Fertility preservation: from endometriosis to ovarian tissue cryopreservation

Brussels, Belgium - September 20-21, 2012
General information

Venue
The Hotel, Brussels
Boulevard de Waterloo 38,
1000 BRUSSELS
http://www.thehotel-brussels.be

Language
The official language of this course will be English

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Fertility preservation: from endometriosis to ovarian tissue cryopreservation

Serono Symposia International Foundation conference on:

Fertility preservation: from endometriosis to ovarian tissue cryopreservation
Brussels, Belgium - September 20-21, 2012

Aim of the course
Fertility preservation is one of the most important challenges for physicians working in the field of human reproduction. Nowadays, the procedures aimed at preserving and restoring fertility in women should not be limited to patients undergoing oncological therapy but should also be available in other clinical conditions in which the reproductive function can be impaired, such as endometriosis, premature ovarian failure and ovarian ageing. Endometriosis, which affects 14 million reproductive-aged women in Europe and 150 million in the world, is one of the leading causes of infertility and it is estimated that 30-40% of women affected by endometriosis is infertile. Nevertheless, the mean time to diagnosis for this pathology is nine years. Indeed, a correct multidisciplinary approach is absolutely mandatory to diagnose endometriosis earlier and to better treat the affected women. This symposium aims to provide a broad and up-to-date review of endometriosis (genetics, pathogenesis, treatment, reproductive outcomes) and an examination of the techniques for oocyte and ovarian tissue cryopreservation that are clinically available as well as those that are still in the experimental phase. To achieve this objective, the meeting has a distinguished panel of speakers who have dedicated their professional life and scientific activity to endometriosis and the preservation of female fertility. In particular, the symposium is a tribute to Prof. J. Donnez, one of the pioneers of female fertility preservation, whose experience and knowledge represent a precious gift for all physicians working in reproductive medicine.

Learning objectives
After attending the meeting, the participants will be able to:

- Apply the principles of early diagnosis and treatment of endometriosis in clinical practice
- Describe the most recent clinical procedures for oocyte and ovarian tissue cryopreservation
- Recognize the challenging future objectives for the experimental research

Target audience
Gynecologists with specific surgery skills, reproductive clinicians and biologists who work in the field of endometriosis and oocyte and ovarian tissue cryopreservation.

Accreditation
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Université Catholique de Louvain
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Utrecht, the Netherlands

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Brussels, Belgium

Robert Fischer  
Fertility Center Hamburg  
Hamburg, Germany

Claus Yding Andersen  
The Fertility Clinic  
Copenhagen University Hospital, Rigshospitalet  
Copenhagen, Denmark

Reinaldo Gonzalez-Ramos  
University of Chile  
Santiago, Chile

Stephan Gordts  
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Outi Hovatta  
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Münster, Germany

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Leuven, Belgium

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Ana Veiga
Institut Universitari Dexeus
Barcelona, Spain
**Scientific program**

**Thursday, September 20 - 2012**

<table>
<thead>
<tr>
<th>Time</th>
<th>Session I</th>
<th>Session II</th>
<th>Session III</th>
</tr>
</thead>
<tbody>
<tr>
<td>08.00</td>
<td>Registration</td>
<td></td>
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<tr>
<td>08.40</td>
<td>SSIF welcome</td>
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<td></td>
<td>R. Fischer, Germany</td>
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<tr>
<td>08.45</td>
<td>Scientific organizer welcome</td>
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<td></td>
<td>M.-M. Dolmans, Belgium</td>
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<tr>
<td>09.00</td>
<td>L1 - Endometriosis and inflammation</td>
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<td></td>
<td>R. Gonzalez-Ramos, Chile</td>
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<tr>
<td>09.30</td>
<td>L2 - Endometriosis and genetics</td>
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<td></td>
<td>D. Barlow, UK</td>
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<td>10.00</td>
<td>L3 - Medical therapy in endometriosis: from the past to the future</td>
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<td></td>
<td>P. Bouchard, France</td>
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<td>10.30</td>
<td>L4 - Endometriosis and the great obstetrical syndromes</td>
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<td></td>
<td>I. Brosens, Belgium</td>
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<tr>
<td>11.00</td>
<td>Coffee break</td>
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<tr>
<td>11.30</td>
<td>L5 - Recent advances in diagnostic biomarkers for endometriosis, including the nerves</td>
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<td></td>
<td>L. Kiesel, Germany</td>
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<tr>
<td>12.00</td>
<td>L6 - Surgery for endometriomas: when and how?</td>
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<tr>
<td></td>
<td>S. Gordts, Belgium</td>
<td></td>
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<tr>
<td>12.30</td>
<td>L7 - The endometriosis surgery challenge: shaving versus rectal resection in deep endometriosis</td>
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<tr>
<td></td>
<td>P. Koninckx, Belgium</td>
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<tr>
<td>13.00</td>
<td>Conclusions and take home messages</td>
<td></td>
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<tr>
<td></td>
<td>J.-B. Dubuisson; S. Gordts; E. Loumaye; J. Squifflet</td>
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<tr>
<td>13.30</td>
<td>Lunch</td>
<td></td>
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<tr>
<td>14.30</td>
<td>L8 - The follicle point of view: from tissue to Petri dish</td>
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<td></td>
<td>E. Telfer, UK</td>
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<tr>
<td>15.00</td>
<td>L9 - The host point of view: from thawing to grafting</td>
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<td></td>
<td>M. Zelinski, USA</td>
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<tr>
<td>15.30</td>
<td>L10 - The researcher point of view: evaluating follicle quality and chances to pregnancy</td>
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<td></td>
<td>D. Albertini, USA</td>
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<tr>
<td>16.00</td>
<td>Coffee break</td>
<td></td>
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<tr>
<td>16.30</td>
<td>L11 - In vivo post-grafting recovery of folliculogenesis and ovulation: the role of pressure</td>
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<tr>
<td></td>
<td>S. Silber, USA</td>
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<tr>
<td>17.00</td>
<td>L12 - Gametes from pluripotent stem cells</td>
<td></td>
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<tr>
<td></td>
<td>A. Veiga, Spain</td>
<td></td>
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<tr>
<td>17.30</td>
<td>L13 - Freeze/drying oocytes (lyophilization) and stem cells as the new frontiers in cryopreservation</td>
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<tr>
<td></td>
<td>P. Patrizio, USA</td>
<td></td>
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<tr>
<td>18.00</td>
<td>Conclusions and take home messages</td>
<td></td>
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<tr>
<td></td>
<td>O. Hovatta; J. Smitz; C. Pirard</td>
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<tr>
<td>18.10</td>
<td>End of the first day</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
## Scientific program

**Friday, September 21 - 2012**

### Session IV  Ovarian tissue cryopreservation – clinics

**Chairmen:** P. Barri, Spain; P. Jadoul, Belgium; S.S. Kim, USA

<table>
<thead>
<tr>
<th>Time</th>
<th>Session</th>
<th>Title</th>
<th>Speaker/Speakers</th>
</tr>
</thead>
<tbody>
<tr>
<td>08.00</td>
<td>Poster awards:</td>
<td>1 selected oral communication on ovarian tissue cryopreservation; 1 selected oral communication on endometriosis</td>
<td>Jury: C. Amorim, Belgium, S. Defrère, Belgium</td>
</tr>
<tr>
<td>08.20</td>
<td>L14</td>
<td>Premature menopause: how to define it?</td>
<td>B. Fauser, The Netherlands</td>
</tr>
<tr>
<td>08.50</td>
<td>L15</td>
<td>Iatrogenic premature menopause and fertility preservation</td>
<td>H. Wallace, UK</td>
</tr>
<tr>
<td>09.20</td>
<td>L16</td>
<td>Can we protect the primordial follicle before aggression?</td>
<td>D. Meir, Israel</td>
</tr>
<tr>
<td>09.50</td>
<td>L17</td>
<td>1986-2011: Fertility matters: from endometriosis to ovarian tissue cryopreservation: 25 years of research</td>
<td>J. Donnez’s Gyne Research Laboratory</td>
</tr>
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<td></td>
<td><strong>Coordinators:</strong> A. Van Langendonckt, Belgium; M.-M. Dolmans, Belgium</td>
<td></td>
</tr>
<tr>
<td>10.20</td>
<td></td>
<td>Conclusions and take home messages</td>
<td>P. Barri; P. Jadoul; S. S. Kim</td>
</tr>
<tr>
<td>10.30</td>
<td></td>
<td>Coffee break</td>
<td></td>
</tr>
</tbody>
</table>

### Session V  Ovarian tissue transplantation - clinics

**Chairmen:** H. Wallace, UK; B. Fauser, the Netherlands

<table>
<thead>
<tr>
<th>Time</th>
<th>Session</th>
<th>Title</th>
<th>Speaker/Speakers</th>
</tr>
</thead>
<tbody>
<tr>
<td>11.00</td>
<td>L17</td>
<td>Ovarian tissue transplantation: myth or reality?</td>
<td>C. Y. Andersen, Denmark</td>
</tr>
<tr>
<td>11.25</td>
<td>L18</td>
<td>Ovarian tissue transplantation: the Belgian experience</td>
<td>M.-M. Dolmans, Belgium</td>
</tr>
<tr>
<td>11.50</td>
<td>L19</td>
<td>Ovarian tissue vitrification: the option of the future?</td>
<td>N. Suzuki, Japan</td>
</tr>
<tr>
<td>12.15</td>
<td>L20</td>
<td>Is the whole ovary transplantation (fresh or frozen) still indicated?</td>
<td>B. Salle, France</td>
</tr>
<tr>
<td>12.40</td>
<td>L21</td>
<td>Final lecture: Oocyte vitrification and social freezing: fertility preservation of the future?</td>
<td>A. Pellicer, Spain</td>
</tr>
<tr>
<td>13.05</td>
<td></td>
<td>Take home messages</td>
<td>H. Wallace, B. Fauser</td>
</tr>
<tr>
<td>13.10</td>
<td></td>
<td>Conclusion</td>
<td>M.-M. Dolmans, Belgium</td>
</tr>
</tbody>
</table>
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- **Jean Squifflet** Declare no potential conflict of interest
- **Nao Suzuki** Declare no potential conflict of interest
- **Evelyn Telfer** Declare no potential conflict of interest
- **Anne Van Langendonckt** Declare no potential conflict of interest
Ana Veiga
Mary Zelinski

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P. Bouchard
MM. Dolmans
J. Donnez
O. Hovatta
D. Meirov
A. Pelliccer
B. Salle
C. Pirard
H. Wallace
Abstracts
Endometrial tissue survival and growth on the peritoneal surface of the pelvic cavity, named peritoneal endometriosis, is associated to a local inflammatory reaction. Increased concentration of activated macrophages, lymphocytes, pro-inflammatory cytokines, adhesion molecules and chemotactant proteins in the pelvic cavity of endometriosis patients characterize this local pro-inflammatory environment. As well, iron overload in peritoneal fluid, macrophages, endometriotic lesions and peritoneum has been shown in the context of endometriosis, leading to increased oxidative stress, tissue damage, cell proliferation and inflammation. One of the main mediators of inflammation, cell proliferation and apoptosis that has been involved in endometriosis pathophysiology is the transcription factor nuclear factor-kappaB (NF-κB). Canonical and atypical activation pathways of NF-κB trigger p65/p50 (NF-κB dimer)-DNA binding, up-regulating the transcription of proinflammatory cytokines, such as IL-1β, TNF-α, IL-8, ICAM-1 and RANTES, between others, that have been involved in endometriosis associated inflammatory response. Additionally, these activation pathways down-regulate endometriotic cell apoptosis, favouring endometriosis maintenance and development. Three major mechanisms are postulated to explain NF-κB dysregulation and chronic inflammation in endometriosis. The first mechanism is related to the Sampson’s theory and iron overload in the pelvic cavity originated from retrograde menstruation. Iron overload and associated oxidative stress are known inducers of NF-κB activation (atypical pathway) in macrophages, increasing the expression of pro-inflammatory proteins which in turns activate NF-κB in macrophages and endometriotic cells, and decreasing macrophage phagocytic activity. The second mechanism involves increased expression of IL-1 receptor in endometriotic cells, which may lead to increased NF-κB activity through activation of the canonical NF-κB pathway. The last mechanism is linked to hormonal modulation of the NF-κB pathway. Estradiol and progesterone receptors (and their respective ligands) have been shown to interact with NF-κB and are known modulators of endometrial and endometriotic cell processes. Estradiol actions and NF-κB could act together in a positive manner to increase endometriotic cell proliferation and inflammation. Progesterone actions, instead, inhibit NF-κB activity, but progesterone resistance in endometriotic cells prevents NF-κB inhibition by progesterone, enhancing NF-κB abnormal activity. These mechanisms and interactions are very complex and cell specific. In vitro and in vivo studies on endometriosis support all these hypotheses, but research efforts still are needed to confirm and complement them.
Throughout decades endometriosis has remained an enigmatic disease in which many aspects of its aetiology and progression remain poorly understood despite a considerable worldwide research effort. At several levels genetics science has contributed to our expanding understanding of endometriosis; initially through familial aggregation studies and twin studies then the application of genetics tools for the study of the disease including genetic association studies, linkage analysis and genome-wide association studies. These approaches have improved our understanding of endometriosis but none has provided the much desired fully defined biological mechanism.

A real problem for genetics approaches to the study of endometriosis is the diversity of potential disease phenotypes and the uncertainty of progression through different manifestations of endometriosis. These make it impossible to be certain that the endometriosis phenotype selected for a study is the most appropriate. Another complicating factor is the impossibility of knowing the true population prevalences of the different endometriosis phenotypes. The problem is that the diagnosis of endometriosis is dependent on invasive investigation, generally by laparoscopy, so there is no possibility of knowing the prevalence of endometriosis in that majority of the population that has not had laparoscopy.

Studies of familial aggregation indicated an increased risk of endometriosis in female relatives of patients with endometriosis and that this risk is higher in first degree than second degree female relatives. There is also a suggestion that those with endometriosis who have an affected relative are more likely to have an increased severity of endometriosis than patients without an affected relative. Studies have indicated some degree of concordance for endometriosis in female twin pairs.

Candidate gene studies have been widely applied in endometriosis. This is on the basis that particular polymorphisms in important relevant genes may be found significantly more often in endometriosis patients than in women without that phenotype. These studies require that candidate genes have been highlighted by the biological features of the disease process, such as the sex hormone dependence and the inflammatory features. For the most studied candidate gene polymorphisms there are multiple published candidate gene studies in different populations and it is noteworthy that generally, for each candidate gene, there are both positive studies supporting the candidacy and negative studies failing to support the candidacy.

The availability of powerful high-throughput genotyping for the screening of entire genomes in large numbers of patients has made possible genome-wide association studies and these are being applied to the study of many diseases likely to have a complex genetic basis. The aim is to identify genetic variants that may be associated with endometriosis without any assumptions having to be made about which genes may be involved. It requires large collections of disease affected families, and affected sister pairs are especially interesting. This necessitates nationwide and international collection of affected families with an agreed definition of the disease phenotype and international collaborations have been established to facilitate this, including an important Oxford-Australian programme. This approach has been fruitful in some diseases that have a complex genetic basis. In endometriosis this approach is suggesting chromosomal loci of importance which can provide a focus on genes and gene products, not necessarily previously identified as linked to endometriosis.

The evolving genetics effort has provided insights into the molecular mechanisms that may be important in endometriosis but despite this effort and the other more directed approaches of cell and molecular biological research a clear definition of the underlying molecular mechanisms in endometriosis is yet to be achieved.
L3 - Medical therapy in endometriosis: from the past to the future

Philippe Bouchard
Université Pierre et Marie Curie Paris
Paris, France

Abstract not in hand at the time of printing.
The myometrial junctional zone (JZ) plays a critical role in human placentation and is characterized by unique vascular plasticity in terms of physiological remodeling of the myometrial spiral arteries in pregnancy as well as complete vascular rejuvenation during the postpartum period. It is well known that partial and absent remodeling of the myometrial spiral arteries or defective deep placentation is associated with major obstetrical disorders such as late miscarriage, preterm birth, fetal growth restriction and preeclampsia. High-resolution imaging studies have demonstrated functional and structural changes in the JZ myometrium in young women with endometriosis. Recent studies have found that both endometriosis and adenomyosis are associated with an increased risk of major obstetrical syndromes such as preterm birth, fetal growth retardation and postpartum haemorrhage. The association with pre-eclampsia is still controversial. The question therefore arises whether the JZ changes in endometriosis and adenomyosis predispose for defective deep placentation.

Recently, it has been suggested that the process of defective deep placentation starts in the late menstrual cycle and triggers a cascade of events resulting in defective remodeling of the spiral arteries. Several uterine vascular factors can be responsible such as defective uterine preconditioning, disruption of the decidualisation process and presence of subclinical cardiovascular disease. Several types of defective deep placentation have been described.

Preliminary data from IVF treatment suggest that pre-treatment pituitary down regulation may be beneficial for the prevention of obstetrical disorders. More studies will be required to evaluate the beneficial effect of pituitary down regulation on the prevention of obstetrical complications in women with endometriosis and adenomyosis.

It is strongly recommended that in the future the diagnosis of endometriosis is complemented by the evaluation of the myometrial JZ in order to estimate the potential impact of the disorder on the obstetrical outcome. Moreover, studies on endometriosis and adenomyosis frequently report on infertility, miscarriage and births, but fail to report on major obstetrical syndromes.
Detection and diagnosis of endometriosis is often delayed, sometimes up to ten years, due to the lack of specific symptomatology and non-invasive tests. The gold standard for diagnosing endometriosis relies on surgery. There is a general need for the development of biomarkers without requirement of surgery. Such a biomarker would ideally require the measurement of peripheral serum marker or another easily accessable sample, i.e. endometrial fluid or tissue.

Major efforts have been put into measuring a great variety of markers in different body fluid samples but only a few have been tested in a preclinical or clinical setting. Among these markers are cytokines, antibodies, immunological cells, various glycoproteins, i.e. tumor markers, growth factors, hormones or molecules related to angiogenesis, apoptosis, cell adhesion, etc. In addition to the necessity to developing non-invasive methods for early diagnosis, there is also an urgent requirement to find new markers for characterizing the biological activity of endometriosis with regard to prognosis of the progression of the disease as well as prediction of the efficacy of medical and surgical therapies. Symptoms of the disease include infertility and chronic pain. The latter has stimulated research on the presence of nerve fibres in eutopic and ectopic endometrium. Various studies have implicated the role of endometriosis associated to nerve fibres in the clinical relevance of the disease. In other entities, i.e. breast cancer, major advances have been made to allow for early diagnosis and more detailed characterization of the disease, which have led to better understanding of prognosis as well as treatment prediction. In many respects future research in endometriosis may follow along this path which has been successful for other diseases.
Since several decades basically the treatment of ovarian endometriosis remained unchanged. It is hardly questionable if we are performing better now. Contradiction remains between believers of cystectomy on one site and those favoring an ablative surgical approach. It is hardly questionable if results of such a complex surgery of a complex disease can be described within the simplicity of “ablative” or “excision”. Imaging techniques with 3-D ultrasound and MRI has to give us all the information about the presence, localization and extent of extra-uterine and uterine adenomyosis. Sampson was the first to suggest that ovarian endometriotic cysts originate from the outside of the ovary and was caused by adhesions and bleeding of surrounding peritoneal implants. Donnez and Nisolle suggested that mesothelial metaplasia is at the origin, but also causing an invagination of the ovarian cortex. As such the ovarian endometrioma differs from other benign ovarian cysts by his extra-ovarian localization. Therefore ovarian volume and AFC (antral follicular count) tend to be decreased after cystectomy. This is in contrast with cystectomy for other benign ovarian cyst like dermoid where there is no intrinsic damage to the ovarian volume post-cystectomy. (Exacoustos). Both ablative and excisional surgery result in a diminished ovarian reserve and in hands of experienced surgeons there will probably be no difference in final ovulatory function between the two techniques. However in hands of inexperienced surgeons it is likely that the damage to the ovarian function will be greater after cystectomy. In an attempt to lower the ovarian damage a two step operative procedure or combination of techniques has been proposed. Factors favoring surgery are the increased fertility after surgery and although fertility will never be completely normalized it offers couples the possibility for a spontaneous conception with reported pregnancy rates between 40-60% most of them occurring in a mean delay of 10 months. Furthermore it offers for most of the patients a relieve of their pain and return of normal sexual live. Before IVF surgery of the ovarian endometrioma is recommended to avoid the risk of infection and abscess formation at the time of follicle aspiration and supplementary it provides a histological diagnosis to exclude malignancy. Clinicians should be aware of the recent publications mentioning the possible association between endometriosis and an increased risk for ovarian carcinoma. The interference of the presence of endometrioma with results of IVF still is a controversial issue. There is lack of consensus amongst studies as to whether ovarian response is adequate or suboptimal in patients with ovarian endometriosis. The conclusion of a recent meta-analysis (Tsoumpou et al.) could not identify statistically significant differences in pregnancy or clinical pregnancy rates per cycle after IVF between women undergoing surgery for endometriomas and women with endometriomas without surgery. Other studies indicating that there is a negative impact and that this impact is directly correlated with the degree of endometriosis. Data of an oocyte donation program suggested an impaired oocyte quality to be responsible for the lower pregnancy rates. Surgery of ovarian endometriomas remains faced with controversial issues. Complexity of the pathology renders conclusions from prospective randomized studies difficult. The results may be biased by selection of patients from groups with major differences in the pathology. Presence and treatment of adenomyosis within the uterus or in the pelvis should be incorporated in the analysis of the post surgical results. Treatment should be individualized taking into account different factors as age of the patient, size, number and localization of the endometriomas, presence of pain, recurrence and the wish to conceive.

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Deep endometriosis can be difficult surgery, especially in larger nodules. Surgery requires expertise especially to identify smaller nodules in the bowel wall and ramifications of the nodule.

It remains debated however, when a bowel resection should be done. In two systematic reviews, we demonstrated that the leakage rate and long-term consequences of bowel resection increase when the resection involves the lower part of the bowel. For sigmoid resection, leaks occur in less than 1% of cases, almost without long-term problems. For low rectal resections, leaks increase to 15% or more and carry a lifelong risk of bowel, bladder and sexual problems of 30%, 30% and 40% respectively. Of almost 2000 bowel resections for endometriosis published to date, the majority were lower resections. It remains unclear what the exact indications were. The size of nodules is rarely indicated, the length of resection ranges from 5 to 25 cm while data on sexuality after surgery are lacking. We should be aware that other aspects influencing the decision of surgery are rarely mentioned. Bowel resection is technically easier and faster than discoid excision; it by definition makes the bowel surgeon co-responsible, which is medico-legally important. In addition, the fact that reimbursement of bowel resection is 5 to 10 times higher than discoid excision might be an argument for some hospitals.

Excision of nodules is feasible in over 90% of cases. Larger nodules often require a suture of the bowel muscularis or a full-thickness resection. We therefore suggest that discoid excision should at least be attempted for all rectosigmoid and rectal nodules. If too difficult for the level of expertise of the surgeon, bowel resection can be performed. For the rectum and rectosigmoid, we (PK) performed less than 1% bowel resections. What is important is that the incidence of bowel resection should progressively decrease with expertise from 15-20% to much less. An incidence of over 50% of bowel resections signals a surgical choice not a technical necessity. For sigmoid lesions, we should be much more liberal with bowel resections. To identify the limits of conservative surgery, we (PK) today perform 5% elective bowel resections for deep sigmoid endometriotic nodules, and another 5% is decided during surgery.

We strongly oppose bowel resection that is decided upon before surgery, except in case of the sigmoid showing signs of extensive occlusion. Indeed, some lesions can be erroneously be judged endometriosis or as invasive endometriosis. Although data are scarce, up to 14% of bowel resections were reported in which no endometriosis was subsequently found by pathology. Moreover, if we consider that in 12% of cases the endometriotic nodule was located outside the muscularis, this adds up to 26% of unnecessary bowel resections. Considering the severe lifelong implications of bowel, bladder and sexual problems following rectal and especially low rectal resection, we suggest that this procedure should not be performed when avoidable. This statement remains valid at least until it is demonstrated that complications for low rectal resection in case of endometriosis are different from those for other indications.
The ability to develop human oocytes from the earliest follicular stages through to maturation and fertilisation in vitro could revolutionise fertility preservation practice. This has been achieved in mouse where in vitro grown (IVG) oocytes from primordial follicles have resulted in the production of live offspring. However, developing IVG systems to support complete development of human oocytes has been more difficult because of differences in scale of timing and size. The aim of our work is to determine whether complete oocyte development can be achieved from human ovarian tissue grown in a multi-step culture system. We have developed a dynamic 3 step culture system that supports the activation of primordial follicles (step 1), growth of multilaminar follicles (step 2) and oocyte growth outwith the large follicular environment (step 3). Using this system a population of oocytes capable of reaching Metaphase II can be obtained. This presentation will focus on the challenge that lies ahead to improve quantity and quality of in vitro grown human oocytes and will discuss the testing and safety checks that will be required before this technology could ever be applied in a clinical setting. Finally, the prospect of using this system to support the differentiation of stem cells to oocytes will be considered.
Heralded from around the globe, twenty human births from cryopreserved ovarian cortex after transplantation have provided a framework from which to pursue this technique as a viable option for fertility preservation in cancer survivors. From the host point of view, the cryopreservation procedure, site of transplantation, and ability to re-vascularize the ovarian cortical transplant to prevent ischemia-reperfusion injury as well as restore gametogenic and endocrine function are collectively important considerations for fertility restoration. All of the reported human pregnancies originated from ovarian tissue cryopreserved using slow-freezing, a technique popular in non-U.S. countries. Despite a plethora of published vitrification protocols for human tissue, very few have assessed subsequent ovarian function, particularly post-transplantation. Given that both slow-freezing and vitrification preserve the morphology of primordial and primary follicles and their enclosed oocytes, and that most ovarian function recurs 3 or more months post-transplantation, the resulting antral follicles most likely originated from primordial or primary follicles. Thus, from a host point of view, ovarian tissues cryopreserved by either method should be viable for transplantation, but live births from vitrified ovarian tissue remain to be demonstrated. While orthotopic transplantation sites can provide an extensive hilar vascular bed, such sites may not be available in young patients who will return for transplantation many years later after having received extensive ovarian damage from chemotherapeutic and/or radiotherapy. Human ovarian cortical tissue cryopreserved via slow-freezing and transplanted to heterotopic sites within the abdomen (subperitoneal pockets, omental grafting) has restored endocrine function, and yielded fertilizable oocytes, and early cleavage stage embryos. The first human live birth from an oocyte derived from cryopreserved tissue transplanted into the abdomen was published just this year by Dr. Donnez and colleagues. Optimal intra-abdominal locations for restoration of antral follicle growth and endocrine function using fresh abdominal transplants in baboons and vitrified-thawed abdominal transplants in macaques were recently reported. Our initial results in macaques revealed vitrified ovarian cortex transplanted heterotopically can yield follicles with oocytes that fertilize and progress to the morula stage in vitro. Vascular endothelial growth factor (VEGF) applied locally to subcutaneous transplant sites (abdomen or arm) in macaques via microspheres elicited sustained delivery for up to one week, resulting in higher tissue concentrations of VEGF compared to a fibrin-hydrogel containing VEGF. However, fibrin VEGF did not increase a) vascular area determined by counting CD31-positive vessels in fresh tissues explanted 1-2 weeks post-transplant, or b) vascular perfusion rate evaluated noninvasively in vivo by dynamic contrast enhanced microbubble ultrasound at vitrified-thawed graft sites months post-transplantation. Qualitatively, VEGF maintained stromal integrity resulting in maintenance of preantral follicles post-transplantation. Future studies, informed by the regenerative medicine field, employing scaffolds and endothelial progenitor cells to improve angiogenesis and the extracellular matrix, as well as evaluating the contribution of anti-apoptotic and “survival” factors to long-term follicle preservation post-transplantation are needed. While some support the view that ovarian tissue transplantation using cryopreserved cortex for the restoration of fertility is no longer experimental, many avenues remain for improving the robustness, efficiency and safety of this procedure such that more patients who have no other options, particularly prepubertal girls, adolescents and young women, may benefit from this emerging technology.

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The field of fertility preservation is becoming more reliant upon technological advancements that will optimize gonadal tissue and gamete functionality prior to, during, and following storage under various temperature conditions. While emphasis has been placed upon “cryo” storage, there is a need to better understand pathophysiological alterations that take place “after the big chill” to gain new insights into stress responses and metabolic adjustments that best serve recovery of ovarian function at a cellular and tissue level. Improving chances to obtain pregnancy after tissue grafting or in vitro follicle culture ultimately relies upon the re-establishment of follicle development at different stages of folliculogenesis and therefore must take into account the physiological mechanisms that normally support metabolic demands of both the somatic and germ cell constituents that comprise the follicle. This presentation is built around the recently appreciated notion of metabolic cooperation from the follicle perspective of nourishment and maintenance oocyte quality that must be achieved following any storage or grafting procedure that hopes to provide patients with a reasonable chance of attaining pregnancy. Emphasis will be placed on the dark side of aerobic metabolism especially as it pertains to dynamic changes in the ovarian vasculature.
The aim of this lecture is to summarize the state-of-the-art of ovarian tissue cryopreservation and transplantation, and Dr. Donnez’s crucial leadership in this field. This clinical experience will then be applied to understanding 1) the mechanism for initiation of primordial follicle development, and 2) why cancer cells rarely settle in the ovarian cortex and, therefore, do not severely limit the transplantation of frozen ovary tissue in cancer patients.

To evaluate the effectiveness of ovary freezing requires a comparison to results with fresh ovary tissue transplantation. Ten of ten fresh ovary transplants were successful, resulting in twelve healthy babies in eight of the recipients. Recipients always reinitiated ovulatory menstrual cycles and normal Day 3 serum FSH levels by 4-1/2 months. Most conceived naturally (three of them twice or three times from the same graft). Duration of function of fresh ovarian grafts, contrary to initial expectation, indicated a very acceptable or minimal oocyte loss from ischemia time. Grafts of just modest portions of ovarian tissue have lasted more than 7 years.

The same surgical techniques were then applied to 4 frozen ovary tissue transplants, up to 14 years after the ovary had been frozen, all resulting in normal ovulation and in 3 more healthy babies. Around the world, the number of healthy babies from ovary grafts has now risen to over 30. Although ovary freezing and transplantation has been referred to as “experimental” for preserving fertility in cancer patients, it should be noted that all of the babies born from fertility preservation for cancer patients, have resulted thus far from ovary tissue freezing, and none thus far from egg freezing.

As to the efficiency of ovarian tissue freezing compared to the use of fresh tissue, slow freeze has resulted in only a 50% loss of oocytes, and vitrification has resulted in no loss. For practical purposes therefore both techniques are reliable. However, in vitro studies in humans, and in vivo studies in bovine, show that vitrification of ovarian tissue, may nonetheless be an improvement over slow freeze.

The basic science concept of vitrification is to completely avoid and ice crystal formation by using a very high concentration of cryoprotectant and a very rapid rate (virtually “instant”) of cooling. This is quite different from classic slow freeze cooling which relies on a partial and very gradual removal of water from the cell by encouraging ice crystal formation preferentially on the outside of the cell, drawing the water out.

A comparison has to be made between vitrification for ovarian tissue versus vitrification for mature eggs and embryos. For mature eggs and embryos, there is first an equilibration in 7,5% EG and 7,5% DMSO followed by a final solution of 15% EG and 15% DMSO with 0,5 molar sucrose. It is very important to allow enough time for full absorption of this more concentrated cryoprotectant solution usually more than one minute, as these solutions, contrary to myth, are not toxic. However, for ovarian tissue, there must be a longer incubation in 7,5% EG and 7,5% DMSO, and then a later incubation in a denser 20% EG and 20% DMSO with 0,5% sucrose, to make certain there is full absorption of cryoprotectant. In all cases, the eggs or embryos or ovarian tissue must not be frozen in a droplet [even a tiny microdroplet] as this would slow the rate of freeze and thaw. The ovarian tissue must have fully absorbed the cryoprotectant, but be “dry” on the outside. Just as thawed ovarian tissue works as well as fresh, thawed embryos have just as good a success rate as fresh embryos. With vitrification, embryos can be frozen with impunity, whereas with slow freeze (like with ovarian tissue) there is some viability loss.

A comparison of the embryology and anatomy of the ovary and the testis is very instructive for two issues, 1) the initiation of follicle development, and 2) the low risk of neoplastic metastasis to the ovary cortex and, therefore, the safety of ovarian cortical cryopreservation and transplantation in cancer patients. The ovarian cortex is actually identical to the tunica albuginea of the testis, except that in the male the germ cell cords do not invade the tunica, and in females they do.

Leukemic cells routinely invade the testes of boys and men with leukemia, but virtually never the tunica albuginea of the testis. This phenomenon explains both of these issues.

It is clearly possible to preserve and restore fertility, using ovary and egg or embryo freezing in young women with cancer who are undergoing otherwise sterilizing chemotherapy and radiation. But this approach can also be used for any woman who wishes to prolong her reproductive lifespan. It may thus eventually obviate the growing worldwide epidemic of female age-related decline in fertility, and even could eliminate menopause.
Stem cell research is one of the most promising areas in biomedicine. Embryonic stem cells (ESC) and induced pluripotent stem cells (iPS) hold pluripotency and self renewal characteristics and have the possibility to differentiate in any cell type. The obtention of gametes through pluripotent stem cells has applications in research, teaching and in ART.

The differentiation from ESC to oocytes has been achieved in vitro in the mouse model. Fetal porcine skin cells have also been used for oocyte generation. As oogenesis in vitro constitutes a very complex process, very few papers have been published in this area of research.

Male gametes have been generated in the mouse model through ESC and iPS cells and viable offspring have been born after the fertilisation with ESC derived mouse sperm even though abnormal imprinting patterns and phenotypic abnormalities have been described. Male meiosis has been reproduced in in vitro conditions with and without the use of specific gene overexpression.

It has been recently demonstrated that pluripotent stem cells have the ability to differentiate in vitro into primordial germ cells that produce functional male gametes that give rise to healthy offspring with normal methylation patterns of imprinted genes.

Gamete derivation from ESC allows the study of genes involved in germ cell production. These gametes constitute an alternative source for research in nuclear transfer, as well as for reproductive purposes. Before clinical use of pluripotent stem cell gametes is considered, improvements in technical and safety aspects are needed.
Storage of cryopreserved oocytes and stem cells in liquid nitrogen is very demanding in terms of maintenance, storage space, equipment and costs. Such demands are predicted to increase as more women are resorting to oocyte cryopreservation as a method to protect their future fertility either before cancer treatment or to postpone their reproductive plans. At the same time more couples are storing, as protective measures, their newborn’s umbilical cord blood samples in the hope of retrieving (should the need arise) haematopoietic precursor stem cells (HSC’s) for re-transplant. Preliminary experiments have been carried out, in parallel, comparing various cooling methods on the recovery and survival after freeze/dry of in vitro-matured MII bovine oocytes (n=68). Some oocytes were cryopreserved with slow freezing (using MTG 1314 device) at a cooling rate of 4°C/minute (group A); some additional ones with rapid freezing (using MTG 516 device), at a cooling rate of 150°C/minute (group B); and the final sample with vitrification using minimum drop size in IMT-4 solution (mix of cryoprotectants and trehalose) at a cooling rate > 20,000°C/minute. The lyophilization process was carried out with the VirTis wizard for 24 hours with shelf temperature of -55°C and vacuum 10 mTorr. The rehydration process took place at room temperature using double distilled water and TC199 supplemented with 0.5M trehalose. Previous experiments with mononucleated cells (MNC) extracted from umbilical cord blood samples and frozen using the multithermal gradient device (MTG 1314) will also be presented. Both oocytes and MNC viability was assessed using the live/dead fluorescent stains Syto-13/PI for membrane integrity. The percentage of viable MNC or oocytes was calculated as follows: % Viable cells= (Live cells after freeze drying/Live cells before freezing) x 100. After rehydration the bovine oocytes had the best recovery and survival when they were vitrified (88% and 77%, respectively) as opposed to the rapid freezing or slow freezing method. The rehydrated MNC were assessed with the colony forming unit (CFU) assay and compared to fresh samples. These preliminary experiments support the concepts that: 1) Lyophilization is a potential groundbreaking innovation for gamete and stem cells bio-banking; 2) Vitrification of oocytes is confirmed as an essential method not only for preservation in liquid nitrogen but also for storage in a dry state; 3) The elimination of the thawing process, often associated with cell damage from recrystallization, is another important advantage of freeze dry storage.
Learning objectives
• Understand that both normal and premature menopause is related to mechanisms underlying primordial follicle pool depletion
• Appreciate that the distinction between normal and premature menopause (i.e. occurring before or after the age of 40 yrs) is arbitrary.
• Appreciate that both “experiments of nature” (involving rare and complex syndromes) along with targeted knock-out animal models allowed the identification of novel genes involved in follicle pool depletion.
• Appreciate that POI has major general female health implications beyond fertility
• Appreciate that care for women with POI is insufficient, requiring a multi-disciplinary approach

Summary
Ovarian failure is currently referred to as primary ovarian insufficiency (POI), suggesting that the primary defect of dysfunction resides within the ovary itself. This condition is also classified as WHO, group 3. The cause of this devastating condition remains unknown in the great majority of cases. Depending on the clinic and referral patterns in up to 30% of cases a clear underlying cause can be identified: Genetic (approximate 10-20% of POI is familial) sometimes associated with rare syndromes involving many phenotypic features, previous chemotherapy or radiotherapy, pelvic surgery, inflammation or auto-immune disease.

Causes of POI may be classified as mechanisms interfering with ovarian follicle depletion (either low initial numbers or accelerated follicle loss), or follicle dysfunction (i.e. signal defects, enzyme deficiency or auto-immunity). Many mutations on the X chromosome (including BMP 15) have been associated with POI. Special attention should be paid to the associated between POI and mental retardation, in relation to fragile X (number of CGG repeats). In addition, a series or rare mutations on autosomes, including FOXL2, FSHR, GDF9, GPR3 and LH beta have been described in these patients.

Gene knock-out studies in rats have provided additional and novel information regarding various factors involved in follicle pool depletion. Much attention is focussed towards assessing the extent of ovarian damage following the use of various chemotherapeutic agents for early age Hodgkin, leucemia and breast cancer. Anti-Mullerian hormone (AMH, produced by pre-antral an early antral follicles) seems to represent the best endocrine marker for the detection of early stages of POI.

The most intriguing questions remains whether from a genetic point of view POI should be considered the extreme of the spectrum of the normal distribution of menopausal age. Contemporary genetic technologies (including genome wide association studies) provide new insight in the possible overlap of factors involved in normal menopause and POI.
With increasing numbers of survivors from cancer at a young age the issue of fertility preservation has assumed greater importance. Female fertility preservation provides significantly different challenges to that for the male. Embryo freezing is now an accepted and well-established procedure in many centres, but is not available for children who do not have a partner. Cryopreservation using vitrification of mature oocytes has become increasingly successful, but requires the patient to go through a course of hormone stimulation and is therefore not appropriate for children and young girls. Ovarian tissue cryopreservation has the potential advantages of preservation of a large number of oocytes within primordial follicles, it does not require hormonal stimulation when time is short, and is appropriate for the pre-pubertal girl. Disadvantages include the need for an invasive procedure, and the uncertain risk of ovarian contamination in haematological and other malignancies. Ovarian tissue cryopreservation in adult women with later re-implantation has resulted in at least 19 live births worldwide. This invasive approach to fertility preservation remains unproven and experimental in children and adolescents. The majority of young patients treated for cancer will have a window of opportunity for natural fertility if they survive their original cancer. I strongly recommend that all young patients with cancer have an assessment made of their fertility prognosis before they commence treatment. We have previously published guidelines for patient selection in young female patients with cancer and in this lecture I will report our practise in a single centre that has offered fertility preservation since 1996. The clinical records of all 407 female patients treated for cancer, leukaemia or brain tumours at the Edinburgh Children’s Cancer Centre who were ≤18 years old at diagnosis between 1st January 1996 and 30th June 2012, were reviewed retrospectively to assess current ovarian function. Ovarian cryopreservation was offered to 9% of our patients, and performed in 5%. The procedure was safe and without complications. All patients who have thus far developed ovarian failure were identified pre-treatment using our criteria.
Ovarian damage and follicle loss are significant complications associated with cytotoxic drug treatments. Clinically, treatments can cause complete ovarian atrophy and premature ovarian failure, however, for many patients ovarian damage is partial, resulting in reduced fertility, menstrual cycle disturbances, higher follicular phase FSH levels, and low anti-mullerian hormone levels. Cytotoxic drugs preferentially target rapidly dividing cells, and as such the oocyte is not a natural target as it exists primarily in dormant form in the primordial follicle. Despite this, however, histological studies have shown that chemotherapy causes a drastic loss of primordial follicle stockpiles. We have recently suggested that the alkylating agent Cyclophosphamide (Cy) activates the growth of the quiescent follicle population, resulting in loss of ovarian reserve. Differential follicle counts on Cy treated mice demonstrated a decrease in primordial follicles and an increase in early growing follicles leading to a “burn-out” effect and follicle depletion. Protein analysis indicated that follicle activation occurs through the PI3K signaling pathway. These results provide a new understanding of the mechanisms involved in chemotherapy-induced loss of ovarian reserve, suggesting that Cy accelerates primordial follicle activation.
A career devoted to reproductive medicine
Jacques Donnez studied medicine and did his internship in Gynecology and Obstetrics at the Université Catholique de Louvain (UCL). He started his scientific research activities under the supervision of Professors Jacques Ferin and Karl Thomas. Ever since, he has devoted his career to understanding the pathophysiological mechanisms underlying female infertility and developing new therapeutic options to restore or preserve women’s health and fertility.
He has also been instrumental in spreading and sharing knowledge in reproductive medicine by promoting numerous PhD theses, organizing international meetings to bring together clinicians and scientists, and publishing over 500 original articles in peer-reviewed journals.

Summary of research activities
Towards a better understanding and clinical management of endometriosis
The UCL Gynecology Unit, led by Prof. Donnez, is recognized worldwide as a reference center in the field of endometriosis. Prof. Donnez has made a major contribution to continued progress in laparoscopic surgical management of endometriosis, as well as the development of new therapies. His morphometric and immunohistochemical studies of endometriotic lesions and set-up of original models have proved groundbreaking in the field. These will be detailed during the presentation and include:
1. Providing the first evidence that pelvic endometriosis, ovarian endometriosis and nodules of the rectovaginal septum are three distinct entities.
2. Developing a new medical device for local endometriosis treatment based on innovative research on inflammatory and angiogenic processes.
3. Clarifying hormonal responses and demonstrating the absence of aromatase in endometriotic lesions.

Pioneer in the field of ovarian cryopreservation and transplantation
In 1995, following a discussion with Professor Edwards, Jacques Donnez decided to embark upon a brand new project on ovarian tissue cryopreservation and transplantation. His team was one of the first to propose cryopreservation of ovarian tissue to patients treated by chemo- and/or radiotherapy (as part of an experimental program approved as early as 1995 by the Ethics Committee of the UCL). The ovarian tissue bank was created in 1996 after setting up a protocol for human ovarian tissue freezing with Prof. Smits. Prof. Donnez published one of the first articles outlining the indications for ovarian cryopreservation, which has since become a point of reference.
One of the first models of human ovarian tissue heterografting to nude mice was established in his laboratory, leading to a better understanding of factors affecting ovarian tissue vascularization and folliculogenesis after grafting.
Ten years after starting the project, Prof. Jacques Donnez achieved the first livebirth after orthotopic transplantation of cryopreserved ovarian tissue in humans. The scientific data, published in the Lancet, describe restoration of endocrine and reproductive function in a patient who had become infertile as a result of chemotherapy for Hodgkin’s lymphoma.
This historic achievement has brought hope to thousands of women facing the prospect of infertility after undergoing gonadotoxic treatment for life-threatening conditions, and paves the way for further research in the field.
Transplantation of frozen/thawed ovarian tissue for fertility restoration and regaining of menstrual cycles is rapidly gaining ground as a valid method alongside cryopreservation of embryos and oocytes. More than 20 healthy children have been born worldwide as a result of this procedure after an estimated 70-80 women have had tissue transplanted. The procedure is most often carried out by excising one ovary or part of an ovary and leaving the remaining ovary in situ in case the treatment does not destroy all follicles. Until recently all babies born resulted from transplantation of frozen/thawed tissue to the remaining postmenopausal ovary. However, despite the fact that the vast majority of women receiving tissue had high postmenopausal FSH levels at the time of transplantation, it is impossible to know whether the developing follicles in the rejuvenated ovary actually resulted from the transplanted tissue or from silent endogenous stores of follicles that became activated at the time of surgery. Or perhaps that follicles from both types of stores became active and contributed to the ovarian activity. Case reports have suggested that residual follicles in the in situ ovary may become activated even after a prolonged period of postmenopausal levels of FSH – especially in young women. It could be speculated that the surgery in connection with transplantation of tissue alters intraovarian conditions and allow for a vascularization that provides a better hormonal environment of possible residing follicles?

However, there is now good evidence to support that the transplanted tissue is active. The presentation will focus on providing support to the notion that follicles present in the transplanted tissue indeed do become active and restore fertility and recreate endogenous hormone secretion by:

1. A relative constant period elapses from transplantation until the tissue become active, also when a second transplant is carried out.
2. Tissue transplanted to a heterotropic site is active.
3. Genetic tests of children born have shown that the oocyte originated in the transplanted tissue.
4. Two children have now been born of women who were bilateral oophorectomised at tissue collection and therefore did not have any ovarian tissue left in the body.
Abstract not in hand at the time of printing.
Summary
Two methods are mainly used to cryopreserve biological tissues, which are slow cooling or vitrification by rapid cooling. Several groups have reported that slow cooling is more successful than vitrification for cryopreservation of human ovarian tissue, thus slow cooling method has now become the standard method for clinical application. On the other hand, vitrification works by trapping aqueous solutions in an amorphous, “vitreous” solid phase that prevents ice crystal formation in tissues. Rapid cooling methods that were developed using mice and monkeys\(^1\) have recently been shown to improve the viability of vitrified ovarian tissues. We have developed a new vitrification device for ovarian tissue which achieves rapid cooling rates as the tissue sections are exposed directly to liquid nitrogen\(^1\). Importantly these ovarian tissues contained ultra-structurally normal oocytes and follicles even with equilibration periods as short as 5 min. Finally, we established a new vitrification method for ovarian tissue in cynomolgus monkey, a non-human primate, as preclinical model\(^2,3\). According to our preclinical data, we have already started to use this new technology to human subjects (over 30 cases) in Japan, and have already succeed in egg collection after the autografting, and acquiring a fertilized egg in human. Although further studies of the conditions for vitrification are required, it is possible that this new method will be beneficial for young women with cancer.

References
L20 - Is the whole ovary transplantation (fresh or frozen) still indicated?

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Abstract not in hand at the time of printing.
The possibility to successfully cryopreserve oocytes has opened new pathways in the field of ART. The demonstration that vitrification provided similar outcomes than fresh oocytes was established by our group in a prospective, randomized, non inferiority study performed in 2010. Since then, many procedures have been simplified such as the management of the oocyte donation waiting lists, the prevention of ovarian hyperstimulation syndrome, among others.

An important issue is the prediction of the ovarian response to ovarian stimulation. Recent data suggest that the ovary is affected by cancer and we must expect around 2 oocytes less per women than their aged-matched controls without malignancies. Serum AMH levels are a good marker for these patients because they positively correlate with the number of oocytes obtained. Our accumulated experience in 324 and 419 cycles has show that we can retrieve a mean of 7.6 mature oocytes per woman in a single stimulation. We have also learned from a different population in whom we accumulate oocytes to increase the chances of becoming pregnant in a single insemination procedure, i.e. the so called low responders, that a mean number of 10 eggs are necessary to freeze to achieve ongoing pregnancy rates close to 60%. In this sense, oncologic patients have relative good chances of becoming pregnant in the future employing frozen and thawed oocytes. In fact, 4 transfers have been currently performed, one resulting in a miscarriage and another is an ongoing pregnancy. Thus, we expect that the near future will bring many other pregnancies as it has been the case in oocyte donation.

The same technique can be employed to preserve fertility for social reasons. We have accumulated experience in 284 patients. The mean age was 36.5 years and the total number of mature eggs retrieved was 7.6. Many of these patients were referred by their gynecologists because they had endometriosis which resulted in unilateral oophorectomy. Others, just consider seriously the effect of age on oocyte’s quality and try with this measure to avoid the deletereous effect of time on their fertility potential. This is seen by many women as a real possibility for total emancipation. In fact, a recent survey in infertile patients showed that at least 50% of them would be interested in social freezing. If the survey is conducted in young workers without any infertility problems, the numbers could be even higher. Thus, fertility preservation through for social reasons is a reality and an ambulatory procedure that is regarded in positive by a majority of women.