STELLENBOSCH UNIVERSITY

# ENDOCRINE FUNCTION AND FERTILITY PRESERVATION IN WOMEN SURVIVING CANCER

A study on cancer treatment and fertility

**Matthys Hendrik Botha**

*Dissertation presented for the degree of Doctor of Medicine at the University of Stellenbosch*

**Promoter: Prof. T.F. Kruger**

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# Declaration

I, Matthys Hendrik Botha, hereby declare that the work contained in this dissertation, is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Signature

Date

**6 October 2010**

# Abstract of thesis


### *Chapter 1*

Chapter 1 is a literature review investigating the incidence of cancer in children and young adults. It describes the most important treatment options including chemotherapy, radiotherapy and surgery and the effect of treatment on future endocrine development and fertility. Different primary cancer sites are discussed in more detail.

### *Chapter 2*

Chapter 2 is a literature review on the effects of cancer surgery in women and the options for fertility sparing. Cervical cancer and pre-cancer are discussed in detail with options for more conservative surgery in selected patients. A summary of the available published cases of trachelectomy with pregnancy outcomes is included. Other gynaecological cancers requiring surgery are also discussed with reference to conservative options.

### *Chapter 3*

Chapter 3 is a literature review about the medical (pharmacological) options for protection of ovarian function in patients undergoing oncotherapy. The role of gonadotrophin releasing hormone analogues and hormonal contraceptives in ovarian suppression is discussed in detail.

### *Chapter 4*

This chapter examines germ cell physiology with reference to cryopreservation. It includes two major parts. Part 1 is the description of germ cell- and follicle

physiology, the principles of cryobiology followed by a review of *oocyte* cryopreservation and *ovarian tissue* preservation. Both slow freezing and vitrification techniques are described. The second part of chapter 4 is a report on a randomised controlled evaluation of two different slow freezing cryopreservation protocols. This experimental study compared ultrastructural changes in fresh and previously cryopreserved ovarian cortical tissue after equilibration and thawing using two different cryoprotectants. This is the first randomised investigation into DMSO and PROH as cryoprotectants.

### *Chapter 5*

Chapter 5 is an investigation into cryopreservation of ovarian tissue as a strategy to protect hormonal function and fertility against gonadotoxic treatment. This chapter consists of two parts. The first part is a thorough literature review of all the published work about grafting of previously cryopreserved ovarian tissue. The largest case series found from a single institution was five patients. Another report of six patients included patients from various sites in Denmark.

Part 2 is a description of a cohort of patients followed up after re-implantation of previously cryopreserved ovarian cortical tissue. Follow-up hormone levels of 13 individual cases are described in detail. This is the largest case series ever reported.

The experimental study described in Chapter 4 and the clinical study described in Chapter 5 was approved by the ethical research committee of the Faculty of Health Sciences, Stellenbosch University, project number N05/10/182.

### *Chapter 6*

Chapter 6 provides an integrated overview of the incidence and treatment of cancer in young women and how its negative effects may be prevented or mitigated. Aspects of chemotherapy, radiotherapy and surgery are evaluated where it may affect future reproductive health. The role of oocyte and ovarian tissue cryopreservation is discussed. Guidelines are provided for clinicians.

## Opsomming van tesis

### *Hoofstuk 1*

Hierdie is ‘n literatuuroorsig wat die insidensie van kanker in kinders en jong volwassenes ondersoek. Dit sluit die mees belangrike behandelingsopsies in, naamlik chemoterapie, radioterapie en chirurgie en die effek wat behandeling mag hê op toekomstige endokriene ontwikkeling en fertiliteit. ‘n Verskeidenheid kanker tipes word in meer detail beskryf.

### *Hoofstuk 2*

Hoofstuk 2 is ‘n literatuuroorsig oor die effekte van kankerchirurgie in vroue en die geleenthede tot beskerming van fertiliteit. Servikale kanker en voorlopers van servikale kanker word bespreek en die opsies vir konserwatiewe chirurgie in uitgesoekte pasiënte word gegee. ‘n Opsomming van die inligting wat beskikbaar is oor tragelektomie en swangerskap uitkomste word ingesluit. Ander ginekologiese kankers wat chirurgie mag benodig, word ook bespreek met verwysing na konserwatiewe hantering.

### *Hoofstuk 3*

‘n Literatuuroorsig oor die mediese (farmakologiese) opsies vir die beskerming van ovariële funksie in pasiënte wat behandeling ontvang vir kanker. Die rol van gonadotropien-vrystellingshormoon-analoë en hormonale kontrasepsie vir ovariële onderdrukking word in detail bespreek.

### *Hoofstuk 4*

Hierdie hoofstuk ondersoek kiemselfisiologie met verwysing na vriesbewaring. Dit is verdeel in twee dele. Deel 1 is ‘n beskrywing van kiemsel- en follikelfisiologie en die

beginsels van vriesbiologie. Dit word gevolg deur ‘n oorsig van oösiet vriesbewaring en ovariële weefselbewaring. Stadige bevriesing en vitrifikasie- metodes word bespreek. Die tweede deel van hoofstuk 4 is ‘n verslag oor ‘n gerandomiseerde, gekontroleerde evaluasie van twee stadige bevriesingsmetodes. Hierdie eksperimentele studie het die ultrastrukturele veranderinge vergelyk in vars en voorheen bevrore ovariële kortikale weefsel na ekwilibrasie en ontdooiing met twee verskillende vriesbeskermers. Dit is die eerste gerandomiseerde studie oor DMSO en PROH as vriesbeskermers.

### *Hoofstuk 5*

Hierdie hoofstuk handel oor ‘n ondersoek na vriesbewaring van ovariële weefsel as ‘n benadering tot beskerming van hormonale funksie en fertiliteit teen gonadotoksiese behandeling. Die hoofstuk bestaan uit twee dele. Die eerste deel is ‘n deeglike oorsig van die literatuur oor al die beskikbare werk wat handel oor terugplasing van voorheen bevrore ovariële weefsel. Die grootste pasiëntreeks van ‘n enkel instelling was slegs vyf pasiënte. ‘n Ander beskrywing van ses pasiënte het pasiënte van verskeie eenhede in Denemarke ingesluit.

Deel 2 is ‘n beskrywing van ‘n groep pasiënte wat opgevolg is na oorplanting van voorheen bevrore ovariële kortikale weefsel. Opvolg hormoonvlakke van 13 gevalle word in detail bespreek. Hierdie is die grootste pasiëntreeks wat tot nog toe beskryf is.

Die eksperimentele studie wat in hoofstuk 4 beskryf word en die kliniese studie wat in hoofstuk 5 beskryf word, is goedgekeur deur die etiese navorsingskomitee van die

Fakulteit Gesondheidswetenskappe van die Universiteit Stellenbosch met die projeknommer N05/10/182

## *Hoofstuk 6*

Hierdie is ‘n geïntegreerde oorsig van die voorkoms en behandeling van kanker in jong vroue en hoe die negatiewe effekte daarvan voorkom of verminder kan word. Aspekte van chemoterapie, radioterapie en chirurgie word geëvalueer ten opsigte van die effek op toekomstige reproduktiewe gesondheid. Die rol van oösiet- en ovariële weefselvriesbewaring word bespreek. Riglyne vir klinici word gegee.

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# Chapter 1: Incidence and survival of childhood and adolescent cancer and the effects of treatment on future fertility and endocrine function.


### *Abstract*

Cancer is not uncommon in children. The reproductive system is an important site for late effects of cancer treatment and normal pubertal development depends on an undamaged hypothalamic-pituitary-gonadal axis. Fertility compromise can occur due to chemotherapy and radiotherapy of the hypothalamic-pituitary-gonadal axis.

This review describes the incidence of malignancies affecting children and young adults. Chemotherapy may cause premature ovarian failure through direct toxicity to germ cells and other mechanisms. Age at treatment and type and total dose of chemotherapy are predictors of risk. Ovarian tissue is very sensitive to radiotherapy induced damage.

## *Introduction*

Cancer is not an uncommon diagnosis in children. The incidence of childhood cancer (generally calculated under and up to the age of 15) is 110 to 130 per million children per annum. [1] It is estimated that the cumulative risk of a child being diagnosed with cancer is slightly higher in boys at 1:444 compared to girls which is one is 1:594. [2] In South Africa accurate figures for childhood cancer are not available. The reported incidence is around 70-80 per million, however it is estimated that one in 600 children will suffer from cancer before they turn 16. Many of these cancers are diagnosed late or may not be diagnosed at all. [3]

The prognosis for patients with cancer diagnosed before the age of 15 has improved dramatically over the last 30 years and there are now more than 80% of cases who survive longer than 5 years and more than 70% will be long-term survivors. Information from cancer statistics during the 1970 to 1980's indicate that in the United States, the cure rate of all childhood cancers combined was between 70-90%. [4] The estimated 5 year survival of children of both sexes improved form 50.4% in 1973 to 79.2% in 1990. [5]

The age at diagnosis of cancer is important. Neuroblastomas and Wilms' tumours are most common in infants less than five years old while Hodgkin's lymphoma and bone tumours usually present in the teenage years and early adult life. Leukaemia may occur at all ages.

| | Incidence 100 000/y | Cure rate (%) |
|---|---|---|
| **ALL* / non Hodgkin's lymphoma** | 5.0-6.0 | 78-80 |
| **Hodgkin's lymphoma** | 0.4 | >90 |
| **Brain tumours** | 4.0 | Depends on type |
| **Wilms' tumour** | 0.9 | 80 |

*Table 1 Leukaemia and non-Hodgkin's lymphoma represents the most common cancers in children. [6] *Acute lymphoblastic leukaemia*

Older children and young adults have historically not been studied to the same extent as young children with regards to cancer incidence. The age range for adolescence for the purpose of this study has been set at 15-19 years. [7] More recently the concept of "Young Adult Oncology" refers to a larger group of young people aged 15-29. [8] This group of young people is at a very important developmental phase particularly for establishment of normal hormonal and sexual function. The spectrum of cancers affecting this group will differ from younger children and from adults.

| Age | 15-19 | 20-24 | 25-29 |
|---|---|---|---|
| **Lymphoma** | 26 | 22 | 16 |
| **Leukaemia** | 12 | 7 | 4 |
| **Central nervous system** | 10 | 7 | 5 |
| **Endocrine system** | 9 | 12 | 11 |
| **Skin** | 8 | 14 | 18 |
| **Male genital** | 8 | 13 | 11 |
| **Female genital** | 8 | 8 | 12 |
| **Bone and joint** | 8 | 3 | 1 |
| **Soft tissue** | 5 | 3 | 2 |
| **Digestive system** | 2 | 3 | 5 |
| **Oro-pharynx** | 2 | 3 | 2 |
| **Respiratory system** | 2 | 2 | 2 |
| **Urinary system** | 2 | 2 | 2 |
| **Breast** | 0 | 2 | 8 |
| **Other** | 2 | 2 | 1 |

*Table 2 The relative frequencies (%) of cancers in adolescents and young adults aged15-29. [9] Data from National Cancer Institute in the USA*

Tumours of the male and female genital tracts become more common in the young adult group. Testicular cancer is the commonest form of solid malignancy amongst the young adult male group and the frequency increases with progressive age from 15 to 29 years. [9] Cure rates for men with seminomas exceeds 90% but non-

seminomatous tumours have poorer outcome. In young women from the developed world, 18% of the total malignancies are of gynaecological origin. [10] Carcinoma of the cervix becomes more frequent and germ cell tumours, particularly dysgerminoma, are also present.

It is estimated that 1:570 adults are cancer survivors and that this will increase to 1:250 by the year 2010. [5] The increased cure rate means that many more patients will reach adulthood with a history of cancer and a fertility wish. The reproductive system is an important site for late effects of cancer treatment and normal pubertal development depends on an undamaged hypothalamic-pituatary-gonadal axis. Practitioners should be aware of the potential harm to the endocrine and reproductive systems after life-saving but potentially toxic chemo- and/or radiotherapy. Fertility compromise can occur due to chemotherapy, due to radiotherapy of the hypothalamic-pituitary-gonadal axis or from surgery. Chemotherapy and radiotherapy may also be used in patients with non-malignant autoimmune diseases like systemic lupus erythematosis and rheumatoid arthritis as well as certain haematological diseases. [11]

### *The effects of chemotherapy on the ovary*

In females the production of sex-hormones requires the presence of germ cells. Young women will experience *endocrine function* loss after chemo-and radiotherapy in childhood and adolescence more often than boys. Unlike men, women have a fixed number of germ cells that gradually diminish with age. At puberty between 200 000 and 400 000 follicles are present which may eventually mature and only 400 to

500 oocytes are produced in a normal reproductive life span. [12] At the age of menopause only a few hundred follicles are left. [13] Anti-cancer therapy may increase the rate of follicular loss and therefore also premature ovarian failure, with subsequent premature menopause which is one of the common toxic side effects of cancer treatment. [14] Chemotherapy may affect the ovary to cause amenorrhoea in between 40 to 68% of cases depending on various factors. [15]

**Mechanisms of damage to ovarian function**

The pharmacological action of chemotherapy is mainly aimed at disrupting the process of DNA synthesis and cell replication. In general the alkylating agents interact with DNA preventing replication and/or transcription. Anti-tumour antibiotics like actinomycin D works on the same principle. Other agents may damage the structure of DNA directly and adriamycin acts by damaging the plasma membrane. The plant-based chemotherapy agents like the taxanes disrupt the function of tubulin that is important in the normal functioning of the microtubules that is critical in the normal mitotic process.

Specific chemotherapy agents, particularly the alkylating compounds e.g. cyclophosfamide and chlorambucil, may cause permanent DNA damage in ovarian follicles. Other chemotherapy agents are less harmful and these include 5-fluorouracil, methotrexate, etoposide and adriamycin. [16] There are various mechanisms of damage to the ovaries. The damage may be directly to the primordial follicles with demise of follicular cells. Human and animal studies demonstrated that chemotherapy can damage ovarian pre-granulosa cells, [17] with

increased apoptosis during oocyte and follicle maturation. [18] Vascular complications associated with antineoplastic agents have been reported. One recognized mechanism for such toxicity includes drug-induced endovascular damage. [19]

In a descriptive study Meirow and co-workers studied histology of ovarian tissue form 17 women exposed to chemotherapy and compared it with 18 patients that were not exposed. [20] The pathologists were blinded for patient characteristics. They found injury of blood vessels and focal fibrosis of the ovarian cortex to be present in ovaries of patients previously exposed to chemotherapy. Circulation for the cortex of the ovary is supplied by an end-artery system and the cortex is a fairly poorly oxygenated tissue. After chemotherapy there is prominent thickening and narrowing of the vessels and neo-vascularisation with abnormal blood vessels to the ovarian cortex is seen on microscopy. There is also cortical fibrosis. Direct damage to the follicles can also be seen after chemotherapy and there may be pre-granulosa cell swelling with increased apoptosis.

More mature follicles are more vulnerable to chemotherapy damage. [21] Certain endocrine mechanisms may play a role in the damage to the ovarian function. Anti-Müllerian hormone (AMH) is mainly secreted by growing follicles and anti-Müllerian hormone levels drop significantly during therapy. [22] AMH may be used as a marker of ovarian reserve. Serum AMH levels can be measured to assess sub-clinical ovarian damage in patients treated with chemotherapy. [23] A drop in AMH may cause a raised recruitment and atresia. A possible mechanism to protect ovarian

function may be to administer anti-Müllerian hormone during treatment with chemotherapy to reduce recruitment of follicles.

Müllerian Inhibiting Substance (MIS), also known as anti-Müllerian hormone, has been successfully isolated and may in future be used clinically in the treatment of various cancers. [24] It is from the transforming growth factor beta (TGF-ß) superfamily and ovarian, prostate and breast cancer cell lines have shown regression after exposure to AMH. A recombinant human MIS/AMH is available for research purposes. [25]

**Age at treatment**

One of the most important clinical factors that may influence the risk for permanent ovarian damage is the age at treatment. The risk for ovarian failure increases with age. [26] [27] [28] [29] This is due to the fact that the number of remaining primordial follicles is far more at a younger age.

Amenorrhoea due to chemotherapy is more commonly found in treated women who were over the age of 30 years (50-89%) compared to younger women where normal menses was preserved in 48-100% of cases. [30] [31] [32] Chemotherapy related amenorrhoea may be transient. However, if the condition is present for more than one year after treatment, less than 11% of women over the age of 40 and 12-15% of women younger than 40 will experience a return to menses. [33]

| Age | Premature ovarian failure |
|---|---|
| **<20 years** | 13% |
| **20–30 years** | 50% |
| **>30 years** | 100% |

*Table 3 The incidence of ovarian failure after cyclophosfamide pulsed chemotherapy according to age [15]*

## *Treatment of young people with cancer*

There are many different malignancies affecting young people. The most common forms are summarised in Table 4. It is clear that multi agent chemotherapy regimens and radiation may contribute to reproductive failure. It is often difficult to determine the individual effect of specific therapies on fertility outcome.

| | Chemotherapy | Cranial Ro | Gonadal Ro |
|---|---|---|---|
| **Acute lymphoblastic leukaemia** | + | ± | ± |
| **Non-Hodgkin's lymphoma** | + | ± | ± |
| **Hodgkin's lymphoma** | + | - | ± |
| **Brain tumours** | ± | ± | ± |
| **Wilms' tumour** | + | - | ± |

*Table 4 Treatment modalities which are commonly used in the treatment of childhood cancers that may affect future fertility. [6]*

**Haematological malignancies**

There are a significant number of reports in the literature about the effects of chemotherapy on subsequent fertility and hormonal function following treatment for haematological malignancies in younger women. Treatment for Hodgkin's lymphoma with MVPP (mechlorethamine, vinblastine, procarbazine and prednisolone), MOPP (mechlorethamine, vincristine, procarbazine and prednisolone) or ChIVPP (chlorambucil, vinblastine, procarbazine and prednisolone) resulted in permanent ovarian failure in 19-63% of cases. [26] [31] [30] Treatment for Acute lymphoblastic leukaemia (ALL), however, had less long-term risk for permanent amenorrhoea. [34] [35] Conditioning with chemotherapy before bone marrow transplantation is usually associated with transient amenorrhoea. Cyclophosfamide doses of 200mg/kg caused amenorrhoea in all women on treatment but all recovered to normal ovarian function after bone marrow transplantation. [36] Doses higher than 200mg/kg may cause premature ovarian failure. [37] Multi-agent combination chemotherapy regimens will have synergistic toxicity and the specific contribution of each agent may be difficult to determine.

**Breast cancer**

In the United States breast cancer is the most common cancer in women of reproductive age (< 40 years of age) and approximately 13% of all breast cancer diagnoses are made in women younger than 45. [38] Alkylating agents (e.g. cyclophosfamide) are often included in the treatment regimes for breast cancer. The

higher the cumulative dose of cyclophosfamide, the higher the risk for premature menopause. In cases treated with CMF (cyclophosfamide, methotrexate and 5-flurouracil) the incidence of amenorrhoea was 61% in patients younger than 40 years and 95% in patients older than 40 years. [29] Slightly higher incidence of amenorrhoea was found with a regime containing FEC (5-flurouracil, epirubicine and cyclophosfamide) compared with CMF (51% vs 42.6%). [39] Anthracycline based regimes had a lower incidence of amenorrhoea. [16] There is very little evidence with regard to taxanes and the risk for subsequent amenorrhoea. There does not appear to be an increased overall risk when it is added to chemotherapy regimes. [40]

**Ovarian cancer**

Maltaris summarized the obstetric outcome in patients with previous epithelial ovarian carcinoma after receiving conservative treatment. [40] A total of eight studies were included in the review and out of the total of 282 patients, 113 pregnancies were described. The number of term deliveries was 87. In this group the number of reported relapses of ovarian carcinoma was 33 and disease related deaths 16. Not all of these patients received chemotherapy.

**Choriocarcinoma**

At present the treatment for choriocarcinoma is surgical removal of the tumour together with multi agent chemotherapy; usually with methotrexate and/or actinomycin D combined with other agents. In a study reported by Newlands, cyclophosfamide was associated with a reduction in the fertility rate when compared with treatment with methotrexate only. [39] 79% of the total number of patients

desiring pregnancy had at least one live birth after cyclophosfamide compared to 86% in those receiving methotrexate only.

## *Radiotherapy damage to hormone production and fertility in women*

Radiation therapy may affect many different sites of the hypothalamic-pituitary-ovarian axis causing hormonal and reproductive failure. Effects on the uterus may also be directly responsible for poor pregnancy outcomes.

### Radiotherapy effects on the ovary

The extent of radiotherapy damage to ovarian function and reproduction is determined by the total dose of radiation, the fractionation schedule and also the age of the patient at the time of treatment. [14] [41] The number of primordial follicles present at the start of treatment will depend on the age of the patient. The number of primordial follicles will be higher the younger the child at the time of radiotherapy and the larger the remaining oocyte pool, the later the eventual age of menopause. The human oocyte is exquisitely sensitive to the damaging effects of radiation and the estimated median lethal dose ($LD_{50}$) is less than 4 Gray. [14] A descriptive study by Wallace found that 37 out of 38 females had ovarian failure after whole abdominal irradiation of 20 to 30 Gray in childhood. 71% had primary amenorrhoea i.e. they never had normal pubertal development and premature menopause occurred in the rest with a median age of 23.5 years. [41] Total body irradiation (TBI) is sometimes used alone or in combination with cyclophosfamide as conditioning for bone marrow

transplantation. This treatment is often associated with infertility and only a small number of patients (9 out of 144) had normal ovarian function after TBI of a dose 9 - 16 Gray combined with cyclophosfamide 120mg/kg before bone marrow transplantation. The effect of age at treatment was also demonstrated in this study with a greater probability of recovery of ovarian function observed in younger girls. [36]

The largest cohort of cancer survivors studied for ovarian failure describe the long term follow up of 3390 cancer survivors. [42] The Childhood Cancer Survivor Study reported on loss of menstrual function within the first five years after diagnosis and excluded from analysis those with cranial radiation of more that 3000cGy, those with tumours of the hypothalamus or pituitary or who had bilateral oophorectomy. 215 Of the survivors developed ovarian failure. The cases of ovarian failure were older at treatment, had a higher incidence of pelvic and/or abdominal radiation or were treated with procarbazine. In multivariate analysis increasing doses of radiation was an independent risk factor. [42]

It is clear from Table 5 that premature ovarian failure is higher if the patient receives treatment at an older age. The number of viable follicles is reduced by the normal physiological processes and the surviving number is therefore less after an insult.

| Dose Gy | Effect on ovarian function |
|---|---|
| **0,6** | No deleterious effect |
| **1,5** | No effect in <40yr<br>Some risk of POF in >40yr |
| **5** | 60% sterile <40yr<br>100% sterile >40yr |
| **8** | 70% sterile <40yr<br>100% sterile >40yr |
| **>8** | 100% sterile |

*Table 5 The effect of radiation on ovarian function [43]*

Ovarian trans-position outside the field of radiotherapy may reduce the dose to the ovary. Howell described how lateral trans-position of the ovaries to the para-colic gutters may reduce the radiotherapy dose by up to 95%. [44] This may protect the sensitive follicles to direct dose related damage. Other reports, however, were less optimistic and found that ovarian trans-position may compromise blood supply and there was mixed success with this technique due to scattered radiation and vascular compromise. [45] Ovarian transposition may have a place for fertility preservation in cases where the pelvic dose of radiotherapy is not high enough to be damaging to the other organs of the reproductive tract.

## Radiotherapy effects on uterine function

The uterus may be damaged by radiotherapy and reduced uterine volume and decreased elasticity of the myometrium can be found in girls who received pelvic-, abdominal- or total body irradiation before puberty. [46] [47]

Even though successful pregnancies following radiotherapy have been reported, there is an increased incidence of miscarriage, intrauterine growth restriction and premature delivery. [34] It is difficult to diagnose uterine damage after exposure of the uterus to radiotherapy but endometrial sampling may help in the assessment of endometrial function. Exact prediction of eventual reproductive outcome is however very difficult.

### Effects on the uterine volume

When girls are irradiated before puberty and at very young age, final uterine volume is significantly lower compared to those irradiated at a later age. [48] Damage to the uterus is usually greater in prepubertal than in pubertal girls. [49] Uterine volume is directly affected by the dose of irradiation as described by Larsen in a large group of 100 childhood cancer survivors. [50] Transvaginal ultrasound was used to evaluate the uterine volume. The children were divided into four groups according to the amount of radiation exposure. In control patients (n = 44) the median uterine volume was 47mls. In those receiving radiation above the diaphragm (n=21) the median uterine volume was 40mls. Young women treated with radiation below the diaphragm (n=19) had volumes of 34mls and in patients treated with direct uterine

radiation (n=16) the median uterine volume was only 13mls. The treatment age was also significantly associated with smaller uterine volume (p=0.02). There was a significant increase in the mid-trimester miscarriage rate in those having exposure to therapy.

Holm followed 12 female patients who had total body irradiation at a median age of 12.7 years for childhood leukaemia. They found a significantly decreased uterine volume - 2.6 standard deviations below that of controls. [51]

Not only is radiotherapy associated with reduced uterine volume but also with impaired blood flow. In a study of 12 leukaemia survivors who received total body irradiation, uterine blood flow was reduced. [51] The reduced blood flow may be due to direct vascular damage or hormonal factors and fibrosis. [48] [46] There is some evidence that uterine blood flow is better in women who have premature ovarian failure due to causes other than radiotherapy which would support a direct radiation linked damage to the vasculature of the uterus. [52]

Delanian described histological changes of the irradiated uterus. [53] Two major features are identified namely

1. Necrosis and atrophy of the endometrium, inflammation and later fibrosis with telangiectasis of the inner layer.
2. Arteriolar sclerosis, fibrosis and muscular atrophy of the myometrium.

Evidence of endometrial necrosis and atrophy is described in MRI follow up studies of patients receiving uterine radiotherapy. [54]

The observed effects on uterine volume, decreased blood flow and endometrial atrophy appears to increase the risk for pregnancy related complications. Case reports of placenta accreta and even uterine rupture in patients with previous total body irradiation and pelvic radiation have been described. [55] [56] Low birth weight infants and placental abnormalities have also been described. [57] Abnormal placentation was described after midtrimester miscarriage in a 23-year old woman who had 70 Gray to the pelvis for treatment of a sarcoma. She had intraperitoneal bleeding and a subsequent hysterectomy. Histology on the removed uterus showed fibrosis and an area where the chorionic villi were implanted directly into the myometrium. [56] Another case report describes uterine rupture occurring at 17 weeks of gestation in a patient with placenta accreta secondary to previous radiation for chronic myeloid leukaemia. [55]

There may be an increased risk for congenital abnormalities in the offspring of childhood cancer survivors. In the Wilms' tumour study group, 10% of offspring of the irradiated group was born with congenital abnormalities compared to 3.2% in the non-irradiated group. [58] The same study also described an increase in premature labour, relative risk (RR) 2.36 [0.93 - 6.02] and also a higher incidence of low birth weight ( <2500 grams at birth) RR 1.85 [1.07 to 3.18] Radiation was an independent risk factor after controlling for factors like age, parity, smoking, alcohol intake and education.

## Strategies to improve uterine function after previous radiation

A combination of direct radiation toxicity to the myometrial and endometrial tissues combined with poor circulating hormone levels will cause low uterine volume and a thin endometrium. Hormone replacement therapy will improve uterine blood flow and endometrial development as well as overall uterine size. [52] Girls exposed to radiation before puberty showed a smaller improvement in uterine volume than girls treated after the menarche. [50] However, those receiving in excess of 30 Gray had no improvement in uterine volume after hormonal treatment. [50]

Pentoxifylline (PTX) and vitamin E in combination have demonstrated the ability to reverse certain effects of chronic radiation damage. [59] This led to a small therapeutic trial of 800mg of PTX combined with vitamin E 1000 IU daily administered for one year to women who previously received high dose ( >45 Gray) radiotherapy in childhood. [60] These patients also received hormone therapy for premature ovarian failure. After adding the additional PTX and vitamin E treatment, vascularity improved significantly. There was also an increase in the endometrial thickness and uterine size. The effect on subsequent pregnancy is still largely unknown but spontaneous pregnancies leading to the birth of healthy children have been described after combination therapy with PTX and vitamin E. [61]

## Effects of cranial radiation

Cranial irradiation may cause hypo-pituitarism in doses over 30 Gray. [62] [63] Up to 60% of patients experienced a gonadotrophin deficiency four years after treatment

with cranial irradiation. [63] Effects on other pituitary hormones like growth hormones have also been reported. [64] In a report by Nygaard, a cranial radiation dose of between 18 and 24 Gray has been identified as a possible risk factor for a significantly lower first birth rate compared to those without any radiation. [65] The presence of a regular menstrual cycle may not be an adequate indication of hypothalamic-pituitary function and women who received cranial radiation with infertility need careful hormonal assessment.

Prophylactic cranial radiation (PCI) was often used as treatment for children with leukaemia. The effect of PCI on future reproduction mainly revolves around two issues namely an increased risk for *precocious puberty* and the *possibility of gonadotrophin impairment*. A population based cohort study from Scandinavia found that women, who were treated as children for acute lymphoblastic leukaemia with PCI doses of 18 to 24 Gray, had a significantly decreased birth rate when compared to those who never received radiation. [65] In another review of long-term survivors of acute lymphoblastic leukaemia after PCI, 12 women who received radiation were compared to healthy controls. [66] Despite the fact that the entire group of young women achieved adult sexual development and menstrual cycles, they had decreased levels of LH secretion and shorter luteal phases when compared to controls. Because of the luteal phase deficiency, prepubertal girls who received low dose PCI may have a higher risk for ovarian failure and a higher miscarriage rate. [67]

There is a link between cranial irradiation and precocious puberty. In a report of 46 children receiving an average of 30 Gray for brain tumours, puberty was earlier for

both boys and girls (girls 8.5 versus 11.2 years and boys 9.2 versus 11.6 years). [68] In another cohort of 36 children treated with high dose cranial irradiation (the hypothalamic pituitary dose was between 30-72 Gray) all the young people were treated before the age of 9 years. [69] In girls the median age of puberty was 9.3 years versus 10.9 in controls and for boys 11 years versus 11.5 years in controls. There was a significant positive correlation between the age at diagnosis and the eventual age of puberty in both sexes.

The effects of cranial irradiation on postpubertal children confirm the negative effect on hormonal function. In a group of 16 women and 16 men treated at an average age of 19 with cranial doses of 40-70 Gray, 70% of females developed oligomenorrhoea and 50% showed low oestrogen concentrations after a mean follow-up of 7 years. [70] There was also an incidence of 50% for hyperprolactinemia. Damage to the hypothalamus and pituitary gland was also confirmed in an observational study of 107 adults who received radiotherapy to the base of the skull. [71] Seventy-two percent of cases developed hyperprolactinemia and 29% developed hypogonadism within 5 years. This increased to 84% for hyperprolactinemia and 36% for hypogonadism after 10 years. It is important to note that there may be a significant period of latency between treatment and subsequent hormonal dysfunction. It is therefore necessary to follow these patients for a long time.

### *Chemotherapy and testicular function*

In men endocrine and exocrine functions of the gonads are separate. The average age of the spermarche is at 13.4 years. The alkylating agents are gonadotoxic with

procarbazine particularly harmful. This is a very useful drug used in the treatment of Hodgkin's lymphoma where repeated courses of alkylating agents are often needed. The Sertoli and germ cells are more sensitive than the Leydig cells; therefore a patient with normal testosterone production may have azoospermia. [72] Azoospermia is likely if the volumes of the post pubertal testes are less than 10ml as measured by the Prader orchidometer.

A combination of chemotherapy for Hodgkin's disease may include the following regimes: MOPP (mechlorethamine, vincristine, procarbazine and prednisolone) or ChlVPP (chlorambucil, vinblastine, procarbazine and prednisolone) or COPP (cyclophosfamide, vincristine, procarbazine and prednisolone). It is clear that in these multi-agent regimes there may be synergistic toxicity of individual agents and it is often very difficult to determine the specific contribution of each agent. Certain agents have been identified as been more gonadotoxic to the testes including the alkylating agents procarbazine, cisplatinum and vinblastine. [73] [74] [75] [76] [77] [78] [79] [80] Newer regimes like the ABVD combination (adriamycin, bleomycin, vinblastine and decarbazine) have been shown to be less gonadotoxic with full recovery after 18 months of treatment in nearly all patients. [79] Cyclophosfamide may cause azoospermia in up to 13% and oligozoospermia in 30% of patients treated with a total dose of 560-840mg/kg. [77] The estimated threshold for impaired spermatogenesis was a total dose of 10g. Ifosfamide is sometimes used for the treatment of sarcomas and a dose of between 84-126mg/m$^2$ was associated with impaired spermatogenesis. [81] The testicular seminiferous epithelium that is responsible for spermatogenesis is very sensitive to the effects of chemotherapy, however, the Leydig cells are more resistant to damage and in certain cases,

although secondary sexual characteristics may develop normally, there may be severe impairment of sperm production. [82] [72] In higher cumulative doses Leydig cells may also be damaged [83] however this rarely occurs in clinical practice.

### *Radiotherapy and testicular function*

Radiotherapy may damage the hypothalamic pituitary axis if the dose is more than 30 Gray to the cranial region. Radiotherapy can also damage the testes directly and the damage may be reversible if the dose is between 20-200 cGray but irreversible azoospermia will develop over 400 cGray. A low production of testosterone will only occur when the dose goes above 1500 cGray.

It is important to consider collecting a semen sample before the initiation of chemotherapy. [81] Cryopreservation of sperm is a well known technique and has excellent outcomes. If pre-pubertal boys cannot produce a sample through masturbation, testicular biopsies are a viable alternative. [84]

Pubertal development is usually normal after treatment with TBI in preparation for bone marrow transplantation. [85] It was found that these boys had slightly higher levels of FSH and that mean testicular volume was lower than normal at an average of 10.5ml. LH was elevated which may indicate subtle hormonal dysfunction of the Leydig cells. [85] Other reported studies found no change in LH levels after preparation for bone marrow transplantation. [86] [87]

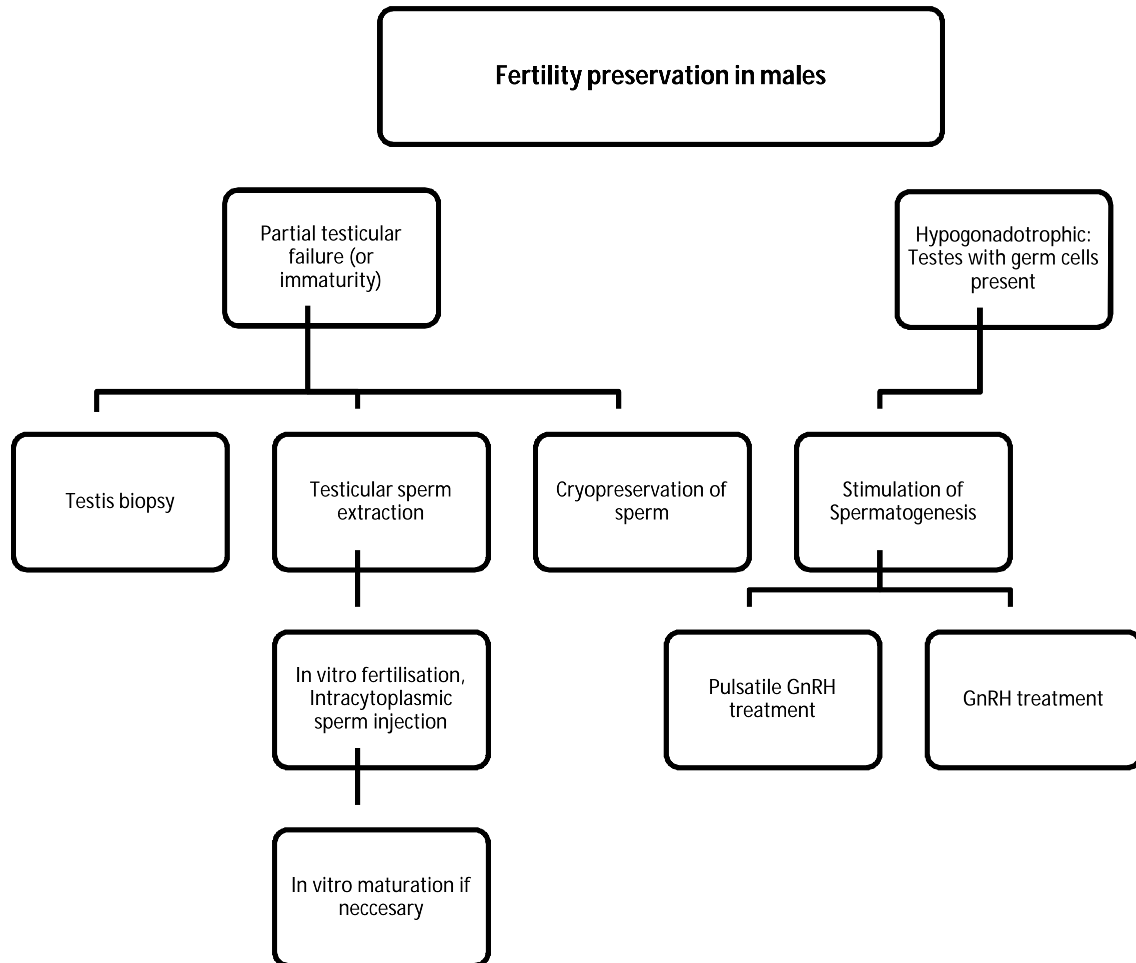


*Figure 1 Schematic view of fertility preservation options in males. Adapted from [84]*

In cases of acquired hypogonadotrophic hypogonadism, gonadotrophin therapy is an option for treating pituitary hormone deficiencies (LH and FSH). [84] Damage to the GnRh production from the hypothalamus will also lead to hypogonadotrophic hypogonadism. GnRh administration in a pulsitile or intermittent regime may induce spontaneous spermatogenesis in selected patients. [88] [89]

Where testicular damage is a high likelihood because of planned treatment, pre-treatment semen samples should be collected for cryopreservation. In young boys

who are unable to produce semen samples testicular tissue can be collected for storage. Studies in animal models have demonstrated the possibility of in vitro maturation of sperm from spermatogonal stem cells. [90] [91] “Testicular sperm extraction and testicular tissue freezing in the prepubertal child is experimental”. [84] [92]

### *Conclusion*

Cancer survivors often are left with long term sequelae after treatment. They are at risk of losing endocrine and exocrine functions of reproduction. The team of clinicians involved in the care of young people with cancer should be aware of the potential harm done by treatment. The clinician can plan strategies to minimize risk and still have a safe oncological outcome. Cryotherapy techniques offer real hope to boys and girls who need gonadotoxic therapy and ovarian tissue, testicular tissue, sperm and ova may be retrieved before treatment is started.

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# Chapter 2: Gynaecological cancer surgery and the options for fertility sparing.

### Abstract

There are various strategies to protect fertility potential against the possibly harmful effects of cancer therapy. Options range from newly emerging pharmacological treatment (e.g. ovarian suppression, apoptotic inhibitors) to cryopreservation techniques (e.g. embryo, oocyte, ovarian tissue).

Surgical interventions for cancer treatment may directly or indirectly harm future fertility potential. New developments in the surgery for tumours are affording different approaches to fertility-sparing options and these surgical approaches can be employed successfully in a large number of situations. This review investigates surgical treatments and its effect on future fertility in women with pre-malignant and invasive cancer of the cervix, uterus and ovary.

## *Introduction*

A significant number of young women are diagnosed with a malignancy during their childbearing years. At present there are various strategies to protect fertility potential against the possibly harmful effects of cancer therapy. The understanding of tumour biology, prognostic factors, epidemiology and behaviour at a microscopic and biochemical level improved over the years. Because of the better understanding of cancer, there are more effective therapies to cure the disease but also to minimise problems associated with treatment. Developments in the surgery for tumours make fertility-sparing options possible in a large number of situations.

Surgical intervention for cancer may directly or indirectly harm future fertility potential. The aim of this chapter is to investigate surgical treatment for pre-malignant disease and cancer of the cervix, uterus and ovary and its effect on future fertility.

## *Cervical disease*

According to the International Agency for Research on Cancer (IARC), cervical cancer accounts for 23% of all new cancers diagnosed in South Africa annually. [1] The age standardized incidence rate for cervical carcinoma in Southern Africa is approximately 35 per 100 000 women years. That is one of the highest incidence rates in the world. An estimated 3 700 deaths in South Africa during 2002 were because of cervical cancer.

**Cervical pre-cancer**

Screening for cervical carcinoma in well-organized programmes has been shown to be effective in reducing the incidence and death rates due to the disease. [2] The aim of a cervical cytology screening programme is to detect pre-malignant lesions of the transformation zone of the cervix. Those patients with abnormal cytological results are then referred for further management. In South Africa the cytological screening programme is not always well organized. [3] Despite this many screening smears are performed. Patients with abnormal cytological results are referred for further management, usually to dedicated colposcopy clinics. The current referral criteria is a single smear with a HSIL (High grade Squamous Intra-epithelial Lesion) or two LSIL (Low grade Squamous Intra-epithelial Lesion) smears. The aim of colposcopy is to detect the most abnormal area on the cervix and to direct the clinician to the area of biopsy.

In many clinics a "see-and-treat" approach is used and a patient with an abnormal smear often gets treatment at her first visit to the colposcopy clinic. [4] If the referral cytology indicates a high grade abnormality and the colposcopic assessment supports the cytological diagnosis, a confirmatory biopsy of the cervix is not needed before excisional treatment is offered. The rationale may be that, in the public sector at least, follow-up rates are poor and that transport to and from clinics is difficult. One has to caution against blanket treatment of all patients purely on cytological results. Certain authors have shown that between five and 40% of all patients with abnormal cytology might not have histological abnormality on LLETZ cone biopsy. [5] It is therefore necessary to do a thorough colposcopic evaluation and to treat only

those patients with a recognizable abnormality. If there is doubt about the severity of the abnormality, a biopsy should confirm a CIN II lesion or higher to justify treatment by destruction or resection of the transformation zone. Over-treatment may jeopardize a patient's future reproductive performance.

### Anatomical considerations

The anatomy of the cervix is an important consideration when discussing potential longer-term side effects of cervical conisation. The cervix has a specialised epithelial layer which is very important to both the cyto-pathologist and the gynaecologist. All the investigations and treatments are aimed at the transformation zone which is the area between the original squamo-columnar junction and the current squamo-columnar junction. This area is very susceptible to the oncogenic effects of the human papilloma virus. On histology the transformation zone consists of ectocervical squamous epithelium covering the underlying stroma with glandular components. The endocervical glands may be involved with intra-epithelial neoplasia and may lie as deep as 7 mm from the surface epithelium. Treatment for intra-epithelial neoplasia should be at least 1 cm deep to include these crypts.

### Treatment for cervical pre-cancer

When treatment is planned for intra-epithelial neoplasia, the treatment should include the whole lesion as visible on colposcopy. It should also include the upper border of the metaplastic epithelium. That might be slightly higher up in the endocervical canal, particularly in peri-menopausal patients. The squamo-columnar junction might not

be visible during colposcopy (incomplete colposcopy). In order to achieve complete excision of the transformation zone in those cases, it is necessary to aim treatment even higher up in the endocervical canal. A LLETZ cone biopsy should be of adequate size to achieve disease free margins and include the whole transformation zone. It has been shown that incomplete excision margins may lead to a higher rate of treatment failure.

Since 1965 it has been shown that locally destructive techniques might be as effective as hysterectomy in preventing the subsequent development of a CIN lesion into cancer. These techniques include cryotherapy, coagulation, conisation and laser treatment. Prendeville, during the late 1980's, popularised the concept of large loop excision of the transformation zone although it was first described by another author a few years previously. [6] The principle of a LLETZ is that a high current is created in a very thin wire loop, which then produces a steam envelope in tissue that has a high water content. This steam envelope cuts the tissue with minimal thermal damage to the surrounding tissue. It is important that the sample produced after a LLETZ cone biopsy has as little thermal damage as possible. Thermal damage adversely affects the histological examination of the specimen. Prendeville, in his original work, suggested that the depth of the cervical cone be between 0.7 and 1.5cm.

A cold knife cone biopsy has the potential advantage that there is a very clear surgical margin. That would make histological examination much more accurate. A cold knife cone biopsy allows tissue to be obtained higher up in the canal without damaging the histological sample. It is therefore preferred in some situations. If the

cytological evaluation suggests an endocervical lesion or if microscopic invasive cancer is suspected, the clinician may choose a cold knife procedure above a Lletz procedure. Because of the more invasive nature of a cold knife cone biopsy, it might cause more long-term morbidity. Whenever the underlying stroma of the cervix is included in the biopsy, the remaining cervix is shortened and the supportive tissue is potentially less capable of supporting an intra-uterine pregnancy.

### Reproductive outcome

Cervical intra-epithelial neoplasia is most often diagnosed in women of reproductive years. [7] When treatment for a CIN lesion is considered, it would be prudent to take note of a patient's future reproductive wishes and to be aware of any adverse effects that cervical conisation might have on her future fertility and obstetrical outcome. Cervical conisation could theoretically have an adverse effect on a patient's fertility and lead to an increase in the incidence of miscarriage, premature rupture of membranes, premature labour, cervical and precipitate labour.

### Infertility

There is limited information in the literature about the effect of cervical conisation on fertility. Buller found no evidence of secondary infertility in a group treated by cold knife conisation. [8] Keijser, Turlington, Bigrigg and Cruikshank did retrospective cohort studies on the effect of LLETZ conisation on subsequent pregnancy outcome. [9] [10] [11] [12] They could show no decrease in pregnancy rate in the treatment groups compared to the cohorts. Similarly Ferenczy, in a prospective cohort study of patients treated by LLETZ, could demonstrate no deleterious effect on fertility. [13]

Kyrgiou in an excellent meta-analysis concluded: "...despite these difficulties, the available evidence suggests that fertility is not impaired after treatment for cervical intraepithelial neoplasia." [14]

These studies provide some reassurance that cervical conisation is not a major cause of infertility. They unfortunately do not have sufficient power to exclude a subtle influence.

Cervical conisation might lead to infertility by causing cervical stenosis or a decrease in the production of cervical mucus. Very rarely an ascending infection, caused by the conisation, might lead to tubal damage. There have been case reports of women presenting with secondary infertility due to cervical stenosis and amucorrhea post LLETZ. [15] Cervical stenosis seems to occur more often after cold knife cone biopsy than laser conisation or LLETZ. With all modalities, a higher cone is associated with a greater occurrence of cervical stenosis. [16] [17]

It is important to remember that the patient who presents with a CIN lesion (caused by sexually transmitted HPV infection) is also at risk for tubal damage due to other sexually transmitted diseases. [8] This will need to be taken into account when any conclusions regarding the effect of cervical conisation on fertility are made.

### Miscarriage

Cervical conisation has not been shown to have any effect on the occurrence of first trimester miscarriages. Midtrimester miscarriages do seem to be significantly more

common after cold knife conisation. Moinian compared the pregnancies in a group of 414 patients before and after cold knife conisation. He found late spontaneous miscarriages to be seven times more frequent after cold knife conisation than before. This complication increases proportionately to the size of the cone biopsy. [18] [19]

Laser conisation and LLETZ do not seem to cause an increase in the incidence of midtrimester miscarriages. This is possibly due to the smaller amount of cervical tissue removed by these methods when compared to cold knife conisation. In a prospective study of 50 pregnancies in 86 patients treated by cold knife conisation, LLETZ or laser conisation, Mathevet observed no late miscarriages. [16] In a group of 54 women treated by laser conisation, Sagot noted no late spontaneous abortions in 71 pregnancies following conisation. [20] Similarly, Althuisius found no second trimester abortions in 56 women delivering after LLETZ. [21] These studies were unfortunately not large enough to detect less overt effects of conisation on the incidence of miscarriage. Nevertheless, it would seem prudent to rather do a LLETZ or laser conisation and only perform a cold knife conisation if it is specifically indicated.

**PPROM and premature labour**

In any study of the effect of cone biopsy on preterm premature rupture of membranes (PPROM) and preterm labour, it is important to bear in mind that CIN lesions have risk factors such as smoking, multiple sex partners and sexually transmitted diseases in common with both PPROM and premature labour. [22] There are a number of mechanisms whereby a prior cone biopsy could lead to

PPROM and preterm labour. The structural change in the cervix after a cone biopsy is important. The cervix might be shortened and the collagen formed in the scar tissue could be more fragile and react in a different manner from normal tissue to the hormonal changes of pregnancy. Especially with the larger cones that remove endocervical glands, the formation of cervical mucus may be impaired. The protective mucus plug and local immunological mechanisms are compromised. This might lead to ascending infections, the release of prostaglandins and PPROM or premature labour. [22]

Kristensen examined a cohort of 14 233 women of whom 170 had a cervical conisation. [22] Women who had cone biopsies for CIN lesions had a significantly increased risk of premature labour before 37 weeks when compared to the general population. This risk is increased *before* conisation, but even *more so* after conisation. The author postulates that the same risk factors that predispose patients to develop CIN lesions are also associated with premature labour, but that cervical conisation has an additive effect.

Other earlier studies found cold knife and laser cervical conisation to be associated with delivery before 37 weeks gestation. [23] [24] [25] There are, however, also a number of studies that found cold knife or laser conisation and LLETZ not to be associated with preterm delivery or PPROM. [8] [12] [16] [20] [26] These conflicting results can be explained by the generally small size of the study groups and the resultant lack of power to detect significant differences in the incidence of preterm birth.

Crane did a systematic review on the effect of LLETZ on subsequent pregnancy outcomes [27] and found LLETZ to be significantly associated with preterm birth before 37 weeks gestation. In a retrospective cohort study of 571 women who delivered after a LLETZ, Samson found LLETZ to be associated with low birth weight, PPROM and preterm delivery before 37 weeks. [28] The increase in delivery before 34 weeks was not significant. Sadler found both laser conisation and LLETZ to be associated with a significantly increased risk of PPROM and subsequent preterm delivery. This effect was more marked with increasing cone height. [29]

In a large meta-analysis, there was a statistically significant association between cold knife conisation and preterm delivery RR 2·59 (1·80–3·72) and low birth weight RR 2·53 (1·19–5·36). [14] LLETZ was also significantly associated with preterm delivery RR1·70 (1·24–2·35) and low birth weight RR 1·82 (1·09–3·06). Laser procedures, both ablation and conisation, were not associated with preterm delivery or low birth weight infants.

While it seems clear that cervical conisation results in a greater risk of PPROM and preterm delivery and that this effect increases with increasing size of the cone, most women will have uncomplicated pregnancies following cervical conisation. It remains to identify those at increased risk of having a complicated pregnancy after cervical conisation. Berghella found a cervical length on ultrasound of less than 25 mm to be predictive of preterm birth in patients with prior cone biopsy. [30] It is unfortunately not yet clear how to best manage these patients. Prophylactic cerclage does not appear to prevent preterm labour in patients with prior cervical conisation. [31]

Despite excitement in the literature about the potential role of progesterone therapy in patients with premature labour, [32] a recent Cochrane review on the topic concluded with “there is insufficient evidence to advocate progestational agents as a tocolytic agent for women presenting with preterm labour”. [33] Even though cervical length shortening was measured as an important outcome measure in the Cochrane review, no mention is made of any factors like previous cervical surgery or LLETZ procedures. It is therefore very difficult to speculate on the effect of progesterone on the pregnancy outcome after LLETZ procedures.

### Precipitate labour and cervical dystocia

Cervical conisation disturbs the structural integrity of the cervix. The resultant scar tissue might not respond appropriately to the hormonal changes of parturition and result in an abnormal pattern of labour. It has been shown that neither precipitate nor prolonged labour is more common after cervical conisation. [23] [27] There is, however, a small increase in the number of caesarean sections done for cervical dystocia. [18] [19] [20]

### Conclusion

Cervical conisation is an indispensable tool in the management of cervical intra-epithelial neoplasia but is not without risks. The risk of intra-operative complications is low and significant postoperative bleeding is rare. It may, however, also have long term adverse effects on a patient’s future fertility and obstetric outcome. Cervical conisation is associated with a small but significant increase in the incidence of

PPROM and premature labour. The greater the amount of tissue removed by the cone, the greater is this effect. In isolated cases cervical conisation might cause cervical stenosis and amucorrhea which could lead to infertility.

It is important to be aware of these possible complications and to appropriately counsel the patient before she undergoes treatment. The indications for conisation should be sound and should be based on a careful colposcopic examination of the cervix. This would make unnecessary surgical intervention a rare event. It would seem prudent to remove as little cervical tissue as will treat the CIN lesion. During pregnancy the patient should be carefully monitored. Measurement of cervical length during the second trimester would identify those at higher risk of premature labour, but it is not yet clear how to best manage those patients. The role of prophylactic cerclage in the patient with a shortened cervix following conisation needs to be elucidated.

### *Invasive cervical cancer*

Invasive cervical cancer remains one of the most common cancers affecting women. In certain parts of the world and in particular southern and eastern Africa, the incidence for cervical cancer is in the region of 30-50 cases per 100 000 women per year. [1] Cervical cancer is the most common cancer of women in South Africa. [34] The chances for developing cervical cancer increases with age, although about a third of cancers occur in patients during their fertile years. Figures from the National cancer registry of South Africa clearly show that the most common cancer of reproductive women in South Africa is cervical cancer.

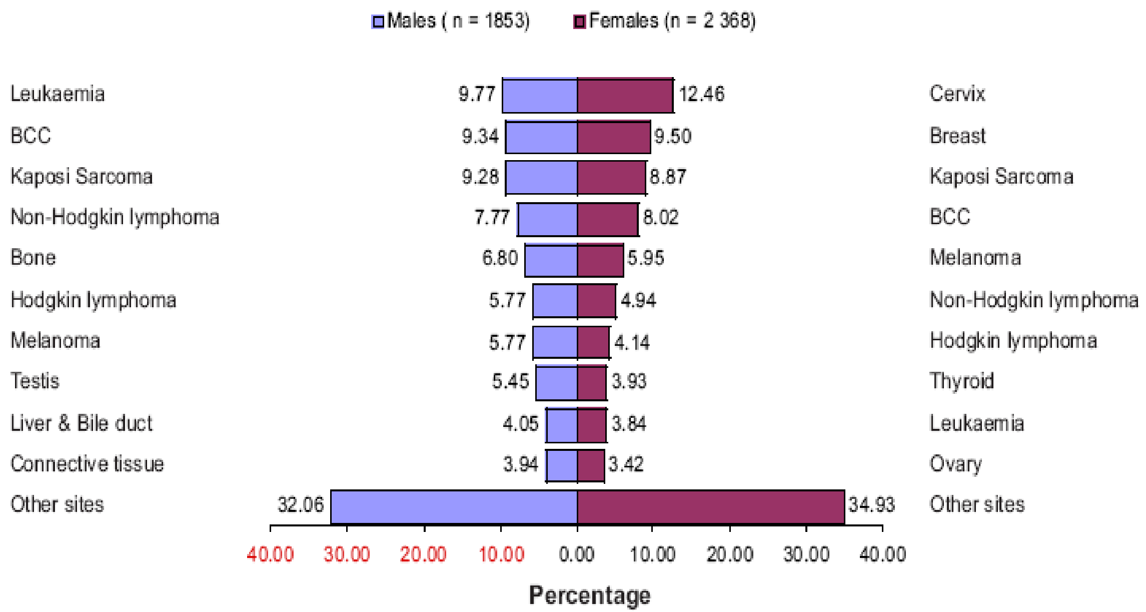


*Figure 2 Percentage distribution of 10 most common cancers by sex, 1998 and 1999, 15-29 years [34]*

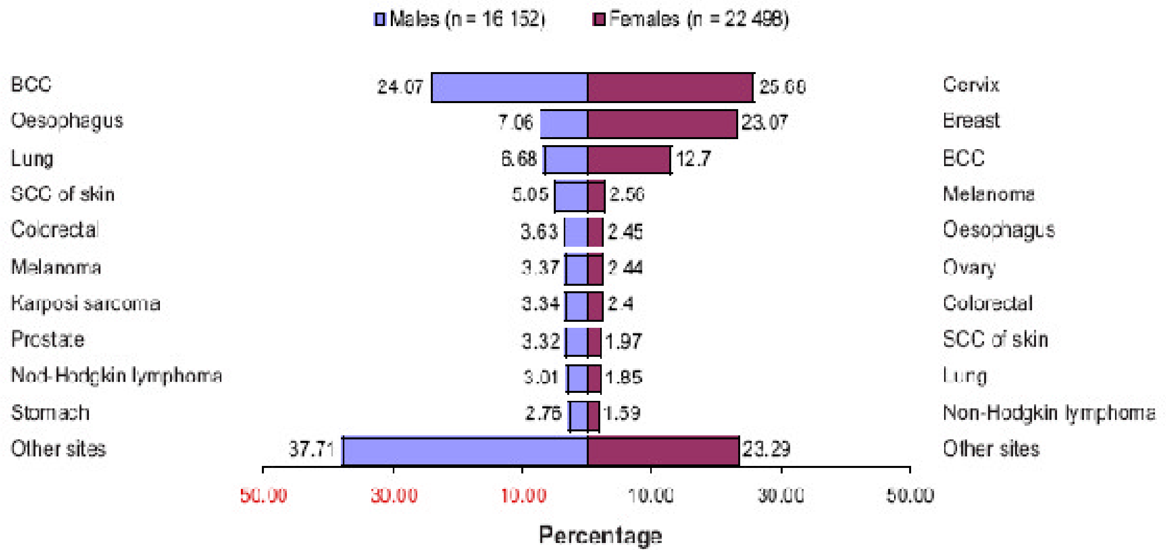


*Figure 3 Percentage distribution of 10 most common cancers by sex, 1998 and 1999, 30-54 years [34]*

A significant number of women may be diagnosed with invasive cervical cancer before they have completed their families. Screening for cancer of the cervix results in a younger age at diagnosis but also often an earlier stage at diagnosis. Many women and their partners prefer to delay childbearing to first establish their careers and this may increase the risk for developing cancer when fertility is still very important. In Africa the HIV epidemic causes rapid progression from cervical cancer precursors to invasive carcinoma and the average age of diagnosis is 15 years younger in HIV positive individuals. [35] The average age of infiltrating cancer in a South African study was 39.8 years in HIV positive individuals versus 55.2 years in HIV negative individuals. Another study from Johannesburg confirmed the earlier age at which HIV infected women develop invasive cervical cancer. In that cohort the average age at presentation of HIV infected women with cervical cancer were 44 years compared to 53 years in non-infected women. [36] An earlier study by Ellerbrock also showed that 20% of HIV positive women would develop biopsy proven CIN within three years. [37] This highlights the effect of HIV infection on the rapid progression from HPV infection to proven CIN lesions. Evidence for the association between cytological abnormalities and HIV is seen in a study performed in Cape Town where an HIV positive population on anti-retroviral therapy was compared to historical control groups. The incidence of HSIL in the HIV group was 29.5% and the incidence of invasive carcinoma a staggering 3.1%. When compared to historical controls (0.84 and 1.8% respectively for HSIL and 0.2% and 0.47% for invasive carcinoma) the effect is very clear. [38]

**Factors affecting prognosis**

1) ***Tumour Volume***

Tumour volume is one of the most important predictors of outcome in cervical cancer. Both adeno- and squamous carcinomas are staged according to the FIGO classification and microscopic disease is staged according to tumour volume. [39] Stage Ia1 indicates stromal invasion of less than 3mm in depth and less than 7mm of horizontal spread on the cervix. Stage Ia2 refers to depth of stromal invasion between the 3mm and 5mm with horizontal spread of less than 7mm. Any measurement above 5mm in depth and more than 7mm on the surface is regarded as a stage Ib tumour. Any tumour visible to the naked eye is also regarded as stage Ib.

| Stage | Pelvic | Para-aortic |
|---|---|---|
| **$Ia_1$** | 0.5 | 0 |
| **$Ia_2$** | 4.8 | <1 |
| **Ib** | 15.9 | 2.2 |
| **IIa** | 24.5 | 11 |
| **IIb** | 31.4 | 19 |
| **III** | 44.8 | 30 |
| **IVa** | 55 | 40 |

*Table 6 Importance of tumour volume (as defined by FIGO stage) as a predictor for lymph node involvement. [40] (Outcomes shown as %)*

Careful pathological examination of early invasive cervical cancer is of utmost importance. During the pathology examination stromal invasion should be measured from the basement membrane of the nearest endocervical gland. Adequate number of sections should be performed in cervical biopsies (LLETZ or cold knife cone) and an adequate number of sections, preferably less than 100 microns apart, should be examined by a dedicated gynaecological pathologist. Lymphovascular space invasion and excision margins should be reported in addition to tumour size.

Depth of stromal invasion in the 1994 FIGO staging divides stage Ia into $Ia_1$ for tumours less than 3mm in depth and $Ia_2$ for tumours invading more than 3mm but less than 5 mm. The horizontal spread must be less than 7mm. The FIGO staging before 1994 did not clearly state depth of "early stromal invasion" and most authors used 1mm as the upper limit. Some earlier studies using the 1985 FIGO staging may therefore have underestimated the risk for lymph node metastases in stage $1a_2$ cancers. [41] A few published series reported on depth of invasion of 3-5mm but horizontal spread was not limited to less than 7mm. In these reports a total of 262 patients had 7.3% risk for lymph node metastases but it varied from 0.0% to 13.8% in different reports. A good review article by Sevin concluded that patients, with a stromal invasion of 3.1-5.0 mm, have a significantly higher risk of up to 7.4% for lymph node involvement and 5.4% for invasive cancer recurrence. [42]

| Author | n | Nodal metastases | Invasive recurrences | Dead of disease |
|---|---|---|---|---|
| **Van Nagell 1983 [43]** | 32 | 3 (9.4%) | 3 | 2 |
| **Hasumi 1980 [44]** | 29 | 4 (13.8%) | NS | NS |
| **Simon 1986 [45]** | 26 | 1 (3.8%) | 0 | 0 |
| **Maiman 1988 [46]** | 30 | 4 (13.3%) | 0 | 0 |
| **Buckley 1996 [47]** | 94 | 7 (7,4%) | 5 | 4 |
| **Creasman 1998 [48]** | 51 | 0 (0.0%) | 0 | 0 |
| **Total** | 262 | 19 (7.3%) | 8 (3.1%) | 6 (2.3%) |

*Table 7 Incidence of lymph node metastases with stromal invasion of 3-5 mm – horizontal dimension not stated. [49]*

Width (or horizontal spread) of the tumour is also measure of tumour volume. FIGO staging incorporate surface spread in the diagnosis of stage Ia tumours by defing the maximum spread as 7mm. See Table 8.

| Horizontal width (stromal invasion <5 mm) | Positive pelvic nodes (%) | Recurrence (%) |
|---|---|---|
| **>7 mm** | 7.4 | 4.2 |
| **<7mm** | 2 | 0.3 |

*Table 8 Importance of width of lesion (as defined by FIGO stage) as a predictor for lymph node involvement. [50]*

### *2) Lymphovascular space involvement*

It is clear that lymph node involvement in cervical cancer conveys a poor prognosis for ultimate disease free survival. The numbers of cases with confirmed lymph node metastases decrease with decreasing stage. It would be ideal to identify those early cases with an increased risk for nodal involvement and a high risk for recurrence. Lymphatic- and/or vascular invasion by tumour indicate a higher risk for a poor prognosis. [51] Lymph node micro-metastases are associated with lymphovascular space involvement. [52] The volume of lymphovascular space involvement, as defined by the percentage of all sections with lymphovascular space involvement and total number of foci with lymphovascular space involvement, is an independent prognostic factor for time to recurrence in women with early-stage squamous carcinoma of the cervix. [53]

| Stage | LVI | Lymph nodes % | Recurrence % |
|---|---|---|---|
| **$1a_1$** | Pos | 4.7 | 4.6 |
| | Neg | 0.5 | 0.6 |
| **$1a_2$** | Pos | 11.1 | 17.4 |
| | Neg | 3.4 | 0.9 |

*Table 9 Importance of lymphovascular space involvement as a predictor for lymph node involvement and recurrence of disease [54]*

Lymphovascular space involvement as an independent indicator of risk may influence the decision to treat early invasive cervical cancer with uterus sparing surgery or not and may necessitate the addition of adjuvant chemo/radiation.

### 3) ***<u>Para-cervical or parametrial invasion</u>***

Not all tumours are surgically treatable and full surgical staging is not possible in more advanced case of cervical cancer. However in early operable disease parametrial invasion in surgical specimens is a significant poor prognostic factor for disease free survival and overall survival. [55] [56] [57] Pre-operative clinical evaluation of parametrial invasion is subjective and there may be a high degree of inter-observer variability. Magnetic resonance (MR) imaging may be the best imaging modality to detect parametrial invasion with the least inter-observer variability. [58] [59] Other authors question whether MRI is cost effective for the evaluation of parametrial invasion because preoperative MRI showed low PPV for detecting LN involvement and parametrial invasion in cervical cancer. [60]

### 4) ***<u>Lymph node metastases</u>***

Lymph node involvement in cervical cancer increases with increasing stage. Within stages, those with involved lymph nodes have a poorer prognosis and shorter disease free survival. [61] [62] Factors that influence lymph node involvement include tumour volume, lymphovascular space involvement, stage and parametrial involvement. [62]

| Stage | n | % Pelvic nodes | % Para-aortic nodes |
|---|---|---|---|
| **$Ia_1$** | 179 | 0.5 | 0 |
| **$Ia_2$** | 84 | 4.8 | <1 |
| **Ib** | 1926 | 15.9 | 2.2 |
| **IIa** | 110 | 24.5 | 11 |
| **IIb** | 324 | 31.4 | 19 |
| **III** | 125 | 44.8 | 30 |
| **IVa** | 23 | 55 | 40 |

*Table 10 Lymph node metastases by stage. From [63]*

### *5) Resection margin status*

Cervical cancer surgery as a single modality for the treatment to safeguard fertility will only be successful if clear surgical margins can be guaranteed. [64] The accepted pathological tumour free margin is 3-5mm. [65] [66] In cases where conservative surgery for fertility preservation is attempted, intra-operative frozen section to evaluate surgical margins is essential. [67] If, during uterus sparing surgery, the specimen is not clear at the resection margin at as determined by frozen section, a further (deeper) resection should be done or a completion operation performed.

| | | |
|---|---|---|
| **Stage 0** | | Carcinoma in situ, cervical intra-epithelial neoplasia grade 3 |
| **Stage I** | | The carcinoma is strictly confined to the cervix (extension to the corpus would be disregarded |
| | **IA** | Invasive carcinoma which can be diagnosed only by microscopy.All macroscopically visible lesions—even with superficial invasion—are allotted to stage IB carcinomas. Invasion is limited to a measured stromal invasion with a maximal depth of 5.0 mm and a horizontal extension of not >7.0 mm. Depth of invasion should not be>5.0 mm taken from the base of the epithelium of the original tissue—superficial or glandular. The involvement of vascular spaces—venous or lymphatic—should not change the stage allotment |
| | **$IA_1$** | Measured stromal invasion of not >3.0 mm in depth and extension of not >7.0 mm |
| | **$IA_2$** | Measured stromal invasion of >3.0 mm and not >5.0 mm with an extension of not >7.0 mm |
| | **IB** | Clinically visible lesions limited to the cervix uteri or preclinical cancers greater than stage IA |
| | **$IB_1$** | Clinically visible lesions not <4.0 cm |
| | **$IB_2$** | Clinically visible lesions >4.0 cm |
| **Stage II** | | Cervical carcinoma invades beyond the uterus but not to the pelvic wall or to the lower third of the vagina |
| | **IIA** | No obvious parametrial involvement |
| | **IIB** | Obvious parametrial involvement |
| **Stage III** | | The carcinoma has extended to the pelvic wall. On rectal examination, there is no cancer-free space between the tumour and the pelvic wall. The tumour involves the lower third of the vagina. All cases with hydronephrosis or nonfunctioning kidney are included unless they are known to be due to other causes |
| | **IIIA** | Tumour involves lower third of vagina with no extension to the pelvic wall |
| | **IIIB** | Extension to the pelvic wall and/or hydronephrosis or non-functioning kidney |
| **Stage IV** | | The carcinoma has extended beyond the true pelvis, or has involved (biopsy proven) the mucosa of the bladder or rectum. A bullous oedema, as such, does not permit a case to be allotted to stage IV |
| | **IVA** | Spread of the growth to adjacent organs |
| | **IVB** | Spread to distant organs |

*Table 11 Carcinoma of the cervix uteri: FIGO nomenclature (Montreal 1994)*

**Treatment for stage $Ia_1$ cervix cancer**

A small volume tumour, FIGO stage $Ia_1$, with depth of invasion of less than 3mm and less than 7mm on the surface, is adequately treated with a cold knife cone biopsy. [68] [69] [70] Laser conisation has also been reported as a safe option. [71] Care should be taken where lymphatic- and or vascular invasion is present on initial biopsy and pelvic lymphadenectomy should be part of surgical management. It is important to consider excision margin status and the cone edges should be negative not only for invasive carcinoma but also for intra-epithelial disease. Ideally pathology should be reviewed at a multi-disciplinary team meeting and after treatment the patient should have careful cytology follow-up on a regular basis. Evaluation of the vulva and vagina is important when micro invasive cervical cancer is diagnosed because of the high incidence of multi-focal human papilloma virus disease.

Even if stage 1a adenocarcinoma of the cervix is diagnosed during pregnancy, a conservative approach may be reasonable. A cone biopsy (also sometimes called a coin biopsy because of flatter shape to avoid membrane rupture) during pregnancy with careful follow-up is acceptable as reported from Japan. [72]

Cold knife conisation may shorten the cervix and may impair the ability of the uterus to sustain a pregnancy. The increased incidence of premature labour and premature rupture of membranes has been discussed earlier.

## Treatment for stage $Ia_2$ to $Ib_1$ cervix cancer

The standard approach for treatment for slightly larger volume disease historically has been radical hysterectomy with pelvic lymph node dissection. [69] However, with radical hysterectomy or pelvic radiotherapy the patient was not only rendered infertile but in addition both neurological and rectal dysfunction was unfortunate complications. [73] Some authors now argue that a simple hysterectomy, or even cone biopsy, with pelvic lymphadenectomy is adequate treatment. [70] [68] This approach afforded at least a 90% cure rate if lymph nodes were found to be negative during surgery.

### The rationale for less aggressive surgery

Less than 31% of all stage $Ib_1$ cases will have parametrial involvement. [57] Another retrospective report found that “positive parametrial involvement in stage Ia and $Ib_1$ cervical cancer is infrequent.” [74] In an attempt to predict which individuals are likely to have parametrial involvement, Bennedetti-Panici further found that parametrial involvement was found in less than 2% of cases if pelvic lymph nodes were negative. [75] Some authors have questioned the need of a complete parametrial excision in early cervical cancer. [76] A suitable approach intra-operatively may therefore be to do a frozen section of the pelvic lymph nodes before continuing with the radical hysterectomy and this approach can predict parametrial invasion with 90% sensitivity and 100% specificity. Those cases that were not accurately predicted had metastases less than 4mm in diameter. [75]

**The history of surgical treatment for cervix cancer**

Hippocrates, in approximately 400 BC, carried out the first known cervixectomy or trachelectomy (from the Greek word t??????? for neck) in order to remove bleeding tumours. This approach was clearly unsatisfactory and had little chance of cure. [77] Radical operations in surgical oncology became popular in the beginning of the 20th century after the hypothesis of Halstead and Meyer became dogma. The initial work was described for the management of breast cancer and the hypothesis was that cancer spread from the primary site through the lymph nodes to distant sites. Removal of the entire breast together with the tumour and also adding healthy adjacent tissue with removal of nearby lymph nodes was the best way to prevent future persistence of disease. In the next 20 years following the initial reports, Halstead and his followers expanded on the hypothesis of "contagious spread" of breast carcinoma to other forms of cancer. A colleague of Halstead called JD Clarke; also working at John's Hopkins in Baltimore, applied the same principles to cervix cancer and did the first radical hysterectomy for cervix cancer in 1895. This was followed in 1896 by Wertheim. Wertheim described a procedure for removing the uterus with the cervical tumour and a wide tumour free margin. The removed tissue included the upper part of the vagina, the para-cervical and parametrial tissue and sampling of the pelvic lymph nodes. Wertheim reported the outcomes of his first 270 patients in 1905. The peri-operative mortality rate was 18% and the major morbidity rate was 31%. [78] Other authors (notably Schauta who was Wertheim's predecessor) described similar procedures that were performed vaginally and achieved lower operative mortality rates than the abdominal approach. Meigs revived interest in the radical surgical approach from 1944 when he developed a

modified Wertheim operation with removal of all pelvic nodes. Meigs reported a much improved survival rate of 75% for patients with stage I disease and demonstrated an operative mortality rate of only 1% when these procedures were performed by a specially trained gynaecologist.

From the late 1940's the concept of pre-malignant disease of the cervix became better known and in 1948 Franz Novak performed surgical removal of the cervix for an intra-epithelial neoplasia. This procedure was the predecessor of modern cone biopsies that led eventually to work of Burghardt and Holzer on micro-invasive carcinoma of the cervix where they stated that it was not always imperative to remove the whole uterus for small early cervical cancers. [79]

Dargent and co-workers described "la trachelectomie elargie" (TE). [80] (reference taken from [77] ) This was a new concept of radical surgery for an early cervical tumour with conservation of the uterine corpus. The technique has subsequently been modified slightly by Shepherd and co-workers. [81] Roy and Plante followed soon after with a report of a successful pregnancy after such treatment. [82] Radical removal of the cervix with conservation of uterine function has become an established treatment option, where expertise is available, for the treatment of early stage cervical cancer in patients with a fertility wish.

**Dargent's operation (radical vaginal trachelectomy)**

Dargent and other authors developed a radical operation for the treatment of early cervical tumours. [83] [84] This operation follows Halstead's principals but aims to

preserve uterine function and makes conception, normal menstruation and even pregnancy possible. The removal of the cancer should include an adequate surgical margin and the resection includes parametrial tissue and pelvic lymph nodes. The aim of the operation is to include the whole of the cervix with a clear 1cm margin beyond the tumour and a vaginal cuff and para-cervical tissues of approximately 2cm.

The first step in Dargent's operation is a pelvic lymph node dissection with a laparoscopic approach. Intra-operative frozen sections are done of the lymph nodes and if these are negative the cervix is removed together with the parametria through the vaginal route. The endocervical margin is also checked for possible malignancy and if the excision margin is positive a further portion of the cervix should be removed. The last aspect of this operation is to include a "cervical" cerclage. With adenocarcinomas, removal of the entire endocervical canal is essential; skip lesions and field change effects may affect the canal higher (and separate from) the initial tumour. [85] Since the first description of vaginal trachelectomy, a few modifications have been published of which an open abdominal procedure was described by Abu-Rustum in 2006. [86] Another approach was to do extra-peritoneal abdominal lymph node dissection, a technique that is described for radical vaginal hysterectomy [87] , and in this way prevent adhesion formation due to manipulation of the Fallopian tubes and the ovaries. [88]

Laparoscopic lymphadenectomy requires advanced laparoscopic pelvic sidewall dissection and training and expertise is not always available. Additional to this, advanced laparoscopy is time consuming and expensive and requires a highly

equipped theatre. In South Africa and other developing countries where the incidence of cervical cancer is high, a more pragmatic approach would be to do an extra-peritoneal lymphadenectomy through an abdominal incision. This will also have the added benefit of protecting the intra-peritoneal environment against infection and adhesion formation.

**Selection criteria for radical trachelectomy**

Radical trachelectomy, whether it is done abdominally or trans-vaginally, should still be considered as a highly specialised procedure that should be performed in centres with the necessary surgical skill. When the decision is taken to be conservative in a patient with invasive cancer, oncological outcome should not be compromised in order to safeguard fertility. Only if the following criteria are met should a trachelectomy be offered: [89]

- Carcinoma of the cervix
- Younger than 40 years of age with fertility desire
- No unfavourable histology (eg neuro-endocrine tumours)
- Stage Ia1 with lymphovascular space invasion
- Stage Ia2 or Ib1 with tumours less than 2cm
- If the endocervix is involved with tumour, colposcopy (with the aid of Kogan's endocervical speculum) or MRI is important to see the upper limit of the tumour
- No radiological evidence of lymph node- or systemic metastases

### Oncological features

The oncological outcome of trachelectomy procedures, as measured by tumour recurrences, compares very favourably with standard radical hysterectomy and is in the region of approximately 4%. [90] [77] Recurrences occurred up to eight years after the surgery, and although the recurrence rates are less than expected for a similar group of patients with stage $Ib_1$ disease, it may be because of the good prognostic small tumours that have been selected for this specific procedure. Despite the fact that the recurrence rates are very low, it is still very important that patients should have careful cytological, colposcopic and clinical follow-up on a regular basis. The oncological features of a total of 520 cases from different centres are summarised in Table 12.

| Authors | Dargent n=95 [91] [92] | Plante, Roy n=72 [93] | Covens n=93 [94] [89] | Burnett n=21 [95] | Schlearth n=10 [96] | Hertel n=108 [97] | Shepherd n=123 [98] | Total n=520 |
|---|---|---|---|---|---|---|---|---|
| **Age** | 32 | 31 | 30 | 30 | 31 | 32 | 31 | 31 |
| **Follow-up (mths)** | 76 (4-176) | 60 (6-156) | 30 (1-103) | 31 (23-41) | 47 (28-84) | - | 45 (1-120) | 48 (1-176) |
| **Squamous cell** | 76 (80%) | 42 (58%) | 42 (48%) | 10 (53%) | 4 (40%) | 75 (69%) | 83 (67.5%) | 332 (60%) |
| **Adenocarcinoma** | 19 (20%) | 30 (42%) | 44 (52%) | 9 (47%) | 6 (60%) | 33 (31%) | 33 (27%) | 174 (40%) |
| **LVSI** | 23 (24%) | 14 (20%) | 31 (36%) | 2 (11%) | 1 (10%) | 38 (35%) | 39 (32%) | 148 (24%) |
| **Size <2cm** | 74 (78%) | 64 (89%) | 85 (91%) | 19 (100%) | 8 (80%) | * | N.R. | 250 (88%) |
| **Size >2cm** | 21 (22%) | 8 (11%) | 8 (9%) | 0 (0%) | 2 (20%) | * | N.R. | 39 (12%) |
| **Op. time (min)** | 163 | 250 | 180 | 220 | N.R. | 253 | N.R. | 213 |
| **Recurrence rates** | 4.2% | 2.8% | 7.3% | 0 | 0 | 4% | 2.7% | 4.2% |
| **Death rates** | 3.1% | 1.4% | 4.2% | 0 | 0 | 2% | 3.3% | 2.8% |

*Table 12 Oncological features of trachelectomy series from different units*

### Pregnancy outcomes for trachelectomy

In the general population, the average accepted fertility rate is in the region of about 85%. [99] Bernardini is of the opinion that the incidence of infertility, in a patient population suitable for conservative cervical cancer surgery, may be higher. [100] There is support for this opinion from Dargent [101] and Shepherd. [85] In an earlier report from Roy and Plante, none of the six patients trying to fall pregnant, could achieve a pregnancy. [82] Possible reasons for a decreased fertility rate in this population may include the fact that human papilloma virus infection is a marker of sexually transmitted diseases. This could also indicate a population that is at higher risk for tubal damage due to previous upper genital tract infections. The trachelectomy procedure in itself may also compromise tubal function. If an open laparotomy is performed for the pelvic lymphadenectomy, manipulation of the uterus and other pelvic organs during surgery may cause adhesions.

Over the last decade, a few larger series have been published about pregnancy outcome in this patient population. The biggest risks for pregnancies after trachelectomy procedures are miscarriage, premature preterm rupture of membranes, early delivery and subsequent premature infants. This pregnancy loss occurs despite prophylactic cervical cerclage. In a review published during 2005 by Shepherd of a total of 406 cases, he described 171 pregnancies which occurred in 118 women, resulting in 109 live births. Seventeen of the live born infants delivered before 32 weeks gestation. [77] Pregnancy loss before viability also is a serious concern. The number of first and second trimester losses from six different authors are summarised in Table 13. It is clear that less than 60% of all deliveries occurred

at term. Approximately 32% of pregnancies ended in loss and before viability. The first trimester losses may be similar to that of the general population, but the second trimester loss rate is much higher than expected.

| Author | Schlaerth [96] | Burnett [95] | Shepherd [85] | Dargent/ Mathevet [16] | Bernardini [100] | Roy/Plante [82] | Total |
|---|---|---|---|---|---|---|---|
| **1st trimester loss %** | 0 | 0 | 29 | 16 | 14 | 16 | 13 |
| **2nd trimester loss %** | 50 | 33 | 7 | 14 | 5 | 4 | 19 |
| **Delivery> 37 Weeks %** | 50 | 50 | 22 | 85 | 67 | 78 | 59 |

*Table 13 Pregnancy loss rates after trachelectomy from different units*

Bernardini speculates that the amount of cervical tissue remaining after trachelectomy may influence the incidence PPROM. [100] The material used for the cervical cerclage may also make a clinical difference. A procedure called "Early Total Cervical Occlusion" (ETCO), has been suggested as a possible solution for PPROM. [102] [100] The specific factors causing premature rupture of membranes have been well studied, but there are no definitive conclusions. Both mechanical and infectious pathways may be involved in post-trachelectomy patients. The mucus plug, which may protect against infection, is usually absent if the patient has a short cervix. The absence of the mucus plug may lead to ascending infection. [85] The use of prophylactic antibiotics between 14 and 16 weeks, with regular bimonthly vaginal swabs for bacterial infections has also been suggested as a possible management strategy. [100] Because of the higher rate of premature delivery, a single dose of corticosteroids, given routinely during the second trimester, may be a useful intervention. [103] In general obstetric literature regular sonographic evaluation for cervical incompetence have also been suggested as a possible early warning sign of premature birth. [104] [105]

Shepherd describes a single case where, as a result of a lower segment transverse caesarean section, extensive bleeding occurred when the incision extended into a uterine vessel. [77] Because of this risk of uterine tears and bleeding, the suggestion is made that these patients should always have an elective caesarean section by a lower *vertical midline* incision (classical caesarean section), as the lower segment is usually not well developed. Shepherd also describes an abdominal cerclage in a

case where the insertion of the initial cerclage was impossible due to the very limited cervical tissue.

### Neo-adjuvant chemotherapy followed by uterus conserving surgery

Plante described in 2006 three cases with locally advanced cervical cancer treated with neo-adjuvant chemotherapy (NACT) and radical vaginal trachelectomy. [106] All these patients were young, pre-menopausal patients who required future fertility. They were treated with:

- Paclitaxol 175mg/m$^2$ on day 1
- Cisplatinum 75mg/m$^2$ on day 2
- Ifosfamide 5g/m$^2$ over 24h and
- Mesna 5g/m$^2$ on day 2 and 3

These cycles are to be repeated every three weeks.

The chemotherapy was followed by laparoscopic pelvic lymph node dissection and radical vaginal trachelectomy. All cases had initial tumour sizes of less than 4x4cm on MRI imaging. All patients had excellent pathological response on chemotherapy with negative nodes and surgical margins during surgery. However, the chemotherapy caused significant bone marrow suppression and there is also a concern about the use of ifosphamide and cisplatinum for ovarian follicle reserve. A successful pregnancy after treatment for invasive cervical cancer treated by NACT followed by conisation has also been described. [107] Although NACT could make more patients suitable for uterus conserving surgery, it may reduce follicular reserve and cause premature ovarian failure.

## *Conservative surgery in endometrial cancer*

Endometrial carcinoma is usually a disease of peri- or post-menopausal women. There are, however, a number of younger women who develop endometrial cancer, who usually present with excess oestrogen associated with obesity, infertility and nulliparity. Overall, 2-14% of endometrial cancers occur in women younger than 40 of age. [108] [109] There are many case reports in the literature of early stage, low-grade endometrial cancers that have been treated using uterus conserving therapies. [110] [111] [112] [113] Hormonal treatment with progesterone, with or without additional gonadotrophin- releasing- hormone analogue (GnRHa), often have the result that these early-stage tumours respond adequately to allow time for pregnancy. Surgery as part of conservative management of endometrial carcinoma or atypical endometrial hyperplasia has been described by Jadoul. [109] A small number of early-stage endometrial cancers were treated with partial hysteroscopic resection followed by GnRHa for three months. [109] Regular endometrial curettage, combined with medroxyprogesterone acetate, has also been used for the treatment of early-stage tumours. [114]

Despite the many case reports of successful conservative management, there remain some doubt about the safety of this approach. There have been isolated reports of metastatic disease in the myometrium or ovaries after conservative treatment. [115] [116]

### *Fertility sparing surgery in ovarian cancer*

Ovarian cancer is the sixth most common form of cancer in women but is the fourth leading cause of cancer death in the more developed regions of the world. [1] The symptoms are usually mild and the cancer is usually only diagnosed in advanced stages of the diseases. It occurs mainly in older women, but below the age of 40 years, the incidence can be as high as 3/ 100 000 women per year. [117] In these young women the question arises whether fertility sparing surgery is possible without compromising survival. Selected patients with Borderline Ovarian Tumours (BOTs) or early stage invasive epithelial cancer (FIGO stage Ia) with well differentiated tumours may be managed with fertility sparing surgery. [117] [118] [119] [120] [121] [122]

Successful pregnancies after conservative surgery for BOTs have been reported by various authors. [120] [119] [117] Selection criteria for conservative fertility-sparing management of BOTs depend on the risk for recurrence with invasive disease. The following factors may influence the risk for invasive disease:

- DNA ploidy status (Aneuploid tumours had 19x higher risk of dying of disease)
- Stage at presentation
- Histological type (serous types have better outcome)
- Age at diagnosis [123]

The presence of micro-invasion together with BOT has been seen as a possible poor prognostic indicator but a large meta analysis showed no increased risk for poor prognosis. [124] The World Health Organization (WHO) classification system states that, in borderline tumours, it is acceptable for stromal micro-invasion up to 5mm of measurement in any single focus or peritoneal implant. [125] Surgery for borderline tumours may include unilateral oophorectomy in patients with unilateral disease or an ovarian cystectomy with resection of all peritoneal and omental deposits in patients with bilateral disease. Careful surgical staging is important. After completion of the family, completion surgery with a total hysterectomy and removal of any ovarian tissue is debatable. However, some authors suggest that late recurrences are reported in retrospective reports and therefore completion surgery is indicated. [117]

Early stage, well-differentiated invasive epithelial ovarian carcinoma can also be treated successfully with fertility sparing surgery. In these patients accurate surgical staging is of the utmost importance. A surgical staging procedure should include peritoneal washings, removal of the primary tumour, multiple random peritoneal biopsies and infracolic omentectomy. A lymph node dissection may be considered in poorly differentiated tumours. Only if, after careful surgical staging, the patient remains a FIGO stage Ia can conservative management and omission of adjuvant therapy be safely advised.

Epithelial ovarian cancer infrequently occurs at a young age. The place for conservative management in invasive epithelial cancer is limited. Colombo and co-workers reported data on 99 patients younger than 40 who had treatment for stage I

ovarian carcinoma. [126] Fifty-six percent of these patients had fertility sparing conservative surgery over a period of 10 years between 1982 and 1992. Of the total of the 99 patients, only 16 received adjuvant chemotherapy consisting of cisplatinum alone before 1988 and carboplatinum after 1989. The recurrence rate among the patients who had conservative surgery and those who had radical surgery is shown in Table 14. The patients were followed up for a median of 75 months.

| | **Surgery** | |
|---|---|---|
| | **Fertility sparing** | **Complete staging (radical)** |
| **Stage 1a** | | |
| **Grade 1** | 1/24 | 0/15 |
| **Grade 2** | 1/8 | 1/5 |
| **Grade 3** | 1/4 | 1/3 |
| **Stage Ib** | 0/1 | 1/4 |
| **Stage Ic** | 0/19 | 2/16 |
| **Total** | **3/56** | **5/42** |

*Table 14 Relapses in patients <40 years according to type of surgery. Data represented as relapses per group. Adapted from [126]*

Colombo et al state that “our experience suggests the possibility of some extension of the traditional conservative approach to patients with unfavourable prognostic factors. When a stage I ovarian carcinoma is found at the time of first laparotomy we believe that conservative surgery can be performed regardless of the histo-type and histological grade”. [126] Many of the patients included in this long-term follow-up

study attempted pregnancy. Of the 17 patients attempting pregnancy all women conceived. A total of 16 healthy babies were born. Two patients had ectopic pregnancies, 4 miscarried and 4 had elective terminations.

In a review on the role of surgery in ovarian carcinoma, the authors state that “in selected patients desiring fertility who have stage Ia G1 or 2 ovarian tumours, unilateral salpingo-oophorectomy with inspection of the contra-lateral ovary and comprehensive staging is an option with a low risk of recurrence”. [127] This approach is supported by the work published by Maltaris where 113 pregnancies resulted after conservative treatment in 282 patients with early invasive ovarian carcinoma. [90] Sixteen of the 282 patients died due to ovarian cancer disease and a total of 33 developed recurrences.

A summary of the literature is presented in Table 15.

| Author | Patients (n) | Pregnancies (n) | Term deliveries (n) | Relapses (n) | Death disease (n) |
|---|---|---|---|---|---|
| **Colombo [126]** | 56 | 25 | 16 | 3 | 2 |
| **Zanetta [128]** | 84 | 33 | 22 | 5 | 3 |
| **Duska [129]** | 6 | 2 | 2 | 1 | 1 |
| **Morice [130]** | 34 | 10 | 7 | 10 | 4 |
| **Schilder [131]** | 52 | 17 | 26 | 5 | 2 |
| **Raspagliesi [132]** | 10 | 3 | 3 | 0 | 0 |
| **Colombo [133]** | 24 | 7 | 6 | 7 | 2 |
| **Park [134]** | 62 | 15 | 22 | 11 | 6 |
| **Kajiyama [135]** | 60 | 13 | 9 | 8 | 7 |
| **Satoh [136]** | 211 | 56 | 56 | 18 | 5 |

*Table 15 Reproductive and oncological outcome in patients with epithelial ovarian cancer after conservative treatment*

Surgery for *non-epithelial* ovarian cancer should be individualized but fertility sparing surgery is a real option. Many of the germ cell and sex cord stromal tumours respond well to surgery alone or a combination of surgery and chemotherapy. [90] [137] Individualization of care after final histological diagnosis will depend on the particular histological type.

### *Surgical ovarian trans-position*

The human oocyte is exquisitely sensitive to the damaging effects of radiation and the estimated LD50 is less than 4 Gray. [138] A descriptive study by Wallace found that 37 out of 38 females had ovarian failure after whole abdominal irradiation of 20 to 30 Gray in childhood. Seventy-one percent had primary amenorrhoea i.e. they never had normal pubertal development and premature menopause occurred in the rest with a median age of 23.5 years. [139] Ovarian trans-position outside the field of radiotherapy may reduce the dose to the ovary. Howell described how lateral trans-position of the ovaries to the para-colic gutters may reduce the radiotherapy dose by up to 95%.[140] This may protect the sensitive follicles to direct dose related damage. Other reports, however, were less optimistic and found that ovarian trans-position may compromise blood supply and there was mixed success with this technique due to scatter radiation and vascular compromise. [141] Ovarian transposition may have a place in cases where the pelvic dose of radiotherapy is not high enough to be damaging to the other organs of the reproductive tract.

## *Conclusion*

Surgery remains an effective treatment option for many gynaecological cancers. The surgeon should be aware of the potential effects of treatment on future reproductive outcome. Procedures for the treatment of pre-malignant cervical disease may cause premature rupture of membranes in subsequent pregnancies. Premature labour and midtrimester miscarriages are also more frequent in women who had cervical surgery.

Small early stage cervical cancers may be suitable for uterus-sparing surgery. Premature rupture of the membranes and premature labour are risks after radical trachelectomy procedures. Ovarian function may be preserved in advanced cases by ovarian cryopreservation.

In selected cases of early, low risk ovarian and endometrial cancers, fertility sparing conservative surgery can be a safe and reasonable option. Quality of life in young cancer survivors may be as important as oncological outcome.

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# Chapter 3: Pharmacological options for the protection of ovarian function in patients undergoing chemotherapy


## *Abstract*

Chemotherapy, particularly alkylating agents, can be toxic to germ cells and may lead to chemotherapy induced amenorrhoea in young women. The age at which chemotherapy is administered is a strong predictor of subsequent premature ovarian failure with older patients at the highest risk.

Smaller primordial follicles may survive the insult of chemotherapy better and suppression of follicle development may protect the germ cell pool. Suppression of follicle development by gonadotrophin-releasing hormone agonists (or antagonists) (GnRHa) or combined oral contraceptives have been reported in the literature. The results are promising but conflicting reports of the protection makes further studies essential.

## *Introduction*

Over the last few decades major improvements have been made in the treatment of childhood and adolescent malignancies. However, treatment modalities may have serious harmful effects on ovarian function and the incidence of premature ovarian failure in patients who received chemotherapy and/or radiotherapy is increased from the general population. [1] [2] The late effects of cancer treatment have been a popular topic for discussion in the medical literature and various attempts have been made to protect young women against the potential harm of chemo- and radiotherapy. Chemotherapy and radiotherapy may permanently harm reproductive ability and a high rate of premature ovarian failure due to damage to germ cells and follicles is widely reported. [1] [3] [4] Age at treatment with chemotherapy remains one of the most important determinants of the incidence of ovarian failure. The risk increases with age of exposure. [5] [6] [7] Increasing age is as an important predictive factor for chemotherapy-induced amenorrhoea. [8] Differing response to chemotherapy was demonstrated between pre-pubertal girls and adult women during treatment for lymphoma.[9]

Many theories have been put forward to explain the age phenomenon. A popular theory is that the number of primordial follicles decreases with age as demonstrated by the Faddy-Gosden differential equation. [10] [11] [12] It is argued that a constant fraction of the primordial follicles will be damaged during a particular cycle of chemotherapy (or radiotherapy). Therefore the remaining number is directly proportional to the initial population. This initial population decreases at a logarithmic rate and hence the older the patient the lower the initial population of primordial

follicles will be. The total number of oocytes in the ovary is a combination of primordial, small primary and intermediary follicles. [13]

### *GnRH agonists*

Almost 30 years ago Glode tested the possibility of testicular protection against cyclophosfamide induced damage in male mice and found a protective effect of GnRH- agonists. [14]

Ataya demonstrated that GnRHa may protect against chemotherapy induced ovarian compromise in a murine model as early as 1985 and other reports from studies in female rats, seemed to confirm this work. [15] [16] [17] [18] The data from Letterie, however, did not show improvement in outcome in the same animal model. [19] In another study, mice were treated with cyclophosfamide and combined oestrogen/progesterone *or* leuprolide acetate. The author concluded that neither method of ovulation suppression protected the mice against gonadal toxicity. Despite the promising work in rats where GnRH-agonists inhibited chemotherapy induced ovarian damage, many authors remain sceptical about its application in humans. [20] [21] A possible reason for the difference between rats and humans may be the lower incidence of GnRHa receptors in human ovarian tissue. [22] [23]

Ataya later showed in three adult menstruating female rhesus monkeys (Macaca Mulatta) treated with GnRHa during chemotherapy that they had more primordial follicles after treatment than those not receiving GnRHa. [24] The possible protective mechanisms postulated were:

- Suppression of the levels of gonadotrophins in the ovary
- A direct influence of GnRHa mediated through receptors in the ovary
- A reduction in the metabolism of the ovary resulting in a decrease in the blood flow to the ovary

Oral contraceptives also may reduce the gonadotrophin levels in the follicular environment and therefore may also create a similar environment for protection. [25]

For ovulation to occur during a particular menstrual cycle a single oocyte within a dominant follicle must develop. This whole process starts when a group of primordial follicles starts to develop. From this group a single follicle is selected to eventually effect ovulation. Growing follicles makes up less than 10% of the total number of follicles but after initiation of the growth process either ovulation or atresia is inevitable. [26] The initial growth is independent of gonadotrophins. [13] It does not make a plausible argument that GnRHa works as a protective mechanism during chemotherapy by keeping follicles outside of this growth phase because the initial process is gonadotrophin independent. Some interesting work found that in human ovarian cells GnRHa receptors are absent before reaching the stage of pre-ovulatory follicle and the corpus luteum; therefore no GnRHa stimulation is possible in the primordial and early follicular stages of development. [27] [28]

Imai, in 2007, demonstrated that Buserelin acetate was protective against damage by doxyrubicine on human granulosa cells. [29] It was an in vitro study of cultured cells exposed to chemotherapy and provided additional support for a protective action of GnRHa on stromal cell damage. The authors found that doxorubicin

damage caused irreversible decreases in oestrogen production and that by adding GnRHa before or during exposure protected granulosa cells. Granulosa cells only showed receptors for GnRHa in mature follicles; therefore the presence or absence of GnRHa receptors as the mechanism through which GnRHa analogues protects against chemotherapy damage also does not make biological sense. It is important to remember that, although this study had as its primary measure to determine GnRHa analogues protection against granulosa cell damage, it was performed *in vitro*; therefore a direct influence on the cell environment may play a role and not the hypo-gonadotrophic environment that would be created in the usual clinical situation.

The cortex of the ovary is a relatively avascular area. GnRHa down regulation of pituitary stimulation may further reduce the blood flow in the ovary. Ultrasound studies have shown reduced perfusion during reduced pituitary stimulation. [30] [31] By reducing blood flow, the exposure of follicles to harmful chemotherapy may be decreased. Although this is plausible, there is conflicting evidence that the blood flow may not significantly change after treatment with GnRHa when measured by Doppler flow studies. A three dimensional doppler flow study of 85 women who were treated with short term Buserelin did not show significant changes in antral follicle count, stromal blood flow or ovarian volume. [32]

GnRH-agonists may up-regulate the production of anti-apoptotic molecules such as sphingosin-1-phosphate. [33] It may also affect molecules like transforming growth factor-b. [34] Other growth factors in the transforming growth factor super-family may also be influenced by GnRH-analogues. However, the complex interaction of all these growth factors is very difficult to show and control in an experimental model.

Some work suggests that the transition between primordial to primary follicle may be influenced by ligands produced by growing follicles (these growing follicles will be under the influence of gonadotrophins). [35] Through this secondary pathway down-regulation of growing follicles by an absence of FSH stimulation may lead to a lesser number of follicles being recruited.

Whatever the mechanism that may be involved in protection, it has been shown convincingly that the prepubescent girls are less likely to develop premature ovarian failure after chemotherapy than older patients. This led to the assumption that an artificial pre-pubertal environment might lead to ovarian protection. This argument may be inaccurate and has not been conclusively shown in experimental models. The reason why young girls may have less obvious functional damage is likely due to the fact that the total number of follicles available is much higher at younger age. This argument is supported by long-term follow-up of young girls who have received chemotherapy and who eventually did have premature menopause. [36] In a large post treatment follow-up study relative risk of menopause in the third decade was 9.2 after treatment with alkylating agents alone [37]

## ***Literature review on ovarian suppression for protection against chemotherapy induced ovarian damage***

| Author and year | GnRHa % | Control % | Follow up years |
|---|---|---|---|
| **Waxman 1987 [38]** | 50.0 | 67 | 2.3 |
| **Blumenfeld 2000 [39]** | 0 | 66 | - |
| **Pereyra Pacheco 2001[40]** | 0 | 100 | <5 |
| **Blumenfeld & Eckman 2005 [3]** | 7 | 54 | |
| **Franke 2005 [41]** | 20 | - | <1 |
| **Dann 2005 [42]** | 0 | 17 | 5.3 |
| **Somers 2005 [43]** | 5 | 30 | - |
| **Elis 2006 [44]** | 0 | 8.7 | - |
| **Del Mastro 2006 [45]** | 3 | - | 1 |
| **Recchia 2006 [46]** | 33 | | 6.3 |
| **Giuseppe 2007 [4]** | 0 | 47 | 2.4 |
| **Castelo-Branco 2007 [47]** | 7 | 77 | - |
| **Blumenfeld 2008 [25]** | 3 | 37 | 8 |
| **Huser 2008 [48]** | 21 | 71 | 1 |

*Table 16 Incidence of ovarian failure in different studies with and without GnRHa administration.*

Two recent review articles on the topic of ovulation suppression to protect against chemotherapy induced ovarian toxicity and amenorrhoea was published in the same issue of the Human Reproduction Update. [34] [49] Beck-Fruchter et al was of the opinion that the currently available information in the literature is not enough to reach

a definite conclusion that GnRH-agonists are beneficial in protecting against chemotherapy induced damage. They argue that on the basis of the current evidence it is possible to draw the wrong conclusions from the published data. There are many drawbacks to the published studies and in their meta-analysis of the literature where a large number of studies were combined, most of the patients included were from non-randomised studies. [34] Very often the studies were not controlled at all or had historical controls. This would suggest that the control groups were more likely to have reached the point where the premature ovarian failure has occurred at the time of the evaluation for the study. There were many treatment protocols, many different indications for chemotherapy and the definition of ovarian function was not always the same. Various parameters were included like the presence or absence of menstruation, hormone levels, ultrasound appearance of the ovaries and even cycle regularity. In most of the published data indications of what is meant by preserved ovarian function and ovarian failure is not clearly outlined. They mention that the two largest studies (those by Castelo-Branco and Huser) [47] [48] had unexpectedly high incidences of premature ovarian failure (POF) in the control groups. This high POF rate was higher than expected when compared to other studies of Hodgkin's lymphoma survivors, which is usually in the region of 32-37%. [50] [51] This high POF rate in the control group of the larger studies will influence any meta-analysis currently performed.

Blumenfeld published a review in the same journal and came to a slightly more positive conclusion. [49] However, they also concluded their review by stating that there is not enough convincing statistical evidence concerning the reduction of POF

to treat young women with GnRH agonists. More large prospective randomised studies are needed.

Embryo cryopreservation is illegal in Italy. That makes other strategies for fertility preservation perhaps even more important. A group from Bari evaluated ovarian reserve in 29 patients treated for Hodgkin's disease. [4] They used serum levels of follicle stimulating hormone, luteinizing hormone, Inhibin B, anti-Mullerian hormone (AMH) and the ultrasound antral follicular count as markers of ovarian reserve after chemotherapy. The patients randomly had GnRHa or not at the time of chemotherapy. It is not clear from the article which specific analogue was used. Of the 14 women who received GnRHa, not one developed amenorrhoea. Seven out of 15 (46%) not receiving GnRHa showed clinical symptoms of ovarian failure "suggesting a possible protective action of GnRH-a". However, when the results of the ovarian reserve parameters were compared, those treated with GnRHa had similar results suggesting that chemotherapy results in depletion of the follicular pool.

A different Italian group from Pescara treated 61 women with triptorelin a gonadotrophin-releasing hormone analogue (GnRHa) given monthly during chemotherapy treatment for patients with Hodgkin's lymphoma. [52] At completion of the study, the majority (81%) recovered normal menses while 6% had irregular cycles and the remaining 12% developed premature ovarian failure. They felt confident enough about the results to recommend that all young women should receive GnRHa co-treatment from the onset of chemotherapy.

A recent report from Korea investigated the incidence of chemotherapy-related amenorrhoea in pre-menopausal women with breast cancer. [53] In this study 238 women received adjuvant endocrine therapy for oestrogen/progesterone receptor positivity. The adjuvant endocrine therapy included selective oestrogen-receptor modulators (toremifene citrate and tamoxifen), aromatase inhibitors (letrozole and anastrozle) or luteinizing hormone-releasing hormone. The two important predictors for amenorrhoea were older age at treatment and the use of endocrine treatment. Interestingly "the use of adjuvant endocrine therapy was *more likely* to result in permanent chemotherapy-related amenorrhoea compared with no use (P = 0.006)".

**Potential problems with GnRHa treatment**

The GnRH-agonists are not without problems. The side effects include postmenopausal symptoms like hot flushes and a decreased bone mineral density. It is also of importance to note that GnRHa receptors have been found in certain tumour cell lines and the effect that GnRHa may have on tumour progression has not been studied carefully. [20] [54] Some reassuring data can be found in a study on 10 women who were treated by Leuprolide acetate before bone marrow transplantation. [55] The authors found no adverse effects of the GnRHa treatment.

With GnRHa 7 to 10 days may be needed for complete ovarian suppression. However, with a GnRH antagonist used alone or together with GnRHa the "induction time" will be reduced. GnRH antagonists block GnRH receptors immediately. The waiting time before chemotherapy could be reduced to less than 4 days. [56]

**Potential (non-fertility) benefits of GnRH treatment**

GnRHa may be beneficial in patients with early breast carcinoma where suppression of oestrogen production will lead to a better outcome. A recent Cochrane review on GnRHa in adjuvant therapy setting for early breast cancer in pre-menopausal women found that the therapy may be of clinical benefit. The authors concluded that the current data strongly supports the continuation of further trials on LhRH agonists in early breast cancer treatment. [57] Similar results were reported from a study on 100 women treated for breast cancer and who received GnRHa. [46] Not only was the treatment tolerated well and there was some long term protection of ovarian function but the authors also stated that the GnRHa "appeared to improve the expected clinical outcome."

Other potential benefits of GnRHa not related to ovarian function include reduction of menorrhagia during chemotherapy especially at the time when myelosuppression may be an important complication of chemotherapy. A recent systematic review has found that GnRH agonist therapy is highly effective for prevention of excessive uterine bleeding in women being treated for haematological malignancies. [58]

## *Contraceptives as a strategy to reduce ovarian damage*

**Combined oral contraceptives**

Suppression of follicle development during the administration of chemotherapy may reduce the risk of follicular damage. A group of 31 young women who received neo-

adjuvant chemotherapy for the treatment of osteosarcoma were observed for chemotherapy-induced menopause. [59] All these patients received high dose ifosfamide, methrotrexate, adriamycin and cisplatinum. They were treated with oral contraceptives for the duration of chemotherapy.

Three out of nineteen patients developed early menopause in the treatment group. They were compared with an historical control group in which three out of 71 patients developed chemotherapy-induced menopause. The authors concluded that oral contraceptives during chemotherapy *do not protect* ovarian function in patients receiving high-dose alkylating chemotherapy and that age at treatment and the total dose of alkylating agents are the most important predictors for ovarian failure. [59]

An earlier retrospective report on the reproductive outcome of women treated for Hodgkin's disease included a total of 44 females followed up after chemotherapy (MVPP). [5] Nine of these patients took combined oral contraceptives throughout the administration of chemotherapy of which four subsequently developed amenorrhoea and a further three developed oligomenorrhoea. It seems as if oral contraceptives *did not protect* these patients against chemotherapy induced ovarian damage.

A report on a small number of women from 1981 evaluated the use of oral contraceptives in six young women treated with MVPP for Hodgkin's disease. [60] They were followed up for between four and twelve months and had post-therapy ovarian biopsies and menstrual history to determine fertility potential. Five out of six patients had normal menstruation after discontinuation of the chemotherapy. The three cases that had ovarian biopsies were 18, 19 and 28 years respectively. The

19-year old had more than 1000 follicles per section after chemotherapy. It is difficult to know whether histological evaluation of follicle numbers will predict eventual menstrual function but despite the small numbers in this study the authors concluded “that suppression of ovarian function by combination oral contraceptives protects the ova against an otherwise certain injury by the chemotherapeutic drugs”. [60]

In a report on the post-treatment fertility status in women who had received aggressive treatment for non-Hodgkin’s lymphoma, a group from Israel found that age was the most important risk factor and that older women were at the highest risk for chemotherapy related amenorrhoea. [44] In their cohort of patients, fertility preserving measures in the form of GnRHa were used in three patients and nine received combined oral contraceptives. When these latter patients were compared to the rest of the group there was no significant difference in the rate of ovarian failure.

The best evidence so far for the potential benefit of oral contraceptives during chemotherapy is from a large retrospective report from Germany. [61] A total of 405 women answered a questionnaire about menstrual status after therapy for Hodgkin’s lymphoma. These patients were all younger than 40 years of age at treatment. The rate of amenorrhoea was significantly higher in women not taking oral contraceptives when compared to women on oral contraceptives during chemotherapy (44.1% versus 10.1%) ($p < .0001$). Looking at all the different factors evaluated in the retrospective evaluation, a multivariate analysis was performed looking at age, chemotherapy regime, stage of disease, the use of oral contraceptives during

chemotherapy and the effect on amenorrhoea. Two-hundred-and-fourteen of the total 405 women had enough information to be included into the multivariate analysis and the following were found to be significant predictors of amenorrhoea:

- Receiving eight cycles of dose escalated BEACOPP when compared to other less toxic regimes
- Amenorrhoea was statistically significantly higher in women with advanced stage ($p < .0001$)
- Age over 30 years at treatment ($p = .0065$)
- Not taking oral contraceptives during chemotherapy ($p = .0002$)

This study provides the strongest evidence so far for the potential effect of oral contraceptives in the protection of ovarian function during chemotherapy.

However, oral contraceptives have never been adequately tested in a randomised controlled trial. [62] A randomised phase II trial performed by the German Hodgkin's lymphoma study group, the protection of ovarian fertility study (PROFE), evaluating the use of GnRHa and oral contraceptives for patients receiving treatment for Hodgkin's lymphoma, was stopped early after only 23 patients enrolled. The reason for the early termination of the study was mainly that most women already received GnRH-analogues as recommended by their gynaecologists. Twelve patients received oral contraceptives and 11 GnRH-analogues. The respective infertility rates were 90% for contraceptives and 100% with GnRHa. The conclusion of the authors was that treatment with GnRHa or oral contraceptives conferred no meaningful ovarian protection.

### Medroxyprogesterone acetate

The only report on the effect of medroxyprogesterone acetate on ovarian protection during chemotherapy is a small descriptive study from Italy. [63] Twelve women had ovarian biopsies of which four received no chemotherapy or MPA, four received no chemotherapy and 250mg MPA and the last four received chemotherapy and 250mg MPA per month. Ovarian biopsies were taken and electron microscopy studies were done. Despite the very small number of cases this did not prevent the authors from stating that "the results presented here demonstrated that the administration of MPA to patients with Hodgkin's disease protects the ovary against an acute effect of chemotherapy". It is not clear from the discussion how the authors concluded that there was a protective effect because the studied follicles of patients receiving chemotherapy and MPA had substantial morphological damage. At present there is no strong argument in the literature to suggest the protective effect of the MPA against chemotherapy induced amenorrhoea.

## *Conclusion*

It is very difficult to reach a conclusion about the use of ovarian suppression during chemotherapy in an attempt to prevent subsequent chemotherapy induced ovarian failure. Most of the evidence in the literature is from non-randomised case reports; often with historical or no controls. Various chemotherapy regimes were used, doses of GnRHa and COC's were not similar and the number of patients was in general very low. There is no randomised controlled trial to evaluate either COC's or GnRHa in this clinical scenario.

The only way to reach consensus would be to test COC's and GnRHa in a properly designed prospective randomised trial.  Numbers of young patients with cancer are usually small in individual institutions and it is extremely important to combine data from multiple sites to reach useful results.

At present the indication for oral contraceptives or GnRHa may be for other non-fertility benefits.  GnRHa and oral contraceptives may be used to reduce menstrual blood loss which is often increased during treatment because of abnormal clotting and platelet function.  Where hormones may be contra-indicated due to hormone sensitive tumours, GnRHa can be very useful.  There is some evidence that GnRHa administration may improve the outcome in oestrogen receptor positive breast carcinoma.

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# Chapter 4: Germ cell physiology with reference to cryopreservation


### *Abstract*

The cryopreservation and transplantation of ovarian tissue have been shown to restore ovarian function temporarily and may also preserve the fertility of young female cancer patients until after their sterilizing cancer treatment. Since tissue samples are large and morphologically complex, the cryopreservation methodology is difficult to optimise and standardise. Ovarian tissue cryopreservation is still in its experimental stages and is not a routine option offered to cancer patients.

This review describes oocyte maturation with reference to follicular development and the possible effects of cryopreservation on normal function. Oocyte cryopreservation, including slow freezing and vitrification are becoming more successful and reproductive outcomes are summarised.

A randomised controlled investigation of 1,2-Propanediol (PROH) and Dimethyl Sulphoxide (DMSO) cryopreserved tissue was performed. The tissue was evaluated with transmission electron microscopy for morphological features of damage. No significant difference was found between the two groups. Slow freezing using DMSO and PROH were associated with ultrastructural changes, but had no significant difference with fresh tissue. Slow freezing and rapid thawing of ovarian cortical tissue is a promising option for protection of fertility.

## *Introduction*

### Ovarian germ cell reserve

The number of oocytes in humans peaks at around six to seven million by 20 weeks of gestation. [1] There is a sharp decline in the number of oocytes from around 20 weeks after gestation to birth when only about one to two million oocytes remain in the ovary. See Figure 4. Further atresia of the oogonia occurs and at the start of the first menstruation. About three hundred to four hundred thousand of the original seven million oocytes remain available for ovulation. Four hundred to five hundred of these will ultimately ovulate and the process of ovulation is carefully controlled by endocrine, autocrine and paracrine factors. No new oogonia are formed after birth and all the available oocytes at birth are in the diplotene (dyctyate) phase of meiosis and will only continue further in the process of meiosis at the time of antral development. At the diplotene stage, the resting phase of the meiotic division, the oogonia becomes surrounded by granulosa cells in a single layer. The oogonia that failed to become surrounded by granulosa cells will undergo atresia.

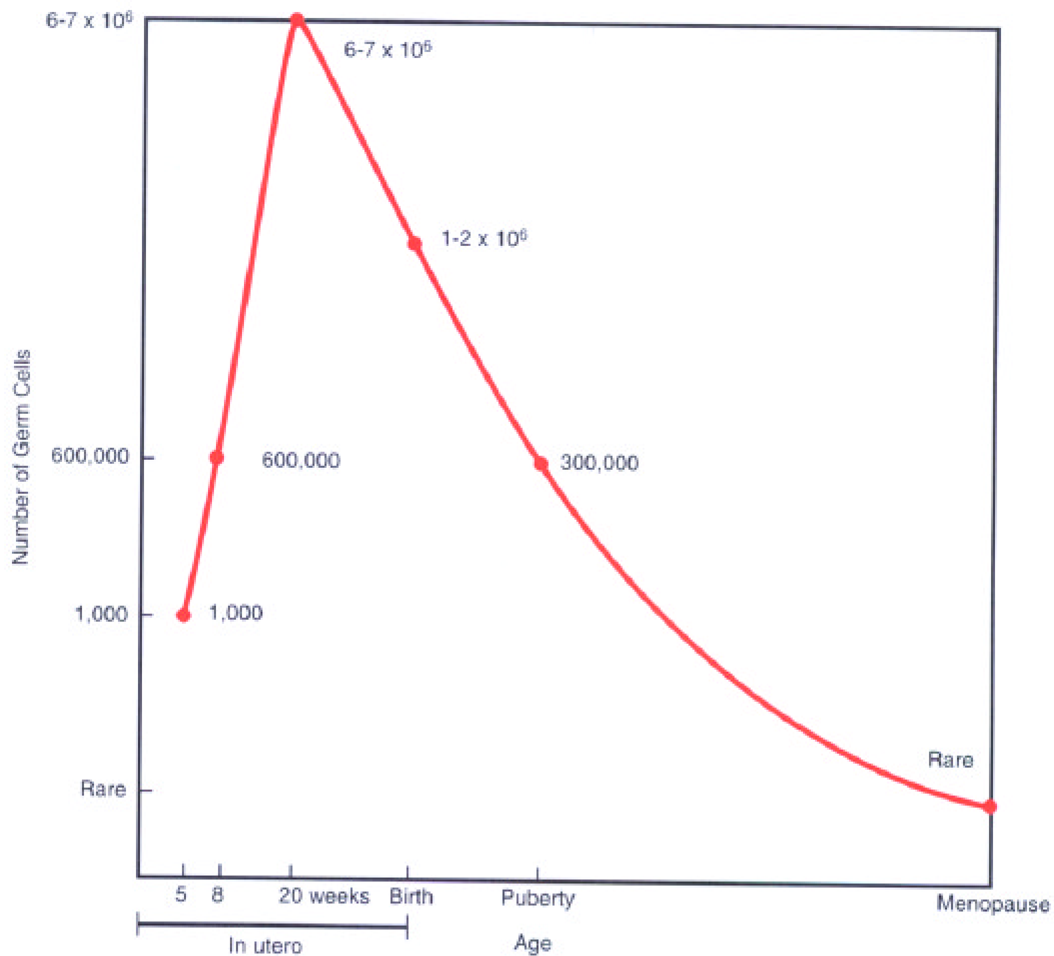


*Figure 4 Follicular reserve (germ cells) at different ages. From [2]*

**Cell division**

Meiosis is usually divided into four stages; prophase, metaphase, anaphase and telophase. The prophase of meiosis is further divided into five stages namely leptotene, zygotene, pachytene, diplotene and diakinesis. As soon as the oogonia begin developing into the meiotic prophase they are known as primary oocytes. [3] This process begins around 8 weeks of gestation and if meiosis does not occur the oogonia will undergo atresia and apoptosis. The oocytes arrested in prophase (that is the late diplotene or dictyate stage) will remain so until the time of ovulation when, during oocyte development, further meiotic processes will occur usually at the time of

the LH surge. This resting phase of the development is believed to be, at least partly, controlled by oocyte maturation inhibitor (OMI) produced by granulosa cells. [4]

**Germ cell physiology**

The relative fecundity of a particular individual varies widely between different species. It depends on the specific reproductive strategy where a different kind of investment is made in the production of oocytes. An example of very efficient oogenesis is broadcast spawners like fish while on the other side of the spectrum primates, including humans, have a highly selective process where oocytes are selected through a process of attrition to allow only a small number of individual oocytes to progress to ovulation throughout the reproductive lifespan. Where damage to the relatively small surviving pool of oocytes occur in relatively low producers of oocytes it is essential to understand the specific mechanisms of follicle development in order to minimise damage by these clinical interventions.

The understanding of germ cell physiology has improved tremendously over the last few decades. Since the advent of assisted reproductive technologies in the early 1970's it is estimated that more than 3 million children have been born using assisted reproductive techniques. [5]

Oogenesis is regulated at many levels. The exact process by which the activation of primordial follicles is achieved is not clearly described. The process through which the granulosa cells proliferate and change their shape from squamous to cuboidal

ends with the establishment of a primary follicle. [6] [7] This transition is under control of many different factors. There are *physical* and *hormonal* contacts between the oocyte and granulosa cells and various autocrine and paracrine factors have been described.

Physical communication between oocytes and granulosa cells are extremely important. The structures that provide connections are called trans-zonal projections and maintain the physical contact between the different cell types. [8] At the end of these trans-zonal projections, gap junctions allow physical connection through groups of proteins called connexins. Connexins are the functional units of the gap junctions and are intercellular membrane channels that allow the sharing of small molecules between neighbouring cells. [9] Specific proteins like connexin 37 is unique to the oocyte-granulosa cell interaction and the absence of these connecting proteins may lead to a failure of ovum maturation. [10] Physical properties of trans-zonal projections include microtubules and actin filaments. [11] [12] *These structural elements may be damaged by cryotherapy.* These physical connections, providing a communication network between the oocyte and the surrounding ovarian stroma, should remain intact in order for complete meiotic division to take place and eventual follicle development. [11] [13]

Many inter-cellular autocrine factors have a role in the development of successful follicles. Anti-Müllerian Hormone (AMH) has been implicated in maintaining primordial follicles in the resting phase. [14] AMH is also a regulator of FSH and may inhibit primordial follicle activation. [15] The Forkhead Transcription factor 03 (FOX 03) is another potential inhibitor of primordial follicle activation. [16] Stimulatory

effects on the activation of the transition from primordial to primary follicle were also demonstrated for compounds like basic fibroblast growth factor (bFGF). [17], Bmp4 [18] Keratinocyte Growth Factor (KGF)(also known as FGF7) [19] and platelet derived growth factor (PdGF) [20]

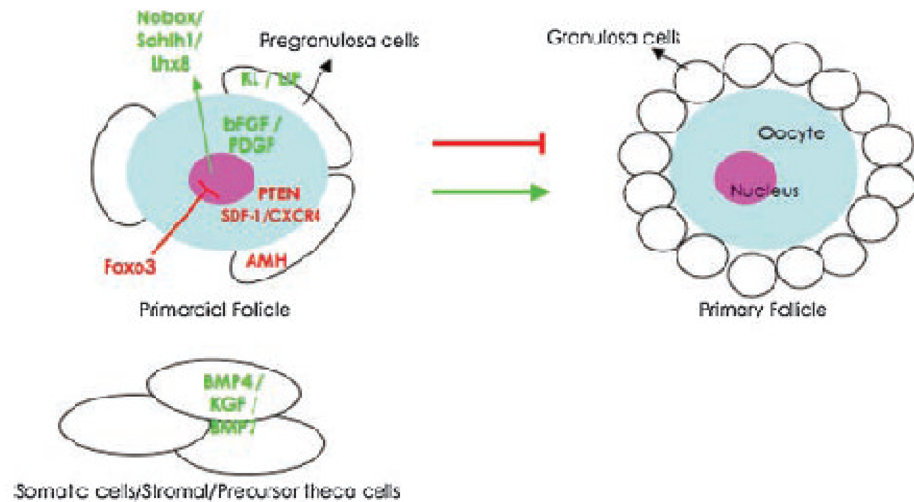


*Figure 5 Schematic representation summarizing the inhibitory and stimulatory factors involved in primordial to primary follicle transition [5]*

## Follicle development

### Primary to multi-layered follicles

The growth of the oocyte in the primary follicle is quite slow and is accompanied by a slow rate of granulosa cell proliferation. The granulosa cell layers increase from one to six or seven in the pre-antral stage. [21] The growth of the follicle and oocyte may be influenced by factors like GDF9 [22] and other oocyte specific factors like BMP15 [23] and GDF9b [24] Activin and Inhibin are both members of the transforming growth factor beta (TGF beta) family and these compounds were both identified in

follicular fluid during development of the antral follicle. Both these compounds have effects of FSH production. [25] [26] Other compounds also affecting FSH production include Follistatin which is a single chain glycoprotein with certain similarities to the alpha and beta subunits of the Inhibin-Activin family. [27] In patients with tissue re-implanted after cryopreservation levels of Inhibin may be similar to pre-menopausal women due to low follicle number. Insulin-like growth factor 1 (IGF1) plays a role in FSH sensitivity in granulosa cells. [28] AMH is also a regulator of FSH and may inhibit primordial follicle activation. [15]

### Antral follicle to ovulation

The second phase of follicogenesis starts when granulosa cells become responsive to FSH. For this FSH receptors need to be present which is under the control of IGF1. [28] Oestrogen acts in a stimulatory fashion by enhancing FSH action on granulosa cells. [29] LH responsiveness in the granulosa cells only develop with the presence of LH receptors. [30] [31] The LH surge begins a process where luteinisation signals the terminal differentiation of the granulosa cells and they stop proliferating. At the same time the oocyte resumes meiosis and ovulation occurs. [32] [31]

### Anatomical considerations

When the anatomy of the ovary is considered, it is important to note that oocytes and therefore also follicles are present just below the peritoneal epithelium. If ovarian tissue is cryopreserved only the cortex needs to be dissected and frozen.

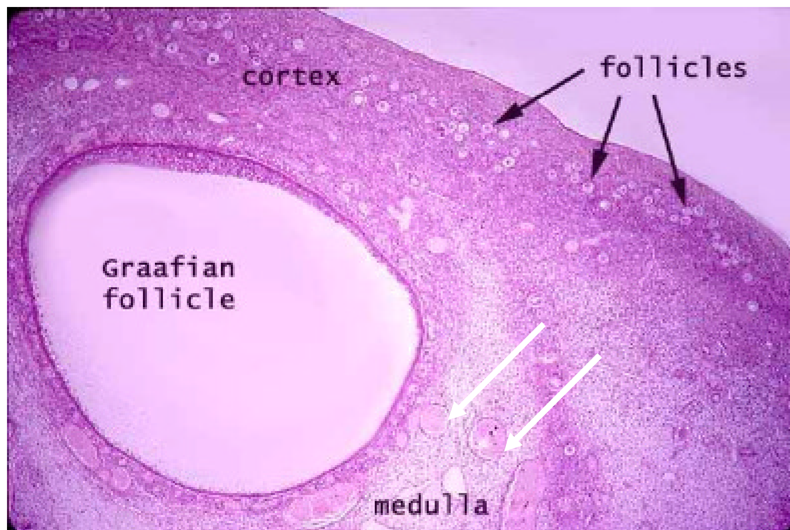


*Figure 6 H&E stain of the cortex containing early follicles (black arrows) and the stroma of the ovary, containing blood vessels (white arrows). (http://www.siumed.edu/~dking2/erg/images/RE002b.jpg)*

The blood supply to the developing follicle is not with direct vessel support but through an end artery system. [33] That means the ovarian cortex is a relatively hypoxic tissue and probably protects oocytes during the process of removal, freezing and thawing before re-implantation.

During follicogenesis the oocyte is dependent on the surrounding tissue for hormonal support. If successful follicle development is required, consideration of the effect of the intervention on granulosa cells, extra-cellular matrix and stromal cells is important.

Relative size of the follicle directly affects the physics of freezing. There is a linear relationship between the volume and complexity of the tissue and the eventual cellular damage. Cryoprotectants fail to penetrate larger tissue samples effectively and heat transfer is impaired. The larger the amount of tissue, the more pronounced the damage. Table 17 shows a summary of the histological characteristics of

different stages of follicle development and Figure 7 demonstrates the increasing volume and complexity of the developing follicle.

| Stage | Description | Size |
|---|---|---|
| **Primordial** | Resting, small, one layer of flat granulosa cells | 0.03-0.05 mm |
| **Primary (germinal vesicle stage)** | Mitotic cells, cuboidal granulosa cells | < 0.1 mm |
| **Secondary** | Presence of theca cells, multiple layers of granulosa cells | 0.2 mm |
| **Early tertiary (Antral)** | Formation of an antrum | Early tertiary follicle:<br>Class 1~ 0.2 mm *<br>Class 2 ~ 0.4 mm<br>Class 3 ~ 0.9 mm<br>Class 4 ~ 2 mm<br>Class 5 ~ 5 mm. |
| **Late tertiary** | Fully formed antrum, no further cell differentiation | Class 6 ~10 mm<br>Class 7 ~ 16 mm<br>Class 8 ~ 20 mm.<br>Non-dominant follicles may grow beyond class 5, but rarely more than one class 8 follicle. |
| **Pre-ovulatory** | Increase oestrogen in dominant follicle and all other follicles atretic | |

*Table 17 Summary of characteristics of follicle development stages. Adapted from [34] *Class refers to Pedersen and Peters classification. [35]*

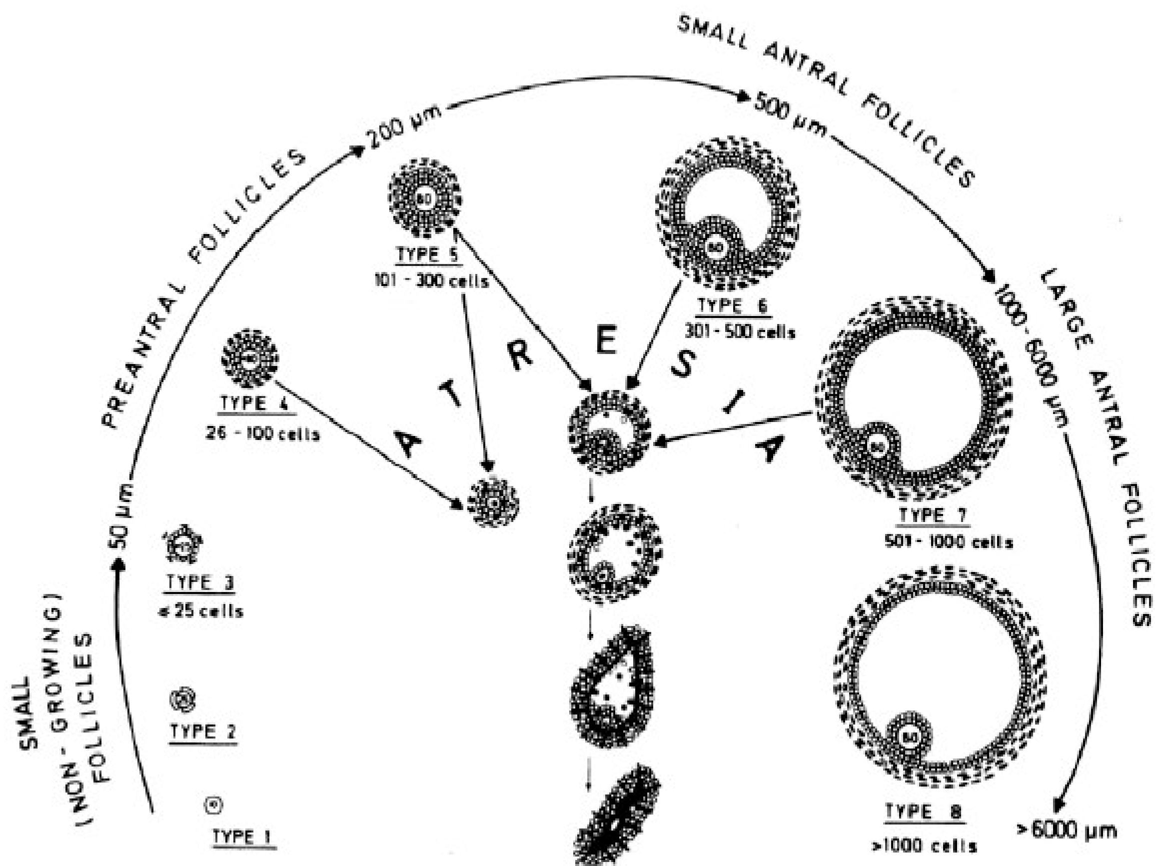


*Figure 7 Diagram of follicular development showing relative sizes of developing follicles. From [1]*

Antral follicles have higher water content and will undergo more pronounced contraction and expansion during freezing. Contraction and distortion of cells may cause breaks in intercellular connections including the gap junctions. Smaller follicles survive the insults of cryopreservation better. [36]

## *Cryobiology*

### Introduction

Preservation of tissue without damage to individual cells is possible only under well controlled conditions. There are three stages of lowering temperature that may affect cell physiology. The first is temperatures below body temperature down to 0º Celsius which may be damaging if the exposure is prolonged. [37] Many cells and tissues can survive low temperatures down to 0º for a limited period of time.

A second stage of cooling is reached between 0 and -40º Celsius where ice crystal formation in water may damage tissues significantly. Most sub-cellular damage will happen at these temperatures. Water freezes with nucleation and crystallization and very few cells will survive exposure to these temperatures for a long period of time.

A third stage of cooling with temperatures between -40° C to the temperature of liquid nitrogen of -196º C is very useful for preservation of tissues and cells and will limit damage to sub-cellular structures since physiological processes and biochemical mechanisms cease at these temperatures. It is often the *process of cooling down* to these very low temperatures and the *warming or thawing* that are the damaging stages and not the storage at very low temperature. [38]

Intra-cellular ice formation is very damaging to cells. Fine ultra-structural properties may be directly damaged due to mechanical stress of ice crystals or may be displaced within the cell due to dehydration. Ice crystal formation also causes expansion of volume which may cause permanent and fatal damage to cell

membranes and certain structures within the cytoplasm. [38] Dehydration can be fatal to a cell. Ice crystals contain only water molecules and as the water freezes solids are concentrated in the remaining cytoplasm. The high solid concentration may reach toxic levels and lead to unfolding and denaturing of enzymes. [38]

To try and minimise the effect of intra-cellular ice formation the following strategies may be used:

- Super cooling and freezing point depression
- Dehydration
- Vitrification

**Super-cooling and freezing point depression**

Super cooling is a freezing avoidance mechanism where solutions are cooled to below the freezing point while remaining in a liquid state. This allows slower than normal metabolism. In practice only small volumes (micro litres of pure liquids) can be super cooled to a few °C below the equilibrium freezing point. Biological tissues are inherently unstable with so many ice nucleators and if only one ice crystal is formed the solution will freeze immediately.

In nature many plants and animals can increase the amount of suspended solutes intra and intercellular in a response to cold conditions. This will decrease the temperature at which the tissue freezes. The process is well described for certain arctic fish species. [39] Water freezes at 0°C but if the temperature of freezing can be artificially lowered in tissue it may allow the scientist to control the process better.

The main aim of freezing point depression is the avoidance of ice crystal formation at relatively warm sub-zero temperatures which is characterised by disorganised and unpredictable crystallization.

Crystal formation of freezing starts with a process called nucleation of water molecules. Just before freezing water molecules with lower kinetic energy will start to arrange into a lattice formation. When this crystal lattice reaches a critical point freezing will start quickly and in a disorganised way. Freezing point depression is a technique that allows a scientist to do manual seeding which introduces crystallisation in an organised predictable way.

By adding cryoprotectants lower freezing points can be achieved. By increasing concentrations of cryoprotectants like dimethyl sulfoxide (DMSO) and 1,2 propylene glycol (PROH) freezing point temperatures can be lowered. [40] Sugars can also be added to reduce freezing temperatures and high concentrations can be tolerated by biological tissues without severe damage. [38]

**Dehydration**

Extra-cellular freezing usually occurs first because the volume is larger. If extra-cellular water freezes it draws liquid water out of the cell causing a rapid change of shape. By increasing extra-cellular osmotic pressure before freezing, water moves out of the cytoplasm resulting in cellular dehydration. This will reduce the amount of water left in the cell to freeze. Many seeds and spores use dehydration to survive extreme low temperatures.

Dehydration with cryoprotectants should not be too severe. The osmotic pressure has to be carefully controlled. Moderate dehydration and cell contraction can be achieved by a cryoprotectant like hydroxyl ethyl starch (HES) or dextrone. [38]

**Vitrification**

Cryopreservation through vitrification attempts to achieve ultra low temperatures without intra-cellular ice crystal formation and membrane damage. If a liquid can be cooled sufficiently fast the viscosity increases rapidly to form a glasslike vitreous solid state. The viscosity of the liquid becomes so high that nucleation (and therefore crystallisation) becomes impossible. A glass is amorphous with no long term crystalline structure but at the same time has the mechanical properties of a solid.

**Rapid Thawing**

Thawing represents another critical period for the tissue. The most harmful period for complex sub-cellular structures is the subzero temperatures near the equilibrium freezing point. At this point crystallization is unpredictable and ice may form randomly in a disorganized way. If extra-cellular tissue thaws there is a sudden decrease in the osmotic pressure which may cause rapid movement of fluids across the cell membrane. Rapid thawing avoids many of the dangers. [38]

## *Oocyte cryopreservation*

### Slow freezing of oocytes

The survival and ultimate normal functioning of an oocyte after the process of cryopreservation has been more difficult to achieve when compared to sperms and embryos. Success is compromised by the complexity of the intra- and sub-cellular organelles which are far more complex than in smaller cells like sperm and pre-implantation embryos. Early work done on frozen murine oocytes showed spindle abnormalities and other damage due to cryopreservation and exposure to cryoprotectants. [41] [42] Metaphase II oocytes are structurally quite complex with the fragile meiotic spindle one of the most vulnerable structures.

Immature oocytes have been successfully cryopreserved and matured in vitro, however, the clinical application of this technique is at present not clear. Some authors postulate that cryopreservation with an earlier oocyte (e.g. human oocytes at the germinal vesicle phase) may have better survival. [43] A study done on the microscopy of metaphase II oocytes compared to germinal vesicle oocytes showed a significant difference regarding spindle and chromosome abnormalities. [43] The oocytes that were cryopreserved at the germinal vesicle stage and then matured in vitro showed less spindle damage. With improved in-vitro maturation techniques this may become a clinical option. The process of in vitro maturation of germinal vesicle oocytes involves the removal of the cumulus cells in order to get a fertilizable egg.

It is postulated that the high lipid content of cell membranes play a role and that improved results may be achieved by manipulating the lipid phase transition where

lipids change from liquids to solid state during the freezing process. [44] Some Antarctic fish contain a natural anti-freeze protein that can shift the lipid phase transition. Adding agents like phosphotadylglycerol may alter these lipid phase transitions. Cryoprotectants in themselves may however also act as toxins to the oocyte and cause irreparable damage even before the start of the freezing protocol. [45]

Intracellular micro-injection of certain cryoprotectants like trehalose at a concentration of 0.15M (molar) showed promising protection against freeze associated stresses. An interesting in-vitro study describes the survival of three different groups of human oocytes of which one was a control group, one group had extra-cellular trehalose and the third had intra-cellular injection of trehalose. The 57 oocytes with injected trehalose had statistically significantly better survival even at very low temperatures. [46] Trehalose is an interesting compound and is a naturally occurring disaccharide found in nature. It can be produced by fungi, plants and even certain insects. The sugar allows plants and animals to withstand long periods of desiccation and allows cells to significantly dehydrate without serious damage. It is commercially used in various food applications and is also extensively used in the pharmaceutical industry.

**Cryoprotectants**

PROH and DMSO have been described as choices for oocyte protection and the first live birth from a DMSO cryopreserved thawed oocyte was reported by Chen in 1986. [47] Two pregnancies from PROH protected oocytes were reported in 1985 but they

did not end in live births. [48] In a report from the late 1980's a comparison between PROH and DMSO found PROH to be superior in terms of oocyte survival. [49] The mechanism by which the cryoprotectants are directly toxic can be due to volume and concentration changes in the intra-cellular milieu as described by Fahy. [45] Exposure to cryoprotectant agents has been implicated in changing in the characteristics of the zona pellucida making it more impenetrable to sperms. All the mechanisms through which failure to fertilize after cryopreservation are not clearly understood but zona pellucida effects may play an important role as demonstrated by Matson in mice. [50] After an experimental study in mice, the investigators concluded that intracytoplasmic sperm injection (ICSI) may be beneficial after cryopreservation of oocytes in humans.

Two years before the animal work by Matson was published, a study from the United Kingdom showed that fertilization and cleavage rates of human oocytes were significantly improved when oocytes were fertilized by ICSI compared to conventional IVF i.e. 13.5% of oocytes fertilized by IVF only compared to 45.9% fertilization in the group with ICSI. [51] This makes ICSI the preferred option for patients wanting to fall pregnant from cryopreserved oocytes. [52] There are indications that with improving technical ability freezing of oocytes is becoming more efficient with increases in survival rates of individual oocytes, better fertilization rates and ultimately an improvement in pregnancy rates. [53]

A meta-analysis published on the efficiency of oocyte cryopreservation found that success rates with slow frozen oocytes were statistically significantly poorer when compared with fertilization of non frozen oocytes. However, the authors

acknowledge possible improvement in outcome after 2005 which may suggest that with improvement in technique slow freezing may have even better fertilization rates in future. [54]

Possible improvements in the slow freezing of oocytes may be achieved by changing cryoprotectant concentration. One example may be to alter the concentration of sucrose as a cryoprotectant as reported by Bianchi. [55] The temperature at which cryoprotectants are introduced may play a role in the eventual survival. If cryoprotectants exposure happens at body temperature it may decrease the injury to the cytoskeleton of the human oocyte. [56]

**Pregnancy results from oocytes preserved by slow freezing**

One of the largest series reported on pregnancies from cryopreserved oocytes demonstrated 11 live births from cryopreservation of 1796 oocytes. Despite promising results in small numbers of patients the overall live birth rate from previously frozen oocytes is only about 2% which is lower than that with IVF using fresh oocytes. [57]

A large meta-analysis confirmed this figure of around 2% of live birth rate after using frozen oocytes. [54] It is important to note that in a report from Porcu [58] their stimulation protocol was used on 18 young women with a mean age of 19 years and a range between 14 and 26 years. The number of days used for stimulation was a mean of 11, ranging from 8 to 14. They could retrieve an average of 15 oocytes per cycle. The stimulation period remains the most problematic aspect of oocyte

cryopreservation because of the delay in the start of treatment for patients with underlying malignancy. In order to reduce the time necessary for stimulation before oocyte retrieval ovarian stimulation may be started in the luteal phase. [59] A very similar time from start to retrieval was achieved in patients starting stimulation in the follicular phase and the luteal phase of the menstrual cycle.

A concern about possible increased incidence of chromosomal abnormalities in cryopreserved oocytes seems to be ungrounded. There is no evidence that chromosomal abnormalities are more frequent when fertilization does occur, even if it is at a lower rate than with fresh oocytes, [60] [61]

## Oocyte Vitrification

### Technique

Recently vitrification (ultra rapid cooling technique) of human oocytes has been described and the success rate after vitrification seems to be improving. High concentrations of cryoprotectant and very quick cooling, in order to prevent crystallization or ice formation, cause the intra-cellular water to form a glassy solid state directly from liquid phase. [62] Usually a very small volume of high concentration cryoprotectant is used and the cooling rate is extremely fast.

A theoretical concern is direct toxicity of liquid nitrogen to the cell. Vitrification of oocytes has recently become safer with the introduction of properly sealed containers like the Cryotop system. [63] This sealed system reduces many of the

concerns about potential contamination of oocytes in an open bath of liquid nitrogen. A concern with vitrification remains the high levels of exposure to cryoprotectants.

A recent review by Nagy gives a good overview of the history of human oocyte vitrification. [64] The authors have a very positive experience of recent improvements in vitrification techniques and describe the following phases in the development of vitrification:

1. Identification of the best solutions for the different stages of vitrification namely equilibration, cooling, warming and rehydration.
2. The establishment of techniques to achieve extremely high cooling and warming rates to minimize oocyte damage.
3. To work out the detail of concentrations and times exposed to cryoprotectants.
4. To eliminate the risk of disease transmission during all phases of cooling and thawing.

From this review it is interesting to note that many developing countries from South America have high success rates with live births after oocytes vitrification. See Table 18 for approximate numbers of births as reported by Nagy.

| Country | Approximate number of Births |
| --- | --- |
| **United States** | 35-40 |
| **Canada** | 35-40 |
| **Italy** | 10-20 |
| **Japan** | 40-60 |
| **Spain** | 40-50 |
| **Mexico** | 100 |
| **Colombia** | 200 |

*Table 18 Number of babies born from vitrified oocytes. From [64]*

The author gives a comprehensive list of indications for human oocyte cryopreservation which include the obvious indication of fertility preservation in cancer survivors but also include non-medical indications. These include a wish to delay motherhood for various reasons like career development, storage of excess donor oocytes, oocyte donation from mother to daughter e.g. in a case with Turner syndrome, severe endometriosis and also poor responders to ovarian stimulation. Some of these indications indeed raise moral questions but the most obvious indication still remains fertility preservation for cancer patients.

The potential safety problems associated with vitrification is discussed in detail. Despite the fact that liquid nitrogen at a temperature of minus 196 degrees Celsius slows down most biological processes to virtual zero, many micro-organisms will survive this environment. There is the possibility of transfer of pathogens and a description of hepatitis B virus contamination among bone marrow transplant

specimens was described in 1995. [65] Direct viral transmission between embryos has also been described by Bielanski. [66] In the review by Nagy and co-workers the suggestion is made that the separate phases of vitrification namely cooling, storage and warming should be separated. [64] They give a detailed description of methodology which may reduce the risk for transmission and included in this description is the filtration of liquid nitrogen and the sterilization of containers with bleach.

Despite the concern this review shows vitrification of oocytes in a very positive light with excellent outcome figures. There is concern, however, that this optimism may not be reflected in everyday practice due to varying standards of quality control.

### Pregnancy outcome with oocytes preserved with vitrification

A pregnancy using previously vitrified oocytes has been described by a group already in 1999. [67] The attraction of using vitrification instead of the traditional slow freezing methods is that expensive slow cooling technology is not necessary and that it is much quicker than the long cryopreservation protocols.

It is difficult to compare cryopreservation of oocytes by slow freezing and vitrification because of many variables involved in each process. However, a group from China reports on their experience with a total of 605 oocytes preserved either with slow freezing or vitrification. The survival rate of the vitrification group was 91.8% compared to 61% in the slow freezing group. However, the fertilization rate was approximately the same for both groups. Of the fertilized oocytes the vitrification

group had higher rates of blastocyst formation and had less damage when examined with microscopy. [68]

## *Cryopreservation of ovarian tissue*

### Introduction

The exact methodology of cryopreservation of human ovarian tissue is not yet standardized and the technique is not routinely offered to patients. [69] [70] Ovarian tissue is morphologically complex due to multiple cell types with different characteristics. Intercellular connections and intracellular micro architecture may be damaged with ice crystal formation and contraction or expansion of volume. Follicles, even at quiescent primordial stage, may sustain injury from cryopreservation. [71]

Ovarian tissue cryopreservation protocols are complex. Ovarian tissue consist of multiple cell types with varying volumes and water permeability which may alter the way cryoprotectants protects against cell damage. Even minor changes may greatly affect the success of the protocol. Cryopreservation of ovarian tissue is similar to whole organ cryopreservation when compared to the preservation of single cells like gametes. [71] [72]

Cryopreservation of ovarian tissue is mainly aimed at optimising the primordial follicle survival rate. [71] [72] [73] [74] Larger, more complex follicles may have too many sites where freezing can harm the ultrastructure and intercellular connections between cells.

**Early functional studies of ovarian transplantation**

Early primate transplant data was published in 2002 by a group from Norfolk. [75] A total of 16 Cynomolgus monkeys were used in an experimental design to show that transplanted ovarian tissue will function to produce hormones and normal menstrual cycles. The monkeys were divided into three groups. The first group had no ovarian tissue replaced (so-called sham group), group two where ovarian tissue was transferred and a third group where the transplanted ovarian tissue was supported by administration of 1 µg of VEGF (vascular endothelial growth factor). The authors did not state the anatomical site where the transplantation was performed but gave a very good description of the hormonal and physiological function in these animals after transplantation. In the group of monkeys that received transplantation without VEGF support, five out of six had functioning transplants and two out of five of the animals with VEGF had functioning ovarian transplants. At a later stage in this study previously cryopreserved ovarian tissue was transplanted into the so-called sham group and 50% (2 out of 4) of the animals developed hormonal function. Despite the low numbers this work supported the potential of this technique to safeguard at least hormonal function of ovarian grafts. The authors concluded that “the administration of VEGF at the dose tested did not appear to improve transplant outcome”. [75]

**Cryoprotectants**

It is clear that two protectant media, Dimethyl sulfoxide (DMSO) and 1,2-Propanediol (PROH) showed better results when used in combination with controlled slow-freezing and rapid thawing when compared to other cryoprotective media. More

normal oocytes with intact cytoplasmic organelles survived when compared to fresh control groups. [70] [71] [73] [74] [76]

Two animal studies compared DMSO and PROH as cryoprotectants in slow-freezing cryopreservation. Similar results with high percentages of morphologically normal follicles were found. [77] [78]

Another animal study on goats using controlled slow-freezing of ovarian tissue found that 1.5M DMSO, when compared to 3M DMSO and corresponding PROH concentrations, resulted in a significantly higher percentage morphological normal primordial follicles. [79]

A summary of clinically relevant ovarian tissue cryoprotectant protocols are included in Table 19.

**Vitrification of ovarian tissue**

Vitrification and other methods of rapid cooling of ovarian tissue have been investigated but only a few studies have been published on rapid cooling methods. [71] [80] [81] Where the volume of the cells are smaller and the structure of the tissue is less complex, vitrification may be more useful.

A recent publication from Germany describes the histological evaluation of xeno-transplanted human ovarian tissue studied for signs of neovascularisation development. [82] The aim of this experimental study was to describe the amount of

revascularisation in three different groups of human ovarian tissue. It was previously suggested that transplantation rather than cryopreservation caused the majority of primordial follicle loss in animal models. [83] To try and demonstrate the validity of this, the authors studied fragments of ovarian tissue from three different treatment groups. Group A was fresh non-treated tissue, group B was tissue previously frozen with a slow freezing protocol and group C was tissue that was previously vitrified. A standard thawing methodology was used for all specimens and the tissue was then transplanted to SCID mice. The tissue was then examined for neovascularisation by staining with anti-mouse platelet epithelial cell adhesion molecule (PECAM-1) at progressively longer intervals. The amount of fluorescence was used as a quantitive indication of neovascularisation. The main findings were that there was no difference in the amount of revascularisation between the types of tissue treatment i.e. the revascularisation was independent of treatment method that is cryopreservation through slow freezing, vitrification or no treatment at all. There was an observed loss of follicles that was more pronounced within the first week after re-implantation (13%) when compared to follicle loss later after transplantation (5%) in the following three weeks. This would support the findings by Oktay [84] that suggests the most important period for follicle loss is in the initial re-vascularisation period where most ischemic damage occurs.

| Reference | Study Sample | Approach | CPA & Base solution | Non-Permeable CPA | Tissue size | Equilibration Time & Temperature | Cooling rate (ºC/min) | Seeding Method | Thawing & Wash out | Result |
|---|---|---|---|---|---|---|---|---|---|---|
| **Gosden 1994 [85]** | Sheep | Slow rate, controlled | 1.5M DMSO in Leibowitz-15 | 0.1M sucrose 10% calf serum | Tissue: 5-15x5mm Vials: 2ml | 15min, on ice | 2 to -7, 0.3 to -40, 10 to 140, plunged | Manual, -7ºC, hold 10min after | In air, water bath,10min in Leibowitz-15 | Live births |
| **Oktay 2000 [86]** | Case study: POF patient | Slow rate, controlled | 1.5M DMSO in D-PBS | 0.1M sucrose 20% serum | Tissue: 1-3x3-10mm | 30min 4ºC | 2 to -7, 0.3 to -40, 10 to -140, plunged | Manual, -7ºC, hold 10min before | In air 30s, 37ºC water, ↓CPA 4x5min | Ovarian function, ovulation |
| **Radford 2001 [87]** | Case study: POF cancer patient | Slow rate, controlled | 1.5M DMSO in Leibowitz-15 | 2.5% HSA | Tissue: 1x5x10mm Vials: 1ml | 30min, 4ºC | 2 to -9, 0.3 to -40, 10 to -140, plunged | Manual, -9ºC | In air 30s, 1min 37ºC water,3x5m Leibowitz-15 | Ovarian function for 2 months |
| **Oktay 2004 [88]** | Case study: POF cancer patient | Slow rate, controlled | 1.5M DMSO in D-PBS | 0.1M sucrose 20% serum | Tissue: 1-3x3-10mm | 30min 4ºC | 2 to -7, 0.3 to -40, 10 to -140, plunged | Manual, -7ºC, hold 10min before | Air 30s, 37ºC water bath, ↓CPA 4x 5min | Ovarian function, ICSI-ET, No pregnancy |
| **Donnez 2004 [89]** | Case study: POF cancer patient | Slow rate, controlled | 1.5M DMSO in Leibowitz-15 | 4mg/ml HSA | Tissue: 2x2mm Vials: 2ml | _ | 2 to -8, 0.3 to -40, 30 to -150, plunged | Manual, -8ºC | In air 2min, 2min 37ºC water,3x in Leibowitz-15 | Ovarian function, Spontaneous Live birth |
| **Meirow 2005 [90]** | Case study: POF cancer patient | Slow rate, controlled | 1.5M DMSO in D-PBS | 15% synthetic serum | Tissue: 1-2x5x5mm, Vials: 2ml | 30min | 1 to -9, 0.3 to -36, 5 to -140, plunged | Manual, -9ºC | In air 30s, 2min 37ºC water, ↓CPA 4x 5min | Ovarian function, IVF-ET, Live birth |
| **Demeestere 2006 [91]** | Case study: POF cancer patient | Slow rate, controlled | 1.5M DMSO in D-PBS | 0.1M sucrose 10% patient's serum | Tissue: 2x5x5mm, Vials: 2ml | 30min, 4ºC | 2 to -7, 0.3 to -40 -10 to 140, plunged | Manual, -7ºC, hold 10min before | In air 2min, 2min 25ºC water, ↓CPA 4x 5m | Ovarian function, Spontaneous pregnancy |

*Table 19 Summary of cryopreservation methods achieving successful results after cryopreservation of ovarian tissue.*

## *Whole Ovary cryopreservation*

Whole ovary cryopreservation may become an option if certain technical difficulties can be overcome. In slow freezing, the precise controlled rate of cooling is important for individual cell survival. Due to the complex structural and physical properties of larger volume tissue, survival and normal function of whole organs after freezing and transplantation is often difficult to achieve. There are signs however that the progress in cryobiology in the late 1990's may overcome some of these difficulties.

An early report of whole ovary cryopreservation and re-transplantation performed on rats was reported in 2002 by Wang. [92] Since then many other primate models, including humans, have followed.

### Whole ovary cryopreservation studies in animals

Work on mammalian ovaries includes a description of cryopreservation of pig ovaries with slow freezing and storage for 3 weeks. [76] The ovaries were then thawed and examined by light and electron microscopy for morphological appearance. Follicles containing intact oocytes were considered viable. The investigators found a survival rate of more than 80% for primordial follicles but conceded that "morphologic methods cannot provide final proof of viability, which clearly requires assessment of the in vivo function of cryopreserved whole ovaries."

A summary of the most important animal work is summarised in Table 20. It is clear that techniques are not standardised and the results are variable.

| Author | Year | Model | Cryo - method | Transplant | Important outcome measures |
|---|---|---|---|---|---|
| **Bedaiwy [93]** | 2003 | 11 sheep | Slow cooling | Vascular anastomosis | • Short term vascular patency 100%<br>• Long term vascular patency 27%<br>• Postoperative FSH similar than pre-operative *if vessels patent* |
| **Revel [94]** | 2004 | 8 sheep | Slow cooling | Vascular anastomosis | • Menstrual cycles after transplant 37.5%<br>• Successful establishment of bloodflow in ovary 62.5% |
| **Imhof [76]** | 2004 | 10 pigs | Slow freezing & vitrification | Not transplanted | • Morphology – 84.4% viable (slow freezing)<br>• Morphology – 21.1% viable (cryopreservation) |
| **Arav [95]** | 2005 | 8 sheep | Slow cooling | Vascular anastomosis | • 3 of 8 sheep had hormone activity 24 to 36 months post-transplantation<br>• In 2 of 8 sheep oocyte retrieval was successful |
| **Imhof [96]** | 2006 | 9 sheep | Slow cooling | Vascular anastomosis | • 4 of 9 sheep had hormonal function<br>• One of 9 sheep conceived after spontaneous intercourse and delivered a healthy lamb 545 days after transplantation<br>• Follicular survival rate was 1.7%–7.6% |
| **Baudot [97]** | 2007 | sheep | Vitrification | Not transplanted | • 61% small follicles viable on dye test after thaw<br>• 48% primordial follicles normal after thaw |
| **Grazul-Bilska [98]** | 2008 | 4 sheep | Slow cooling | Vascular anastomosis | • 2 of 8 ovaries with showed developing follicles<br>• Morphology similar to control group |
| **Qi [99]** | 2008 | 10 rats | Slow cooling | Vascular anastomosis | • 100% survival on histological evaluation<br>• 80% hormone production recovery |
| **Courbiere [100]** | 2008<br>2009 | 5 sheep | Vitrification | Vascular anastomosis | • 1 of 5 sheep recovered endocrine function after 6 months<br>• 100% follicle loss in all sheep on *histological evaluation* |
| **Arav [101]** | 2010 | 3 sheep | Slow cooling | | • 2 of 3 sheep had follicles 6 years after transplantation<br>• 3 of 3 sheep had intact vascular supply |
| **Onions [102]** | 2009 | 8 sheep | Slow freezing | Vascular anastomosis | • 7 of 8 sheep had vascular patency after implantation<br>• 3 of 8 sheep resumed hormone production |

*Table 20 Summary of animal data on whole ovary cryopreservation.*

In a review by Bromer, a strong argument is made about ischemia that may occur at the time of re-implantation of previously cryopreserved ovarian cortical strips. [103] The authors argue that small tissue fragments need to establish neovascularisation after grafting and that this process may require at least seven days during which time many cell populations undergo hypoxic damage with a significant loss in primordial follicle numbers. In work previously published by the group of Gosden, [104] the loss of primordial follicles after transplantation of previously cryopreserved sheep ovarian cortical tissue was calculated to be due to ischemia in 60-70% of follicles after the transplantation but only 7% due to the cryopreservation procedure. Bromer and Patrizio support the argument of follicular loss after transplantation with the fact that the resumption of endocrine function only appears after a few months and that the life span of the transplanted tissue in terms of hormone production is limited to two to three years. [103] Cryopreservation of the whole ovary on a vascular pedicle may limit some of the ischemic time after transplantation and limit the primordial follicle loss. Perfusion of the whole ovary will be almost immediate after transplantation. [105] [106]

Studies on human ovary freezing and transplantation are promising. In all the published work, slow cooling was used. [105] [106] [107] [108] DMSO was used in most of the freezing protocols and follicle viability was assessed by evaluation of apoptosis and microscopy. In an experimental study published by Martinez Madrid in 2004, whole human ovaries were perfused with Heparin and cryoprotectants before slow cooling to -80° C. [105] After thawing, 75% of follicles were considered to be viable on microscopy. There are no reports yet of transplantation of previously

cryopreserved whole intact ovaries in a human model. Live follicle rates after thawing ranged from 75.1% [105] to 78%. [107]

Bromer and Patrizio concluded with the statement that “cryopreservation of the whole ovary could potentially provide an alternative to patients requiring this method of fertility preservation”. The fact that numerous animal studies have had positive results makes it likely that this option for fertility preservation will be investigated as another option in humans.

**Whole ovary transplantation with vascular anastomosis**

There are very few reports in the literature about successful whole ovary transplantation. *Fresh* whole ovary transplantation was described from the United States in a patient with premature ovarian failure. [109] The donor was a monozygotic twin sister. During the removal of the donor ovary the ovarian veins could easily be identified but the ovarian artery was not visible with the naked eye. With an operating microscope vascular anastomosis was created between the recipient vessels using 10.0 Nylon sutures. A figure from Silver is shown (Figure 8).

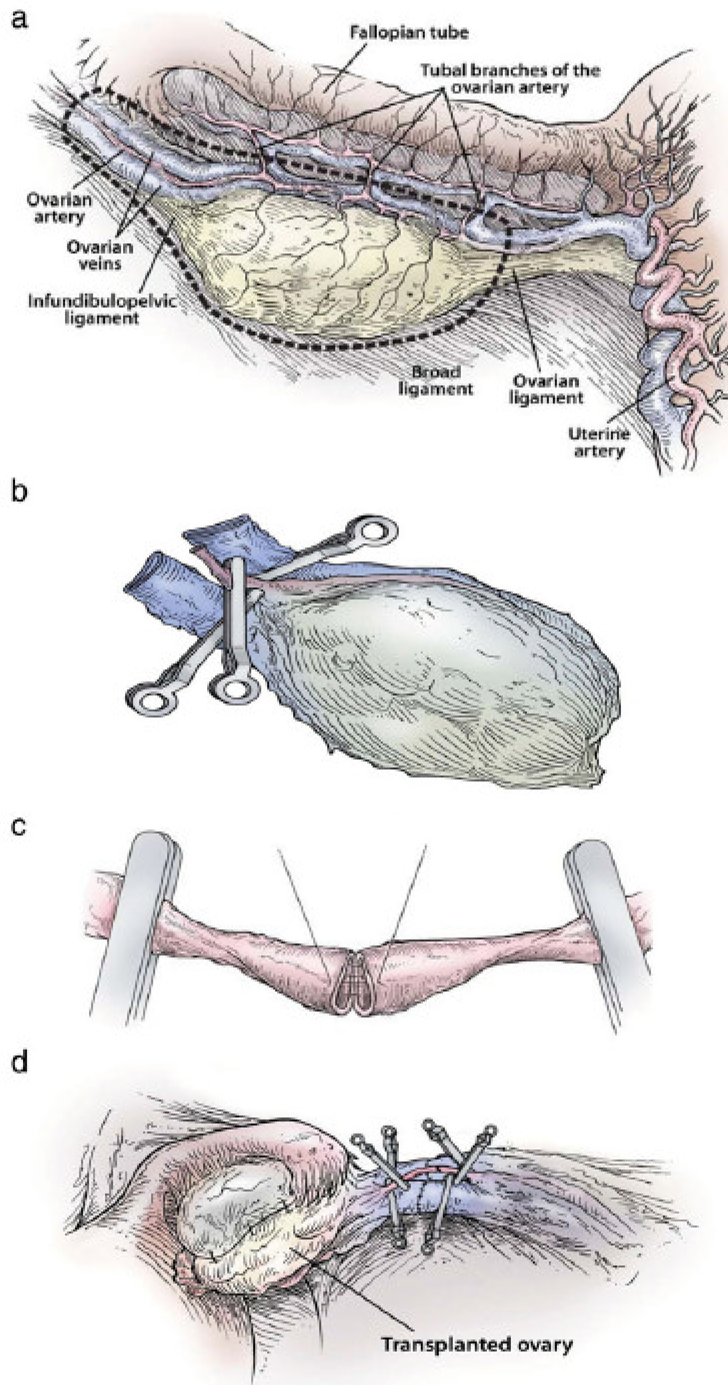


**Figure 3:** Steps in the procedure of intact ovary microvascular transplantation: (**a**) depiction of donor oophorectomy, (**b**) microsurgical isolation of donor ovary blood supply, (**c**) end-to-end anastomosis of ovarian blood vessel, (**d**) completed anastomosis of ovarian artery and veins

*Figure 8 Diagrams from [109] demonstrating transplantation of whole human ovary.*

After transplantation the patient resumed normal menstrual cycles but it is not stated in the report how long after transplantation the endocrine function returned. The authors stated that "we suspect follicle ischemia was minimal". Unfortunately this description was not detailed enough about preoperative assessment of premature ovarian failure and the condition of the contra-lateral ovary. The patient previously had two normal pregnancies and in a table from the same report it is apparent that she was on oral contraceptives. Whether the menstrual cycles were indeed due to the transplanted ovary is difficult to confirm or evaluate from this. Mhatre & Mhatre claim to have performed a surgical fresh whole human ovary transplantation on a vascular pedicle in an orthotopic position on 29 March 2002. [110] An end-to-end anastomosis of the ovarian artery to the recipient inferior epigastric artery is described with venous drainage directly into the external iliac vein. It is difficult to understand exactly where the donor ovary was positioned because the authors state that "having achieved the vascular anastomosis extra-peritoneally the ovary is placed orthotopically in close proximity to the uterus and Fallopian tube.

Another description of the same case clearly states that "the donor ovary was placed orthotopically and supported with multiple 3/0 Vicryl sutures in the round ligament and the peritoneum". [111] The patient then had immune suppression with a combination of cyclosporine and prednisolone which were weaned over time. It is interesting to note that the recipient was discharged only on day 15 postoperatively. The patient had almost immediate recovery of hormonal function and still had spontaneous menstruation, ovulation and development of secondary sexual characteristics. The patient was a 17-year old with Turner's syndrome and the donor was a "biologically related sister" aged 26 with two living children.

The obvious advantage of rapid re-perfusion after transplantation implies that the time between ligation of the ovarian pedicle and the cryopreservation procedure should be as short as possible. This concept is supported by interesting basic scientific work by Coskun and co-workers where they aimed to determine the ischemic time necessary for tissue damage to the ovary. [112] This group studied 36 rats divided into four experimental groups. Each group had an explorative laparotomy and were randomised to one of four intra-operative procedures. Group 1 had no ovarian manipulation, group 2 was subjected to ischemia of the ovary by twisting the pedicle for one hour and then re-perfusion of one hour, group 3 had induced ischemia for two hours and re-perfusion for one hour and group 4 had ischemia for three hours and then re-perfusion for one hour. After the laparotomy and ischemic event (or no ischemic event in the case of group 1) the ovaries were removed and sent for histopathological evaluation. The authors concluded that the critical ischemic time for rat ovaries was two hours when early signs of haemorrhage indicated early necrosis. [112] This important basic scientific work gives some reassurance that there is at least a small window of opportunity for cryopreservation before ischemic damage occurs. This window may be as long as two hours, at least in the rat model.

## *Randomised controlled evaluation comparing ultrastructural damage of ovarian tissue after two slow freezing ovarian tissue protocols*

**Aim**

The ultrastructural effects after cryopreservation with two well-known cryoprotectants, dimethyl sulfoxide (DMSO) and 1,2-propylene glycol (PROH), on early human follicles were investigated and compared to fresh tissue.

**Ethics approval**

This study was approved by the local institutional review board of the Faculty of Health Sciences, Stellenbosch University. *Project number: N05/10/182*

## Materials and methods

Eleven women with advanced cervical cancer had oophorectomy before the initiation of cancer treatment. Ovarian tissue was obtained by laparoscopic oophorectomy. After dissection of the ovarian cortex, fresh stromal tissue was sent for metastatic analysis (by histological evaluation). Fresh cortical tissue samples were also taken as control. Remaining dissected ovarian cortical tissue sections of each patient were equally divided into the two cryoprotectant groups. Samples were taken from each cryoprotectant group, after equilibration before and after freezing. Five resulting groups could be compared:

i. Fresh tissue (control group)

ii. Tissue equilibrated in DMSO
iii. Tissue equilibrated in PROH
iv. Tissue equilibrated and cryopreserved in DMSO
v. Tissue equilibrated and cryopreserved in PROH.

Five tissue samples per patient were fixed for standard histological haematoxylin and eosin (HE) staining and transmission electron microscopy (TEM). Tissue samples showing early follicles on HE slides were evaluated by TEM.

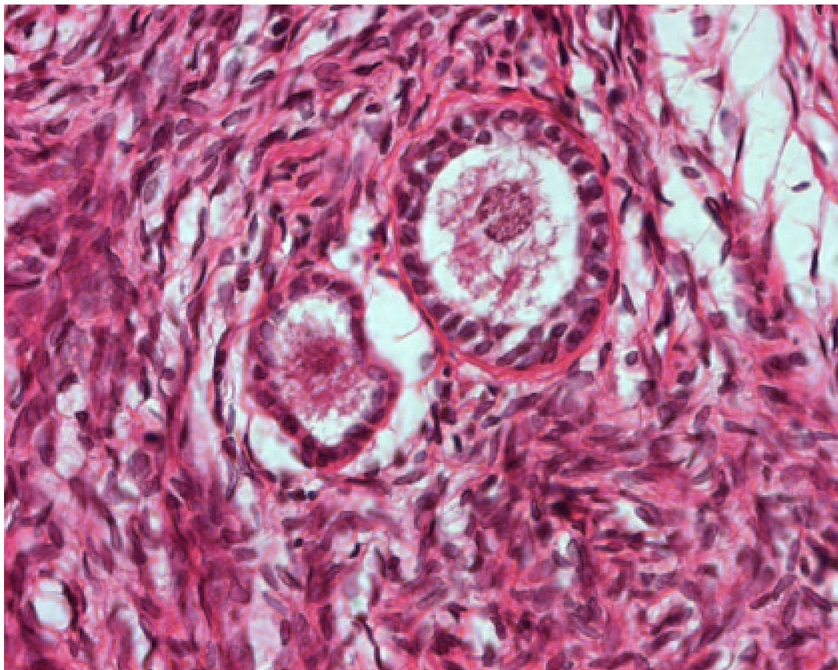

*Figure 9 Two primordial follicles under light microscopy on H&E stained slide. x20.*

Ultrastructural studies on micrographs of primordial and primary follicles were assessed according to a scoring system which gave an indication of follicular health. Appropriate statistical tests were applied to analyse the mean scores and P=0.05 was considered as significant.

**Protocol for ovarian tissue freezing in current study**

After laparoscopic oophorectomy the ovaries were transported either in a laparoscopic endobag or were transported in a standard sterile 90ml specimen container with 40ml Quinn's advantage medium with HEPES. The ovary was dissected in a laminar flow cabinet under sterile conditions by first bisecting the ovary and removing the ovarian stroma. The dissection was performed in Quinn's advantage medium with HEPES at room temperature. The cortex of the ovary was dissected into small strips with as little manipulation of the tissue as possible. After dissection the tissue was handed over to the scientist.

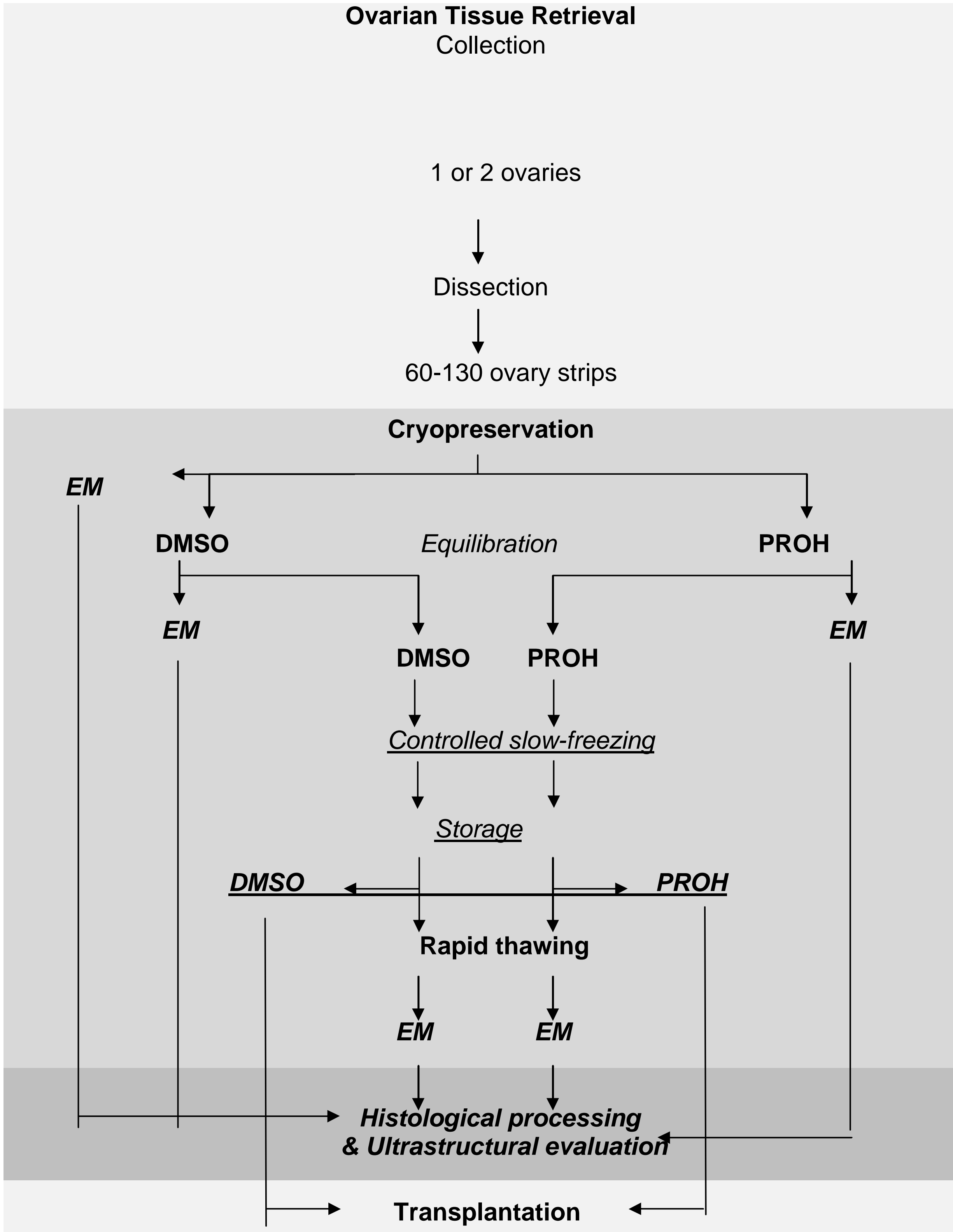


*Figure 10 Flow diagram of cryopreservation protocol. (EM indicates where tissue was taken and prepared for ultrastructural study)*

## *Protocol for DMSO and PROH*

**(Details of chemical compounds, mediums and consumables used attached as addendum B)**

The ovarian tissue strips were washed in 10ml of Quinn's advantage medium with HEPES and then counted and divided into two groups. Half of the tissue was in the DMSO group and the other half in the PROH group. The final equilibration and cryopreservation in the PROH group resulted in 0.2 micro millilitre medium containing 1.5M 1.2 propanediol plus 0.1M sucrose plus 10mg per millilitre Quinn's advantage human serum albumin in DPBS. [113] The equilibration cryopreservation of each vial in the DMSO group contained 0.2 micro-millilitres sterile filtered DMSO medium containing 1.5M, DMSO 0.1M sucrose and DPPS pre-cooled to 4 °C. The PROH group was equilibrated for 90 minutes at room temperature and the DMSO group for 30 minutes. See Figure 11.

*Figure 11 Accurate timing of equilibration time of tissue in cryoprotectants*

**Slow freezing of ovarian tissue**

Slow freezing of the tissue was achieved by using a specialised electronic cryochamber. The Cryologic freeze control system (see Figures 12 and 13) run by cryo-genesis version V software allowed customisation of the slow freezing protocol accurately. See Figure 8

*Figure 12 The CryoLogic Freeze Control® System*

An example of the screen print from the cryo-genesis software is included below. This figure shows how the drop in temperature was carefully controlled from room temperature to 4 °C at a constant cooling rate of 2°C per minute. (See Figure 13) At 4 °C the DMSO vials were added and then from 4 °C to -7 °C again cooled at a constant rate of 2 °C per minute.

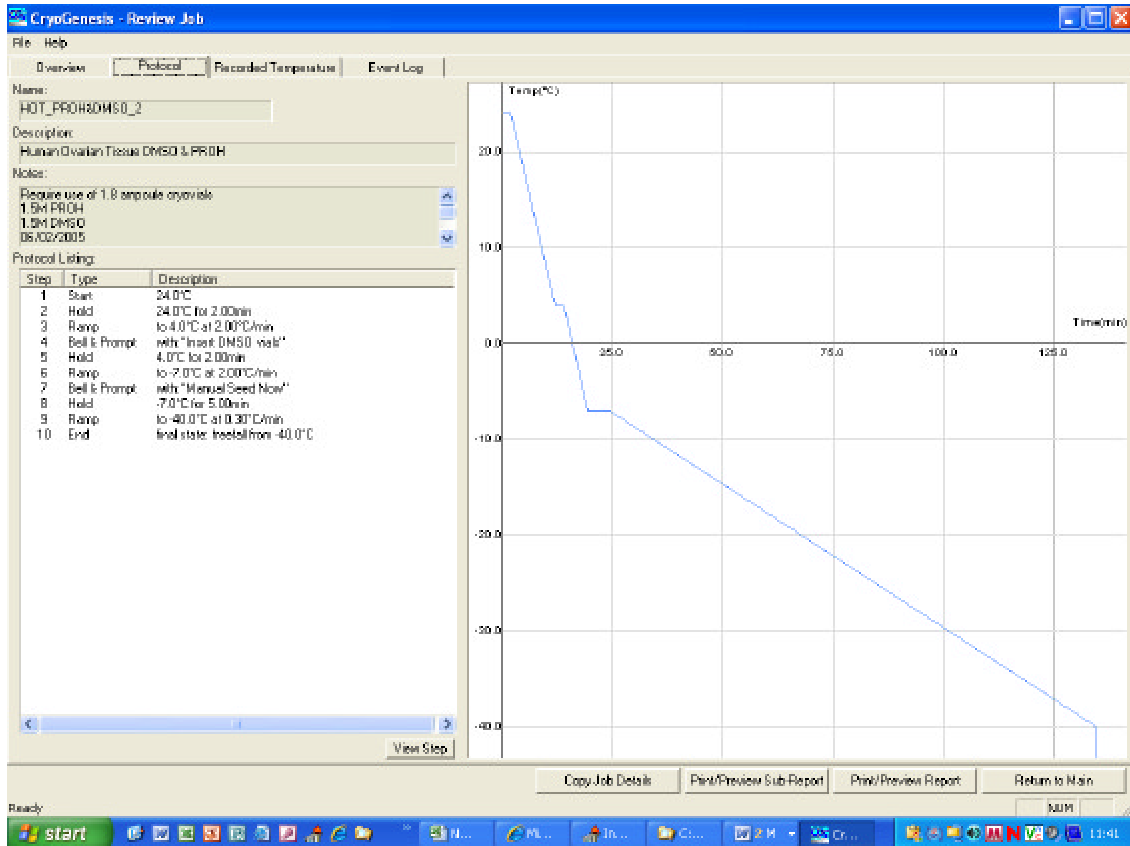


*Figure 13 A cryopreservation protocol is programmed and saved in the CryoGenesis™ software.*

At minus 7 °C freezing was started in a controlled way through a process called manual seeding where the side of the vial was touched by an ultra cooled cotton swab that was recently dipped in liquid nitrogen. It was previously demonstrated in an experimental study that "there was a significant difference in primordial and primary follicle density" between tissue samples that were allowed to crystallize spontaneously and those that were started through a process of seeding. [114] The ice crystal formation started in an organised fashion to try and prevent unnecessary ice crystal formation. From minus 7°C the temperature was reduced at a slower rate of 0.3 °C per minute to minus 40°C. The temperature was then allowed to free fall until minus 60 °C at which point the samples were plunged into the liquid nitrogen.

The tissue samples were stored in liquid nitrogen until they were required for use. See Figure 14.

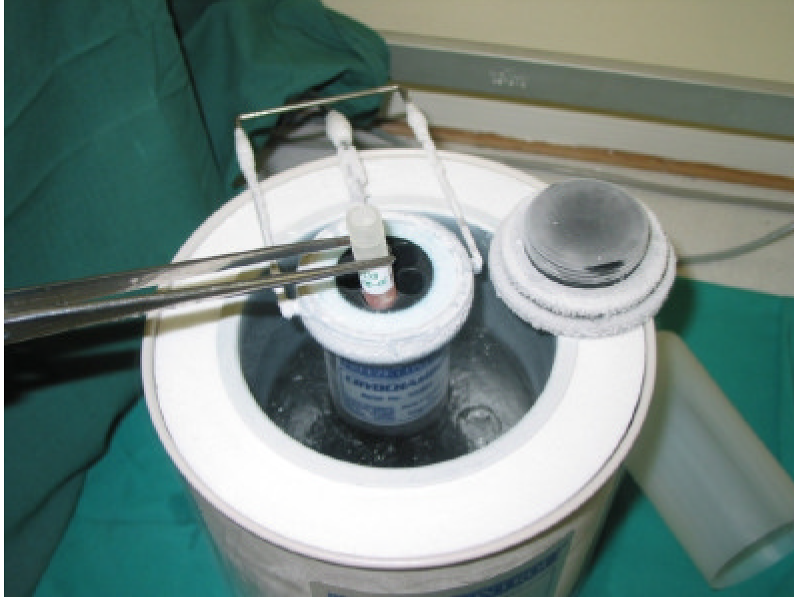

*Figure 14    Cryovials packed in cryochamber for controlled slow freezing*

**Rapid thawing**

Tissue for evaluation was thawed through a rapid process to minimise reheating injury. The vial was unclipped and kept at room temperature for a few seconds to remove excess liquid nitrogen. [113] Vials were transferred into sterile water bath at ±37°C. The vials were gently swirled while completely immersed for ±5 minutes until the medium and the tissue sample were completely thawed. See Figure 15.

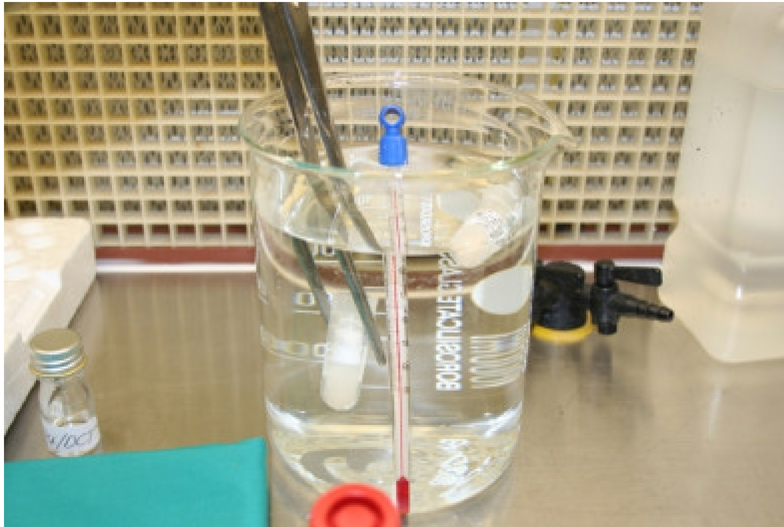

*Figure 15 Cryovial thawed in water bath*

Tissue sample were thereafter decanted into a small sterile culture dish and strips were transferred to wash-out media with sterile forceps. The samples were washed step-wise in lower concentrations of cryoprotectant media at room temperature to remove all cryoprotectant.

The PROH group was washed for 5 minutes in 10ml sterile-filtered medium containing 0.75M PROH + 0.2M + 10mg/ml Quinn's Advantage™ HSA in D-PBS and then in 10ml 0.2M sucrose + 10mg/ml Quinn's Advantage™ HSA in D-PBS for 10 minutes

The DMSO group was washed for 5 minutes in 10ml sterile-filtered medium containing 0.75M DMSO + 0.25M sucrose in D-PBS and then in 10ml 0.25M sucrose in D-PBS for 10 minutes

*Figure 16 Storage of tissue in liquid nitrogen.*

The ultra structural evaluations were performed after micrographs were printed and delivered to Prof M Sousa in Spain. The follicles were examined for various characteristics and evaluation was done using a novel scoring system.

### *Ultrastructural evaluation*

Ultrastructural evaluation was done on original printed micrographs. Micrographs were individually evaluated and the evaluator was blinded for the group. The following ultrastructures of each primordial or primary follicle were evaluated on the micrographs:

- **Stroma**:
  - Stromal cells: membrane, nucleus, cytosol, organelles.
  - Extra-cellular matrix.
  - Basal lamina.
- **Granulosa cell layer**:
  - Nucleus: nuclear envelope, nuclear pore complexes, nuclear lamina, heterochromatin, euchromatin, nucleolus.
  - Cytoplasm: membrane, interdigitations /microvilli, pinocytosis, receptor mediated endocytosis, exocytosis, desmosomes, gap junctions, mitochondria, peroxisomes, primary lysosomes, refractile bodies, Golgi complexes, secretory vesicles, smooth endoplasmic reticulum, rough endoplasmic reticulum, lipid droplets, centrosome, microfilaments, intermediary filaments, microtubules, cytosol.
- **Oocyte**:
  - Nucleus: nuclear envelope, nuclear pore complexes, nuclear lamina, heterochromatin, euchromatin, nucleolus.
  - Cytoplasm: membrane, interdigitations / microvilli, pinocytosis, receptor mediated endocytosis, exocytosis, desmosomes, gap junctions,

mitochondria, peroxisomes, primary lysosomes, refractile bodies, Golgi complexes, cortical vesicles, smooth endoplasmic reticulum, rough endoplasmic reticulum, annulate lamellae, nuage, lipid droplets, centrosome, microfilaments, intermediary filaments, microtubules, cytosol.

- Perivitelline space.
- Zona Pellucida.

Evaluation was done using the following scoring system:

1 = Poor, degenerative;

2 = Fair, severe damage;

3 = Average, recoverable damage;

4 = Good, little damage;

5 = Excellent, normal, no damage.

No scores were awarded in cases where a structure or organelle was absent or could not be seen clearly enough for evaluation on the micrograph. Examples of electron micrographs are attached as Addendums A 1-4.

After cryopreservation many of the examined cells showed ultra structural damage including lytic damage to cell membranes, organelles and general degeneration. Due to relatively low number of specimens and low number of evaluated follicle structures that could be compared, no statistically significant changes were seen between the

different groups. This may suggest that both cryopreservation protocols were equally effective. However, in a post-hoc analysis of the results where p-values were below the 10% threshold, a possible superior result for DMSO is described. [115] The results are summarised in Table 21.

| Evaluated Follicle Structure | *P* | Result |
|---|---|---|
| **Ovarian Stroma** | | |
| **Stroma (overall)** | 0.85 | NS |
| **Stromal cells** | 0.57 | NS |
| **Extracellular matrix** | 0.15 | Trend |
| **Basal lamina** | 0.74 | NS |
| **Granulosa cells** | | |
| **Granulosa cells (overall)** | 0.34 | Trend |
| **Cytoplasm** | 0.35 | Trend |
| **Mitochondria** | 0.60 | NS |
| **Smooth and rough endoplasmic reticulum** | 0.13 | Trend |
| **Nuclei of granulosa cells** | 0.09 | Trend |
| **Oocytes** | | |
| **Oocyte (overall)** | 0.62 | NS |
| **Perivitelline space** | 0.32 | Trend |
| **Cytoplasm of oocytes** | 0.54 | NS |
| **Mitochondria of oocytes** | 0.91 | NS |
| **Smooth and Rough endoplasmic reticulum of oocytes** | 0.69 | NS |
| **Nuclei of oocytes** | 0.14 | Trend |

*Table 21 Summary of the treatment effect (equilibration and cryopreservation) test results on follicle structures compared to the control sample.*

| Compared Follicle Structures | | *r* (correlation coefficient) | *P* | Relationship |
|---|---|---|---|---|
| **Stroma** | **Granulosa cells** | 0.60 | <0.01 | Positive linear |
| **Stroma** | **Oocyte** | 0.68 | <0.01 | Positive linear |
| **Granulosa cells** | **Oocyte** | 0.77 | <0.01 | Positive linear |
| **Nuclei of granulosa cells** | **Nuclei of oocytes** | 0.86 | <0.01 | Positive linear |
| **Cytoplasm of granulosa cells** | **Cytoplasm of oocytes** | 0.68 | <0.01 | Positive linear |
| **Mitochondria of granulosa cells** | **Mitochondria of oocytes** | 0.62 | <0.01 | Positive linear |
| **ER of granulosa cells** | **ER of oocytes** | 0.65 | <0.01 | Positive linear |
| **Stroma** | **Extracellular matrix** | -0.18 | 0.44 | NS |

*Table 22 Summary of the Spearman rank correlation test results on follicle structures.*

The follicle scoring data was examined to determine whether certain cell types were affected to a greater or lesser extent by the cryopreservation procedure. Spearman's rank correlation coefficient is a statistical description of correlation between two non-parametric variables. When the variables are not arranged in a direct linear distribution but rather in a monotonic relationship, the Spearman correlation should be used instead of the Pearson correlation coefficient. If the two variables (the dependant and independent) behave similarly e.g. structure Y will change in the same way as structure X under the same circumstances, the correlation will be positive. The strong correlation in the scores when different cellular structures were compared indicates that different tissue elements responded in similar ways to the environment.

## *Conclusion*

Oocyte numbers are limited and decrease rapidly with age. Women near the age of menopause have depleted ovarian follicle reserve and they are at the highest risk for developing premature ovarian failure due to cancer treatment. Oocytes are complex cells with complex intra-cellular substructures that may be damaged by freezing. It is however not only *intra*-cellular damage but also damage to *inter*-cellular and *extra*-cellular structures that may influence the eventual survival of germ cells after cryopreservation. The intimate association between oocytes and granulosa cells can easily be damaged by freezing. These include autocrine and paracrine hormonal disruptions or damage to very specialised connecting proteins and gap junctions between cells.

Oocyte preservation with slow freezing is a useful technique in certain selected clinical scenarios. Where there is a risk of metastatic spread of a malignancy to the ovary it may be possible to retrieve single oocytes for future use. This will allow microscopy evaluation before the re-implantation of the preserved tissue. However, many problems exist with oocyte freezing particularly in the scenario of a patient with cancer diagnosis. There is an inevitable delay before follicle aspiration because of ovarian hyper-stimulation that takes a few days and perhaps even a few weeks. This will delay the onset of chemotherapy. Additionally only a limited number of oocytes can be retrieved in a single stimulation cycle.

Vitrification of single oocytes has become more successful recently and when fertilization is performed with ICSI, pregnancy outcomes are reasonable. There is,

however, still a lower pregnancy rate with vitrified oocytes when compared to fresh oocytes.

The randomised investigation into DMSO and PROH cryopreserved ovarian tissue showed no significance difference in electron microscopic evaluation of the tissue. There was also no significant difference between both treatment groups together and fresh controls. That would indicate that cryopreservation using either of the slow freezing protocols did not cause significant damage. A possible superior result with DMSO was found in post-hoc analysis but due to the low number of cases it is difficult to be certain of a true difference between the two groups. One possible advantage of DMSO is the fact that the equilibration time was shorter. A total cryopreservation time of around seven hours was one hour shorter with DMSO compared to PROH.

Slow freezing of ovarian cortical strips is technically feasible in a South African fertility laboratory. After the initial cost of the acquisition of the new equipment, the cost per individual patient was reasonable. The most important cost factor in this study was the electron microscopy evaluations and the start-up equipment expenses.

Ovarian cortical tissue cryopreservation is a practical and useful technique for patients wanting to preserve ovarian function after gonadotoxic treatment.

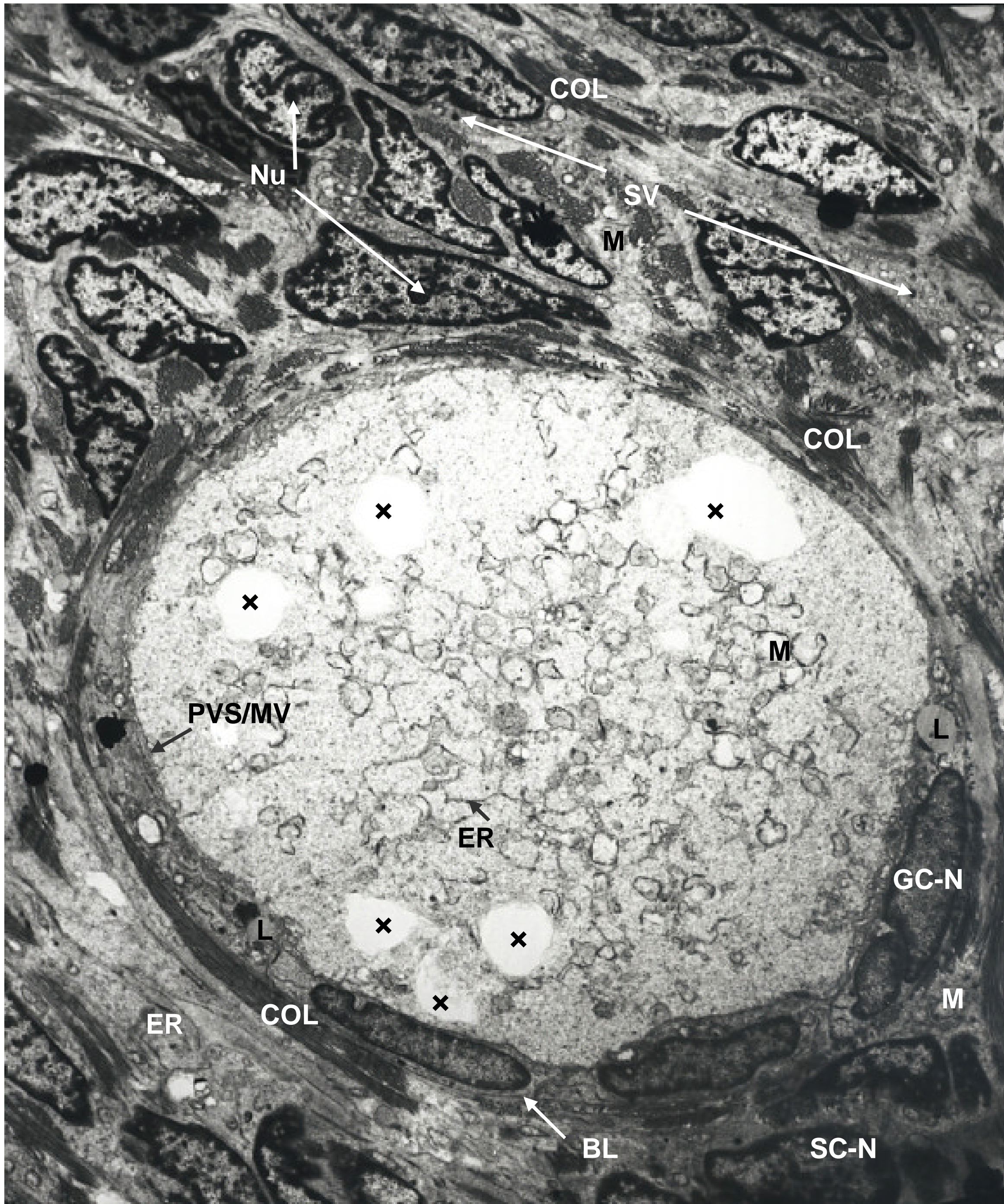


*Figure 17 Micrograph of primordial follicle with large areas of lysis (×) in the oocyte cytoplasm. Ultrastructures present for evaluation were stromal nuclei (SC-N) with nucleoli (Nu), secretory vesicles (SV), mitochondria (M) and endoplasmic reticulum (ER). The granulosa layer contained granulosa cell nuclei (GC-N), lipid droplets (L) a thin basal lamina (BL). Minimal microvilli (MV) and perivitelline space (PVS) were visible.*

## *Addendum A 1*

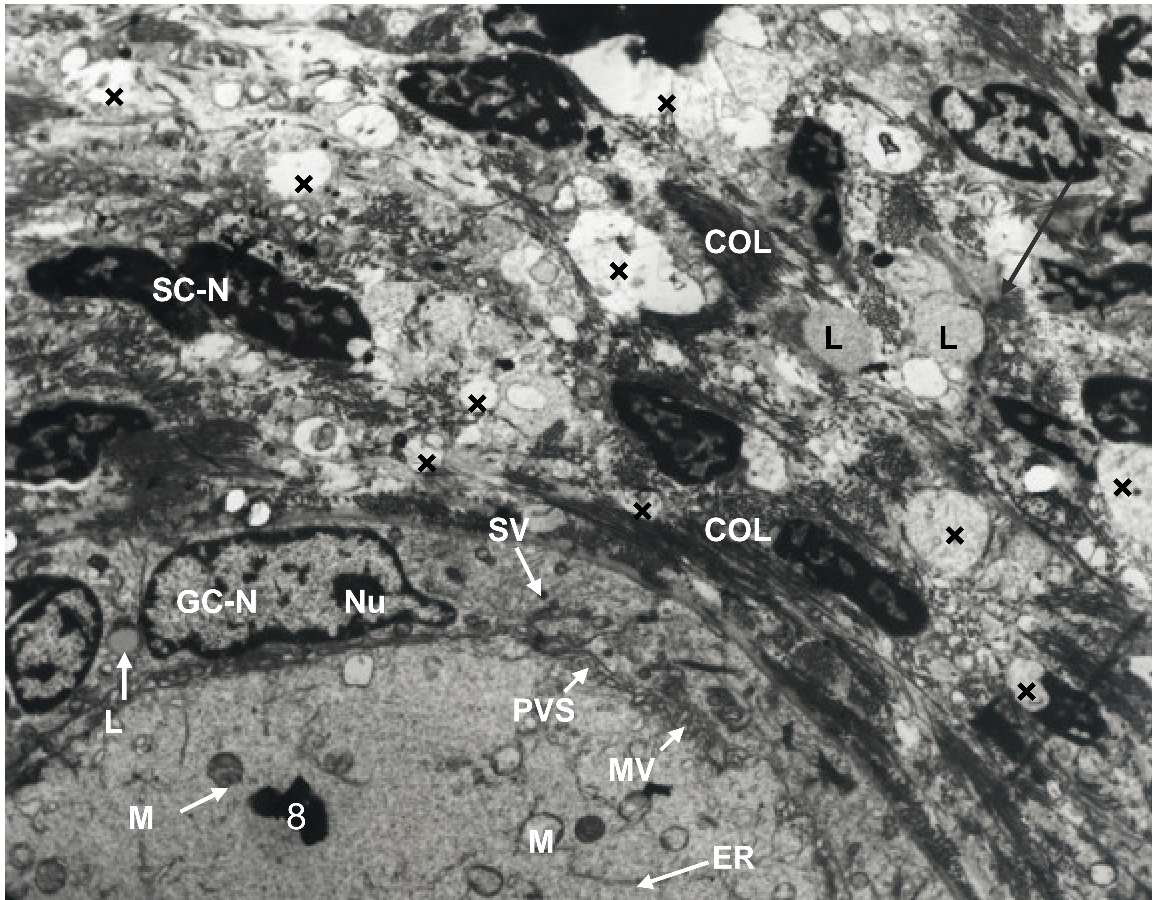


*Figure 18 A primordial follicle with lytic areas (**x**) in the stroma. Stromal cell nuclei (SC-N), collagen bundles (COL) and lipid droplets (L) were present in the stroma. Lipid droplets (L), secretory vesicles (SV), perivitelline space (PVS), microvilli (MV), mitochondria (M) and endoplasmic reticulum (ER) were observed in the follicle. TEM artefacts are indicated (8 ).x2000-6000.*

## *Addendum A 2*

## *Addendum A 3*

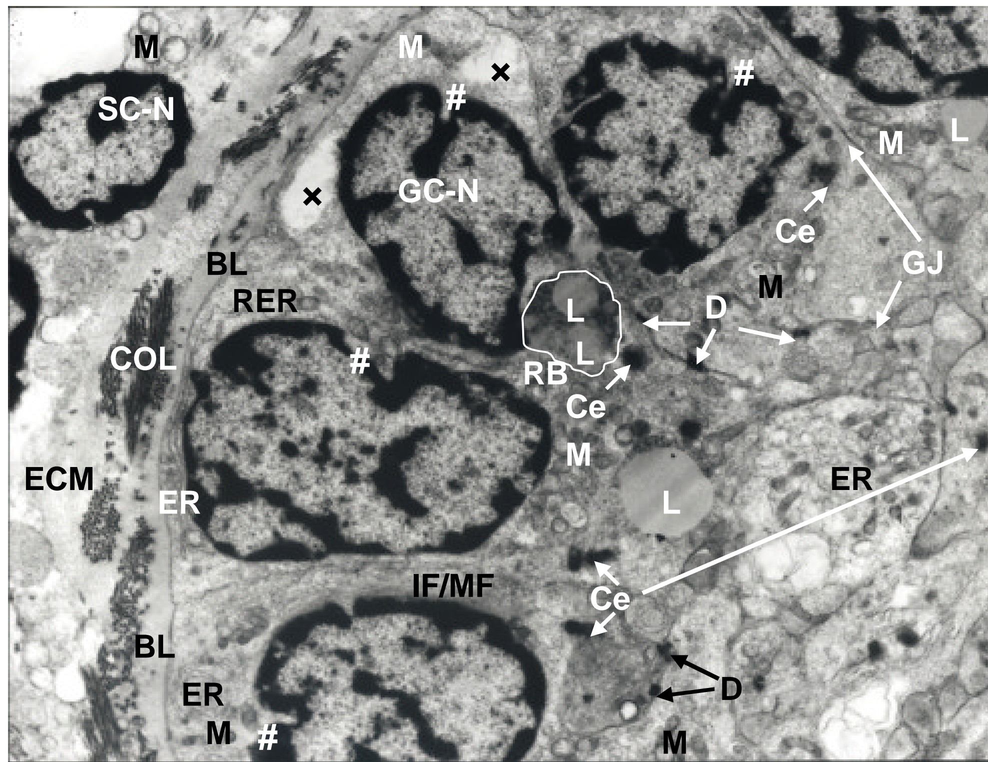


*Figure 19 Higher magnification of granulosa layer of follicle. Desmosomes (D) and centrosomes (Ce) are abundant. Gap junctions (GJ) are demonstrated. Several lipid droplets (L), some in a refractile body (RB) are demonstrated. Areas of lysis (**x**) and granulosa nuclei indentations (#) are indicated. The basal lamina (BL), mitochondria (M), endoplasmic reticulum (ER & RER), intermediary- and microfilaments (IF/MF) are demonstrated. Stromal nuclei (SC-N), collagen bundles (COL) and extracellular matrix (ECM) are demonstrated.x12000.*

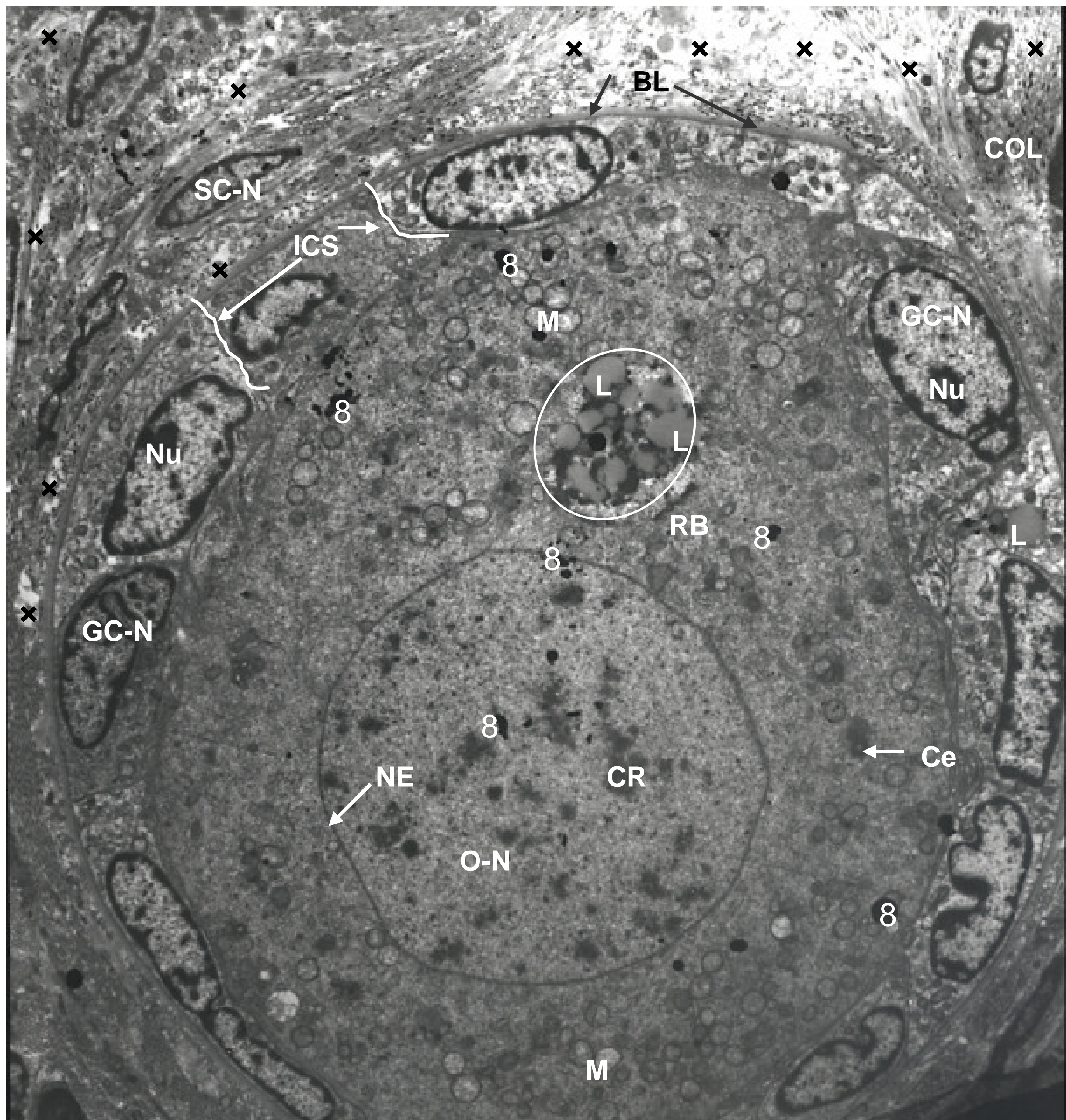


*Figure 20 Micrograph of primordial follicle with small areas of lysis (×) in the stroma. TEM artefacts are indicated (8). The stromal nuclei (SC-N), collagen bundles (COL) and the basal lamina (BL) are visible. The granulosa layer contained granulosa cell nuclei (GC-N) and lipid droplets (L). The intracellular separations (ICS) by the granulosa cell membranes are clearly seen. Abundant mitochondria (M) and endoplasmic reticulum (ER) are present in the oocyte as well as a centrosome (Ce) and a giant refractile body containing several lipid droplets (L).x1500-4500.*

## *Addendum A 4*

## *Addendum B*

# Details of chemical compounds, mediums and consumables

### Chemical compounds

Cryoprotectants:

**Dimethyl Sulphoxide** (DMSO) Hybri-Max™ D2650. Sterile filtered. Sigma® Sigma-Aldrich Company Ltd., Irvine, Ayrshire KA128NB, UK.

Molecular formula: $C_2H_6OS$

Molar mass: 78.13g/mol

IUPAC Name: Dimethyl sulfoxide

Other names: Methyl sulfoxide; Methyl sulfinylmethane; DMSO

Melting point: 16-19°C (292K)

Boiling point: 189°C (462K)

Density: 1.1004g/cm$^3$

**1,2-Propanediol** *Art.* 7478 *Ph Eur, B P, USP*. Merck, Darmstadt, Germany

Molecular formulae: $C_3H_8O_2$

IUPAC name: Propane-1,2-diol

Other names: Propylene glycol; *1,2-Propylenglycol*; 1,2-Propanediol (*1,2-propylene glycol*); Propanediol-1,2 (*propylèneglycol-1,2*); Propandiol-(1,2) (*1,2-propilenglicole; 1,2-propilenoglicol*); 1,2-Propanodiol (*1,2-propilenglicol*)

Molar mass: 76.10g/mol

Melting point: -59°C

Boiling point: 188.2°C

Density: 1.036g/cm$^3$

**Sucrose** >99.5% (GC). S1888. Cell culture tested.

Manufacturer: Sigma® Sigma-Aldrich Company Ltd., St. Louis, MO 63103, USA.

### MEDIUMS

Base solution:

**Dulbecco's Phosphate-Buffered Saline** with Calcium and Magnesium without Phenol Red (D-PBS + $CaCl_2$ + $MgCl_2$) liquid. Sterile.1178.

Manufacturer: Gibco™Invitrogen™Auckland, N.Z.

Melting point: No data available.

Boiling point: No data available.

Mediums:

**Quinn's Advantage™ Media with HEPES**. Sterile.

Manufacturer: SAGE Assisted Reproduction Products™ A Cooper Surgical Company. In-Vitro Fertilization, Inc., Trumbull, CT 06611, U.S.A.

Quinn's Advantage™ HSA (Human Serum Albumin). Sterile. Manufacturer: SAGE Assisted Reproduction Products™ A Cooper Surgical Company. In-Vitro Fertilization, Inc., Trumbull, CT 06611, U.S.A.

Solution: 100mg/ml total protein (weight/volume) in saline solution.

pH 7.4±0.2

Osmolality: 280±10mOsm/kg water

**Consumables**

Cryovials:

**Cryo.s**™PP, 2ml conical round-based internal thread with screw cap

Manufacturer: Greiner Bio-One GmbH, 72636 Frickenhausen, Germany

Petri dishes:

Falcon®3003, 100x20mm, Becton Dickinson Labware, NJ, USA.

Manufacturer: Cellstar®, 35x10mm Greiner Bio-One GmbH, 72636 Fickenhausen, Germany.

Sterilizing micro filters:

Manufacturers: Ministart®, 0.20µm, Sartorius, Vivascience, Hannover, Germany

# *Addendum C*

## PATIENT FORMS

### Patient Information and Consent forms

**Hierdie vorm is ook in Afrikaans beskikbaar**

### Information Sheet for Ovarian Biopsy and Cryopreservation

We are providing you with the following information in order for you to be able to make an informed decision about whether you wish to freeze your ovarian tissue. We want to be sure you understand this fully before you formally agree to participate. Be sure to ask any questions you have about the information that follows and we will do our best to explain and to provide any further information you require.

In order to treat the illness from which you are suffering, you will require surgery, chemotherapy and/or radiotherapy which are likely to cause permanent damage to your ovaries such that they will no longer be able to produce eggs. By removing some ovarian tissue before you start this treatment and carefully freezing it, it will be possible to protect some of your eggs from the harmful effects of the chemotherapy or radiotherapy you are shortly to undergo.

Ovarian tissue is collected at a minor operation called a laparoscopy. This is performed under general anaesthesia, when a telescope, about the thickness of a pencil, is placed through your navel and a small piece of ovarian tissue is removed through two smaller incisions, lower down on your abdomen.

There are potential risks involved with this procedure, of which you should be aware:
There is a very small risk of a reaction to the anaesthetic agents; a small chance of infection; damage to the other pelvic organs; and if you have abnormal blood clotting mechanisms, a chance of bleeding after the procedure.

Some women undergoing abdominal surgery for their condition may be able to have ovarian tissue removed at the time of surgery and so avoid the need for a laparoscopy. A piece or pieces of ovarian tissue will be removed. In some cases it may be necessary to remove a whole ovary, in order to maximize your chances of saving a useful number of eggs.

You will not be asked to take any drugs, make any extra visits to the hospital or to have any additional blood tests.

The use to which we can put your ovarian tissue is complicated by the fact that only very immature eggs in the ovary will survive the freezing process. The frozen tissue may, at a later stage, be re-implanted into your body and possibly restore ovarian function. The benefits of normal ovarian function may be hormonal. Hormones secreted by the ovaries protect the body against ageing and in particular the strength of your bones.

Some people have expressed concern that the re-implantation may risk reintroducing some untreated cancer cells back into your body. There may be other risks associated with ovarian re-implantation that we do not know about at present. Research is presently on-going to look at both these risks and ways of reducing the potential harm.

At this stage you are consenting only to collect ovarian tissue for storage. If and when you decide you wish to use the tissue, the details of any procedure will be explained to you and you will be asked to sign a separate consent form. Your participation is entirely voluntary and, whatever your decision, it will not affect your treatment in any way. We understand that this is a difficult time for you and that you may wish to discuss the implications of the storage of your ovarian tissue.

In the event of your death, the stored ovarian tissue will either be destroyed or be used for research purposes. You will be asked to sign a separate consent form instructing us what to do.

If you require any further information regarding the proposed research or have any concerns regarding any potential side-effects, please feel free to discuss them with your doctor.

## Information Sheet for Ovarian Tissue Re-implantation

We are providing you with the following information in order for you to be able to make an informed decision about whether you wish re-implant your stored ovarian tissue. We want to be sure you understand this fully before you formally agree to participate. Be sure to ask any questions you have about the information that follows and we will do our best to explain and to provide any further information you require.

In order to treat the illness from which you are suffering, you required surgery, chemotherapy and/or radiotherapy which could cause permanent damage to your ovaries. Part of your ovarian tissue was stored and can now be re-implanted.

The re-implantation is done under local anaesthesia except when you may require general anaesthesia for another reason. A small cut of less than 1 centimetre is made in your forearm and the piece of tissue is then inserted and sutured with absorbable sutures. The cut is made on the inner aspect of the arm. Sometimes it may be necessary for the tissue to be implanted in the abdominal cavity. This will happen during a laparoscopy. Your doctor will inform you about the most suitable place for your implantation.

Some people have expressed concern that the re-implantation may risk reintroducing some untreated cancer cells back into your body. There may be other risks associated with ovarian re-implantation that we do not know about at present. Research is presently on-going to look at both these risks and ways of reducing the potential harm. There is a very small risk that cancer cells may be re-implanted with the ovarian tissue. The tissue is carefully examined before re-implantation to prevent this problem.

You will not be asked to take any medication after re-implantation.

The benefits of re-implantation may be hormonal. Hormones secreted by the ovaries protect the body against ageing and in particular the strength of your bones.

At this stage you are consenting to re-implantation of ovarian tissue. Your participation is entirely voluntary and, whatever your decision, it will not affect your treatment in any way.

If you require any further information regarding the proposed research or have any concerns regarding any potential side-effects, please feel free to discuss them with your doctor

Hierdie vorm is ook in Afrikaans beskikbaar

## PARTICIPANT INFORMATION LEAFLET AND CONSENT FORM

## TITLE OF THE RESEARCH PROJECT:

**A STUDY TO EVALUATE OVARIAN CRYOPRESERVATION TO REDUCE LONG TERM HORMONAL DYSFUNCTION ASSOCIATED WITH CANCER TREATMENT IN WOMEN**

**REFERENCE NUMBER: N05/10/182**

**PRINICIPAL INVESTIGATOR:** Dr MH Botha

**ADDRESS:** Dept. Obstetrics & Gynaecology, Tygerberg Hospital, TYGERBERG

**CONTACT NUMBERS:**

Tel: 021 9385696
Cell: 0844024023
Fax: 021 9384648
Email: mhbotha@sun.ac.za

You are being invited to take part in a research project. Please take some time to read the information presented here, which will explain the details of this project. Please ask the study staff or doctor any questions about any part of this project that you do not fully understand. It is very important that you are fully satisfied that you clearly understand what this research entails and how you could be involved. Also, your participation is **entirely voluntary** and you are free to decline to participate. If you say no, this will not affect you negatively in any way whatsoever. You are also free to withdraw from the study at any point, even if you did agree to take part.

This study has been approved by the Committee for Human Research at Stellenbosch University and will be conducted according to the ethical guidelines and principles of the international Declaration of Helsinki, South African Guidelines for Good Clinical Practice and the Medical Research Council (MRC) Ethical Guidelines for Research.

**What is this research study all about?**

- Please read the information sheet "Information Sheet for Ovarian Biopsy and Cryopreservation" that will be provided to you, carefully.

**Why have you been invited to participate?**

- Your participation in this project will result in the broadening of the knowledge of preservation of ovarian function after cancer treatment.

**Will you benefit from taking part in this research?**

- By removing some ovarian tissue before you start this treatment and carefully freezing it, it may be possible to protect some of your eggs from the harmful effects of the chemotherapy or radiotherapy you are shortly to undergo. The frozen tissue may, at a later stage, after thawing be re-implanted into your body and may possibly restore ovarian function. The benefits of normal ovarian function may be hormonal. Hormones secreted by the ovaries protect the body against ageing and in particular the strength of your bones. This procedure may sometimes restore fertility.

**Are there in risks involved in your taking part in this research?**

- The possible risks are clearly described in the information sheet - Please read the information sheet that will be provided to you, carefully.

**If you do not agree to take part, what alternatives do you have?**

- Participation is voluntary, and you may refuse to participate in the project and may at any time withdraw your participation from the project. Refusal or withdrawal from the project will in no way affect your present or future treatment at the clinic. The researcher may also withdraw you from the project if he/she considers it to be in your best interest.

**Who will have access to your medical records?**

- All information collected will be treated confidentially. The results will be used for publication in human fertility related journals, without revealing the identity of any individual. On completion of the project, the final outcomes of the project will be available to you.

**What will happen in the unlikely event of some form of injury occurring as a direct result of your taking part in this research study?**

- At this stage you are consenting only to collect ovarian tissue for storage. If and when you decide you wish to use the tissue, the details of any procedure will be explained to you and you will be asked to sign a separate consent form. The freezing and storage of ovarian tissue will not cause any injury to you.

**Will you be paid to take part in this study and are there any costs involved?**

- No, you will not be paid to take part in the study and there will be no costs involved for you, if you do take part.

**Is there any thing else that you should know or do?**

- You should inform your family practitioner or usual doctor that you are taking part in a research study.
- You can contact **Dr MH Botha at Tel 021 9385696** if you have any further queries or encounter any problems.
- You can contact the **Committee for Human Research at 021-938 9207** if you have any concerns or complaints that have not been adequately addressed by your study doctor.
- You will receive a copy of this information and consent form for your own records.
- You will sign a consent form to instruct us that in the event of your death, frozen ovarian tissue will either be destroyed or used for research purposes.

***By Signing below, I………………………….. agree to take part in a research study entitled:-***

***A STUDY TO EVALUATE OVARIAN CRYOPRESERVATION TO REDUCE LONG TERM HORMONAL DYSFUNCTION ASSOCIATED WITH CANCER TREATMENT IN WOMEN***

I declare that:

- I have read or had read to me this information and consent form and it is written in a language with which I am fluent and comfortable.
- I have had a chance to ask questions and all my questions have been adequately answered.
- I understand that taking part in this study is **voluntary** and I have not been pressurised to take part.
- I may choose to leave the study at any time and will not be penalized or prejudiced in any way.
- I may be asked to leave the study before it has finished, if the study doctor or researcher feels it is in my best interests, or if I do not follow the study plan, as agreed to.

Signed at (*place*)……………………………on (*date*) ……………………………….. 2…………

………………………….. ……………………                                        ………………………
Signature of Participant     Date of birth                                        Signature of Witness

**Declaration by Investigator**

I (name ) ………………………………………………declare that:-

- I explained the information in this document to …………………………..
- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understands all aspects of the research, as discussed above
- I did/did not use a translator. (*If a translator is used then the translator must sign the declaration below.*

Signed at (*place*)…………………………..on (*date*) ………………………….. 2……….

………………………….. ………………………
Signature of Investigator Signature of Witness.

Declaration by Translator

I (*name* ) ………………………………………………declare that:-

- I assisted the investigator (*name*)…………………………. to explain the information in this document to (*name of participant*)………………………….. using the language medium of Afrikaans/Xhosa/Other ……………..…………...
- We encouraged him/her to ask questions and took adequate time to answer them.
- I conveyed a factually correct version of what was related to me.
- I am satisfied that the participant fully understands the content of this informed consent document and has had all his/her question satisfactorily answered.

.
Signed at (*place*)…………………………..on (*date*) ………………………….. 2……

………………………….. ………………………
Signature of Translator. Signature of Witness.

## WRITTEN CONSENT FOR HANDLING OF OVARIAN TISSUE

### Ovarian Cryopreservation Project for Oncology Patients

Name of Patient: ..............................  Date:…………………………..
Address: …………………………………
…………………………………
…………………………………

I have read the attached information on the experimental freezing programme for ovarian tissue. I have been given a copy to keep. I have had the opportunity to discuss the details and ask questions about this information. I understand that I must undergo the operation of laparoscopic ovarian biopsy in order that some of my ovarian tissue may be cryopreserved (frozen) and stored for my possible future use. I understand that this tissue may not survive the freezing and thawing process and that the Hospital cannot be held responsible for this. I understand that this technique is new and experimental and thus I understand that the technique may not work.

However, I am willing to undergo the operation accepting these reservations and I understand that no guarantee can be given to me regarding future success.

I understand that in the event of my death, or should I no longer require my ovarian tissue, it will be:-
a) disposed of *
b) made available for research purposes*
(* delete as appropriate)

I have been given the opportunity to take part in counseling regarding the implications of the proposed storage of my ovarian tissue.

**PATIENT'S** NAME *(BLOCK CAPITALS)* . ..........................................

PATIENT'S SIGNATURE …………………………………… …

PATIENT'S DATE OF BIRTH ..........................................

**PATIENT'S WITNESS'** NAME *(BLOCK CAPITALS)* ………………………………………….

WITNESS' SIGNATURE ………………………………………….

**DOCTOR'S** NAME *(BLOCK CAPITALS)* ………………………………………….

DOCTOR'S SIGNATURE ………………………………………

## *Addendum D*

### PATIENT DETAILS FORM

***Project no:N05/10/182***

***Note: 1. To be completed for/by every patient entering this project!***
***2. Attach 2 copies of signed cryopreservation consent forms to this form!***
***3. Place TBH patient stickers on every form and/or page!***

Please complete in block letters, as accurate and thorough as possible.

**Personal Details**

| Date: | |
|---|---|
| Surname: | |
| Full names: | |
| Date of Birth: | Place of Birth: |
| ID no. (Identification book no.): | |
| Hospital no.: | |
| Local Clinic / Doctor: | |
| Occupation: | |
| Do you have any children? | Ages: |
| Any previous serious illnesses?(specify): | |
| Any previous serious operations?(specify): | |
| Any prescription medication past 90days?(specify): | |
| Tel(h): (w) | |
| Cell: | |
| Home Address: | |
| | |
| | |
| | |
| Postal Address: | |
| | |
| Next of kin: | Relationship: |
| Tel(h): (w) | |
| Cell: | |
| Home Address: | Postal Address: |
| | |
| | |
| | |
| Possible personal reason(s) for entering the study: | |
| To have children: To regain hormones: | Both: |
| Other (specify): | |
| | |

**Completed by** ______________________ **at** _______________________________
**Patient Signiture** ________________________ **Name** _______________________
**Medical Doctor** _________________________ **Name** ________________________

# PATIENT & PROCEDURE INFORMATION FORM

***Project no:N05/10/182***

***Note: 1. To be completed for every patient entering the project!***
***2. Attach 2 copies of signed cryopreservation consent forms to this form!***
***3. Place TBH patient stickers on every form and/or page!***

**Patient**

| Surname: | |
|---|---|
| Full names: | |
| DOB: | ID no: |
| Hospital no: | Cell: |

**Diagnosis and Pre-Operation Details**

| Date diagnosed: | | Treatment: | |
|---|---|---|---|
| Cancer type: | | | |
| Location: | | | |
| Stage: | | Treatment duration: | |
| Date: | FSH (IU/l): | LH (IU/l): | $E_2$ (pmol/l): |
| | | | |
| | | | |
| | | | |
| Any other illness? Specify: | | | |
| Additional medication prescribed: | | | |

**Surgical Procedure**

| Date: | Theatre: |
|---|---|
| Procedure: | Time procedure started: |
| Specimen: | Time procedure finished: |
| Collection holder: | Time tissue retrieved: |

**Cryopreservation**

| Sample no: | Dissection by: |
|---|---|
| Time tissue recieved: | Cryo by: |
| Time freezing started: | Total strips in storage: |
| Initial total strips: | % Strips for storage: |
| Total vials in storage: DMSO: | PROH: |
| Storage place: DMSO: | PROH: |
| Colour of vials: DMSO: | PROH: |
| Date cryopreservation results report sent: | |

**Pre-Transplantation Details – Dr MH Botha**

| Date treatment completed: | | | |
|---|---|---|---|
| Metastasis present?: Yes / No | | Date: | |
| Date: | FSH (IU/l): | LH (IU/l): | $E_2$ (pmol/l): |
| | | | |
| | | | |
| | | | |
| Any other illness during/after treatment? Specify: | | | |
| If yes, other prescription medication taken: | | | |

# PATIENT & PROCEDURE INFORMATION FORM

***Project no:N05/10/182***

***Note:1. To be completed for every patient recieving transplantation!***
***2. Attach 2 copies of signed transplantation consent forms to this form!***
***3. Place TBH patient stickers on every form and/or page!***

**Thawing Details**

| Date: | Vials: | Strips: | Start: | End: | Media: | Reason: | Done by: |
|---|---|---|---|---|---|---|---|
| | | | | | | | |
| | | | | | | | |
| | | | | | | | |
| | | | | | | | |
| | | | | | | | |
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| | | | | | | | |

**Histology**

| Date sent: | Tissue sent: | Date results recieved: | Done by: |
|---|---|---|---|
| | | | |
| | | | |
| | | | |
| | | | |
| | | | |
| | | | |

**Transplantation Details – Dr MH Botha**

| Date: | Strips: | Location: | Anaesthetic: | Start: | End: |
|---|---|---|---|---|---|
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |

**Post-Transplantation Details – Dr MH Botha**

| Date: | FSH (IU/l): | LH (IU/l): | $E_2$ (pmol/l): | Follicles? |
|---|---|---|---|---|
| | | | | |
| | | | | |
| | | | | |
| | | | | |
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| | | | | |
| | | | | |

**Medical Scientist:** ______________________ **Name:** ________________________

**Medical Doctor:** ________________________ **Name:** ________________________

**CRYOPRESERVATION DETAILS**

***Project no:N05/10/182***

***Note:1. To be completed for every patient having ovarian tissue cryopreserved.***
***2. Place TBH patient stickers on every form and/or page!***

| **Date:** | **Sample no:** |
|---|---|
| **Patient:** | **DOB: Age:** |
| **Specimen:** | **Est. time retrieved: :** |
| **Total strips counted:** | **Time taken to dissect:** |
| **Batches: 1 / 2** | **% Strips for storage:** |

**Batch 1**: n= Time started: _____:______

| Control n= BCode | | | |
|---|---|---|---|
| DMSO n= | | PROH n= | |
| DMSOeq n= BCode | | PROHeq n= BCode | |
| DMSO freeze n= | | PROH freeze n= | |
| **Vial no.** | Vial colour: | **Vial no.** | Vial colour: |
| **1**. n= | Bcode<br>Tank | **1**. n= | Bcode<br>Tank |
| **2**. n= | Tank | **2**. n= | Tank |
| **3**. n= | Tank | **3**. n= | Tank |
| **4**. n= | Tank | **4**. n= | Tank |
| **5**. n= | Tank | **5**. n= | Tank |

**Batch 2**: n= Time started: _____:______

| Control n= BCode | | | |
|---|---|---|---|
| DMSO n= | | PROH n= | |
| DMSOeq n= BCode | | PROHeq n= BCode | |
| DMSO freeze n= | | PROH freeze n= | |
| **Vial no.** | Vial colour: | **Vial no.** | Vial colour: |
| **1**. n= | Bcode<br>Tank | **1**. n= | Bcode<br>Tank |
| **2**. n= | Tank | **2**. n= | Tank |
| **3**. n= | Tank | **3**. n= | Tank |
| **4**. n= | Tank | **4**. n= | Tank |
| **5**. n= | Tank | **5**. n= | Tank |

Please mark block with ✓when tissue thawed and/or sent for histology.

**Medical Scientist:** ________________________**Name:** __________________________

**<u>RESULTS REPORT OF FREEZING OF OVARIAN TISSUE</u>**

***Project no: N0/10/182***

***Note: 1. To be completed for every patient entering the project!***
***2. Attach 1 copy of consent forms to this form!***

**Date: ______________________**

**Dear __________________________________________**

**Tygerberg Hosiptal number: _____________________**

**Your ovarian tissue were frozen and stored.** **Yes / No**

**If yes, please complete:**

| | | |
|---|---|---|
| Date of freezing: | | |
| Ovarian tissue recieved for freezing: | | |
| % Ovarian tissue kept in storage *: | | |
| Vial colour identification: | | |
| Storage place: | | |
| | | |
| | | |
| Referring Dr. | | |

* Amount of ovarian tissue left in storage available for future treatment, for example transplantation, expressed as % of original tissue sample.

**If not, please explain: _____________________________________________**
______________________________________________________________________
______________________________________________________________________
______________________________________________________________

**Signed at ________________________ on ______ of _____________20___.**

**Medical Scientist: ______________________ Name: ___________________**

**Senior Medical Scientist: ________________ Name: ___________________**
**Medical Doctor: ________________________ Name: ___________________**

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# Chapter 5: Cryopreservation of ovarian tissue as a strategy to protect hormonal function and fertility against gonadotoxic treatment


### *Abstract*

With modern treatment 70% or more of children will survive their cancer diagnosis. Many treatments have serious, harmful late effects including an effect on future fertility and hormone production. Ovarian function plays a key role in the regulation of sexual development during puberty, the regulation of normal menstrual function and peripheral effects on bone and other tissues. Ovulation is essential for normal reproduction. Chemotherapy may be very toxic to ovaries. Radiotherapy, even in low doses of 10 to 15 Gray will deplete the number of primordial follicles and will cause premature ovarian failure.

This review describes the indications, technique and outcome of grafting after cryopreservation of ovarian cortical tissue.

A detailed description of 13 cases with grafting of cryopreserved ovarian tissue is included. This is the largest group reported to date. Pre-menopausal patients with locally advanced cervical squamous carcinoma had pre-radiotherapy laparoscopic oophorectomy. Ovarian dissection to remove the cortex was performed and the cortex then divided into small strips. Ovarian tissue cryopreservation was done with

a slow freezing protocol. After completion of chemo-radiotherapy, thawed tissue was introduced back into the patients.

Return to hormone production took more than 6months after re-implantation of tissue. Levels of circulating gonadotrophins remained high despite estrogen production and follicle formation.

Ovarian cryopreservation is a relatively simple technique to safeguard hormonal function (and in certain cases fertility) in children and young women with cancer who are at risk of ovarian failure due to treatment.

### *Introduction*

Normal ovarian function produces endocrine and exocrine effects. Endocrine ovarian function plays a key role in regulation of sexual development during puberty, regulation of normal menstrual function, and peripheral effects on bone, breast, heart, brain and other tissues. Exocrine function in the form of ovulation is essential for ovum production and normal reproduction.

Many young girls and women survive the diagnosis of cancer due to modern treatment modalities. Many of these treatment modalities have serious, harmful, late-effects including ovarian failure. [1] Ovarian toxicity due to chemotherapy is related to the age of treatment and younger patients have better post-treatment ovulation figures. It may be beneficial to protect the ovaries during chemotherapy cycles with combined oestrogen and progesterone therapy. [2]

Radiotherapy, even in modest doses of 10-15Gy, will deplete primordial follicles which will impair ovarian function. In a group of 38 patients treated with radiotherapy up to a total dose of 30Gy, only one retained normal ovarian function. [3]

Various options for ovarian sparing have been proposed of which transposition of the ovaries is one option. Surgery is performed to transpose ovaries from their normal position in the pelvis to a site outside the field of pelvic radiotherapy. This technique has had mixed success due to scatter radiation and vascular compromise. [4] [5] [6] [7] However, transposition does not offer any protection against the potential late effects of chemotherapy. Alkylating agents, used in the management of

haematological malignancies in young girls, are particularly toxic to ovarian function. [8]

There are a few reports on transplantation of *fresh* ovarian tissue or complete ovaries to a location outside the field of radiotherapy. Animal studies demonstrated the potential for vascular anastomoses of ovaries in locations distant from the original. [9] Transplantation may be effective where extended fields of radiotherapy may make transposition on the original vascular supply impossible. It will, however, not protect against toxic systemic chemotherapy.

Where transposition or transplantation of fresh tissue will be of no benefit due to systemic chemotherapy an option may be to remove ovarian tissue for the period of therapy, preserve the tissue and to replace ovarian tissue when toxic treatment is finished. Cryopreservation of spermatozoa has long been successfully used when men were treated with toxic chemotherapeutic regimes. The first live birth from cryopreserved *ovarian tissue*, which was re-implanted after sterilising cancer treatment, was recently reported from Belgium. [10]

The American Society of Reproductive Medicine practice committee categorised the use of ovarian tissue cryopreservation with transplantation at a later stage as an experimental technique. [11] It should only be performed after approval by a local ethics committee. They suggested that it should always be performed under controlled conditions and be carefully monitored.

**Who may benefit from ovarian tissue cryopreservation?**

The obvious candidates for ovarian cryopreservation are young girls with haematological cancer who need aggressive chemotherapy regimes. When bone marrow transplants are considered, children often receive whole body irradiation with doses adequate to cause premature ovarian failure. There are, however, many other forms of cancer where treatment directly affects ovarian function.

Most reported cases of frozen-thawed ovarian tissue transplantation focus on protection of fertility in cases of cancer in young women. However, there may be other reasons as well for using stored ovarian tissue for transplantation. Ovarian dysgenesis and twins discordant for premature ovarian failure may be treated with donated heterologous transplantation after matching the patient and donor. [12] [13] [14] Another (as yet unpublished) indication may be to extend the fertile period in older healthy women.

Despite the experimental nature of ovarian tissue cryopreservation, ovarian tissue harvesting and banking has been offered to many patients in clinical practice over the last two decades. [15] [16] [17] Although a number of reports have been published on ovarian tissue re-implantation there is very little information on the reproductive outcomes after successful transplantation. This may be due to the small number of patients, the various techniques used, the volume of ovarian tissue that is re-implanted and the limited availability of the technology.

Many different strategies have been reported using a variety of tissues sizes. The use of cortical strips has been reported for fresh and frozen samples and whole ovary transplantation has been attempted to the ovarian fossa.

Tissue transplantation to the original site or to a transplantation location near the original site is referred to as *orthotopic* transplantation. Transplantation to an intra-abdominal site has been achieved with either laparoscopy or laparotomy. Several authors described ovarian transplantation to the ovarian stump or the peri-ovarian region. [10]

Transplantation to sites distant from the original is referred to as it *heterotopic*. Many alternative heterotopic sites have been tested for re-implantation including the arm, rectus abdominus muscle, the anterior abdominal wall, and the supra-pubic area. [18]

Cervical carcinoma is the most common cancer of women in South Africa [19] and patients often present with advanced disease where ovary sparing surgery is not an option. Standard therapy for advanced cervical carcinoma involves high doses of pelvic radiotherapy with concomitant chemotherapy as radio-sensitiser. The doses of radiotherapy needed for tumour sterilisation are invariable high enough to cause rapid ovarian failure. [20] There is an abrupt drop in oestrogen levels due to an absence of follicogenesis. Young patients with cervical carcinoma receiving radical radiotherapy will undergo immediate menopause with severe menopausal symptoms and potential long-term sequelae including osteoporosis and premature ageing. A high proportion of these patients are cured and the survivors of cervical carcinoma

thus present a unique challenge for the preservation of endocrine ovarian function. The use of hormone replacement therapy has many difficulties particularly in a resource-poor environment. Compliance is generally poor where access to health care is difficult. If a non-invasive procedure is available to preserve endocrine function it will improve not only quality of life but also the general health. There are reports in the literature where ovarian cryopreservation was used to preserve ovarian function in patients with advanced squamous cervical carcinoma. [18] [21]

When cancer therapy for malignancy is not toxic to the endometrial cavity and the uterus, pregnancy from cryopreserved ovarian tissue is possible.

**Ethical issues**

Harvesting and storage of human tissue and in particular gonadal tissue may present ethical problems. The technique of ovarian tissue cryopreservation must still be considered as experimental, but it may reduce anxiety about future fertility at a time when many other difficult decisions must be made. [22] Primum non nocere can be translated from Latin as "First, do no harm." Non-maleficence, which is the ethical principle that flows from this, is a fundamental principle in medical ethics. The risk of the operation to obtain ovarian tissue and the potential risk to re-introduce cancer cells at transplantation of frozen-thawed tissue should be carefully considered. Cancer is by definition a local *and* systemic disease. At a certain point during the disease process, a tumour may become metastatic and involve organs distant from the primary tumour. Microscopic metastases are difficult to diagnose with routine imaging and even careful microscopic examination of tissue may not identify

individual cells or small groups of cells. It is therefore very important to consider the risk for metastatic disease to the ovary very carefully before removal and storage of such ovarian tissue for future re-implantation.

The histological examination of fresh and thawed ovarian tissue has been described and it should be part of routine practice to evaluate harvested tissue for the presence of microscopic metastases. [18] [23] If there is any doubt about possible re-introduction of carcinoma, it remains prudent not to store the tissue in the first instance. The risk for ovarian metastases from haematological malignancies in children is usually very low with the exception of leukaemia. [23]
The risk for ovarian metastases from squamous cell carcinoma of the cervix is also very low. [24] [25] No reports have been published at present where cancer cells have been re-introduced through preservation of ovarian tissue.

**Consent**

Patient *autonomy* at the time of taking consent should be a high priority. Care should be taken when the procedure is offered that coercion is avoided. Family members may pressurise a young women to “preserve the family line”. Due to the paucity of good quality information about nearly all aspects of ovarian cryopreservation, it is nearly impossible to give well-informed, structured and evidence-based counselling to a potential patient before undergoing ovarian cryopreservation. [26] It is important to underline the experimental nature of the procedure without giving patients and their parents false hope about future fertility.

Dudzinski (2004) states that: “Clinically and ethically, cancer treatment takes priority over potential restoration of fertility, as the latter has no value to the woman who has died of cancer”. [22] Despite this small risk, techniques of ovarian cryopreservation and thawing are rapidly developing and may provide the only option for a patient to retain some hope of future fertility.

In the counselling of minors, extra care should be taken. The Royal College of Obstetricians and Gynaecologists working party report on ovarian tissue storage suggested assessment of Gillick competence. [26] Gillick competence is a British legal term which refers to the ability of a child younger than 18 years of age to give consent for medical procedures. In clinical practise the child should be involved with the decision making and be fully informed about the expected benefits and potential problems.

Thawing and use of the cryopreserved tissue should be discussed at initial taking of consent. The further storage or destruction of the tissue, in case of death of the tissue donor, should also be discussed carefully.

When information is given about the expected benefit of ovarian tissue cryopreservation it is important to remember another principle of medical ethics namely “making clinical decisions that are best for the patient” (*Beneficence*). The information about success rates and pregnancy outcomes is at present still limited and the procedure should be regarded as experimental.

**Removal of ovarian tissue**

Removal of ovarian tissue should preferably be done with the least invasive available technique. In certain cases a patient may require general anaesthetic for an examination as part of cancer staging. Laparoscopic oophorectomy or multiple ovarian biopsies may be scheduled at the same time to reduce the risks involved in repeated general anaesthesia. If there is doubt whether the cancer treatment will cause ovarian failure, only one ovary should be removed and the other left in-situ. Harvesting of gonadal tissue must be done within the required guidelines as stipulated in the National Health Act of South Africa.

**Re-implantation**

Re-implantation of ovarian tissue may be immediate after completion of treatment if the aim is to restore endocrine function only. If the aim is to restore fertility, it is preferable to wait for a disease free period of around two years since most malignancies reoccur during the fist 24 months after completion of treatment. This will only be feasible in hormone insensitive tumours and where further systemic chemotherapy will not harm the re-implanted tissue. As soon as the risk of ovarian toxicity due to therapy has passed, tissue strips can be replaced in orthotopic or heterotopic positions. Many heterotopic sites have been proposed including the neck, femoral triangle, forearm, breast and abdominal wall. [9] If the ovarian tissue is replaced with the aim of *restoring fertility*, it may be best to allow a disease-free period of at least two years before attempts at pregnancy are made.

## *Literature review on ovarian function after re-implantation of previously cryopreserved ovarian tissue*

| Author and year | Number of cases | Measure of function |
|---|---|---|
| **Oktay 2000 [27] [28]** | 1 | Hormone production |
| **Radford 2001 [29]** | 1 | Hormone production |
| **Callejo 2001 [30]** | 1 | Hormone production |
| **Oktay 2004 [31]** | 1 | Embryo development after ART |
| **Donnez 2004 [10]** | 1 | Live birth (orthotopic transplant) |
| **Kim 2004 [18]** | 1 | Hormone production |
| **Wolner-Hanssen 2005 [32]** | 1 | Hormone production |
| **Meirow 2005 [33] [34]** | 1 | Live birth |
| **Tryde Schmidt 2004 [35]** | 3 | Oocyte retrieval |
| **Donnez 2006 [36]** | 1 | Hormone production |
| **Oktay 2006 [37]** | 1 | Live birth (heterotopic transplant) |
| **Demeestre 2006 [38] [39]** | 1 | Pregnancy (miscarriage + live birth) |
| **Rosendahl 2006 [40]** | 1 | Pregnancy (biochemical) |
| **Andersen 2008 [41]** | 6 | Hormone production in 6<br>4 Pregnancies after ART, 2 live births |
| **Silber 2008 [42]** | 1 | pregnancy |
| **Dittrich 2008 and 2009 [43] [44]** | 1 | Hormone production |
| **Sánchez 2008 [45]** | 4 | Hormone production in 2 patients |
| **Donnez 2008** (including patients form earlier reports) **[46]** | 5 | Hormone production in all 5<br>Live birth in 1 (see 2006 and 2004) |
| **Kim 2009** (including patients form earlier reports) **[47]** | 4 | Hormone production in 4<br>Follicle aspiration in 2 |
| **Sánchez-Serrano 2009 [48]** | 1 | Live birth twins after oocyte vitrification as well) |

*Table 23 Summary of reported cases of outcome after transplantation cryopreserved ovarian tissue*

Table 23 shows a summary of the reported cases of re-implantation of cryopreserved ovarian tissue. This summary excludes all transplantations of *fresh ovarian tissue* and shows how the authors evaluated function of the transplants.

The first report on transplantation of previously cryopreserved ovarian tissue was announced in a letter to the editor of the New England Journal of Medicine in June 2000. [27] A 29-year old woman was reported who had a right salpingo-oophorectomy at the age of 17 with a wedge resection of the ovary for a diagnosis of benign cystic teratomas. She later had the remaining ovary removed because of severe menometrorrhagia. Eighty pieces of tissue were sutured onto a cellulose membrane and returned to the peritoneal cavity by laparoscopy. She had stimulation protocol with menopausal gonadotrophins that was started 15 weeks after transplantation that led to hormone production.

The procedure of re-implantation was further described in a general article published in June 2001. [28] From this description it is unclear why high doses of human menopausal gonadotrophin (HMG) were necessary to stimulate the grafts. The authors stated that the patient had “hypothalamic amenorrhoea”.

Radford followed in April 2001 with the first case report of a patient receiving the implantation after cancer therapy. [29] This 36-year old patient had a single ovary removed before chemotherapy and 19 months after treatment, when the hormone profile confirmed premature ovarian failure; two strips of cortical tissue were re-implanted. The authors describe that the cortex was dissected into 1x0.5cm strips

and that seven strips could be harvested from one ovary. She started producing oestrogen about seven months after re-implantation with ultrasonic confirmation of the follicular development.

A report from Spain included one patient where ovarian tissue was cryopreserved before re-implantation. [30] The patient volunteered for the transplantation and had a bilateral salpingo-oophorectomy together with hysterectomy done for uterine leiomyomata. She was 47 years of age and had the ovarian tissue re-implanted in the rectus abdominus muscle. The authors describe the process of “mincing of 1 cubic cm of ovarian cortical tissue into 40 to 45 fragments” which were then placed into a muscle pocket in the rectus abdominus. This patient produced oestrogen after “three to four months” and had an ovarian follicle of 16mm in diameter demonstrated by ultrasound.

The first report of fertilization of an oocyte aspirated from previously cryopreserved tissue was published in the Lancet in March 2004. [31] The patient was a 30-year old with premature ovarian failure of her remaining ovary after high-dose chemotherapy for a stage IIb breast cancer. She also had bone marrow transplantation but the reason for this was not explained in the paper. All the available cortical strips (15 in total) were implanted into the patient’s anterior abdominal wall. In total she had eight separate oocyte retrievals producing a total of 20 oocytes of which eight were acceptable for IVF. Two embryos resulted from this of which one had abnormal morphology. The last remaining embryo was transferred to the patient’s uterus but failed to progress to a pregnancy.

The first successful live birth was described in 2004. [10] At that stage the reporting team from Belgium already had 146 patients who had ovarian cryopreservation before the onset of chemotherapy of which only two had re-implantation. The pregnancy resulted after ovarian cortical biopsies were taken in a 25-year old woman before she received treatment for Hodgkin's lymphoma. The patient also had 38 Gray of radiotherapy but it is not clear from the report which part of the body was irradiated. Biochemical menopause followed treatment for cancer. Re-implantation was done during a series of three laparoscopy procedures and at the third procedure all remaining tissue was re-implanted. Approximately six months after the second implant the patient had a rise in Beta HCG levels. The pregnancy was achieved without any ovarian stimulation but the pregnancy was supported with 600mg progesterone vaginally daily. A healthy girl with good Apgar score was born.

A report from South Korea in 2004 showed hormone production 14 weeks after transplantation in a patient with cervical cancer stage Ib. [18] It is not clear from the report why both ovaries were removed at the time of radical hysterectomy before the final decision about radiotherapy was made. Nonetheless the patient received radiotherapy as well and after exclusion of ovarian metastases, 40 strips were transplanted into a space below the breast between the breast tissue and the pectoralis muscle. A second group of tissue strips was transplanted in the anterior abdominal wall. By 14 weeks the first increase in serum oestrogen was detected and ultrasound confirmed a follicle of 16x11mm in the abdominal transplant site.

A case report from Sweden described re-implantation of cryopreserved tissue after stem cell transplantation and chemotherapy as treatment for red cell aplasia. [32]

Here a site 8cm distal to the anti-cubital fossa in the forearm was used for transplantation. Follicle formation was confirmed with ultrasound up to a maximum diameter of 12.6mm.

A successful pregnancy was also described in a letter to the editor of the New England Journal in a patient who had been treated for non-Hodgkin's lymphoma by first and second line chemotherapy before ovarian tissue was removed for cyro-preservation. [33] This was followed by high-dose chemotherapy which led to premature ovarian failure. After re-implantation of the ovarian tissue into the remaining ovary, oocyte aspiration was performed with in vitro fertilization. A normal healthy girl was born after a single embryo was transferred. The authors state that "we cannot rule out the possibility that the egg was derived from the native ovary, we consider this possibility very unlikely".

A single metaphase II oocyte was retrieved after a stimulation protocol after transplantation of the frozen thawed tissue in a 32-year old patient with Hodgkin's lymphoma. Fertilization did not occur after intra cytoplasmic sperm injection (ICSI) but follicular activity remained. [35]

An interesting case report describes a live birth after "heterotopic ovarian tissue transplantation". [37] This 52-year old patient was treated for Hodgkin's lymphoma four years before which did not cause ovarian failure. She developed a relapse and was offered heterotopic stem cell transplantation. Before she started chemotherapy in preparation for the stem cell transplantation the left ovary was removed laparoscopically. After completion of treatment she did not menstruate for two-and-

a-half years and the hormone levels were in the menopausal range. However, shortly after heterotopic transplantation of frozen thawed tissue (two months after the transplant) her hormone levels indicated ovulation. At that time the patient already had a six week intra-uterine pregnancy with an absent foetal heart. The pregnancy was evacuated. She was pregnant very shortly after this pregnancy and delivered a healthy female child. The authors note that the most likely explanation is recovery of normal ovarian function in the ovary that was left behind; however, another explanation may be a new production of primordial follicles through a possible re-activation of germ cells is also a theoretical possibility.

A case report from Brussels describes the transplantation of cryopreserved ovarian tissue in a patient after treatment for stage IV Hodgkin's disease. [39] What is interesting is that this patient had combined heterotopic and orthotopic transplantation. The ovarian transplant was done according to the initial description of Donnez. [36] Some tissue was re-implanted in the atrophic remaining ovary and the rest was implanted in a peritoneal window near the ovary. The remaining tissue was implanted in the abdominal wall just below the trocar port incision. The ovarian site produced the largest follicles and this lead the authors to suggest that "the local environment and/or the vascularisation in the ovary could favour the restoration of ovarian function from transplanted cortical ovarian tissue". This patient had a spontaneous pregnancy which unfortunately was followed by a miscarriage.

A further report on the patient initially described by Demeestre describes a second orthotopic and heterotopic transplantation about one year after the first

transplantation. [38] This second transplantation was followed by the spontaneous pregnancy during the fifth menstrual cycle. A healthy girl was delivered.

A case report from Denmark described re-implantation of frozen thawed tissue in a patient after treatment for stage IIb Hodgkin's disease. [40] Tissue was re-implanted in three sites namely the atrophic ovary, the right pelvic side wall and in the anterior abdominal wall. A metaphase II oocyte was aspirated at the third attempt from the ovary and ICSI resulted in an embryo. A serum ß-HCG confirmed a chemical pregnancy, however, this pregnancy failed to progress. Another oocyte was aspirated from the abdominal wall and was successfully fertilized with ICSI. Transfer of the embryo did not result in a pregnancy. The authors of this case report are sceptical about the origin of the oocytes that resulted in the live birth reported by Donnez and Meirow. "However in both cases the origin of the fertilized oocytes can be questioned. Although unlikely, it cannot be ruled out that the fertilized oocytes originated from the remaining ovarian tissue rather that in the thawed ovarian transplants".

A combined report from three centres in Denmark reports on six women after re-implantation of frozen thawed ovarian tissue. [41] Out of the six cases all had orthotopic transplantation of between 20 and 100% of the stored tissue. Of the six two women delivered one healthy child each and a third had an early miscarriage at seven weeks gestation. The fourth patient had a biochemical pregnancy. Two patients did not become pregnant. All the patients had assisted reproduction and the two live births followed after transfer of embryos.

A paper by Silber describes a series of monozygotic twins treated with ovarian tissue transplantation from one sister to the other when the first has become menopausal. [42] This was done to treat premature ovarian failure and a total of eight such pairs of twins where transplantation had taken place were described. In one pair of twins fresh tissue was originally transplanted with normal ovarian function for 36 months after the transplant. The patient then experienced menopausal symptoms and stored frozen-thawed tissue was then transplanted. This tissue originated from the patient's sister. She conceived five months later but there is no detail about the outcome of the pregnancy in the paper.

In 2009 an interesting histological evaluation of transplanted tissue was published from Germany. [44] A patient treated for anal carcinoma had pre-treatment ovarian tissue cryopreservation. [43] Re-implantation was performed two-and-a-half years after remission into peritoneal pockets near the ovary. At approximately four months oestrogen levels started to rise but the grafts stopped functioning at around 11 months. A second laparoscopy was performed to re-implant more tissue but at the same time some of the old tissue strips were removed and examined histologically. It was found that despite the drop in hormone production primordial follicles were still present in some of the tissue.

Of the 200 women who received ovarian cryopreservation in the Vallencia programme for fertility preservation only four had re-implantation of tissue. [45] Although this follow-up is very short two out of the four had hormonal evidence of ovarian function.

A recent publication by Donnez outlines three further cases not previously reported in the literature. The indications for cryopreservation were advanced Hodgkin's lymphoma, advanced non-Hodgkin's lymphoma and Wegener's granulomatosis. [46] Two patients had a laparotomy and one had a laparoscopy for orthotopic transplantation. In all three patients GnRh antagonist was started in combination with oestrogen and progesterone for a period of 8 weeks before transplantation. This was done in order to reduce circulating FSH and LH concentrations. High FSH and LH concentrations may induce significant activation of follicles soon after ovarian tissue being transplanted. [49] The authors do not mention the details of two other cases of transplantation that did not result in hormone production.

A case report describes the twin pregnancy of a patient previously treated for breast cancer. [48] She had a laparoscopic right ovarian cortex extraction after which she completed 6 cycles of chemotherapy and 25 fractions of radiotherapy. Approximately two years after completion of treatment, ovarian tissue was transplanted onto the remaining left medulla. After initial poor response to ovarian stimulation a total of 14 oocytes were retrieved during three cycles. Nine of the fourteen oocytes were vitrified and all 14, including the 9 vitrified oocytes, were fertilized by ICSI. Eventually only two embryos, both resulting from the vitrified oocytes, were transplanted. It led to the birth of two boys delivered by caesarean section at approximately 33 weeks gestation. This is the first report of cryopreserved tissue that was stimulated and the aspirated oocytes re-frozen through a process of vitrification that led to a successful pregnancy.

A systematic review of the transplantation of ovarian tissue was published at the end of 2008. [50] Included in the review were 25 reports about ovarian transplantation with the primary aim to describe the return to ovarian function, including the maintenance of ovarian function in the short- and long-term after transplantation. *Fresh* and *previously cryopreserved* tissue was included in the study. Details about 46 patients could be included and the median age of the patients were 36 (range 15 to 49). Out of the total of 46 patients, 3 had one or more fresh, whole ovary transplanted with or without anastomoses of the small blood vessels. [51] [52] [12] Two of these cases had transplantation with own ovaries to the upper limb outside the field of pelvic radiation for Hodgkin's lymphoma, and another case for cervical cancer. Ovarian function was confirmed after transplantation.

Ovarian transplantation was reported in 23 patients who were confirmed to be in ovarian failure before transplantation; FSH levels >30 IU/l. [50] Confirmation of return to ovarian function was by using follicular growth and return to menstruation in 19 cases and by detection of follicles in the remaining four cases (these four cases all had hysterectomy previously). The median time to return of ovarian function was 120 days with range from 60 to 244. The median follow up after returning to ovarian function was only 210 days and during this period, 4 out of the 23 women again developed ovarian failure, with reduced oestrogen production. All those individuals with ovarian failure were older, with a median age of 37.5 years compared to 33 years for those who did not have recurrent ovarian failure. All four women who had the recurrent ovarian failure had transplants of small cortical strips.

In an effort to describe the pregnancy rate after ovarian tissue transplant the authors of the review looked at 25 women with intact uteri and found a total of 11 pregnancies in nine women. Two of these pregnancies were subsequently excluded from the final analysis because the transplanted tissue was in the anterior abdominal wall outside the peritoneal cavity in the presence of another ovary in a normal anatomical position. Six pregnancies in five women were achieved spontaneously, and the remaining three pregnancies were achieved through IVF.

In the discussion of the results from this review article, the authors states that "ovarian tissue transplantation, using cryopreserved tissue is not yet equivalent to that of a fresh grafts". The authors also conclude that "transplantation of ovarian tissue can re-establish ovarian function after premature ovarian failure. However, evidence is not sufficient to evaluate the long-term efficacy and longevity of ovarian grafts." [50]

From this review it is clear that various factors will influence the success of re-establishment of ovarian function, longevity of the grafts and the hormone levels achieved. Older women, using *previously frozen* tissue and transplanting only a portion of the tissue may all negatively influence the eventual outcome.

## *Clinical study: Ovarian tissue transplantation after cryopreservation*

### Aim

This descriptive study is a combined experimental (cryopreservation of ovarian tissue) and clinical (transplantation of frozen-thawed ovarian tissue) study. Ovarian tissue was obtained for cryopreservation from pre-menopausal patients with advanced cervical cancer. The standard therapy for these patients was radical chemo-radiation. In the experimental part of the study tissue samples were randomised into two groups and preserved according to different cryopreservation protocols. This part of the study is described in more detail in Chapter 4.

The clinical part of the study evaluated preservation of endocrine function after transplantation of frozen-thawed ovarian tissue. After radiotherapy, frozen-thawed ovarian tissue was transplanted back into patients to evaluate preservation of endocrine function.

### Ethics approval

This study was approved by the local institutional review board of the Faculty of Health Sciences, Stellenbosch University. *Project number: N05/10/182*

## Materials and methods

### Patients

- A sample of convenience was taken from a group of patients who received their consultations, surgery and therapy for advanced squamous cervical carcinoma at the unit for gynaecological oncology, Tygerberg Hospital.
- Patients with advanced squamous cervical carcinoma planned for curative chemo-radiotherapy
- The patients were entered into the study on basis of voluntary informed consent
- Patients younger than 45 years of age with normal FSH and E2 levels
- HIV non-infected

### Procedures

- Laparoscopic oophorectomy during examination under anaesthesia for cancer staging
- Preparation of cortical strips under sterile conditions.
- Histological evaluation of tissue strips for evaluation of metastatic disease
- Cryopreservation of ovarian tissue
- Thawing
- Histology of thawed ovarian tissue for evaluation of follicle survival.
- Re-implantation
- Follow-up of hormonal function.

**Detailed methodology**

Tissue was removed with laparoscopic oophorectomy in all cases. The patient was put in the supine position and catheterised preoperatively. An examination under anaesthesia was performed as part of the tumour staging. A sub-umbilical incision was made through the skin and a Verres needle was passed. The peritoneal cavity was inflated with 2 ½ litres of $CO_2$, after which a 10 mm trocar was inserted. Two additional 5 mm ports were inserted; one near Palmer's point and the other approximately 5 cm away infero-laterally. A third, 5 mm trocar was placed in the right iliac fossa. This configuration of ports gave the ideal approach to both ovaries. The individual ovaries were elevated out of the pelvis with a grasper and the vascular pedicle was cauterised carefully and then cut. After complete removal of the first ovary it was placed in the vesico-uterine pouch.

The second ovary was then elevated in a similar fashion and removed after careful cauterisation of the vascular pedicle. The second ovary was then also placed in the vesico-uterine pouch. Up to this point the 10mm primary trocar at the infra-umbilical site have been the port for a 10mm 30$^o$ laparoscope which was now removed and a 5mm 30$^o$ laparoscope was placed through the Palmer's point 5mm trocar. An endobag was passed through the 10mm port and the two dissected ovaries were placed within the endobag and subsequently removed from the abdomen. In case of larger sized ovaries it was necessary to increase the size of the incision in the sheath to accommodate the size of the ovaries. After removal of the ovaries it was kept in the sterile endobag and transported to the laboratory where the dissection was performed in a laminar flow cabinet in a completely sterile fashion to prevent contamination.

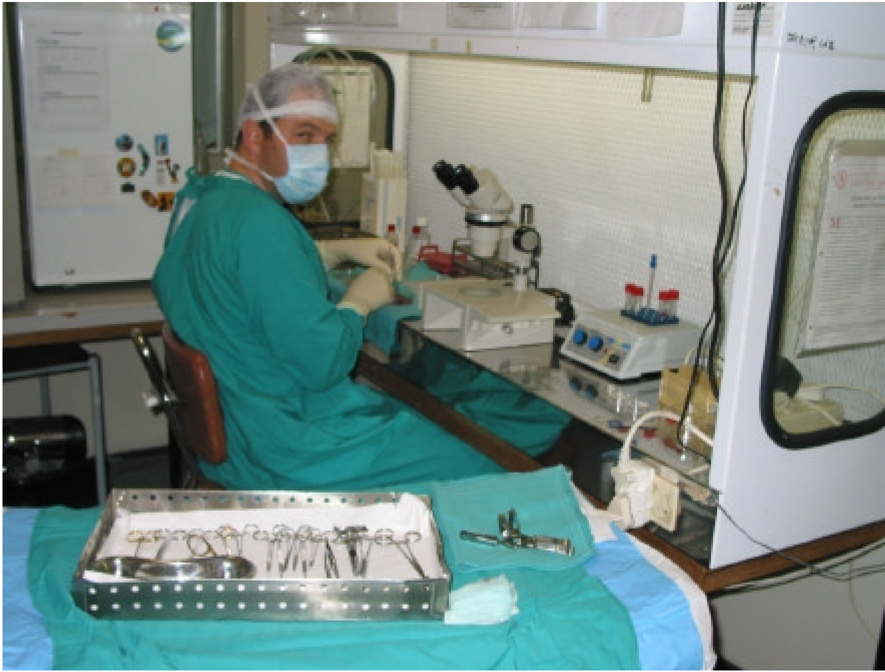


*Figure 21    Dissection of tissue in laminar flow cabinet*

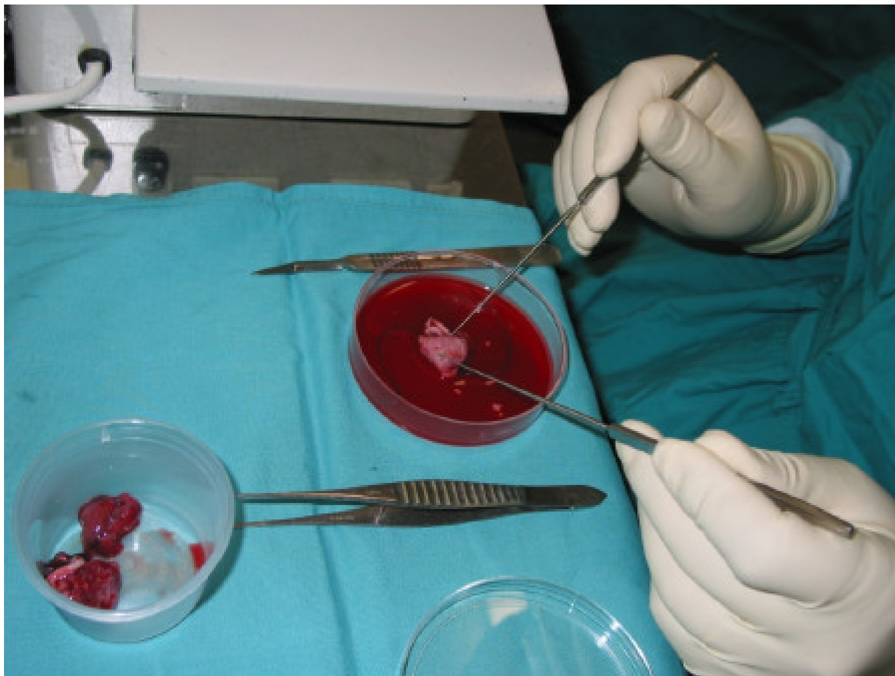


*Figure 22    Dissection of ovarian cortex from underlying stroma*

The dissection of the ovarian strips was performed by first bisecting the ovary and removing most of the cortex. See Figure 22. The cortex was divided into 2mmx5mm strips. See Figure 23.

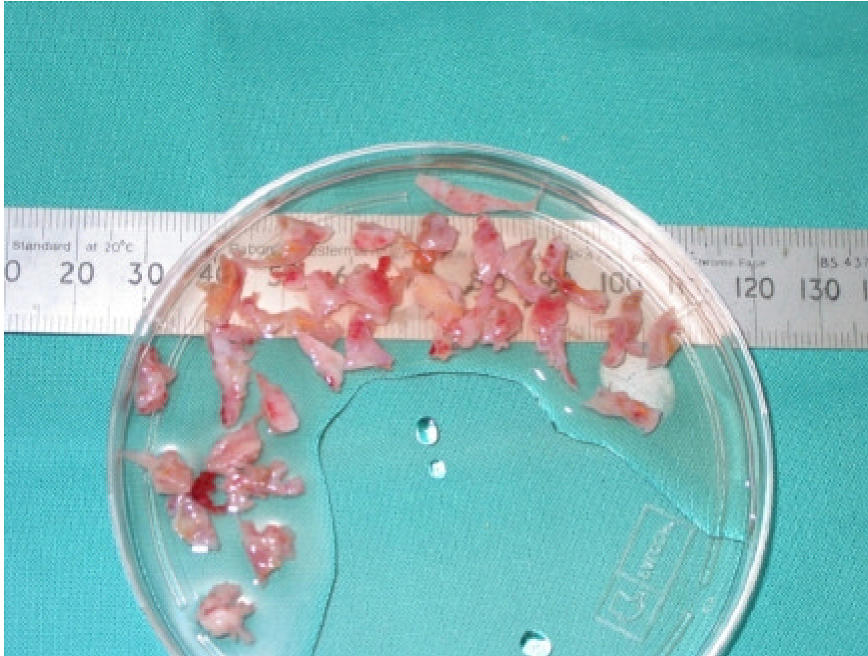



*Figure 23 Ovarian strips after dissection*

The remaining tissue of the ovary which was mostly the stroma of the ovary was sent for normal light microscopy. Tissue strips were then divided into separate containers and prepared for cryopreservation. See Figure 24.

*Figure 24 Cortical strips in cryovials*

### *Re-implantation (grafting)*

When the tissue was needed for re-implantation it was thawed to room temperature.

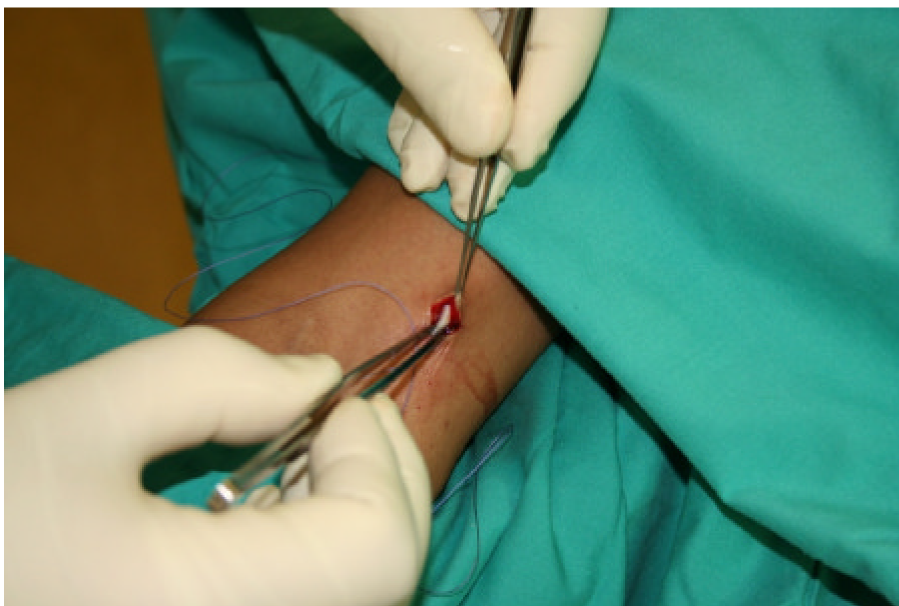


*Figure 25 Tissue strips inserted under skin of upper arm*

In the current study the contents of two cryo vials were used for every single transplant. This represented between 10 and 20 strips per transplant. At present there is no clear indication in the literature about the optimal amount of tissue that is necessary for oestrogen production and/or follicle formation. The volume of tissue used in our study was also partly determined by the re-implantation site in the arm because the volume of tissue would have been too much if all the tissue was re-implanted at the same time.

The anterior aspect of the upper arm was used for re-implantation. See Figure 25. The implantation was scheduled to coincide with the insertion of brachytherapy (under general anaesthesia). Otherwise the re-implantation was done under local anaesthesia after the completion of systemic treatment that may harm the transplanted tissue. A small incision of less than a centimetre was made and the tissue strips placed in a subcutaneous tunnel. The skin was closed with an absorbable suture. See Figure 25.

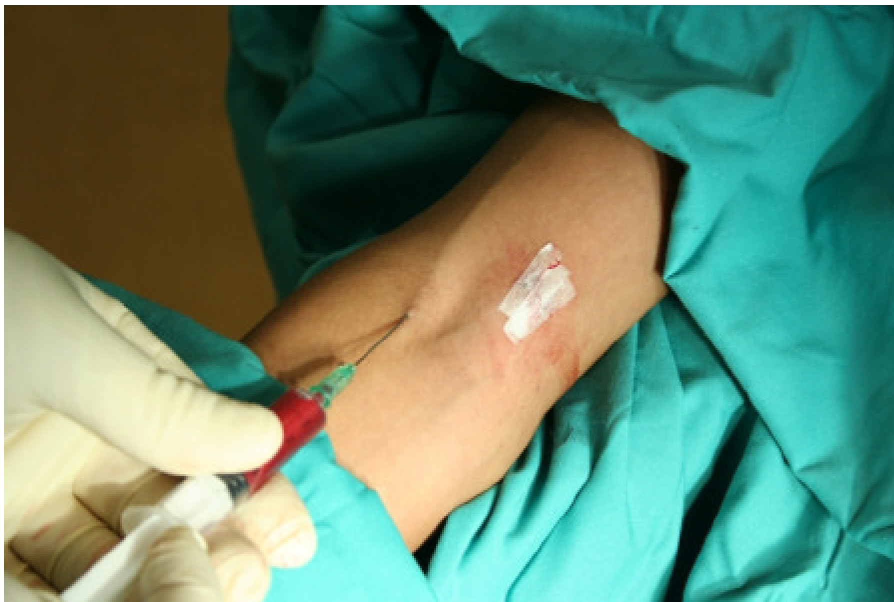


*Figure 26 Blood taken at transplantation for hormone profile*

**Results**

In the current study all the participants had advanced cervical cancer, which necessitated whole pelvic irradiation with subsequent total ovarian failure. All the patients also required systemic chemotherapy as part of treatment, and therefore the only option was to do cryopreservation of tissue until after completion of the treatment. Both ovaries were removed in all but one of the described cases and all the patients in this series had confirmed ovarian function before removal of ovarian tissue and had confirmed ovarian failure at the time of the re-implantation. All the patients had no evidence of metastatic tumour on light microscopy in ovarian tissue of the remaining stromal tissue.

The local laboratory defined oestrogen levels below 37pmol/l as non-detectable. In the hormone graphs all the values reported as <37 pmol/l was changed to 5 pmol/l to make the graphs easier to read.

All patients who had tissue transplantation between June 2006 and June 2008 are included in the report. A total of 17 patients were available for transplantation during this period. 3 patients declined transplantation after counselling and one patient that received transplantation had no follow-up at all. She was from a small village outside Oudtshoorn and could not be traced. The other 13 patients are described in more detail below.

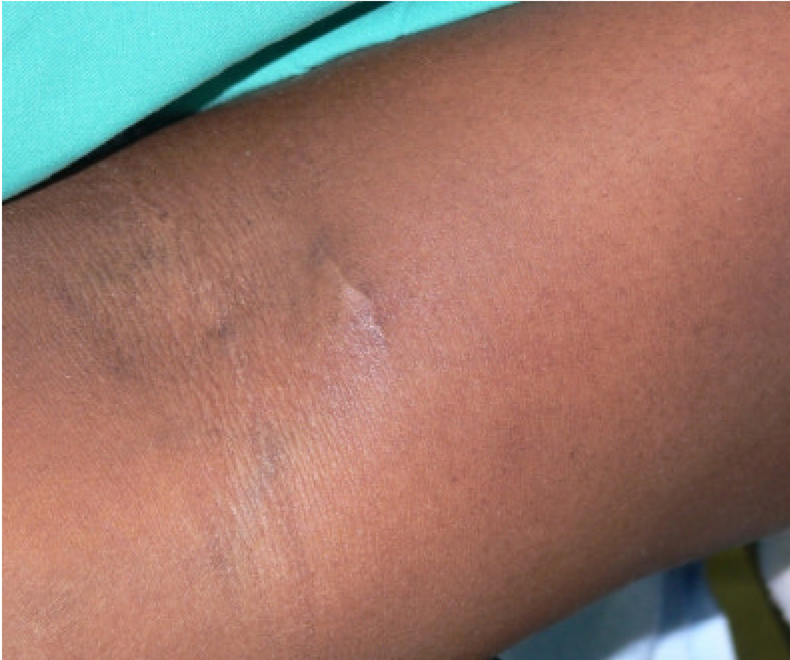


*Figure 27 Scar of implantation site one month after the procedure*

## Individual patient details

### Patient 1

Patient number 1 was a 33-year old G5 P3 with one previous miscarriage and one previous termination of pregnancy that presented with a bulky stage IIb cervical cancer. For this she was offered whole pelvic irradiation with concomitant chemosensitization and brachytherapy to the cervix. She had a laparoscopic oophorectomy without complications and both ovaries were removed. In total 102 strips were available after dissection of the cortical tissue. Ninety of the strips were available for cryopreservation after the necessary samples were prepared for histology and electron microscopy studies.

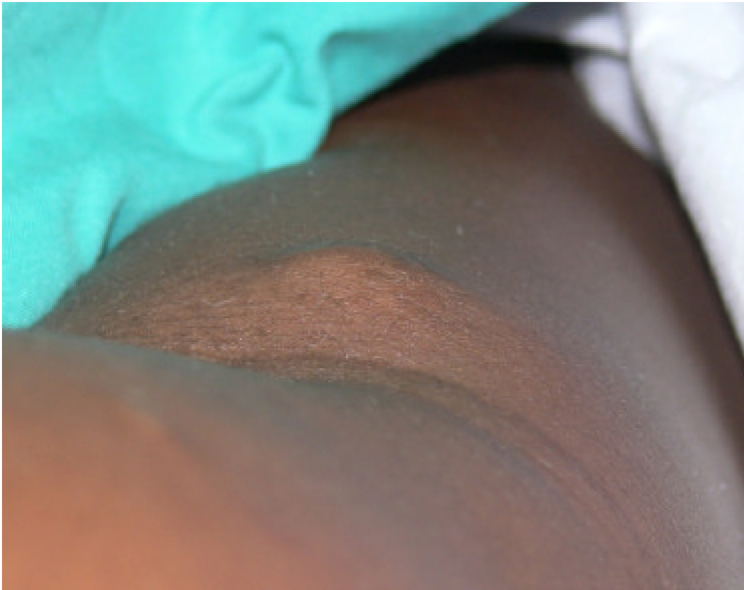
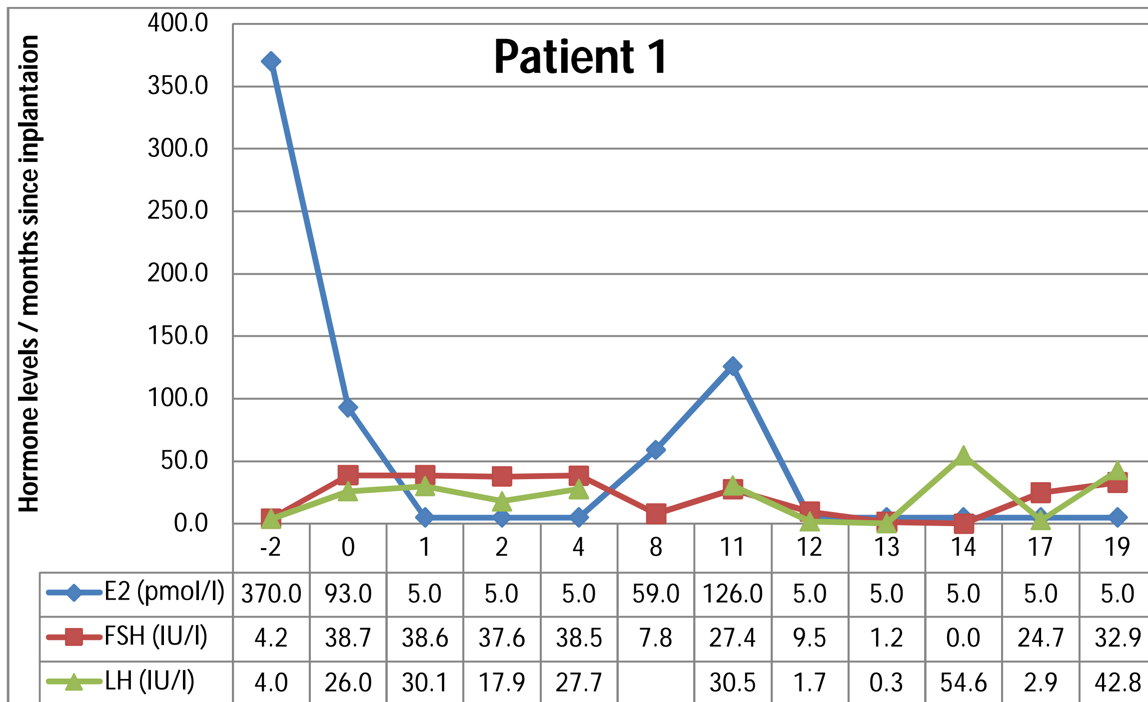


*Figure 28 Visible swelling in upper arm due to follicle formation*

Before oophorectomy the oestrogen level was 370 pmol/l, FSH 4.2 IU/l and the LH 4.0 IU/l. At insertion of the brachytherapy under general anaesthesia after chemotherapy was completed, 11 strips were re-inserted in the right upper arm about 2cm above the cubital fossa. The strips were randomly selected from the DMSO group.

This patient was HIV negative at inclusion to the study and at the start of radiotherapy. When pelvic progression was confirmed at around 11 months after completion of radiotherapy, a follow-up HIV test was positive. The patient was started on highly active retroviral therapy with a good initial virological response.

The first sign of oestrogen production was noted eight months after transplantation with a peak level at month 11 of 126 pmol/l. See Figure 28 and Table 24. Unfortunately the patient died 25 months after re-implantation due to renal failure caused by ureteric obstruction secondary to progressive pelvic disease.




| | -2 | 0 | 1 | 2 | 4 | 8 | 11 | 12 | 13 | 14 | 17 | 19 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| E2 (pmol/l) | 370.0 | 93.0 | 5.0 | 5.0 | 5.0 | 59.0 | 126.0 | 5.0 | 5.0 | 5.0 | 5.0 | 5.0 |
| FSH (IU/l) | 4.2 | 38.7 | 38.6 | 37.6 | 38.5 | 7.8 | 27.4 | 9.5 | 1.2 | 0.0 | 24.7 | 32.9 |
| LH (IU/l) | 4.0 | 26.0 | 30.1 | 17.9 | 27.7 | | 30.5 | 1.7 | 0.3 | 54.6 | 2.9 | 42.8 |

*Table 24  Summary of hormone profile patient 1*

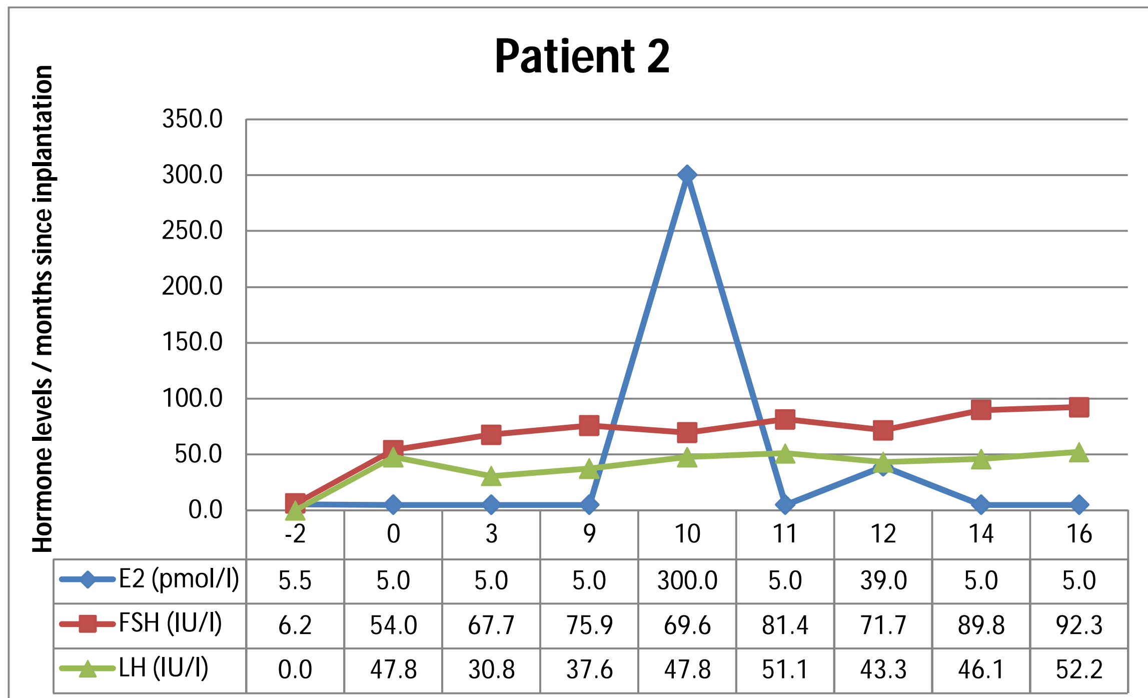
**Patient 2**

Patient number 2 was a 36-year old G4 P3 M1 who presented with a locally advanced stage IIIb cervical cancer for which she was offered whole pelvic irradiation and concomitant chemosensitization and cervical brachytherapy. She had a pre-treatment laparoscopic oophorectomy and both ovaries were removed. Surgery was uncomplicated.

A total of 116 strips were available after dissection of the ovarian cortex. One hundred and eight of these strips were cryopreserved after tissue for histology and electron microscopy was removed.

Re-implantation was done at insertion of brachytherapy after chemotherapy has been completed. A total of 12 strips were re-inserted in the right upper arm. These strips were from the PROH group.

Preoperative measurements were E2 198.8 pmol/l, FSH 6.2 IU/l and LH 8.2 IU/l. Despite relatively high and rising levels of gonadotrophins she had evidence of follicle formation at month 10 with an oestrogen level of 300 pmol/l. There was another rise in E2 at month 12. See Table 25. The high FSH and LH levels could possible be due to the patient's age and also due to the small number of strips re-inserted (only 12).




| | -2 | 0 | 3 | 9 | 10 | 11 | 12 | 14 | 16 |
|---|---|---|---|---|---|---|---|---|---|
| E2 (pmol/l) | 5.5 | 5.0 | 5.0 | 5.0 | 300.0 | 5.0 | 39.0 | 5.0 | 5.0 |
| FSH (IU/l) | 6.2 | 54.0 | 67.7 | 75.9 | 69.6 | 81.4 | 71.7 | 89.8 | 92.3 |
| LH (IU/l) | 0.0 | 47.8 | 30.8 | 37.6 | 47.8 | 51.1 | 43.3 | 46.1 | 52.2 |

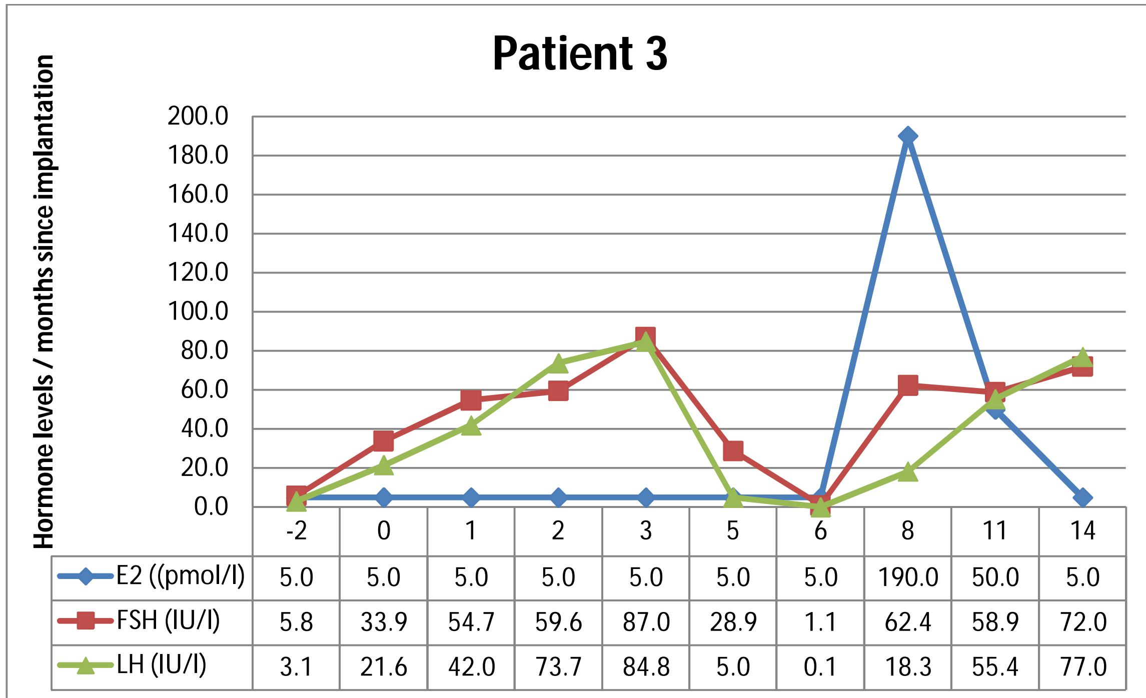
*Table 25 Summary of hormone profile patient 2*

**Patient 3**

Patient number 3 was a 27-year old G2 P2 that presented with stage IIb squamous carcinoma of the cervix with parametrial invasion on the left. For this she had neo-adjuvant chemotherapy and a radical hysterectomy. Histology confirmed a poorly differentiated tumour with a positive surgical margin. She continued to have complete whole pelvic irradiation as well. She had a laparoscopic oophorectomy before the start of the radiotherapy and both ovaries were removed without any difficulty.

A total of 129 strips have been removed of which 122 were cryopreserved after histology and electron microscopy were performed.

Re-implantation was performed at commencement of brachytherapy in the left upper arm. A total of 14 strips were re-implanted. Pre-operative oestrogen level was less than 37pmol/l but FSH was 5.8 IU/l and the LH 3.1 IU/l. She had evidence of oestrogen production at month eight with a sudden decrease in the FSH and LH levels from month five after the re-implantation after an initial rise to 87 (FSH) and 84 (LH) respectively. However, from month eight the FSH and LH started to rise again and continued to rise until her death due to locally advanced tumour month 18 after the re-implantation. See Table 26.




| | -2 | 0 | 1 | 2 | 3 | 5 | 6 | 8 | 11 | 14 |
|---|---|---|---|---|---|---|---|---|---|---|
| E2 ((pmol/l) | 5.0 | 5.0 | 5.0 | 5.0 | 5.0 | 5.0 | 5.0 | 190.0 | 50.0 | 5.0 |
| FSH (IU/l) | 5.8 | 33.9 | 54.7 | 59.6 | 87.0 | 28.9 | 1.1 | 62.4 | 58.9 | 72.0 |
| LH (IU/l) | 3.1 | 21.6 | 42.0 | 73.7 | 84.8 | 5.0 | 0.1 | 18.3 | 55.4 | 77.0 |

*Table 26 Summary of hormone profile patient 3*

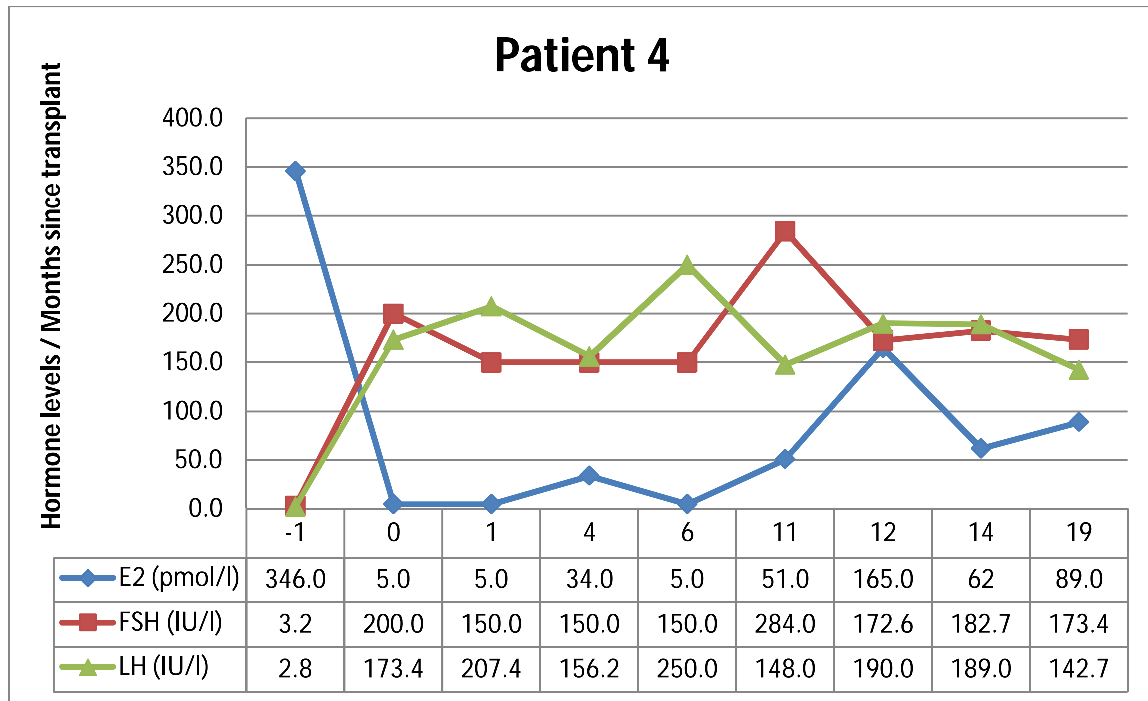
**Patient 4**

Patient number 4 was a 38-year old G4 P4 who presented with stage IIIb cervical cancer. For this she had radical radiotherapy with chemosensitization.

This patient had bilateral oophorectomy but only 54 strips were available after dissection due to small volume ovaries. Only 36 strips were available for cryopreservation after some of the strips were deemed inadequate because of a large corpus luteum distorting the anatomy.

Pre-operative E2 was 346 pmol/l, FSH was 3.2 IU/l and LH was 2.8 IU/l. Re-implantation was performed at the end of radiotherapy with insertion of the brachytherapy and 10 strips were inserted in the upper arm. These strips were from the PROH group.

Despite very high levels of FSH and LH she started producing oestrogen from month 11. The levels peaked at month 12 around 165 pmol/l. The FSH and LH stayed over 150 IU/l after removal of the ovaries. See Table 27. This is perhaps due to the patient's age and the low number of strips re-implanted.




| | -1 | 0 | 1 | 4 | 6 | 11 | 12 | 14 | 19 |
|---|---|---|---|---|---|---|---|---|---|
| E2 (pmol/l) | 346.0 | 5.0 | 5.0 | 34.0 | 5.0 | 51.0 | 165.0 | 62 | 89.0 |
| FSH (IU/l) | 3.2 | 200.0 | 150.0 | 150.0 | 150.0 | 284.0 | 172.6 | 182.7 | 173.4 |
| LH (IU/l) | 2.8 | 173.4 | 207.4 | 156.2 | 250.0 | 148.0 | 190.0 | 189.0 | 142.7 |

*Table 27 Summary of hormone profile patient 4*

**Patient 5**

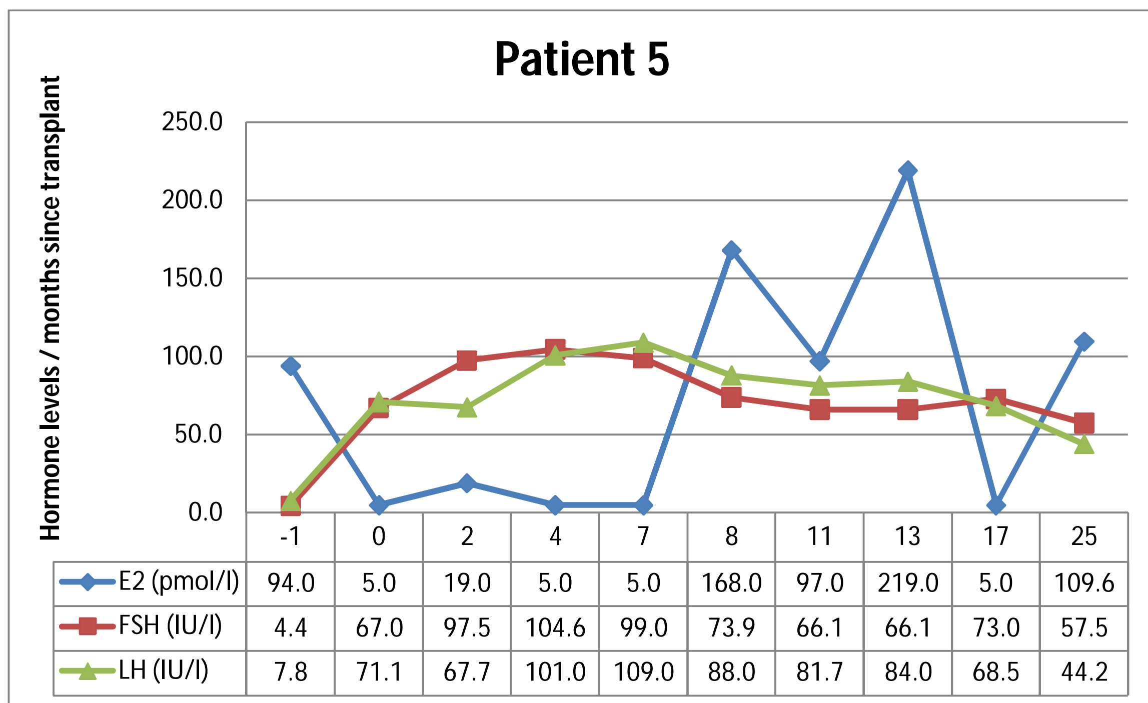
Patient number 5 was a 41-year old gravida 6 para 6 that presented with stage IIIb squamous carcinoma of the cervix. Pre-treatment E2 was 94 pmol/l and the FSH was 4.4 IU/l and the LH 7.8 IU/l. She had a laparoscopic oophorectomy of both ovaries without any complications.

This patient had a total of 75 strips after dissection of which 68 were available for cryopreservation. A 50-minute delay in one batch of the tissue was due to a

software problem after a USB mouse was inserted into the computer. This caused in the software programme to stop responding.

Re-insertion was done and at insertion of the brachytherapy and a total of 12 strips preserved in PROH was done on the left arm. These strips were not from the batch with the delay in the cryotherapy protocol.

She had high levels of FSH of around 100 which steadily declined over the following months. The first production of oestrogen was detected at month 8 and continued past month 25. See Table 28.




| | -1 | 0 | 2 | 4 | 7 | 8 | 11 | 13 | 17 | 25 |
|---|---|---|---|---|---|---|---|---|---|---|
| E2 (pmol/l) | 94.0 | 5.0 | 19.0 | 5.0 | 5.0 | 168.0 | 97.0 | 219.0 | 5.0 | 109.6 |
| FSH (IU/l) | 4.4 | 67.0 | 97.5 | 104.6 | 99.0 | 73.9 | 66.1 | 66.1 | 73.0 | 57.5 |
| LH (IU/l) | 7.8 | 71.1 | 67.7 | 101.0 | 109.0 | 88.0 | 81.7 | 84.0 | 68.5 | 44.2 |

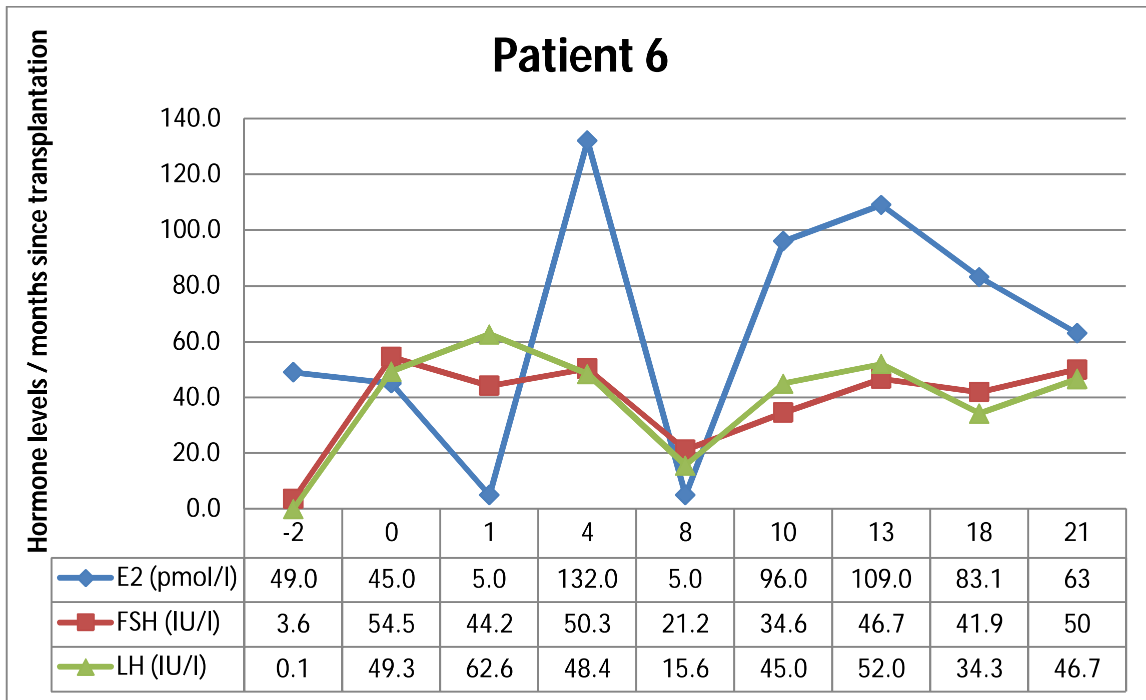
*Table 28 Summary of hormone profile patient 5*

**Patient 6**

Patient number 6 was a 40-year old G2 P2 that presented with a stage IIIb squamous carcinoma of the cervix. She was offered radical radiotherapy with chemosensitization and had a pre-treatment oestrogen level of 49 pmol/l, FSH of 3.6 IU/l and a LH of 0.1 IU/l.

A total of 100 strips were available for cryopreservation. Re-implantation was done in the right upper arm under general anaesthesia with insertion of the brachytherapy. A total of 15 strips preserved in PROH were re-inserted.

At month four there was already a peak in oestrogen with level of 132 pmol/l. The FSH and LH also decreased to levels below 50. She continues to produce oestrogen for at least the first 21 months after re-implantation. See Table 29.




| | -2 | 0 | 1 | 4 | 8 | 10 | 13 | 18 | 21 |
|---|---|---|---|---|---|---|---|---|---|
| E2 (pmol/l) | 49.0 | 45.0 | 5.0 | 132.0 | 5.0 | 96.0 | 109.0 | 83.1 | 63 |
| FSH (IU/l) | 3.6 | 54.5 | 44.2 | 50.3 | 21.2 | 34.6 | 46.7 | 41.9 | 50 |
| LH (IU/l) | 0.1 | 49.3 | 62.6 | 48.4 | 15.6 | 45.0 | 52.0 | 34.3 | 46.7 |

*Table 29  Summary of hormone profile patient 6*

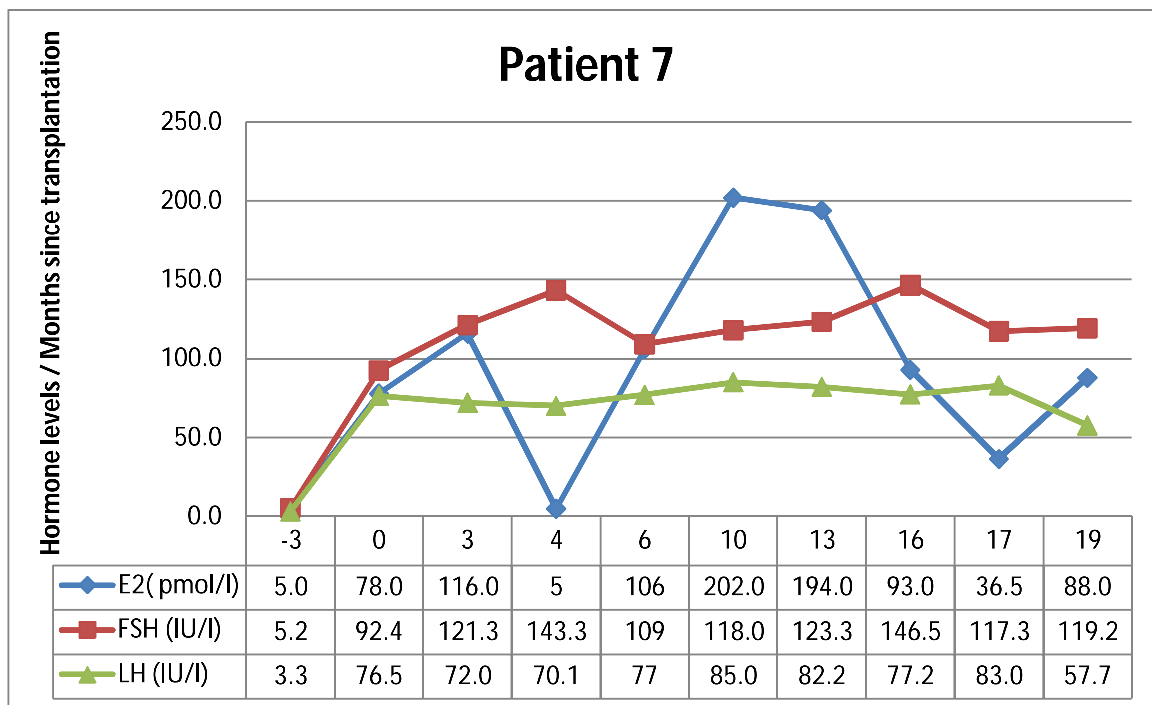
**Patient 7**

Patient number 7 was a 37-year old G3 P3 who presented to us with a squamous cell carcinoma of the cervix stage IIIb.  She had a laparoscopic oophorectomy without any difficulties and her pre-operative oestrogen level was < 37 pmol/l, FSH was 5.2 IU/l and the LH 3.3 IU/l.

A total of 72 strips were available after dissection of which 68 were available for cryopreservation.  She had an implant under local anaesthesia 6 weeks after completion of radiotherapy and chemotherapy.  A total of 13 strips cryopreserved in DMSO were re-inserted.

At month 16 after the first transplantation she complained of menopausal symptoms and requested an additional transplantation.

She had a high oestrogen level at month three after the implantation of 116 pmol/l which is difficult to explain, however, from month 6 onwards she had sustained levels of oestrogen above 100 pmol/l which declined around month 16. This despite continuously elevated levels of FSH over 100 IU/l and LH over 70 IU/l. See Table 30. The second transplantation was a little bit higher up in the left upper arm and 14 DMSO preserved strips were re-inserted.




| | -3 | 0 | 3 | 4 | 6 | 10 | 13 | 16 | 17 | 19 |
|---|---|---|---|---|---|---|---|---|---|---|
| E2( pmol/l) | 5.0 | 78.0 | 116.0 | 5 | 106 | 202.0 | 194.0 | 93.0 | 36.5 | 88.0 |
| FSH (IU/l) | 5.2 | 92.4 | 121.3 | 143.3 | 109 | 118.0 | 123.3 | 146.5 | 117.3 | 119.2 |
| LH (IU/l) | 3.3 | 76.5 | 72.0 | 70.1 | 77 | 85.0 | 82.2 | 77.2 | 83.0 | 57.7 |

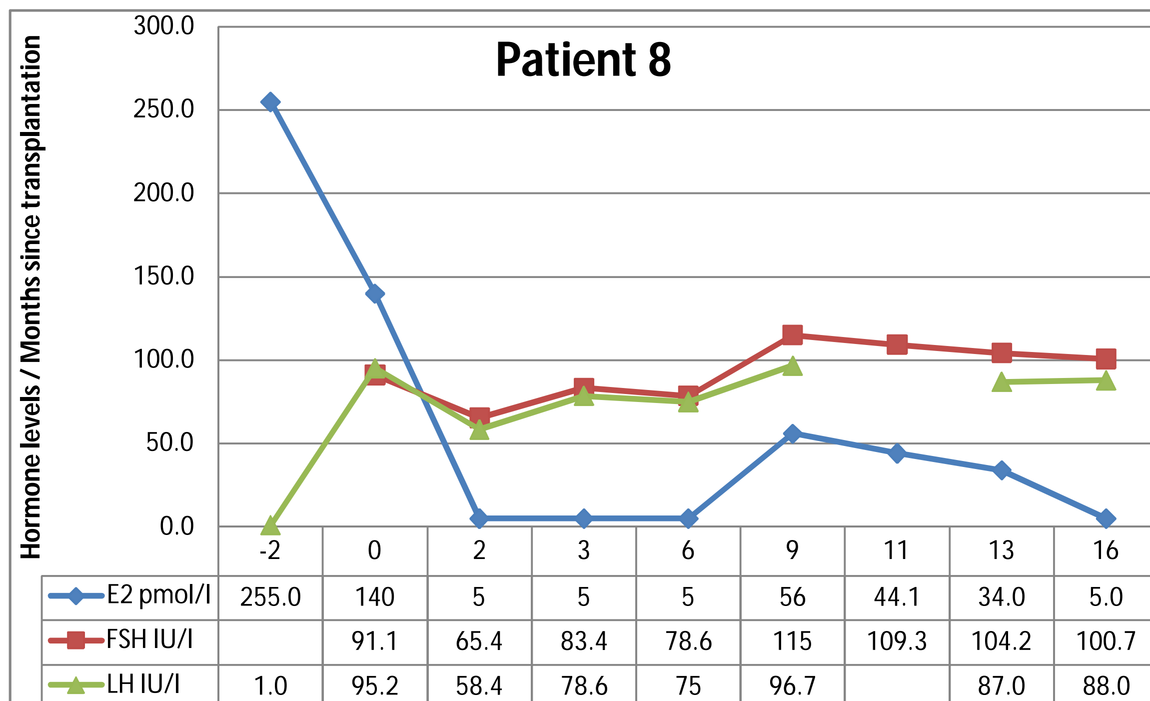
*Table 30 Summary of hormone profile patient 5*

**Patient 8**

Patient number 8 was a 38-year old woman. She had a pre-operative oestrogen of 255 pmol/l, the LH was 1 IU/l and the FSH was not recorded. The patient had 110 strips available after laparoscopic oophorectomy and dissection of tissue. A total of 106 were available for cryopreservation.

The re-implantation was done under general anaesthesia at the time of insertion of the brachytherapy 10 strips cryopreserved in DMSO were inserted in the right upper arm.

This patient showed very low oestrogen response with levels just over 50 at month 9 with high FSH levels throughout. See Table 31. It is unsure whether she produced any follicles. Ten strips were re-implanted.




| | -2 | 0 | 2 | 3 | 6 | 9 | 11 | 13 | 16 |
|---|---|---|---|---|---|---|---|---|---|
| E2 pmol/l | 255.0 | 140 | 5 | 5 | 5 | 56 | 44.1 | 34.0 | 5.0 |
| FSH IU/l | | 91.1 | 65.4 | 83.4 | 78.6 | 115 | 109.3 | 104.2 | 100.7 |
| LH IU/l | 1.0 | 95.2 | 58.4 | 78.6 | 75 | 96.7 | | 87.0 | 88.0 |

*Table 31 Summary of hormone profile patient 8*

**Patient 9**

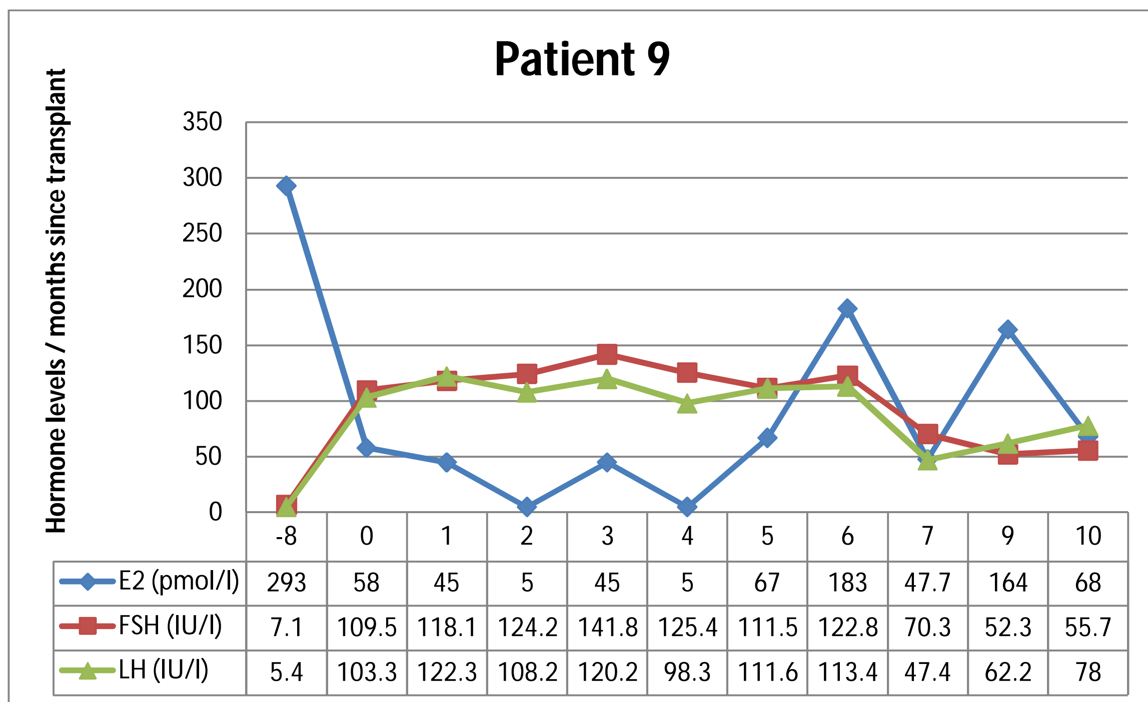
Patient number 9 was a 30-year old G1 that presented with a stage IIb squamous carcinoma of the cervix. For this she had neo-adjuvant chemotherapy, radical hysterectomy and post-operative whole pelvic irradiation due to poor prognostic factors. Pre-treatment E2 was 293 pmol/l, FSH was 7.1 IU/l and LH 5.4 IU/l. A laparoscopic oophorectomy was performed and a single ovary was removed before the start of the neo-adjuvant chemotherapy. She had a laparoscopic oophorectomy for only one ovary because the primary treatment was neo-adjuvant chemotherapy and possible surgery that could result in possible normal ovarian function in the remaining ovary.

Only one ovary was removed and 42 strips were available for cryopreservation.

However, after completion of full dose chemotherapy a radical hysterectomy was performed and due to poor prognostic indicators on the histology including a positive surgical margin, she was offered whole pelvic irradiation as well. After treatment with radiotherapy she was in premature ovarian failure.

Re-implantation was done under local anaesthesia 8 months after the original cryopreservation. Twenty-one DMSO preserved strips were available for re-implantation but during the re-implantation procedure one tissue strip became unsterile and was not useable. The strips were implanted into the left arm. At the time of re-implantation the other ovary was in premature ovarian failure with an FSH level of 109.5 IU/l and an LH level of 103.3 IU/l and an E2 level of only 58 pmol/l.

This dropped to less than detectable at month four. However, from month five the oestrogen levels started rising and at month six a follicle was present with an oestrogen level of 183 pmol/l. She continued to produce oestrogen and the FSH levels came down to 52.3 IU/l and the LH levels came down from over 100 IU/l to 62.2 IU/l. See Table 32.




| | -8 | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| E2 (pmol/l) | 293 | 58 | 45 | 5 | 45 | 5 | 67 | 183 | 47.7 | 164 | 68 |
| FSH (IU/l) | 7.1 | 109.5 | 118.1 | 124.2 | 141.8 | 125.4 | 111.5 | 122.8 | 70.3 | 52.3 | 55.7 |
| LH (IU/l) | 5.4 | 103.3 | 122.3 | 108.2 | 120.2 | 98.3 | 111.6 | 113.4 | 47.4 | 62.2 | 78 |

*Table 32 Summary of hormone profile patient 9*

**Patient 10**

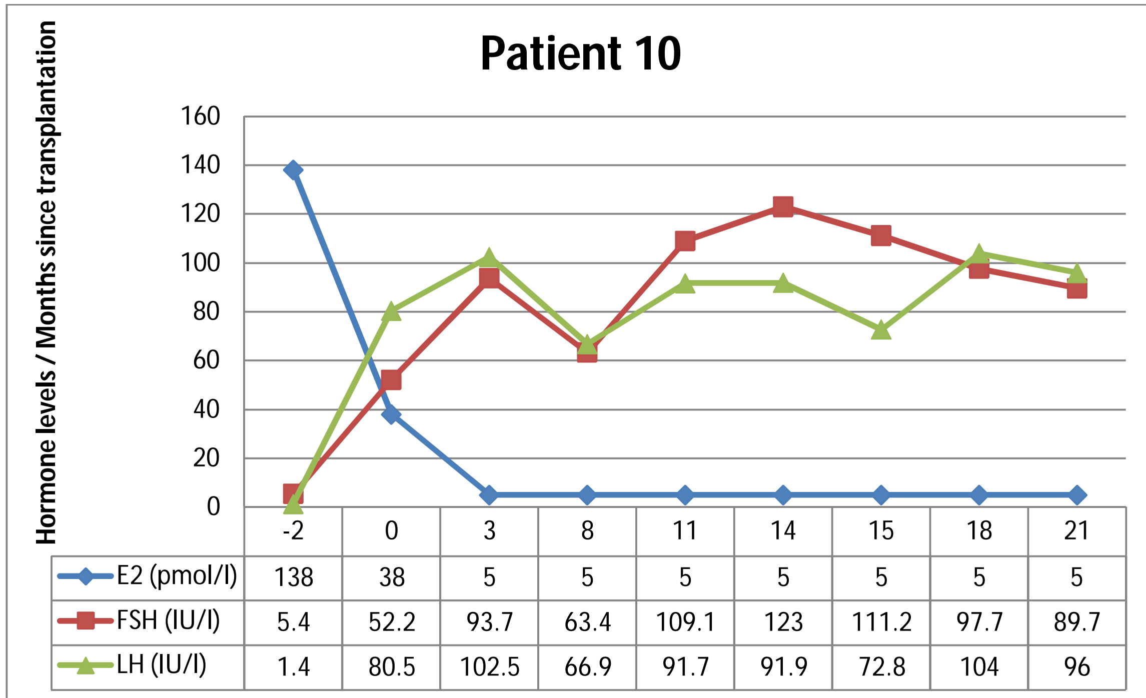
Patient number 10 was a 38-year old G3 P3 that presented with stage IIIb cervical cancer. She was on treatment for underlying hypertension and diabetes on oral

medication. For the cancer she was offered radical radiotherapy with concomitant chemosensitization. Pre-treatment her E2 was 138.0 pmol/l and the FSH was 5.4 IU/l and the LH 1.4 IU/l. Two ovaries were removed with laparoscopic oophorectomy without any complications.

A total of 82 strips were available for cryopreservation in the second batch of tissue there were slight delays at minus 19.5 degrees Celsius and again at minus 24 degrees Celsius due to a computer problem.

Re-implantation was performed under general anaesthesia in the left upper arm during insertion of the brachytherapy. Twelve DMSO strips from three vials in the first batch were re-inserted. These strips were not from the delayed batch.

Twelve strips were re-implanted. The re-implantation was performed after completion of treatment. The oestrogen level stayed low and the FSH and LH levels continued to rise until it reached a plateau around month seven. She never produced any follicles for at least 21 months after re-implantation. The oestrogen levels continue to stay low without any evidence of follicle formation and she declined another implant. See Table 33.




| | -2 | 0 | 3 | 8 | 11 | 14 | 15 | 18 | 21 |
|---|---|---|---|---|---|---|---|---|---|
| E2 (pmol/l) | 138 | 38 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| FSH (IU/l) | 5.4 | 52.2 | 93.7 | 63.4 | 109.1 | 123 | 111.2 | 97.7 | 89.7 |
| LH (IU/l) | 1.4 | 80.5 | 102.5 | 66.9 | 91.7 | 91.9 | 72.8 | 104 | 96 |

*Table 33  Summary of hormone profile patient 10*

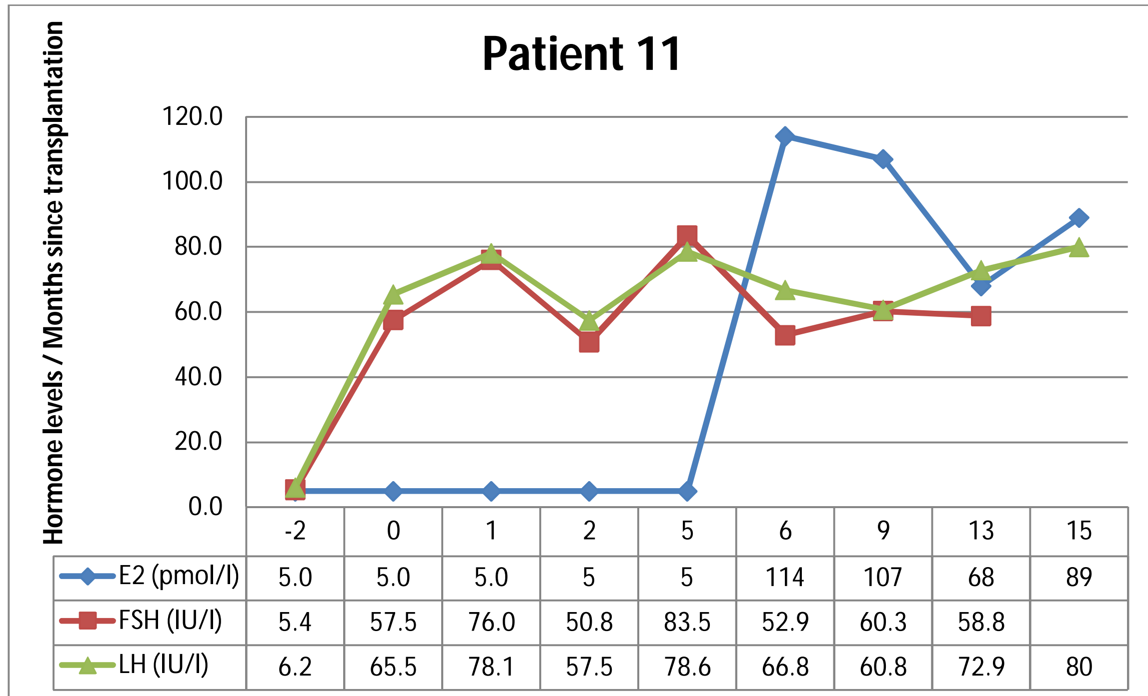
**Patient 11**

Patient number 11 was a 27-year old G2 who presented with a stage IIb cervical cancer. She was offered radical radiotherapy with chemosensitization. Pre-operative E2 level was < 37 pmol/l and her FSH was 5.4 IU/l and LH 6.2 IU/l. She had a laparoscopic oophorectomy before the commencement of treatment.

This patient had 144 strips available for cryopreservation. Re-implantation was done with 14 strips preserved in DMSO in the left upper arm.

At month six after transplantation she started producing a follicle with oestrogen levels of 114 pmol/l. The FSH and LH stayed high despite the oestrogen production.

She had a peak of oestrogen at month six after re-implantation and continued to produce oestrogen to least month 15; this despite high levels of gonadotrophins in the region between 60 and 80 IU/l. See Table 34.




| | -2 | 0 | 1 | 2 | 5 | 6 | 9 | 13 | 15 |
|---|---|---|---|---|---|---|---|---|---|
| E2 (pmol/l) | 5.0 | 5.0 | 5.0 | 5 | 5 | 114 | 107 | 68 | 89 |
| FSH (IU/l) | 5.4 | 57.5 | 76.0 | 50.8 | 83.5 | 52.9 | 60.3 | 58.8 | |
| LH (IU/l) | 6.2 | 65.5 | 78.1 | 57.5 | 78.6 | 66.8 | 60.8 | 72.9 | 80 |

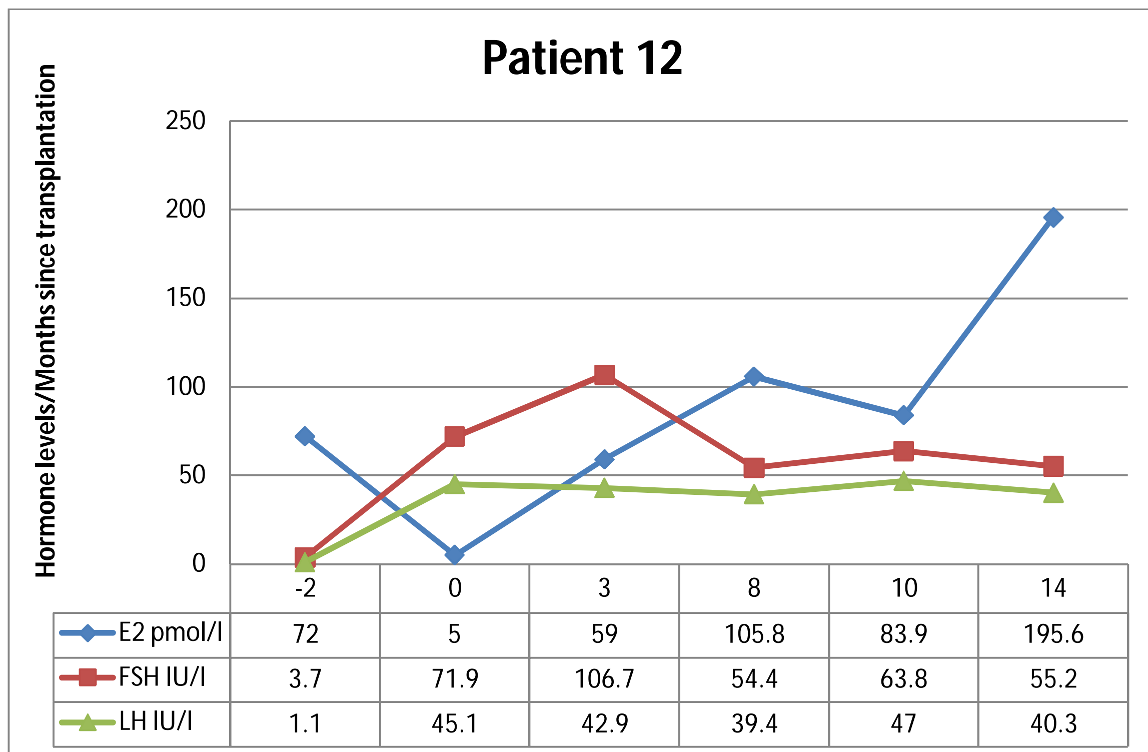
*Table 34 Summary of hormone profile patient 11*

**Patient 12**

Patient number 12 was a 33-year old who presented with stage IIIb cervical carcinoma for which she was offered radiotherapy with chemosensitization. Pre-treatment she had a laparoscopic oophorectomy with removal of both ovaries. Unfortunately the patient had anaphylactic reaction to the anaesthetic and was admitted postoperatively into the Intensive Care Unit. She recovered fully.

This patient had a total of 68 strips available for cryopreservation. She had very small ovaries. Re-insertion was done at the same time as the brachytherapy under general anaesthesia.

Re-insertion of 14 strips cryopreserved in DMSO was performed. At insertion of the brachytherapy she had an oestrogen level at month three of 59 pmol/l and a peak of over 100 pmol/l at month eight. She continued to produced oestrogen at month 14 but was then lost to follow-up. See Table 35.




| | -2 | 0 | 3 | 8 | 10 | 14 |
|---|---|---|---|---|---|---|
| E2 pmol/l | 72 | 5 | 59 | 105.8 | 83.9 | 195.6 |
| FSH IU/l | 3.7 | 71.9 | 106.7 | 54.4 | 63.8 | 55.2 |
| LH IU/l | 1.1 | 45.1 | 42.9 | 39.4 | 47 | 40.3 |

*Table 35 Summary of hormone profile patient 12*

**Patient 13**

Patient number 13 was a 25-year old G3 P3 that presented with stage IIIb cervical carcinoma. She had a bilateral oophorectomy pre-treatment and she was offered radical radiotherapy and chemosensitization.

This patient had 64 strips available for cryopreservation. Twelve DMSO cryopreserved strips were re-implanted six weeks after completion of radical radiotherapy under local anaesthesia.

Unfortunately no blood sample for hormone profile was taken on the day of re-implantation.

She had a relatively short follow-up period but started producing oestrogen at month eight and continued to have elevated oestrogen levels until at least month thirteen. She had a steady increase in the gonadotrophins levels after month eight. See Table 36.

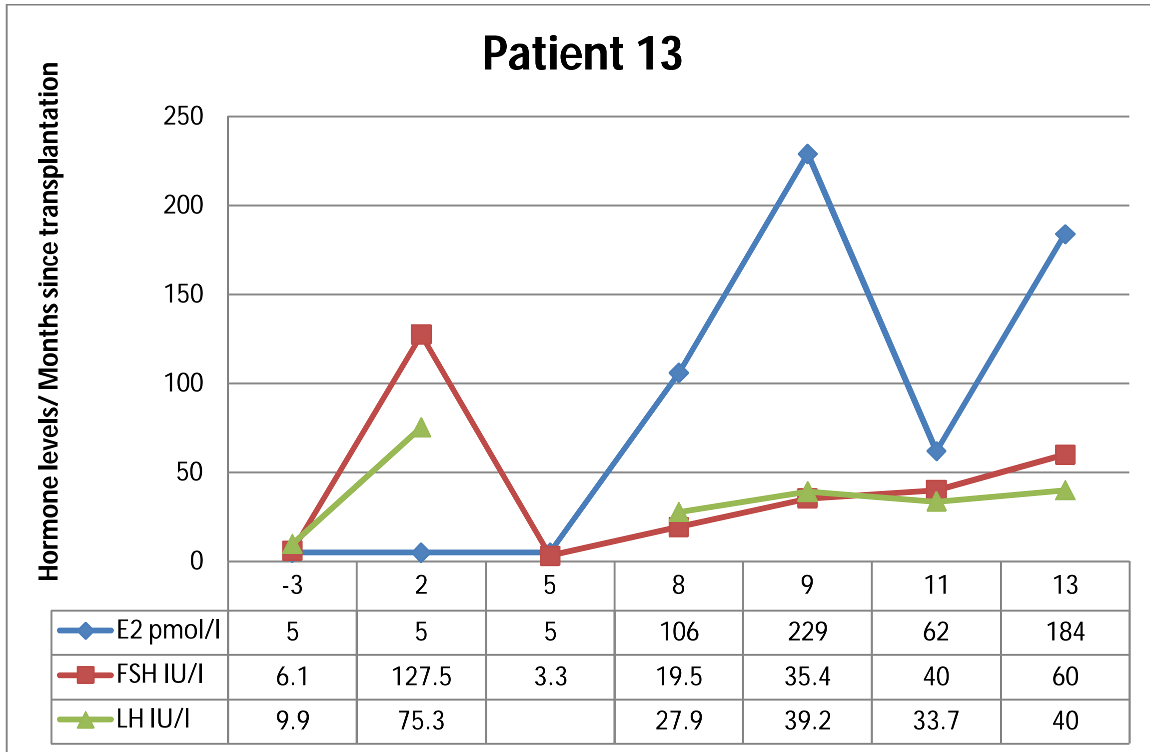



*Table 36  Summary of hormone profile patient 13*

## *Discussion*

This is the largest cohort of patients reported from one centre with implantation results after cryopreservation of cryopreserved ovarian tissue. It confirms that ovarian tissue slow freezing is a useful tool to protect follicles against irreversible damage caused by cancer therapy. Patients were very positive about the possibility of safeguarding their future hormone production.

Laparoscopic oophorectomy was in general without any complications.  One of the patients had only one ovary removed due to abnormal adhesions of the right ovary to the pelvic side. This patient is not included in the detailed description, because she declined transplantation of cryopreserved tissue. The oophorectomy procedure

became easier with time as experience improved. In the beginning the positioning of the trocars made it difficult to orientate the endobag but after a few cases, the procedure took less than 45 minutes in total. All the specimens were removed through the primary 10 mm sub umbilical port but it was sometimes necessary to increase the size of the incision slightly.

Re-implanted ovarian cortex may require 4-5 months before follicogenesis resume. [10] [21] This may be due to the fact that only primordial follicles survive the freezing process and that more developed (larger) germ cells undergo wastage due to intracellular ice formation and disruption of the microtubules. The earliest indication of oestrogen production occurred in two patients during month three. The laboratory defined oestrogen levels below 37 pmol/l as non-detectable. Of the 13 patients reported here, only patient, number 10 did not have any evidence of oestrogen production. See Table 37. The routine imaging of the ovarian tissue was not performed at every follow-up visit. However, patient number 1, 3, 5, 7, 9 and 13 all had visible distension of the transplanted tissue, suggesting follicle formation.

The term menopause is defined as the permanent cessation of menstruation after the loss of ovarian activity. In clinical practice, the diagnosis of menopause is usually based on a history of amenorrhoea and can be confirmed with a hormone profile. In the peri-menopause, the menstrual cycle will increase in length, and the level of FSH usually increases significantly. [53] Inhibin levels are usually lowered but oestrogen and LH may still be at normal levels. With normal ageing the follicular reserve starts to decline at around 35 years of and Inhibin may be a better marker of ovarian reserve than follicle stimulating hormone. [54]

| Patient | Age | Number strips | Cryoprotectant | E2 Production ( >37pmol/l) | Length of follow-up |
|---|---|---|---|---|---|
| **1** | 35 | 11 | DMSO | Months 8 and 11 | 19 |
| **2** | 37 | 12 | PROH | Months 10 and ?12 | 16 |
| **3** | 28 | 14 | DMSO | Months 8 and 11 | 14 |
| **4** | 38 | 10 | PROH | Months 11,12,14,19 | 19 |
| **5** | 42 | 12 | PROH | Months 8,11,13,25 | 25 |
| **6** | 40 | 15 | PROH | Months 4,10,13,18,21 | 21 |
| **7** | 37 | 13 | DMSO | 6,10,13,16 | 19 |
| | | 14 | DMSO | 19 | |
| **8** | 28 | 10 | DMSO | 9 and ?11 | 16 |
| **9** | 30 | 20 | DMSO | ?3,5,6,7,9,10 | 10 |
| **10** | 39 | 12 | DMSO | No oestrogen production | 21 |
| **11** | 27 | 14 | DMSO | 6,9,13,15 | 15 |
| **12** | 33 | 14 | DMSO | 3,8,10,14 | 14 |
| **13** | 25 | 12 | DMSO | 8,9,11,13 | 13 |

*Table 37  Summary of individual patient details*

Anti-Mullerian Hormone can serve as a marker for the evaluation of the ovarian reserve. AMH is expressed in human follicles immediately after recruitment right up to the selection stage (4-6mm diameter). Levels in serum of AMH in normal cycling women decline with age and become undetectable in menopausal women.  [55]

It is very difficult to pinpoint an exact level of FSH and LH, which corresponds with clinical menopause. A level of 30 international units per litre is often quoted as a marker of biochemical menopause. Some authors report an early return of FSH to pre-menopausal levels after re-implantation of cryopreserved ovarian tissue. Others report of post re-implantation hormone levels that FSH and LH levels remained elevated. [46] [30] In the case series reported here nearly all the levels of gonadotrophins were above the 30 international units per litre despite oestrogen production. The amount of tissue grafted may play an important role, because in all cases reported here only a small fraction of the dissected ovarian cortex was implanted. The reduced number of follicles in the transplanted tissue may not produce high enough levels of Inhibin to control gonadotrophin production. The effect of reduced Anti Mullerian Hormone produced by the transplanted tissue is also possibly responsible for persistent high gonadotrophin levels.

High levels of circulating gonadotrophins may recruit a high number of follicles soon after implantation. This may increase the number of follicles that is destined to become artretic. It may be beneficial to control the gonadotrophin secretion by administering gonadotrophin releasing hormone antagonists before tissue re implantation. [46] In animals a high number of mitoses in granulosa cells were noted shortly after transplantation and the number of primordial (resting) follicles decreased rapidly due to recruitment of large numbers of follicles. [56]

After careful electron microscopic study of five early cases a possible improved outcome was found in the DMSO group. After the results became available and despite the fact that differences were not statistically significant, a decision was

made to only use DMSO cryopreserved tissue for transplantation. However, all four cases that had transplanted tissue from the PROH protocol produced oestrogen. This indicates that despite ultrastructural damage the effect was not lethal, and that tissue survival was adequate for clinical hormone production.

Other groups reported functional grafts up to 21 and 25 months after re-implantation. [21] The follow-up in the current series of patients reported here is relatively short but at least three patients were still producing detectable levels of oestrogen more than 19 months after implantation.

## *Conclusion*

This clinical study confirms that ovarian tissue cryopreservation with two different cryprotectant protocols was successful in restoring low level oestrogen production in the majority of patients. The aim of this prospective clinical study was not to restore fertility because all the patients described here received whole pelvic irradiation which made normal pregnancy impossible. However, by demonstrating the successful hormone production of tissue implanted in a heterotopic position, a proof of principle was demonstrated for use in many different clinical situations.

There was no time delay for the initiation of chemotherapy or radiotherapy. That makes the storage of ovarian cortex a very practical option compared to oocyte retrieval and preservation of embryos or oocytes. It is inevitable that the stimulation cycle to obtain multiple follicles for aspiration will delay the onset of treatment. This makes oocyte cryopreservation problematic in many clinical scenarios. The number

of primordial follicles in ovarian tissue cryopreservation is substantial and may offer a better chance of subsequent fertility when compared to oocyte or embryo freezing.

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# Chapter 6: Integrated approach to fertility sparing in cancer treatment for young women


## *Abstract*

An integrated approach for fertility preservation is necessary and clinicians should be aware of their respective roles in the management of young women with cancer.

The incidence and treatment of cancer in young women and how its negative effects may be prevented or mitigated is discussed. Aspects of chemotherapy, radiotherapy and surgery are evaluated where it may affect future reproductive health. The role of oocyte and ovarian tissue cryopreservation is discussed. Guidelines are provided for clinicians.

## *Introduction*

"First do no harm" is the translation of *primum non nocere*; a precept of Hippocrates (around 460- 377 B.C.). *Non-maleficence*, which is the ethical principle that flows from this, is fundamental to medical ethics. *Beneficence* refers to actions that promote the wellbeing of others. In the management of serious disease like cancer there is often a conflict between these two moral imperatives. The obligation *not to harm* others is usually more stringent than the obligation to help others. In an effort to cure cancer (an act of beneficence) the treatment itself may at the same time also cause significant harm. Many treatments have serious, harmful late effects including an effect on future fertility and hormone production. Every young patient deserves to get safe and oncologically effective treatment but with the least long term negative effects as possible. A multi-disciplinary team approach involving the oncologist, surgeon and counsellor should include a reproductive health specialist if fertility may be compromised by cancer therapy. Clinicians should consider the long-term side-effects of treatment before mapping out a treatment plan and use fertility sparing techniques where appropriate.

William Harvey was a physician to King Charles I in England. He was a keen hunter and often accompanied the king on his weekly hunting trips but still had enough time to publish a great work called De Generatione Animalium (on the generation of animals) in 1651. On the front page there was a drawing of Zeus sitting on his throne holding an egg with the shell open to reveal the emergence of various animals, birds and insects including fallow deer that he enjoyed hunting. On the egg the inscription was "Ex ovo omnia" (everything comes from the egg). Harvey

speculated that all life forms started in an egg but he found it difficult to substantiate this belief from his experience as a hunter. He believed that the seed of the male would coagulate the menstrual blood in order to form an egg which would produce the foetus. He could not demonstrate this in fallow or red deer because of their very short breeding season and no menstruation. At that point he could see no use for “female testicles”. Despite these difficulties he continued to elaborate in his thesis on the theory that the egg was the source of all living things. It was only a few years later that Neils Stensen of Denmark described that fact that “female testicles” of mammals contained eggs and should rather be called ovaries. According to some historians he was undoubtedly the unsung hero of the ovary “we should be referring to ovarian follicles as Svensen’s follicles not Graafian follicles”. [1]

**Cancer in young people**

The incidence of childhood cancer (generally calculated under and up to the age of 15) is 110 to 130 per million children per annum. [2] It is estimated that the cumulative risk of a child being diagnosed with cancer is slightly higher in boys at 1:444 compared to girls which is one is 1:594. [3] In South Africa accurate figures for childhood cancer are not available. The reported incidence is around 70-80 per million, however it is estimated that one in 600 children will suffer from cancer before they turn 16. Many of these cancers are diagnosed late or may not be diagnosed at all. [4]

With modern treatment methods the majority of children will survive cancer. The most prevalent tumours before the age of 15 include acute lymphoblastic leukaemia /

non Hodgkin's lymphoma, Hodgkin's lymphoma, brain tumours and Wilms' tumour. [5] The prognosis for patients diagnosed before the age of 15 improved dramatically over the last three decades and more than 80% of cases survive longer than 5 years. More than 70% will be long-term survivors. Information from cancer statistics during the 1970 to 1980's indicate that in the United States, the cure rate of all childhood cancers combined was between 70-90%. [6] The estimated 5 year survival of children of both sexes improved from 50.4% in 1973 to 79.2% in 1990. [7]

Older children and young adults have historically not been studied to the same extent as young children with regards to cancer incidence. The age range for adolescence for the purpose of oncology studies has been set at 15-19 years. [8] "Young Adult Oncology" refers to a larger group of young people between the ages of 15-29 years. [9] This group of young people is at a very important developmental phase particularly for establishment of normal hormonal and sexual function. The most important tumours in the United States are summarised in Table 38. Figures from the National cancer registry of South Africa indicate that the most common cancer of reproductive women aged 15-29 in South Africa is cervical cancer followed by breast cancer and Kaposi's sarcoma. [10]

| Age | 15-19 | 20-24 | 25-29 |
|---|---|---|---|
| **Lymphoma %** | 26 **(1)** | 22 **(1)** | 16 **(2)** |
| **Leukaemia %** | 12 **(2)** | 7 | 4 |
| **Central nervous system %** | 10 **(3)** | 7 | 5 |
| **Endocrine system %** | 9 | 12 | 11 |
| **Skin %** | 8 | 14 **(2)** | 18 **(1)** |
| **Male genital %** | 8 | 13 **(3)** | 11 |
| **Female genital %** | 8 | 8 | 12 **(3)** |

*Table 38 The relative frequencies (rank) of cancers in adolescents and young adults in the United States aged 15-29. [11]*

Anti-cancer therapy may increase the rate of follicular loss and therefore premature ovarian failure, with subsequent premature menopause which is one of the common toxic side effects of cancer treatment. [12] Chemotherapy causes amenorrhoea in 40-68% of cases depending on various factors. [13] Chemotherapy may damage primordial follicles directly with demise of follicular cells. Human and animal studies also showed that chemotherapy damage ovarian pre-granulosa cells, with increased apoptosis during oocyte and follicle loss. [14] [15] Ovarian stromal cell damage plays a key role due to their importance in hormonal function of follicles and perhaps also in the repair process after chemotherapy treatment. [16] Vascular complications associated with anti-neoplastic agents have been reported. One recognised mechanism for such toxicity include drug-induced endovascular damage. [17]

**Aspects to consider before chemotherapy**

One of the most important clinical factors that may influence the risk for permanent ovarian damage is the age at treatment. The risk for ovarian failure increases with age. [18] [19] [20] [21] This fact should be kept in mind when advice is given to women who need chemotherapy after the menarche.

Certain anti-neoplastic agents cause more ovarian damage. The risk associated with the use of some agents are summarised in Table 39. In certain circumstances muti-drug regimes may be tailored to decrease the risk for ovarian failure. In a study on primordial follicle counts after chemotherapy it was found that "patients treated with alkylating regimens had significantly lower primordial follicle counts when compared with those who received non- alkylating agents and with those who did not receive any chemotherapy." [22]

| High Risk | Moderate Risk | Low or No Risk |
|---|---|---|
| Cyclophosfamide | Cisplatin | Methotrexate |
| Busulfan | Adriamycin | 5- Fluorouracil |
| Melphalan | Paclitaxel (limited evidence) | Actinomycin D |
| Procarbazine | | Bleomycin |
| Nitrogen Mustard | | Vincristine |
| Chlorambucil | | |
| Vinblastine | | |
| Cytarabine | | |

*Table 39 Risk for gonadotoxicity associated with different chemotherapy agents. Adapted from [16] and [23]*

Smaller primordial follicles may survive the insult of chemotherapy better and suppression of follicle development during treatment may protect the ovary. Suppression of follicle development by gonadotrophin-releasing hormone agonists (or antagonists) (GnRHa) or combined oral contraceptives have been reported in the literature. Two recent review articles on the topic of ovulation suppression with GnRHa to protect against chemotherapy induced ovarian toxicity and amenorrhoea summarises the lack of a definite answer. [24] [25] The first was of the opinion that the currently available information is not enough to reach a definite conclusion and the second also concluded their review by stating that there is not enough convincing statistical evidence concerning the reduction of premature ovarian failure (POF) to treat young women with GnRH agonists. Conflicting reports of the protection makes

further studies essential. The best evidence so far for the potential benefit of oral contraceptives during chemotherapy is from a large retrospective report from Germany. [26] However, oral contraceptives have never been adequately tested in a randomised controlled trial. [27]

## Aspects to consider before radiotherapy

The estimated LD50 (median lethal dose) of an oocyte is is less than 4 Gray. [12] Shielding of ovaries may protect against radiation induced failure. [28] [29] The uterus may be damaged by radiotherapy. Reduced uterine volume and decreased elasticity of the myometrium can be found in girls who received pelvic-, abdominal- or total body irradiation before puberty. [30] [31] Cranial irradiation may cause hypo-pituitarism in doses over 30 Gray. [32] [33] Effective shielding during treatment reduce the dose to healthy tissue.

Ovarian trans-position outside the field of radiotherapy may reduce the dose to the ovary. [34] Lateral trans-position of the ovaries to the para-colic gutters can reduce the radiotherapy dose by up to 95%.[35]

## Aspects to consider before surgery

Treatment for cervical pre-cancer can cause long-term reproductive sequelae. One has to caution against blanket treatment of all patients purely for abnormal cytology results. We know that between five and 40% of all patients with abnormal cytology might not have histological abnormality on LLETZ cone biopsy. [36] It is therefore

necessary to do a thorough colposcopic evaluation and to treat only those patients with a recognizable abnormality. If there is doubt about the severity of the abnormality, a biopsy should confirm a CIN II lesion or higher to justify treatment by destruction or resection of the transformation zone. Over-treatment may jeopardize a patient's future reproductive performance. Cervical conisation might lead to infertility by causing cervical stenosis or a decrease in the production of cervical mucus. [37] Cervical stenosis seems to occur more often after cold knife cone biopsy than laser conisation or LLETZ. A larger cone is associated with a greater occurrence of cervical stenosis. [38] [39] Other risks associated with previous cervical surgery include spontaneous miscarriages [40], premature labour before 37 weeks [41] and an increase in the number of caesarean sections done for cervical dystocia. [40] [42] [43]

Small early stage cervical cancers may be suitable for uterus-sparing surgery. [44] Dargent and others developed radical trachelectomy for the treatment of early cervical tumours.[45] [46] Even though subsequent pregnancies are sometimes complicated by miscarriage, premature preterm rupture of membranes, early delivery and premature infants it offers a solution for patients with early tumours who would like to retain the chance for pregnancy. [47]

There are a number of young women who develop endometrial cancer. They usually present with excess oestrogen associated with obesity, infertility and nulliparity. There are many case reports of early stage, low-grade endometrial cancers that have been treated using uterus conserving therapies. [48] [49] [50] [51] A small

number of early-stage endometrial cancers were treated with partial hysteroscopic resection followed by GnRHa for three months. [52]

Selected patients with Borderline Ovarian Tumours (BOTs) or early stage invasive epithelial cancer (FIGO stage Ia) with well differentiated tumours may be managed with fertility sparing surgery. [53] [54] [55] [56] [57] [58] In these patients accurate surgical staging is of the utmost importance. Only if, after careful surgical staging, the patient remains a FIGO stage Ia can conservative management and omission of adjuvant therapy be safely advised.

### *Cryopreservation of oocytes or ovarian tissue as an option for fertility sparing*

In certain clinical situations the ovaries of a young woman will be exposed to high doses of toxic cancer therapy. If surgical transposition will be of no benefit due to systemic chemotherapy an option may be to remove ovarian tissue for the period of therapy. Preservation of ovarian tissue before cancer treatment and later transplantation of the tissue (when toxic treatment is finished) may restore hormonal function and sometimes even fertility.

The self-evident candidates for ovarian tissue or oocyte cryopreservation are young women and girls with haematological cancer who need aggressive chemotherapy regimes. Children often receive whole body irradiation with doses adequate to cause premature ovarian failure in cases where bone marrow transplants are

prepared. There are, however, many other forms of cancer where treatment directly affects ovarian function.

**Oocyte cryopreservation**

The survival and ultimate normal functioning of an oocyte after the process of cryopreservation has been more difficult to achieve when compared to sperms and embryos. Success is compromised by the complexity of the intra- and sub-cellular organelles which are far more complex than in smaller cells like sperm and pre-implantation embryos. Early work done on frozen murine oocytes showed spindle abnormalities and other damage due to cryopreservation and exposure to cryoprotectants. [59] [60] Metaphase II oocytes are structurally quite complex with the fragile meiotic spindle being one of the most vulnerable structures.

There are indications that, with improving technical ability, slow-freezing of oocytes is becoming more efficient with increases in survival rates of individual oocytes, better fertilization rates and ultimately an improvement in pregnancy rates. [61] Recently vitrification (ultra rapid cooling technique) of human oocytes has been described and the success rate after vitrification seems to be improving. [62]

The ovarian stimulation period before oocyte retrieval remains the most problematic aspect of oocyte cryopreservation because of the delay in the start of treatment for patients with underlying malignancy. In order to reduce the time necessary ovarian stimulation may be started in the luteal phase. [63] A very similar time from start to retrieval was achieved in patients starting stimulation in the follicular phase and the

luteal phase of the menstrual cycle. Another concern about possible increased incidence of chromosomal abnormalities in cryopreserved oocytes seems to be ungrounded. There is no evidence that chromosomal abnormalities are more frequent when fertilization does occur, even if it is at a lower rate than with fresh oocytes, [64] [65]

The number of oocytes retrieved in a cycle is usually low. In a report on 18 young women with a mean age of 19 years (range 14-26), an average of 15 oocytes were retrieved per cycle. [66] The success of stimulation protocols is lower in older women and, if we consider that only a fraction of the thawed oocytes will fertilise, it may be better to preserve ovarian tissue where a larger pool of oocytes will survive. The advantage of oocyte freezing is a potentially lower risk of reseeding of cancer cells because a much smaller volume of tissue is transplanted.

**Ovarian tissue cryopreservation**

Ovarian tissue samples are relatively large and morphologically complex. Cryopreservation methodology is difficult to optimise and standardise. Ovarian tissue cryopreservation is still in its experimental stages and is not a routine option offered to cancer patients. Despite the experimental nature of ovarian tissue cryopreservation, ovarian tissue harvesting and banking has been offered to many patients in clinical practice over the last two decades. [67] [68] [69] The histological examination of fresh and thawed ovarian tissue for metastatic tumour should be part of routine practice to evaluate tissue for the presence of microscopic metastases. [70] [71]

Re-implantation of ovarian tissue may be immediate after completion of treatment if the aim is to restore endocrine function only. If the aim is to restore fertility, it is preferable to wait for a disease free period of around two years since most malignancies recurs during the first 24 months after completion of treatment. Tissue transplantation to the original site or to a transplantation location near the original site is referred to as *orthotopic* transplantation. Transplantation to an intra-abdominal site has been achieved with either laparoscopy or laparotomy. Several authors described ovarian transplantation to the ovarian stump or the peri-ovarian region. [72] Transplantation to sites distant from the original is referred to as *heterotopic*. Many alternative heterotopic sites have been tested for re-implantation including the arm, rectus abdominus muscle, the anterior abdominal wall, and the supra-pubic area. [70]

Many small case series have been published about hormonal and reproductive outcome after re-implantation of frozen-thawed ovarian tissue. In a study from South Africa we described hormonal function in 12 out of 13 patients who had heterotopic transplantation of frozen-thawed ovarian tissue. After laparoscopic oophorectomy ovarian cortical tissue strips were made by first bisecting the ovary and removing most of the cortex. The cortex was then divided into 2x5 mm strips. The strips were preserved using a controlled slow freezing method. After cancer treatment rapid thawing of the tissue and heterotopic transplantation was performed to the upper arm. The study is a proof of principle and shows the way forward for further study. Many questions still remain including:

- What is the optimal freezing and thawing protocol for tissue strips
- How much tissue should be transplanted at one time
- How long will tissue continue to function?
- Should we suppress gonadotrophins before implantation as suggested by others? [73]
- Can we use other markers of ovarian reserve like Anti-Mullerian Hormone and Inhibin to predict graft longevity and function?

## *Conclusion*

The diagnosis of cancer usually comes as a tremendous shock to any person. It is even more unexpected in children and young people and there is a lot of anxiety and fear around the time of diagnosis and planning of treatment. Clinicians involved in the care of these young people cannot escape the pressure of the situation and often focus solely on the survival and cure of the patient without the necessary concern about the long-term effects of the proposed treatment plan. There may be completely safe and effective treatment options that will minimise late harmful effects, including hormonal dysfunction and infertility. To improve the chances of taking a good decision about treatment it is important to involve a multidisciplinary team, including a fertility expert. At the time of diagnosis the focus will invariably be on cure of the disease but a calm, informed discussion about future fertility can go a long way to inform the patient and the family members about the risks involved in a particular treatment plan and possible solutions.

All medical personnel involved in the care of young people with cancer should be educated about the available fertility sparing options.

*Medical oncologists* need to take into consideration the risk for gonadotoxicity with each chemotherapy agent or combination of agents that will be used. Effective regimes with lower risk are often available. Combined oral contraceptives and gonadotrophin suppression may offer some protection of follicular reserve but at present there is not enough evidence to support this strategy entirely. Gonadotrophins may be beneficial for other reasons in certain hormone sensitive tumours including oestrogen dependent breast cancers. If the risk for premature ovarian failure is high, e.g. an older patient receiving ovarian-toxic treatment, cryopreservation of ovarian tissue constitutes a viable option for future re-implantation.

*Radiation oncologists* should be aware of the risk of ovarian and uterine damage due to radiotherapy, whether it is because of direct exposure or scattered radiation. Shielding is often a fairly simple way of protecting ovarian and uterine function although it can be time-consuming. Pre-exposure surgical transposition may be of help in selected cases. However, there may still be a risk for some damage due to scatter radiation and vascular compromise. If the risk for ovarian failure is high, cryopreservation of ovarian tissue or oocytes should be considered. At present, there is no convincing evidence that suppression of ovarian function with contraceptives or gonadotrophins make any difference whatsoever in the risk for future ovarian failure after radiotherapy exposure.

*Surgeons* should think and plan carefully before any surgical intervention taking into consideration the longer term risks associated with the planned surgery. Even small procedures performed for pre-cancer lesions may have profound effects on future pregnancies. In early invasive disease a conservative operation with retention of future fertility is often available.

*Infertility specialists* must be well-informed about the different options for fertility preservation. They should be able to give suitable advice, not only to the patient and family, but also to the other care givers in the team. Cryopreservation of embryos and semen samples has long been an integral part of assisted reproductive techniques. It is now important to also develop the laboratory protocols for cryopreservation of oocytes and ovarian tissue.

In the South African environment we have now demonstrated that the laboratory facilities are able to offer cryopreservation of ovarian tissue as an effective service to patients. By investigating two different cryopreservation protocols and showing no significant difference in the ultra structure of the tissue it is clear that there may be more than one effective protocol for successful storage.

Developing the necessary laboratory capacity by training of laboratory technicians in the new techniques are of the utmost importance. A well controlled clinical environment with very high safety and ethical standards is essential as the only way to protect the cryopreserved tissue, and ultimately the patient. Histological evaluation of tissue for possible metastatic disease prior to preservation must always

be part of the process. Centres of excellence need to be established with expertise in this rather low-frequency service.

In a South African setting cryopreservation of ovarian tissue can be done effectively. The re-implantation of previously cryopreserved tissue has been performed successfully in an adequately large group of patients to demonstrate the principle. Although this technique is still experimental, we can now argue that cryopreservation of ovarian tissue should become part of good clinical practice in suitable patients.

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