A European Perspective On Testicular Tissue Cryopreservation For Fertility Preservation In Prepubertal And Adolescent Boys

Title

A European Perspective On Testicular Tissue Cryopreservation For Fertility Preservation In Prepubertal And Adolescent Boys

Running Title

Testicular Tissue Cryobanking In Boys And Adolescents

Authors

Helen M Picton[1], Christine Wyns[2] , Richard A Anderson[3], Ellen Goossens[4], Krisi Jahnukainen[5], Sabine Kliesch[6], Rod T Mitchell[3], G Pennings[7] , Natalie Rives[8], Herman Tournaye[9], Ans MM Van Pelt[10] , Ursula Eichenlaub-Ritter[11], ~~Etienne Van den Abbeel[12]~~, Stefan Schlatt[6]

On behalf of the ESHRE Task Force On Fertility Preservation In Severe Diseases

Addresses

[1] Division of Reproduction and Early Development, Leeds Institute of Cardiovascular and Metabolic Medicine, Faculty of Medicine and Health, University of Leeds, Clarendon Way, Leeds, UK.

[2]Université Catholique de Louvain (UCL), Pôle de Recherche en Gynécologie, Institut de Recherche Expérimentale et Clinique (IREC), 1200 Brussels, Belgium and
Department of Gynecology-Andrology, Cliniques Universitaires Saint-Luc, Avenue Hippocrate, 10, 1200 Brussels, Belgium

[3]MRC Centre for Reproductive Health, Queens Medical Research Institute, University of Edinburgh, Edinburgh UK.

[4]Research Group Biology of the Testis (BITE), Vrije Universiteit Brussel, Laarbeeklaan 103, 1090 Brussels

[5]Children´s Hospital, Helsinki University Central Hospital, Helsinki, Finland and Department of Women's and Children´s Health, Karolinska Institute and University Hospital, Stockholm

[6]Centre of Reproductive Medicine and Andrology, University Münster, Domagkstraße 11, 48149 Münster, Germany

[7]Bioethics Institute Ghent (BIG), Faculty of Philosophy and Moral Science, Ghent University, Belgium

[8]Laboratoire de Biologie de la Reproduction – CECOS, Research team EA 4308 "Gametogenesis and gamete quality", IRIB, Rouen University Hospital, University of Rouen, 76031 Rouen cedex, France

[9]Centre for Reproductive Medicine, University Hospital of the Brussels Free University, Laarbeeklaan 101, 1090 Brussels , Belgium

[10]Center for Reproductive Medicine, Academic Medical Center, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands

[11]Faculty of Biology, Gene Technology/Microbiology, University of Bielefeld, Bielefeld 33501, Germany.

~~[12]IVF lab- Center for Reproductive Medicine, University Hospital Gent, De Pintelaan 185, 9000 Gent, Belgium.~~

55 **ESHRE Task Force On Fertility Preservation In Severe Disease**

56

57 **Corresponding Author**

58 HM Picton, Division of Reproduction and Early Development, Leeds Institute Cardiovascular and

59 Metabolic Medicine, Faculty of Medicine and Health, University of Leeds, Clarendon Way, Leeds,

60 UK. LS2 9JT

61 Email: H.M.Picton@leeds.ac.uk, Fax +44 (0)113 343 6603

62

63

Abstract

This Task Force paper explores the evidence base for fertility preservation in prepubertal boys and adolescents. It provides an overview of the current best clinical practices, patient management strategies and experimental methods used to preserve and restore the fertility for young male patients. Collection and cryopreservation of semen is acknowledged as the proven first line treatment for fertility preservation in adolescent males. However, semen retrieval is not possible in prepubertal boys and may not always be feasible in adolescents. For these young patients practitioners are already looking towards the experimental treatments of testicular tissue cryopreservation and the harvesting and banking of isolated spermatogonial stem cells as viable means of preserving fertility. Results of a recent survey of testis freezing practices in young patients from 24 European and Israeli university hospitals prior to December 2012 indicate that more than 260 young patients (age range less than 1 year old to 16 years of age), had already undergone testicular tissue retrieval and storage for fertility preservation. In the greater majority of these cases tissue was cryobanked for boys prior to onset of oncological treatments. In light of this evidence, the ethical and legal challenges and outstanding clinical and research questions are discussed and an algorithm for the cryopreservation of sperm and testicular tissue in pre-pubertal and adolescent patients at high risk of infertility is proposed.

Key words: Adolescents**,** boys**,** fertility preservation**,** testis**,** spermatogonial stem cell

~~1.~~ Introduction

Cancer is a major cause of non-accidental mortality in children and adolescents. However, as a result of the remarkable improvements in treatments, childhood and adolescent cancer mortality rates are now declining with significant declines being recorded for multiple cancer types (Smith *et al.,* 2014). Results from European and American data suggest that long-term survival can be expected in approximately 80% of the children and adolescent diagnosed with cancer (Desandes, 2007; Hudson 2010). Indeed, recent estimates suggest that approximately 1 in 530 young adults between the ages of 20 and 39 is a childhood cancer survivor (Ward *et al.,* 2014). Unfortunately, just like the rapidly dividing malignant cells that are their primary targets, proliferating spermatogonial stem cells (SSCs) in the testis are damaged by exposure to chemotherapy agents and radiation treatments. Thus the treatments used to cure the cancer may render the patients temporarily or permanently infertile. Furthermore, gonadotoxic treatments are increasingly used to cure a range of non-malignant conditions in children. Finally, underlying genetic causes such as Klinefelter's syndrome may lead to premature germ line stem cell loss in boys (Gies *et al.*, 2012; Van Saen *et al.*, 2012; Rives *et al.,* 2013).

Formatted: Font: Italic

Formatted: Font: Italic

~~As the loss of fertility in adult life following childhood illness is becoming increasingly unacceptable different~~ Different strategies ~~are being~~have been developed to safeguard the fertility of these young patients. Cryopreservation of spermatozoa is routinely used to preserve fertility in men (~~for example,~~ Sharma 2011), and there is an increasing evidence base documenting the efficacy of sperm cryopreservation as the first line fertility preservation treatment in adolescents (Daudin *et al.,* 2015). However, for some adolescents it may not be possible to recover sperm prior to the onset of ablative therapies and semen production is clearly not an option for prepubertal boys. Testicular tissue and SSC cryopreservation are therefore now being considered as experimental strategies for fertility preservation in those young individuals who are facing the prospects of loss of their SSCs as a result of exposure to gonadotoxic therapies or for genetic conditions. This summary paper will

~~provide a comprehensive~~ review ~~of the best clinical practices and~~the ~~evidence base for~~current practices used for fertility preservation in prepubertal boys and adolescents. It will provide insights into the state of the art of SSC and testicular tissue cryopreservation as means to preserve the future fertility in young patients~~.~~. The reader is referred to accompanying long version of this paper for detailed overview of the cytotoxic impact of chemotherapy and radiations treatments on the testis and subsequent disruption of future fertility in boys and adolescents that underpins the need for fertility preservation in these young patients.

~~2. The Gonadotoxicity Of Chemotherapy And Radiation Treatments~~

~~Chemotherapy agents and radiation treatments have been shown to adversely affect the male germinal epithelium. The amount of damage depends on the regimen and the cumulative dosage of treatments used (Van der Meer *et al.*, 1993; Wyns *et al.*, 2010). Testicular cells and especially dividing germ cells (spermatogonia) are highly sensitive to cytotoxic treatments. Low doses of these treatments deplete the pool of differentiating spermatogonia, while proliferating SSCs may initially survive and spermatocytes and spermatids continue their maturation into sperm (van Alphen *et al.*, 1988). Testicular involution after such gonadotoxic damage is a slow process that takes several weeks in sexually mature men until temporary or permanent azoospermia ensues.~~

~~The recovery of sperm production after a cytotoxic insult in adulthood or at puberty depends on the ability of quiescent SSCs to survive and resume mitotic activity to produce differentiating spermatogonia (van Alphen *et al.*, 1988). If the damage is severe all SSCs may commit to apoptosis and the patient will become permanently sterile. Spermatogonia have been shown to be susceptible to such depletion at all stages of life (Whitehead *et al.*, 1982; Relander *et al.*; 2000; Jahnukainen *et al.*, 2011b). Indeed, the presence of a steady turnover of spermatogonia that undergo spontaneous degeneration before the haploid stage is reached (Muller and Skakkebaeck, 1983; Kelnar *et al.*, 2002; van Alphen *et al.*, 1988) may explain why the prepubertal state does not offer any protection~~

against the deleterious effect of chemotherapy and irradiation. Furthermore, patient age, previous testicular disorders and the individual susceptibility to cancer treatment toxicity may also influence the potential of the seminiferous epithelium to support spermatogenesis after treatment (Rives *et al.*, 2012). In addition, the different cellular compartments in the testis respond to toxic insults in different ways.

Chemotherapy-induced damage to the testis

The impact of chemotherapy agents on the spermatogenic epithelium is dependent on the type, dosage of and combination of drugs used (Wallace *et al.*, 2005; Aubier *et al.*, 1989; Siimes and Rautonen, 1990; Wyns *et al.*, 2010, Figure 1); in some circumstances even one course of cytotoxic chemotherapy can lead to azoospermia. Acute leukaemia, the most common cancer type in children, involves treatment with antimetabolites and vinca-alkaloids that inhibit DNA and RNA synthesis and mitosis. Long-term follow-up of childhood leukaemia survivors indicates that treatment with these cell cycle specific cytotoxic drugs, without a high dose of alkylating agents such as cyclophosphamide, does not totally deplete SSCs and that spermatogenesis may be reinitiated from the surviving SSCs (Jahnukainen *et al.*, 2011c; Nurmio *et al.*, 2009). The threshold dose of cyclophosphamide, in relation to infertility, has been shown to be between 7.5 and 10 g/m^2 (Aubier *et al.*, 1989; Meistrich *et al.*, 1992; Rivkees and Crawford 1988). Slow recovery of spermatogenesis may occur even after such high doses whereas permanent sterility may appear after 19-20 g/m^2 dose of cyclophosphamide (Gurgan *et al.*, 2008; Jahnukainen *et al.*, 2011c). Other alkylating agents and platinum-containing compounds such as carboplatin and cisplatin have also been reported to have a threshold dose above which there is an increased incidence of testicular failure (Figure 1, Meistrich *et al.*, 1992; Gurgan *et al.*, 2008; Bokemeyer *et al.*, 1994; Wyns *et al.*, 2010; Lampe *et al.*, 1997; Meistrich *et al.*, 1989). Both alkylating- and platinum-containing- agents cause direct DNA and RNA damage and so can affect even non-dividing, reserve stem cells. Germ cell toxicity is therefore a significant late effect of conditioning for hematopoietic stem cell transplantation such

that germ cell depletion with raised serum FSH and decreased testicular growth in puberty is observed among most male patients (van Casteren *et al.*, 2009; Anserini *et al.*, 2002). Long term spermatogenetic recovery is more likely after chemotherapy than after total body irradiation based conditioning regimens (Anserini *et al.*, 2002). Evidence for impairment of Sertoli cell function following chemotherapy has also been reported (Bar-Shira Maymon *et al.*, 2004).

Chemotherapy induced Leydig cell failure resulting in androgen insufficiency and requiring testosterone replacement therapy is rare (Sklar 1999). The majority of males undergo a normal puberty and most produce normal adult levels of testosterone. Compensated Leydig cell failure has been reported in patients treated with a combination of mustine and procarbazine for Hodgkin disease and also after treatment with high doses of cyclophosphamide (Meistrich *et al.*, 1992; Heikens *et al.*, 1996; Bramswig *et al.*, 1990). In contrast, young boys and adolescent males who receive 200 mg/kg (6.7 g/m^2) cyclophosphamide or a combination of busulphan and cyclophosphamide as conditioning therapy for bone marrow transplantation appear to retain normal Leydig cell function (Sarafoglou *et al.*, 1997).

Irradiation-induced damage to the testis

The germinal epithelium is very susceptible to radiation induced damage. The extent of these effects depends on the field of treatment, total dose and fractionation (Brauner *et al.*, 1983; Sklar *et al.*, 1990; Van der Meer *et al.*, 1993). Differentiating spermatogonia are radiosensitive to scattered doses as low as 0.1 Gy leading to short term cessation of spermatogenesis (Rowley *et al.*, 1974). Doses of 2–3 Gy also affect SSCs and cause long term azoospermia (van Alphen *et al.*, 1989), whereas doses in excess of 6 Gy deplete the SSC pool and can lead to permanent infertility (Rowley *et al.*, 1974; Centola *et al.*, 1994). Fractionation of radiotherapy increases the germ cell toxicity because repeated hits to activated reserve stem cells (Centola *et al.*, 1994; Ash 1980) and total body irradiation (TBI), used as conditioning for haematological stem cell transplantation, is associated

with significant germ cell failure (Sarafoglou *et al.*, 1997). Indeed, following TBI (10 or 13 Gy), azoospermia has been reported in 85% of men with oligozoospermia (Anserini *et al.*, 2002) and spermatogenesis did not recover until the 4th year after transplantation. Furthermore, azoospermia after hematopoietic stem cell transplantation may be overestimated if sperm samples are evaluated too early after transplantation (Anserini *et al.*, 2002).

Leydig cells are susceptible to damage from external irradiation although the dose required is much higher than that needed to cause germ cell failure. Leydig cell damage is dose dependent and is inversely related to patient age at treatment, such that pubertal or younger males receiving 24Gy for testicular leukaemia are at a high risk of delayed sexual maturation associated with decreased testosterone levels and they require androgen replacement therapy (Brauner *et al.*, 1983; Sklar et al. 1990). In contrast, the majority of males receiving 20Gy of fractionated testicular irradiation appear to retain their ability to produce normal amounts of testosterone (Sklar 1999; Sarafoglou 1997); elevated LH in these patients may indicate compensated Leydig cell failure as with some chemotherapy regimens.

Sperm damage following chemotherapy or radiation treatment

Apart from the cytotoxic effects detailed above, anti-neoplastic treatments have also been shown to have genotoxic effects (i.e. aneugenic, clastogenic and mutagenic) on male germ cells at different stages of maturation (Russo *et al.*, 2000; Adler *et al.*, 2012). Cancer treatments may induce a range of chromosome abnormalities in mature spermatozoa including: aneuploidy; structural rearrangements and alterations of chromosomes including sister chromatid exchanges; simple or double strand breaks; mutations; and micronucleus formation (Russo *et al.*, 2000; Arnon *et al.*, 2001; Adler *et al.*, 2012). Sperm DNA damage may have a negative impact on male fertility and although in many cases these spermatozoa will not be able to fertilize oocytes when fertilization does occur, genetic damage may be transmitted to the next generation (Arnon *et al.*, 2001) as

manifest by: (i) abnormal embryo development; (ii) undetected and detected spontaneous abortion; (iii) malformations in offspring; or (iv) diseases later in life of the progeny.

While much of the evidence demonstrating the genotoxic effects of cancer treatments on spermatozoa have been derived from animal studies (Adler *et al.*, 2012), reports on sperm DNA damage in humans have also been obtained following exposure to multiple drugs or treatment regimens used for common malignancies. For example, data have been derived both from young adult males with testicular cancer, Hodgkin's lymphoma or non Hodgkin's lymphoma (Tempest *et al.*, 2008; Burello *et al.*, 2011; Smit *et al.*, 2010) and from survivors of childhood cancer (Thomson *et al.*, 2002; Romerius *et al.*, 2010). These studies are frequently retrospective with different time frames of follow-up and the number of patients involved is often low. Furthermore, treatment regimens are variable and depend on the type of malignancy and sperm DNA damage is often evaluated using only one parameter of DNA alteration such as aneuploidy, defective chromatin compaction, or sperm DNA fragmentation. Very few prospective studies have directly investigated sperm DNA damage prior to, during and/or after treatment (Tempest *et al.*, 2008; Burello *et al.*, 2011; Smit *et al.*, 2010) and in general the data lack consistency. For example, sperm DNA breaks and low chromatin compaction were detected for up to 24 months post chemotherapy in survivors of testicular cancer and Hodgkin's lymphoma (O'Flaherty *et al.*, 2012). In contrast, DNA damage was significantly reduced in post-treatment semen samples after 1.1 years in another study (Smit *et al.*, 2010) whereas sperm DNA breaks were not increased in 23 childhood cancer survivors when compared to the healthy population (Thomson *et al.*, 2002) but were impaired in young survivors treated with radiotherapy or surgery only (Romerius *et al.*, 2010). In the case of testicular cancer and Hodgkin's lymphoma in young adults, chemotherapy has been shown to significantly increase aneuploidy rates in sperm 6 months after the initiation of treatment. Thereafter, chromosomal errors generally declined to pre-treatment levels around 12 (Burrello *et al.*, 2011) to 18 months after treatment onset (Tempest *et al.*, 2008). Increased aneuploidy rates have been shown to persist in

some chromosomes for up to 24 months after drug exposure but other studies have reported no increased aneuploidy frequency after 24 months but these data are based on small patient numbers (reviewed by Tempest *et al.*, 2008). Another confounding factor is the influence of the cancer itself since it has been shown that both testicular cancer and Hodgkin's lymphoma can exert a detrimental effect on chromosome segregation during the meiotic process prior to treatment and so can also lead to an increased rate of sperm aneuploidy prior to treatment (Tempest *et al.*, 2008).

One explanation for the discrepancies between studies is the observation that differentiating premeiotic and meiotic germ cells are more sensitive to the induction of mutations than SSCs. Thus the risk of transmission of genetic errors to the next generation is temporary and lasts for only a few months after treatment. In contrast, surviving SSCs containing mutations will continue to produce sperm with these mutations and if not repaired these may be transmitted to the next generation for the life time of the individual (reviewed in Meistrich 2009). Finally, epigenetic changes such as abnormal sperm methylation patterns are known to be associated with infertility and trans-generational effects (Kobayashi *et al.*, 2009). Examination of rodent sperm after exposure to a combination chemotherapy regimen normally used for testicular cancer treatment, demonstrated that the anti-neoplastic treatment altered spermatozoa DNA methylation (Chan, 2011). Largely due to the lack of large prospective studies that simultaneously evaluate the impact of chemotherapy and radiation treatment on the risk of genetic and epigenetic alterations of spermatozoa, the consensus regarding fertility preservation is therefore that gamete storage should take place before the beginning of medical anti-neoplastic treatment (Menon *et al.*, 2009; Rives *et al.*, 2012; Nahata *et al.*, 2013).

3. Risks To The Offspring Of Male Survivors Of Childhood Cancer

While the potential for reproductive loss or congenital abnormalities in the children born of cancer

~~survivors remains a concern, there are now sufficient populations of childhood cancer survivors who have reached their reproductive years to address this issue directly. Beyond the impact of sperm DNA damage on early embryo development, there are two main areas to address: risks associated with pregnancy i.e. stillbirth, and the possibility of increased risk of congenital abnormalities, genetic disease or neonatal death in the offspring of male childhood cancer survivors. The risk of stillbirth (defined in the US as occurring after 20 weeks of pregnancy) or neonatal death among the offspring of men who had survived childhood cancer have been reported in a retrospective cohort analysis within the Childhood Cancer Survivor Study (CCSS) (Signorello *et al.*, 2010). Among 1148 men who had survived childhood cancer, there were 2031 pregnancies. Irradiation of the testes (16 [1%] of 1270 men; adjusted relative risk 0·8 [95% CI 0·4–1·6] and chemotherapy with alkylating drugs (10 [1%] of 732 men, adjusted relative risk 1·2 [0·5–2·5]) were not found to be associated with an increased risk of stillbirth or neonatal death. The risks of stillbirth (from 28 weeks of gestation), neonatal disease and genetic disease have been reported in a population-based cohort of 472 male and female Danish survivors of cancer in childhood and adolescence, that included 1,037 pregnancies (Winther *et al.*, 2012). This study revealed that the risk of genetic disease was similar among the children of irradiated survivors compared with children born of non-irradiated survivors (RR 1.02, 95% CI 0.59 to 1.44) and was unchanged amongst those who received alkylating agents both when compared with those who did not receive chemotherapy (RR 0.9, 95% CI 0.5 to 1.3) and those without any potential mutagenic treatment (RR 0.8, 95% CI 0.3 to 2.1).~~

~~The risk of congenital abnormality or genetic disease in the children of male childhood cancer survivors is low. A Danish population-based cohort study of 1715 children born of 3,963 cancer survivor parents examined the association between gonadal radiation and risk of malformation in the offspring, including malformations diagnosed later in life (median follow-up time 8.2 years; Winther *et al.*, 2009). The prevalence of congenital malformations in the offspring of cancer survivors was not increased when compared to either sibling controls or the general population,~~

either overall or when male and female cancer survivors were separated. The prevalence proportion ratio was 1.1 (95% CI 0.8 to 1.5) compared with sibling controls, and the observed to expected ratio was 1.2 (95% CI 0.9 to 1.6) when compared with the general population (Winther *et al.*, 2009). There was also no detectable relationship with dose of gonadal radiotherapy. These reassuring findings support the recent data from the CCSS on 4,699 children of 1128 male and 1627 female childhood cancer survivors, which again provides strong evidence that neither chemotherapy nor gonadal radiotherapy increase the risk of congenital abnormality in offspring (Signorello et al., 2012). The prevalence of abnormality after testicular radiotherapy was 1.9%, vs. 1.7% following treatment with alkylating agents, and 2.1% in the offspring of men who did not receive radiotherapy. There was no relationship between testicular radiation dose and risk of congenital abnormality (OR 1.01; 95% CI, 0.36 to 2.83 for >5cGy). These data support previous analyses from the CCSS (Green *et al.*, 2009). In contrast, a recent Swedish/Danish birth register study of 8670 children whose fathers had cancer, compared with over 1.7 million children who did not, suggests a small increased risk of birth abnormalities, from 3.2% to 3.7% (RR 1.17, 95% CI 1.05 to 1.31) (Stahl *et al.*, 2011). The increased risk applied to both natural and assisted conceptions and was higher if the child was born within 2 years of the father's cancer diagnosis. The use of birth registers does not permit the analysis of the effects of specific cancer treatments. While these data demonstrate that the direct risks to the offspring of male childhood cancer survivors, both in terms of congenital abnormality and genetic disease, do not seem to be increased by either chemotherapy or radiotherapy, these studies only evaluated the risks to the offspring of long-term cancer survivors, they do not contradict the evidence presented for an increased risk in the short term i.e. during and for the first 2 years after therapy. This important issue requires further investigation.

4. Current Practices Of Fertility Preservation In Prepubertal Boys And Adolescents

The current interventions used to preserve fertility in males range from the use of validated clinical procedures such as semen collection and sperm cryopreservation to the adoption of experimental methodologies such as slow freezing or vitrification of immature testicular tissue or the use of research-based drug therapies that reduce or shield the testis from the gonadotoxic impact of chemotherapy or radiation treatments (Wyns *et al.*, 2010). Hormonal approaches to conserve fertility have not proven to be useful in males (for review see Shetty and Meistrich, 2005) and anti-apoptotic agents such as spingosine-1-phosphate have been shown to be of limited value (Suomalainen *et al.,* 2003). Co-administration of the immunomodulating compound AS101 during cyclophosphamide treatment appears to provide protection against cytotoxic damage without attenuating the anticancer effect in animal studies. AS101 may act via Akt/GSK-3beta phosphorylation (Carmely *et al.,* 2009). Whether AS101 has a similar protective effect in primate testes has yet to be evaluated.

Formatted: Font: Italic

Sperm cryopreservation and storage for adolescent patients

The fertility preservation strategy that has been used for many decades to safeguard the future fertility of adults (Crabbé *et al.,* 1999; ~~for review see~~ Sharma 2011) and adolescents (Daudin *et al.,* 2015) is the cryopreservation and long-term storage of ejaculated or testicular spermatozoa. With regard to adolescent patients recommendations advocate that patients are informed of their need for fertility preservation and the options available to them as early as possible during the planning of their treatment (Lee *et al.,* 2006). Indeed the presence of a cryostorage depot facility for spermatozoa has been shown to contribute positively to the patient's psychological health and confidence in post-survival fatherhood in both adults and adolescents (Saito *et al.,* 2005; Edge *et al.,* 2006). Despite the fact that cryopreservation of spermatozoa is recognized as the only effective fertility preservation technique for males facing gonadotoxic treatments, a study performed in the US revealed that only about 50% of physicians offered cryopreservation to a quarter of their patients prior to the start of potentially gonadotoxic therapies (Schover *et al.,* 2002). A recent

study of 23 French regional sperm banks recorded considerable inter-centre variation in practices involving young patients seeking to preserve their fertility before cancer therapy (Daudin *et al*., 2015). Indeed, it is mostly young adults who undertake sperm storage and the mean age varies depending on the underlying disease. Stable partnerships are rare in the younger males (up to 24%) (Kliesch *et al.,* 2010, Behringer *et al.,* 2012) and are not relevant when considering fertility preservation in prepubertal boys and adolescents. The most common malignant diseases for semen storage are testicular tumours, Hodgkin or Non-Hodgkin lymphoma, leukaemia or bone tumours with some additional non-malignant conditions with indications for gonadotoxic treatments (Kliesch *et al.,* 2010; Daudin *et al.,* 2015). Semen characteristics vary with both patient age and type of cancer (Daudin *et al.,* 2015). However in testicular cancer patients, semen parameters may be significantly reduced at time of diagnosis compared to other malignancies with oligozoospermia occurring in up to 60% of cases. Approximately 60% of males with lymphoma or leukaemia are normozoospermic, but 14% of testis cancer patients are azoospermic (with an additional 5% with anejaculation) compared to only 3% azoospermia in lymphoma (Kliesch *et al.,* 2010; van der Kaaij *et al.,* 2009).

Semen can be cryopreserved for adolescent boys in more than 80% of cases (Bahadur *et al.,* 1999; Kliesch *et al.,* 1996; van Casteren *et al.,* 2008a; Menon *et al.,* 2009; Daudin *et al.,* 2015). The rate of azoospermia varies between 2.6 to 18% of patients (Menon *et al.,* 2009; van Casteren *et al.,* 2008a). However, up to 15% of adolescent or adult patients may either fail to produce a semen sample or have insufficient spermatozoa present in the collected semen (see Table 1). In adolescents, measurements of testicular volume have been shown to be helpful in predicting the chance for successful retrieval of spermatozoa and semen production (Kliesch *et al.,* 1996; Kamischke *et al.,* 2004). As soon as spermatogenesis has been induced, semen parameters can be comparable to those of adult patients irrespective of the underlying disease (Kliesch *et al.,* 1996; Kamischke *et al.,* 2004) (Table 1).

The rules and recommendations for fertility preservation in males differ between countries. There are no strict limitations concerning semen quality or sperm numbers for fertility preservation strategies and there are no international guidelines for the duration of storage of spermatozoa, whether ejaculated or testicular. While standard semen evaluation and documentation according to World Health Organisation (WHO, 2010) criteria prior to sperm cryopreservation is valuable for fertility preservation patients, if vital sperm can be recovered even in small numbers then sperm storage is possible, as ARTs post-thaw will facilitate the selection and use of viable sperm for insemination (Nordhoff *et al.,* ~~2012~~2013). However, it must be noted that if there are fewer than 0.1 $x10^6$ sperm/ml present in the semen sample on freezing, then the success of semen cryopreservation is likely to be significantly reduced. Most cancer patients have reduced semen parameters at the time of cryopreservation that will cause a decline in sperm quality after thawing. Never-the-less successful inseminations with samples stored for cancer patients have been documented and range from 5 to 16% of patients (van Casteren *et al.,* 2008b) provided that semen quality is high post-thaw. When IVF or ICSI are applicable using cryopreserved spermatozoa success rates are comparable to standard IVF and ICSI procedures in infertile couples. Indeed, depending on the Centre, pregnancy rates of 23-57% have been recorded for fertility preservation patients, (Agarwal *et al.,* 2004; Freour *et al.,* 2012; Hourwitz *et al.,* 2008; Schmidt *et al.,* 2004; van Casteren *et al.,* 2008b). To date no adverse effect of the combination of cryopreservation of semen and subsequent ART has been reported concerning the health of the offspring.

For patients with non-obstructive azoospermia, severe oligozoospermia, necrozoospermia or ejaculation disorders, testicular sperm extraction (TESE) and storage are often the only acceptable means of tissue retrieval for fertility preservation. The same techniques can be applied in oncological (adolescent or adult) patients with azoospermia with good results prior to cancer treatment. Post-therapeutically, TESE has also been used successfully to obtain sperm in up to 50%

of cases of persistent azoospermia with previous failure of cryopreservation or when cryopreservation had not been considered (Hsiao *et al.,* 2011) (Table 2). The TESE procedure requires surgical intervention, either with local or general anaesthesia with higher recovery rates obtained following microsurgical techniques (Donoso *et al.,* 2007; Ramasamy *et al.,* 2009; Colpi *et al.,* 2009). If microsurgery is not available, multifocal testicular biopsies from different sites of the testis can be used to increase the chance to detect focal spermatogenesis (Tournaye *et al.,* 2006; Dieckmann *et al.,* 2007). However, this procedure may have a negative impact on the vascularisation of the testis following the surgery. In patients with ejaculation disorders either medical or interventional treatments have been described (Sonksen and Ohl, 2002) but how widely they are used is unclear.

Testicular tissue preservation for young patients

There is increasing evidence of the use of testicular tissue cryopreservation as a means to preserve the fertility of prepubertal and peripubertal boys of up to 16 year-old (Wyns *et al.,* 2011). This statement is supported by the findings of a recent questionnaire from the ESHRE Task Force on Fertility Preservation that was distributed to 24 European and Israeli University hospitals prior to December 2012. Of the 14 respondents, half (n=7) were actively offering testis tissue cryobanking for fertility preservation in boys and adolescents, the remainder were considering the implementation of a tissue-based fertility preservation program for boys undergoing oncological treatments (Table 3). At the time of the survey, more than 260 young patients had already undergone testicular tissue retrieval for fertility preservation although the number of cases reported between Centres was highly variable (range 8-98) (Table 3). The age range of patients who had banked tissue was comparable between Centres and ranged from less than 1 year to 16 years of age. With very few exceptions, the greater majority of preserved tissue samples were still in cryostorage at the time of survey. While the majority (n=6) of Centres had cryobanked testicular tissue from boys prior to oncological treatments for the indications detailed in Table 4, the remaining 4 Centres

had also preserved testicular tissue from patients with non-malignant indications that carried a high risk for fertility loss. One Centre had exclusively collected testicular tissue from Klinefelter patients. All Centres preserving testicular tissue in this survey had used slow (equilibrium) freezing protocols to preserve tissue integrity during long-term storage at liquid nitrogen temperatures. The majority of Centres preserving tissue used Dimelthyl Sulphoxide (DMSO) combined with sucrose as the preferred cryoprotective agents. Only 1 Centre had used an ethylene glycol-based protocol.

~~5.~~ Management Of Fertility Preservation In Prepubertal Boys And Adolescents

Fertility preservation management ~~is a multidisciplinary task employing~~requires a specialist team of highly trained physicians and nurses involved in both oncology and reproductive medicine. I~~ndeed,~~ ~~i~~dentifying and educating key staff capable of initiating discussions on fertility preservation is vital to the success of fertility preservation strategies (Nagel and Neal, 2008). ~~Information flow must be accurate and timely and is vital to the uptake of fertility preservation services by young patients and their parents. Pediatric oncologists, together with oncology nurses and reproductive medicine specialists will have multiple interactions with patients and parents prior to the initiation of treatment and are therefore in an ideal position to discuss the late effects of gonadotoxic treatment, quality of life and survivor issues with patients and their families (Vadaparampil *et al.*, 2007). Close collaboration between pediatric, oncologic and fertility specialists (gynecologists, andrologists and reproductive biologists) is essential for the preparation of sperm or testicular tissue for cryopreservation. Informative letters and/or presentations during medical meetings allow oncologists to be aware of spermatogenesis physiology, onset of spermarche and potential fertility preservation approaches related to age and pubertal status. Clear instructions on who to contact in the infertility department to discuss the matter on an individual patient basis, unrestricted access to educational patient information and rapid and flexible access to medical consultation and surgical biopsy for tissue recovery and storage should be provided to accommodate the short time scales~~

infertility specialist by pediatric hematologists and oncologists before gonadotoxic treatment is initiated (Redig *et al.,* 2011). ~~Therefore, oncologists face a challenging decision making process that must take into account the possibility of delaying therapy and achieving optimal curative success rates. Delaying therapy by a few days is only appropriate when there is a good prognosis, combined with a high risk of permanent infertility and no evidence of decreased success rate of treatment. Such decisions should be guided by an institutional policy and shaped by physicians, mental health professionals and an ethical board. During this emergency consultation with a fertility specialist, children (when applicable, usually from the age of 5 with adapted words) and adolescents should receive adapted counseling and appropriate information on sexual maturation, including pubertal events and testicular maturation and reproduction (with content linked to age, physical examination and previous history).~~ It is essential that the clinical team has a detailed knowledge of the hormonal events and testicular physiology around puberty in order to provide patients /parents with accurate information. Parents need to be made aware of- and be receptive to- fertility preservation options while young patients must also be receptive to discussions about fertility preservation, suitable to their age, and be made aware of their health status, as appropriate. Access to institution guidelines, human resources and appropriate educational materials are also vital (Vadaparampil *et al.,* 2008). There is currently some debate as to whether testicular tissue should be frozen in conjunction with sperm freezing as discrepancies may also be found in the presence/absence of spermatozoa between intraoperative analyses and definitive anatomopathological observations (Wyns *et al.,* 2011). Furthermore, the protocols used to preserve mature germ cells differ from those used to preserve spermatogonia. This raises the question of whether testicular tissue should be cryopreserved using both protocols during peri-pubertal life from the age of 12. Such a recommendation is based on concerns about the reproductive potential of immature, haploid germ cells retrieved at early pubertal stages. Indeed, although *in vitro* maturation of round spermatids from adult testicular tissue has already led to the birth of healthy offspring

(Tesarik *et al,* 1999), the fertilization competence of immature haploid cells retrieved from peri-pubertal tissue still remains to be proven.

Where there is a risk of gonadal damage and fertility loss, patients should be referred to the infertility specialist by pediatric hematologists and oncologists before gonadotoxic treatment is initiated (Redig *et al.,* 2011). ~~Therefore, oncologists face a challenging decision-making process that must take into account the possibility of delaying therapy and achieving optimal curative success rates. Delaying therapy by a few days is only appropriate when there is a good prognosis, combined with a high risk of permanent infertility and no evidence of decreased success rate of treatment. Such decisions should be guided by an institutional policy and shaped by physicians, mental health professionals and an ethical board. During this emergency consultation with a fertility specialist, children (when applicable, usually from the age of 5 with adapted words) and adolescents should receive adapted counseling and appropriate information on sexual maturation, including pubertal events and testicular maturation and reproduction (with content linked to age, physical examination and previous history).~~ It is essential that the clinical team has a detailed knowledge of the hormonal events and testicular physiology around puberty in order to provide patients /parents with accurate information. Parents need to be made aware of- and be receptive to- fertility preservation options while young patients must also be receptive to discussions about fertility preservation, suitable to their age, and be made aware of their health status, as appropriate. Access to institution guidelines, human resources and appropriate educational materials are also vital (Vadaparampil *et al.,* 2008). There is currently some debate as to whether testicular tissue should be frozen in conjunction with sperm freezing as discrepancies may also be found in the presence/absence of spermatozoa between intraoperative analyses and definitive anatomopathological observations (Wyns *et al.,* 2011). Furthermore, the protocols used to preserve mature germ cells differ from those used to preserve spermatogonia. This raises the question of whether testicular tissue should be cryopreserved using both protocols during peri-pubertal life

from the age of 12. Such a recommendation is based on concerns about the reproductive potential of immature, haploid germ cells retrieved at early pubertal stages. Indeed, although *in vitro* maturation of round spermatids from adult testicular tissue has already led to the birth of healthy offspring (Tesarik *et al,* 1999), the fertilization competence of immature haploid cells retrieved from peri-pubertal tissue still remains to be proven.

Cryopreservation of spermatozoa for boys and adolescents

The collection and cryopreservation of spermatozoa is the only validated, ~~–~~clinical technique available currently to safeguard the future fertility of peripubertal boys and adolescents (Figure 2). ~~Therefore, sperm~~ Sperm banking should always be ~~used~~ offered as the first line treatment in those young patients who can produce a semen sample since live births can be obtained after ICSI even when only a few spermatozoa are available (Palermo *et al.,* 1992). Although semen samples can be obtained from boys from the age of 12 onwards (Bahadur *et al.,* 2006) the onset of sperm production (spermarche) in boys can be very difficult to predict. Spermatogenesis is known to start at very early stages of pubertal development (Muller and Skakkebaeck, 1983; Hovatta, 2001) and may occur before the ability to produce an ejaculate (Nielsen *et al.,* 1986). Moreover, gonadal maturation in boys is not characterized by critical visible events as is the case in girls, and defining the age below which the experimental immature testicular tissue cryopreservation would be the best choice for fertility preservation is not easy because of the great variability in age at spermarche (Ji and Oshawa, 2000). At the onset of spermarche there also appears to be a wide variation in both testicular size and secondary sex characteristics (Nielsen *et al.,* 1986). Spermarche may occur when little or no pubic hair has developed and when the testicular volume has increased only slightly. Indeed, the presence of spermatozoa (based on spermaturia, as a marker for spermarche) was found in 5% of clinically prepubertal boys and in 50% of boys between Tanner stage II and III for pubic hair pattern. Serum hormone levels are not useful to predict sperm production since at the onset of

spermaturia, gonadotrophin and testosterone concentrations are low and only start to increase after Tanner stage II (Radicioni *et al.,* 2005; van Casteren *et al.,* 2008a). Correlations between spermaturia and clinical parameters have been established (Schaefer *et al.,* 1990), but do not allow clear cut-offs for allocating a boy to either sperm banking or spermatogonial preservation. The detection and preservation of sperm extracted from morning urine is not considered an appropriate therapy because of its time-consuming nature. In cases of failure to produce a semen sample by masturbation, assisted ejaculation techniques such as penile vibratory stimulation or electro-ejaculation under general anesthesia should be considered as a second-line treatment option. These methods may have advantages over experimental techniques such as immature testicular tissue sampling as the former will facilitate collection and storage of mature sperm. Since there is no reliable sensitive estimate for the presence of spermatozoa in the testes, intra-operative examination of testicular tissue (Wyns *et al.,* 2011) should be carried out to determine the presence of either spermatozoa or late spermatids in order to choose an appropriate freezing protocol.

~~Once collected, liquefied ejaculates should be analysed according to the guidelines of the World Health Organization (WHO, 2010) and standard parameters of sperm concentration total number of spermatozoa, morphology and motility determined prior to cryopreservation. According to WHO recommendations, if possible sufficient specimens should be stored to provide 10 or more inseminations, in order to ensure a good chance of pregnancy. However, when sperm preservation is conducted in an emergency situation in response to a young patient's disease it is not always possible to adhere to WHO recommendations. For samples with severely reduced semen quality and/or only a few motile spermatozoa and sperm suspensions from surgically retrieved spermatozoa, it may be necessary to concentrate the sperm into a minimum volume by centrifugation before the addition of cryoprotective agents and sample preservation according to local practises. At the time of writing, the evidence base for vitrification of spermatozoa or testicular tissue using specialist vitrification devices is limited but this approach may prove useful~~

Formatted: Tab stops: Not at 6 cm

~~in the future (Isachenko *et al.,* 2004; 2005; Poels *et al.,* 2013). The storage of individual sperm in empty zona pellucida may be advocated in some situations (Cohen *et al.,* 1997).~~ In all cases, the ~~best~~ cryobiology practises used for the preservation and long-term storage of samples ~~are advocated but the procedures used~~ will be informed by the physical principles and the specific properties and nature of the cells/tissues to be stored (Benson *et al.,* 2012).

Cryopreservation of testicular tissue in prepubertal boys and adolescents

In cases where no semen can be collected, the experimental techniques of cryopreservation of testicular tissue or suspensions of immature testicular cells including SSCs should be considered (Figure 2). To minimise trauma to the patient, the surgical recovery of testicular tissue should be combined with other interventions requiring anaesthesia, such as bone marrow sampling or implantation of venous ports. ~~Close interdisciplinary cooperation between paediatric oncologist and urologists or paediatric surgeons is therefore required.~~ To date 4 freezing protocols for human immature testicular tissue have been described using cryoprotective agents that that range from 1.5M ethylene glycol and sucrose (Kvist *et al.,* 2006), 0.7M DMSO (Keros *et al.,* 2005; 2007) or 0.7M DMSO and sucrose (Wyns *et al.,* 2007; 2008; Poels *et al.,* 2014). Expensive bio-freezers may not be essential for the cryopreservation of human testicular tissue (Baert *et al.,* 2013). Indeed, evaluation of human immature testicular tissue following xenotransplantation into nude mice suggests that vitrification may be as effective for tissue preservation as slow freezing methods (Curaba et al., 2011; Poels *et al.,* 2013). To maximise the quality and viability of human testicular tissue post thaw all aspects of the tissue collection and processing, the type and concentration of cryoprotectants used as well as the cooling and warming protocols must be fully optimised. Since the reproductive potential of cryopreserved immature testicular tissue has still to be proven in humans, the technique remains experimental and no one preservation protocol has been shown to be

superior over any other published method (Kvist *et al.,* 2006; Keros *et al.,* 2007; Wyns *et al.,* 2008; Baert *et al.,* 2013; Goossens *et al.,* 2013; Poels *et al.,* 2013).

Biosecurity and long-term storage of tissues for fertility preservation

The long-term storage of fertility preservation samples, whether in the form of semen sperm, epididymal sperm or testicular tissue samples, requires that the patient and/or his parents maintain a contract with the host institution to guarantee the continued storage of tissue and that the storage facility adheres to national guidelines and international recommendations for good tissue banking practices. Annual tissue banking charges may apply according to local practices. The associated costs may be covered by the patient or their family, or be borne by health insurance or the hospital or an institutional grant (Table 3). Provided optimal low temperatures are maintained throughout long-term freeze-banking there is no obvious deterioration of sperm quality with time. Indeed, children have been born from semen stored for over 28 years (Feldschuh *et al.,* 2005). ~~However, maintenance of the physical- and bio- security of fertility preservation samples is key if samples are to be stored for many decades. Adherence to national guidelines and international recommendations for good tissue banking practices is essential from the outset in terms of witnessing, sample processing and labelling, infection screening , and the need for regular cryobank audit and patient follow-up. There is also a significant chance that fertility preservation samples will need to be transported at some point in their history either for continuation of storage if patients move or prior to their use for fertility restoration in later life.~~

~~6.~~ Fertility Restoration Using Cryopreserved Testicular Tissues And Stem Cells

Development of the procedures used for the preservation of SSCs and testicular tissues from boys and adolescents is far more advanced than research into the methods needed to realise the fertile potential of these cells and these techniques have yet to be proven to be safe for clinical use. In

summary, fertility restoration strategies include the auto-transplantation of a suspension of SSCs by injection into the testis to restore spermatogenesis or auto-transplantation of frozen-thawed testicular grafts ~~back into the testis or an ectopic site. Where there is any risk of reintroduction of malignant cells via the transplant then the only option is to~~ and the growth and maturation of~~e the~~ SSCs *in vitro*. ~~These issues are discussed in full below.~~

Propagation and autotransplantation of spermatogonial stem cells

Currently, SSC injection is considered the most promising tool for fertility restoration in pre-pubertal cancer patients. The technique was originally described in the mouse (Brinster and Zimmerman, 1994) and it has been successfully used to infuse SSC through the efferent duct into the rete testis of sterile recipients with the resultant reinstatement of spermatogenesis and the restoration of fertility ~~(Brinster and Averbock 1994)~~. However, because of differences in anatomy and consistency and the larger testis size, injection of SSC via the rete testis, has proved to be a better treatment site for species such as the bovine, primate, and human (Schlatt *et al.,* 1999; Ning *et al.,* 2012).

If SSCs are to be used to restore male fertility, then they first need to be isolated and propagated *in vitro* before they can be autotransplanted in the numbers required to efficiently recolonize the testis and reinstate spermatogenesis. For example, it has been demonstrated that only 5-10% of transplanted SSCs result in colony formation in the recipient testis and the extent of donor-derived spermatogenesis is directly related to the number of transplanted cells (Dobrinski *et al.,* 1999). Furthermore, murine studies have indicated that factors such as glial cell line derived neurotrophic factor (GDNF)–which facilitates self-renewal of the SSCs and supports SSC replication *in vitro* (Kanatsu-Shinohara *et al.,* 2003) are essential for SSC propagation. This evidence has been replicated in several species (Honaramooz *et al.,* 2002, ~~Izadyar *et al.,* 2003,~~ Schlatt *et al.,* 1999, Nobrega *et al.,* 2010, Aponte *et al.,* 2008). Importantly, in the context of human fertility restoration

adult and prepubertal human SSCs have been successfully grown *in vitro*, without losing their stem cell capacity or ability to colonize the seminiferous tubules upon xenotransplantation (Sadri-Ardekani *et al.,* 2009, Sadri-Ardekani *et al.,* 2011). A number of other studies, mostly in mice, have evaluated the recovery of fertility after non-cultured SSC injection. Transplanted mice were able to produce live born offspring with normal birth weights, growth rates and fertility (Goossens *et al.,* 2009). No numerical chromosomal aberrations were detected in spermatozoa from transplanted males, or in their offspring (Goossens *et al.,* 2010). Importantly, studies of methylation patterns and histone modifications in post-transplantation germ cells revealed that apart from two minor alterations, epigenetic marks following uncultured mouse SSC injection were not different compared to control spermatogenesis (Goossens *et al.,* 2009; Goossens *et al.,* 2011). Most recently Rhesus monkey SSCs have been injected under slow constant pressure into the rete testis under ultrasound-guidance, both autologously and allogeneically and into both adult and prepubertal rhesus monkeys sterilised by alkylating chemotherapy. Following the completion of spermatogenesis *in vivo*, sperm cells that were able to fertilize oocytes by ICSI were found in the ejaculate of recipients (Hermann *et al.,* 2012). While the demonstration of functional donor spermatogenesis following SSC transplantation in primates is an important milestone towards using SSC to restore human fertility it remains vitally important to prove that the epigenetic programming and stability of SSC are not compromised following cryopreservation, culture and transplantation in humans (Struijk *et al.,* 2013).

Restoration of fertility by autotransplantation of testicular tissue

Transplantation of fragments of testicular tissue provides an alternative strategy to the use of SSC suspensions. This approach maintains the SSCs within their non-exposed natural niche, thus preserving the interactions between the germ cells and their supporting somatic cells. Nutrients and hormones from the body will reach the graft and induce spermatogenesis and the resultant sperm can be extracted and used in ICSI procedures. Autologous transplantation of the testicular biopsy

back into the testis (Van Saen *et al.,* 2009), scrotum (Wyns et al., 2007) or ectopically under the skin (Jahnukainen *et al*., 2007) can however only be used to restore spermatogenesis if the presence of malignant cells can be excluded. In initial research using mouse models, testis grafts were placed at ectopic sites such as in the peritoneal space, on the ear or under the back skin (Boyle *et al.,* 1975; Schlatt *et al.,* 2002). However, these grafts become sclerotic or showed meiotic arrest. Autologous grafting to several locations in the irradiated primate body also showed that spermatogenesis could only be re-established when the graft was placed in the scrotum but the efficiency of fertility restoration remained poor (Jahnukainen *et al.,* 2012). Transplantation of the tissue under the tunica albuginea of the testis (intra-testicular grafting) might improve results as, in mice, this technique has proved to be highly efficient with the re-establishment of full spermatogenesis in all of the grafts (Van Saen *et al.,* ~~2008~~2009). At the time of writing, little is known about the functionality of the sperm generated in such grafts as only a few groups have addressed this important question using mouse and rabbit donor tissue. However, with sperm retrieved from ectopic and intra-testicular mouse allografts, insemination studies using ICSI have demonstrated that the spermatozoa so derived were able to support full-term development of the progeny (Schlatt *et al*., 2003; Ohta and Wakayama, 2005). It was also possible to obtain offspring using rabbit sperm that had developed in intra-testicular transplanted xenografts (Shinohara *et al.,* 2002). Normal blastocyst development has been achieved *in vitro* following ICSI with sperm from ectopic porcine and monkey xenografts (Honaramooz *et al.,* 2004; 2008; Nakai *et al.,* 2010).

In vitro spermatogenesis

The major hurdle which must be overcome in patients with a prior haematological malignancy when restoring fertility by autotransplantation of propagated SSCs or testicular tissue is the risk of reintroducing residual malignant cells via the transplanted tissue. While it is possible to avoid the transfer of malignant cells by using testicular xenografts, the risk of zoönosis means that xenografting of human testicular tissue is unlikely to provide an acceptable clinical solution for

679 fertility restoration. Although, positive and negative cell sorting strategies have the potential to
680 target and remove cells from cultured mouse SSC populations and after xenografting (Dovey *et al.,*
681 2013; Hermann *et al.,* 2011). Sorting protocols using magnetic activated cell sorting (MACS),
682 FACS or differential plating have been found to have variable efficiency when used to enrich
683 human SSCs (Geens *et al.,* 2006; ~~Geens *et al.,*~~ 2011; Nickkholgh *et al.,* 2014a). Thus, at the time of
684 writing autotransplantation of cell suspensions or tissues still runs the risk of reintroducing cancer
685 via the graft.
686
687 The risk of reintroduction of malignant cells via the autograft may be circumvented by *in vitro*
688 spermatogenesis. *In vitro*-derived spermatozoa that are free from residual disease can then be used
689 to inseminate oocytes using ICSI. Strategies which support the *in vitro* growth and differentiation of
690 germ cells include the 3 dimensional (3D) culture of testicular cells (Stukenborg *et al.,* 2008) or
691 organ culture (Sato *et al.,* 2011). The main difference between the two approaches lies in the fact
692 that in organ culture the testicular biopsy remains intact and is layered upon an island of agar that is
693 maintained in a liquid medium. In 3D culture, the germ cells are dissociated from their somatic cells
694 prior to culture and they are then suspended in medium containing 35 and 50% agar, the so-called
695 Soft-Agar-Culture-System. In both systems SSCs are co-cultured with somatic cells from the same
696 biopsy so resembling the *in vivo* situation and supporting two-way communication between the
697 different cellular compartments. In the mouse model, *in vitro* spermatogenesis has been successful
698 up to the elongated spermatid stage of spermatogenesis but so far offspring have only been
699 generated with sperm derived following organ culture (Sato *et al.,* 2011). Although encouraging
700 results have recently been obtained regarding the genetic and epigenetic stability of human SSCs
701 during long-term culture (Nickkholgh *et al.,* 2014b), the fertility of *in vitro* derived sperm have still
702 to be established before the clinical value of this type of experimental approach can be fully
703 assessed. When no germ cells are available in the initial testis biopsy, an alternative option may be
704 the *in vitro* derivation of sperm cells from the patient's somatic cells, such as skin fibroblasts, by

induced pluripotency or transdifferentiation of these cells (Yang *et al.,* 2012). This approach is however still in its infancy.

~~7.~~ Follow-Up Of Patients At Risk Of Gonadal Dysfunction Following Treatment For Childhood Cancer

Predicting the likelihood of gonadal dysfunction in individual patients who are survivors of childhood disease may be difficult. Guidance on this topic has recently been published (Wallace *et al.,* 2013). Measurements of gonadotrophins and testosterone in pre-pubertal patients are unlikely to be helpful as the hypothalamo-pituitary-gonadal axis is not active priori to puberty (Mann and Fraser 1996). Therefore, the accurate clinical assessment of growth during childhood using appropriate growth charts is very important, particularly in the context of pubertal staging as puberty may be delayed (or occasionally advanced) following cancer treatment. Treatment for childhood cancer may result in central effects on the hypothalamus and/or pituitary that will affect gonadotrophin production, or primary testicular failure may result from direct damage to the testis (Mitchell *et al.,* 2009). Leydig cell damage may reduce testosterone production and hence delay or arrest puberty (>14y), whilst effects on Sertoli cells and germ cells of the seminiferous epithelium may impair spermatogenesis and decreased adult testicular size. Normal pubertal development with full hair- and penis- growth indicates normal Leydig cell function, irrespective of testicle size. T~~As described earlier, t~~he seminiferous epithelium is more sensitive to the effects of cancer treatment than the Leydig cells and patients may still have small adult testis size and impaired fertility despite having undergone a normal puberty with sufficient testosterone production (Jahnukainen *et al.,* 2011~~a~~).

Assessment of male pubertal development should include: (i) measurement of testicular volume; (ii) Tanner staging of secondary sexual development; (iii) measurement of serum FSH, LH,

testosterone and inhibin B (if available); (iv) yearly bone age x-ray from any signs of initiation until completion of puberty. For patients with delayed or arrested puberty (>14y), treatment with increasing doses of testosterone should be considered (Kenney *et al.,* 2012). Once puberty has been established, measurement of testicular volumes, and FSH and inhibin B levels may also indicate effects on the seminiferous epithelium and hence spermatogenesis (Lahteenmaki *et al.,* 2008). ~~Azoospermia is likely if the testicular volumes are <10ml and the FSH is >10 IU/L (Muller *et al.,* 1996; Siimes *et al.,* 1995).~~ Where possible and, as requested by the patient himself, semen analysis can be performed and the patients referred for ART, as appropriate. Should semen analysis reveal azoospermia, it is worth repeating the test annually, as the recovery of surviving stem cells (spermatogonia) may take several years.

Post-surgical complications

~~While the collection and storage of testicular tissue can be proposed as a means of fertility preservation in young patients who are unable to produce a semen sample, the procedure is experimental and invasive and~~ The evidence from testicular biopsy in adults (Schlegel and Su 1997; Manning *et al.,* 1998) suggest that ~~the~~ risk of the biopsy procedure itself should not be overlooked in younger patients (Mitchell *et al.,* 2009). Immediate surgical complications include bleeding and infection where as later complications may be indicative of damage to the remaining testis. ~~In a study of 64 men with non-obstructive azoospermia undergoing biopsy for sperm extraction, 82% had hypoechoic lesions indicative of haematoma 3 months after biopsy, which had resolved by 6 months leaving linear scars (Schlegel and Su 1997). Another study in adults with obstructive azoospermia who underwent a large-bore needle, testicular biopsy demonstrated no difference in LH, FSH or testosterone levels at 4 weeks of follow-up compared to the pre-biopsy levels (Steel *et al.,* 2001). In contrast, a large open testicular biopsy has been associated with a significant reduction in testosterone at 6 months post biopsy; although testosterone concentrations increased by 12 months but did not not revert to original levels (Manning *et al.,* 1998).~~

The evidence base concerning the effects of testicular biopsy in prepubertal patients is limited. In a US study of 24 boys, 14 underwent testis tissue biopsy without any short term complications and no post-operative orchitis or reports of excessive pain (Ginsberg *et al.,* 2010). In a series of 62 prepubertal and peripubertal patients under 16 years-old, who underwent unilateral testicular biopsy for fertility preservation, no short-term post-surgical complications were observed (Wyns *et al.,* 2011). Longer-term follow up of patients undergoing testicular biopsy has been reported in cryptorchid boys undergoing orchidopexy (Patel *et al.,* 2005). In this study 112 boys were followed up for a mean of 11 years post-surgery (age range 18-29). None of the patients required re-operation for bleeding, received treatment for post-operative orchitis or sustained loss of a testis. An ultrasound scan at follow-up revealed no cases of testicular atrophy or biopsy related damage to the testis, or development of anti-sperm antibodies (Patel *et al.,* 2005). In a study of 23 patients who underwent an open wedge testis biopsy during treatment or on cessation of treatment in childhood for acute lymphoblastic leukaemia, 8 patients receiving standard risk therapy had FSH, inhibin B and testosterone levels comparable to the general population (Nurmio *et al.,* 2009).

Future fertility

Determining Tthe effects impact of prepubertal testicular biopsy on on future fertility is difficult to predict. for prepubertal patients, given the limited amount of tissue taken and the favourable results regarding post-operative complications. To date the evidence base suggests that the procedure itself is unlikely to result in a significant impairment of fertility. Nevertheless, Meticulous record keeping and monitoring of young patients who have undergone a biopsy is vital to ensure that there are no complications related to the procedure including any damage to the remaining testis tissue. In addition, meticulous records must be kept by institutions undertaking these procedures and the outcomes reported in the literature. Multi-centre studies to facilitate data collection fromon these

relatively rare patients ~~will~~ are needed to provide clearer insights into the requirements for long-term follow-up ~~needed~~.

~~8.~~ The Ethical and Legal Frameworks For Fertility Preservation In Prepubertal Boys And Adolescents

The setting for making decisions and developing and implementing fertility preservation strategies in young boys and adolescents are heavily influenced by life-changing and life-threatening diagnoses and treatment options that not only distress patients, parents and physicians but also raise a raft of complex ethical and legal issues. The main ethical justification for interventions associated with fertility preservation is the need to safeguard the best interests of the child. ~~This is generally expressed in a favorable risk-benefit ratio. This balancing is highly complex due to a number of factors such as: the intervention is performed on incompetent minors; there is a lack of scientific and clinical evidence of the efficiency and safety of fertility preservation technologies such as testis and SSC cryopreservation and no proof of principle about their feasibility in humans; and the need for tissue recovery from young patients generates a highly emotional and stressful situation for both patients and parents. In the absence of evidence regarding future success, utilization rate, psychological consequences etc., the current position seems to be largely determined by 2 elements: (i) the importance attributed to the wish to have genetically related children (patient autonomy), and (ii) the optimism regarding the development of medical technology in the future (Murphy, 2010).~~

A key ~~The first~~ question that must be addressed in consideration of fertility preservation strategies is to whom storage of sperm and/or testicular tissue should be offered (Murphy 2010). Indeed, recent surveys suggests that the issue of sterility is hardly discussed with parents of boys undergoing chemotherapy (Lee *et al.,* 2006; Anderson *et al.,* (2008). There are 2 schools of thought. It can be argued that as paediatric oncology teams treat a patient with the intent to cure, then

Formatted: Space After: 0 pt

Formatted: Space After: 0 pt

fertility preservation strategies should be discussed with all young patients and their families. The alternative view, however, is that young patients and/or their parents should only be approached to discuss fertility preservation options if there is ~~both~~ not only a good prognosis ~~and~~ but also a high risk of permanent infertility (Wallace *et al.,* 2005).

~~The diagnosis of genetic disease or cancer in a child inevitably creates a highly distressing and stressful situation that may seriously hamper rational decision-making. For many, cancer treatment will have to start as soon as possible, resulting in considerable time pressure. Moreover, other intervening psychological forces such as feelings of guilt and panic and mechanisms such as anticipated decision regret should be taken into account (Van Den Broecke *et al.,* 2001). Still, careful counseling can lead to a well-considered informed consent from either the patient or the parents (Ginsberg *et al.,* 2010).~~ In all cases i~~I~~nformed consent from parents or legal guardians should be taken before tissue is harvested. Even when minors are legally incompetent, an effort should be made to inform them about the implications of the procedure (at a level appropriate for their age and maturity) and to obtain assent (Bahadur *et al.,* 2001). The consent form must include sections on safety (mentioning the possibility of both expected and unexpected adverse events) and on the experimental nature of testis freezing and SSC preservation and that the research methods for fertility restoration in animals have not yet been successfully translated to humans.

The risks of fertility loss must be balanced against the potential for fertility restoration from stored samples and explained to each individual child and his parents to make sure that they understand that there is no guarantee of success. In this context it is useful to consider the procedure as a two-step process. Phase 1 involves the collection and storage of semen as the priority or the recovery of testicular tissue if semen collection is impossible. Phase 2 incorporates the replacement and/or subsequent use of the material for fertility restoration. The risks associated with these two phases differ. While t~~T~~he collection and cryopreservation of semen for fertility preservation is an

established, non-invasive, technology for adolescents with cancer (Daudin *et al.,* 2015~~). In marked contrast, ~~), the recovery of a testicular biopsy from boys in whom sperm is not yet produced must be regarded as ~~an~~ experimental ~~procedure~~ as key issues such as how much tissue to collect, which preservation and fertility restoration techniques to use, and the potential risk of reintroduction of malignant cells during fertility restoration etc. all remain to be resolved. The direct costs of phase 1 (general anesthesia, pain etc.) are relatively small, especially when they can be combined with necessary cancer-related interventions. ~~Nevertheless, attention should be paid to unknown risk factors such as complications after testis biopsy in prepubertal boys and the possibility of reintroduction of cancer in the patient or defects in the offspring.~~ Both the beneficence and the non-maleficence principle imply that the cost-benefit balance should be maximized. This means that the least harmful and the most beneficial intervention(s) should be chosen, taking into account the other aspects of the intervention. It should be made clear to the patient and his parents that storage does not guarantee that he has a right to have the material replaced in the future.

~~When calculating the benefits for the patient, one should not only look at the possibility to have one's own genetic children in the future but also at the psychological benefits that having testicular tissue in storage may have on the patient both now and in the future. Stored material may put patients at ease, give them hope, promote recovery and create less stress (Crawshaw and Sloper, 2010). However, emphasis should be put on the experimental nature of the intervention to avoid therapeutic misconception (the failure to distinguish between research and treatment) and unrealistic expectations. If the cryostored material proves to be useful for reproductive purposes in the future, it will most likely be with limited success.~~ Clinics offering cryobanking are morally obliged to participate in data collection and follow-up research in order to improve information provision and decision-making.

Formatted: English (United Kingdom)

~~9. The Legal Context Of Fertility Preservation In Boys and Adolescents~~

The ~~application~~ development and uptake of fertility preservation strategies in prepubertal boys ~~assumes that the~~ needs be supported by the creation of suitable legislat~~ion~~ory~~/~~ and regulatory frameworks~~ion~~. Legal rules should cover key points such as: differences associated with the handling and storage of gametes vs. gonadal tissue; maximal storage period- storage for several decades may be required; and tissue disposal in the event of death. The possibility of (partial) reimbursement of treatment and storage costs through some form of insurance and rules about proxy consent by parents or legal guardians regarding tissue collection and storage may also need to be considered. Further discussion of the ethical and legal issues surround fertility preservation in boys and adolescents is provided in the accompanying long version of this paper. ~~within each country covers a number of points including: the possibility to store the material (gametes and/or gonadal tissue) for several decades if necessary; a regulation detailing how to dispose of the material in the event of death; the possibility of (partial) reimbursement of treatment and storage costs through some form of insurance; and rules about proxy consent by parents or legal guardians regarding tissue collection and storage. The results of the current survey of the uptake of testicular tissue cryopreservation for boys and adolescents indicated that testicular tissue collection and cryopreservation is already in limited use either with approval of the local ethics committee of the University or Hospital or according to the current bioethics law as for example in France. In all cases procedures were considered to be experimental such that no costs were charged to the patients. The costs for tissue retrieval and cryobanking as well as for analysis of tissue samples prior to and after cryobanking were covered either by public health insurance or by hospital research grants or grants from public or private funding agencies. One Centre charged patients a yearly fee for continued cryostorage of samples.~~

~~The legal rules relevant for fertility preservation vary on 3 points: differences regarding handling~~

Formatted: Tab stops: Not at 1.75 cm

10. Future Challenges For Fertility Preservation In Boys and Adolescents

The development of strategies for fertility preservation ~~of fertility~~ in prepubertal boys and adolescents is still in its ~~early~~ infancy and represents a balance between biological, clinical and technical knowns, technological unknowns and ethical and legal questions. Progress in this field is ~~very~~ encouraging and ~~our ability to~~ it has enabled us to design treatment algorithms that have the potential to safeguard the future fertility of these young patients ~~by the cryopreservation of sperm when present or the use of new technologies of SSC and/or testicular tissue freezing when sperm are absent~~ (Figure 2). The algorithm is built on a detailed understanding of human spermatogenesis combined with significant improvements in cancer treatments and advances in cryobiology and stem cell technology. However, ~~although the field is advancing there are~~ many important questions ~~that~~ remain unanswered (Table 5). Experimental techniques such as SSC and testicular tissue freezing, while promising, require further validation as efficient and safe methods for clinical use before they can be fully integrated into routine treatment strategies and the decision making process used to ensure the most effective use of cryopreserved tissues for the future restoration of fertility in these patients.

Authors Roles

HM Picton led on the preparation, drafting and editing of this comprehensive review**.** C Wyns, RA Anderson, E Goossens, K Jahnukainen, S Kliesch, R T Mitchell, G Pennings, N Rives, H Tournaye, AMM Van Pelt, all contributed to manuscript drafting and critical review. S Schlatt distributed and analysed the survey data and contributed to manuscript drafting and critical review. U Eichenlaub-Ritter ~~and E Van den Abbeel~~ provided a critical overview from ESHRE on the manuscript contents.

Acknowledgements

We are grateful to all of the Centres who contributed to the data collected in the current survey. Our thanks are extended to: Academic Medical Center, Amsterdam, Netherlands; Cliniques Universitaires Saint Luc, Université Catholique de Louvain, Brussels, Belgium; Hopitaux de Rouen, France; Karolinska Hospital, Stockholm, Sweden; Rigshospitalet Kopenhagen, Denmark; MRC, University of Edinburgh, United Kingdom, Royal Hospital for Sick Children, University Edinburg, United Kingdom, Sheba Medical Center Tel-Hashomer, University Tel-Aviv, Israel, Centrum für Reproduktionsmedizin und Andrologie, Universitätsklinikum Münster, Universität Münster, Germany, Universitätsspital Bern, Universität Bern Switzerland, Universität Klinikum Heidelberg, Universität Heidelberg, Germany, Universitair Ziekenhuis Brussel, Brussels, Belgium, Children's Hospital, Helsinki University Central Hospital, University of Helsinki, Finland.

The authors acknowledge the support of their respective departments during the preparation of this manuscript: HM Picton Division of Reproduction and Early Development, University of Leeds; C Wyns UCL, IREC and Department of Gynecology-Andrology, Cliniques Universitaires Saint-Luc; RA Anderson and RT Mitchell MRC Centre for Reproductive Health, University of Edinburgh; E Goossens Research Group Biology of the Testis, Vrije Universiteit Brussel; K Jahnukainen Children´s Hospital, Helsinki University Central Hospital; S Kliesch and S Schlatt Centre of Reproductive Medicine and Andrology, University Münster; G Pennings, Bioethics Institute Ghent, Ghent University; N Rives, Laboratoire de Biologie de la Reproduction – CECOS, Rouen University Hospital; H Tournaye Centre for Reproductive Medicine, University Hospital of the Brussels Free University; AMM Van Pelt, Center for Reproductive Medicine, Academic Medical Center, Amsterdam; U Eichenlaub-Ritter, Faculty of Biology, University of Bielefeld~~, E Van den Abbeel, IVF lab- Center for Reproductive Medicine, University Hospital Gent.~~

The work was funded by the European Society of Human Reproduction and Embryology (ESHRE)

References

~~Adler ID, Pacchierotti F, Russo A. The measurement of induced genetic change in mammalian germ cells. *Methods Mol Biol* 2012;**817**: 335-375.~~

Agarwal A, Ranganathan P, Kattal N, Pasqualotto F, Hallak J, Khayal S, Mascha E. Fertility after cancer: a prospective review of assisted reproductive outcome with banked semen specimen. *Fertil Steril* 2004;**81**: 342-348.

~~Aksglaede L, Wikström AM,Rajpert De Meyts E, Dunkel L, Skakkebaek NE, Juul A. Natural history of seminiferous tubule degeneration in Klinefelter syndrome. *Hum Reprod Update* 2006 ; **12** : 39-48.~~

Anderson RA, Weddell A, Spoudeas HA, Douglas C, Shalet SM, Levitt G and Wallace WHB. Do doctors discuss fertility issues before they treat young patients with cancer ? *Hum Reprod* 2008, **10** : 2246-2251

~~Anserini P, Chiodi S, Spinelli S, Costa M, Conte N, Copello F, Bacigalupo A. Semen analysis following allogeneic bone marrow transplantation. Additional data for evidence-based counselling. *Bone Marrow Transplant* 2002;**30**: 447-51.~~

Aponte PM, Soda T, Teerds KJ, Mizrak SC, van de Kant HJ, de Rooij DG. Propagation of bovine spermatogonial stem cells in vitro. Reproduction 2008;136: 543-57

~~Ash P. The influence of radiation on fertility in man. Br J Radiol. 1980 Apr;53(628):271-8. Sklar CA. Growth and neuroendocrine dysfunction following therapy for childhood cancer. *Pediatr Clin North Am* 1997;**44**: 489-503.~~

Aubier F, Flamant F, Brauner R, Caillaud JM, Chaussain JM, Lemerle J. Male gonadal function after chemotherapy for solid tumors in childhood. *J Clin Oncol* 1989; **7**:304–309.

Formatted: desc2, Left

963 Baert Y, Van Saen D, Ning L, Haentjens P, Tournaye H, and Goosens E What is the best
964 cryopreservation protocol for human testicular tissue banking? *Hum Reprod* 2013; **Epub ahead**
965 **of print**;**28**: 1816-1826.
966 Bar-Shira Maymom B, Yogev L, Marks A, Hauser R, Botchan A, Yavetz H. Sertoli cell
967 inactivation by cytotoxic damage to the human testis after cancer therapy. *Fertil Steril* 2004; **81**:
968 1391–1394.
969 Bahadur G, Ralph D. Gonadal tissue cryopreservation in boys with paediatric cancers. *Hum Reprod*
970 1999; **14**:11-17.
971 Bahadur G, Spoudeas H, Davies MC, Ralph D. Factors affecting sperm banking for adolescent
972 cancer patients *Arch Dis Child* 2006; **91**;715–716.
973 Bahadur G, Whelan J, Ralph D, Hindmarsh P. Gaining consent to freeze spermatozoa from
974 adolescents with cancer: legal, ethical and practical aspects. *Hum Reprod* 2001; **16**: 188-193.
975 Behringer K, Müller H, Goergen H, Thielen I, Eibl AD, Stumpf V, Wessels C, Wiehlpütz M,
976 Rosenbrock J, Halbsguth T, Reiners KS, Schober T, Renno JH, von Wolff M, van der Ven K,
977 Kuehr M, Fuchs M, Diehl V, Engert A, Borchmann P. Gonadal function and fertility in survivors
978 after Hodgkin lymphoma treatment within the German Hodgkin Study Group HD 13 to HD 15
979 trials. *J Clin Oncol* 2012; **31**: 231-239.
980 Benson JD, Woods EJ, Walters EM, Critser JK. The cryobiology of spermatozoa. *Theriogenology*
981 2012; **78**:1682-99.
982 Bokemeyer C, Schmoll HJ, van Rhee J, Kuczyk M, Schuppert F, Poliwoda H. Long-term gonadal
983 toxicity after therapy for Hodgkin's and non-Hodgkin's lymphoma. *Ann Hematol* 1994;68:105–110.
984 Boyle PF, Fox M, Slater D. Transplantation of interstitial cells of the testis: effect of implant site,
985 graft mass and ischaemia. *Br J Urol* 1975; **47**: 891-898.
986 Bramswig JH, Heimes U, Heiermann E, Schlegel W, Nieschlag E, Schellong G. The effects of
987 different cumulative doses of chemotherapy on testicular function. Results in 75 patients treated for
988 Hodgkin's disease during childhood or adolescence. *Cancer* 1990; **65**:1298–1302.

Formatted: Font: Bold
Formatted: Font: Bold, Highlight
Formatted: Font: Bold

~~Brauner R, Czernichow P, Cramer P, Schaison G, Rappaport R. Leydig cell function in children~~ *Natl Acad Sci USA* 1994:**91**: 11298-11302.

Brinster RL, Zimmermann JW. Spermatogenesis following male germ-cell transplantation. *Proc Natl Acad Sci USA* 1994:**91**: 11298-11302.

~~Burrello N, Vicari E, La Vignera S, Romeo G, Campagna C, Magro E, Giuffrida D, D'Agata R, Calogero AE. Effects of anti-neoplastic treatment on sperm aneuploidy rate in patients with testicular tumor: a longitudinal study. *J Endocrinol Invest* 2011; **34**: e121-5.~~

~~Centola GM, Keller JW, Henzler M, Rubin P. Effect of low dose testicular irradiation on sperm count and fertility in patients with testicular seminoma. *J Androl* 1994;**15**: 608-613.~~

~~Chan D, Delbès G, Landry M, Robaire B, Trasler JM. Epigenetic alterations in sperm DNA associated with testicular cancer treatment. *Toxicol Sci* 2012; **125**: 532-43.~~

Carmely A, Meirow D, Peretz A, Albeck M, Bartoov B, Sredni B. Protective effect of the immunomodulator AS101 against cyclophosphamide-induced testicular damage in mice. *Hum Reprod* 2009; **24**:1322-9.

~~Cohen J, Garrisi GJ, Congedo-Ferrara TA, Kieck KA, Schimmel TW, Scott RT. Cryopreservation of single human spermatozoa. *Hum Reprod* 1997; **12**: 994-1001.~~

Colpi GM, Colpi EM, Piediferro G, Giacchetta D, Gazzano G, Castiglioni FM, Magli MC, Gianaroli L. Microsurgical TESE versus conventional TESE for ICSI in non-obstructive azoospermia: a randomized controlled study. *Reprod Biomed Online* 2009;1**8**: 315-319.

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**: 43-8.

~~Crawshaw, M.A. and Sloper, P. 'Swimming against the tide' - the influence of fertility matters on the transition to adulthood or survivorship following adolescent cancer. *Eur J Cancer Care* 2010; **19**: 610-620.~~

1014 Curaba M, Poels J, Van Langendonckt A, Donnez J, Wyns C. Can prepubertal human testicular
1015 tissue be cryopreserved by vitrification? Fertil Steril 2011; 95: 2123.e9-12.
1016 Damani MN, Master V, Meng MV, Burgess C, Turek P, Oates RD. Postchemotherapy ejaculatory
1017 azoospermia: fatherhood with sperm from testis tissue with intracytoplasmic sperm injection. *J Clin*
1018 *Oncol* 2002; **20**: 930-936.
1019 Daudin M, Rives N, Walschaerts M, Drouineaud V, Szerman E, Koscinski I, Eustache F, Saïas-
1020 Magnan J, Papaxanthos-Roche A, Cabry-Goubet R, Brugnon F, Le Lannou D, Barthélémy C, Rigot
1021 JM, Fréour T, Berthaut I, Giscard d'Estaing S, Touati F, Mélin-Blocquaux MC, Blagosklonov O,
1022 Thomas C, Benhamed M, Schmitt F, Kunstmann JM, Thonneau P, Bujan L. Sperm
1023 cryopreservation in adolescents and young adults with cancer: results of the French national sperm
1024 banking network (CECOS). *Fertil Steril* 2015; **103**: 478-486.
1025 Desandes E. Survival from adolescent cancer. *Cancer Treat Rev* 2007; **33**: 609-15.
1026 Dieckmann KP, Kulejewski M, Pichlmeier U, Loy V. Diagnosis of contralateral testicular
1027 intraepithelial neoplasia (TIN) in patients with testicular germ cell cancer: systematic two site-
1028 biopies are more sensitive than single random biopsy. *Eur Urol* 2007; **51**:175-185.
1029 Dobrinski I, Ogawa T, Avarbock MR, Brinster RL. Computer assisted image analysis to assess
1030 colonization of recipient seminiferous tubules by spermatogonial stem cells from transgenic donor
1031 mice. *Mol Reprod Dev* 1999; **53**:142–148.
1032 Donoso P, Tournaye H and Devroey P. Which is the best sperm retrieval technique for non-
1033 obstructive azoospermia? A systematic review. *Hum Reprod Update* 2007;**13**:539-549.
1034 Dovey SL, Valli H, Hermann BP, Sukhwani M, Donohue J, Castro CA, Chu T, Sanfilippo JS,
1035 Orwig KE. Eliminating malignant contamination from therapeutic human spermatogonial stem
1036 cells. J Clin Invest 2013;123:1833-43.
1037 Edge B, Holmes D, Makin G. Sperm banking in adolescent cancer patients. *Arch Dis Child* 2006;
1038 **91**:149-152.

Feldschuh J, Brassel J, Durso N, Levine A. Successful sperm storage for 28 years. *Fertil Steril* 2005 **84**:1017.

Freour T, Mirallie S, Jean M, Barriere P. Sperm banking and assisted reproductive outcome in men with cancer: a 10 years´ experience. *Int J Clin Oncol* 2012;**17**: 598-603.

Geens M, De Block G, Goossens E, Frederickx V, Van Steirteghem A, Tournaye H, Spermatogonial survival after grafting human testicular tissue to immunodeficient mice, *Hum Reprod 2006;* **21**: 390-396.

~~Geens M, Van de Velde H, De Block G, Goossens E, Van Steirteghem A, Tournaye H. The efficiency of magnetic-activated cell sorting and fluorescence-activated cell sorting in the decontamination of testicular cell suspensions in cancer patients. Hum Reprod 2007; 22:733-42.~~

Geens M, Goossens E, Tournaye H. Cell selection by selective matrix adhesion is not sufficiently efficient for complete malignant cell depletion from contaminated human testicular cell suspensions. Fertil Steril 2011; 95:787-91.

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.

Gies I, De Schepper J, Goossens E, Van Saen D, Pennings G, Tournaye H. Spermatogonial stem cell preservation in boys with Klinefelter syndrome: to bank or not to bank, that's the question. *Fertil Steril* 2012; **98**:284-9.

Goossens E, Bilgec T, Van Saen D, Tournaye H. Mouse germ cells go through typical epigenetic modifications after intratesticular tissue grafting. *Hum Reprod* 2011:**26**; 3388-3400.

Goossens E, De Rycke M, Haentjens P, Tournaye H. DNA methylation patterns of spermatozoa and two generations of offspring obtained after murine SSC transplantation. *Hum Reprod* 2009; **24**: 2255-2263.

Field Code Changed

Goossens E, de Vos P, Tournaye H. Array comparative genomic hybridization analysis does not show genetic alterations in spermatozoa and offspring generated after SSC transplantation in the mouse. *Hum Reprod* 2010; **25**:1836-1842.

~~Goossens E, Frederickx V, De Block G, Van Steirteghem AC, Tournaye H. Reproductive capacity of sperm obtained after germ cell transplantation in a mouse model. Hum Reprod 2003;18;1874-1880.~~

Goossens E, Van Saen D and Tournaye H Spermatogonial stem cell preservation and transplantation: from research to clinic. *Hum Reprod* 2013; **28**: 897-907. ~~epub Feb 20 2013~~

~~Green DM, Sklar CA, Boice JD, Jr., Mulvihill JJ, Whitton JA, Stovall M and Yasui Y Ovarian failure and reproductive outcomes after childhood cancer treatment: results from the Childhood Cancer Survivor Study. *J Clin Oncol* 2009; **27**: 2374-2381.~~

~~Gurgan T, Salman C, Demirol A. Pregnancy and assisted reproduction techniques in men and women after cancer treatment. *Placenta* 2008;**29 Suppl B**:152-159.~~

~~Heikens J, Behrendt H, Adriaanse R, Berghout A. Irreversible gonadal damage in male survivors of pediatric Hodgkin's disease. *Cancer* 1996;**78**: 2020-2004.~~

Hermann BP, Sukhwani M, Winkler F, Pascarella JN, Peters KA, Sheng Y, Valli H, Rodriguez M, Ezzelarab M, Dargo G, Peterson K, Masterson K, Ramsey C, Ward T, Lienesch M, Volk A, Cooper DK, Thomson AW, Kiss JE, Penedo MC, Schatten GP, Mitalipov S, Orwig KE. Spermatogonial stem cell transplantation into rhesus testes regenerates spermatogenesis producing functional sperm. Cell Stem Cell 2012; 11:715-26.

Hermann BP. Sukhwani M. Salati J, Sheng Y. Chu T. Orwig KE, Separating spermatogonia from cancer cells in contaminated prepubertal primate testis cell suspensions. Hum Reprod 2011 26: 3222-31.

Hibi H, Ohori T, Yamada Y, Honda N, Hashiba Y, Asada Y. Testicular sperm extraction and ICSI in patients with post-chemotherapy non-obstructive azoospermia. *Arch Androl* 2007;**53**: 63-65.

Formatted: Not Highlight

Formatted: Font: Bold, Not Highlight

Formatted: Not Highlight

Honaramooz A, Cui XS, Kim NH, Dobrinski I. Porcine embryos produced after intracytoplasmic sperm injection using xenogeneic pig sperm from neonatal testis tissue grafted in mice. *Reprod Fertil Dev* 2008:**20**;802-807.

~~Honaramooz A,. Megee S. O and Dobrinski I.Germ cell transplantation in pigs, *Biol Reprod 2002*, vol. 66 (1) 21-28~~

Honaramooz A, Li MW, Penedo MC, Meyers S, Dobrinski I. Accelerated maturation of primate testis by xenografting into mice, *Biol Reprod 2004;* **70**: 1500-1503.

Hovatta O. Cryopreservation of testicular tissue in young cancer patients. *Hum Reprod Update* 2001; **7**: 378-383.

Hsiao W, Stahl PJ, Osterberg EC, Nejat E, Palermo GD, Rosenwaks Z, Schlegel PN. Successful treatment of postchemotherapy azoospermia with microsurgical testicular sperm extraction: the Weill Cornell experience. *J Clin Oncol* 2011; **29**:1607-1611.

Hourvitz A, Goldschlag DE, Davis OK, Veeck Gosden L, Palermo GD, Rosenwaks Z. Intracytoplasmic sperm injection (ICSI) using cryopreserved sperm from men with malignant neoplasm yields high pregnancy rates. *Fertil Steril* 2008; **90**:557-563.

Hudson MM. Reproductive outcomes for survivors of childhood cancer. *Obstet Gynecol* 2010; **116**: 1171-83.

~~Isachenko V, Isachenko E, Katkov II, Montag M, Dessole S, Nawroth F, Van Der Ven H.~~ cryopreservation of immature non-human primate testicular tissue on spermatogonial stem cell potential in xenografts, *Hum Reprod* 2007; **22**: 1060-1067.

~~Isachenko V, Isachenko E, Montag M, Zaeva V, Krivokharchenko I, Nawroth F, Dessole S, Katkov II, van der Ven H. Clean technique for cryoprotectant-free vitrification of human spermatozoa. Reprod Biomed Online 2005, 10:350-4~~Izadyar D. O. K, Creemers F, Posthuma LB, Parvinen G ~~M,De Rooij DG., Proliferation and differentiation of bovine type A spermatogonia during long-term culture. *Biol Reprod* 2003; **68**: 272-281.~~

Formatted: Highlight

Jahnukainen K., Ehmcke J.,. Hergenrother S.D, 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.

Jahnukainen K, Ehmcke J, Hou M, Schlatt S. Testicular function and fertility preservation in male cancer patients. *Best Pract Res Clin Endocrinol Metab* 2011~~a~~ ; **25**: 287-302.

Jahnukainen K, Ehmcke J, Nurmio M, Schlatt S. Autologous ectopic grafting of cryopreserved testicular tissue preserves the fertility of prepubescent monkeys which receive a sterilizing cytotoxic therapy. *Cancer Res* 2012; **72**: 5174-8.

~~Jahnukainen K, Ehmcke J, Quader MA, Saiful Huq M, Epperly MW, Hergenrother S, Nurmio M, Schlatt S. Testicular recovery after irradiation differs in prepubertal and pubertal non-human primates, and can be enhanced by autologous germ cell transplantation. *Hum Reprod* 2011b; **26**:1945-54.~~

~~Jahnukainen K, Heikkinen R, Henriksson M, Cooper TG, Puukko-Viertomies LR, Mäkitie O. Semen quality and fertility in adult long-term survivors of childhood acute lymphoblastic leukemia. *Fertil Steril* 2011c; **96**: 837-42.~~

Ji C-Y, Ohsawa S. Onset of the release of spermatozoa (spermarche) in Chinese male youth. *Am J Hum Biol* 2000; **12**: 577-587.

Kamischke A, Jürgens H, Hertle L, Berdel WE, Nieschlag E. Cryopreservation of sperm from adolescents and adults with malignancies. *J Androl* 2004; **25**:586-592.

Kanatsu-Shinohara,. Ogonuki MN, Inoue K, Miki H, Ogura A, Toyokuni S, Shinohara T., Long-term proliferation in culture and germline transmission of mouse male germline stem cells, *Biol Reprod* 2003; **69**: 612-616.

~~Kelnar CJ, McKinnell C,Walker M, Morris KD,Wallace WH, Saunders PT, Fraser HM, Sharpe RM. Testicular changes during infantile 'quiescence' in the marmoset and their gonadotrophin dependence:a model for investigating susceptibility of the prepubertal human testis to cancer therapy? *Hum Reprod* 2002;**17**:1367–1378.~~

Field Code Changed

Kenney LB, Cohen LE, Shnorhavorian M, Metzger ML, Lockart B, Hijiya N, Duffey-Lind E, Constine L, Green D, Meacham L.Male reproductive health after childhood, adolescent, and young adult cancers: a report from the Children's Oncology Group. *J Clin Oncol* 2012; **30**: 3408-3416.

Keros V, Rosenlund B, Hultenby 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, Hultenby K, Borgstro¨m B, Fridstro¨m 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.

Kliesch S, Behre, HM, Nieschlag E. Cryopreservation of semen from adolescent patients with malignancies. *Medic Pedc Oncol* 1996; **26**: 20-27.

Kliesch S, Kamischke A, Cooper TG, Nieschlag E. Cryopreservation of semen. In: Nieschlag, E., H.M. Behre, S. Nieschlag (Hrsg) Andrology. 3. ed, Springer, Berlin, Heidelberg, ch **24**, 515-531, 2010.

~~Kobayashi H, Hiura H, John RM, Sato A, Otsu E, Kobayashi N, Suzuki R, Suzuki F, Hayashi C, Utsunomiya T, Yaegashi N, Arima T. DNA methylation errors at imprinted loci after assisted conception originate in the parental sperm. *Eur J Hum Genet* 2009;**17**: 1582-91.~~

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.

Lahteenmaki PM, Arola M, Suominen J, Salmi TT, Andersson AM, Toppari J. Male reproductive health after childhood cancer. *Acta Paediatr* 2008; **97**: 935-942.

~~Lampe H, Horwich A, Norman A, Nicholls J, Dearnaley DP. Fertility after chemotherapy for testicular germ cell cancers. *J Clin Oncol* 1997;**15**:239–345.~~

Lee SJ, Schover LR, Partridge AH, et al.: American Society of Clinical Oncology recommendations on fertility preservation in cancer patients. *J Clin Oncol* 2006;**24**: 2917-2931.

Mann DR, Fraser HM. The neonatal period: a critical interval in male primate development. *J Endocrinol* 1996;**149**: 191-197.

Manning M, Junemann KP, Alken P. Decrease in testosterone blood concentrations after testicular sperm extraction for intracytoplasmic sperm injection in azoospermic men. *Lancet* 1998 **352**: 37

~~Meistrich ML. Male gonadal toxicity. *Pediatr Blood Cancer* 2009;**53**: 261-266.~~

~~Meistrich ML, Chawla SP, Da Cunha MF, Johnson SL, Plager C, Papadopoulos NE, Lipshults LI, Benjamin RS. Recovery of sperm production after chemotherapy for osteosarcoma. *Cancer* 1989; **63**: 2115–2123.~~

~~Meistrich ML, Wilson G, Brown BW, da Cunha MF, Lipshultz LI. Impact of cyclophosphamide on long-term reduction in sperm count in men treated with combination chemotherapy for Ewing and soft tissue sarcomas. *Cancer* 1992;**70**:2703–2712.~~

Menon S, Rives N, Mousset-Siméon N, Silbert L, Vannier JP, Mazurier S, Massé L, Duchesne V, Macé B. Fertility preservation in adolescent males: experience over 22 years at Rouen University Hospital. *Hum Reprod* 2009;**24**: 37-44.

Meseguer M, Garrido N, Remohí J, Pellicer A, Simón C, Martínez-Jabaloyas JM, Gil-Salom M. Testicular sperm extraction (TESE) and ICSI in patients with permanent azoospermia after chemotherapy. *Hum Reprod* 2003;**18**:1281-1285.

Mitchell RT, Saunders PT, Sharpe RM, Kelnar CJ, Wallace WH. Male fertility and strategies for fertility preservation following childhood cancer treatment. *Endocr Dev* 2009;**15**: 101-134.

~~Muller HL, Klinkhammer-Schalke M, Seelbach-Gobel B, Hartmann AA, Kuhl J. Gonadal function of young adults after therapy of malignancies during childhood or adolescence. Eur J Pediatr 1996; **155**: 763-769.~~

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.

Murphy T. Parents' choices in banking boys' testicular tissue. *J Med Ethics* 2010; 36: 806-9.

Nagel K, Neal M. Discussions regarding sperm banking with adolescent and young adult males who have cancer. *J Pediatr Oncol Nurs* 2008; **25**:102-6.

~~Nahata L, Cohen LE, Lehmann LE, Yu RN. Semen analysis in adolescent cancer patients prior to bone marrow transplantation: when is it too late for fertility preservation? *Pediatr Blood Cancer.* 2013; **60**:129-32.~~

Nakai M, Kaneko H, Somfai T, Maedomari N, Ozawa M, Noguchi J, Ito J, Kashiwazaki N, Kikuchi K. Production of viable piglets for the first time using sperm derived from ectopic testicular xenografts. *Reproduction* 2010:**139**; 331-335.

Nickkholgh B, Mizrak SC, Korver CM, van Daalen SK, Meissner A, Repping S, van Pelt AM. Enrichment of spermatogonial stem cells from long-term cultured human testicular cells. *Fertil Steril* 2014a; **102**:558-565.

Nickkholgh B, Mizrak SC, van Daalen SK, Korver CM, Sadri-Ardekani H, Repping S, van Pelt AM. Genetic and epigenetic stability of human spermatogonial stem cells during long-term culture. *Fertil Steril*. 2014b: **102**:1700-7.

Nielsen CT, Skakkebaek N, Richardson DW, Darling JA, Hunter WM, Jørgensen M, Nielsen A, Ingerslev O, Keiding N, Müller J. Onset of release of spermatozoa (spermache) in boys in relation to age, testicular growth, pubic hair and height. *J Clin Endocrinol Metab* 1986; **62**: 532-535.

Ning L, Meng J, Goossens E, Lahoutte T, Marichal M, Tournaye H. In search of an efficient injection technique for future clinical application of spermatogonial stem cell transplantation: infusion of contrast dyes in isolated cadaveric human testes. *Fertil Steril* 2012; **98**:1443-1448.

Nobrega R. H., Greebe C. D., van de Kant H., Bogerd J., de Franca L. R., Schulz RW. Spermatogonial stem cell niche and spermatogonial stem cell transplantation in zebrafish, *PLoS One* 2010; **5**: 2010.

Nordhoff V, Schüring AN, Krallmann C, Zitzmann M, Schlatt S, Kiesel L, Kliesch S. Optimizing TESE-ICSI by laser-assisted selection of immotile spermatozoa and polarization microscopy for selection of oocytes. *Andrology* 2013;**1**: 67-74.

1215 Nurmio M, Keros V, Lahteenmaki P, Salmi T, Kallajoki M, Jahnukainen K. Effect of childhood
1216 acute lymphoblastic leukemia therapy on spermatogonia populations and future fertility. *J Clin*
1217 *Endocrinol Metab* 2009; **94**: 2119-2122.
1218 ~~O'Flaherty CM, Chan PT, Hales BF, Robaire B. Sperm chromatin structure components are~~
1219 ~~differentially repaired in cancer survivors. *J Androl* 2012; **33**: 629-36.~~
1220 Ohta H, Wakayama T. Generation of normal progeny by intracytoplasmic sperm injection
1221 following grafting of testicular tissue from cloned mice that died postnatally. Biol Reprod
1222 2005:73;390-395.
1223 Palermo G, Joris H, Devroey P, Van Steirteghem AC. Pregnancies after intracytoplasmic injection
1224 of single spermatozoon into an oocyte. Lancet 1992:340;17-18.
1225 Patel RP, Kolon TF, Huff DS, Carr MC, Zderic SA, Canning DA,Snyder HM 3rd. Testicular
1226 microlithiasis and antisperm antibodies following testicular biopsy in boys with cryptorchidism. *J*
1227 *Urol* 2005; **174**: 2008-2010.
1228 Poels J, Abou-Ghannam G, Herman S, Van Langendonckt A, Wese F-X, Wyns C. In search of
1229 better spermatogonial preservation by supplementation of cryopreserved human immature testicular
1230 tissue xenografts with N-acetylcysteine and testosterone. *Front Surg*. 2014; 1: 47.
1231 Poels J, Van Langendonckt A, Many MC, Wese FX, Wyns C. Vitrification preserves proliferation
1232 capacity in human spermatogonia. *Hum Reprod* 2013; **28**: 578-89.
1233 Radicioni AF, Anzuini A, De Marco E, Nofroni I, Castracane VD, Lenzi A. Changes in serum
1234 inhibin B during normal male puberty. *Eur J Endocrinol* 2005; **152**: 403-409.
1235 Ramasamy R, Lin K, Veeck Gosden L, Rosenwaks Z, Palermo GD, Schlegel PN . High serum FSH
1236 levels in men with nonobstructive azoospermia does not affect success of microdissection testicular
1237 sperm extraction. *Fertil Steril* 2009;**92**: 590-593.
1238 Redig AJ, Brannigan R, Stryker SJ, Woodruff TK, Jeruss JS. Incorporating fertility preservation
1239 into the care of young oncology patients. *Cancer* 2011; **117**: 4-10.

~~Relander T, Cavallin-Ståhl E, Garwicz S, Olsson AM, Willén M. Gonadal and sexual function in~~ The feasibility of fertility preservation in adolescents with Klinefelter syndrome. *Hum Reprod*. 2013; **28**, 1468-1479.

~~Rives N, Perdrix A, Hennebicq S, Saïas-Magnan J, Melin MC, Berthaut I, Barthélémy C, Daudin M, Szerman E, Bresson JL, Brugnon F, Bujan L. The semen quality of 1158 men with testicular cancer at the time of cryopreservation: results of the French National CECOS Network. *J Androl* 2012; **33**:1394-401.~~

Rives N, Milazo JP, Perdrix A, Castanet M, Joly-Hélas, Sibert L, Bironneau A, Way A and Macé B. The feasibility of fertility preservation in adolescents with Klinefelter syndrome. *Hum Reprod*. 2013; **28**, 1468-1479.

~~Rivkees SA, Crawford JD. The relationship of gonadal activity and chemotherapy-induced gonadal damage. *JAMA* 1988; **259**: 2123–2125.~~

~~Romerius P, Ståhl O, Moëll C, Relander T, Cavallin-Ståhl E, Gustafsson H, Löfvander Thapper K, Jepson K, Spanò M, Wiebe T, Lundberg Giwereman Y, Giwereman A. Sperm DNA integrity in men treated for childhood cancer. *Clin Cancer Res* 2010;**16**: 3843-50.~~

~~Rowley MJ, Leach DR, Warner GA, Heller CG. Effect of graded doses of ionizing radiation on the human testis. *Radiat Res* 1974; **59**:665-678.~~

~~Royere D, Barthelemy C, Hamamah S, Lansac. Cryopreservation of spermatozoa: a 1996 review. *Hum Reprod Update*. 1996; **2**:553-9.~~

Sadri-Ardekani H, Akhondi MA, van der Veen F, Repping S, van Pelt A M. In vitro propagation of human prepubertal spermatogonial stem cells, *JAMA* 2011; **305**: 2416-2418.

Sadri-Ardekani H, Mizrak SC, van Daalen SK, Korver CM, Roepers-Gajadien HL, Koruji M, HovinghS, de Reijke TM, de la Rosette JJ, van der Veen F, de Rooij DG, Repping S, van Pelt AM. Propagation of human spermatogonial stem cells in vitro, *JAMA* 2009; **302**: 2127-2134.

Saito K, Suzuki K, Iwasaki A, Yumura Y, Kubota Y. Sperm banking before cancer chemotherapy helps in the emotional battle against cancer. *Cancer* 2005;**104**: 521-524.

Sarafoglou K, Boulad F, Gillio A, Sklar C. Gonadal function after bone marrow transplantation for vitro production of fertile sperm from murine spermatogonial stem cell lines. *Nat Commun* 2011; **2**: 472,

Sato T, Katagiri K, Yokonishi T, Kubota Y, Inoue K, Ogonuki N, Matoba S, Ogura A, Ogawa T, In vitro production of fertile sperm from murine spermatogonial stem cell lines. *Nat Commun* 2011; **2**: 472,

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, Honaramooz A, Boiani M, Schöler HR, Dobrinski I. Progeny from sperm obtained after ectopic grafting of neonatal mouse testes. *Biol Reprod* 2003; **68**: 2331-2335.

Schlatt S, Kim SS, Gosden R. Spermatogenesis and steroidogenesis in mouse, hamster and monkey testicular tissue after cryopreservation and heterotopic grafting to castrated hosts. *Reproduction* 2002;**12**4: 339-346.

Schlatt S, Rosiepen G, Weinbauer GF, Rolf, C Brook PF, Nieschlag E. Germ cell transfer into rat,bovine, monkey and human testes. *Hum Reprod* 1999; **14**: 144-150.

Schlegel PN, Su LM. Physiological consequences of testicular sperm extraction. *Human Reprod* 1997; **12**: 1688-1692.

Schmidt KLT, Larsen E, Bangsboll S, Meinertz H, Carlsen E, Andersen AN. Assisted reproduction in male cancer survivors: fertility treatment and outcome in 67 couples. *Hum Reprod* 2004;**19**:2806-2810.

Schover LR, Brey K, Lichting A, Lipshultz LI, Jeha S. Oncologists´ attitudes and practices regarding banking sperm before cancer treatment. *J Clin Oncol* 2002;**20**:1890-1897.

Sharma V. Sperm storage for cancer patients in the UK: a review of current practice. *Hum Reprod 2011* **26**: 2935-2943.

Shetty G, Meistrich ML. Hormonal approaches to preservation and restoration of male fertility after cancer treatment. *Natl Cancer Inst Monogr* 2005;**34**:36–39.

Shinohara T, Inoue K, Ogonuki N, Kanatsu-Shinohara M, Miki H, Nakata K, Kurome M, Nagashima H, Toyokuni S, Kogishi K, Honjo T, Ogura A. Birth of offspring following transplantation of cryopreserved immature testicular pieces and in-vitro microinsemination. Hum Reprod 2002;**17**: 3039-3045.

Signorello LB, Mulvihill JJ, Green DM, Munro HM, Stovall M, Weathers RE, Mertens AC, Whitton JA, Robison LL and Boice JD, Jr. Stillbirth and neonatal death in relation to radiation exposure before conception: a retrospective cohort study. Lancet 2010; 376: 624-630.

Signorello LB, Mulvihill JJ, Green DM, Munro HM, Stovall M, Weathers RE, Mertens AC, Whitton JA, Robison LL and Boice JD, Jr. Congenital anomalies in the children of cancer survivors: a report from the childhood cancer survivor study. *J Clin Oncol* 2012; **30**: 239-245.

Sharma V. Sperm storage for cancer patients in the UK: a review of current practice. *Hum Reprod 2011* **26**: 2935-2943.

Shetty G, Meistrich ML. Hormonal approaches to preservation and restoration of male fertility after cancer treatment. *Natl Cancer Inst Monogr* 2005;**34**:36-39.

Siimes MA, Rautonen J. Small testicles with impaired production of sperm in adult male survivors of childhood malignancies. *Cancer* 1990; **65**:1303-1306.

Siimes MA, Rautonen J, Makipernaa A, Sipila I. Testicular function in adult males surviving childhood malignancy. *Pediatr Hematol Oncol* 1995; **12**: 231-241.

Sklar C. Reproductive physiology and treatment-related loss of sex hormone production. *Med Pediatr Oncol* 1999;**33**:2-8.

Sklar CA, Robison LL, Nesbit ME, Sather HN, Meadows AT, Ortega JA, et al . Effects of radiation on testicular function in long-term survivors of childhood acute lymphoblastic leukemia: a report from the children cancer study group. *J Clin Oncol* 1990; **8**:1981-1987.

Smit M, van Casteren NJ, Wildhagen MF, Romijn JC, Dohle GR. Sperm DNA integrity in cancer patients before and after cytotoxic treatment. *Hum Reprod* 2010;**25**:1877-83.

Smith MA, Altekruse SF, Adamson PC, Reaman GH, Seibel NL. Declining childhood and adolescent cancer mortality. Cancer. 2014; **120**: 2497-506.

Sonksen J, Ohl DA. Penile vibratory stimulation and electroecjaculation in the treatment of ejaculatory dysfunction. *J Androl* 2002; **25**:324-323.

~~Stahl O, Boyd HA, Giwercman A, Lindholm M, Jensen A, Kjaer SK, Anderson H, Cavallin-Stahl E and Rylander L. Risk of birth abnormalities in the offspring of men with a history of cancer: a cohort study using Danish and Swedish national registries. *J Natl Cancer Inst* 2011; **103**: 398-406.~~

~~Steele EK, Ellis PK, Lewis SE, McClure N. Ultrasound, antisperm antibody, and hormone profiles after testicular Trucut biopsy. *Fertil Steril* 2001; **75**: 423-428.~~

Struijk RB, Mulder CL, van der Veen F, van Pelt AM, Repping S. Restoring fertility in sterile childhood cancer survivors by autotransplanting spermatogonial stem cells: are we there yet? Biomed Res Int 2013; Article ID 903142, 1-12

Stukenborg J., Wistuba J. Luetjens C, Elhija M., Huleihel M., Lunenfeld E, Gromoll J, Nieschlag E, Schlatt S., Coculture of spermatogonia with somatic cells in a novel three-dimensional soft-agar-culture-system. *J Androl 2008;* **29**: 312-329.

Suomalainen L, Hakala JK, Pentika¨inen V, Otala M, Erkkila¨ K, Pentika¨inen MO, Dunkel L. Shingosine-1-phosphate in inhibition of male germ cell apoptosis in the human testis. *J Clin Endocrinol Metab* 2003;**88**:5572–5579.

~~Tempest HG, Ko E, Chan P, Robaire B, Rademaker A, Martin RH. Sperm aneuploidy frequencies analysed before and after chemotherapy in testicular cancer and Hodgkin's lymphoma patients. *Hum Reprod* 2008;**23**: 251-8.~~

Tesarik J, Bah Ceci M, Ozcan C, Greco E, Mendoza C. Restoration of fertility by *in vitro* spermatogenesis. *Lancet* 1999; **353**: 555-556.

~~Thomson AB, Campbell AJ, Irvine DC, Anderson RA, Kelnar CJ, Wallace WH. Semen quality and spermatozoal DNA integrity in survivors of childhood cancer: a case-control study. *Lancet* 2002; **360**: 361-7.~~

Formatted: Font: 12 pt

Formatted: Left, Don't adjust space between Latin and Asian text, Don't adjust space between Asian text and numbers

Tournaye H, Liu J, Nagy PZ, Camus M, Goossens A, Silber S, Van Steirteghem AC, Devroey P. Correlation between testicular histology and outcome after intracytoplasmic sperm injection using testicular sperm. *Hum Reprod* 1996; **11**:127–32.

Vadaparampil ST, Clayton H, Quinn GP, King LM, Nieder M, Wilson C. Pediatric oncology nurses' attitudes related to discussing fertility preservation with pediatric cancer patients and their families. *J Pediatr Oncol Nurs* 2007; **24**:255-63.

Vadaparampil S, Quinn G, King L, Wilson C, Nieder M. Barriers to fertility preservation among pediatric oncologists. *Patient Educ Couns* 2008; **72**: 402-10.

~~Van Alphen MM, van de Kant HJ, de Rooij DG. Depletion of the spermatogonia from the seminiferous epithelium of the rhesus monkey after X irradiation. *Radiat Res* 1988;**113**:473-486.~~

~~Van Alphen MM, van de Kant HJ, Davids JA, Warmer CJ, Bootsma AL, de Rooij DG. Dose-response studies on the spermatogonial stem cells of the rhesus monkey (Macaca mulatta) after X irradiation. Radiat Res 1989;119: 443-51.~~

~~Van Casteren NJ, van der Linden GH, Hakvoort-Cammel FG, Hählen K, Dohle GR, van den Heuvel-Eibrink MM. Effect of childhood cancer treatment on fertility markers in adult male long-term survivors. *Pediatr Blood Cancer* 2009;**52**:108-112.~~

Van Casteren NJ, Dohle GR, Romij JC, de Muinck Keizer-Schrama SMPF, Weber RFA, van den Heuvel-Eibrink MM. Semen cryopreservation in pubertal boys before gonadotoxic treatment and the role of endocrinologic evaluation in predicting sperm yield. *Fertil Steril* 2008a;**90**:119-1125.

Van Casteren NJ, van Santbrink EJP, van Inzen W, Romijn JC, Dohle GR. Use rate and assisted reproduction technologies outcome of cryopreserved semen from 629 cancer patients. Fertil Steril 2008b;90:2245-2250

~~Van den Broecke, R., Pennings, G., Van der Elst, J., Liu, J. and Dhont, M. Ovarian tissue cryopreservation: therapeutic prospects and ethical reflections. *Reprod BioMedi Online* 2001; **3**: 179-184.~~

van der Kaaij MA, Heutte N, van Echten-Arends J, Raemaekers JM, Carde P, Noordijk EM, Fermé C, Thomas J, Eghbali H, Brice P, Bonmati C, Henry-Amar M, Kluin-Nelemans HC. Sperm quality before treatment in patients with early stage Hodgkin's lymphoma enrolled in EORTC-GELA Lymphoma Group trials. *Haematologica* 2009; **94**:1691-1697.

Van Saen D, Gies I, De Schepper J, Tournaye H, Goossens E . Can pubertal boys with Klinefelter syndrome benefit from spermatogonial stem cell banking? *Hum Reprod* 2012; **27**: 323-330

~~Van der Meer Y, Huiskamp R, Davids JA, de Rooij DG. Differential effects of fractionated X irradiation on mouse spermatogonial stem cells. *Radiat Res* 1993;**135** :222-8.~~

Van Saen D, Goossens E, De Block G, Tournaye H. Regeneration of spermatogenesis by grafting testicular tissue or injecting testicular cells into the testes of sterile mice: a comparative study. *Fertil Steril* 2009; **91**: 2264-2272.

Wallace WH, Anderson RA, Irvine DS. Fertility preservation for young patients with cancer: who is at risk and what can be offered? *Lancet Oncol* 2005; **6**: 209-218.

Wallace WHB, Thomson L and Anderson RA Long-term follow up of survivors of childhood cancer: summary of updated SIGN guidance. *2013;* **346**: f1190.

Ward E, De Santis C, Robbins A, Kohler B, Jemal A. Childhood and adolescent cancer statistics, 2014. CA: *A Cancer Journal for Clinicians* 2014; **64**:83-103.

~~Winther JF, Boice JD, Jr., Frederiksen K, Bautz A, Mulvihill JJ, Stovall M and Olsen JH Radiotherapy for childhood cancer and risk for congenital malformations in offspring: a population-based cohort study. *Clin Genet* 2009; **75**: 50-56.~~

~~Winther JF, Olsen JH, Wu H, Shyr Y, Mulvihill JJ, Stovall M, Nielsen A, Schmiegelow M and Boice JD, Jr. Genetic disease in the children of Danish survivors of childhood and adolescent cancer. *J Clin Oncol* 2012; **30**: 27-33.~~

~~Whitehead E, Shalet SM, Jones PH, Beardwell CG, Deakin DP. Gonadal function after combination chemotherapy for Hodgkin's disease in childhood. *Arch Dis Child*. 1982; **57**:287-91.~~

Formatted: Font: Times New Roman, 12 pt

Formatted: Font: Times New Roman, 12 pt

Formatted: Font: Times New Roman, 12 pt, Not Italic

Formatted: Font: Times New Roman, 12 pt, Not Italic

Formatted: Font: Times New Roman, 12 pt, Bold, Not Italic

Formatted: Font: Times New Roman, 12 pt, Not Italic

Formatted: Font: Times New Roman, 12 pt

World Health Organization. WHO laboratory manual for the examination and processing of human semen, 5th ed. World Health Organization, Geneva, 2010.

Wyns C, Curaba M, Petit S, Vanabelle B, Laurent P, Wese JF, Donnez J. Management of fertility preservation in prepubertal patients: 5 years' experience at the Catholic University of Louvain. *Hum Reprod* 2011; **26**:737-47.

Wyns C, Curaba M, Martinez-Madrid B, Van Langendonckt 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 Langendonckt A, Wese F-X, Donnez J, Curaba M. Long-term spermatogonial survival in cryopreserved and xenografted immature human testicular tissue. *Hum Reprod* 2008;**23**:2402–2414.

Wyns C, Curaba M, Vanabelle B, Van Langendonckt A, Donnez J. Options for fertility preservation in prepubertal boys. *Hum Reprod Update* 2010;**16**:312-328.

Yang S, Bo J, Hu H, Guo X, Tian R, Sun C, Zhu Y, Li P, Liu P, Zou S, Huang Y, Li Z. Derivation of male germ cells from induced pluripotent stem cells in vitro and in reconstituted seminiferous tubules. Cell Prolif. 2012; 45:91-100.

Zorn B, Virant-Klun I, Stanovnik M, Drobnic S, Meden-Vrtovec H. Intracytoplasmic sperm injection by testicular sperm in patients with aspermia or azoospermia after cancer treatment. *Int J Androl* 2006;**29**:521-527.