

Management of fertility preservation in prepubertal patients: 5 years' experience at the Catholic University of Louvain

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BACKGROUND: Since prepubertal boys cannot benefit from sperm banking, a potential alternative strategy for fertility preservation involves immature testicular tissue (ITT) banking aimed at preservation of spermatogonial stem cells. Survival of spermatogonia has been demonstrated after ITT freezing, which is considered ethically acceptable. We report the results of a pilot program set up for fertility preservation in prepubertal boys.

METHODS: All boys undergoing ITT cryobanking from May 2005 were identified from our clinical register. Data were collected from medical files.

RESULTS: Testicular tissue was retrieved from 52 prepubertal patients under 12 years of age and 10 peripubertal patients aged between 12 and 16 years, in whom no spermatozoa were identified in testicular biopsies. Malignant disease accounted for 80.6% of cases; the remaining patients suffered from benign disorders requiring gonadotoxic treatments. Mean ages, Tanner stages and occurrence rates of urogenital pathology were 6.43 ± 3.32 and 14 ± 1.23 years, I and I–IV, and 13.5 and 20% for pre- and peripubertal patients, respectively. Mean volumes of removed tissue were 20.1 ± 8.6 and 42.4 ± 15.6 mm³ for pre- and peripubertal patients, respectively. No complications occurred during or after tissue retrieval and 93.5% of referred patients accepted ITT storage. The presence of spermatogonia, and thus the potential for later tissue use, was established in all of these patients.

CONCLUSIONS: The majority of cryopreserved samples showed reproductive potential. Storage was accepted by most parents. All parents and children considered this fertility preservation strategy a positive approach.

Key words: immature testicular tissue / human / cryopreservation / fertility preservation

Introduction

The growing success of oncological treatments and extension of such gonadotoxic therapies to benign pathologies make routine implementation of techniques aimed at post-treatment preservation of reproductive function an increasingly important factor for quality of life. The American Society of Clinical Oncology recently highlighted the fact that more education is required to encourage increased use of fertility preservation methods in young cancer patients (Lee *et al.*, 2006).

For prepubertal boys who do not yet produce spermatozoa, freezing of immature testicular tissue (ITT), with a view to preservation of spermatogonial stem cells for future use, has been suggested and is

considered ethically acceptable (Bahadur *et al.*, 2000; Tournaye *et al.*, 2004). Several teams have developed cryopreservation protocols for human ITT (Kvist *et al.*, 2006; Keros *et al.*, 2007; Wyns *et al.*, 2007). Although this approach remains experimental, the capacity of human spermatogonia to survive after freezing has been demonstrated *in vitro* (Keros *et al.*, 2007) and their ability to proliferate and initiate differentiation has been observed in xenografting experiments (Wyns *et al.*, 2008). Studies conducted in animals on fertility restoration approaches after freezing of ITT pieces are encouraging, with live births reported after grafting of thawed tissue fragments in mice and rabbits (Shinohara *et al.*, 2002). However, a number of unresolved issues remain, relating essentially to the actual cryopreservation

of the tissue and its use to restore fertility. Indeed, in non-human primates, a negative impact of freezing has been demonstrated in marmosets, since none of the cryopreserved tissue survived after autografting compared with 70% of survival in fresh tissue grafts (Luetjens et al., 2008), although grafting of frozen ITT did lead to complete spermatogenesis in rhesus monkeys (Orwig and Schlatt, 2005). Furthermore, a significant decrease in the percentage of seminiferous tubules containing spermatogonia was observed after xenografting of cryopreserved monkey biopsies compared with fresh tissue grafts (Jahnukainen et al., 2007). In humans, the survival rate of spermatogonia was found to be reduced and abnormal differentiation was encountered after xenotransplantation of cryopreserved ITT (Wyns et al., 2007, 2008).

All potential options for fertility restoration from frozen tissue and their limitations were recently reviewed (Wyns et al., 2010). While none of these approaches have proved successful in a clinical set-up as yet, human preclinical *in vitro* studies using cadaver or surgically removed testes have nevertheless demonstrated the feasibility of transplanting germ-cell suspensions into testes (Schlatt et al., 1999; Brook et al., 2001).

Hence, on the basis of encouraging results of spermatogonial preservation in animals, and after obtaining approval from the Ethics Committee, we set up a pilot program for fertility preservation in prepubertal boys in May 2005. The main objective of this report is to provide information about: (i) the feasibility of ITT banking for fertility preservation in prepubertal and peripubertal boys; (ii) the risk/benefit balance of such an approach; and (iii) the acceptance of this strategy by patients and their parents.

Materials and Methods

Patients

All boys undergoing ITT cryobanking between May 2005 and June 2010 in the andrology department of the Cliniques Universitaires Saint-Luc were included in the study. Clinical data and previous history were assessed for each patient: cryptorchidism, inguinal hernia, hydrocele, varicocele, genital and urinary infectious disease, scrotal injury and testicular torsion. Indications for fertility preservation were recorded according to pediatric and oncological information provided.

Patient management

Patients were referred to our department by pediatric hematologists and oncologists before gonadotoxic treatment was initiated. Collaboration between pediatricians and oncologists was established through informative letters and presentations (including spermatogenesis physiology, onset of spermatogenesis and potential fertility preservation approaches related to age and pubertal status) during medical meetings. Clear instructions were given on who to contact to discuss the matter on an individual basis, with two specially appointed doctors from the department of reproductive medicine. Information on fertility preservation was initially given to parents and children by pediatricians and oncologists, who immediately contacted fertility specialists. Rapid and flexible access to medical consultation and surgical biopsy prior to tissue storage was provided to accommodate acutely ill young patients and their parents. Every child and his parents then had an emergency consultation with a fertility specialist to investigate the possibility of obtaining an ejaculate by masturbation or penile vibratory stimulation (for boys close to 12 years or older), and to explain potential testicular content based on physical examination and

previous history. All children and adolescents received appropriate information on sexual maturation, including pubertal events, testicular maturation with potential content linked to age, sperm emission and reproduction. During the consultation, boys were encouraged to ask questions freely. For peripubertal boys, a trained nurse from the unit of reproductive medicine explained the procedure applied to collect sperm, and accompanied the patient without his parents, which reduces the stress of talking about masturbation in their presence. All patients, in whom surgery was contemplated, received adapted counseling and information, as well as explanation of and access to assisted ejaculation techniques before surgery was scheduled. Potential fertility restoration approaches were explained to each individual child and his parents, making sure they understood that with stored ITT there is no guarantee of success as yet. In all cases, parents or legal guardians gave their signed informed consent for cryobanking, as well as the young boys themselves, if they were mature enough to understand the implications of the procedure.

Tissue sampling

Tissue samples were surgically retrieved under general anesthesia during the same anesthesia as for central line placement used for administration of chemotherapeutic drugs. Testicular sampling was exclusively performed when no previous gonadotoxic treatment had been administered. Only one testis was biopsied in each child and <5% of the testicular volume was removed. Biopsied testicular tissue was cut into pieces, with size determined by exact measurements ($\sim 2\text{--}4\text{ mm}^3$), cryopreserved and stored in the cryobank for later clinical use.

A small tissue sample was sent for anatomopathological analysis, which identified spermatogonia on hematoxylin–eosin-stained (H–E) sections based on histological characteristics (Clermont, 1963). The Clermont classification provides a clear description of germ cells and is used to facilitate identification of cellular associations. Briefly, Sertoli cells are identified by their polymorphous-shaped nucleus with a typically folded nuclear membrane and large nucleolus, and are easily distinguished from spermatogonia, which show a spherical or ovoid nucleus with fine chromatin granulations. One or more nucleoli are contained in vacuolar spaces or attached to nuclear membrane in type A spermatogonia. Chromatin clumps and a more centrally located nucleolus are observed in some type B spermatogonia. Unlike spermatogonia, which lie in close proximity to the basement membrane, primary spermatocytes are usually detached from the tubular limiting membrane. When chromatin granulations assume a filamentous texture, the cell is identified as a leptotene primary spermatocyte. The zygotene step of the meiotic prophase is characterized by the presence of coarser filaments, which progressively shorten and thicken to give the typical pachytene configuration. Newly formed spermatids have a spherical nucleus containing a few heavily stained granules, often depressed by a small acrosomic vacuole. As spermatids complete their maturation, the nucleus reduces in size and acquires its definitive shape. From a side view, the nucleus is pear-shaped. From the front, it looks paddle-shaped, the thinner anterior half being more lightly stained than the caudal half.

Observations on H–E sections were confirmed by immunohistochemistry (IHC) with MAGE A4 mouse anti-human monoclonal antibody purified from hybridoma 57B (Yakirevich et al., 2003; Wyns et al., 2007). Qualitative and semi-quantitative (percentage of seminiferous tubules showing spermatogonia) observations were recorded. Samples were considered rich in spermatogonia if more than 50% of seminiferous tubules contained one or more spermatogonia, and poor if the figure was less than 25%. Tumoral cell detection was not carried out unless required by the oncologist. A tissue sample for research purposes was requested only when both testes were present. If accepted, specific signed consent for this was sought from the parents and child

where applicable, and a small piece of tissue ($\sim 1\text{--}3\text{ mm}^3$) was used according to prior approval from the Ethics Committee. Fragments were collected in Falcon tubes containing Hank's balanced saline solution (HBSS) at 4°C and placed on ice.

In subjects over 10 years of age, intraoperative tissue analysis was performed in order to detect the potential presence of spermatozoa for using the testicular sperm extraction (TESE) technique (Verheyen *et al.*, 1995) since, according to Hovatta (2001), spermatids and/or spermatozoa may be found in patients' tissue, even if they are azoospermic in terms of ejaculate (where available).

Freezing procedure

For boys below the age of 10, a previously described ITT protocol aimed at preservation of spermatogonia was applied (Wyns *et al.*, 2007). Briefly, 0.7-mol/l dimethylsulfoxide (DMSO) was supplemented with 0.1-mol/l sucrose and 10-mg/ml human serum albumin (HSA) as cryoprotectants. Tissue pieces were placed in 1-ml freezing medium at 4°C in 2-ml cryovials. Using a programmable freezer (Minicool 40 PC Air Liquide, Marne-la-Vallée, France), the vials were maintained at 0°C for 9 min, cooled at a rate of $0.5^\circ\text{C}/\text{min}$ to -8°C and then held for 5 min before seeding manually at -8°C . After holding for a further 15 min at -8°C , a cooling rate of $0.5^\circ\text{C}/\text{min}$ was used from -8 to -40°C before final dehydration for 10 min at -40°C . After cooling at $7^\circ\text{C}/\text{min}$ to -80°C , the vials were transferred to liquid nitrogen.

For boys above the age of 10, allocation to the appropriate freezing protocol was confirmed after intraoperative examination of the tissue. When haploid cells were observed, half of the retrieved tissue was preserved with an adapted protocol (mature testicular tissue, MTT) routinely used for preservation of mature gametes (Crabbé *et al.*, 1999). However, because of the scarcity or immobility of mature germ cells in the tissue, limiting its potential for later fertility restoration, half of the tissue was also frozen with the protocol specific for ITT. Figure 1 summarizes sample allocation to cryopreservation protocols.

Results

Population studied

We identified 62 pre- and peripubertal patients eligible for cryopreservation of ITT. Indications for ITT preservation are shown in Fig. 2.

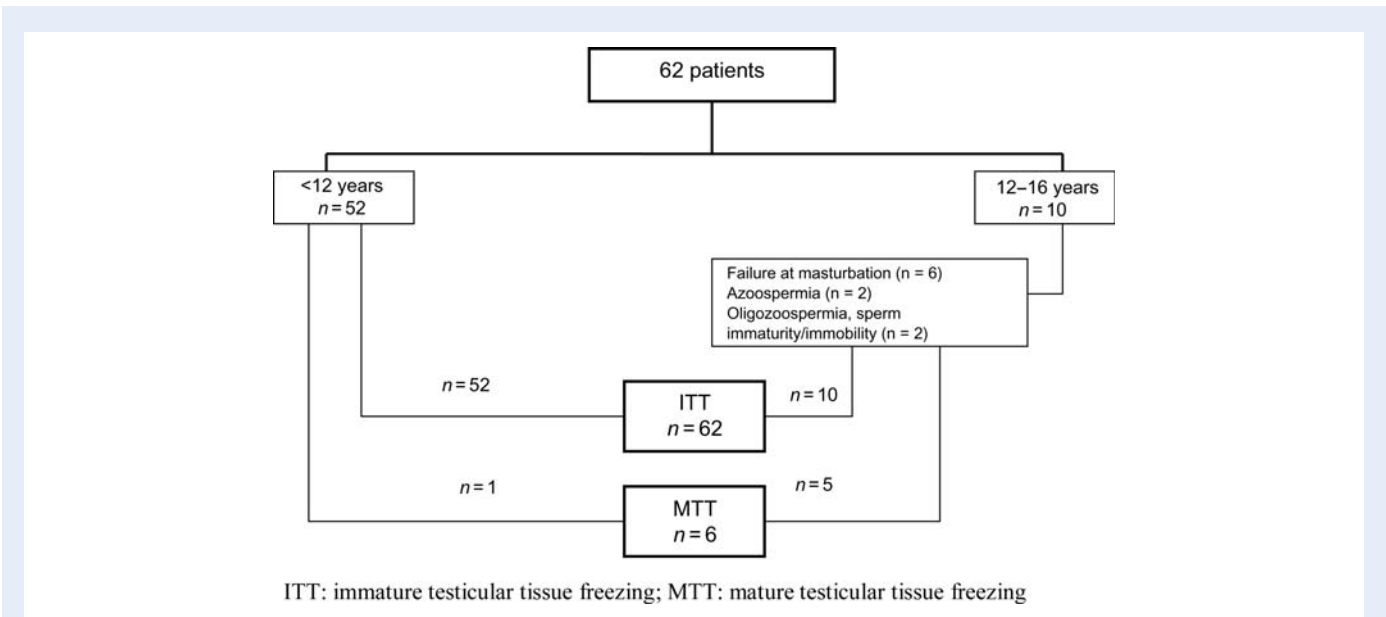
Malignant disease accounted for 80.6% of patients ($n = 50/62$) treated with polychemotherapy, combined with radiotherapy in 28% ($n = 14/50$) of cases. Conditioning chemotherapy for bone marrow transplantation was carried out in 24.2% ($n = 15/62$) of subjects. Table 1 shows the treatment details of patients classified into low, intermediate and high-risk groups according to gonadotoxicity.

There were 52 prepubertal patients under 12 years of age who underwent ITT cryobanking.

Their mean age was 6.43 ± 3.32 years (range: 0.92–11.92), Tanner stage was I, and 13.5% of them had a history of urogenital disorders.

There were 10 patients between 12 and 16 years of age who also benefited from ITT storage as they could not provide a semen sample ($n = 6$), were azoospermic ($n = 2$), or presented with extremely severe oligozoospermia after scrupulous examination of their semen samples following washing and centrifugation ($n = 2$). These patients had no ($n = 5$) or very few ($n = 5$) spermatozoa in their testicular tissue after TESE performed during testicular sampling. Patient characteristics and reasons for ITT preservation in this group of peripubertal boys are summarized in Table II.

Patients 1 and 2 were unable to provide a semen sample because of their immaturity. Patients 4 and 9 could not provide a semen sample either by masturbation or after two attempts or one attempt respectively with penile vibratory stimulation. Patient 5 could not provide a semen sample because of severe acute pain. Patient 8 could not provide a semen sample by masturbation and refused penile vibratory stimulation. Except for Patients 1 and 2, at least two attempts to collect sperm were made. The mean age of these patients was



ITT: immature testicular tissue freezing; MTT: mature testicular tissue freezing

Figure 1 Allocation of samples to cryopreservation protocols. ITT, immature testicular tissue freezing; MTT, mature testicular tissue freezing.

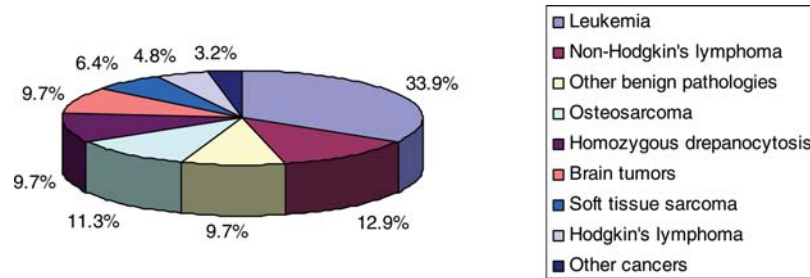


Figure 2 Indications for ITT preservation. Other cancers were one hepatoblastoma and one nasopharyngeal carcinoma. Other benign diseases were hemolytic anemia, beta-thalassemia, chronic granulomatous disease, Kostmann's syndrome, lymphohistiocytosis or ganglioma of the posterior fossa.

14 ± 1.23 years (range: 12.42 ± 16.08), Tanner stage ranged from I to IV, and 20% had a history of urogenital disorders.

Tissue samples

The mean volume of removed tissue was 20.1 ± 8.6 mm³ (range: 6–46 mm³) and 42.4 ± 15.6 mm³ (range: 28–58 mm³) for pre- and peripubertal patients, respectively, depending on the testicular volume of each individual child, in order to remain below 5% of total testicular volume. No complications occurred during or after tissue retrieval.

Anatomopathological analysis of the tissue was carried out in 93.5% of cases. Reasons for unavailability of information on tissue histology were loss of fragments during the embedding procedure or absence of seminiferous tubules in the biopsy (mainly consisting of tunica albuginea) in one case. Data on anatomopathological results of testicular biopsies of boys from the age of 10, related to clinical patient characteristics and urogenital history, are presented in Table III. In prepubertal patients, spermatogonia were observed in all samples, and 18% of samples showed seminiferous tubules rich in spermatogonia, while 51.3% did not. One of the patients, presenting with a history of orchiopey, had very few spermatogonia. Occasional spermatids were seen in one patient. In peripubertal patients, spermatogonia were also observed in all samples, with 66.7% of samples showing seminiferous tubules rich in spermatogonia, and 33.3% not. Data obtained from H–E sections were corroborated by IHC with MAGE A4 antibody, since qualitative evaluation showed positive immunostaining of spermatogonia in boys of all ages (Fig. 3). Direct intraoperative examination of tissue from the peripubertal boys revealed a few isolated spermatozoa in the testicular tissue of five patients and an absence of spermatozoa in five patients (Tables II and IV). Table IV shows the tissue characteristics observed during surgery, anatomopathological analysis after surgery, and the freezing protocol applied in the peripubertal patients.

ITT cryobanking was performed in 93.5% of referred patients. Discontinuation of tissue storage was requested in three cases, because of patient death. A small sample for research purposes was obtained from 42 patients (67.7%).

Discussion

Loss of fertility in adult life is a major psychologically traumatic consequence of fertility-threatening therapies. Indeed, in a quality-of-life

analysis of former oncological patients, about 80% viewed themselves as potential parents (Schover et al., 1999).

For prepubertal boys, very few options exist to protect their fertility, besides choosing therapies that are less toxic to gonads (Wyns et al., 2010). Advances in assisted reproduction technologies and increasing interest in *in vivo* and *in vitro* gamete maturation have focused on preserving immature gametes, and thus germ stem cells, before sterilizing treatments, in the hope of developing new techniques allowing their use in the future. Since the aim is to cryopreserve spermatogonial stem cells, which are much larger than spermatids or spermatozoa, appropriate freezing protocols need to be used.

For cryopreservation of ITT, we selected a low concentration of DMSO (0.7 M) in order to reduce its well-known toxicity (Martino et al., 1996; Zambelli et al., 1998) and because use of a low DMSO concentration (0.7 M) has already been recommended for cryopreservation of human hematopoietic stem cells (Fleming and Hubel, 2006). This was also the underlying rationale of Keros et al. (2005) when they set out to demonstrate that human spermatogonial survival could be achieved with 0.7 M DMSO. Although in non-human primates, it has been shown that this dose is suboptimal compared with 1.4 M, the freezing medium was not supplemented with a non-membrane-permeating cryoprotectant, and 1.4 M DMSO was only found to be optimal when a non-controlled freezing protocol was used (longer dehydration time with 0.7 M DMSO than 1.4 M DMSO; Jahnukainen et al., 2007). We chose to add sucrose, a non-membrane-permeating cryoprotectant, to the freezing medium to increase tissue protection against cryoinjury by promoting cell dehydration during the procedure. The addition of sucrose was previously shown to improve the cryopreservation outcome of bovine spermatogonial stem cells (Izadyar et al., 2002). We also applied a controlled freezing protocol with an induced ice-nucleation step, because cooling rates have been found to influence cryopreservation outcomes. Indeed, when 0.7 M DMSO was used, slow-controlled cooling rates yielded better morphological preservation of human spermatogonia (with a very high proportion of intact spermatogonia), Sertoli cells, and interstitial tissue after 24 h of culture than rapid cooling rates (Keros et al., 2007), probably because slower cooling increases cell dehydration, which can minimize the risk of ice crystal formation.

A controlled slow-freezing protocol including an induced ice-nucleation step may even yield better results at low DMSO

Table 1 Planned future treatment related to best estimated risk of gonadotoxicity (after testicular biopsy).

Patient numbers	Pathology	Chemotherapy	Radiotherapy
Low risk			
6	ALL	Asparaginase, MTX, mercaptopurine, vincristine, thioguanine (+-daunorubicin)	
12	ALL	Cyclophosphamide (3 g/m ²), cytarabine, daunorubicin, doxorubicin, etoposide, thioguanine, MTX, mercaptopurine, vindesine, vincristine	
2	ALL	Cyclophosphamide (3 g/m ²), cytarabine, daunorubicin, doxorubicin, etoposide, thioguanine, MTX, mercaptopurine, vindesine, vincristine	18 Gy, 10 fractions, brain
1	ALL		24 Gy, 12 fractions, brain up to C2
1	T-lymphoblastic lymphoma	Cyclophosphamide (3 g/m ²), cytarabine, daunorubicin, doxorubicin, etoposide, thioguanine, MTX, mercaptopurine, vindesine, vincristine	Total body irradiation
Intermediate risk			
1	Nasopharyngeal carcinoma	Cisplatin (100 mg/m ²), 5-FU	46 Gy, 23 fractions, for cervical and retropharyngeal lymph nodes, increased to 70 Gy, 30 fractions, for nasopharynx and invaded lymph nodes
1	Cutaneous lymphoblastic lymphoma	Cyclophosphamide (3 g/m ²), cytarabine, daunorubicin, doxorubicin, etoposide, Thioguanine, MTX, mercaptopurine, vindesine, vincristine	
1	Hodgkin's lymphoma stage IV	Vincristine, doxorubicin, etoposide; cyclophosphamide (3.5 g/m ²)	
1	Mature B lymphoma	Cyclophosphamide (6.3 g/m ²), cytarabine, doxorubicin, MTX, vincristine	
1	Burkitt lymphoma	Cyclophosphamide (6.3 g/m ²), cytarabine, doxorubicin, MTX, etoposide, vincristine	
1	Burkitt lymphoma	Cyclophosphamide (<3 g/m ²), doxorubicin, vincristine	
2	Burkitt lymphoma	Cyclophosphamide (6.3 g/m ²), cytarabine, doxorubicin, MTX, vincristine	
1	Anaplastic lymphoma	Cyclophosphamide (6.4 g/m ²), ifosfamide (12 g/m ²), MTX, cytarabine, doxorubicin	
3	Medulloblastoma	Lomustine (600 mg/m ²), cisplatin (560 mg/m ²), vincristine	23.4 Gy, 13 fractions, for cerebrospinal axis + 50.8 Gy for posterior fossa
1	Posterior fossa ganglioma	Vincristine, carboplatin (+-etoposide)	
1	Hepatoblastoma	Carboplatin, cisplatin (570 mg/m ²), doxorubicin	
High risk			
1	Neuroblastoma stage IV	Vincristine, etoposide, cisplatin (320 mg/m ²), cyclophosphamide (4 g/m ²), busulfan, melphalan (140 mg/m ²)	
1	Pinealoblastoma stage IV	Etoposide (1 g/m ²), carboplatin (1.6 g/m ²), melphalan (200 mg/m ²), cisplatin (200 mg/m ²), thiotepa (720 mg/m ²)	
1	Hodgkin's lymphoma stage III scleronodular	Doxorubicin, procarbazine (9 g/m ²), vincristine, cyclophosphamide (12 g/m ²)	20 Gy, 10 fractions for mediastinum and subclavicular area + lumboaortic area and spleen
1	Hodgkin's lymphoma stage IIE	Doxorubicin, procarbazine (9 g/m ²), vincristine, cyclophosphamide (12 g/m ²)	20 Gy, 10 fractions, for mediastinum, subclavicular area and supradiaphragmatic lymph nodes + 36 Gy, 18 fractions, for hypermetabolic focus in mediastinum
1	Rhabdomyosarcoma	Actinomycin, doxorubicin, ifosfamide (54 g/m ²), vincristine (+-cyclophosphamide) (4.2 g/m ²)	42 Gy for popliteal area + 41 Gy for primitive tumor
1	Rhabdomyosarcoma	Actinomycin, doxorubicin, ifosfamide (54 g/m ²), vincristine (+-cyclophosphamide) (4.2 g/m ²)	50.4 Gy, 28 fractions, for left inguinal iliac and lumboaortic area

Continued

Table I Continued

Patient numbers	Pathology	Chemotherapy	Radiotherapy
I	Rhabdomyosarcoma	Actinomycin, doxorubicin, ifosfamide (54 g/m ²), vincristine (+-cyclophosphamide) (4.2 g/m ²)	
I	Rhabdomyosarcoma	Actinomycin, doxorubicin, ifosfamide (54 g/m ²), vincristine (+-cyclophosphamide) (4.2 g/m ²)	45 Gy, 25 fractions, petrous pyramid
I	Ewing sarcoma (petrous localization)	Actinomycin, doxorubicin, etoposide, ifosfamide (102 g/m ²), vincristine	
I	Ewing sarcoma (sacral localization)	Actinomycin, doxorubicin, etoposide, ifosfamide (102 g/m ²), vincristine	45 Gy, 25 fractions, for L5-S1 area + 54 Gy, 30 fractions, right sacrum + S1
I	Osteosarcoma	Vinorelbine (25 mg/m ²) cyclophosphamide (25 mg/m ²)	
3	Osteosarcoma	Cisplatin (480 mg/m ²), doxorubicin, MTX (+-ifosfamide) (50 mg/m ²), etoposide	
I	Osteosarcoma	Cisplatin (480 mg/m ²), doxorubicin, MTX (+-ifosfamide) (50 mg/m ²), etoposide	54 Gy, 30 fractions, pelvis
6	Drepanocytosis	Busulfan (16 mg/kg), cyclophosphamide (200 mg/kg)	
I	Chronic familial granulomatosis	Busulfan (8 mg/kg), fludarabine (7.5 mg/kg)	
I	Hemolytic anemia (pyruvate kinase deficiency)	Busulfan (16 mg/kg), cyclophosphamide (200 mg/kg)	
I	Kostmann's syndrome	Busulfan (16 mg/kg), cyclophosphamide (200 mg/kg)	
I	Beta-thalassemia	Busulfan (16 mg/kg), cyclophosphamide (200 mg/kg)	
I	Lymphohistiocytosis	Melphalan (140 mg/m ²)	

MTX, methotrexate; ALL, acute lymphoblastic leukemia.

Table II Clinical characteristics and reasons for ITT preservation in peripubertal patients.

Patient number	Age (years)	Tanner stage	Pathology	Indication for testicular biopsy	Presence of spermatozoa (intraoperative TESE)
1	12	I	Drepanocytosis	No semen	No
2	12	I–II	Drepanocytosis	No semen	No
3	13	II–III	Osteosarcoma	Poor quality semen sample ^a	Few
4	13	II–III	Osteosarcoma	No semen	Few
5	13	IV	Osteosarcoma	No semen	Few
6	14	III	Lymphohistiocytosis	Azoospermia	Few
7	14	II	Non-Hodgkin's lymphoma	Azoospermia	No
8	14	III	Nasopharyngeal carcinoma	No semen	No
9	15	I	Drepanocytosis	No semen	No
10	16	IV	Leukemia	Poor quality semen sample ^b	Few

^aFewer than 50 motile spermatozoa.

^b100% immotile spermatozoa.

concentrations. With a low DMSO concentration, sucrose supplementation and a slow-controlled freezing protocol with induced ice nucleation, we observed survival of human spermatogonia able to proliferate and initiate differentiation in frozen-thawed grafts after 6 months (Wyns et al., 2008), while no germ cells were detected after 5 months with 0.7 M DMSO and a non-controlled freezing protocol (Jahnukainen et al., 2007).

Furthermore, results achieved using 1.5 M DMSO as a cryoprotectant in marmosets were not encouraging, since none of the cryopreserved tissue survived after autotransplantation, in contrast to fresh transplants, which showed more than 60% graft survival (Luetjens et al., 2008). This strongly suggests that the cryotolerance of ITT may be species-specific but, as different freezing protocols were applied in these studies, other parameters might also have influenced the outcomes.

Table III Anatomopathological analysis related to clinical patient characteristics and urogenital history.

Patient age	Tanner stage	Urogenital history	Testicular volume (ml)	Anatomopathological analysis of testicular biopsy
10	I	Circumcision	≤4	SG
10	I	Right orchidopexy (6 years)	≤4	SG
10	I	Orchidectomy for testicular atrophy	≤4	SG–SPC
11	I	/	≤4	SG
11	I–II	/	≤4	SG
11	II	/	6	SG–SPC–SPT–SPZ
11	I	Circumcision	≤4	SG
11	I–II	/	6	SG–SPC–SPT
11	I	/	≤4	SG
12	I	/	≤4	SG–SPC–SPT–few SPZ
12	I–II	/	≤4	SG–SPC
13	II–III	/	6	SG–SPC–SPT–SPZ
13	II–III	/	8	SG–SPC–SPT–SPZ
13	IV	Right inguinal hernia + epispadias	15	SG–SPC–SPT–few SPZ
14	III	/	15	SG–SPC–SPT–SPZ
14	II	/	6	SG–SPC–SPT–SPZ
14	III	Circumcision	10	SG–SPC
15	I	Repeated cystitis	≤4	SG–SPC–rare SPT
16	IV	/	15	SG–SPC–SPT–SPZ

SG, spermatogonia; SPC, spermatocyte; SPT, spermatid; SPZ, spermatozoa.

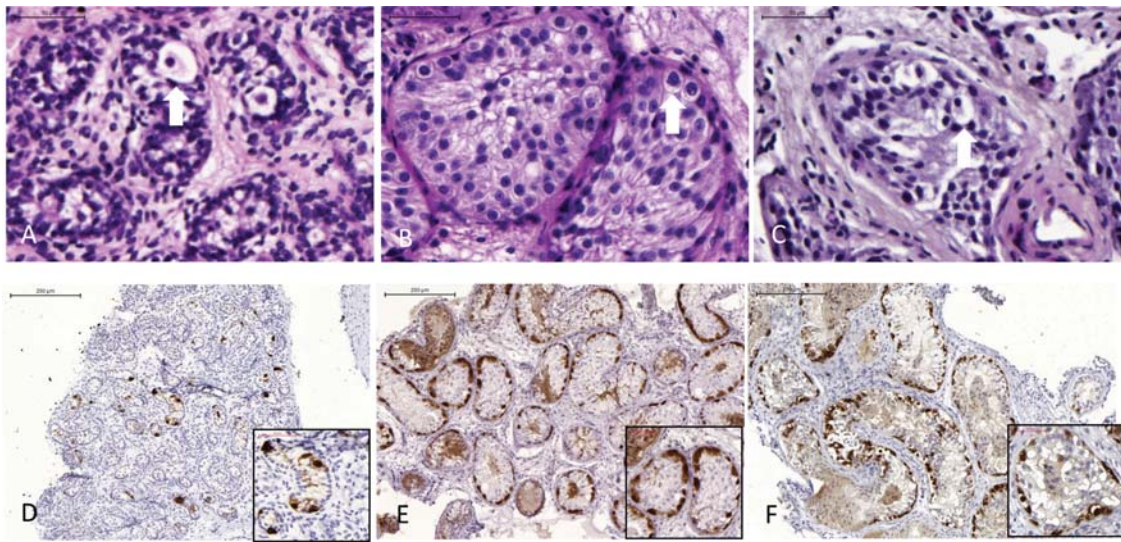


Figure 3 H–E (A–C) and MAGE A4 (D–F) immunostained sections of testicular tissue from a 2-year-old, 12-year-old and 14-year-old boy of Tanner stage I, II–III and III, respectively. Magnification 100× (insets 400×). Arrows show spermatogonia.

Unfortunately, the lack of availability of normal ITT constitutes a serious limiting factor for valid comparative studies on spermatogonial stem cell survival and functionality in different cryopreservation conditions.

Therefore, we cannot rule out the possibility that other cryopreservation methods and protocols might result in even better outcomes. Studies on optimization of our cryopreservation protocol for human ITT are still ongoing and the best approach needs to be defined by

Table IV Tissue characteristics during surgery, anatomopathological analysis after surgery and cryopreservation protocol applied in prepubertal patients.

Patient number	Intraoperative TESE	Anatomopathological analysis after surgery	Cryopreservation protocol
1	–	+	ITT
2	–	–	ITT
3	+	+	ITT + MTT
4	+	+	ITT + MTT
5	+	+	ITT + MTT
6	+	+	ITT + MTT
7	–	+	ITT
8	–	–	ITT
9	–	+	ITT
10	+	+	ITT + MTT

+, presence of spermatozoa; –, absence of spermatozoa; ITT, immature testicular tissue; MTT, mature testicular tissue.

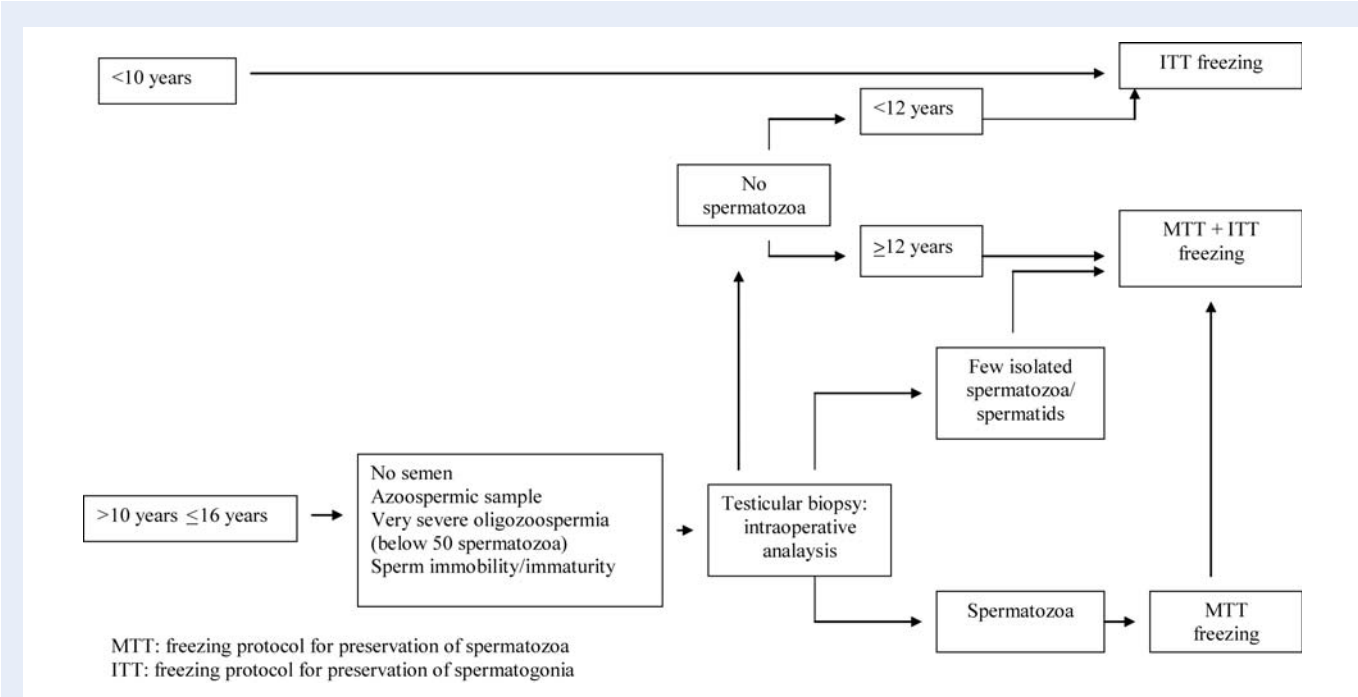


Figure 4 Strategy for fertility preservation in pre-and peripubertal patients.

future experimental models to provide our patients with the best care.

While for prepubertal boys, cryopreservation of spermatogonial stem cells is the only way of preserving their fertility, for peripubertal boys, testicular tissue content must be established because freezing of spermatozoa should be a priority. Indeed, at present, it is the only validated fertility preservation option.

Intraoperative examination of testicular content must therefore be carried out to determine the presence of spermatozoa or late spermatids in order to choose an adapted freezing protocol. Since live births may be achieved in humans after ICSI even with a limited number of spermatozoa (Palermo et al., 1992), their cryopreservation should be

considered even when only a few spermatozoa are available after thawing. Two of our peripubertal patients showing poor-quality semen samples with no motile sperm (Patient 10) or very few motile sperm (Patient 3) underwent sperm cryopreservation. As there was a high probability that no viable sperm would be found after thawing, since cryotolerance of sperm is below 10% in severe oligoasthenozoospermia (unpublished data), they also benefited from testicular biopsy with intraoperative examination. Because of the same concerns about post-thaw availability of sperm, additional tissue was frozen with the protocol for ITT. For boys aged between 12 and 16 years who failed to provide a semen sample or were azoospermic in terms of ejaculate, intraoperative analysis of testicular tissue

showed the presence of spermatozoa in three of eight patients, allowing use of a validated method for fertility preservation (Table II). The relevance of intraoperative analysis of testicular tissue lies in the absence of absolute predictive factors for the presence of haploid germ cells in testes of peripubertal boys, while spermatogenesis is known to occur to some extent at very early stages of pubertal development (Muller and Skakkebaeck, 1983; Hovatta, 2001) and before being able to produce an ejaculate (Nielsen *et al.*, 1986).

Since gonadal maturation in boys is not characterized by a critical visible event as in girls, obtaining a reliable and sensitive estimate of the onset of sperm production (spermarche) is difficult (Hirsch *et al.*, 1985). Defining the age from which ITT cryopreservation is the best choice is not feasible, since there is great variability in age at spermarche. Indeed, a large study conducted in China based on spermatia and age at first ejaculation as markers of spermarche found the onset of sperm production in 3.3, 7.5, 24.3, 46.5, 71.2 and 88.1% of boys aged 11, 12, 13, 14, 15 and 16 years, respectively (Ji and Oshawa, 2000).

Spermatia (Baldwin, 1928; Richardson and Short, 1978; Hirsch *et al.*, 1985; Nielsen *et al.*, 1986) has also been used for spermarche determination. Age at spermatia appears to be earlier than age at first ejaculation, explaining why, in some of our boys who could not provide a semen sample, mature gametes were found in testicular biopsies. Detection and/or preservation of sperm extracted from urine was not performed, as it is time-consuming and has no place in an emergency context, and because it has a high rate of false negatives, underestimating the rate of spermatogenesis (Hirsch *et al.*, 1985; Ji and Oshawa, 2000). Correlations between spermatia and clinical parameters have been established (Schaefer *et al.*, 1990), but do not allow clear cut-offs for allocation to sperm or spermatogonial preservation. Indeed, the onset of spermarche (based on spermatia) was observed in 50% of boys between Tanner stage II and III for pubic hair, although a few cases of spermatia (<5%) were even observed in clinically prepubertal boys (Schaefer *et al.*, 1990). There appears to be wide variation in testicular size and secondary sex characteristics at onset of spermarche (Nielsen *et al.*, 1986), which may occur when little or no pubic hair has developed and the testes have grown only slightly. Moreover, serum hormone levels do not predict sperm production, as shown by van Casteren *et al.* (2008). Testosterone evaluation was not performed in this study since, at the onset of spermatia, testosterone levels are actually low and thus not useful as predictors of spermarche. Indeed, testosterone levels start to increase after Tanner stage II (Radicioni *et al.*, 2005) and urinary testosterone levels do not reach maximum levels until ~2 years after spermarche (Nielsen *et al.*, 1986).

Discrepancies were found in the presence/absence of spermatozoa between intraoperative analyses and definitive anatomopathological observations, which led, in three cases, to cryopreservation with the protocol for spermatogonial preservation rather than sperm preservation. These patients were 12, 14 and 15 years of age and at Tanner Stages I, II and I, respectively. Since clinical characteristics do not appear to be helpful for decision management in these patients, this raises the question of possibly freezing testicular tissue with both protocols from the age of 12 onwards. Such a recommendation is also based on concerns about the reproductive potential of immature haploid germ cells retrieved at early pubertal stages. Indeed, although *in vitro* maturation of the later stages of spermatogenesis

using adult testicular tissue has already led to the birth of healthy offspring (Tesarik *et al.*, 1999) and looks to be a promising approach, the fertilization competence of immature haploid cells retrieved from peripubertal tissue still remains to be proven.

On the basis of our clinical experience and tissue-sample analysis during this 5-year period, we outlined a strategy for fertility preservation in pre- and peripubertal patients not able to provide a sperm sample, or presenting with azoospermia or an insufficient number of spermatozoa to obtain viable spermatozoa after thawing (Fig. 4).

Ethical concerns have been expressed about ITT cryopreservation, highlighting the importance of the risk/benefit balance (Bahadur and Ralph, 1999). Safety and effectiveness of fertility preservation and restoration procedures are therefore essential issues.

Because of the small size of testes in prepubertal children, immature gonadal tissue sampling may be considered too invasive a procedure. However, in the three available studies on testicular tissue harvesting in young cancer patients (Keros *et al.*, 2007; Wyns *et al.*, 2007; Ginsberg *et al.*, 2010), as well as our 5-year clinical study, no major surgical complications occurred during testicular biopsy. Nevertheless, since fertility restoration after gonadotoxic therapy remains unpredictable, we performed (where applicable) and strongly recommend unilateral sampling to limit potential risks of testicular tissue damage. In addition, since there is evidence that the prepubertal state does not protect the gonads from damage in case of gonadotoxic therapy (Rivkees and Crawford, 1988; Mackie *et al.*, 1996; Kenney *et al.*, 2001), testicular biopsy should only be considered before cytotoxic therapy, at least until there is proof of the benefits of such a procedure in terms of fertility restoration. Regarding general anesthesia, since testicular biopsy is usually performed under the same anesthesia as that used for placement of the central line for chemotherapy, there is no additional risk involved.

When considering the effectiveness of tissue harvesting, the availability of sufficient numbers of germ stem cells to contemplate fertility restoration is crucial. In our study, the mean volume of retrieved biopsies was about 5% of total testicular volume, which, according to morphological studies (Muller and Skakkebaeck, 1983), should provide enough germ cells for fertility preservation. Furthermore, the presence of spermatogonia in the tissue, and therefore the potential for its later use, was established in 100% of patients. However, in one patient with a history of urogenital disease, only a few spermatogonia were observed on H-E sections. In such cases, the use of MAGE A4 antibody facilitates their identification. On the basis of qualitative observations, there was a good correlation between MAGE A4 immunostaining and classic histological evaluation, and we did not find any additional benefits of using this specific immunostaining. Since there is no guarantee of the presence or functionality of spermatogonia before puberty, this consideration should be discussed in relation to the urogenital history of the child, and be taken into account in the decision-making process.

Although fertility restoration methods with ITT look promising in the light of animal studies, and offer the prospect of several realistic applications (for review, see Wyns *et al.*, 2010), none have proved feasible in humans to date. A clinical trial initiated in the UK some years ago (Radford, 2003) has unfortunately yielded no further information on patient follow-up as yet. Children and their parents should therefore be informed of the experimental nature of this approach and the fact that there is no guarantee of fertility restoration (Bahadur, 2004; Tournaye *et al.*, 2004; Jahnukainen *et al.*, 2006).

Informed parental consent and the child's assent, meaning he was given the opportunity to discuss the procedure, should be sought. We found that all children from the age of 5 years were able to participate in the discussion when appropriate words were used. As obtaining fully informed consent from children is difficult, substituted consent from parents should for now be limited to the safekeeping of tissue (Bahadur and Ralph, 1999; Bahadur et al., 2000).

During this 5-year study period, 93.5% of referred patients accepted ITT cryobanking, despite being clearly advised about the absence of proven reproductive potential of frozen tissue. Reasons for refusal were essentially religious issues and fear of cancer transmission to offspring. In one case, scheduled sample retrieval was cancelled due to the severity of acute illness in the patient.

Refusal and cancellation of tissue harvesting do not therefore appear to be related to the experimental nature of the procedure. Moreover, all parents and children viewed this strategy of fertility preservation in a positive light since, beside uncertainties surrounding issues of effectiveness and safety, discussion of fertility with cancer patients places the emphasis firmly on the future and provides reassurance that curative treatment is the aim.

In conclusion, the majority of ITT samples showed reproductive potential based on the presence of spermatogonia and, although the procedure was presented as still experimental, storage was accepted by most parents after appropriate explanation.

Over the next few years, research should focus on how to extend successful experiments in animals to young boys, and on identification of the ideal microenvironment for spermatogonial stem cell development. Considering the success of fertility restoration approaches with ovarian tissue cryopreservation (Donnez et al., 2004), there is every hope that in the next 20–30 years, various fertility restoration options will become available for today's prepubertal boys who were given the opportunity to bank their tissue prior to gonadotoxic therapy.

Authors' roles

C.W. was responsible for medical consultations and wrote the manuscript. M.C. performed cryopreservation of tissue samples. S.P. collected clinical data from medical files. P.L. conducted medical consultations. J.-F.X.W. performed surgical biopsies. J.D. reviewed the manuscript.

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References

- Bahadur G. Ethics of testicular stem cell medicine. *Hum Reprod* 2004; **19**:2702–2710.
- Bahadur G, Ralph D. Gonadal tissue cryopreservation in boys with paediatric cancers. *Hum Reprod* 1999; **14**:11–17.
- Bahadur G, Chatterjee R, Ralph D. Testicular tissue cryopreservation in boys. Ethical and legal issues. *Hum Reprod* 2000; **15**:1416–1420.
- Baldwin B. The determination of sex maturation in boys by a laboratory method. *J Comp Psychol* 1928; **8**:39–43.
- Brook P, Radford J, Shalet S, Joyce A, Gosden R. Isolation of germ cells from human testicular tissue for low temperature storage and autotransplantation. *Fertil Steril* 2001; **75**:269–274.
- Clermont Y. The cycle of the seminiferous epithelium in man. *Am J Anat* 1963; **122**:35–51.
- Crabbé E, Verheyen G, Tournaye H, Van Steirteghem A. Freezing of testicular tissue as a minced suspension preserves sperm quality better than whole-biopsy freezing when glycerol is used as cryoprotectant. *Int J Androl* 1999; **22**:43–48.
- Donnez J, Dolmans MM, Demylle D, Jadoul P, Pirard C, Squifflet J, Martinez-Madrid B, van Langendonck A. Livebirth after orthotopic transplantation of cryopreserved ovarian tissue. *Lancet* 2004; **364**:1405–1410.
- Fleming KK, Hubel A. Cryopreservation of hematopoietic and non-hematopoietic stem cells. *Transfus Apher Sci* 2006; **34**:309–15.
- Ginsberg JP, Carlson CA, Lin K, Hobbie WL, Wigo E, Wu X, Brinster RL, Kolon TF. An experimental protocol for fertility preservation in prepubertal boys recently diagnosed with cancer: a report of acceptability and safety. *Hum Reprod* 2010; **25**:37–41.
- Hirsch M, Lunenfeld B, Modan M, Ovadia J, Shemesh J. Spermatogenesis: The age of onset of sperm emission. *J Adolesc Health Care* 1985; **6**:35–39.
- Hovatta O. Cryopreservation of testicular tissue in young cancer patients. *Hum Reprod Update* 2001; **7**:378–383.
- Izadyar F, Matthijs-Rijsenbilt JJ, Den Ouden K, Creemers LB, Woelders H, De Rooij G. Development of a cryopreservation protocol for type A spermatogonia. *J Androl* 2002; **23**:537–545.
- Jahnukainen K, Ehmcke J, Söder O, Schlatt S. Clinical potential and putative risks of fertility preservation in children utilizing gonadal tissue or germline stem cells. *Pediatr Res* 2006; **59**:40R–47R.
- Jahnukainen K, Ehmcke J, Hergenrother SD, Schlatt S. Effect of cold storage and cryopreservation of immature non-human primate testicular tissue on spermatogonial stem cell potential in xenografts. *Hum Reprod* 2007; **22**:1060–1067.
- Ji C-Y, Ohsawa S. Onset of the release of spermatozoa (spermatogenesis) in Chinese male youth. *Am J Hum Biol* 2000; **12**:577–587.
- Kenney L, Laufer MR, Grant FD, Frier H, Diller L. High risk of infertility and long term gonadal damage in males treated with high dose cyclophosphamide for sarcoma during childhood. *Cancer* 2001; **91**:613–621.
- Keros V, Rosenlund B, Hulténby K, Aghajanova L, Levkov L, Hovatta O. Optimizing cryopreservation of human testicular tissue: comparison of protocols with glycerol, propanediol and dimethylsulphoxide as cryoprotectants. *Hum Reprod* 2005; **20**:1676–1687.
- Keros V, Hulténby K, Borgström B, Fridström M, Jahnukainen K, Hovatta O. Methods of cryopreservation of testicular tissue with viable spermatogonia in pre-pubertal boys undergoing gonadotoxic cancer treatment. *Hum Reprod* 2007; **22**:1384–1395.
- Kvist K, Thorup J, Byslov AG, Hoyer PE, Mollgard K, Yding Andersen C. Cryopreservation of intact testicular tissue from boys with cryptorchidism. *Hum Reprod* 2006; **21**:484–491.
- Lee SJ, Schover LR, Partridge AH, Patrizio P, Wallace WH, Hagerty K, Beck LN, Brennan LV, Oktay K. American Society of Clinical Oncology. American Society of Clinical Oncology recommendations on fertility preservation in cancer patients. *J Clin Oncol* 2006; **24**:2917–31.
- Luetjens CM, Stukenborg JB, Nieschlag E, Simoni M, Wistuba J. Complete spermatogenesis in orthotopic but not in ectopic transplants of autologously grafted marmoset testicular tissue. *Endocrinology* 2008; **149**:1736–47.

- Mackie EJ, Radford M, Shalet SM. Gonadal function following chemotherapy for childhood Hodgkin's disease. *Med Pediatr Oncol* 1996;**27**:74–78.
- Martino M, Morabito F, Messina G, Irrera G, Pucci G, Iacopino P. Fractionated infusions of cryopreserved stem cells may prevent DMSO-induced major cardiac complications in graft recipients. *Haematologica* 1996;**81**:59–61.
- Muller J, Skakkebaeck N. Quantification of germ cells and seminiferous tubules by stereological examination of testicles from 50 boys who suffered sudden death. *Int J Androl* 1983;**6**:143–156.
- Nielsen CT, Skakkebaeck N, Richardson DW, Darling JA, Hunter WM, Jørgensen M, Nielsen A, Ingerslev O, Keiding N, Müller J. Onset of release of spermatozoa (spermarche) in boys in relation to age, testicular growth, pubic hair and height. *J Clin Endocrinol Metab* 1986;**62**:532–535.
- Orwig KE, Schlatt S. Cryopreservation and transplantation of spermatogonia and testicular tissue for preservation of male fertility. *J Natl Cancer Inst Monogr* 2005;**34**:51–54.
- Palermo G, Joris H, Devroey P, Van Steirteghem AC. Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. *Lancet* 1992;**340**:17–18.
- Radford J. Restoration of fertility after treatment for cancer. *Horm Res* 2003;**59**:21–23.
- Radicioni AF, Anzuini A, De Marco E, Nofroni I, Castracane VD, Lenzi A. Changes in serum inhibin B during normal male puberty. *Eur J Endocrinol* 2005;**152**:403–409.
- Richardson D, Short R. Time of onset of sperm production in boys. *J Biosoc Sci* 1978;**5**:15–25.
- Rivkees SA, Crawford JD. The relationship of gonadal activity and chemotherapy-induced gonadal damage. *J Am Med Assoc* 1988;**259**:2123–2125.
- Schaefer F, Marr J, Seidel C, Tilgen W, Schärer K. Assessment of gonadal maturation by evaluation of spermaturia. *Arch Dis Child* 1990;**65**:1205–1207.
- Schlatt S, Rosiepen G, Weinbauer C, Rolf C, Brook P, Nieschlag E. Germ cell transfer into rat, bovine, monkey, and human testes. *Hum Reprod* 1999;**14**:144–150.
- Schover LR, Rybicki LA, Martin BA, Bringelsen KA. Having children after cancer. A pilot survey of survivors' attitudes and experiences. *Cancer* 1999;**86**:697–709.
- Shinohara T, Inoue K, Ogonuki N, Kanatsu-Shinohara M, Miki H, Nakata K, Kurome M, Nagashima H, Toyokuni S, Kogishi K et al. Birth of offspring following transplantation of cryopreserved immature testicular pieces and in-vitro microinsemination. *Hum Reprod* 2002;**17**:3039–3045.
- Tesarik J, Bah Ceci M, Ozcan C, Greco E, Mendoza C. Restoration of fertility by *in vitro* spermatogenesis. *Lancet* 1999;**353**:555–556.
- Tournaye H, Goossens E, Verheyen G, Frederickx V, De Block G, Devroey P, Van Steirteghem A. Preserving the reproductive potential of men and boys with cancer: current concepts and future prospects. *Hum Reprod Update* 2004;**10**:525–532.
- van Casteren N, Dohle G, Romijn J, de Muinck Keizer-Schrama S, Weber R, van den Heuvel-Eibrink M. Semen cryopreservation in prepubertal boys before gonadotoxic treatment and the role of endocrinologic evaluation in predicting sperm yield. *Fertil Steril* 2008;**90**:1119–1125.
- Verheyen G, De Croo I, Tournaye H, Pletincx I, Devroey P, Van Steirteghem AC. Comparison of four mechanical methods to retrieve spermatozoa from testicular tissue. *Hum Reprod* 1995;**10**:2986–2959.
- Wyns C, Curaba M, Martinez-Madrid B, Van Langendonck A, Wese F-X, Donnez J. Spermatogonial survival after cryopreservation and short-term orthotopic immature human cryptorchid testicular tissue grafting to immunodeficient mice. *Hum Reprod* 2007;**22**:1603–1611.
- Wyns C, Van Langendonck A, Wese F-X, Donnez J, Curaba M. Long-term spermatogonial survival in cryopreserved and xenografted immature human testicular tissue. *Hum Reprod* 2008;**23**:2402–2314.
- Wyns C, Curaba M, Vanabelle B, Van Langendonck A, Donnez J. Options for fertility preservation in prepubertal boys. *Hum Reprod Update* 2010;**16**:312–28.
- Yakirevich E, Sabo E, Dirnfeld M, Sova Y, Spagnoli GC, Resnick MB. Morphometrical Quantification of Spermatogonial Germ Cells With the 57B Anti-MAGE-A4 Antibody in the Evaluation of Testicular Biopsies for Azoospermia. *Appl Immunohistochem Mol Morphol* 2003;**11**:37–44.
- Zambelli A, Poggi G, Da Prada G, Pedrazzoli P, Cuomo A, Miotti D, Perotti C, Preti P, Robustelli della Cuna G. Clinical toxicity of cryopreserved circulating progenitor cells infusion. *Anticancer Res* 1998;**18**:4705–4708.