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A European perspective on testicular tissue cryopreservation for fertility preservation in prepubertal and adolescent boys†

Helen M. Picton[1,*], Christine Wyns[2,3], Richard A. Anderson[4], Ellen Goossens[5], Kirsi Jahnukainen[6,7], Sabine Kliesch[8], Rod T. Mitchell[4], G. Pennings[9], Natalie Rives[10], Herman Tournaye[11], Ans M.M. van Pelt[12], Ursula Eichenlaub-Ritter[13], and Stefan Schlatt[8] on behalf of the ESHRE Task Force On Fertility Preservation In Severe Diseases

[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 LS2 9JT, UK [2]Université Catholique de Louvain (UCL), Pôle de Recherche en Gynécologie, Institut de Recherche Expérimentale et Clinique (IREC), 1200 Brussels, Belgium [3]Department of Gynecology-Andrology, Cliniques Universitaires Saint-Luc, Avenue Hippocrate, 10, 1200 Brussels, Belgium [4]MRC Centre for Reproductive Health, Queens Medical Research Institute, University of Edinburgh, Edinburgh, UK [5]Research Group Biology of the Testis (BITE), Vrije Universiteit Brussel, Laarbeeklaan 103, 1090 Brussels, Belgium [6]Children's Hospital, Helsinki University Central Hospital, Helsinki, Finland [7]Department of Women's and Children's Health, Karolinska Institute and University Hospital, Stockholm, Sweden [8]Centre of Reproductive Medicine and Andrology, University Münster, Domagkstraße 11, 48149 Münster, Germany [9]Bioethics Institute Ghent (BIG), Faculty of Philosophy and Moral Science, Ghent University, Ghent, Belgium [10]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 [11]Centre for Reproductive Medicine, University Hospital of the Brussels Free University, Laarbeeklaan 101, 1090 Brussels, Belgium [12]Center for Reproductive Medicine, Academic Medical Center, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands [13]Faculty of Biology, Gene Technology/Microbiology, University of Bielefeld, Bielefeld 33501, Germany

*Correspondence address. Division of Reproduction and Early Development, Leeds Institute Cardiovascular and Metabolic Medicine, Faculty of Medicine and Health, University of Leeds, Clarendon Way, Leeds LS2 9JT, UK. Fax: +44-113-343-6603; E-mail: h.m.picton@leeds.ac.uk

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STUDY QUESTION: What clinical practices, patient management strategies and experimental methods are currently being used to preserve and restore the fertility of prepubertal boys and adolescent males?

SUMMARY ANSWER: Based on a review of the clinical literature and research evidence for sperm freezing and testicular tissue cryopreservation, and after consideration of the relevant ethical and legal challenges, an algorithm for the cryopreservation of sperm and testicular tissue is proposed for prepubertal boys and adolescent males at high risk of fertility loss.

WHAT IS KNOWN ALREADY: A known late effect of the chemotherapy agents and radiation exposure regimes used to treat childhood cancers and other non-malignant conditions in males is the damage and/or loss of the proliferating spermatogonial stem cells in the testis. Cryopreservation of spermatozoa is the first line treatment for fertility preservation in adolescent males. Where sperm retrieval is impossible, such as in prepubertal boys, or it is unfeasible in adolescents prior to the onset of ablative therapies, alternative experimental treatments such as testicular tissue cryopreservation and the harvesting and banking of isolated spermatogonial stem cells can now be proposed as viable means of preserving fertility.

STUDY DESIGN, SIZE, DURATION: Advances in clinical treatments, patient management strategies and the research methods used to preserve sperm and testicular tissue for prepubertal boys and adolescents were reviewed. A snapshot of the up-take of testis cryopreservation as a means to preserve the fertility of young males prior to December 2012 was provided using a questionnaire.

PARTICIPANTS/MATERIALS, SETTING, METHODS: A comprehensive literature review was conducted. In addition, survey results of testis freezing practices in young patients were collated from 24 European centres and Israeli University Hospitals.

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MAIN RESULTS AND THE ROLE OF CHANCE: There is increasing evidence of the use of testicular tissue cryopreservation as a means to preserve the fertility of pre- and peri-pubertal boys of up to 16 year-old. The survey results indicate that of the 14 respondents, half of the centres were actively offering testis tissue cryobanking as a means of safeguarding the future fertility of boys and adolescents as 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. The remaining centres were considering the implementation of a tissue-based fertility preservation programme for boys undergoing oncological treatments.

LIMITATIONS, REASONS FOR CAUTION: The data collected were limited by the scope of the questionnaire, the geographical range of the survey area, and the small number of respondents.

WIDER IMPLICATIONS OF THE FINDINGS: The clinical and research questions identified and the ethical and legal issues raised are highly relevant to the multi-disciplinary teams developing treatment strategies to preserve the fertility of prepubertal and adolescent boys who have a high risk of fertility loss due to ablative interventions, trauma or genetic pre-disposition.

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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 US data suggest that long-term survival can be expected in ∼80% of the children and adolescent diagnosed with cancer (Desandes, 2007; Hudson, 2010). Indeed, recent estimates suggest that ∼1 in 530 young adults between the ages of 20 and 39 years 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).

Different strategies have been developed to safeguard the fertility of these young patients. Cryopreservation of spermatozoa is routinely used to preserve fertility in men (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 review the 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 of young patients. The reader is referred to the online version of this paper for a detailed overview of the cytotoxic impact of chemotherapy and radiation 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.

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 sphingosine-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 (Carmely et al., 2009). 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.

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; 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

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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 USA 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's or Non-Hodgkin's 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 with 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 with only 3% azoospermia in lymphoma (van der Kaaij *et al.*, 2009; Kliesch *et al.*, 2010).

Semen can be cryopreserved for adolescent boys in more than 80% of cases (Kliesch *et al.*, 1996; Bahadur and Raiph, 1999; Bahadur *et al.*, 2006; Van Casteren *et al.*, 2008a; Menon *et al.*, 2009; Daudin *et al.*, 2015). The rate of azoospermia varies between 2.6 and 18% of patients (Van Casteren *et al.*, 2008a; Menon *et al.*, 2009). 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 I). 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 I).

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 Organization (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 the assisted reproductive technique (ART) post-thaw will facilitate the selection and use of viable sperm for insemination (Nordhoff *et al.*, 2013). However, it must be noted that if there are fewer than 0.1×10^6 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. Nevertheless, 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; Schmidt *et al.*, 2004; Hourvitz *et al.*, 2008; Van Casteren *et al.*, 2008b; Freour *et al.*, 2012). 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 II). The TESE procedure requires surgical intervention, either with local or general anaesthesia with higher recovery rates obtained following microsurgical techniques (Donoso *et al.*, 2007; Colpi *et al.*, 2009; Ramasamy *et al.*, 2009). If microsurgery is not available, multifocal testicular biopsies from different sites of the testis can be used to increase

Table I Semen parameters of adolescents compared with adults at the time of cryopreservation.

Patients	Age (years)	Sperm concentration (mill/ml)	Forward motility (%)	Cryopreservation performed (%)	Author
Adults (*n* = 740)	20–39	34 ± 6 (mean ± SD)	50 ± 3 (mean ± SD	88	Kliesch *et al.* (1996) and Kamischke *et al.* (2004)
Adolescents (*n* = 111)	14–20	48 ± 18 (mean ± SD)	48 ± 5 (mean ± SD)	96	Kliesch *et al.* (1996) and Kamischke *et al.* (2004)
Adolescents (*n* = 80)	14–19	9 ± 7 (median ± SEM)	26 ± 2 (median ± SEM)	66	Van Casteren *et al.* (2008a)
Adolescents (*n* = 156)	13–20	35 ± 3 (mean ± SEM)	30 ± 1 (mean ± SEM)	3	Menon *et al.* (2009)
Adolescents (*n* = 4004)	11–14	42 ± 66 (mean ± SD)	33 ± 21 (mean ± SD)	81	Daudin *et al.* (2015)
	15–17	52 ± 71 (mean ± SD)	37 ± 21 (mean ± SD)	91	
	18–20	49 ± 73 (mean ± SD)	39 ± 21 (mean ± SD)	95	

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the chance to detect focal spermatogenesis (Tournaye et al., 1996; Tournaye, 2006; Dieckmann et al., 2007). However, this procedure may have a negative impact on the vascularization 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 years old (Wyns et al., 2011). This statement is supported by the findings of a recent questionnaire from the European Society for Human Reproduction and Embryology (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 programme for boys undergoing oncological treatments (Table III). 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 12–98) (Table III). 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 IV, four 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 dimethyl sulphoxide (DMSO) combined with sucrose as the preferred cryoprotective agents. Only one centre had used an ethylene glycol-based protocol.

Table II Results of testicular sperm extraction (TESE) in oncological patients with post-treatment azoospermia.

Study	Patients with TESE after chemotherapy	Positive sperm retrieval from TESE samples
Damani et al. (2002)	23	15/23 (65%)
Meseguer et al. (2003)	12	5/12 (42%)
Zorn et al. (2006)	30	13/30 (43%)
Hibi et al. (2007)	5	3/5 (60%)
Hsiao et al. (2011)	73	27/73 (37%)
Total	182	80/181 (44%)

Management of fertility preservation in prepubertal boys and adolescents

Fertility preservation management requires a specialist team of highly trained physicians and nurses involved in both oncology and reproductive medicine. Identifying and educating key staff capable of initiating discussions on fertility preservation is vital to the success of fertility preservation strategies (Nagel and Neal, 2008). Where there is a risk of gonadal damage and fertility loss, patients should be referred to the infertility specialist by paediatric haematologists and oncologists before gonadotoxic treatment is initiated (Redig et al., 2011). 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

Table III Survey results of centres conducting testicular tissue banking for fertility preservation in boys and adolescents across Europe.

	Number of centres offering the service	Number of cases/ samples (total)	Range of cases/samples (lowest-highest)
(1) How many immature patients underwent testicular tissue retrieval?	7	266	12–98
(2) How many samples are currently stored?	7	264	12–98
(3) What was the age range of patients?	0.8–16 years		
(4) Do you also recruit non-malignant patients?	Yes (6 centres), No (1 centre)		
(5) Which cryopreservation protocol was used?	Slow Freezing (all centres)		
(6) Which cryoprotectant was used?	DMSO (6 centres) EG (1 centre)		
(7) Who pays for the associated costs?	Hospital/research grant (6 centres) Patients (1 centre)		
(8) Do legal restrictions apply for retrieval/storage?	Yes (4 centres), no (3 centres)		

DMSO, dimethyl sulphoxide; EG, ethylene glycol.

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Table IV Results of survey of indications for testicular tissue banking in boys and adolescents.

Malignant diseases	Non-malignant diseases
Acute myeloid leukaemia	Hematopoietic stem cell transplantation in case of
Acute lymphoblastic leukaemia	
Testicular cancer	Drepanocytosis
Neuroblastoma	Thalassemia
Ependymoma	Idiopathic medulla aplasia
Hodgkin lymphoma	Granulomatous disease
Non-Hodgkin lymphoma	
Osteosarcoma	
Large B-cell lymphoma	Risk of testicular degeneration
Primitive neuroectodermal tumour (PNET)Rhabdomyosarcoma	Klinefelter's syndrome Vasectomy
Hepatoblastoma	
Cranial germinoma	
Medulloblastoma	
Anaplastic ependymoma	
Burkitt lymphoma	
Ewing Sarcoma	
Nasophyarynx carcinoma	
Undifferentiated sarcoma	
Ganglioma	
Pinealoblastoma	

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 intra-operative 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 years. 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 off-spring (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 (Fig. 1). Sperm banking should always be 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 years 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 electroejaculation under general anaesthesia should be considered as a second-line treatment option. These methods may have advantages over experimental techniques such as immature testicular tissue sampling as penile vibratory stimulation and electroejaculation both 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. In all cases, the cryobiology practices used for the preservation and long-term storage of samples 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 (Fig. 1). To minimize 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. To date, four freezing protocols for human immature testicular tissue have been described using cryoprotective agents that range from 1.5 M ethylene glycol and sucrose (Kvist *et al.*, 2006) to 0.7 M DMSO (Keros *et al.*, 2005, 2007) or 0.7M DMSO and sucrose (Wyns *et al.*, 2007, 2008;

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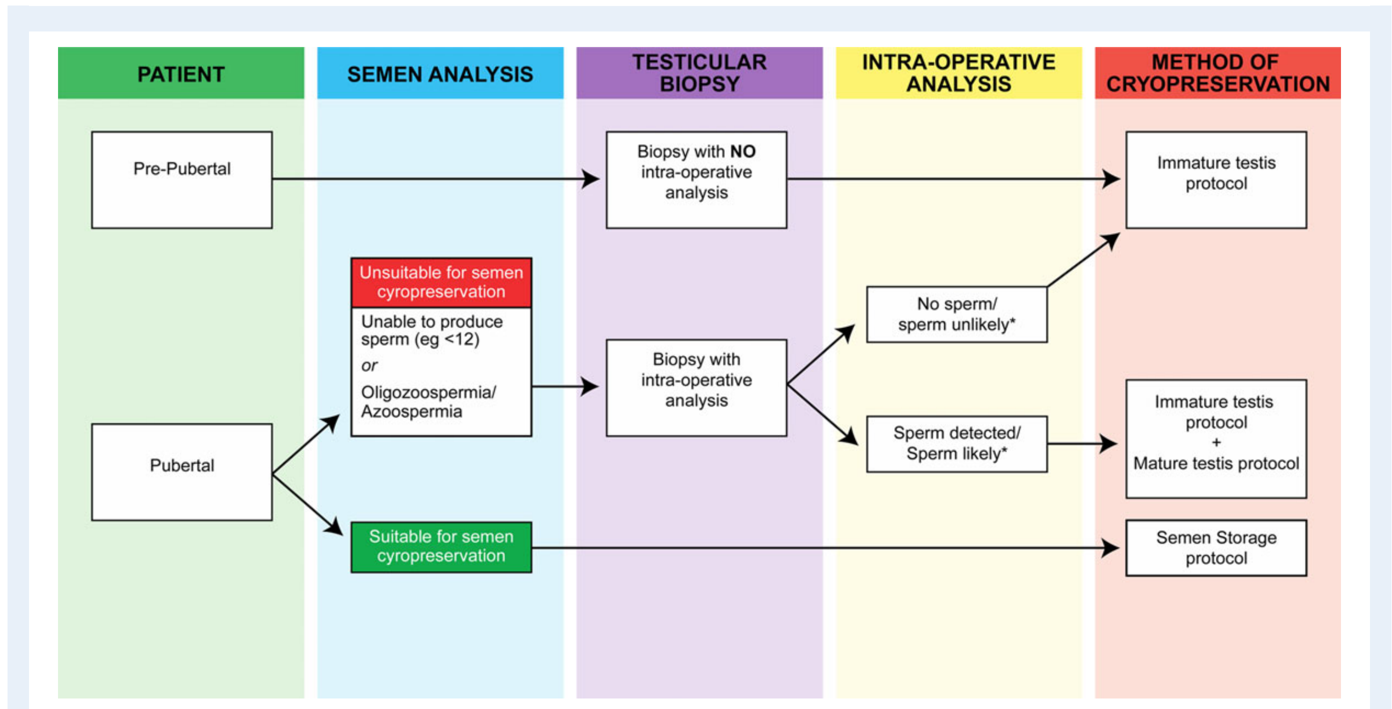

Figure 1 Algorithm for the cryopreservation of testicular tissue/sperm in prepubertal and adolescent patients at high risk of infertility. Clinical assessment for puberty should be carried out by a clinician with experience in pubertal assessment. It must be stressed that no clinical parameter can accurately predict the presence of sperm. The proven treatment option for pubertal and adolescent boys who are considered capable of producing a semen sample is semen collection and cryopreservation. If sufficient sperm are recovered the gametes can be banked using commercial glycerol-based sperm cryomedia. For those young patients who are clinically prepubertal and for whom semen cryopreservation is not possible the fertility preservation strategy should include collection of a testicular biopsy by an experienced surgeon. The tissue should be cryopreserved with a protocol optimized for preserving immature germ cells (immature testis protocol). Patients who are pubertal but are unable to produce a suitable semen sample may proceed to testicular biopsy, with intra-operative analysis. Techniques for intra-operative analysis may vary between institutions but should be aimed at identifying tissue containing (or likely to contain) sperm. This should be carried out by an individual with experience in analysis of testicular tissue (e.g. surgeon, embryologist or andrologist). When sperm are not identified or deemed unlikely the tissue should be frozen with the immature testis protocol used for prepubertal patients. For patients in whom sperm are identified or considered likely to be present, tissue should be split into two portions for storage. One portion should be cryopreserved using the immature testis preservation protocol, whilst the second portion should be stored using a protocol aimed at preserving mature sperm cells with glycerol as the main cryoprotectant. As stated in the text at present there are several protocols for cryopreservation of immature testicular tissue and there is no clear evidence at the time of writing to demonstrate which is optimal.

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 maximize 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 optimized. 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 III). 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).

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

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research into the methods needed to realize 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 autotransplantation of a suspension of SSCs by injection into the testis to restore spermatogenesis or autotransplantation of frozen-thawed testicular grafts and the growth and maturation of SSCs *in vitro*.

Propagation and autotransplantation of SSCs

Currently, SSC injection is considered the most promising tool for fertility restoration in prepubertal cancer patients. The technique was originally described in the mouse (Brinster and Zimmermann, 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. 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 (Schlatt *et al.*, 1999; Honaramooz *et al.*, 2002; Aponte *et al.*, 2008; Nobrega *et al.*, 2010). 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, ,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 birthweights, 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 with control spermatogenesis (Goossens *et al.*, 2009, 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 sterilized 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 (intratesticular 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.*, 2009). At 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 intratesticular 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 intratesticular 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 fertility restoration. However, positive and negative cell sorting strategies have the potential to target and remove cells from cultured mouse SSC populations and after xenografting (Hermann *et al.*, 2011; Dovey *et al.*, 2013). Sorting protocols using magnetic activated cell sorting, a fluorescence- activated cell sorter or differential plating have been found to have variable efficiency when used to enrich human SSCs (Geens *et al.*, 2006, 2011; Nickkholgh *et al.*, 2014a). Thus, at time of writing, autotransplantation of cell suspensions or tissues still runs the risk of reintroducing cancer via the graft.

The risk of reintroduction of malignant cells via the autograft may be circumvented by *in vitro* spermatogenesis. *In vitro*-derived spermatozoa that are free from residual disease can then be used to inseminate oocytes using ICSI. Strategies which support the *in vitro* growth and

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differentiation of germ cells include the three dimensional (3D) culture of testicular cells (Stukenborg et al., 2008) or organ culture (Sato et al., 2011). The main difference between the two approaches lies in the fact that in organ culture the testicular biopsy remains intact and is layered upon an island of agar that is maintained in a liquid medium. In 3D culture, the germ cells are dissociated from their somatic cells prior to culture and they are then suspended in medium containing 35 and 50% agar, the so-called Soft-Agar-Culture-System. In both systems SSCs are co-cultured with somatic cells from the same biopsy so resembling the *in vivo* situation and supporting two-way communication between the different cellular compartments. In the mouse model, *in vitro* spermatogenesis has been successful up to the elongated spermatid stage of spermatogenesis but so far offspring have only been generated with sperm derived following organ culture (Sato et al., 2011). Although encouraging results have recently been obtained regarding the genetic and epigenetic stability of human SSCs during long-term culture (Nickkholgh et al., 2014b), the fertility of *in vitro*-derived sperm have still to be established before the clinical value of this type of experimental approach can be fully assessed. When no germ cells are available in the initial testis biopsy, an alternative option may be 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.

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 prepubertal 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 (>14 years), 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. The 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).

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 (>14 years), 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). 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

The evidence from testicular biopsy in adults (Schlegel and Su, 1997; Manning et al., 1998) suggests that risk of the biopsy procedure itself should not be overlooked in younger patients (Mitchell et al., 2009). Immediate surgical complications include bleeding and infection whereas later complications may be indicative of damage to the remaining testis. 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), where 112 boys were followed up for a mean of 11 years post-surgery (age range 18–29 years). 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 antisperm 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).

The impact of prepubertal testicular biopsy on future fertility is difficult to predict. To date the evidence suggests that the procedure itself is unlikely to result in a significant impairment of fertility. 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. Multi-centre studies on these relatively rare patients are needed to provide clearer insights into the requirements for long-term follow-up.

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 is 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

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safeguard the best interests of the child. A key 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 suggest that the issue of sterility is hardly discussed with parents of boys undergoing chemotherapy (Lee *et al.*, 2006; Anderson *et al.*, 2008). There are two schools of thought. It can be argued that as paediatric oncology teams treat a patient with the intent to cure, then 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 not only a good prognosis but also a high risk of permanent infertility (Wallace *et al.*, 2005). In all cases informed 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 the collection and cryopreservation of semen for fertility preservation is an established, non-invasive technology for adolescents with cancer (Daudin *et al.*, 2015), the recovery of a testicular biopsy from boys in whom sperm is not yet produced must be regarded as experimental 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 anaesthesia, pain etc.) are relatively small, especially when they can be combined with necessary cancer-related interventions. 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. Clinics offering cryobanking are morally obliged to participate in data collection and follow-up research in order to improve information provision and decision-making.

The development and uptake of fertility preservation strategies in prepubertal boys needs be supported by the creation of suitable legislatory and regulatory frameworks. Legal rules should cover key points such as: differences associated with the handling and storage of gametes versus 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 online version of this paper.

Table V Clinical and research priorities for fertility preservation strategies in boys and adolescents.

Clinical priorities for fertility preservation in boys and adolescents
1. Optimization of protocols for the collection and cryopreservation of testicular tissue
2. Optimization of the timing of testicular tissue collection and cryopreservation relative to the onset of gonadotoxic treatments.
3. Optimization of the age range/pubertal status of boys for preservation of testicular tissue
4. Optimization of protocols for the management and transportation of tissue between the procurement site and cryopreservation site
5. Assessment of the need for quality assurance and testing of tissue before and after storage
6. Development of accessible information on fertility preservation in boys and adolescents
7. Assessment of the counselling and support needs for patients and parents before tissue freezing and at the time of fertility restoration
Research priorities for fertility preservation in boys and adolescents
1. Evaluation of the effect of pretreatment with gonadotoxic therapies on the efficacy of testicular freezing and autotransplantation and spermatogonial stem cells (SSC) autotransplantation for fertility restoration
2. Optimization of cryopreservation protocols for immature testicular tissue
3. Development of protocols for minimal residual disease testing of testicular tissue
4. Development of protocols for cell sorting method for SSC to exclude cancer cells
5. Optimization of cell expansion protocols for generation of SSC for fertility restoration
6. Optimization of autotransplantation methodologies for testicular tissue and SSCs for use in fertility restoration
7. Development of protocols for the *in vitro* maturation of SSCs
8. Assessment of the fertilizing capacity of sperm after autologous grafting/transplantation of SCCs or *in vitro* maturation of SSCs
9. Further evaluation of the genetic and epigenetic stability and hence safety of cryopreserved, cultured and transplanted human SSCs and *in vitro*-derived sperm

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Future challenges for fertility preservation in boys and adolescents

The development of strategies for fertility preservation in prepubertal boys and adolescents is still in its infancy and represents a balance between biological, clinical and technical knowns, technological unknowns and ethical and legal questions. Progress in this field is encouraging and it has enabled us to design treatment algorithms that have the potential to safeguard the future fertility of these young patients (Fig. 1). 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, many important questions remain unanswered (Table V). 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.

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Authors' roles

H.M.P. led on the preparation, drafting and editing of this comprehensive review. C.W., R.A.A., E.G., K.J., S.K., R.T.M., G.P., N.R., H.T., A.M.M.V.P., all contributed to manuscript drafting and critical review. S.S. distributed and analysed the survey data and contributed to manuscript drafting and critical review. U.E.-R. provided a critical overview from ESHRE on the manuscript contents.

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Conflict of interest

None declared.

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