The GYNE unit has focused its research activities on four main topics related to male and female infertility:

1. Ovarian tissue and ovarian follicle cryopreservation and transplantation in order to preserve fertility in female cancer patients. Development of a transplantable artificial ovary.
2. Pathogenesis of endometriotic nodules, which are among the most frequent benign gynecological diseases affecting women of reproductive age.
4. Andrology: Testicular tissue cryopreservation and transplantation, in vitro maturation of spermatogonial stem cells, and development of an artificial testis.

In the GYNE unit, a pluridisciplinary team (gynecologists, molecular biologists, clinical biologist and veterinary surgeon) investigate reproductive tissue physiology at the molecular and cellular level, both on patient biopsies and in experimental animal models. The team involved in these projects works in close cooperation with the gynecology, hematology and oncology departments of the Cliniques Universitaires Saint-Luc.
Ovarian tissue cryopreservation is offered to young women at risk of premature menopause and sterility after gonadotoxic therapies such as chemo- and radiotherapy. Cryopreservation and transplantation of ovarian tissue is a promising approach to preserve fertility in young cancer patients undergoing gonadotoxic treatment and the only option for prepubertal patients and patients who have no time to undergo stimulation for embryo or oocyte cryopreservation. Transplantation of cryopreserved ovarian tissue allowed restoration of ovarian function, and fertility in more than 100 patients so far worldwide, with 13 babies for Saint-Luc. The ovarian tissue bank at Cliniques Universitaires St Luc (one of the first and largest in the world) contains tissue from more than 600 patients, with around 100 patients having donated their tissue for research purposes and 500 for fertility preservation and long-term cryopreservation. Pathologies are various and include both malignant and benign diseases requiring chemotherapy. The most frequent indications are hematological malignancies and breast cancer.

A review of our cohort of patients who underwent ovarian tissue cryopreservation was recently published. Premature ovarian failure rate is as high as 31.5%, proving a good referral from the oncologists. So far, the return rate for autotransplantation is estimated at 4.4% and the pregnancy rate after autotransplantation is 33%. Each year, a workshop "Course on cryopreservation and transplantation of human ovarian tissue and preantral follicle isolation and in vitro culture" is organized in close collaboration between the research laboratory and the Clinics.

B) Assembling a transplantable artificial ovary

C. Chiti, P. Asiabi, E. Ouni, MM. Dolmans, C.A. Amorim

AIM
The aim of this project is to develop a bioinspired artificial ovary that offers an environment in which follicles can survive and grow. It is essential to bear in mind that just like the natural ovary, the artificial ovary should maintain the original structure of follicles, preserving contact between granulosa cells and oocytes and preserving follicular interaction with the extracellular matrix (ECM). In other words, the artificial ovary should spatially and temporally mimic the ECM. In order to do so, it needs to include certain design specifications, such as interaction with cells, physical support of follicles, porosity and biodegradability, which are all interconnected and influence each other. It must also be biocompatible and, from a practical point of view, capable of being sterilized and handled.

BACKGROUND
Cryopreservation and transplantation of ovarian tissue is a promising approach to preserve fertility in young cancer patients undergoing gonadotoxic treatment. Transplantation of cryopreserved ovarian tissue allows restoration of ovarian function and fertility. Although safe xenotransplantation of ovarian tissue from lymphoma patients has been reported in SCID mice, the possibility of reintroducing tumor cells into cancer patients by autografting of ovarian tissue cannot be excluded for other indications, such as leukemia. To avoid transferring malignant cells, grafting of isolated follicles may be considered. We encapsulated isolated mouse follicles and ovarian cells in fibrin matrix and autotransplanted to immunocompetent and immunodeficient mice. After one week of transplantation, we observed that secondary follicles seem to survive better than small primordial and primary follicles. Moreover, these larger follicles were able to increase vascularization in the graft.

Based on our promising results with mouse follicles, we decided to test fibrin matrix to encapsulate isolated human preantral follicles. An artificial ovary prototype made of fibrin or fibrin-hyaluronic acid, isolated human follicles and ovarian cells was xenografted to immunodeficient mice. After one week of xenotransplantation, we found a high follicle recovery rate in both matrices (around 22%). Such encouraging results demonstrated that the artificial ovary can be a feasible option to restore fertility in cancer patients. It is also important to highlight that the ultrastructure of the fibrin matrix was similar to human ovarian tissue (Fig. 1). This is a great advantage for our artificial ovary because we can easily prepare the prototype matrix without the need to use complex strategies, such as 3D printing.
C) Improving human ovarian grafting outcome
D. Manavella, L. Cacciottola, M-M Dolmans

The main mechanism provoking loss of follicles after ovarian tissue transplantation is ischemia, since initiation of graft reperfusion takes place only on day 5 post-transplantation. Preparation of the transplantation site prior to grafting is also very important and needs to be further investigated. Our studies in cynomolgus monkeys (2015) demonstrated that grafting to a freshly decorticated ovarian hilum yields good follicular survival. Therefore, in order to optimize grafting procedures with the ultimate goal of increasing follicle survival and hence pregnancy rates after ovarian tissue transplantation we aim to use Adipose-derived stem cells (ASCs) to prepare the grafting site prior to ovarian tissue transplantation.

Our objective is to increase follicle survival after ovarian tissue transplantation by enhancing angiogenesis in the transplantation site introducing a novel two-step transplantation procedure. This procedure consists in preparing a peritoneal transplantation site by grafting ASCs inside a fibrin matrix in a first step and the ovarian tissue in a second step, after 14 days. In this project, human ovarian tissue is grafted to SCID mice.

D) Minimal disseminated disease in the ovary
R. Masciangelo, C.A. Amorim, MM. Dolmans

AIM
(i) evaluate the risk of disease retransmission through the graft
(ii) obtain disease-free ovarian follicle suspensions from ovarian tissue of leukemia patients for grafting

BACKGROUND
In most centers, including ours, hematological disease represents the most frequent indication for ovarian tissue cryopreservation. For leukemia, our experimental studies showed that ovarian tissue reimplantation cannot be safely performed in young women with the acute lymphoblastic form because of the risk of reintroducing the disease. For ovarian tissue from hematologic cancer patients, it is therefore of primordial importance to identify minimal disseminated disease (MDD) before ovarian transplantation. Markers used to detect MDD in ovarian tissue are disease-specific, requiring a patient-oriented case-by-case approach. Given the presence of leukemic cells and the possibility of disease transmission, reimplantation of ovarian tissue in young women with the acute form of leukemia is not recommended. One option to restore fertility in these patients could be the grafting of isolated preantral follicles, through the so-called artificial ovary.

Safe follicle isolation in leukemia patients
We have recently investigated the safety of our follicle pick-up procedure in a model of ovarian tissue suspension artificially contaminated with leukemic cells. We showed that this procedure was not safe in case of a relatively important contamination. However, 3 additional washes proved effective in eliminating the leukemic cells taken along with the follicles. This improved follicle isolation technique has been applied to follicle suspensions obtained from the ovarian tissue of 10 leukemia patients. Suitable markers to be used for detecting leukemia cells in each patient has been determined with the help of Pr. Pascale Saussoy, hematology laboratory, Cliniques Universitaires St. Luc) and the disease free nature of these follicles was confirmed by PCR. This series of 10 patients with leukemia who had their ovarian tissue frozen was recently published in BJH.
SELECTED PUBLICATIONS


• Soares M, Sahra K, Chiti MC, Amorim CA, Ambroise J, Donnez J, Dolmans MM. The best source of isolated stromal cells for the artificial ovary: medulla or cortex, cryopreserved or fresh? Hum Reprod. 2015;30:1589-98.


ETIOLOGY OF THE ADENOMYOTIC NODULE OF THE RECTOVAGINAL SEPTUM

J. Garcia, J. Squifflet, O. Donnez, MM. Dolmans

Endometriosis is one of the most commonly encountered benign pathologies in gynecological practice all over the world, affecting approximately 10% of reproductive-age women and 35-50% of women presenting with pelvic pain and infertility. Dysmenorrhea (painful menstruation), dyspareunia (painful sexual intercourse), chronic pelvic pain and infertility are the most frequent symptoms found in this disease. It is now well established that there are three distinct forms of endometriosis in the pelvis that can be considered separate entities: peritoneal endometriosis, ovarian endometriosis and deep endometriotic nodules of the rectovaginal septum. Most deep endometriotic nodules originate from the posterior part of the cervix (types II and III) and secondarily infiltrate the anterior wall of the rectum (type III). Surgery is the gold standard for the management of rectovaginal endometriotic nodules, but treatment so far is sometimes disappointing, highlighting the importance of developing new treatment strategies.

However, considering the number of variables involved in the pathogenesis of endometriosis and the often late diagnosis, an experimental model is required for more in-depth study of the progression of the disease. Our group previously induced deep nodular endometriosis in baboons, obtaining endometriotic lesions with innervation and invasion of surrounding tissue after both 6 months and 1 year. Invasive glands were found to express a more aggressive phenotype after 1 year than after 6 months, characterized by thin glands maintaining cell-cell attachment during the migration process and showing higher proliferation rates. We are currently comparing these lesions with human deep nodular endometriotic samples to ascertain if this phenotype is also present in human disease.

There is increasing evidence in the literature of the presence of nerve fibers in deep nodular endometriosis. Moreover, deep endometriosis has been described as the most innervated type of endometriosis and also the most painful, hence indicating possible involvement of nerve development in the chronic pain that these patients suffer.
In our model, we detected a significant increase in nerve fibers positive for the ubiquitous neuronal marker protein gene product (PGP9.5) and a significant decrease in levels of nerve growth factor (NGF), in established lesions one year after induction, compared to lesions after 6 months.

These results suggest that this growth factor could well be a potent stimulator of nerve fiber genesis in endometriotic lesions themselves, with higher levels of NGF expression in deep-infiltrating lesions inducing a greater density of nerve fibers. We are currently working on the hypothesis that NGF could act as a positive chemotaxin for neurons, and may therefore facilitate their contact with target tissues through interaction with its receptors.

Finally, light-CT scanning technology (recently acquired by Professor Dolmans’s laboratory through an FNRS equipment grant) will be used for direct and noninvasive assessment of the location and shape of glands, generating a 3D image reconstruction, generating a novel database of human endometriotic samples.

SELECTED PUBLICATIONS

Uterine myomas (also referred as fibroids) are the most common benign tumors of the female reproductive tract and derive from the myometrium. Generally asymptomatic, they can be responsible for abnormal uterine bleeding leading to anemia, abdominal pain, urinary frequency and infertility depending on their number, size and location in the uterus. Stopping or reducing bleeding, moderating pain and decreasing myoma and uterine size are thus essential to improve patient health and quality of life, with particular consideration for the potential of less invasive surgery.

Since uterine myoma growth depends on progesterone, targeting progesterone receptor (PR) appeared as a potential strategy for non-surgical management. Indeed, blocking sex steroid secretion by treatment with GnRH agonist efficiently reduces myoma volume but cannot be proposed for long-term therapy because of important side effects (ie. reduced levels of circulating estrogen similar to castration cause osteoporosis). Furthermore, this therapy does not provide long-lasting reduction on the myoma volume.

A novel class of molecules, the selective PR modulator (SPRM), produce specific effects in PR-expressing tissues (uterus, cervix, ovaries, hypothalamus), being either agonist, antagonist, or with mixed activity probably depending on the presence of PR cofactors. Among this class, ulipristal acetate (UPA, Esmya® for uterine myoma treatment or EllaOne® for emergency contraception) was tested to manage the symptoms caused by uterine myomas. In a phase III study, it was demonstrated that a 3-month course of UPA treatment effectively controlled excessive bleeding due to uterine myomas and reduced the size of myomas. Moreover, daily oral doses of UPA were at least as efficient as once-monthly injections of leuprolide acetate (GnRH agonist) for controlling uterine bleeding, and were significantly less likely to cause hot flushes. Furthermore, unlike GnRH agonists, UPA does not cause an initial flare-up resulting in an additional episode of bleeding. In a long-term study of intermittent (from 2 to 4 courses) and repeated doses of UPA demonstrated that in most cases (~80%) the volume of myomas was considerably reduced after treatment. The reduction was maximized after more than one course and maintained after treatment cessation, demonstrating long-lasting benefits.

UPA efficacy in terms of abnormal uterine bleeding management and safety was also confirmed. Pregnancies were achieved after this therapy and no tumor regrowth was observed, even after ovarian stimulation, confirming the sustained effect on myoma volume regression. The molecular mechanisms leading to myoma volume reduction remain poorly understood however. Our study aims to elucidate the molecular action of UPA in uterine myoma, with the hope of better know the pathogenesis of this disease.

We already identified that UPA treatment (i) reduces proliferation of myoma cells and (ii) increases the apoptotic index of myoma cells. However, apoptosis mainly occurred in short-term-treated. Most importantly, we highlighted an essential reduction of the ECM fraction. This suggested that proteases such as matrix metalloproteinase 2 (MMP-2) may participate in the ECM resorption. Taken these findings together, we proposed that a dynamic and multifactorial combination of factors were involved in myoma size reduction (Figure 1).

Few studies, exclusively in vitro, proposed some key factors to explain UPA mechanism of action. They included PR and isoforms, PR nuclear cofactors, prosurvival factor Bcl-2, and Akt. Unexpectedly, no difference were found in their expression, distribution and ratios, hence tempering extrapolations based on in vitro studies.

To better understand the molecular basis of myoma volume regression after UPA treatment, we investigate the ability of UPA to control the expression of apoptosis-associated and ECM-related genes in myomas that reduced and myomas that did not respond to treatment. This comparison identified new factors which were differently expressed in responsive myoma vs unresponsive.

In conclusion, this work aims to clarify the molecular mechanisms involved in myoma volume reduction after UPA treatment, better know the fundamentals of this pathology which remain poorly understood and identify new therapeutic applications of this molecule.
SELECTED PUBLICATIONS

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ANDROLOGY

Due to remarkable advances in cancer therapies, we have seen great improvements in survival rates of pediatric and reproductive-age male patients. Unfortunately, fertility in adult life may be severely impaired by these treatments. Gonadotoxic therapy is also used to cure a variety of non-malignant disorders, such as hemoglobinopathies, aplastic anemia and autoimmune diseases, resulting in a growing population affected by fertility-threatening therapies. Knowledge and understanding of fertility preservation and restoration approaches therefore warrants broader dissemination in clinical practice.

Our research focuses on four main axes:

> A Optimization of fertility preservation methods for prepubertal boys by cryopreservation of immature testicular tissue (ITT).

> B Development of fertility restoration techniques from cryopreserved ITT by autotransplantation and in vitro maturation.

> C Creation of a bioengineered artificial testicle to be used as an in vitro study model for the spermatogonial stem cell niche pathophysiology.

> D Optimization of the transplantation technique of immature testicular tissue.

A) Fertility preservation and restoration from cryopreserved immature testicular tissue (ITT)

J. Poels, F. De Michele, M. Vermeulen, F. Del Vento, C. Wyns

We developed a slow-freezing protocol for prepubertal human testicular tissue that has yielded good structural integrity of cells and tissue after evaluation in an in vivo xenografting model.

Consequently, indications for spermatogonial stem cells banking were established and banking of ITT from prepubertal boys undergoing gonadotoxic treatments was initiated. Patient/parent satisfaction and decisional factors were also evaluated.

Further assessment of the functional capacity of cryopreserved human ITT after long-term xenografting was subsequently performed. Although seminiferous tubule integrity and the ability of spermatogonial cells to proliferate were well preserved, complete normal spermatogenic differentiation could

Fundings

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not be achieved. Indeed, spermatids were slightly smaller than in situ controls and spermatozoon-like cells with small heads and short tails were observed. In addition, a high proportion of spermatogonial cells were lost. Studies aimed at optimizing cryopreservation protocols were therefore conducted. The potential of vitrification (a technique preventing ice crystal formation by use of high concentrations of cryoprotectants and ultrafast cooling velocity that could minimize cellular damage) was evaluated. Vitrification of non-human primate ITT allowed survival of spermatogonia able to proliferate and functional Leydig cells. Moreover, in humans, integrity of seminiferous tubules and survival and proliferation of spermatogonia were observed in long-term organotypic culture, showing vitrification to be a promising alternative strategy to slow-freezing in the emerging field of ITT cryopreservation. Unexpectedly, our comparative studies of cryopreservation methods in our in vivo xenotransplantation model led to the conclusion that the grafting method and transplantation environment were at least partially responsible for spermatogonial cell loss and their incomplete differentiation, highlighting the urgent need to develop a robust controlled environment for transplanted tissue before considering autotransplantation of cryopreserved ITT in our patients.

Successful fertility restoration with frozen-thawed ITT in humans has not yet been reported.

Our current research focuses on three different fertility restoration strategies from cryopreserved ITT:

> A Autotransplantation of the stored tissue for patients in whom there is no risk of contamination of the tissue by cancer cells. Avascular transplantation of ITT showed limited spermatogonial survival (14.5% and 3.7% three weeks and 6 months after transplantation, respectively). Addition of antioxidant agents and testosterone did not improve transplantation efficiency in terms of spermatogonial survival. To improve the transplantation outcome, we aimed at reducing tissue hypoxia occurring before revascularization of the graft. We demonstrated that encapsulation of mice ITT in hydrogels supplemented with VEGF nanoparticles improved vascular density, VEGFR2 activation, endothelial proliferation and that spermatogonial survival following encapsulation of ITT in alginate doubled. However, vascular density was not maintained after 21 days of grafting suggesting a lack of neovessel stabilization. Further studies will now focus on further improvement of spermatogonial survival and angiogenesis in grafted tissue, which are essential for spermatogenesis initiation. Two strategies will be explored: supply of vascular factors to improve neovascularization of the tissue and addition of antinecrotic agents to decrease ischemia-induced damages.

> B In vitro maturation of the spermatogonial stem cells contained in the stored tissue yielding in vitro-derived male haploid gametes available for ICSI. This procedure circumvents the risk of reintroducing malignant cells, making this approach potentially highly beneficial in cancer patients. A short time organotypic culture system was firstly designed for evaluating the functionality of the cryopreserved tissue after thawing. Recently, the culture system has been further improved and allowed Sertoli cell maturation mimicking the passage from pre- to postpuberty as well as testosterone production by Leydig cell during a 139 day culture period, providing thus some of the paracrine and molecular processes required to achieve the spermatogenic process. This is the first time that human ITT was studied in a long-term organotypic culture system, opening the possibility of a deeper understanding of the SSC molecular niche before puberty and during the pubertal transition phase.

> C Elaboration of a porcine bioengineered testicular scaffold to incorporate sorted human testicular cells with a view to be transplanted to the patient and achieve differentiation of isolated and cultured spermatogonia in the presence of testicular somatic cells. By comparing different decellularization protocols of pig ITT, we designed a method to produce a testicular tissue scaffold allowing Sertoli cell attachment, proliferation and functionality. Further studies will compare the testicular cell behavior in a solid and in a gelified scaffold in order to obtain organoids with a potential of spermatogonial renewal and differentiation.
SELECTED PUBLICATIONS


