell types that provide for exocrine, endocrine, and metabolic functions, the essential elements of an envisioned bioengineered kidney would include multiple hPSC-derived cell types supported in a perfusable scaffold that provides appropriate cellular segregation and compartmentalisation. Two scaffolding approaches have been undertaken, kidney decellularisation and a 3D-printed framework.[66,67]

Decellularised kidney approaches preserve the extracellular matrix (ECM) of distinct kidney compartments, retaining matrix-associated signals and growth factors, and conserving the vascular tree and branched collecting duct network. Mammalian kidneys have been decellularised, using the detergent sodium dodecyl sulfate and the cell membrane toxicant Triton X-100, with haematoxylin and eosin stains confirming the removal of cellular material and immunohistochemistry demonstrating the preservation of native ECM.[68] Surgically implanted decellularised vertebrate kidneys remain perfusable and lack blood extravasation, but completely thrombose due to denuded ECM.[69] Seeding decellularised rat kidneys with rat fetal kidney cells via the ureter, and endothelial cells via the renal artery, enabled the production of a small amount of urine when perfused by the recipient's circulation following orthotopic transplantation.[66] However, the urinary filtrate was negligible and vasculature rapidly thrombosed. Similarly, murine ESCs were seeded into decellularised rat kidneys via the renal artery and ureter.[70] Cells lost their pluripotent phenotype, expressed kidney markers, but were limited by small vessel thrombosis on perfusion testing *in vivo*. Decellularised vasculature *ex vivo* lined with the biocompatible polymer, poly(1,8-octanediol citrate), and functionalised with heparin, reduced platelet adhesion and whole blood clotting.[71] Given their advantage of maintained architecture, decellularised kidney approaches may provide a valuable resource in efforts to create a bioengineered kidney.

Biologic applications of 3D printing have gained notoriety and credibility with the 'organ-on-a-chip' series, modelling lung, gut, the kidney proximal tubule, and bone marrow.[72-75] While these early organ chips employ a soft lithography method

first published nearly two decades ago,[76] recent advances in 3D printing have enabled faithful manufacturing of micrometre scale, multicomponent 3D structures. However, current commercially available 3D printing resins, for both stereolithography and multi-jet modelling, demonstrate poor biocompatibility.[77,78] To overcome obstacles of resin cytotoxicity and the need for a vascular network in tissue engineering, the Lewis lab employed biocompatible collagen-based resins to develop vascular networks.[79] Human umbilical vein endothelial cells, embedded in a sacrificial Pluronic® F127 hydrogel, were printed in channels and surrounded by photocurable gelatin methacrylate. Removal of the fugitive ink yielded tubular channels consisting of a confluent monolayer of human umbilical vein endothelial cells. Using a similar methodology, the proximal tubule-on-a-chip has evolved from incorporating a cellular monolayer into tubular structures containing a perfusable lumen.[67]

## DISCUSSION

Acclaim for human stem cell-based research has been based on its potential to influence patient care through translational applications. 'Hope-based' notoriety has preceded evidence-based credibility. Clinical trials using hiPSC-derived tissues are currently underway in patients with retinal disease and acute spinal cord injury. Concerning kidneys, recent protocols to generate 3D, multicellular, and multi-compartmentalised kidney tissue from hPSC has reinvigorated the promise of human stem cell work to revolutionise the fields of drug discovery, disease modelling, and regenerative medicine.

Nephrotoxicity is a common cause for failures during drug development, accounting for 19% of Phase III clinical trial failures.[56] The proximal tubule is the predominant site of toxicity, where endogenous and exogenous toxin excretion is mediated by organic anion transporters (OAT) and organic cation transporters (OCT). Current methods to assess nephrotoxicity, involving immortalised human cell lines and animal models, are limited by inadequate expression and interspecies heterogeneity among OAT/OCT transporters, respectively. Use of hPSC-derived kidney tissue *in vitro* may prove to be an improved model. Future work to determine the OAT/OCT expression profiles of hPSC-derived kidney tissue, generated from the various directed differentiation protocols, may identify an optimal method to predict nephrotoxicity in preclinical studies, thereby preventing late phase failures. Confirmatory functional assays may include the reproduction of cisplatin proximal tubule toxicity, mediated by OCT2, with reversal using cimetidine, an OCT2 inhibitor. Reporter lines for KIM-1, a proximal tubule injury marker, and neutrophil gelatinase-associated lipocalin, a distal nephron injury marker, may permit real-time toxicity monitoring. Adapting human kidney tissue *in vitro* to perfusable systems, simulating *in vivo* conditions, may improve the sensitivity and specificity of identifying nephrotoxins through increased drug transporter expression. Additionally, perfusable systems would permit toxicity testing of individual compounds across increasing concentration gradients, greatly consolidating the test conditions required by preclinical testing methods.

Human kidney tissue *in vitro* is capable of modelling human genetic disease, whether by using patient-derived iPSC or by introducing mutations through genome editing techniques such as CRISPR/Cas9 or transcription activator-like effector nucleases (TALENS). The paucity of therapeutics for PKD, which accounts for 10% of ESKD, provides motivation for clinical-trials-in-a-dish using hPSC-derived PKD models. ARPKD, while less prevalent, may be a better model than ADPKD owing to its developmental phenotype. Alternatively, an ADPKD model could be used to identify mechanisms by which a dominant disease can engender a delayed age of onset, such as the second hit hypothesis. Future work to determine the directed differentiation protocol for terminal UB may provide a better tissue model for PKD, as cysts are generally concentrated in the collecting system. Coculture of UB and MM would represent an ideal model for studying congenital anomalies of the kidney and urinary tract, which often arises from failure of the reciprocal induction between the two tissues. Experiments using kidney tissue generated from patients with *APOL1*-associated focal segmental glomerulosclerosis may elucidate the concerted factors that transition carriers of the *APOL1* risk variant to primary focal segmental glomerulosclerosis.

In the future, non-genetic kidney diseases may be modelled in stem cell-based human tissue. Severe or recurrent acute injury to hPSC-derived kidney tissue, possibly with fibrogenic cells and/or macrophage coculture, may recapitulate the pathophysiology leading to kidney fibrosis, a common hallmark of varied causes of progressive

chronic kidney disease. Using kidney-on-a-chip techniques to generate perfusable glomerular structures, perfusion with isolated immune complexes from patients' serum may permit modelling of lupus glomerulonephritis, cryoglobulinemia, post-streptococcal glomerulonephritis, and immunoglobulin A nephropathy. The application of anti-phospholipase-A2-receptor antibodies may simulate membranous nephropathy. Culturing organoids in a hypoxic incubator may model kidney ischaemia. Disease modelling in human tissue *in vitro* represents a formidable tool for drug screening, aiding the identification of novel therapies in human disease.

Regenerative medicine strategies employing existing methods for hPSC-derived kidney tissue face challenges related to the lack of a perfusable vascular tree and organised collecting system. Advances in biomedical engineering include the generation of stable vascular networks in 3D printed ECM-based hydrogels. The coculture of developing stem cell-based human kidney tissue with a pre-existing vascular network may generate a perfusable vascular tree. Determining the optimal timing along the directed differentiation protocol to conduct coculture and composition of the ECM-based hydrogel may facilitate glomerular vascularisation. Perfusion of glomerular capillary tufts *in vitro* may elicit a filtrate, for which sieving coefficients could be determined using dextran particles of differing size and charge. A collecting duct system has been previously generated using isolated mouse MM and UB *in vitro*. Using similar methods, the coculture of hPSC-derived MM and UB tissue may induce a common branched collecting duct system. Alternatively, the decellularisation-recellularisation technique represents a pre-formed scaffolding approach towards bioartificial kidney creation. One such approach involves dissociating multicellular hPSC-derived kidney organoids to single cells and seeding decellularised mammalian kidney via the renal artery, renal vein, and ureter. The influence of existing compartmentalised kidney ECM, and the retained growth factors, may facilitate cellular attachment and proliferation in proper compartments. The increasing need for novel forms of renal replacement therapy will surely lead to considerable advances towards a bioartificial kidney, as necessity breeds innovation.

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# OOCYTE DONATION: AN OVERVIEW

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## ABSTRACT

The use of donor oocytes has expanded the scope of assisted reproductive technology (ART) for women with poor oocyte quantity and quality. *In vitro* fertilisation with oocyte donation (IVF-OD) is considered to give better implantation, pregnancy, and livebirth rates compared to IVF with autologous oocytes. Maternal age, infertility factors, BMI, smoker status, and ethnicity reduce reproductive outcome. An increasing demand and a good success rate with oocyte vitrification programmes have led to the formation of oocyte banks, reducing the need for donor–recipient cycle synchronisation and allowing egg sharing. Obstetric and neonatal complications with donor oocytes are significantly increased in comparison to autologous IVF and spontaneous pregnancies. The risk of pregnancy-induced hypertension (PIH), pre-eclampsia (PE), prematurity, low birth weight and very low birth weight are increased, as is the need for operative delivery. The age group of these patients and the increase in obstetric and neonatal complications associated with multiple pregnancy, dictates the use of single embryo transfer. As increasingly older women enter these programmes, concerns for maternal and fetal health necessitate guidelines to set an age limit for offering the procedure. Advanced paternal age is also raising concerns in long-term follow-up studies in neonates.

Keywords: Oocyte donation, *in vitro* fertilisation (IVF), pregnancy rate, complications, age, male factor.

## INTRODUCTION

*In vitro* fertilisation with the use of donor oocytes (IVF-OD) has become an integral part of infertility treatment today. The procedure is used to achieve pregnancy in women with premature and age-related ovarian failure, poor ovarian reserve (due to disease or advanced age), Turner's syndrome, recurrent implantation failure due to poor oocyte quality, and recurrent abortions.[1,2] Couples also opt to use donated oocytes to avoid transmission of severe genetic diseases.[3]

Results achieved in recipients surpass those attained with use of autologous oocyte IVF[4-6] in good prognosis patients, resulting in an exponential rise in the procedure. In the USA, the annual number of donor oocyte cycles increased from 10,801 to 18,306 between 2000 and 2010,[7] whilst the European IVF Monitoring (EIM) consortium for the European Society of Human Reproduction and Embryology (ESHRE)[4] reported 7,171 IVF-OD cycles in 2006 and 33,605 cycles in 2012. Indeed, couples will travel outside their country if local laws forbid oocyte donation. The procedure is so popular that oocyte banks[8] have been established to allow for sharing of oocytes from a donor, reduced waiting times involved in sourcing and screening donors, and circumventing donor–recipient synchronisation. Cross-border reproductive care too is gaining attention, as assisted reproduction technology (ART) practices and obstetric care may differ between countries.[9]

The donor–recipient model has provided an insight into various aspects of ART, such as the importance of oocyte age in implantation,[10] endometrial receptivity,[11] and the contribution of male factors to IVF failure.[12] Perhaps the most thought-provoking realisation is the knowledge that the reproductive system can function perfectly in the absence of a genetic connection.[2]

## REPRODUCTIVE PERFORMANCE IN RECIPIENTS

### Pregnancy and Implantation Rate

The use of young (<35 years), healthy donors has resulted in high pregnancy rates (PR) and implantation rates (IR) in recipients. The EIM consortium report of 2012[4] on reproductive outcome with use of donor oocytes, reported a PR of 48.4% per fresh embryo transfer, 35.9% per frozen embryo transfer (FET), and 45.1% using frozen oocytes. In comparison, use of autologous oocytes resulted in a clinical PR (CPR) of 29.4% per aspiration, 33.8% per transfer, and 23.1% with FET, and an overall multiple PR of 17.9%. The rate of twin pregnancy was also significantly higher in women with IVF-OD compared to IVF and spontaneous pregnancy (39.4% versus 15.0% with IVF and 2.5% with spontaneous, p<0.001).[13]

A comparison of pregnancy outcomes in 15,037 fresh donor oocyte versus 11,420 autologous IVF cycles in women aged 20–30 years (in both groups) between 2008 and 2010 reported that, despite similar demographics, stimulation, and embryo parameters, the odds of implantation, (odds ratio [OR]: 1.33, 95% confidence interval [CI]: 1.26–1.40), clinical pregnancy (CP) (OR: 1.43, 95% CI: 1.35–1.52), and live birth (LB) (OR: 1.26, 95% CI: 1.18–1.33) were significantly higher in donor cycles after adjusting for patient age, number of oocytes retrieved, and number of embryos transferred. A sub-group analysis revealed higher odds of implantation, CP, and LB in intracytoplasmic sperm injection (ICSI) cycles, ICSI with male factor, unexplained infertility (UI), cleavage stage transfer, blastocyst transfer, and elective single blastocyst transfer with donor eggs. The odds for LB, which is considered the best measure of outcome, were also higher in IVF-OD sub-groups; ICSI (OR: 1.13, 95% CI: 1.01–1.26), ICSI with male factor (OR: 1.37, 95% CI: 1.28–1.48), and UI (OR: 1.47, 95% CI: 1.20–1.81). Women with conditions that could affect the uterine environment or lead to interference with implantation were excluded from the study.[14]

### Effect of Maternal Age

As more and more women delay motherhood, there is an increasing demand for donor cycles, paralleled by a rise in recipient age. Does a woman's age per se affect implantation? Is menopause a disadvantage? What are the maternal complications? How does the neonate fare? These are critical issues that need to be examined, particularly in relation to women of very advanced age. It would appear that uterine senescence reduces the capacity for implantation, perhaps due to age-associated uterine factors such as fibroids and reduced vascularity. Decreased PR in recipients beyond 49 years have been reported by Check et al.[15] A poorer pregnancy and live birth rate (LBR) has also been reported by Paulson et al.[16] in women >50 years old (range: 50–63). Recent data from the Society for Assisted Reproduction Technology (SART) registry also suggest that donor oocyte recipients have stable rates of pregnancy (CPR: 62.8%) before the age of 45 years, after which there is a small but steady and significant decline (CPR: 59.9%).[17] Ameratunga et al.,[18] comparing PR in women with premature ovarian failure (Group A) and physiological menopause (Group B) found similar cumulative PR; Group A (75%) versus Group B (72%). The average number of stimulated cycles for each woman to produce a LB was 1.75 and 1.4 in Group A and B, respectively.

### Effect of Paternal Age

The effect of advanced paternal age on fertility, pregnancy, and neonatal outcome has received very little attention. Although there is no clearly accepted definition of advanced paternal age, an age >40 is often used as a cut-off. Conflicting results have been reported in literature regarding the contribution of advanced paternal age. Gallardo et al.[19] stated that age ≤64 years did not affect embryo development *in vitro* as well as implantation in recipient uteri. On the other hand, Campos et al.[20] reported that paternal age had a detrimental effect on reproductive outcome of oocyte donation cycles when both men and recipient are ≥39 years old. Frattarelli et al.[21] evaluated 1,023 infertile couples undergoing an anonymous oocyte donation cycle. After controlling for donor age, they reported significantly lower PR when male age was >50 years. The LBR and miscarriage rates were 56.0% versus 41.3%, and 24.4% versus 41.5% in men ≤50 and >50 years (p<0.01), respectively.

It is possible that recipient age and age-related maternal factors such as uterine fibroids may bias results. A systemic review and meta-analysis including 12,538 oocyte-donation cases concluded that the available evidence did not suggest an association between advanced paternal age and adverse reproductive outcome in donor oocyte cycles, although the quality of evidence was suboptimal.[22] It is possible that DNA repair mechanisms within young oocytes corrects

exogenous and endogenous paternal DNA damage, thus overriding the effect of the ageing sperm. Long-term follow-up studies have found an association between advanced paternal age and an increase in *de novo* autosomal dominant disorders, autism, schizophrenia, impaired neurocognitive development, and increased risk of malignancy.[23]

## Oocyte Sharing

Oocyte sharing involves a woman sharing some of her eggs with another patient in exchange for free or reduced-cost fertility treatment.[24] This concept has generated considerable interest, especially in countries where commercial egg donation is not allowed or there is paucity of egg donors. The concern that reproductive outcome may be reduced in these patients has been negated, with similar PR being reported in both donors and recipients.[25] It is important to bear in mind, however, that such good results require meticulous donor screening for ovarian reserve, age, and cause of infertility. If the number of eggs recovered is lower or of poor quality, both donor and recipient's cycles may be compromised with neither having sufficient good quality embryos to transfer and/or cryopreserve, necessitating a fresh IVF cycle with its inherent costs. Oocyte vitrification and banking has changed the concept of oocyte sharing from one that involved sharing of oocytes between two subfertile couples to one where the eggs of a commercial donor are shared.

## Oocyte Banking

Reports of good PR with cryopreserved embryos[26,27] and the reduced constraints of donor-recipient synchronisation has encouraged the use of frozen embryo transfers. The availability of frozen oocytes and success of oocyte vitrification[9] has added to convenience, increasing the number of cryopreserved cycles. Data from a national registry show a trend towards the increase in use of frozen embryos: 26.7% to 40.3% from the years 2000–2010.[8]

Though a lower LBR with cryopreserved oocytes was shown by Kushnir et al.,[28] recent larger studies have shown a significant improvement in results. A retrospective cohort study compared pregnancy outcome using embryos generated from fresh versus frozen donor oocytes.[29] After adjusting for significant covariates and looking at overall cycles, those using a cryopreserved oocyte had lower PR (51.1% versus 58.5%; adjusted risk ratio [aRR] 0.88, 95% CI: 0.81–0.95), and LBR (43.0% versus 49.4%; aRR: 0.87, 95% CI: 0.80–0.95) compared to fresh oocyte cycles. However, looking only at cycles that reached embryo transfer, there was no evidence of differences in IR, PR, or LBR. Lower number of cycles were cancelled before embryo transfer with frozen oocytes (aRR: 0.74, 95% CI: 0.57-0.96). A decreased rate of miscarriage was seen with the transfer of one rather than two embryos. Cobo et al.[8] projected an oocyte-to-baby rate of 6.5% and found that the probability of achieving a baby increased progressively with the number of vitrified oocytes used, a plateau being reached at 25 oocytes.

## Clinical Factors Affecting Endometrial Receptiveness in Oocyte Donation Cycles

Apart from recipient age, the presence of hydrosalpinx, high BMI,[12] and tobacco consumption[30] are associated with a poorer outcome, while endometriosis and adenomyosis are not.[12] The effect of obesity was addressed in this study and the authors observed lower IR and PR, and higher miscarriage rates in women with a BMI ≥30 kg/m$^2$. A totally contradictory conclusion was drawn in a systematic review and meta-analysis undertaken to look at the effect of obesity on the chance of pregnancy in recipients. Evaluating >4,000 patients, the authors could not find an association between obesity (BMI: ≥30 kg/m$^2$) and chance of pregnancy after IVF-OD (risk ratio [RR]: 0.98, 95% CI: 0.83–1.15). Additional analysis assessing associations between recipient obesity and embryo implantation, miscarriage, and LB also failed to show a negative effect. However, most studies included were small and showed heterogeneity.[31] Interestingly, an increasing oocyte donor BMI is associated with a reduction in CP and LBR.[32]

## Racial and Ethnic Differences

Asian and Hispanic women undergoing oocyte donation did not have a reduction in CPR or LBR compared to white women. Black women, however, had a reduced chance of pregnancy and a trend toward lower LBR suggesting the contribution of uterine factors to reproductive outcome.[33]

## Maternal Complications

The literature suggests that infertility itself is a risk factor for maternal and perinatal complications, and women who conceive through use of 'high technology infertility treatments' are at an even higher risk.[34] Concerns about antenatal complications associated with the use of donor eggs were expressed from the early days of this

treatment. One of the earliest reports suggesting a very high rate of pre-eclampsia (PE) came from 1987, in a study by Serhal et al.[35] that included just 10 recipients. Apart from an increased risk of pregnancy-induced hypertension (PIH) and PE,[36-38] studies demonstrate an increased occurrence of first-trimester bleeding, gestational diabetes,[39] placental abnormalities,[40] intrauterine growth restriction (IUGR), preterm delivery,[40,41] prolonged maternal hospitalisation after delivery, and increased prevalence of caesarean section.[42] Stoop et al.,[43] in a study including 205 donor oocyte and 205 autologous oocyte pregnancies, stated that oocyte donation was associated with an increased risk for PIH (matched OR: 1.502, CI: 1.024–2.204) and first trimester bleeding (matched OR: 1.493, CI: 1.036–2.15), independent of the recipients' age, parity, and plurality, and independent of the age of the donor or the partner. Although the observed incidences for PE (11.8% versus 6.4%), HELLP syndrome (0.98% versus 0.59%) or gestational diabetes (7.4% versus 3.4%) were almost twice as high in recipient pregnancies, these differences were not statistically significant. No differences were observed between the two matched groups with regard to gestational age, mean birth weight and length, head circumference, and Apgar scores. Risk of complications increases with age and multiple pregnancy. The incidence of PE reported in different studies ranges from 9.8–12%[14,44] to 13–35%.[45,46]

In a national retrospective cohort case study,[42] a comparison was made between the obstetric outcome of women who conceived with donated oocytes (n=76), non-infertile nulliparous women who conceived spontaneously (n=115), and 63 women who conceived after non-donor IVF. Women who conceived with OD had a higher risk of hypertensive disorders (adjusted overall response [aOR]: 2.84, 95% CI: 1.04–7.81), oligohydramnios (aOR: 12.74, 95% CI: 1.24–130.49), postpartum haemorrhage (aOR: 7.11, 95% CI: 2.02–24.97), and retained placenta (aOR: 6.71, 95% CI: 1.58–28.40) when compared to women who conceived spontaneously, after adjusting for relevant variables. A similar trend was noticed when IVF-OD was compared to autologous IVF, although this was not statistically significant. More recipients had induction of labour (aOR: 2.80, 95% CI: 1.10–7.08) and caesarean delivery (aOR: 5.20, 95% CI 2.21–12.22) than women with autologous IVF. There were no differences in gestational length between the groups.

A systematic review and meta-analysis including 11 retrospective cohort studies[47] also reported a 3-fold increase in the likelihood of developing PE in pregnancies with IVF-OD compared to those achieved with autologous oocytes. The prevalence of PE was 17.2% (9–29%) in OD pregnancies, while it was 5.7% in IVF pregnancies (0–13%) ($p<0.001$). The meta-regression analysis showed that neither multiple pregnancies, nor patient age significantly explained the variability of the effect of oocyte donation on PE. Statistical evaluation ruled out heterogeneity between the studies. Although studies show a discrepancy in the clinical manifestation of hypertensive disorder (PIH/PE) the underlying factor appears to be placental dysfunction.

## Possible Causes of Pregnancy-Induced Hypertension/Pre-eclampsia in *In Vitro* Fertilisation with Oocyte Donation

PE and PIH are believed to be the result of an altered feto-maternal immune response resulting in reduced trophoblastic invasion of the spiral arteries, a precursor to the placental pathology witnessed in PIH/PE. It is postulated that the trophoblastic HLA-C in donated oocytes is less recognisable to the maternal immune system, being completely allogeneic. This possibly leads to an altered functionality of the uterine natural killer cells and consequently an altered maternal blood supply to the placenta.[48-50] Additionally, autoantibodies are associated with premature ovarian failure[51] and it is postulated that autoantibodies could lead to disruption of trophoblastic invasion. One must also bear in mind that there is an independent risk of nulliparity and age associated factors such as pre-existing hypertension and gestational diabetes. The contribution of family history of idiopathic hypertension may also be a predisposing factor.[52,53]

## Neonatal Complications

The donor oocyte risk rates for neonatal complications are higher than those found with use of autologous IVF. Neonatal complications include an increased risk of prematurity, extreme prematurity, small for gestational age (SGA), low birth weight (LBW), and very LBW (VLBW).[43,54,55] The increased rate of preterm delivery is probably responsible for the increased rate of LBW babies.[56] A good perinatal outcome in IVF-OD was reported in 27.5% recipients using fresh embryos,[8] with good perinatal outcome being defined as a singleton live-born infant delivered at ≥37 weeks and weighing ≥2,500 g. A comparison of singleton birth after

IVF with autologous oocytes and donor oocytes adjusted for maternal age and infertility factor, reported an increased risk of LBW (aOR: 1.21, 95% CI: 1.13–1.30), VLBW (aOR: 1.28, 95% CI: 1.10–1.49), and SGA with IVF-OD.[55] Results of a meta-analysis of 23 studies comparing perinatal health outcomes in pregnancies with autologous and donor oocytes showed similar perinatal outcomes.[54] The risk ratio for preterm (<37 weeks) births was 1.26 (CI: 1.23–1.30) and for preterm with LBW was 1.24 (CI: 1.19–1.29). LBW outcomes were improved in term donor oocyte neonates (RR: 0.86, CI: 0.8–0.93). Regarding perinatal mortality, similar rates are reported between IVF and IVF-OD singletons.[47]

### Prognostic Factors

Elective single embryo transfer and blastocyst transfer improved the perinatal outcome by an OR of 2.32 (95% CI: 1.92–2.80) and 1.17 (95% CI: 1.04–1.32), respectively. Infertility factors and ethnicity are also associated with perinatal outcome. The odds of good outcome decreased in tubal and uterine factor infertility and in non-Hispanic, black recipients. Surprisingly, recipient age was not associated with the likelihood of good perinatal outcome.[8] Twin pregnancies, pre-existing chronic pathologies, and development of obstetrics complications led to poorer perinatal outcome.[57]

## SHOULD THERE BE AN AGE LIMIT FOR RECIPIENTS? PREGNANCY IN WOMEN OF ADVANCED AGE

Judicious use of technology forms the core of good medical practice. The age at which women are attempting pregnancy is increasing and there are reports of births above the age of 60,[19] raising serious concerns on maternal safety. Based on longevity and health-related diseases, most physicians limit IVF-OD treatment to an age of 50 years. Guesdon et al.[46] reported on the obstetric's outcome in women below (45–49) and above 50 years. The rates of PIH and IUGR in singleton pregnancies was statistically higher in the older than in the younger group (19.2% versus 5.5%, and 30.7% versus 14.3%, respectively). Complication rates with twins were higher compared to singletons, but similar between groups.

The Committee of the American Society for Reproductive Medicine (ASRM),[58] after a review of existing literature, concluded that healthy women in the age range of 50–54 years who are well-prepared for parenting, are candidates to receive donated eggs. A thorough medical evaluation to assess physical fitness is mandatory before attempting transfer of embryos to any woman >45 years.

## CONCLUSION AND FUTURE RECOMMENDATIONS

The use of donor oocytes has expanded the scope of ART for women with poor oocyte quantity and quality. Implantation, pregnancy, and LBR are higher compared to IVF with autologous oocytes. This has encouraged increasingly older women to attempt pregnancy without understanding the inherent risks. Oocyte banking is becoming popular due to improved results achieved with vitrified oocytes. The global rise in viral infections underpin the importance of a mandatory quarantine period for oocytes. Use of vitrified oocytes from oocyte banks should be the way forward, in terms of both safety and procedure efficiency. A move towards single embryo transfer and a pre-procedure comprehensive health check will go a long way in reducing obstetric and neonatal complications. Although maternal age is more relevant to outcome, paternal age does appear to have an association with long-term neonatal health and further studies are required in that area.

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# TARGETING MAST CELLS AS A VIABLE THERAPEUTIC OPTION IN ENDOMETRIOSIS

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## ABSTRACT

Endometriosis is a chronic condition that affects ~10% of young women worldwide. Pain and infertility are the two most common features of the disease. The condition appears to be sex hormone-dependent, although a subset of females with the condition still experience symptoms post-menopause. The aetiology of endometriosis induction still remains elusive, and surgery to remove the lesions often fails to cure the condition, as the lesions often reappear. The lesions contain stromal cells, blood vessels, nerves, and numerous mast cells. In some respects, endometrial lesions resemble a chronic fibrotic scar-like tissue that does not resolve. Studies in other fibrotic abnormal healing conditions have revealed that targeting mast cells, as a central component of what is called a 'neural–mast cell–fibroblast' axis, by repurposing asthma drugs can prevent induction of the abnormal healing phenotype. Given the similarities between conditions with abnormal healing phenotypes and endometrial lesions, it is postulated that taking a similar approach to target endometrial lesion mast cells could exert a benefit for patients with endometriosis. This review also outlines approaches to assess the likelihood that targeting mast cells could lead to clinical trials using such 'repurposed' mast cell targeted drugs.

Keywords: Mast cells, mast cell stabilisers, endometriosis, cell biology of endometriosis, neuroinflammatory pathways, biochemistry of endometriosis.

## PREAMBLE

This review is intended to focus on the rationale and feasibility of targeting mast cells in endometriosis, and discussing selected findings in the literature related to this focus. Due to space limitations, it is not intended to be an exhaustive review of all aspects of endometriosis. Thus, the reader is encouraged to read the large number of recent reviews in the literature which address other aspects of this complex disease to complement the discussion below.

## THE CLINICAL PROBLEM

Endometriosis is an inflammatory condition that affects ~10% of young women of reproductive age.[1] The disease appears to be sex hormone-dependent as symptoms can vary across the menstrual cycle, with pain as a prominent symptom. The condition is also frequently accompanied by dysmenorrhoea and infertility, with the presence of pelvic abdominal lesions. Conservative treatment with anti-inflammatory drugs is often ineffective, and surgical removal of the lesions becomes a viable alternative.[2] However, after surgery, the condition can reappear with pain and associated lesions. As the condition is chronic, it can be accompanied by epigenetic changes that may complicate the effectiveness of treatment.[3,4]

The disease is characterised by the growth of endometrial elements outside of the uterine cavity. Why this occurs is currently unknown, and what factors predispose or contribute to this very common condition in a subpopulation of younger females is not yet evident. It may result from retrograde menstruation,[5,6] with attachment and

growth of endometrial tissue in this environment. The tissue associated with endometriosis contains blood vessels, nerves, mast cells, myofibroblasts, and macrophages,[7-10] and, thus, appears to be a fibrotic lesion that in some respects resembles a progressive scar. As endometriosis tissue can be influenced by sex hormones,[11-13] and possibly neuroinflammatory elements,[14] it may resemble abnormal scarring such as occurs following a burn injury (hypertrophic scar), or following a traumatic elbow injury leading to a joint contracture.[15,16] Thus, the initiating events may differ but the cellular and molecular interactions leading to fibrotic progression may share a number of commonalities. Interestingly, endometriosis has also been linked with risk for other chronic diseases such as asthma,[17,18] in which mast cells also play a central role.

## PRECLINICAL MODELS

Endometriosis can occur naturally in horses[19] and non-human primates,[20] and can be induced in rats by auto-transplantation of uterine tissue to the abdomen, or in nude mice by transplantation of human endometrial tissue.[21,22] Such models can provide some insights into aspects of endometriosis development and progression, as well as insights into potential interventions, but, thus far, the aetiology of endometriosis in humans and in non-human primates remains undefined.

## ROLE OF SEX HORMONES IN ENDOMETRIOSIS

As discussed above, endometriosis is a sex hormone-mediated inflammatory disease. With regard to endometriosis, oestrogen has been implicated in the activity of macrophages,[10] fibroblasts and myofibroblasts,[13] nerves,[10,12] and mast cells.[7,23] It is also clear that oestrogen is a regulator of inflammatory processes, and activation of inflammatory cells can contribute to nerve fibre-mediated pain.[14] In addition, sex hormones, such as oestrogen, are known to contribute to wound healing in response to injury.[24,25] Both oestrogen and progesterone have been implicated in mast cell degranulation.[26]

After menopause, inflammatory processes decline, wound healing is compromised, and endometriosis in most patients becomes latent.[5,27] However, 2–4% of women still continue to experience endometriosis after menopause.[5] Therefore, either alternative mechanisms could potentially allow the disease to continue in this subpopulation (e.g. due to epigenetic modifications[4,28]) or local production of oestrogen could continue to contribute to disease activity in the post-menopausal state.

While endometriosis is considered oestrogen-dependent, in part based on menstrual cycle dependency, a role for progesterone is also likely.[29] Furthermore, the impact of sex hormones could be a secondary sequela of hormone level changes (e.g. water retention with increased turgor of endometrial lesions contributing to pain).

## REGULATION OF PROTEINASE EXPRESSION IN ENDOMETRIOSIS

### Role of Sex Hormones and Their Receptors

Proteinases such as tissue factor,[30] elements of the fibrinolytic system,[31] cathepsins,[32] mast cell tryptase and chymase,[33] and matrix metalloproteinases (MMP)[34] have all been implicated in endometriosis. As these enzymes can facilitate extracellular matrix turnover, activate pro-enzymes and other molecules, and serve other functions related to inflammation, coagulation, collagen deposition, and angiogenesis, they are likely central to endometriosis progression. In this regard, their role is likely not different from their roles in other fibrogenic processes and wound healing.

Much research has focussed on the MMP, their expression in endometriosis, and their regulation by progesterone[35,36] and oestrogen.[34] Progesterone appears to limit MMP expression,[35,36] while oestrogen is reported to enhance MMP-9 expression,[37] and MMP-9 levels are enhanced in endometriosis.[38] As oestrogen functions primarily via estrogen receptor (ER)-alpha and ER-beta in cells, receptors known to be expressed in endometrial tissue,[39,40] this response is potentially contributing to the symptoms of endometriosis and its progression. However, the role of oestrogen receptors in endometriosis is somewhat controversial,[39,40] with ER-beta a more prominent variant in endometriosis than normal tissue.[39]

Interestingly, oestrogen receptors in the absence of oestrogen can also regulate expression of some MMP[41-43] and these include MMP-1 and MMP-13. The addition of oestrogen actually depressed expression rather than enhancing expression. ER-beta was more effective than ER-alpha in many of these responses, and, interestingly, genetic variants of the *MMP-1* promoter region were also

differentially regulated. Some of these same variants, as well as those for *MMP-2*, *7*, and *9* were found to be associated with risk of endometriosis.[44] Intriguingly, splice variants of ER-beta that do not bind oestrogen were also effective in upregulating expression of *MMP-1*,[45] and splice variants have also been detected in endometriosis tissues.[46]

## MAST CELLS IN ENDOMETRIOSIS

Mast cells, often thought of in the context of allergies or asthma, are present in most tissues throughout the body, including connective tissues and many organs.[7,15,16] Mast cells are also very prevalent in normal and abnormal healing environments, with elevated numbers, in abnormal healing conditions.[7,15,16] Mast cells are very prevalent in endometriosis tissue, and many of them appear to be activated and degranulated.[47-49] In addition, many of the mast cells were localised very close to neural elements,[49] and, as such, could play an active role in neuroinflammatory processes.[7,15,16] We have previously seen neural elements ending in normal dense connective tissues very close to a mast cell, implying that nerve-mast cell co-localisation may also play a role in normal tissue functioning as well.[7,15,16]

Mast cells and their products could contribute to several features of endometriosis. Mast cell tryptase can activate protease activated receptors (PAR), particularly PAR-2.[50] PAR are known to be expressed in endometrium and endometriosis.[51] Activation of PAR-2 on cells may participate in pain processing[52] and angiogenesis.[53] Mast cell tryptase can also activate myofibroblasts and contribute to fibrosis.[15,16] Histamine released from mast cells has been studied for decades and has multiple activities, including enhancing tissue oedema and many other effects. Activated mast cells can also release a number of pro-inflammatory cytokines, mediators, and growth factors, and thus, can have a very potent and varied impact on a target tissue, particularly one that appears to be abnormally regulated, as in endometriosis. The enhanced presence of mast cells, many of which appear to be activated, in endometriosis tissue has led to the proposal that mast cells should be targeted with drug interventions to assist in controlling endometriosis progression and symptoms.[7,23,54,55] Based on the findings that activated mast cells are present in endometrial tissues, oestrogen/progesterone can influence mast cells, mast cells can release many biologically active molecules, and mast cell numbers are increased in endometrial tissues, this cell may be an excellent cell to target in endometriosis, alone or in combination with other targets.

## MAST CELL STABILISERS

Mast cell stabilisers are drugs which inhibit or prevent mast cell degranulation. These include two drugs, ketotifen and sodium cromoglycate, that have a long history of use in the treatment of asthma. While ketotifen is now off patent protection, it has been used safely for >40 years in adult and paediatric populations. It is not totally targeted specifically for mast cells, and is also reported to inhibit degranulation of polymorphonuclear leukocytes.[17,18] Thus, with the development of new potential indications (e.g. endometriosis), there may be an impetus for industry to develop newer versions that may be more specific for mast cells, or more active in specific diseases or conditions.

## USE OF MAST CELL STABILISERS IN ABNORMAL FIBROPROLIFERATIVE CONDITIONS

Abnormal healing follows induction of skin injuries in the red Duroc pig[56,57] and following trauma and immobilisation of a knee injury in a rabbit model.[58] The former exhibits characteristics of hypertrophic scarring and also fibrogenic scarring.[59] In the red Duroc pig, this abnormal scarring response appears to have a genetic component.[60] The rabbit model exhibits cellular and molecular characteristics of joint contractures that occur in a subset of humans who experience an elbow injury.[15,16]

In these two models, elevated numbers of mast cells, nerves, and myofibroblasts have been observed leading to the conclusion that the cells contribute to fibroproliferative dysfunction. These cells have been postulated to form a cellular 'axis' (Figure 1) in which the mast cell plays a central role.[7,15,16] In this proposed axis, the fibroblasts and myofibroblasts are the effector cells which release fibrotic molecules, such as collagens, and contract the fibrotic matrix. Neuropeptides from nerves or, in the case of endometriosis, possibly sex hormones, impact mast cells, which then release molecules that enhance the activity of fibroblasts and myofibroblasts. Thus, the mast cells function as accelerators or amplifiers of the fibrotic environment. This axis is postulated to be

dysfunctional in the two models, but whether the initiator of the dysfunction is only due to the nerves (e.g. neuroinflammation), or some other cell type or stimulus, remains to be determined. Treatment of skin wounds on red Duroc pigs with the asthma drug ketotifen from the time of injury prevented development of the abnormal fibrogenic response to injury and led to a more typical healing response.[57]

If treatment was delayed until 28 days post-injury, the drug was without effect. Stopping the drug treatment after epithelisation of the wounds did not lead to a reactivation of the abnormal fibrogenic response. Thus, this mast cell stabiliser appeared to exert its influence early in the response to injury. Interestingly, treatment with ketotifen led to a decline in detectable nerves, mast cells, and myofibroblasts in the skin scar tissue, which likely also indicated that the postulated axis was not unidirectional and interfering with mast cell degranulation impacted other elements of this axis. Finally, treatment of skin wounds with ketotifen in Yorkshire pigs that healed normally was without effect and did not influence the number of cells in the axis that could be detected. Thus, the drug did not appear to influence normal healing of skin wounds.

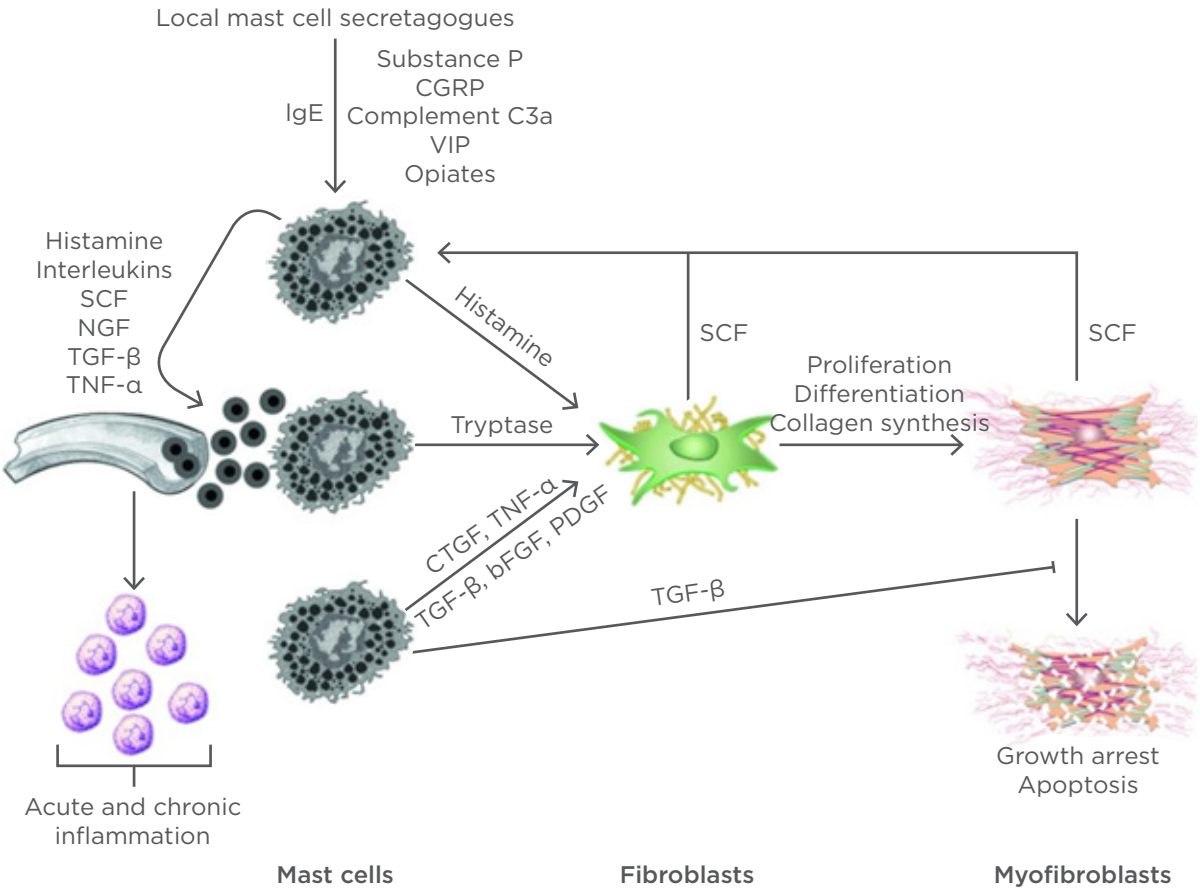


**Figure 1: Mast cells mediated inflammation and fibrosis.**

Mast cells circulate as CD34-positive precursor cells and terminally differentiate in connective tissues. Both IgE-dependent and independent mechanisms can activate mast cells causing the release of preformed and newly synthesised pro-inflammatory mediators. Many of these mediators increase vascular permeability and promote the recruitment of other inflammatory cells and additional mast cell precursors. SCF is also secreted by activated fibroblasts and myofibroblasts, further potentiating mast cell recruitment and proliferation. TGF-β is a potent fibroblast mitogen and stimulator of myofibroblast differentiation. It also impedes myofibroblast apoptosis.

bFGF: basic fibroblast growth factor; CGRP: calcitonin gene-related peptide; CTGF: connective tissue growth factor; IgE: immunoglobulin E; NGF: nerve growth factor; PDGF: platelet-derived growth factor; SCF: stem cell factor; TGF-β: transforming growth factor beta; TNF-a: tumor necrosis factor alpha; VIP: vasoactive intestinal peptide.

*Adapted from Monument et al.*[16]

**Table 1: Potential clinical pathway to confirming mast cells as a therapeutic target in endometriosis.**

| |
|---|
| **Phase 1: Amass evidence for mast cell activation in patients with endometriosis** |
| Assess mast cell tryptase in serum of age matched individuals with and without established endometriosis at three defined points during their menstrual cycle using a validated ELISA.[63] If indeed mast cell degranulation is involved in endometriosis activity and symptoms across the menstrual cycle, the ELISA results should parallel symptoms. |
| As surgery is recommended for many patients with endometriosis, one could assess serum mast cell tryptase levels before and post-surgery during specific points in the menstrual cycle to address the question of whether serum tryptase levels can be used as a biomarker for a return of the endometriosis. |
| Histologic assessment of the nerve–mast cell–myofibroblast axis (Figure 1) in tissue obtained at the time of surgery, using immunolocalisation protocols developed for assessing human joint contracture tissues.[58] |
| *In vitro* explant studies using tissue obtained at the time of surgery. Such tissues could be incubated plus or minus ketotifen and supernatant levels of tryptase assessed using an ELISA.[63] A search of the literature did not reveal whether such surgery is preferentially performed at specific times in the menstrual cycle of patients, but certainly this may impact the results of the studies outlined. |
| **Phase 2: Short-term ketotifen trial in endometriosis patients scheduled for surgery** |
| Patients would be randomly assigned to a ketotifen arm or a sham solution arm of the protocol (oral dosing of ketotifen or sham solution) in a blinded fashion. Daily diaries for symptoms would be maintained for the 3 months prior to surgery, as well as serum levels for mast cell tryptase (blood draws once per month at the optimal timing). Tissue obtained at the time of subsequent surgery could be assessed for nerves, mast cells, myofibroblasts, and macrophage subsets, by immunolocalisation techniques, as well as RNA isolated from the tissue by RT-qPCR, and incubation of culture supernatants for detection of relevant proteins by established array technologies. |
| **Phase 3: Large scale clinical trials of ketotifen (single-centre and multicentre)**<br>These trials could take many designs, optimal designs determined by clinical experts to yield the most information and maintain patient safety. |
| Ketotifen pilot trial in patients: pain assessments (diary) for 3 months prior to drug or sham initiation and then daily for 3 months. Washout for 3 months and then cross over for 3 months. |
| Ketotifen implementation trial: Cohort of patients to receive ketotifen or sham for 12 months and then stop and follow for a defined period of time to determine whether any improvements revert following cessation of treatment. |
| Large-scale RCTs: Double-blind multicentre trials |

ELISA: enzyme-linked immunosorbent assay; RCT: randomised controlled trial; RT-qPCR: quantitative reverse transcription polymerase chain reaction.

Similar studies in the rabbit model of post-traumatic joint contractures using ketotifen treatment also led to improvement in the healing process and significant declines in the extent of the joint contractures (˜50%), as well as cells of the nerve–mast cell–myofibroblast axis.[61,62] The suggestion that ketotifen was likely inhibiting mast cell degranulation, arose from recent studies demonstrating that serum levels of mast cell tryptase were significantly depressed in the treated rabbits.[63]

The mast cell stabiliser sodium cromoglycate has also shown effectiveness in a rat model of experimental endometriosis.[55] In this model, transplantation of endometrial tissue to the abdominal wall followed by a 2-week treatment regimen with sodium cromoglycate led to significant declines in activated mast cells, as well as tissue levels of mast cell tryptase and serum levels of tumour necrosis factor alpha. However, the size of the lesions was not apparently influenced by the treatment. As myofibroblasts have also been identified in endometriosis lesions,[9] as well as nerves,[49] it would be of interest to assess the influence of mast cell stabiliser treatment of the transplanted tissues on levels of these two cells as well, particularly since mast cells and nerve elements have been detected in close proximity in human endometriosis tissues.[49] Interestingly, close proximity of nerves and mast cells have been noted in unrelated tissues, and it appeared that neuropeptides released from nerves could impact the mast cells (Figure 1), leading to an amplified impact on the target tissue.

From our studies using ketotifen,[57,61,62] and those of Zhu et al.[55] with sodium cromoglycate, inhibition of mast cell degranulation may be a viable direction to explore. In contrast, D'Cruz and Uckun[54] proposed using a janus kinase (JNK) 3 inhibitor, JANEX-1, and recently a JNK inhibitor has been reported to cause regression of endometriotic lesions in

rodent models.[64] While targeting mast cells using these approaches has not yet led to clinical trials in endometriosis, a clinical trial is currently underway using ketotifen to prevent joint contracture development following traumatic elbow injuries.[65]

## Are There Commonalities in Dysregulated Fibrogenic Responses to Inflammation and Injuries?

Joint contracture development following a trauma to the elbow occurs in 10–15% of those injured.[15,16] Development of a contracture is accompanied by a fibrotic response in which the healing process is dysregulated and does not proceed through to maturation and remodelling of the scar. In many cases, the patients undergo surgery to release the contracture, but often with only partial recovery.[15,16]

Similarly, development of a hypertrophic scar after a severe burn injury is also a fibrotic response to the injury, with excessive matrix deposition, myofibroblasts, and mast cells. For many patients, the hypertrophic scars have to be surgically removed. Interestingly, female sex is also a risk factor for hypertrophic scar development.[66] The abnormal fibrogenic skin wound healing, in the previously discussed porcine models, also exhibits some similarities with hypertrophic scarring.

Given these similarities, perhaps much of the uniqueness of endometriosis is associated with where the abnormal fibrotic tissue is located, its sex hormone dependency, and the sequelae of the fibrosis, rather than any intrinsic uniqueness in the cellular aspects of the dysregulated processes contributing to progression and chronicity. Thus, some of what has been learned from these other diseases or conditions, as well as from the preclinical models discussed, could provide insights into focussing research directions forward with mast cells as a target.

## Future Directions for Assessment of Efficacy and Implementation of Mast Cell Stabilisers in Patients with Endometriosis

To undertake a clinical trial of mast cell stabilisers, such as ketotifen or sodium cromoglycate, in populations of young females with endometriosis, investigators must proceed with caution as many of these individuals are of reproductive age, and mast cells have been implicated in normal ovulation events and others related to reproduction in some species.[67] However, the complexity of endometriosis in patient populations could be approached in phases to strengthen the link between mast cell activity and symptoms and then identify those best suited to participate in pilot trials using the mast cell stabilisers (Table 1).

One of the current gaps in endometriosis research is a lack of good biomarkers, such as serum components, that can be used to monitor disease activity, assess the impact of interventions, or assess the return of disease activity following surgery prior to overt symptoms being evident. Serum mast cell tryptase levels, as assessed by an enzyme-linked immunosorbent assay[63] could contribute to filling this gap.

Finally, it is clear that epigenetic changes occur during progression of endometriosis.[3,4,28] These changes (DNA methylation, histone modifications, and alterations to miRNA profiles) can lead to alterations in cell responsiveness to interventions. In some chronic diseases (e.g. rheumatoid arthritis), these changes can occur in fibroblasts and other cells. Thus, endometrial lesions early in the disease may respond differently to targeting mast cells than those with more advanced disease. Such factors may need to be considered when assessing effectiveness.

## CONCLUSIONS

Based on the above discussion, there is considerable circumstantial evidence to support the use of mast cell targeted drug interventions in the treatment of endometriosis. There is an advantage for using known drugs such as ketotifen and sodium cromoglycate, both with long track records of use for the treatment of asthma in a variety of populations. Thus, their safety and efficacy is well documented. However, repurposing these drugs is a viable approach, but one that will require a systematic analysis in patient populations (Table 1). Given the large population of females with endometriosis (5–15% of females worldwide), and the impact of the condition on these young women, new approaches that could impact their quality of life should be entertained. However, this approach using mast cell targeted drugs may address the how of the disease, but not why the disease occurs, so it would only be a stop-gap approach until new information arises as to why it develops, and what is unique about the subset of women who experience the disease (e.g. genetics, epigenetics, exposure to environmental stimuli, and stochastic events).

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# PARENTAL PROTEIN MALNUTRITION PROGRAMMES OF OFFSPRING GROWTH AND VASCULATURE TO INCREASE RISK OF CARDIOVASCULAR, PANCREATIC, AND METABOLIC DISEASE. LESSONS LEARNED FROM ANIMAL STUDIES

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**Search Strategy:** The literature used to inform this narrative review was searched using PubMed and Web of Science using the keywords: maternal, paternal, protein, growth, vasculature, cardiac, cardiovascular, pancreas, metabolic disease, animal, mouse, and rat. The topic is broad and this manuscript is not a full, systematic review: simply a narrative review informed by the authors' own work in the area. Due to space and time constraints, the authors acknowledge that many good-quality papers have not been referenced.
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## ABSTRACT

It is well known that consumption of a balanced diet throughout adulthood is key toward maintenance of optimal body weight and cardiovascular health. Research using animal models can provide insights into the programming of short and long-term health by parental diet and potential mechanisms by which, for example, protein intake may influence fetal development, adolescent health, and adult morbidity/mortality. Malnutrition, whether consumption of too many or too few individual nutrients or energy, is detrimental to health. For example, in Westernised societies, one of the principal factors contributing towards the global epidemic of obesity is over-consumption of calories, relative to the expenditure of calories through physical activity. A large body of evidence now suggests that many chronic diseases of adulthood, such as obesity and diabetes, are linked to the nutritional environment experienced by the fetus *in utero*. Maternal consumption of a poor-quality, nutritionally unbalanced diet can programme offspring to become obese, develop high blood pressure and diabetes, and to experience premature morbidity and mortality. More recently, paternal diet has also been shown to influence offspring health through effects carried via the sperm that affect post-fertilisation development. Mechanisms underpinning such developmental programming effects remain elusive, although early development of the microvasculature in the heart and pancreas, particularly after exposure of the mother (or father) to a protein restricted diet, has been proposed as one mechanism linking early diet to perturbed adult function. In this brief review, we explore the longer-term consequences of maternal and paternal protein intakes on the progeny. Using evidence from relevant animal models, we illustrate how protein malnutrition may 'programme' lifelong health and disease outcomes, especially in relation to pancreatic function and insulin resistance, and cardiac abnormalities.

Keywords: Protein restriction, accelerated growth, cardiac, insulin, pancreas.

## THE IMPACT OF MATERNAL NUTRIENT INTAKE ON THE HUMAN FETUS

Food intake and appetite are driven primarily by the need to ensure sufficient protein intake (the reference range for 'normal' human protein consumption is 80–120 g/day or 15–20% of total calories ~1,500–2,500 kcal/day).[1] Dietary protein dilution through consumption of nutrient-poor, energy-rich food engenders increased energy intake. Although undernutrition has historically been of greater concern to developing or low-income nations, a recent study showed that even in countries with a high gross domestic product rating, 38% of pregnant women were not reaching the 16% recommended protein intake.[2] Indeed, rather surprisingly, the World Health Organization (WHO) estimates that there were 1.1 million children ≤2 standard deviations weight-for-height (an index of protein energy malnutrition [PEM]) in the USA.[3] Insufficient supply of vital nutrients, such as amino acids and metabolites generated by amino acid oxidation, during the reproductive years, can have a plethora of direct effects on the mother and her developing fetus and indirectly affect the subsequent individual as they grow toward adulthood.[4]

## JUSTIFICATION AND LIMITATIONS OF ANIMAL MODELS

Ever since Barker first proposed that adult disease, especially heart disease, could be linked to low birthweight via fetal nutrition,[5,6] a number of clinical[7] and many laboratory animal studies[8,9] have been carried out to confirm the biological validity of the hypothesis. Animal models are widely used as potential mechanistic insights can be achieved more readily than in human epidemiological studies. Environmental and genetic[10] variation may be controlled whilst individual nutrients, such as protein, are manipulated through pregnancy and/or lactation. In addition, longer-term follow-up is possible in a relatively short time (a rat reaches middle age ~18 months of age). Animal models therefore allow for adult interventions to be tested to assess potential amelioration of the programmed phenotype, including multi-generational transmission over a reasonable timescale: a response not possible in clinical cohorts. As a case-in-point, 12 generations of protein malnutrition in rats (i.e. 6.8% protein as a percentage of total calories when protein adequacy in pregnant rats is considered to be 12–18% protein intake) had diverse effects on offspring, including lower birth weight, body size, and fertility; the effects reversed within three generations of nutritional rehabilitation.[11] Each laboratory or farm animal used to 'model' a given experimental intervention with potential resonance for human cohorts needs to define why that animal is appropriate whilst accepting the potential limitations. These issues are too broad for a full discussion here, but readers should consider the following reviews.[12,13]

## THE IMPACT OF MATERNAL MALNUTRITION ON THE ANIMAL FETUS

The first animal studies to model maternal protein-energy malnutrition in the lab showed that the offspring were physiologically compromised in many ways, such as having higher blood pressure[14] and reduced glucose tolerance.[15] An alternative experimental approach that generated intra-uterine growth restricted offspring (a common sequelae of PEM in developing countries) through bilateral uterine artery ligation from Day 19 of a 65–70 day gestation in the guinea pig, resulted in lower birth weight offspring that demonstrated subsequent accelerated catch-up growth.[16] At 15 weeks postnatal age, pancreatic beta cell mass in nutrient restricted guinea pig offspring was 50% that of controls, and by 20 weeks offspring were obese with glucose intolerance that, by 26 weeks, had developed into insulin resistance, a hallmark of Type 2 diabetes mellitus.[16] Hence, malnutrition *in utero* can increase susceptibility to Type 2 diabetes mellitus, partially mediated by accelerated childhood catch-up growth and a tendency toward obesity. Subsequent clinical epidemiological studies validated early accelerated growth as a predictor of cardiovascular mortality.[17] Lower birth weight was also identified as a possible outcome when maternal caloric intake (as oppose to more severe uterine artery ligation) was restricted in rats,[18] an effect also observed in human cohort studies.[19-21] Furthermore, obesity was common in those individuals exposed to malnutrition during pregnancy.[22] For further in-depth reviews of the developmental programming of metabolic syndrome, see references.[8,13,23]

As these studies became more refined in either the nutritional intervention or timing of malnutrition during gestation/lactation/post-weaning, a number of more direct effects of PEM have been observed: in late gestation, protein dilution tends to result in reduced birthweights with the offspring exhibiting catch-up growth post-natally. A number of theories have been proposed to account for this

phenomenon, with the 'thrifty' phenotype remaining popular.[24,25] Such a growth trajectory, relative restriction followed by un-restricted growth, has been proposed to underpin accelerated age-related decline, with individuals having shorter telomeres (in particular in the kidney).[26] For example, Mortensen et al.[4] found that murine newborn pups had 40% lower birth-weight when dams were fed 8% casein by weight throughout gestation, as compared to controls (20% casein). Similarly, intrauterine growth appeared impaired in mice at embryonic day (E)14.5 (a 16.5% reduction in weight) and E18.5 (a 13% reduction in weight) when fed 9% versus 18% protein (casein used as protein source) up to that point in gestation.[27] A number of studies found that protein malnourished (9% casein) laboratory rodents have a shorter lifespan.[23,28] Such early effects of maternal protein malnutrition on the developing fetus, when demand is unlikely to exceed supply of amino acids, suggests that the effects of maternal malnutrition are uncoupled from a simple supply of nutrients for energy. Indeed, in a recent study in sheep, 50% restriction of protein intake (a reduction from 18% protein to 9%; the dietary reference range for pregnant sheep is 10–12% crude protein) from Day 0 to Day 65 (0.45 gestation) had no effect on maternal, amniotic, or fetal plasma amino acid concentrations, with the exception of urea, which was significantly reduced, suggesting reduced protein turnover.[29]

## EFFECTS OF MATERNAL MALNUTRITION ON THE PLACENTA

The greatest proportion of cell differentiation, fetal organogenesis, and angiogenesis occur during the first trimester of pregnancy. Before establishment of the placenta, possible nutrient or endocrine effects on the blastocyst can only be histotrophic. Thereafter, adverse placental development during the first trimester has the potential to impact second and third trimester development of the fetus, long after the nutritional conditions have changed.[30] Placental weight, size, and vascularity can be considered indicators of placental function, and have a close relationship with fetal weight.[31] Maternal diet has been shown to affect these placental factors in a number of species.[27]

## EFFECTS OF MATERNAL MALNUTRITION ON PROGRAMMING OF OFFSPRING METABOLIC HEALTH

A number of studies have been conducted into the effects of maternal protein restriction on the fetus in relation to metabolism, insulin resistance, and obesity. Fernandez-Twinn et al.[32] discovered that maternal protein restriction (rats: 8% versus 20% protein; gestation and lactation) led to hyperinsulinaemia and reduced muscle insulin-action i.e. peripheral insulin resistance in the 21-month old offspring. In protein restricted exposed mice where accelerated postnatal catch-up growth was not observed (8% versus 20% casein, conception to weaning), no differences in adult glucose tolerance were noted relative to protein replete controls.[33] These effects of protein dilution on offspring metabolic phenotype appear highly influenced by sex; young males appear unaffected but exhibit a rapid age-related decline, whereas females are relatively unaffected throughout adulthood (at least to 21 months of age).[25,32,34] Second-generation rat studies have also shown persistent adverse effects on glucose and insulin metabolism.[35] From the first animal studies to have specifically investigated the developmental programming paradigm circa 1994 until 2010, the clear focus was on the delayed developmental consequences of maternal malnutrition, with almost complete disregard to any paternal contribution (despite contributing 50% of the fetal genome). The rationale for this was clear: the fetus developed in the maternal environment and early studies had illustrated how it is the maternal environment that had the greatest impact on offspring.[36] However, in 2006 Pembrey et al.[37] suggested for the first time that developmental programming effects may be passed transgenerationally down the paternal lineage. There was experimental confirmation of an exclusive male-line effect in 2010,[38,39] as outlined below.

## EFFECT OF PATERNAL MALNUTRITION ON PROGRAMMING OF OFFSPRING HEALTH

In a series of independent studies it was shown that paternal diet alone could influence metabolic health of the next (F1 generation) and further generations (F2, F3) of offspring.[38,39] Further studies subsequently outlined paternal programming of metabolic control[40] and hepatic repair processes,[41]

obesity,[42] diabetes,[43] and cardiovascular function.[44] Mechanisms for paternal programming via maternal protein malnutrition are currently being elucidated, but by definition must be transmitted via sperm. Recent work has implicated alterations to the methylation of ribosomal DNA in sperm,[45] an effect most pronounced during early specification of the gonads, as oppose to during adolescence.[46] Collectively, these reports demonstrate non-genetic, intergenerational transmission/programming of an 'acquired phenotype' after paternal malnutrition alone. They illustrate that the environmental burden on offspring phenotype is not just the territory of the mother. The implication for future generations is clear: malnutrition during early development can exert its deleterious effects for many generations to come, even after correction of the nutritional inadequacy. The likely mechanism is epigenetic inheritance. Jean-Baptiste Lamarck would be proud.

## PROGRAMMING OF OFFSPRING CARDIAC FUNCTION

Cardiac dysfunction has been observed in rats subjected to maternal protein malnutrition (9% versus 18% casein prior to mating, throughout pregnancy until weaning).[7,47,48] Effects were seen in newborn offspring (altered cardiomyocyte apoptosis, depressed cardiac ejection fraction) and in aged male and female adults (40 weeks of age) that had left ventricular hypertrophy and increased pulse pressure.[7] Hypertension has also been observed in 7 week old offspring (rat: 9% versus 18% casein prior to mating, throughout pregnancy), and although the maternal protein restricted offspring were born smaller, pup weights were unaltered in comparison to controls at 7 weeks of age.[49] Elevated systolic blood pressure after maternal protein restriction appears to be a relatively consistent outcome regardless of the period of nutritional restriction even when protein malnutrition is confined to the period prior to mating or very early during gestation[50] although others have noted reduced systolic arterial pressure with elevated sympathetic tone in rats 90 days postpartum (8% versus 17% casein; gestation and lactation).[51] Tappia et al.[47] also observed maternal protein restricted-exposed rats to have depressed ejection fractions in the first 4 weeks of life, increased left ventricular internal diameter and posterior cardiac wall thickness during both diastolic and systolic phases of the cardiac cycle to 8 weeks of age, together with a multitude of changes to cardiac gene expression. Recently, maternal protein restricted (8% versus 20% throughout gestation; rat) induced accelerated postnatal growth to 22 days postnatal age that was accompanied by increased cardiac nitrosative and oxidative-stress with concomitant increases in DNA damage and base-excision repair enzyme expression.[52] Indeed, greater effects on the heart appear to be observed when maternal protein restriction is followed by accelerated post-natal growth during adolescence.[53,54] Furthermore, it is Increasingly becoming apparent that protein-malnutrition induced programming of cardiovascular function is highly sex-specific[55] with males, in general, being more susceptible than females. Exact mechanisms have not been elucidated, although age-related changes in sex-hormone status and cellular action, coupled with programmed alterations to hypothalamic-pituitary-adrenal axis sensitivity are central to the effect.[56,57]

## CONCLUSIONS

Maternal or paternal consumption of a protein-restricted diet can have a range of physiologic outcomes in the offspring. In general, lower weight at birth is followed by accelerated post-natal growth. The latter has become designated as catch-up but may simply be regression to the mean. Nevertheless, accelerated early growth appears as powerful a programming stimulus as disproportionate fetal growth. The consequences for the 'programmed' individual have now extended beyond concern for the F1 generation to the F2 and F3 generations as epigenetic inheritance of an acquired phenotype becomes a reality. The fetal origins hypothesis matured into the developmental programming of adult health or disease, to encapsulate the multivariate effects on the fetus, the neonate and the growing adolescent individual. The paradigm has recently been extended to include paternal diet and in the years to come will no doubt be extended further to include grandmaternal and grandpaternal effects as the children of prospectively studied cohorts age and have families of their own. What is obvious is that the dire effects of early malnutrition will have an impact on populations for many generations to come.

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# New Policy Statement Issued on Human Germline Gene Editing

> "While germline genome editing could theoretically be used to prevent a child being born with a genetic disease, its potential use raises a multitude of scientific, ethical, and policy questions. These questions cannot be answered by scientists alone, but also need to be debated by society."

A NEW policy statement on germline genome editing was recently issued by an international collection of 11 organisations, including the International Genetic Epidemiology Society, the National Society of Genetic Counselors, and the American Society of Human Genetics, all of which have genetic expertise. Although this policy supports the research endeavour to further comprehend the extent to which germline editing can be applied clinically, it recommends against the performance of germline genome editing that could result in human pregnancy.

Since its introduction in 2013, the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 gene editing technique has become increasingly widely implemented, due to its customisability and efficacy across cell types and species. Therefore, this policy aims to address CRISPR/Cas9 editing in germline cells. In this regard, there are complex ethical considerations, as editing this cell type would ultimately affect future generations, not just the treated individual.

Lead author Prof Kelly E Ormond, Department of Genetics, Stanford Center for Biomedical Ethics, Stanford University, Stanford, California, USA, commented on the collaborative efforts during the drafting of the policy: "Our workgroup on genome editing included experts in several subfields of human genetics, as well as from countries with varying health systems and research infrastructure." She continued: "Given this diversity of perspective, we are encouraged by the agreement we were able to reach and hope it speaks to the soundness and wider acceptability of our recommendations."

The final policy statement covered the following positions:

- At this time, it is inappropriate to perform germline gene editing that culminates in human pregnancy; and
- There is currently no reason to prohibit *in vitro* germline genome editing research, with appropriate oversight and consent, or to prohibit public funding for such research[1]

When considering future clinical application of human germline genome editing, they concluded that it should not proceed unless there is:

- A compelling medical rationale
- An ethical justification
- An evidence base to support its clinical use
- A transparent public process to solicit and incorporate stakeholder input[1]

"While germline genome editing could theoretically be used to prevent a child being born with a genetic disease, its potential use raises a multitude of scientific, ethical, and policy questions. These questions cannot be answered by scientists alone, but also need to be debated by society," explained Dr Derek T Scholes, American Society of Human Genetics (ASHG), Bethesda, Maryland, USA.

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# Freezing Embryos During IVF Improves Pregnancy Rates

FREEZING embryos after fertilisation in *in vitro* fertilisation (IVF) and then transferring them back to the uterus at a later date can result in improved outcomes for certain groups of women, according to a recent study. With embryo freezing techniques growing increasingly sophisticated and widely used, especially as they allow increased embryo screening, it is pertinent to understand any potential impact freezing might have on pregnancy rates.

> "This finding is important because it may suggest a group of women that benefits more from freeze-all IVF cycles."

At the beginning of IVF, the patient is injected with reproductive hormones to stimulate egg growth. Eggs are then collected and fertilised in a laboratory, before being transferred back into the patient within a few days, a process known as known as a 'fresh transfer'. Alternatively, the embryos can be frozen before being transferred back into the patient during a subsequent hormone cycle.

During this new study, a 100,000-strong data set maintained by Celmatrix was used to collate data from 12 fertility treatment centres across the USA.

A total of 2,910 IVF cycles were selected and split into two cohorts, frozen before implantation (n=1,455) and fresh transfers (n=1,455); making this study the largest ever to compare frozen and fresh embryo transfer. Data from 'leftover' eggs used for second implantation were not included.

Data showed that 52% of frozen embryo transfers resulted in pregnancy, compared to 45.3% of fresh transfers. The results were further analysed to compare women with varying progesterone levels; those with lower progesterone levels experienced little difference (regardless of age), whereas those with a higher progesterone levels at the moment of egg retrieval experienced higher pregnancy rates from frozen embryo transfers. The assessment was

extended to evaluate the effect of age; the greatest improvement in pregnancy rates was for women >35 years with high progesterone levels, where the odds of pregnancy were 73% greater if frozen embryos were used compared to fresh embryo transfer (48.4% and 35.2% implantations resulting in pregnancy, respectively).

Lead author Dr Ange Wang, Stanford University School of Medicine, Stanford, California, USA, commented: "This finding is important because it may suggest a group of women that benefits more from freeze-all IVF cycles," and went on to explain: "Higher progesterone levels may make it more difficult for embryos to implant (that is, adhere to the wall of the uterus to establish pregnancy), possibly due to premature maturation of the uterine lining." Researchers mused that freezing embryos and waiting for transfer during another cycle might allow progesterone levels to fall to more hospitable levels for implantation.

## Human *In Vitro* Fertilisation Treatment Revolutionised Using Piglet Study

A UNIQUE liquid medium used to enhance the growth of porcine embryos may transform the human *in vitro* fertilisation (IVF) technique and drastically reduce the associated costs. Those undergoing IVF will typically spend between $12,000 and $15,000 on each round of IVF, and therefore this novel discovery may provide hope to many seeking children through this method.

> "It improved every aspect of the whole process and almost doubled the efficiency of oocyte maturation."

This special liquid medium was designed by a study group led by Prof R. Michael Roberts, Bond Life Sciences Center, and Prof Randall Prather, College of Agriculture, Food and Natural Resources, both from the University of Missouri, Columbia, Missouri, USA. The team's work focusses on investigating stem cells with pigs, and this particular study aimed to find a method of efficient embryo production. Commenting on the low percentage of original oocytes (1–2%) that successfully generate a piglet, Prof Roberts said: "Normally, researchers overcome this low success rate by implanting large numbers of embryos, but that takes a lot of time and money." Therefore, researchers analysed various growth factors to find an efficient method of growing high-quality embryos.

When fibroblast growth factor 2 and leukaemia inhibitory factor were combined with another chemical named insulin-like growth factor, these compounds created a fluid environment in which the oocytes became competent for fertilisation and the researchers were therefore able to develop high-quality embryos before implantation, leading to successful pregnancy. The team named this medium 'FLI'. Highlighting the importance of this finding to the field of human reproductive health, Prof Roberts said: "It improved every aspect of the whole process and almost doubled the efficiency of oocyte maturation." He added: "Now it's possible that FLI medium could become important in bovine embryo work and possibly even help with human IVF."

It is hoped that the new method of embryo development will be compatible with clinical settings and potentially revolutionise human IVF treatments. By creating large numbers of embryos at a low cost, this novel cell culture medium may provide a less expensive option compared to conventional IVF techniques, greatly improving the technique's financial accessibility.

# Fertility Preservation: Ovarian Tissue Freezing as an Alternative to Oocyte Freezing?

OVARIAN tissue freezing may be a successful alternative option to preserve fertility in women who are unsuitable candidates for oocyte cryopreservation. A new study has shown that 37.7% of women who have undergone ovarian tissue freezing are able to have children later in life. Until recently, this procedure, which can be performed in an outpatient capacity, has been considered principally investigational.

Dr Fernanda Pacheco and Dr Kutluk Oktay, Innovation Institute for Fertility Preservation and IVF, New York City, New York, USA, explained: "Despite the clinical progress within the last two decades, the procedure still remains in the experimental realm."

In 1999, Dr Oktay himself performed the first ovarian tissue cryopreservation procedure. Now, to determine the procedure's current rate of success, Oktay and Pacheco examined data from 1999–2016.

The researchers conducted a literature review that included cases of autologous ovarian tissue transplantation (OTT) with previously banked human tissue. Excluded cases included those involving fresh OTT and instances whereby OTT was performed to treat idiopathic premature ovarian insufficiency or failure.[1]

Meta-analysis revealed that, of the 309 ovarian tissue freezing procedures identified, 84 live births resulted and 8 pregnancies lasted beyond the first trimester. In 113 cases, the woman's age had been recorded at the time of the ovarian tissue cryopreservation, and the average was 27 years of age. The OTT procedure, in 63.9% of women, restored reproductive function and reversed menopause. Examples of restored function included ovarian follicular growth, natural fertility, and resumed menstruation. Whilst restoration of natural fertility occurred for the majority, 37.6% of cases required *in vitro* fertilisation (IVF) to conceive.

"Now, women considering this procedure to preserve fertility and postpone childbearing, have more information at their disposal. Given these recent data, ovarian tissue cryopreservation should be considered as a viable option for fertility preservation," explained the study authors.

Dr Oktay concluded: "Our procedure is superior to egg freezing as it can also reverse menopause and restore natural fertility." Commenting on future directions he stated: "The next frontier is to explore the procedure's potential in delaying childbearing among healthy women, not just cancer patients."

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> "Our procedure is superior to egg freezing as it can also reverse menopause and restore natural fertility."

# Western Men's Sperm Count Halved

SPERM count in Western countries has more than halved in recent decades, according to a recently published review. This study is the largest of its kind and has reported intriguing results, with the West's decline in sperm count not being reflected in other countries.

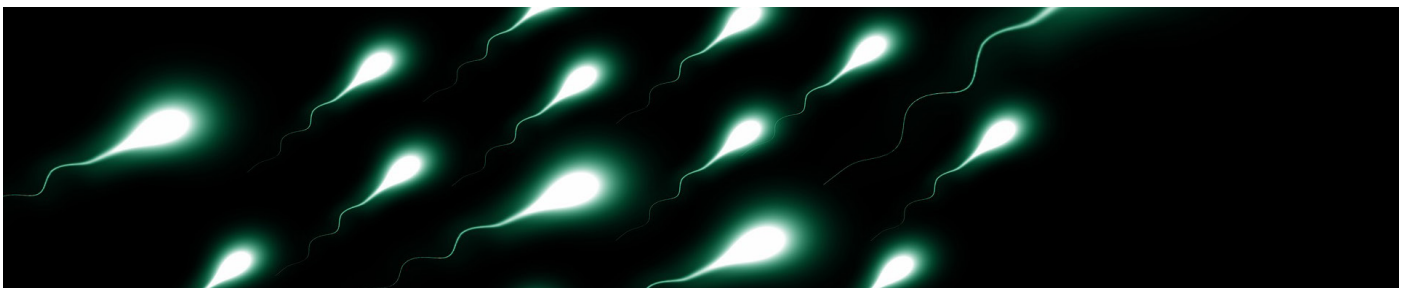
> "Given the importance of sperm counts for male fertility and human health, this study is an urgent wake-up call for researchers and health authorities around the world to investigate the cause of the sharp ongoing drop in sperm count, with the goal of prevention."

The decline in sperm count has been rigorously debated by the scientific community over the years, with no hard data available to direct a conclusion. A reduced sperm count impacts reproduction, but, furthermore, it acts as an indicator for morbidity and mortality within the male population. A study published in 2014 suggested there was a potential link between infertility and mortality and hypothesised that males with reduced sperm counts had a higher risk of death. Reduced sperm count is also associated with other morbidities including hypospadias, cryptorchidism, and testicular cancer.

Researchers carried out a large scale systemic review and meta-analysis of sperm count trends, screening 7,500 studies and conducting a mega-regression analysis on 185 studies from 1973–2011. The results were astounding; men from Europe, North America, Australia, and New Zealand had a decrease in sperm concentration by 52.4% and a 59.3% decline in total sperm count. Even more notable was that Asian, South American, and African men had no significant decline at all; however, there were fewer studies in these areas.

To ensure the results were robust, researchers controlled varying risk factors, such as abstinence time, how sperm was collected and counted, and the age of participants. As a result of this study's detail and breadth, spanning 39 years and 50 countries, these results are reliable and concerning. Lead author Dr Hagai Levine, Mount Sinai Medical Center, New York City, New York, USA, commented: "Given the importance of sperm counts for male fertility and human health, this study is an urgent wake-up call for researchers and health authorities around the world to investigate the cause of the sharp ongoing drop in sperm count, with the goal of prevention."

Although this study did not assess why sperm counts have decreased, there has been much previous speculation, with many making links to pesticides, heat, diet, smoking, stress, and high BMI scores. Further research is much needed to assess the decline more thoroughly and to identify preventative measures. The authors concluded: "research on causes and implication of this decline is urgently needed."



# Analysing Sperm Function Before Fertilisation

PIONEERING research has revealed a novel technique to investigate the functioning of human sperm before being inseminated into an egg. Presented at the European Society of Human Reproduction (ESHRE) annual conference in Geneva last month, this study reported, for the first time, the use of a technique to analyse the functioning of human sperm before an assisted reproduction cycle in order to select the male gametes with the highest chance of successful fertilisation. With development halting in 30% of fertilised eggs during early cell division, scientists believe functional defects in the sperm cell could be responsible, such as problematic pronuclear fusion or incorrect construction of the mitotic spindle.

With the aim of developing a technique to verify the correct development of sperm function, the research team studied 20 semen samples *ex vivo* in the eggs of the African clawed frog (*Xenopus laevis*), bringing the sperm into contact with the frog oocyte cytoplasm. With this methodology, it was possible to analyse the ability of the sperm cells to initiate construction of the bipolar mitotic spindle and additional functions of cell division. Additionally, the researchers were able to examine how the visible characteristics of the sperm cells (including morphology, concentration, and motility), which are usually observed in seminograms, related to their functional capacity in the first stages of embryo development.

The authors concluded that there was an apparent relationship between the characteristics of the selected sperm cells and their ability to successfully generate an embryo and hoped that the results would pave the way for further research in this field. Summarising the implications of the results, Farners Amargant, the first author of the study, Centre for Genomic Regulation, Barcelona, Spain, said: "The technique developed will allow us to observe more closely the incidence of this type of defect in order to understand if they influence the proper development of the embryo." In the future, this pioneering methodology of sperm cell analysis may help to increase the success rate of assisted reproductive techniques, achieving more positive pregnancy outcomes.


> "The technique developed will allow us to observe more closely the incidence of this type of defect in order to understand if they influence the proper development of the embryo."

## What's New Feature

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# UPCOMING EVENTS

## 27th World Congress on Ultrasound in Obstetrics and Gynecology

*16th–19th September 2017*

*Vienna, Austria*

Hosted in the beautiful city of Vienna, this event features a varied and fascinating scientific programme. Attendees can look forward to live scan demonstrations, masterclass sessions with international experts, and a myriad of oral presentations. Keynote speakers include Mary Norton, Professor and Interim Chair in the Department of Obstetrics, Gynecology and Reproductive Sciences, University of California, San Francisco, California, USA.

## 2017 Annual American Society for Reproductive Immunology (ASRI) Meeting

*17th–20th September 2017*

*Chicago, Illinois, USA*

The theme of this unique event is 'Bridging immunity with infection, inflammation, and implantation for better reproductive health'. Discussion on the similarities between pregnancy-associated immunosuppression and tumour-induced immunosuppression will dominate to encourage interdisciplinary collaboration on this issue. There is also a workshop on writing grant proposals to help researchers to secure funding for further vital research in this area.

## 19th World Congress on In Vitro Fertilization

*4th–8th October 2017*

*Antalya, Turkey*

Presented by the International Society of Infertility and In Vitro Fertilization (ISIVF) and the Society of Reproductive Medicine and Surgery (SRMS), this event will bring together all of the most up-to-date research and data on assisted reproductive technology as well as the future directions based on these recent discoveries. The organising committee hopes to enrich the understanding and daily practice of all attendees.

## 12th Congress of the European Society of Gynecology (ESG)

*18th–21st October 2017*

*Barcelona, Spain*

This year's ESG annual meeting will be held in sunny Barcelona and feature a plethora of abstract presentations on topics including fertility preservation, abortion, high-risk pregnancy, stem cells, and endometriosis. The ESG aims to welcome an international audience to the congress, providing an unrivalled opportunity for attendees to network with peers from across Europe and the world and advance their own knowledge and daily practice.

## The Royal College of Midwives (RCM) Annual Conference

*31st October–1st November 2017*

*Manchester, UK*

The programme for this 2-day event has been created from input by RCM members as well as Emma Godfrey-Edwards, editor of Midwives magazine. It is free to attend for all RCM members and the committee hopes to foster a collaborative atmosphere in which colleagues can discuss the most pertinent topics in midwifery today. There is also a dedicated session for student midwives to assist with learning and development.

## Medical Complications in Pregnancy

*15th–17th November 2017*

*London, UK*

This specialist course is designed for both consultants and trainees whose patients are pregnant and suffering from medical disorders. The course, held at the Royal College of Physicians, has a long history and proves popular each year. It covers conditions that create complications in pregnancy, delivery, and the puerperium including both existing and pregnancy-induced disorders such as renal, respiratory, skin, and liver diseases, alongside a workshop on pre-eclampsia.

## 22nd Annual Winter Conference on Clinical Issues in OB/GYN

*14th–17th February 2018*

*St. Maarten, Caribbean*

Sun, sand, and sea provide the backdrop to this meeting involving a range of topics related to obstetrics and gynaecology. The topics for this year's conference have been carefully chosen to address common gaps in knowledge in medical practice, determined through surveys and evaluation of outcomes data from past conferences. These topics include polycystic ovary syndrome, gestational diabetes, and urinary incontinence.

## 34th Annual Meeting of the European Society of Human Reproduction and Embryology (ESHRE)

*1st–4th July 2018*

*Barcelona, Spain*

If you enjoyed our congress review of this year's ESHRE meeting, be sure to keep a note of next year's event in your diary, which promises to be the biggest event on the European reproductive health calendar. Attendees will be treated to presentations, discussions, and symposia on a plethora of topics guaranteed to reinvigorate your passion for reproductive health and inspire hearty debate with peers and colleagues. Be sure to say hello if you spot our congress team there!

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