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ORIGINAL ARTICLE ***Infertility***

Ninety-five orthotopic transplantations in 74 women of ovarian tissue after cytotoxic treatment in a fertility preservation network: tissue activity, pregnancy and delivery rates

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STUDY QUESTION: What is the success rate in terms of ovarian activity (menstrual cycles) as well as pregnancy and delivery rates 1 year after orthotopic ovarian transplantations conducted in a three-country network?

SUMMARY ANSWER: In 49 women with a follow-up > 1 year after transplantation, the ovaries were active in 67% of cases and the pregnancy and delivery rates were 33 and 25%, respectively.

WHAT IS KNOWN ALREADY: Cryopreservation of ovarian tissue in advance of cytotoxic therapies and later transplantation of the tissue is being performed increasingly often, and the total success rates in terms of pregnancy and delivery have been described in case series. However, published case series have not allowed either a more detailed analysis of patients with premature ovarian insufficiency (POI) or calculation of success rates based on the parameter 'tissue activity'.

STUDY DESIGN, SIZE, DURATION: Retrospective analysis of 95 orthotopic transplantations in 74 patients who had been treated for cancer, performed in the FertiPROTEKT network from 2008 to June 2015. Of those 95 transplantations, a first subgroup (Subgroup 1) was defined for further analysis, including 49 women with a follow-up period > 1 year after transplantation. Of those 49 women, a second subgroup (Subgroup 5) was further analysed, including 40 women who were transplanted for the first time and who were diagnosed with POI before transplantation.

[†] These authors contributed equally to this paper.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Transplantation was performed in 16 centres and data were transferred to the FertiPROTEKT registry. The transplantations were carried out after oncological treatment had been completed and after a remission period of at least 2 years. Tissue was transplanted orthotopically, either into or onto the residual ovaries or into a pelvic peritoneal pocket. The success rates were defined as tissue activity (menstrual cycles) after 1 year (primary outcome) and as pregnancies and deliveries achieved.

MAIN RESULTS AND THE ROLE OF CHANCE: The average age of all transplanted 74 women was 31 ± 5.9 years at the time of cryopreservation and 35 ± 5.2 at the time of transplantation. Twenty-one pregnancies and 17 deliveries were recorded. In Subgroup 1, tissue was cryopreserved at the age of 30 ± 5.6 and transplanted at 34 ± 4.9 years. Ovaries remained active 1 year after transplantation in 67% of cases ($n = 33/49$), the pregnancy rate was 33% ($n = 16/49$) and the delivery rate was 25% ($n = 12/49$). In Subgroup 5, tissue was cryopreserved at the age 30 ± 5.9 years and transplanted at 34 ± 5.2 years. Ovaries remained active 1 year after transplantation in 63% of cases ($n = 25/40$), the pregnancy rate was 28% ($n = 11/40$) and the delivery rate was 23% ($n = 9/40$). The success rates were age dependant with higher success in women who cryopreserved at a younger age. In Subgroup 5, tissue was exclusively transplanted into the ovary in 10% ($n = 4/40$) of women and into a peritoneal pocket in 75% ($n = 30/40$), resulting in spontaneous conceptions in 91% of patients ($n = 10/11$).

LIMITATIONS, REASONS FOR CAUTION: The data were drawn from a retrospective analysis. The cryopreservation and transplantation techniques used have changed during the study period. The tissue was stored in many tissue banks and many surgeons were involved, leading to heterogeneity of the procedures. However, this does reflect the realistic situation in many countries. Although patients with POI were evaluated before transplantation to allow specific analysis of the transplanted tissue itself, the possibility cannot be excluded that residual ovarian tissue was also reactivated.

WIDER IMPLICATIONS OF THE FINDINGS: This is the largest case series worldwide to date and it confirms that cryopreservation and transplantation of ovarian tissue can be a successful option for preserving fertility. Persistent tissue activity 12 months after transplantation suggests that the pregnancy and delivery rates may increase further in the future. As transplantation into the peritoneum results in a high success rate, this approach may be an alternative to transplantation into the ovary. However, in order to establish the best transplantation site, a randomized study is required.

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Key words: fertility preservation / ovarian tissue / transplantation / ovary / peritoneum / chemotherapy / radiotherapy / pregnancy rate / delivery rate

Introduction

Since the first births that were achieved after transplantation of ovarian tissue (Donnez *et al.*, 2004; Meirow *et al.*, 2005), cryopreservation of ovarian tissue has become an increasingly widely used technique for preserving fertility in advance of cytotoxic treatments. At least 60 live births have been reported worldwide (Donnez and Dolmans, 2015; Jensen *et al.*, 2015). The procedure has also been incorporated into numerous national and international networks and programmes (Oncofertility Consortium; FertiPROTEKT; International Society for Fertility Preservation; Rosendahl *et al.*, 2011). In the FertiPROTEKT network, cryopreservation of tissue is performed more often than ovarian stimulation and cryopreservation of oocytes (von Wolff *et al.*, 2015), a technique which is no longer experimental according to the criteria by the ESHRE Special Interest Group 'Ethics and Law' (Provoost *et al.*, 2014).

In Germany, ~400 ovarian tissue cryopreservation procedures are carried out each year, representing a total figure of more than 2500 cryopreservations to date (von Wolff *et al.*, 2015). In Denmark, the figure is 70 cryopreservations per year, representing a total of 800 (C. Y. Andersen, personal communication). These figures represent ~5 cryopreservations per million population per year in Germany and ~13 per million in Denmark. Extrapolated to the population of 500 million in the European Union, this would represent 2500–6500 cryopreservations of ovarian tissue per year.

These figures clearly indicate that the technique has in the meantime come into widespread use; but they also show that it is extremely important not only to clarify the indication for cryopreservation, but also to optimize the process of cryopreservation and the transplantations that result. Numerous recommendations have been published (von Wolff *et al.*, 2011; ISFP Practice Committee *et al.*, 2012; Loren *et al.*, 2013; Practice Committee of American Society for Reproductive Medicine, 2013). National programmes with centralized cryobanks have been established in several countries (Rosendahl *et al.*, 2011; von Wolff *et al.*, 2011) in order to concentrate cryopreservation in centres with demonstrated expertise. The obvious advantages of either centralized or network-organized cryopreservation and of transplantation are that the available expertise is pooled to allow optimal implementation of the individual treatment steps involved. Larger case series have accordingly been reported by established networks in particular (Donnez *et al.*, 2013; Dittrich *et al.*, 2015).

These case series have proved the principle of achieving pregnancies following the transplantation of ovarian tissue. However, due to the size and heterogeneity of the groups of patients concerned, it has until now been difficult to obtain reliable and practical information. For example, it has not been possible to carry out systematic analyses, classifying women as with or without premature ovarian insufficiency (POI), also known as premature ovarian failure, or after initial and repeated transplantations—limiting the validity of the studies conducted.

Since no prospective studies are available yet, it seems appropriate to investigate transplantations within a large multicentre network that has a certain degree of homogeneity with regard to ovarian tissue harvesting and the cryopreservation process, on the basis of largely standardized recommendations (von Wolff *et al.*, 2011, 2015). The FertiPROTEKT network meets these requirements, as the tissue is mainly stored in two central cryobanks and the majority of the transplantations are carried out in specialized centres. The large number of transplantations included in the FertiPROTEKT network thus makes it possible for the first time to go beyond pure description of the successful transplantations carried out so far.

The aim of the present study was therefore not only to present a comprehensive and transparent account of all transplantations carried out, but also to conduct subgroup analyses based on various criteria. In the main analysis, ovarian activity 1 year after transplantation was defined as the primary target criterion. For the subgroup analyses, only transplantations that took place before September 2014—i.e. with a follow-up period ≥12 months—were included in the further analysis.

Materials and Methods

The FertiPROTEKT network and the data registry

The FertiPROTEKT network (www.fertiprotekt.com) was founded in 2006 as an association of university and non-university reproductive medicine centres. Approximately 40 centres were members in 2007, and in 2015 there are now 101. The cryopreservations and transplantation of ovarian tissue conducted in these centres have been documented and analysed annually since 2007 (von Wolff *et al.*, 2015). Annual 2-day meetings of the network members, with obligatory attendance, and published (von Wolff *et al.*, 2011) and unpublished recommendations by the network ensure as far as possible that tissue is harvested and cryopreserved in as standardized a protocol as possible. The ~2500 cryopreserved tissue samples obtained to date have mainly been stored in the central cryobanks in Germany in Bonn (n = 1400, July 2015) and Erlangen (n = 500, July 2015), in Austria in Innsbruck (n = 170, July 2015) and in Switzerland in Berne (n = 100, July 2015). Tissue has also been stored in other centres, but specific data on the number of centres and the number of tissue samples do not exist. Storage in Bonn, Germany is mainly (in 91% of cases) carried out after overnight transport of the tissue at ~4°C.

Tissue from 41 of the 74 patients in whom transplantations were carried out was cryopreserved and stored in the Bonn cryobank; tissue from 10 patients was cryopreserved and stored in the Erlangen cryobank; and tissue from 23 patients was stored in other cryobanks. The 74 women who underwent transplantation had surgery in Erlangen, Germany (n = 33), Neuss, Germany (n = 8), Heidelberg, Germany (n = 7), Bonn, Germany (n = 5), Düsseldorf, Germany (n = 4), Bremen/Würzburg, Germany (n = 3), Baden-Dättwil, Switzerland (n = 2), Bern, Switzerland (n = 2), Hamburg, Germany (n = 2) and Innsbruck, Austria (n = 2). One patient each underwent transplantation in Germany in Freiburg, Jena, Lübeck, Mannheim, and Tübingen and in Switzerland in St. Gallen.

The data on patients, transplantation techniques and results were stored in anonymized form in the FertiPROTEKT registry.

Data analysis and patient characteristics

Up to the end of June 2015, a total of 95 transplantations had been carried out in 74 women. The average age of the women was 31 ± 5.9 years at the time of cryopreservation and 34 ± 4.9 years at the time of transplantation. The specific ages at the time of cryopreservation were as follows: 1 × 17, 4 × 20, 2 × 21, 2 × 24, 5 × 25, 2 × 26, 4 × 27, 8 × 28, 6 × 30, 3 × 31, 3 × 32, 5 × 33, 8 × 34, 4 × 35, 2 × 36, 6 × 37, 3 × 38, 2 × 39, 1 × 40, 1 × 41, 1 × 42 and 1 × 44 years. The resulting pregnancies achieved in all 74 women are listed in Table I primary target criterion of 'tissue activity 1 year after transplantation' required a follow-up period of 1 year, all 27 transplantations conducted after June 2014 were, therefore, excluded from all further subgroup analyses (Fig. 1).

The 68 transplantations remaining were defined as Subgroup 1 and were further analysed in Tables II, IV and VI. Several more subgroups were excluded from Subgroup 1, such as 19 repeated transplantations (Subgroup 2), resulting in Subgroup 3. From Subgroup 3, women transplanted for the first time but without POI (Subgroup 4) were excluded, resulting in Subgroup 5. Subgroup 5 included only those remaining 40 women undergoing transplantation for the first time and in which POI had been presented before the transplantation. This group was also further analysed in Tables III, V and VII. POI was regarded as being present when there was amenorrhoea for at least 6 months before the transplantation. Detailed data on the patient characteristics and outcomes in Subgroups 1 and 5 are shown in Tables II and III.

Transportation of the tissue

The ovarian tissue was harvested using surgical laparoscopy without heat coagulation, in order to avoid damage to the ovarian tissue. After harvesting, the tissue was immediately transferred to a previously prepared transport tube filled with an organ perfusion solution (Custodiol; Dr. Franz Köhler Chemie Ltd, Bensheim, Germany) or a phosphate-buffered saline (PBS) solution. Overnight transportation was carried out in special isolated transportation containers with precise temperature documentation (Delta T Gesellschaft für Medizintechnik Ltd, Fernwald, Germany). The mean period between harvesting and final cryopreservation was 18 h to a maximum of 24 h, and the mean temperature of the transported tissue was ~4 C, maximum 8°C (Isachenko *et al.*, 2009; Dittrich *et al.*, 2015).

Cryopreservation of ovarian tissue

Ovarian tissue was exclusively cryopreserved by slow freezing, before the women underwent chemotherapy and radiotherapy. In the Bonn and Berne cryobanks, tissue was processed as follows: immediately after the arrival of the cortex biopsies, the medulla of the ovary was gently removed and cortex strips (8 mm × 4 mm × 1 mm) were prepared on a Petri culture dish filled with dissection medium (Custodiol medium in Bonn; Dulbecco's PBS in Erlangen) and placed on a precooled preparation desk (4°C) using precision forceps and scalpels. The slow-freezing protocol was based on a procedure described by Gosden *et al.* (1994) and Isachenko *et al.* (2007, 2012). Nunc CryoTubes (Sigma-Aldrich, St. Louis, MO, USA) were filled with 1.7 ml Leibovitz's L-15 GlutaMAX medium (Gibco, Carlsbad, CA, USA), 10% CryoSure-DMSO (Wak-Chemie Medical Ltd, Steinbach, Germany) and 10% serum substitute supplement (SSS; Irvine Scientific, Santa Ana, CA, USA) and precooled to 2°C in special cooling blocks. Dimethylsulphoxide (DMSO, 10%) as the cryoprotectant is also used by other groups (Donnez *et al.*, 2004). After the ovarian tissue pieces had been placed in the precooled medium inside the CryoTubes, the tubes were stored in a programmed freezer (IceCube 14S-A, SY-LAB, Neupurkersdorf, Austria). The process started with a precooling process/equilibration period at 2°C for 30 min, and after this the freezer cooled down the tubes in a controlled fashion at 2°C/min until automatic seeding at −6°C. After successful ice nucleation, the CryoTubes were further cooled (0.3°C/min to −40°C; 10°C/min to −140°C) and finally stored at −150°C in MVE Vapor phase storage tanks (MTG Medical Technology Ltd, Bruckberg, Germany).

Table I Characteristics of women who became pregnant following transplantation of ovarian tissue.

No	Diagnosis	Age at cryopreservation	Age at transplantation	Amount (%) of cryopreserved tissue (100% = 1 ovary)	Overnight transportation before cryopreservation	Primary ovarian insufficiency (POI) before transplantation	Graft site	Amount (%) of transplanted tissue (100% = 1 ovary)	Retransplantation	Tissue active 1 year after transplantation	Occurrence of pregnancy after transplantation (month)	Number of pregnancies	Number of deliveries	Ovaries/ tissue still active after pregnancy (regular or irregular cycle)
1	Non-Hodgkin's lymphoma	28	33 (March 2010)	50	No	No	Ovary, Peritoneum—fossa ovarica	25	No	Yes	2	3	1 (1 miscarriage, 1 tubal pregnancy)	Yes
2	Hodgkin's lymphoma	27	32 (October 2010)	50	Yes	Yes	Peritoneum—fossa ovarica	20	No	Yes	7	1	1	Yes
3	Breast cancer	28	31 (August 2011)	50	No	Yes	Ovary, Peritoneum—fossa ovarica	20	No	Yes	2	2	2	Yes
4	Hodgkin's lymphoma	35	37 (June 2012)	50	Yes	Yes	Peritoneum—fossa ovarica	20	No	Yes	9	1	1	Yes
5	Breast cancer	36	37 (August 2012)	50	Yes	Yes	Peritoneum—fossa ovarica	10	No	Yes	6	1	1	Yes
6	Hodgkin's lymphoma	21	27 (December 2012)	50	Yes	Yes	Peritoneum—fossa ovarica	15	No	Yes	8	1	1	Yes
7	Hodgkin's lymphoma	20	29 (February 2013)	50	No	Yes	Peritoneum—fossa ovarica	No data	No	Yes	16	1	0 (1 tubal pregnancy)	Yes
8	Ovarian carcinoma	30	35 (April 2013)	25	No	No	Peritoneum—fossa ovarica	15	No	Yes	6	2 (IVF)	2	Yes
9	Breast cancer	34	38 (April 2013)	50	Yes	Yes	Peritoneum—fossa ovarica	20	No	Yes	4	2	1 (1 miscarriage)	Yes
10	Cystadeno-fibroma	20	27 (July 2013)	25	Yes	Yes	Peritoneum—fossa ovarica	15	No	Yes	14	1	1	Yes
11	Hodgkin's lymphoma	33	37 (October 2013)	50	Yes	Yes	Peritoneum—fossa ovarica	20	No	Yes	5	1	1	Yes
12	Breast cancer	33	36 (December 2013)	50	Yes	No	Peritoneum—fossa ovarica	10	No	Yes	2	1	1	Yes
13	Breast cancer	30	36 (July 2014)	66	Yes	Yes	Peritoneum—fossa ovarica	15	No	Yes	3	1 (IVF)	1	Yes
14	Breast cancer	38	43 (September 2014)	33	Yes	Yes	Peritoneum—fossa ovarica	15	No	Yes	3	1	1	Yes
15	Hodgkin's lymphoma	30	32 (December 2014)	50	Yes	Yes	Ovary, Peritoneum—fossa ovarica	15	No	Yes	8	1	1	Yes
16	Ewing Sarcoma	26	29 (March 2015)	50	Yes	Yes	Peritoneum—fossa ovarica	15	No	Yes	2	1	1	Yes

The cases were extracted from all 95 transplantations in 74 women (in chronological order).

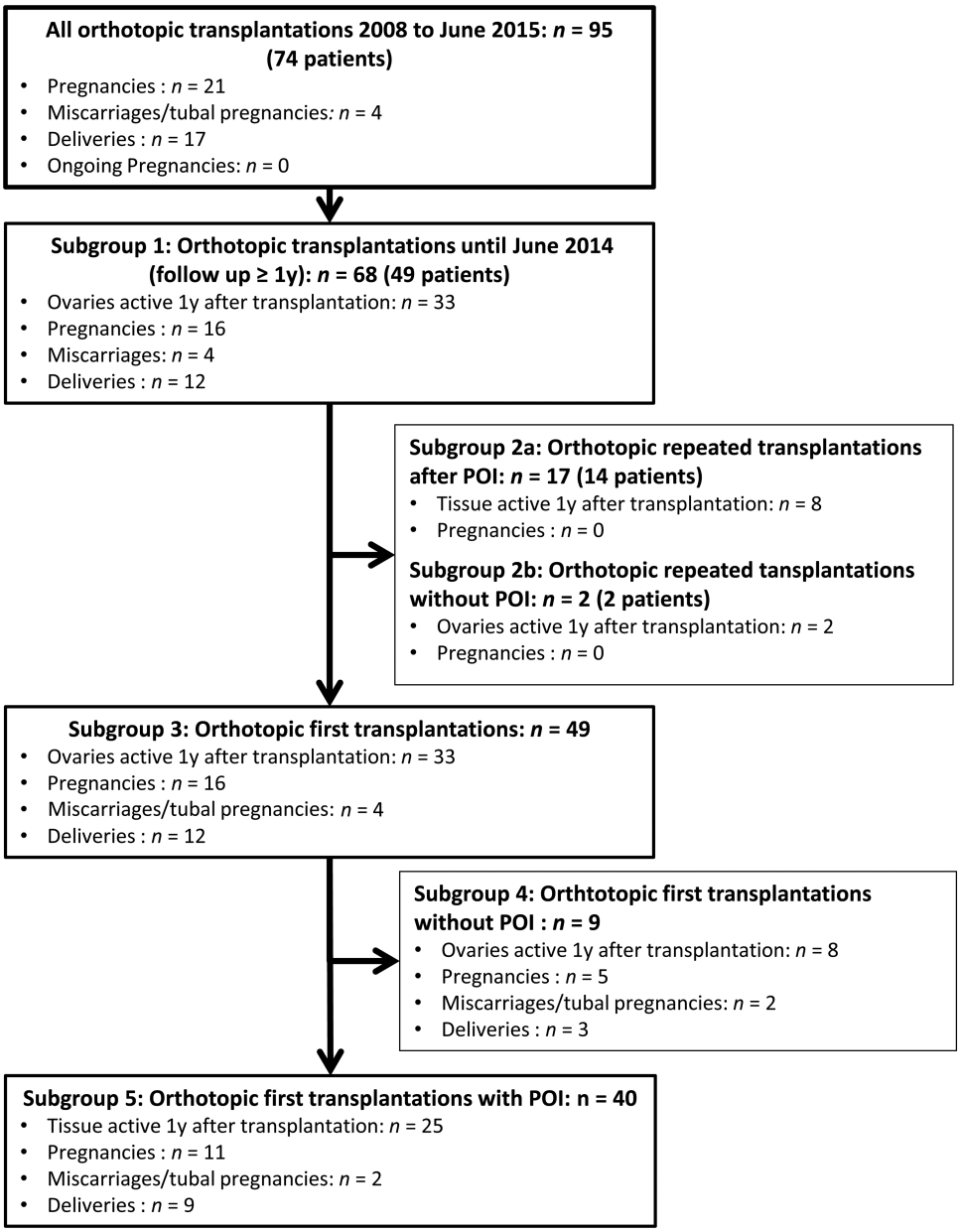

Figure 1 Summary of all orthotopic ovarian tissue transplantations. Orthotopic transplantation was defined as transplantation into or onto the ovaries or into a peritoneal pocket of the pelvic peritoneum. To reduce heterogeneity, analysis focused on those patients with a follow-up of at least 1 year after transplantation (Subgroup 1) and those who were transplanted for the first time and were diagnosed with primary ovarian insufficiency (POI) (Subgroup 5). These two groups were further analysed in Tables II–VII.

The tissue was processed similarly in the Erlangen cryobank. In contrast to the Bonn protocol, ethylene glycol was used as a cryoprotectant. In brief, pieces of ovarian tissue (3 mm × 3 mm × 1 mm) were equilibrated for 30 min in 1.5 mol/l ethylene glycol and 0.1 mol/l sucrose in PBS in 2-ml standard cryovials (Simport T309-2A) on a tilting table on ice and then loaded into the open freezing system (CTE-920, CTE). Ethylene glycol 91.5 mol/l) plus 0.1 mol/l sucrose as the cryoprotectant is also used by other groups (Rosendahl *et al.*, 2011). The following cooling programme was used: 2°C/min to −1°C, 0.5°C/min to −5°C, 0.3°C/min to −9.3°C, 10 min of soaking, then 0.3°C/min to −40°C and 10°C/min to −140°C, at which temperature the samples were plunged into liquid nitrogen at −196°C (Dittrich and Maltaris, 2006). The other centres also used slow cooling methods with slight modifications of the protocol.

Transplantation technique

The transplantations were carried out after oncological treatment had been completed and after a remission period of at least 2 years. Tissue was also transplanted in those women without POI who did not get pregnant spontaneously and who asked for transplantation to increase their pregnancy chances.

Before transplantation, after a short incubation period at room temperature, the CryoTubes were transferred to a 37°C warm-water bath. The

Table II Characteristics and outcome of women in Subgroup 1 comprising women with a follow-up period >1 year after transplantation.

	Subgroup 1 49 patients (68 transplantations)
Age at cryopreservation (years) Ø ± SD	30 ± 5.6
Age at transplantation (years) Ø ± SD	34 ± 4.9
Breast cancer *n*/total (%)	15/49 (30.6%)
Hodgkin's lymphoma *n*/total (%)	16/49 (32.7%)
Other malignancies *n*/total (%)	16/49 (32.7%)
Benign diseases *n*/total (%)	2/49 (4.1%)
Chemotherapy *n*/total (%)[a]	44/49 (89.8%)
Radiotherapy of the pelvis *n*/total (%)[a]	5/49 (10.2%)
Active tissue 1 year after transplantation *n*/total (%)[b]	33/49 (67.3%)
Pregnancies *n*/total (%)	16/49 (32.7%)
Deliveries *n*/total (%)	12/49 (24.5%)

Ø, average.
[a]Chemo and radiotherapy was started after cryopreservation of tissue.
[b]Active tissue defined as the presence of menstrual cycles.

Table III Characteristics and the outcome of women in Subgroup 5 comprising women undergoing transplantation for the first time and with POI present before the transplantation.

	Subgroup 5 (40 patients)
Age at cryopreservation (years) Ø ± SD	30 ± 5.9
Age at transplantation (years) Ø ± SD	34 ± 5.2
Breast cancer *n*/total (%)	14/40 (35.0%)
Hodgkin's lymphoma *n*/total (%)	10/40 (25.0%)
Other malignancies *n*/total (%)	14/40 (35.0%)
Benign diseases *n*/total (%)	2/40 (5.0%)
Chemotherapy *n*/total (%)[a]	35/40 (87.5%)
Radiotherapy of the pelvis *n*/total (%)[a]	5/40 (12.5%)
Active tissue 1 year after transplantation *n*/total (%)[b]	25/40 (62.5%)
Pregnancies *n*/total (%)	11/40 (27.5%)
Deliveries *n*/total (%)	9/40 (22.5%)

Ø, average.
[a]Chemo and radiotherapy was started after cryopreservation of tissue.
[b]Active tissue defined as presence of menstrual cycles.

tissue pieces then underwent a dehydration and rehydration process with sucrose in order to remove antifreeze agent and to restore the cell volume.

During the transplantation operation, the patency of the uterine tubes was initially checked in most cases. The tissue was transplanted into a peritoneal pocket and/or into one of the ovaries. In Subgroup 5, transplantation was carried out directly onto the remaining ovary ($n = 4$), into a peritoneal pocket in the area of the peritoneal ovarian fossa ($n = 30$), or to both locations ($n = 6$).

Table IV The outcome for women in Subgroup 1 in relation to age.

Age group	49 patients (68 transplantations)
<30 years	
Number of patients (*n*)	22
Number of transplantations (*n*)[a]	32/22
Radiotherapy of the pelvis *n*/total (%)	2/22 (9.1)
Active tissue 1 year after transplantation *n*/total (%)	15/22 (68.2)
Pregnancies *n*/total (%)	9/22 (40.9)
Deliveries *n*/total (%)	6/22 (27.3)
30–34 years	
Number of patients (*n*)	14
Number of transplantations (*n*)[a]	18/14
Radiotherapy of the pelvis *n*/total (%)	2/14 (14.3)
Active tissue 1 year after transplantation *n*/total (%)	11/14 (78.6)
Pregnancies *n*/total (%)	5/15 (33.3)
Deliveries *n*/total (%)	4/14 (28.6)
35–39 years	
Number of patients (*n*)	11
Number of transplantations (*n*)[a]	13/11
Radiotherapy of the pelvis *n*/total (%)	0/11 (0)
Active tissue 1 year after transplantation *n*/total (%)	6/11 (54.5)
Pregnancies *n*/total (%)	2/11 (18.2)
Deliveries *n*/total (%)	2/11 (18.2)
≥40 (40 and 44 years)	
Number of patients (*n*)	2
Number of transplantations (*n*)[a]	4/2
Radiotherapy of the pelvis *n*/total (%)	0/2 (0)
Active tissue 1 year after transplantation *n*/total (%)	1/2 (50.0)
Pregnancies *n*/total (%)	0/2 (0)
Deliveries *n*/total (%)	0/2 (0)

[a]Some women were transplanted twice.

For transplantation into a peritoneal pocket, the peritoneum was opened below the ovary. The tissue pieces were introduced in such a way that the upper side of the cortex was directed towards the abdominal cavity. The peritoneum was closed with absorbable sutures when necessary. For transplantation into the ovaries, an incision was made into the ovaries, the tissue pieces were introduced into the resulting pocket and the pocket was closed with absorbable sutures. Transplantation onto the ovary was carried out after an incision into the surface of the ovary, with the tissue pieces being attached to the wound surface with sutures. The transplantation techniques used in each of the successful pregnancies are shown in Table III.

Follow-up after transplantation

Follow-up of the patients included menstrual monitoring, ultrasonography and measurement of the estradiol and FSH concentrations. For the primary target criterion of 'tissue activity 1 year after transplantation,' ovarian function

Table V The outcome for women in Subgroup 5 in relation to age.

Age group	40 patients
<30 years	
Number of patients (*n*)	21
Number of transplantations (*n*)	21
Radiotherapy of the pelvis *n*/total (%)	2/21 (9.5)
Active tissue 1 year after transplantation *n*/total (%)	14/21 (66.7)
Pregnancies *n*/total (%)	6/21 (28.6)
Deliveries *n*/total (%)	5/21 (23.8)
30–34 years	
Number of patients (*n*)	9
Number of transplantations (*n*)	9
Radiotherapy of the pelvis *n*/total (%)	2/9 (22.2)
Active tissue 1 year after transplantation *n*/total (%)	7/9 (77.8)
Pregnancies *n*/total (%)	3/9 (33.3)
Deliveries *n*/total (%)	2/9 (22.2)
35–39 years	
Number of patients (*n*)	8
Number of transplantations (*n*)	8
Radiotherapy of the pelvis *n*/total (%)	0/8 (0)
Active tissue 1 year after transplantation *n*/total (%)	3/8 (37.5)
Pregnancies *n*/total (%)	2/8 (25.0)
Deliveries *n*/total (%)	2/8 (25.0)
≥40 (40 and 44 years)	
Number of patients (*n*)	2
Number of transplantations (*n*)	2
Radiotherapy of the pelvis *n*/total (%)	0/2 (0)
Active tissue 1 year after transplantation *n*/total (%)	1/2 (50.0)
Pregnancies *n*/total (%)	0/2 (0)
Deliveries *n*/total (%)	0/2 (0)

Table VI The outcome for women following tissue transplantation into a peritoneal pocket in Subgroup 1 (*n* = 38).

Age at cryopreservation (years) Ø ± SD	30 ± 5.5
Age at transplantation (years) Ø ± SD	35 ± 4.6
Breast cancer *n*/total (%)	12/38 (31.6%)
Hodgkin's lymphoma *n*/total (%)	12/38 (31.6%)
Other malignancies *n*/total (%)	12/38 (31.6%)
Benign diseases *n*/total (%)	2/38 (5.3%)
Chemotherapy *n*/total (%)	33/38 (86.8%)
Radiotherapy of the pelvis *n*/total (%)	4/38 (10.5%)
Active tissue 1 year after transplantation *n*/total (%)	26/38 (68.4%)
Pregnancies *n*/total (%)	11/38 (28.9%)
Deliveries *n*/total (%)	9/38 (23.7%)

Ø, average.

Table VII The outcome for women following tissue transplantation into a peritoneal pocket in Subgroup 5 (*n* = 30).

Age at cryopreservation (years) Ø ± SD	30 ± 5.8
Age at transplantation (years) Ø ± SD	34 ± 4.9
Breast cancer *n*/total (%)	7/30 (23.3%)
Hodgkin's lymphoma *n*/total (%)	10/30 (33.3%)
Other malignancies *n*/total (%)	12/30 (40.0%)
Benign diseases *n*/total (%)	1/30 (3.3%)
Chemotherapy *n*/total (%)	25/30 (83.3%)
Radiotherapy of the pelvis *n*/total (%)	4/30 (13.3%)
Active tissue 1 year after transplantation *n*/total (%)	19/30 (63.3%)
Pregnancies *n*/total (%)	9/30 (30.0%)
Deliveries *n*/total (%)	7/30 (23.3%)

Ø, average.

was assessed 1 year after transplantation. The minimum criterion used to identify an active transplant following POI (Subgroups 2a and 5) was regular or irregular menstruation.

Data analysis

Ovarian activity 1 year after transplantation was defined as the primary target criterion and the rates of pregnancy and birth were defined as secondary target criteria. In view of the small numbers of patients, statistical comparison of the subgroups was deliberately dispensed with.

Results

Pregnancy rates, delivery rates and tissue activity

Twenty-one pregnancies and 17 births were observed after the 95 transplantations (Fig. 1 and Table I). All of these women received chemotherapy after cryopreservation of ovarian tissue and none received radiotherapy of the pelvis. The time to pregnancy following tissue transplantation was 6.1 ± 4.2 months. Eleven pregnancies and nine births were observed in Subgroup 5, in all cases following a first orthotopic transplantation and previously confirmed POI (Table III). Success rates were highest in women who cryopreserved their tissue at the age of 34 years and younger (Tables IV and V).

As the follow-up period would otherwise have been too short, ovarian activity 1 year after transplantation was not analysed for all transplantations, but only for Subgroups 1 and 5. In Subgroup 1, the transplants were still active 1 year after transplantation in 67.3% of the patients (Table II). In Subgroup 5, the figure was 62.5% (Table III).

An analysis of the success rates in relation to age revealed highest success rates in women who were 34 years and younger at the time of tissue cryopreservation (Tables IV and V).

Transplantation into a peritoneal pocket

In 38 of the transplantations analysed in Subgroup 1, the tissue was transferred exclusively into the pelvic peritoneum (Tables VI and VII).

In Subgroup 5, 30 transplantations into the peritoneum were carried out. Following transplantation into the pelvic peritoneum, ovarian activity was noted 1 year after transplantation in 68% of cases ($n = 26/38$) in Subgroup 1. In Subgroup 5, in which it is possible to assess the activity of the transplanted tissue due to previous POI, the tissue was still active in 63% of cases ($n = 19/30$). The numbers of pregnancies and births achieved in Subgroup 1 were 29% ($n = 11/38$) and 24% ($n = 9/38$), respectively, and in Subgroup 5 the corresponding figures were 30% ($n = 9/30$) and 23% ($n = 7/30$). All of the pregnancies in Subgroup 5 were spontaneous pregnancies without the use of assisted reproductive techniques. In Subgroup 1, 2 women underwent 3 IVF cycles.

Transplantation into a peritoneal pocket was also performed in one patient diagnosed with ovarian carcinoma (Table I, Patient No. 8). This transplantation site was chosen following careful counselling and as the ovary from which the tissue was removed was left *in situ*. Therefore it could be assumed that the risk of a relapse in the transplanted tissue was no higher than in the remaining ovary. Furthermore, it was assumed that the transplanted tissue could easily be removed following a pregnancy.

Pregnancies and deliveries achieved

Among the pregnancies, the patients' maximum age at the time of tissue harvesting was 38 years. Fifty-three percent of the pregnancies ($n = 10/19$) were recorded in cases in which there had been overnight transport of the tissue, and 13 after previously confirmed POI. All 21 pregnancies were observed after the first transplantation. The pregnancy developed spontaneously in 19 cases, and in 3 cases following IVF. Fifteen pregnancies developed after transplantation into the pelvic wall, none after transplantation into or onto the ovary, and six pregnancies developed after a combination of transplantation into the pelvic wall and into or onto the ovary. Overall, 12 births were achieved in Subgroup 1 and 9 in Subgroup 5. Only one patient with breast cancer had a local relapse, which is not regarded as having been caused by the transplantation of the ovarian tissue. Seven out of the 74 transplanted women received chemotherapy and radiation of the pelvis (5 in Subgroup 1, 5 in Subgroup 5), 5 with anal carcinoma, 1 with carcinoma of the cervix, 1 with carcinoma of the uterus. None of these women were pregnant at time of writing.

Discussion

Due to the large number of cases included, the present case series made it possible for the first time to classify the transplantations into subgroups, allowing limited assessment in relation to the aspects of 'first transplantation' versus 'retransplantation' and of 'transplantation with POI' versus 'transplantation without POI.' The new criterion of 'tissue activity 1 year after transplantation' was also introduced to evaluate the success of transplantation, as it is independent of the duration of the follow-up period and is a more sensitive way of assessing the success rate. With the reservation that it might also be possible for residual ovarian tissue to be reactivated, this made it possible to exclusively assess the activity of the transplanted ovarian tissue (Oktay *et al.*, 2011).

In relation to the cryopreservation technique used (before cancer treatment in all cases), there was little heterogeneity in the group, as the tissue from 51 of the total of 74 patients who underwent transplantation was stored in one of two cryobanks with standardized freezing protocols. However, the transplantations were carried out in a total of 16 centres, so that some heterogeneity with regard to the surgical technique used must be assumed. However, this might also be an advantage for the interpretation of the overall chances of success in this study, as it reflects the real chances in a given country, rather than the chances in technically standardized conditions. On the other hand, specific issues such as that of the surgical technique are more difficult to assess.

In the present study, tissue activity (defined as menstruation) after 1 year in all transplantations (Fig. 1, Subgroup 1) was 67%, the pregnancy rate was 33% and the birth rate was 25% per patient with a transplant. In first transplantations in patients with previous POI (Subgroup 5), the figures were tissue activity in 63%, and a 28% pregnancy rate and 23% birth rate per transplantation.

Previous studies analysed groups of patients corresponding to the definition of Subgroup 1 in the present study, i.e. women with first and repeated orthotopic transplantations with and without POI. In a study including 20 transplantations of tissue that had been stored in several cryobanks but was only transplanted in a single centre, Dittrich *et al.* (2015) reported a pregnancy rate of 35% and a birth rate of 20% per transplantation. Those transplantations were also included in the present study. The study by Donnez *et al.* (2013) included 60 transplantations conducted in Belgium, Denmark and Spain. The success rates for pregnancy and birth were 18 and 10% per transplantation. However, more children were born, as 6 of the transplantations led to a total of 12 children. In a review article by Stoop *et al.* (2014), the authors present what they describe as all births reported at that time worldwide after successful transplantations. Although the analysis does not state the success figures absolutely correct (Andersen, 2015), it includes the transplantations from the publications by Dittrich *et al.* (2015) and Donnez *et al.* (2013). According to the review, a total of 121 transplantations have been carried out and 35 children have been born. However, the study only gives a rough overview, without any subanalysis either of the birth rates per transplantation or of clinically relevant factors such as first transplantation or retransplantation, transportation of the tissue, the freezing technique used, storage, surgical technique etc.

All of these studies show that ovarian tissue transplantation can in principle lead to pregnancies and births, but they do not include any further analysis of the relevant factors mentioned. Nevertheless, it is notable that the success rate in terms of the birth rate per transplantation is ~20%. In the present study, the birth rate was 25% per patient receiving a transplant (Subgroup 1). Even after exclusion of retransplantations and women without POI (Subgroup 5), the data presented here for the first time confirm a birth rate of 23%. As the transplanted tissue was still active after 1 year in 63% of the transplantations in Subgroup 5, a further increase in these birth rates can be expected.

Another study which also provides more reliable data on the success of ovarian tissue transplantation has recently been published by Jensen *et al.* (2015). They reported 53 transplantations in 41 women in Denmark. A total of 32 women wanted to become pregnant and 10 women delivered at least 1 child, resulting in a 31% success rate per transplanted women. As some of these women were transplanted more than once, 31% is a cumulative pregnancy rate per transplanted women, whereas in our study the success rate per first transplantation was calculated.

It needs to be stated that the study by Jensen *et al.* (2015) summarizes cases which all cryopreserved their ovarian tissue in a single centre and that the surgery was limited to a few surgeons. This is in contrast to our study in which tissue was stored in many tissue banks and many surgeons were involved. This is definitely a weakness of our study as it led to more heterogeneity of the cryopreservation and transplantation

procedures. On the other hand, it reflects the realistic situation in many, especially large countries, in which several centres are involved.

It is still unknown at what age ovarian tissue can still be cryopreserved with a successful outcome. Many clinicians state that the tissue should not be cryopreserved from women older than 35 years as most pregnancies resulted from young women. Indeed, success rates seem to be highest in women younger than 35 years, as shown in our study. However, as pregnancies and deliveries were still found in women between 35 and 39 years of age, the upper age limit for cryopreservation of ovarian tissue should probably not to be too strict but should rather depend on other factors such as the ovarian reserve.

It is still unknown if tissue could also be cryopreserved after chemotherapy. Abir *et al.* (2008) found that the number of pre-antral follicles was still high after chemotherapy but only in those women $\leq$20 years of age. Therefore, cryopreservation in young women could theoretically be considered even after chemotherapy but clinical data on pregnancy rates still do not exist.

It is unclear as yet which surgical technique is preferable. Various technical options in ovarian tissue transplantation have been described to date. Laparotomy and laparoscopy are possible for surgical access. Laparotomy, as carried out by Silber and Gosden (2007) and in Denmark (Rosendahl *et al.*, 2011), with the use of a surgical microscope, allows microsurgical attachment of tissue fragments to the decorticated ovary. This type of operation is also possible laparoscopically, but then requires considerable surgical expertise. Techniques for attaching the transplants using Interceed® or fibrin glue have also been used (Donnez *et al.*, 2011). A surgically simpler method is transplantation into a peritoneal pocket lateral to the Fallopian tube below the ovary, which can be carried out without problems as a one-step procedure laparoscopically. The peritoneum is opened and after the tissue pieces have been introduced, it is usually closed with single sutures (Dittrich *et al.*, 2012; Donnez *et al.*, 2012).

In the series reported here, two transplantations were carried out using a laparotomy (at the Infertility Centre in Baden, Switzerland), and all of the others were performed laparoscopically. The tissue was introduced either into a pocket in the ovary after a cortical incision or was applied and attached to the ovary after decortication. Alternatively, transplantation was carried out subperitoneally in the pelvic wall into a peritoneal pocket located lateral to and in the immediate vicinity of the fimbrial funnel. The pocket was closed with one or two sutures. Following transplantation into the pelvic wall, all nine pregnancies developed spontaneously (Subgroup 5). The question arises of whether these were genuine pregnancies resulting from folliculogenesis in the pelvic wall. The women had had amenorrhoea—making POI probable, but not confirming it. According to the European Society of Human Reproduction and Embryology (ESHRE) 'ESHRE Guideline: management of women with premature ovarian insufficiency' (ESHRE Guideline Group on POI *et al.*, 2016), a definitive diagnosis of POI requires oligomenorrhoea or amenorrhoea for at least 4 months and FSH concentrations $>$25 IU/l, with two assessments at an interval of at least 4 weeks. In the present study, it was not possible to wait for several months of amenorrhoea without hormone replacement therapy in order to diagnose POI, due to the associated risk of loss of bone mass and possible menopausal symptoms. The diagnosis of POI here was therefore not definitive, but highly probable. It is very unlikely, however, that all nine pregnancies in Subgroup 5 after transplantation into the pelvic wall occurred as a result of activation of the ovaries. In the final analysis, the development of pregnancy after transplantation into the pelvic wall could only be definitively confirmed if both ovaries had previously been removed. As this will only rarely be the case, and also because ovariectomy would probably lead to loss of functionally relevant structural support for the Fallopian tube, this type of evidence will probably never be available.

In principle, however, it is very likely that folliculogenesis in the pelvic wall can also lead to ovulation. During a Caesarean section carried out after a spontaneous pregnancy resulting from transplantation into the pelvic wall, Müller *et al.* (2012) inspected the transplant in the pelvic wall. Small follicles were visible at the transplant site, which were bulging through the peritoneum into the abdominal cavity. Anatomically, the possibility of spontaneous pregnancy is also quite plausible. The transplant is located in the immediate vicinity of the fimbriae of the fallopian tubes. As the fallopian tubes are able to gather oocytes from all areas of the ovary, it can be assumed that the oocyte collection mechanism can also be functional at the pelvic wall. However, with ovulation in the pelvic wall, it is the parietal peritoneum rather than the visceral peritoneum that has to be penetrated. However, the structures of the visceral and parietal peritoneum are largely similar. They both consist of serosal mesothelial cells arranged in single layers, lying on a basal membrane. However, the subserosal connective tissue in the parietal peritoneum is thicker, at 90–130 μm, than in the visceral peritoneum, at 45–70 μm. The difference in thickness is probably hardly of any relevance, since during normal ovarial ovulation a much thicker layer of ovarian connective tissue has to be penetrated.

It is however still an open question whether transplantation into or onto the ovary, or into the pelvic wall, leads to a greater likelihood of folliculogenesis and thus to a higher probability of pregnancy. This can only be clarified using intraindividual comparison of the two transplantation sites in women with established POI. A prospective randomized study on this topic has been initiated by the FertiPROTEKT network (ClinicalTrials.gov, NCT 02780791).

Both for prospective studies of this type and also for purposes of systematic follow-up in a large number of patients, a network structure of the type existing in individual countries such as Denmark and in the FertiPROTEKT network is required. This type of network structure also makes it possible to combine expertise. In Germany, for example, the first birth following transplantation of ovarian tissue took place after the tissue had been harvested in the city of Dresden, cryopreserved in the cryobank in Bonn following overnight transportation at ~4°C, and transplantation in Erlangen; the birth was in Dresden (Dittrich *et al.*, 2012; Isachenko *et al.*, 2012). Such structures can involve centralized cryobanks, which in a smaller country such as Denmark require transportation of the tissue for several hours (Schmidt *et al.*, 2003; Rosendahl *et al.*, 2011) and in a larger international association such as the FertiPROTEKT network often requires overnight tissue transportation.

To date, the tissue has been transported at temperatures of just under ~4°C in an organ perfusion solution (Custodiol medium; Dr Franz Köhler Chemie, Bensheim, Germany). This procedure was adopted by analogy with the transportation of organs for whole organ transplantation. It will need to be clarified in future whether this is the ideal form of transportation and whether transportation for several hours or overnight is capable of causing relevant damage to the tissue. Ten pregnancies among the 19 presented here (Table VII) were achieved after transplantation of tissue that had been transported overnight. These figures, and in particular the pregnancies in Subgroup 5 (following POI), confirm that overnight transportation seems to have no particularly

adverse effects on the tissue. However, a statistical comparison between transplantations with and without overnight transport was not performed in the present study, as the large number of possible influencing factors and low numbers of samples hindered any comparison.

It is also still unclear whether the probability of success in retransplantation is as high as or lower than that in a first transplantation. The case series published to date show that pregnancies and births are possible after retransplantation (Rosendahl *et al.*, 2011), but they do not provide any information about how high the chances are during retransplantation in comparison with a first transplantation. The present study might theoretically provide an initial answer to this question, as the retransplantations in Subgroups 2 and 4 were reported separately. Both, the shorter follow-up period with retransplantation and the negative selection resulting from transplantation mainly in patients with early functional insufficiency in the transplant, however, do not allow reliable conclusions to be drawn. Even larger numbers of cases are therefore needed to answer this question, in order to allow matching of first transplantations and retransplantations relative to follow-up time, as well as amount of tissue transplanted, and other relevant clinical factors.

Another open question is whether ovarian tissue should be cryopreserved for women aged >35 years. In the pregnancies in this series, the highest age at which tissue was harvested was 38 years. Transplantation was carried out in a total of 17 women who cryopreserved tissue aged >35 years (age 36, $n = 2$; age 37, $n = 6$; age 38, $n = 3$; age 39, $n = 2$ and age >39, $n = 4$). Of those, nine women belonged to Subgroup 1 with a follow-up ≤1 year (age 36, $n = 2$; age 37, $n = 5$; age 40, $n = 1$; age 44, $n = 1$). Of those, the tissue was still active in five women (55%) (age 36, $n = 1$, age = 37, $n = 3$, age 40, $n = 1$).

It can be assumed that individual ovarian reserve and thus follicle density at the time of tissue harvesting have greater influence than merely the patient's age at tissue harvesting. Although at the age of 38 years the rate of aneuploidy in 5-day old embryos is 45%, in comparison with 35% in 35-year-old women (Franasiak *et al.*, 2014), the difference should only slightly reduce the likelihood of pregnancy when there is sufficiently high tissue activity and should not make it impossible.

In summary, the present study for the first time makes it possible to quantify the live birth rate after a first transplantation and POI, with a figure of 23%. The persistent tissue activity after 1 year in the great majority of the transplants means that this success rate can be expected to increase. Finally, the study confirms the proof of principle for both transplantation into the pelvic wall and overnight transportation of the tissue. Prospective and controlled studies will however be required in order to provide definitive confirmation of these techniques, and transplantations should therefore continue to be carried out only in the framework of research studies.

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Authors' roles

All authors contributed to the design of the research study. H.V.d.V., J.L., M.v.W. and R.D. prepared and analysed the data and wrote the paper. All authors contributed to the data collecting and the revision of the final manuscript.

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Conflict of interest

None declared.

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