Testicular Tissue Freezing For Fertility Preservation In Boys Facing Infertility-Causing Diseases or Treatment Regimens

TABLE OF CONTENTS

1.0 Background

2.0 Specific Aims

3.0 Significance

4.0 Primary Studies

5.0 Patient Eligibility

6.0 Methods

   6.1 Informed Consent and Survey
   6.2 Collection of Testicular Tissue
   6.3 Methods of Testicular Cryopreservation
   6.4 Testicular Tissue Storage
   6.5 Clinical Data to be Collected
   6.6 Research

7.0 Statistical Considerations and Data Management

8.0 Risks and Benefits

9.0 Reimbursements and Research Costs

References

Appendices
Abstract

The cure rate of cancer in children, adolescents and young adults continues to increase with advances in chemotherapy and/or radiation protocols. As more oncology patients become long-term survivors, the consequences of their treatment on their quality of life have become an important focus of research in clinical oncology and reproductive medicine. One of the most common and most devastating side effects of cancer treatment is infertility. Many chemotherapy and radiation-containing regimens for cancer or prior to bone marrow transplantation can cause sterility in children and young adults. In addition, some human disease conditions (e.g., Klinefelter’s) are associated with infertility. Semen cryopreservation is available as a fertility-preserving option for post pubertal boys and adult men, but many do not take advantage of this option due in part to lack of information, illness, and/or time constraints relative to their treatment plan. Currently, no fertility-preserving options are available for prepubescent boys who are not yet producing sperm. However, experimental techniques are currently being developed to provide future alternatives for patients that preserve their testicular tissue/cells. In order to take advantage of these and future technologies, patients must harvest and preserve their testicular tissue prior to disease or treatment associated fertility decline. This study will be available to males of all ages who have a disease or will undergo a treatment that can cause infertility. The primary objective of the proposed study is to 1) Optimize techniques for cryopreserving testicular tissue, 2) Assess malignant contamination in testicular tissues and 3) develop methods to enrich spermatogonial stem cells and remove malignant contamination from testicular tissue. In addition, this study will process and cryopreserve tissue and/or cells for participating patients as a resource for future elective procedures to attempt fertility restoration.

The study will provide research tissue to address the following Specific Aims:

1. To optimize techniques for cryopreservation of testicular cells, including spermatogonial stem cells, from patients at high risk for infertility due to disease or prior to the initiation of therapy. Efficacy of cryopreservation techniques will be determined.

2. To assess malignant cell contamination in harvested patient testicular tissues and cells.

3. To develop strategies to isolate/enrich spermatogonial stem cells and/or eliminate malignant contamination in patient testicular cells.

This study will provide a pool of research tissue that will be used to develop and test methods for manipulation and cryopreservation of testicular tissue. Progress in these investigations may open up a range of new fertility preservation techniques to patients that currently have no options. At the same time, a substantial portion of the patient’s tissue will be cryopreserved and reserved for his own future use.
INTRODUCTION – BACKGROUND AND RATIONALE:

Over the last 30 years, advances in the survival of oncology patients have been made through the work of cooperative protocol-driven clinical research, particularly in young patient categories. Now that the overall event-free survival (EFS) rate for child, adolescent and young adult cancer patients surpasses 75%, attention is focused on quality of life and long-term consequences of therapy. In particular, patients receiving chemotherapy and radiotherapy for cancer or other conditions are often at risk for infertility, placing fertility preservation at the forefront of these concerns. Progress to minimize the unwanted side effects of current treatment regimens without decreasing their effectiveness has allowed many cancer survivors to have children following spontaneous recovery of fertility (van den Berg et al., 2004). However, some oncological diseases require rigorous treatment regimens which will almost always lead to permanent infertility of the patient.

The primary causal factor for the risk of infertility in males is considered the treatment modality (i.e. the specific chemotherapy or radiotherapy regimen). Most of the available outcome data relating to fertility sequelae are from studies that examined the effects of single treatment agents.

In men, treatment with some chemotherapeutic agents and regimens induced prolonged azoospermia (complete absence of sperm in the ejaculate). The effects are likely the result of cytotoxicity to the spermatogonial stem cells that are responsible for maintaining spermatogenesis, possibly resulting in permanent infertility (Meistrich et al., 2005). In particular, alkylating chemotherapeutic agents such as procarbazine, busulfan, cyclophosphamide, chlorambucil, and melphalan, along with cisplatin are the most likely to produce prolonged infertility (Meistrich et al., 2005). Radiation fields that include the testes also produce prolonged and often permanent damage to spermatogenesis (Dubey et al., 2000; Meistrich and van Beek, 1990; Sandeman, 1966; Speiser et al., 1973). Other agents, particularly topoisomerase inhibitors (e.g., amsacrine), antimetabolites (e.g., methotrexate), and microtubule inhibitors can have additive effects on infertility risk when given with the highly gonadotoxic agents listed above (Meistrich et al., 1989). Combinatorial therapies, such as the busulfan-cyclophosphamide (BuCy) conditioning for bone marrow transplant, often result in permanent infertility (Socie et al., 2003). Furthermore, some agents that are administered in repetitive “fraction” treatments are more toxic in sum than single larger doses, and thus, for these agents a lower cumulative dose can lead to permanent infertility (Pont and Albrecht, 1997).

There is a paucity of data about the risk of infertility in prepubertal male patients. Anti-mitotic therapies (i.e. chemotherapy, radiation) cause infertility by targeting proliferating germ cells (e.g. spermatogonia, spermatocytes), the same mechanism by which they target neoplastic cells. In the prepubertal testis, these agents affect proliferating undifferentiated spermatogonia that are proliferating, but not yet producing complete spermatogenesis and sperm (Simorangkir et al., 2005). Rodent studies concur with this scenario and indicate that germ cells in the fetal, neonatal, prepubertal and adult testis are sensitive to chemotherapy (Brinster et al., 2003). While quantitative clinical data demonstrating the relative risk of male infertility between adults and children are not available, it is our best estimate that prepubertal patients exhibit similar sensitivities to potentially gonadotoxic agents as adults.
Since most oncological treatments involve multiple agents, often administered in complex cocktails and/or fractionated regimens, the best estimates of risk of infertility in these cases are based on broad categories of patients stratified based on the treatment agents employed. The risks of infertility (prolonged azoospermia) from treatment with various chemotherapeutic and radiation regimens have been categorized for males based on the treatment agents employed (Green et al., 2009; Green et al., 2014; Mitchell et al., 2009; Wallace et al., 2005). Patients who will undergo potentially gonadotoxic treatment are categorized as:

- High Risk (≥80% risk of prolonged azoospermia).
- Intermediate risk (21-79% risk of prolonged azoospermia).
- Low Risk (≤20% risk of prolonged azoospermia).

A brochure from “Fertile Hope” (Fertile Hope – Risks of Azoospermia) outlines the specific treatment regimens that fall into each category (see Appendix 1). Risk of azoospermia will also be calculated using the Summed Alkylating Agent (SAA) dose score (Green et al., 2009; Appendix 9) and the Cyclophosphamide Equivalent Dose (CED) method (Green et al., 2014; Appendix 10).

For the purposes of this study, only pediatric patients in the “High Risk” category (≥80% risk of long-term azoospermia) will be considered eligible. Adult patients (≥18 years old) in either the high risk or intermediate risk (21-79% risk of long-term infertility) will be considered eligible. During the informed consent process, patients will be presented with the brochure from Fertile Hope that outlines the risks of infertility with their particular treatment regimen (or similar regimens).

The latency of fertility data in child, adolescent and young adult patients treated with these regimens, together with ever-changing treatment modalities necessitate additional ongoing, prospective fertility surveillance studies in these patients. In contrast to the efficient treatment regimens the clinician can choose to cure the patient’s primary disease, very few and limited options are available to prevent the loss of fertility. However, experimental techniques to provide future alternatives for patients that preserve their testicular tissue or germ cells prior to oncologic treatment are currently in development.

For men and boys who are making sperm, cryobanking of semen before the initiation of treatment is possible and allows for future in vitro fertilization (IVF), including intracytoplasmic sperm injection (ICSI), but this is a finite resource and does not allow for natural conception. Furthermore, some males are not able to provide an adequate semen sample at the time of diagnosis. For these patients, testicular sperm extraction (TESE) is an option. In the TESE procedure, a biopsy is obtained surgically from the testicular parenchyma, the tissue is divided into several aliquots which are minced in buffered solutions and any aliquots (tissue and buffer supernatant) containing sperm are identified by microscopic examination. Often, sperm can be identified, prospectively isolated by micromanipulation, and used for intracytoplasmic sperm injection (ICSI) to generate embryos for uterine implantation or cryopreservation. Other tissue and buffer aliquots containing sperm are frozen for future ICSI in the embryology lab. However, these approaches (sperm banking and ICSI) are not options for prepubertal boys who are not yet

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producing sperm. For men and prepubescent boys, autologous transplantation of spermatogonial stem cells (SSCs) is an approach that may permanently restore natural fertility following successful treatment of their underlying disease. Spermatogonial stem cells are the adult tissue stem cells of the testes that give rise to sperm through the process of spermatogenesis. In animal models (rodents, pigs, goats, dogs, sheep and monkeys), transplantation of SSCs into the testes of infertile males can lead to restoration of spermatogenesis (Brinster et al., 2003; Brinster and Avarbock, 1994; Hermann et al., 2012; Herrid et al., 2009; Honaramooz et al., 2003; Kim et al., 2008; Mikkola et al., 2006; Nagano et al., 2001; Ogawa et al., 2000; Orwig and Schlatt, 2005; Shinohara et al., 2001; Valli et al., 2014a). Stem cells from all ages, newborn to adult, are competent to produce complete spermatogenesis following transplantation into recipient testes (Ryu et al., 2003; Shinohara et al., 2001). In addition SSCs from a variety of species can be cryopreserved and retain spermatogenic function upon recovery and transplantation (Brinster, 2002; Hermann et al., 2012). Dr. Kyle Orwig, investigator on the current study, has demonstrated in nonhuman primates that spermatogonial stem cells can be frozen, thawed and transplanted to restore sperm production in infertile males (Hermann et al., 2012) and is translating these methods for human application.

**SPECIFIC AIMS:**
The “Testicular Tissue Cryopreservation” study is open to a subset of patients facing disease or treatment regimens that could lead to infertility.

For some of these patients, experimental testicular tissue cryopreservation is the only fertility preservation option available. This study will harvest testicular tissues from eligible patients. Separate portions of the harvested tissue and/or derivative cells will be 1) designated for research and 2) cryopreserved and maintained for participating patients as a resource for future elective procedures to attempt fertility restoration. Research tissue will be de-identified using a recruitment site-specific identification number.

The study will provide research tissue to address the following Specific Aims:

1. Optimize techniques for cryopreservation of testicular cells, including spermatogonial stem cells, from patients at high risk for infertility due to disease or prior to the initiation of therapy. Efficacy of cryopreservation techniques will be determined.

2. Assess malignant cell contamination in harvested patient testicular tissues and cells.

3. Develop strategies to isolate/enrich spermatogonial stem cells and/or eliminate malignant contamination in patient testicular cells.

**SIGNIFICANCE:**
For male patients who currently have no options for fertility preservation, this research proposal will enable optimization of testicular tissue procurement and processing, cryopreservation, and diagnosis/elimination of malignant cell contamination to ensure safety for future fertility-restoring treatments. While results from animal models and human organ
donor experiments support the efficacy of testicular tissue/cell cryopreservation for fertility preservation and subsequent restoration, rigorous safety and efficacy data in human patients who will undergo infertility-causing therapies is lacking. However, the patients being recruited for this study currently have no options for future therapies aimed at fertility preservation without the preservation of their testicular tissue/cells prior to treatment. Thus, the current study will 1) address these deficits by testing methods for testicular tissue cryopreservation in patients that will undergo gonadotoxic treatments and 2) provide a potential resource for future fertility restoration. Lastly, the safety aspects of diagnosis/elimination of malignant cell contamination in testicular tissue can only be performed with patient tissue, and thus, the current protocol will generate unique and valuable safety and feasibility data.

SUBJECT RECRUITMENT AND INFORMED CONSENT PROCEDURES:
Inclusion Criteria
  1. Be male at any age.
  2. Be scheduled to undergo surgery, chemotherapy, drug treatment and/or radiation for the treatment or prevention of a medical condition or malignancy with risk of causing permanent and complete loss of subsequent testicular function. Risk categories based on treatment regimens are indicated below. Investigators will utilize three sources to calculate risk: 1) “Fertile Hope – Risks of Azoospermia” brochure that details typical agents and treatment regimens in each risk category (Appendix 1), 2) the Summed Alkylating Agent dose score (Appendix 9; Green et al., 2009) or 3) the Cyclophosphamide Equivalent Dose method (Appendix 10; Green et al., 2014). Because of the complexity of many treatment regimens, patient risk categorization will be at the discretion of the investigators.
    a. High Risk
      i. ≥80% risk of prolonged azoospermia, (Appendix 1; Fertile Hope Brochure).
        1. Total body radiation (TBI)
        2. Testicular radiation ≥2.5 Gy in men and ≥ 6 Gy in boys
        3. Protocols containing procarbazine
        4. Alkylating chemotherapy for transplant conditioning
        5. Any alkylating agent with TBI, pelvic or testicular radiation
      ii. Summed alkylating agent dose score ≥3. (Appendix 9; Green et al., 2009)
      iii. Cyclophosphamide equivalent dose ≥4,000 mg/m². (Appendix 10; Green et al., 2014; Green et al., 2014)
    b. Intermediate risk (21-79% risk of prolonged azoospermia, Appendix 1 Fertile Hope).
      i. Bleomycin, etoposide, cisplatin x 2-4 cycles
      ii. Cumulative cisplatin dose < 400 mg/m²
      iii. Cumulative carboplatin dose ≤ 2 g/m²
      iv. Testicular radiation dose 1-6 Gy (due to scatter from abdominal/pelvic radiation)
    c. For adult subjects (≥18 years old), eligibility is limited to patients in High and Intermediate risk categories.
d. For children subjects (<18 years old), eligibility is limited to patients in the High risk category.
3. Or have a medical condition or malignancy that requires removal of all or part of one or both testicles.
4. Or have newly diagnosed or recurrent disease. Those who were not enrolled at the time of initial diagnosis (i.e., patients with recurrent disease) are eligible if they have not previously received therapy that is viewed as likely to result in complete and permanent loss of testicular function.
5. Have two testicles if undergoing elective removal of a testicle for fertility preservation only. *Note: removal of both testicles will limit fertility preservation options.*
6. Sign an approved informed consent and authorization permitting the release of personal health information. The patient and/or the patient’s legally authorized guardian must acknowledge in writing that consent for specimen collection has been obtained, in accordance with institutional policies approved by the U.S. Department of Health and Human Services.
7. Consent for serum screening tests for infectious diseases [HIV-1, HIV-2, hepatitis B, hepatitis C, RDR (syphilis), CMV, HLTV-1, and HTLV-2], to be performed at the time of testicular tissue harvesting.
8. Undergo a full history and physical examination and obtain standard pre-operative clearance (based on the most recent ACC/AHA Guideline for Perioperative Cardiovascular Evaluation for Noncardiac Surgery) as determined by their primary surgeon.

Eligibility will be recorded using a written checklist based on the criteria listed above and will be verified by the PI or co-investigator prior to initiating experimental interventions.

**Exclusion Criteria**
Patients will be ineligible for participation in this study if they are:
1. Diagnosed with psychological, psychiatric, or other conditions which prevent giving fully informed consent.
2. Diagnosed with an underlying medical condition that significantly increases their risk of complications from anesthesia and surgery.
3. Patients with known bilateral testicular involvement (cancer) will not be eligible for the study if they are not able to have a wedge biopsy of normal tissue.

**Informed Consent:**
Consent forms will be given to the patient immediately after being identified by their physician as meeting eligibility criteria and before any research procedures are performed.

Reconsent at age 18: Subjects who were initially consented for study participation before age 18 will need to be re-consented for continued participation when they turn 18. The PI or co-Investigator will meet with these subjects when they turn 18 to obtain consent for their continued participation. Relevant procedures will be explained to the subjects, including continued storage of their tissue for possible future experimental fertility restoration procedures and yearly recontact to assess general health, disease status, and fertility. The experimental nature of these future
procedures will be emphasized. The costs of continued participation (i.e., cost of tissue storage) will be explained.

**Process to Ensure the Subjects are Fully Informed About the Research:**

1. The PI or a co-investigator will obtain informed consent of the patient or guardian and document the informed consent process.
2. The person to provide consent or permission will either be the patient (if 18 or older) or the parents/legal guardians.
3. The information communicated will be everything presented in the comprehensive informed consent form. In particular, the consenting investigator will emphasize the purpose of the study, research procedures involved, the risks and potential benefits of participation, and the rights of the research study subject.
4. The time between informing the patient or the patient/guardian of the patient and obtaining consent will be flexible based upon the individual patient's clinical situation. As much time as possible will be allotted for patients and/or their parents/legal guardians to review the consent materials and discuss the study with their physician and/or consenting investigator.
5. We will use the teach-back method to ensure adequate comprehension of the study goals, risks, benefits and alternative options.
6. For child subjects, parental consent will be required from both parents. This two-parent consent requirement will be waived under two circumstances: 1) When only one parent is reasonably available or 2) when only one parent has legal responsibility for and custody of the child.

**How to Ensure that Subjects Have Sufficient Time to Decide Whether to Participate and Minimize Possibility of Coercion or Undue Influence:**

The research team will exhaust all avenues to ensure that subjects have as much time as possible to decide whether to participate in this study. As is true for all experimental interventions, the amount of time available for potential subjects to decide whether to participate depends on the patient's clinical situation. In most cases, the decision to participate will need to be made within one week. In some rare cases it is conceivable that patients and/or their parents will have one day to make a decision about participation in the study, depending on their diagnosis. In all cases, the patient and his/her family will be given as much time as they need to review the materials and ask questions within the context of good patient care. During discussion of the study with prospective patients and/or their parents, we will emphasize that the decision whether or not to participate in study will have no bearing on the patient's medical care or the attitude of the medical team towards that patient. This approach will minimize the possibility of coercion or undue influence. While we appreciate that patients and/or their parents may be faced with the necessity to make a rather quick decision and could represent a vulnerable population under duress, the primary consideration MUST be the appropriate timeline for clinical management of the patient’s primary disease.
DESCRIPTION OF RESEARCH ACTIVITIES:

Timeline: Patients who opt for experimental testicular tissue cryopreservation will be consented (using age-specific consent forms) and enrolled in the study. Patients will then be scheduled for surgery at Lurie Children’s to remove testicular tissue on an expedited schedule in consultation with the primary physician and considering the treatment plan for the primary disease or condition. Upon surgical procurement of testicular tissue, a small piece of the testicular tissue will be provided to pathology for routine histological evaluation, and the remaining tissue will be processed for cryopreservation. If pathology finds evidence of cancer in the testicular tissue provided at surgery, they may request that all of the patient tissue (even tissue that has been frozen for patient use) be returned to pathology for a more detailed examination. In this case, no tissue will be available for the patient to use for fertility preservation purposes. The remaining tissue will be immediately placed in a sterile container with media on ice and shipped (typically within a few hours of removal from the patient) to the Center for Fertility and Reproductive Endocrinology (CFRE) at Magee-Womens Hospital, Pittsburgh, PA where it will be processed and allocated for research and cryopreservation.

Testicular Tissue Harvesting:

Patients in three categories will participate in this study:

Category 1: Patients who are having all or part of one or both testicles removed for the treatment of a disease.
   a. Clinical indications for removal of all or part of one or both testicles include (but are not limited to) the following: Advanced stage/grade testicular cancers; testicular metastases; Treatment of hormonally sensitive cancers (i.e., prostate) that necessitate bilateral orchiectomy
   b. Note: removal of both testicles will limit options for fertility preservation.

Category 2: Patients who are having all or part of one or both testicles removed for the prevention of a disease.
   a. Clinical scenarios for prophylactic bilateral orchiectomy include (but are not limited to) the following: Carriers of genes that predispose to hereditary cancers of the testicles or prostate; Patients with increased risk or personal history of hormonally sensitive cancers.
   b. Note: removal of both testicles will limit options for fertility preservation.

Category 3: Patients having all or part of one testicle removed solely for the purpose of fertility preservation in the setting of a medical or surgical condition where the clinically indicated treatment is likely to cause infertility.
   a. Clinical scenarios include (but are not limited to) the following: high- and intermediate-risk chemotherapy or radiation treatments for a variety of neoplastic and malignant disorders; conditioning for bone marrow transplantation for malignant diseases and non-malignant disorders.

Patients in Categories 1 and 2 will have testicular tissue removed for a clinically-indicated purpose. Only patients in Categories 1 and 2 may have both testes removed, which will only
occur in clinically-indicated scenarios. Bilateral orchiectomy will not be performed for patients in Category 3 who are having testicular tissue removed solely for fertility preservation. The amount of tissue removed for clinical purposes will depend on the diagnosis and can include all or some of one or both testes. Each subject’s surgeon will decide on a case-by-case basis if additional testicular tissue should be excised for the research purposes outlined in this protocol. Presence and extent testicular pathology in the clinically indicated portion of the gonad removed will help to determine whether additional tissue can or should be removed for the purposes of the research proposed in this protocol. The goal will be to remove healthy tissue for research and future patient use without compromising the health of any remaining tissue. This will be at the discretion of the surgeon and will be educated by discussion with the laboratory researchers listed as investigators on this protocol. Estimates of the amount of tissue that will be removed for fertility preservation only (for future patient use and the research pool) is as follows:

- Testicular tissue from pre-adolescent and adolescent patients: Between 100-500mg of testicular parenchyma.
- Testicular tissue from adult patients: Between 3-6g of testicular parenchyma (more tissue is obtained from adults because cellular yields are lower and spermatogonial stem cells are diluted by differentiating germ cells during spermatogenesis).

The surgical approach for removal of testicular tissue will be performed using the methods determined by the surgeon based on the medical/surgical diagnosis or treatment (see below). For instance, a trans-scrotal approach will be used for testicular tissue retrieval except in cases where an inguinal approach is not indicated (radical orchiectomy). Furthermore, surgery to harvest testicular tissue may be coordinated with another procedure such as placement of a central venous catheter for future chemotherapy, tumor biopsy, or laparotomy for another purpose.

Testicular tissue designated for research will be de-identified and labeled with a recruitment site-specific identification number.

Timing of the Surgery and Starting Other Therapy: Whenever possible, surgery to remove testicular tissue will be coordinated with other surgical procedures (e.g., central line placement). Whenever possible, surgery to obtain testicular tissue will be performed prior to any potentially gonadotoxic therapy (e.g., chemotherapy or radiation). Patients with previous exposure to gonadotoxic therapy may still be eligible for this protocol if the previous exposure was not associated with high risk of infertility (see section on inclusion criteria). For patients who will receive chemotherapy or radiation for treatment of their primary disease, the patients’ surgeon(s) will determine hemostasis and provide surgical clearance for initiation of therapy. Subjects will begin their treatments on a time-frame dictated by clinical management of their primary disease or condition, typically within one week. It has been reported in some cases that chemotherapy or radiation treatments can begin as early as one day following testicular biopsy surgery (Bahadur et al., 2000).

Surgical Procurement of Testicular Tissue: If a male patient or his guardians elect to participate and provides informed consent, the patient will be screened to determine eligibility. At early stages of technology development, simple orchiectomy (removal of one entire testicle) may give the best chance of preserving sufficient cells for effective therapy. However, incisional biopsy of up to 25% of tissue from one testis (wedge resection) will also be presented to the patient as an alternative option. The amount of testicular parenchyma removed will be at the
discretion of the surgeon. The duration of surgical testicular tissue procurement is likely to be between 1 and 2 hours. The recovery time required prior to resuming normal activities or initiating other treatments (e.g., chemotherapy or radiation) is expected to be 2-3 days.

Wedge Resection (incisional biopsy) - Scrotal Approach
Incision is made with scalpel in scrotum in direction of rugae. Dartos muscle is divided by electrocautery and the tunica vaginalis is divided sharply. The tunica albuginea is incised sharply with a scalpel and up to 25% of the testicular parenchyma is excised. The tunica albuginea is closed with a 5-0 absorbable suture. Then the tunica vaginalis is closed over the testicle with a 4-0 absorbable suture. Then the skin and dartos muscle are closed in a single layer with a 4-0 absorbable suture in a subcuticular fashion.

Wedge Resection (incisional biopsy) - Subinguinal Approach
Incision with scalpel is made 0.5 cm below external inguinal ring. The subcutaneous fat is divided by electrocautery. The spermatic cord is visualized and freed from its investing fascia by sharp dissection. The testicle is then delivered through the inguinal canal, leaving the gubernacular attachments intact. The tunica albuginea is incised sharply with a scalpel and up to 25% of the testicular parenchyma is excised. The testicle is returned back to its normal anatomic position. Scarpa’s fascia is then closed with a 4-0 absorbable suture and the skin is closed with a 4-0 absorbable suture.

Simple Orchiectomy - The incision is made with scalpel in scrotum in direction of rugae. The dartos muscle is divided by electrocautery. The testicle and spermatic cord are then delivered through the incision. The cord is divided into 2 packets: one packet contains the vas deferens and the other contains the spermatic cord vessels. Each packet is tied off with a 2-0 non-absorbable suture. The skin and dartos are closed in a single layer with a 4-0 absorbable suture in a subcuticular fashion.

Residual Adult Testicular Tissue from TESE - Adult men have better access to current fertility-preserving options than all other groups because they can simply cryopreserve semen. However, some men are not able to provide an adequate semen sample at the time of diagnosis. For these men, testicular sperm extraction (TESE) is an option. In the TESE procedure a biopsy is obtained surgically from the testicular parenchyma and the excised testicular biopsy specimen is placed in human tubal fluid culture medium supplemented with 6% Plasmanate. The tissue is divided into several segments and individual tubules from the mass of coiled testicular tissue are isolated by initial dispersal of the testis biopsy specimen with two sterile glass slides, stretching the testicular parenchyma to isolate individual seminiferous tubules. Subsequently, mechanical disruption of the tubules is accomplished by mincing the extended tubules with a sterile scissors in HTF/Plasmanate medium. Additional dispersion of tubules is achieved by passing the suspension of testicular tissue through a 24 gauge angiocatheter. In the IVF embryology laboratory, A "wet preparation" of the suspension is examined under phase contrast microscopy at 100x and 400x power to identify spermatozoa. Subsequent processing of the testicular tissue suspension, including microdissection of the specimens is also performed in the IVF laboratory. Aliquots of tissue containing spermatozoa are also processed for cryopreservation and some spermatozoa may be used immediately for ICSI. Aliquots of tissue that do not contain sperm are discarded. These aliquots may contain spermatogonial stem cells that could be used to address
the aims of this protocol. We propose in this protocol to process and freeze the aliquots of TESE tissue/cells that do not contain sperm (rather than discarding these samples). These samples will provide material for research as well as a “backup” resource for patients. We acknowledge that the number of adult males for whom this technique is a viable option is low, but not zero. However, we want to provide an option for these men and utilize residual material for research. There is no additional risk to the patient because the patients are undergoing a clinically-indicated surgery for tissue accrual and the residual tissue would otherwise be discarded after the TESE procedure.

**Blood collection for infectious disease screening and testing:**

Tissue banking and subsequent use of testicular tissue is currently regulated by the Food and Drug Administration (FDA). In order to comply with current tissue banking regulations and to be prepared for any future changes in regulations while these testicular tissues are in storage, patients will be tested and screened for a number of infectious diseases prior to banking testicular tissue. 4 vials of blood (6 ml each) will be collected, including 1 red top tube for serum and 3 purple top tubes (EDTA) for plasma. 1 red top and 2 purple top tubes will be sent to Memorial Blood Centers for infectious disease testing. Plasma from 1 purple top tube will be frozen and sent with the patient’s frozen testicular tissue/cells to Reprotech to be stored with the tissue to allow for future testing if FDA regulations change. The immediate testing will include but not be limited to testing for Hepatitis B and C and HIV. The screening and testing that will be performed are the same as would be performed on an anonymous reproductive tissue donor and include a physical examination and questions regarding potential high risk behaviors. The testing that will be performed will be testing that is mandated for donors of leukocyte rich tissues and must be performed within 7 days of tissue procurement. In addition, a sample of the patient’s blood plasma will be stored with the testicular tissue to permit any future testing required under federal tissue banking regulations. In spite of storing blood plasma, it is still possible that federal regulations may change and therefore, it may not be possible to perform the appropriate testing to permit heterologous use of the tissue in the future. Infectious disease testing is performed in this study to permit patient use of his own tissue and not for the purposes of research tissue or research study.

**Pathology:** A segment of each testicular specimen (~5%) will be removed, fixed in formalin, and sent to the Pathology Department to assess for contamination by neoplastic (malignant) cells at the institution where the surgery is performed. A full Pathology report detailing results of the histological and morphological examination of each tissue specimen will be included in the patient’s medical record to provide information to counsel patients on the likelihood that the tissue obtained could be used for future fertility restoration. The Pathology report will also be de-identified and included in the research record using the same coding to de-identify the gonadal research tissue in order to protect patient privacy. In cases where surgeons order intraoperative pathological examination of the patient’s testicular tissue, additional tissue will not be reserved for pathological examination.

**Tissue transport:** Testicular specimens will be rapidly submerged in sterile ice-cold shipping medium (lactated ringer solution). The tissue container will be sealed and placed in double-redundant zip lock bags. The testicular tissue specimen and one purple top blood plasma tube will be placed in a Styrofoam shipping container with ice packs for shipment to the coordinating...
center in Pittsburgh (see shipping address below). Tissue and blood samples will be de-identified at Lurie Children’s and labeled with a site specific identification number. No patient identifying information will be shipped to the Pittsburgh coordinating center. The Fertility Preservation Program in Pittsburgh is located in the Center for Fertility and Reproductive Endocrinology (CFRE) at Magee-Womens Hospital in Pittsburgh, PA. CFRE is an FDA-compliant and American Association of Tissue Banks-accredited facility for processing and storage of reproductive tissues and is FDA-registered as a HCT/P manufacturer.

**Tissue processing:** Testicular tissue and blood samples will be processed in the CFRE at Magee-Womens Hospital. Upon arrival at CFRE, testicular tissues will be weighed. The tissue will be minced and cryopreserved as tissue fragments or digested to produce a cell suspension (see below). Approximately 75% of the resulting tissue/cell suspension will be designated for patient use and 25% will be de-identified and designated for research. The Absolute amounts of testicular tissue/cells designated for research and patient use will depend on the actual weight of tissue obtained.

**Cryopreservation:** Testicular tissue will be cryopreserved as minced pieces or as a cell suspension using methods that have been described previously (Baert et al., 2013; Bellve et al., 1977; Dovey et al., 2013; Hermann et al., 2007; Hermann et al., 2009; Hermann et al., 2012; Keros et al., 2007; Nagano et al., 2002; Valli et al., 2014b). Optimal freezing approach may be modified based on research outcomes. The procedure for testicular tissue/cell processing and cryopreservation may be modified as improvements become available.

**Cryopreservation of testicular tissue:** Tissue pieces 2-5mm³ in size will be placed into sterile 1.8ml cryovials containing 1.5ml freezing medium (Modified Human Tubule Fluid). Vials will be equilibrated on ice for 30 minutes and then frozen in a Crysalys PTC-9500 programmable controlled-rate freezer (Biogenics; CA, USA) and stored in liquid nitrogen.

**Cryopreservation of testicular cells:** Testicular cells will be generated from tissue using a modified two-step enzymatic digestion procedure essentially as described previously (Dovey et al., 2013; Valli et al., 2014b). Experiments in the laboratory will test the relative efficacy of different clinical-grade enzymes (mammalian tissue free and GMP production) in this procedure [for example, recombinant bovine pancreatic Trypsin (e.g., TrypZean, Sigma-Aldrich), recombinant human DNase I (Pulmozyme, Genetech), purified collagenase blend (Liberase MTF, Roche)]. Cells will be resuspended at up to 40 x 10⁶/ml in freezing media, aliquotted into sterile cryovials and an equal volume of freezing medium will be added drop-wise. Vials will be frozen in a Crysalys PTC-9500 programmable controlled-rate freezer (Biogenics; CA, USA) and stored in liquid nitrogen. A small portion of the tissue and/or cell suspension may be used for research prior to cryopreservation if the amount of obtained tissue is greater than the minimum outlined above.

**Testicular Tissue/Cell Storage:** Testicular tissues and cells designated for research use will be stored for a short time at the Center for Fertility and Reproductive Endocrinology (CFRE) at Magee-Womens Hospital, Pittsburgh, PA and will be subsequently transferred to Magee-Womens Research Institute (MWRI; Pittsburgh, PA) for research use. Research cells/tissue will not be stored with tissue designated for patient use. Cryopreserved testicular tissue/cells
designated for patient use will be transferred to Reprotech, Ltd. (RTL) in Roseville, MN for storage and subsequent release. RTL is an FDA-compliant and American Association of Tissue Banks accredited long term storage facility for reproductive tissues. Based on the extended periods of time that these testicular tissues/cells are likely to be stored (patients may wait for five years from cancer treatment to be considered cancer free and begin a family; some may wait longer based on age), RTL provides maximum flexibility for the patients involved. In this way, patients are permitted to store their testicular tissues/cells as long as they wish and ship them to a fertility treatment center of their choice at the time of use. The patient can determine how the testicular tissue designated for his use will be utilized as technology changes and based on his unique circumstances. RTL does not perform fertility treatments and is not affiliated with any fertility center so there is no potential conflict of interest. Patients will execute a separate storage agreement with RTL which defines the length of storage, shipping requirements, infectious disease, screening and disposition of the tissues in the event of their death. In some circumstances, as determined by the subjects, it is possible for patient tissues to be donated to research prior to transfer to Reprotech, at which time the de-identified samples will be transferred to MWRI for storage and research use. Donation of subject tissue to research after transfer to Reprotech is governed by the subjects’ agreement with Reprotech.

**FOLLOW-UP PROCEDURES:**
1) Routine postoperative checkups will be performed in all subjects to help assess the safety of testicular tissue harvesting and monitor for adverse events. For patients in Categories 1 and 2, this will be performed as a part of their normal clinical management by their surgeon. For patients in Category 3 who are undergoing elective tissue harvesting, postoperative follow-up will be performed by their surgeon in the same manner, but under the auspices of the study. Surgical complications for patients in Category 3 will be reported as adverse events related to the study. Adverse events will be identified using the Common Toxicity Criteria for Adverse Events (CTCAE). A copy of the CTCAE can be downloaded from the CTEP home page (http://ctep.info.nih.gov).

2) For the purposes of the current “Testicular tissue cryopreservation” protocol, investigators will contact participants (or their parents) by phone or mail yearly. Patient re-contacts will occur indefinitely or until participants’ written request to withdraw from the study have been received. Contact with patients will be used to follow their medical and fertility status over time, ask questions about any future use of their frozen tissue and the outcome(s) of fertility preservation and “restoration” treatment(s), if applicable. The information collected at each subsequent patient contact will include: name, date of birth, gender, current status of primary diagnosis, additional therapies employed (type of chemotherapy drugs, radiation, and surgery), dates of additional therapies, disease or treatment co-morbidities, death, new diagnoses, marital status, sexual history (adolescents and adults), pregnancies and children, and fertility interventions (e.g., IVF).

3) All pre-pubescent subjects would ordinarily require monitoring for hormonal deficiency after their indicated clinical therapy (e.g., chemotherapy, radiation, gonadectomy) to ensure adequate sex hormone production through anthropomorphic measurements, growth velocity curves, and Tanner Staging. This is standard of care for all general pediatric well-visits, and is especially
important for pre-pubertal participants in this study who are at high risk of testicular failure and disrupted puberty due to the treatment for their primary disease (e.g., chemotherapy or radiation treatment). Thus, these follow-up procedures will be part of the pre-pubescent participant’s clinically-indicated surveillance, and will not be provided by the investigators as part of this study. In the event that premature testicular failure, delayed puberty, or short stature results, the child should be referred to Pediatric Medical Endocrinology and/or be provided with hormonal treatment as clinically indicated.

In the event that premature testicular failure, delayed puberty, or short stature results, the child should be referred to Pediatric Medical Endocrinology and/or be provided with hormonal treatment as clinically indicated.

4.) Post-pubertal subjects will also require monitoring to ensure adequate post-operative sex hormone production. Once again, this is standard clinical management of patients facing potentially gonadotoxic treatments and will not be provided by the investigators as part of this study. In the event that a subject is diagnosed with testicular failure, based on post-operative symptoms, the benefits of hormonal replacement therapy should be addressed and prescribed as clinically indicated.

**RESEARCH AND OUTCOME VARIABLES:**

1) **Optimization of testicular cryopreservation techniques:** Testicular tissue will be minced and cryopreserved in toto or used to generate a suspension of testicular cells using a series of enzymatic digestions, washes, and filtrations. Testicular tissue/cells donated to the research pool will be frozen using varying cryopreservation methods and thawed to determine the efficacy of the freeze/thaw techniques. The concentration and number of recovered spermatogonial stem cells in the thawed sample will be determined using molecular markers and a human-to-nude mouse xenotransplantation assay. Recovery of spermatogonial stem cells will be compared to the concentration and number prior to cryopreservation using the same assays. Data gathered from this research will assist in identifying and overcoming some of the challenges to successful freezing and thawing of cells for future use by the patient.

2) **Assessment of malignant cell contamination in harvested patient testicular tissue/cells:** A segment of each testicular specimen (<5%) will be removed using aseptic technique and sent to surgical pathology to assess for contamination by neoplastic (malignant) cells. In some cases, testicular cell suspensions will be evaluated prior to cryopreservation for malignant cell contamination by xenotransplantation into the testes of nude mice, a bioassay for malignant contamination. The endpoint of the xenotransplantation assay for malignant cell contamination will be development of histologically-recognizable solid tumors in nude mice (Jahnukainen et al., 2001; Dovey et al., 2013).

3) **Develop strategies to eliminate malignant contamination in patient testicular cells:** In cases where malignant cell contamination is detected in testicular cell suspensions prior to cryopreservation or in patients diagnosed with malignancies that carry a high-risk for peripheral metastases, efficacy of separating malignant cells from therapeutic spermatogonial stem cells by fluorescence-activated cell sorting (FACS) will be assessed. Patient testis cell suspensions (with known or unknown malignant contamination) will be thawed and subjected to phenotypic analysis using flow cytometry. This flow cytometry analysis will be tailored to the patient according to the outcomes of patient diagnostic information (i.e., flow cytometry performed to diagnose disease) and subsequent analyses of research bone marrow sample by flow cytometry,
if available. The results of these analyses will be used to fractionate patient testis cell suspensions using the best sorting strategies available to each given patient. Unsorted and sorted cell fractions will be collected and analyzed by immunocytochemistry and/or polymerase chain reaction for cancer markers. Unsorted and sorted fractions may also be transplanted to the testes of nude mice to assay spermatogonial stem cell and malignant potential and to determine whether these two activities can be separated.

7.0 STATISTICAL CONSIDERATIONS AND DATA MANAGEMENT

7.1 Accrual

Up to 15 patients per year are estimated to be enrolled at the Lurie Children’s Hospital site. Appropriate statistical approaches (e.g., t-tests, ranked-sign tests and analysis of variance) will be used to reveal significant effects of freezing paradigms and efficacy of methods for stem cell isolation and cancer cell removal.

7.2 Data Collection and Access

Participation in this research is confidential. All research tissues will be de-identified; participants will be identified by number, not name. No information by which the patient can be identified will be released or published in connection with this study. Only the PI and co-investigators will have access to files matching the patient with tissue specimen numbers. Data will be permanently stored by the PI and co-investigators. The researchers using the research portion of the tissue will not have access to any identifying information regarding that tissue.

8.0 RISKS

Risks of general:

Blood Draw
- Common Risks: pain can occur
- Infrequent Risks: bleeding can occur

Confidentiality
- Common Risks: none
- Infrequent Risks: Breach of confidentiality, participation in this research is confidential and to minimize the risk of breach of confidentiality, all paper research records that contain identifiable information will be stored in a locked filing cabinet in Lurie Children’s that is only accessible by the Study Coordinator, study investigators, and research personnel involved in this study. Electronic research records that contain identifiable information will be stored in a secure, encrypted, password-protected database at Lurie Children’s. Personnel involved in this study are expected to protect the security and confidentiality of identifiable information. Furthermore, all tissues designated for research will be de-identified; participants will be identified by number, not name and linkage codes will be kept in a separate, secure location.

Testicular Tissue Harvesting
- Common Risks: none
• Infrequent Risks:
  o General anesthesia: the patient’s risk of death from anesthesia is less than 1 in 100,000 in children older than 3 years and less than 1 in 10,000 in children less than 3 years (Arbous et al., 2001; Gibbs and Borton, 2006; Kawashima et al., 2003).
  o Simple Orchiectomy: Risks of simple orchiectomy are the same as other surgical procedures, including infection and bleeding as a result of surgical incision. The chance of the patient requiring hospitalization for complication(s) is less than 1%. The patient's chance of dying as a result of such complication(s) is less than 1 in 10,000.
  o Testicular Wedge Resection: Risks of testicular wedge resection are also the same as other surgical procedures, including infection and bleeding as a result of surgical incision. It is possible that the surgery itself could cause scar tissue or damage to the remaining testicular tissue, so that chances for producing sperm from that testicle could be reduced. Surgery in the pelvic region or on the testicles can damage the nerves that cause ejaculation. There is also a risk of bleeding within the resected testicle resulting from the surgical removal of tissue. The chance of the patient requiring hospitalization for complication(s) is less than 1%. The patient's chance of dying as a result of such complication(s) is less than 1 in 10,000.
  o Removal of a Testicle: There is a theoretical risk that the patient may experience a reduction in fertility due to the removal of a testicle, although the remaining testicle typically compensates for loss of one gonad. In that case, the surgery to remove testicular tissue would then have been unnecessary. Surgery in the pelvic region or on the testicles can damage the nerves that cause ejaculation. Removal of one testicle can lead to temporary reduction in production of testosterone, 90-95% of which is produced by the testicles (the balance is produced by the adrenal glands). The most common side-effects of reduced testosterone levels in adult mean include lost or reduced sexual desire, impotence, hot flashes similar to those in menopausal women, mood swings or depression, enlargement and tenderness in the breasts, weight gain, osteoporosis, and fatigue. To address the potential psychological consequences of removing a testicle, some men opt to have a testicular prosthesis, or artificial testicles, placed inside the scrotum to replace the testicles removed during surgery. The prosthesis makes the scrotum look much as it did before surgery.
  o Beginning therapy 2-3 days after surgery: Surgery will always occur prior to any potentially gonadotoxic therapy (e.g., chemotherapy or radiation). Therefore, subjects will begin their treatments on a time-frame dictated by clinical management of their primary disease or condition, typically within one week after surgery. For patients who will receive chemotherapy or radiation for treatment of their primary disease, the patients’ surgeon(s) will determine hemostasis and provide clearance indicating lack of complications prior to initiating therapy. It has been reported in some cases that chemotherapy or radiation treatments can begin as early as one day following testicular biopsy surgery (Bahadur et al., 2000).
Delaying a patient’s primary therapy: In nearly all cases, there is no indication that there is an increased risk of delaying a patient’s primary therapy for a window of time to permit surgical removal of testicular tissue and recovery (e.g., one day to one week).

Testicular Tissue/Cell Cryopreservation:
- **Common Risks:** none
- **Infrequent Risks:** Testicular tissue/cells will be cryopreserved following removal from subjects and, following an extended period of cryogenic storage, may be used for future procedures to attempt restoration of fertility. Although care will be taken, damage to the removed testicular tissue may occur during any part of the cryopreservation (freezing) or storage process. The exact method to be employed is unknown and is outside the scope of this protocol. The risk of birth defect(s) and/or genetic damage to any child who may be born following cryopreservation and long term storage of human testicular tissues is unknown. However, thousands of children have been born worldwide from frozen embryos and there only isolated reports of minor increased risk of some specific birth defects in these children (e.g., Angelman syndrome, Prader-Willi syndrome, Beckwith-Wiedemann syndrome). However, the potential risk of genetic mutations that could contribute to birth defects can only occur if subject tissues are used for experimental procedures to restore fertility, which is outside the scope of this protocol. Subjects will not be at direct risk during participation in this study. The testicular tissue removed may not yield usable gametes (i.e., functional spermatogonial stem cell from the testes), or pregnancy may not result when the gametes or their progenitors are ultimately used. Some patients may have particular risks associated with their underlying disease. If a cancer or other disease already affects the testicles, it may be impossible to use the tissue in the future. This may not be known until the patient wishes to use their tissue. Tissue could be lost or made unusable due to equipment failure, or unforeseeable natural disasters beyond the control of this program.

**Steps to Prevent or to Minimize the Severity of Potential Risks:**
All blood draws, surgical procedures, bone marrow aspirations, and tumor biopsies will be performed by skilled, experienced technicians/surgeons in a controlled environment at Lurie Children’s.

Testicular tissue will be shipped to Pittsburgh for processing and cryopreservation in the Andrology and Embryology laboratories in the Center for Fertility and Reproductive Endocrinology (CFRE) at Magee-Womens Hospital by certified technicians with experience processing testicular tissue. CFRE is an FDA-compliant and American Association of Tissue Banks-accredited long term storage facility for reproductive tissues and is FDA-registered as a HCT/P manufacturer, and thus, is an appropriate facility in which to process testicular tissue for potential future use by subjects. All tissue processing will be performed in accordance with good clinical practices, good laboratory practices (GLPs) and current good tissue practices (CGTPs) to minimize the risks for testicular tissue processing and cryopreservation. We have communicated our testicular tissue processing protocol to the Office of Cellular, Tissue and Gene Therapy at the FDA’s Center for Biologics Evaluation and Research, which indicated that our protocol would
be appropriate for the described homologous reproductive purpose under 21 CFR 1271 regulations. In all cases, suitable reagents and disposables will be employed for tissue processing in accordance with FDA recommendations.

Participation in this research is confidential. All research tissues will be de-identified by the individual centers; participants will be identified by number, not name. The Pittsburgh coordinating center will receive de-identified enrollment information, tissue and blood that are identified with a site-specific identification number. No information by which the patient can be identified will be published in connection with this study. Only the individual site PI and co-investigators will have access to files matching the patient information with tissue specimen numbers. Tissue and blood samples will be de-identified by the individual sites, but in such a way that the Pittsburgh coordinating center will know which site sent the tissue (e.g., CHOC-001 from Children's Hospital of Orange County or LCH-001 from Lurie Children’s Hospital). Only the individual site PI, co-investigators and research staff will have access to their own files and these will not be available to Pittsburgh or other individual sites. Authorized representatives of the USDA and the office for human research protections (OHRP) may review and/or obtain identifiable health information for the purpose of monitoring the accuracy of research data and to ensure that the research is being conducted according to FDA regulations. Authorized representatives of Memorial Blood Centers will have access to data, documents and blood samples in association with the FDA-mandated infectious disease screening. Testicular tissue/cells designated for patient use will be stored at Reprotech, LTD, a third party tissue bank. Authorized representatives from Reprotech will have access to data, documents, blood plasma and tissue/cells generated by the study. Patients will sign a separate agreement with Reprotech.

**Steps Taken in the Event that a Clinically Significant, Unexpected Disease or Condition is Identified during the Conduct of the Study:**

If a subject is found to have a positive screen for an infectious disease (e.g., HIV), he will be informed and referred to the appropriate specialist. Infectious disease status will not be determined until after study enrollment. The storage of specimens designated for patient use from potentially infectious subjects (subjects for whom testing show a potential for an infectious disease) require certain additional safeguards for potentially infectious specimens only.

**Endpoints:**

Since this is an observational study, there are no experimental endpoints that impact continued study participation. Continued storage of testicular tissue/cells designated for patient use is governed by the Reprotech agreement and is not dependent upon continued study participation. Disposition of tissue/cells designated for patient use at their death is also determined by the Reprotech agreement.

**Potential for Direct Benefit:**

Established fertility preserving therapies are available for males that have undergone puberty, but these therapies are not accessible or appropriate for all adolescent or adult patients. Currently there are no therapies to preserve the future fertility of preadolescent boys. However, new reproductive therapies are under development and may one day offer “fertile hope” to those survivors that do not currently have access to fertility preserving therapies. When no established fertility sparing or preserving options are available, it is reasonable to offer harvesting and
cryopreservation of testicular tissue as a possible means of fertility preservation. In this case, the potential direct benefits to the subject are two-fold, regardless of diagnosis or age. First, each subject will have tissue cryopreserved and dedicated for their own future use, a scenario that offers hope for patients that currently have limited prospects for future fertility. Retrospective studies indicate that most parents are interested in preserving fertility on behalf of their children with cancer (Ginsberg, 2011; van den Berg et al., 2007; Wyns et al., 2011). Thus, there is perceived acceptability and desire to undergo experimental therapy to preserve fertility, as long as treatment for the primary disease is not compromised (Oosterhuis et al., 2008). There is also likely a psychological benefit to the patient in terms of raising issues relating to their life after cure from their primary disease (e.g., cancer). Second, the subject may have the opportunity to utilize their stored testicular tissue or cells for fertility restoration procedures in the future.

**Data and Safety Monitoring Plan:**
The Pittsburgh coordinating center will serve as the central data safety monitoring board (DSMB) for this study for the multicenter sites. The affiliated sites will send their minutes and their adverse events to the coordinating center. The coordinating center will review this data at the bimonthly meeting and provide a summary or a central DSMB report which will be sent to all the centers.

Dr. Orwig together with the other co-investigators and research team members listed on this protocol will meet on a bimonthly basis to conduct the data safety monitoring review for the Pittsburgh site. All affiliated sites will send their data safety monitoring meeting minutes to the center and they will also be reviewed at the bimonthly meeting. A DSMB report from all affiliated sites will be submitted to the IRB at the time of annual renewal.

Adverse events and surgical complications after an elective orchiectomy (Category 3 Patients-those not requiring surgery for clinical management of their primary disease) will be identified using the Common Toxicity Criteria for Adverse Events (CTCAE). A copy of the CTC version 4.0 can be downloaded from the CTEP home page (http://ctep.info.nih.gov). All appropriate treatment areas should have access to a copy of the CTCAE version 4.0. The severity of the event should then be graded using the CTCAE criteria. Determination of whether the event was related to the surgical procedure and whether the adverse event was expected or unexpected will be made. Any instances of grade 3 or 4 adverse events are reported immediately to the University of Pittsburgh IRB using the standard forms and procedures established by the IRB.

**Precautions to Ensure Subject Privacy is Respected:**
As with all medical discussions, the research protocol will be discussed in a private room. The collection of sensitive information about subjects will be limited to the amount necessary to achieve the aims of the research. Therefore no unneeded sensitive information is being collected as part of the research. We will utilize drapes and other barriers for subjects who are required to disrobe.

**Precautions Used to Maintain the Confidentiality of Identifiable Information**
Participation in this research is confidential. All research tissues will be de-identified by the individual centers; participants will be identified by number, not name. The Pittsburgh
coordinating center will receive de-identified enrollment information, tissue and blood that is identified with a site-specific identification number. No information by which the patient can be identified will be published in connection with this study. Only the individual site PI and co-investigators will have access to files matching the patient information with tissue specimen numbers. Tissue and blood samples will be de-identified by the individual sites, but in such a way that the Pittsburgh coordinating center will know which site sent the tissue (e.g., CHOC-001 from Children's Hospital of Orange County or LCH-001 from Lurie Children’s Hospital). Only the individual site PI, co-investigators and research staff will have access to their own files and these will not be available to Pittsburgh or other individual sites. Authorized representatives of the USDA and the office for human research protections (OHRP) may review and/or obtain identifiable health information for the purpose of monitoring the accuracy of research data and to ensure that the research is being conducted according to FDA regulations. Authorized representatives of Memorial Blood Centers will have access to data, documents and blood samples in association with the FDA-mandated infectious disease screening. Testicular tissue/cells designated for patient use will be stored at Reprotech, LTD, a third party tissue bank. Authorized representatives from Reprotech will have access to data, documents, blood plasma and tissue/cells generated by the study. Patients will sign a separate agreement with Reprotech.

What Happens to the Subject’s Research Data or Biological Specimens if They Withdraw from the Study:
All research records will be rendered anonymous should the subject decide to withdraw from study participation. All data collected to this point will be maintained permanently by the investigators. Testicular tissue designated for research will be maintained for future laboratory research. This tissue is de-identified and it is indicated to subjects on the informed consent forms that this tissue will be maintained for research in the event of subject withdraws. Prior to shipment to Reprotech, disposition of stored testicular tissue designated for patient use when a patient withdraws from the study is governed by patient wishes as expressed in the informed consent form. The patient will decide from the following three options:

1) Continue tissue storage for the patient’s own future use at the patient’s expense
2) Donate all stored tissue to research. Stored testicular tissue designated for the patient will be de-identified and transferred to the research pool.
3) Destroy all stored testicular tissue that is designated for patient use.

After transfer to Reprotech, disposition of stored testicular tissue designated for patient use is governed by the Reprotech Cryostorage Agreement (see attached document).
References


Testicular Tissue Cryopreservation for Fertility Preservation


accessibility. Proceedings Of The National Academy Of Sciences Of The United States Of America 98, 6186-6191.


Appendix 1: Fertile Hope – Risk of Azoospermia

Appendix 2: Fertile Hope – Cancer and Fertility General Information
# Appendix 3: Reprotech registration forms

**REGISTRATION**

### PATIENT INFORMATION

<table>
<thead>
<tr>
<th>Name</th>
<th>Date of Birth</th>
<th>SS#</th>
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<tr>
<th>Address</th>
<th>Home Phone (</th>
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<table>
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<tr>
<th>Street</th>
<th>City</th>
<th>State</th>
<th>Zip</th>
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<table>
<thead>
<tr>
<th>Name of Partner (if applicable)</th>
<th>Partner’s SSN</th>
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<thead>
<tr>
<th>Work Phone (</th>
<th>Cell Phone Number(s)</th>
<th>Email Address</th>
</tr>
</thead>
</table>

| Have you ever tested positive for HIV, Hepatitis B, Hepatitis C, or HTLV I & II? |

If yes, please specify: ____________________________________________________________________________

| What month(s) and year(s) were your specimen cryopreserved? |

Please enter your PIN (may be Social Security Number):

To whom, other than yourself, may we release information about your account (Print name & relationship):

### PERSON RESPONSIBLE FOR THIS ACCOUNT

<table>
<thead>
<tr>
<th>Name</th>
<th>Relationship to patient</th>
<th>Home Phone</th>
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<table>
<thead>
<tr>
<th>Address</th>
<th>Work Phone</th>
<th>SS#</th>
</tr>
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</table>

### PAYMENT POLICY

Please indicate the billing interval for storage fees that you elect. Unused storage fees are non-refundable. Storage and shipping fees must be prepaid.

[ ] Quarterly  [ ] 1 year  [ ] 2 year  [ ] 3 year

### CREDIT CARD AUTHORIZATION:

Your signature here authorizes Reprotech, Ltd. to charge your credit card for shipping and storage fees. Check here if you are only authorizing RTL to use your credit card for the first annual or multi-year storage period and the shipping fees.

<table>
<thead>
<tr>
<th>Signature:</th>
<th>Date:</th>
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<table>
<thead>
<tr>
<th>Account Number</th>
<th>Name on Card</th>
<th>Expiration Date</th>
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</table>

### PHYSICIAN/CLINIC WHERE SEMEN/TESTICULAR TISSUE IS STORED

<table>
<thead>
<tr>
<th>Name</th>
<th>Telephone</th>
<th>Fax</th>
</tr>
</thead>
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<table>
<thead>
<tr>
<th>Address</th>
<th>City</th>
<th>State</th>
<th>Zip</th>
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</table>

### PATIENT SIGNATURE BELOW IS REQUIRED

Your signature below acknowledges acceptance of our payment and privacy policies and agreement to keep Reprotech, Ltd. updated with current address and contact information.

<table>
<thead>
<tr>
<th>Signature of Patient</th>
<th>Date:</th>
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</table>

If the Patient above is a minor, a parent or guardian of the minor must sign below:

| Signature of Parent or Guardian, if applicable: |

---

**The Cryostorage & Compliance Experts**

Florida 888-935-9669 • Fax 954-332-6655  
Minnesota 888-489-8944 • Fax 651-489-0442  
Nevada 888-831-2765 • Fax 702-284-2799

C A C Q 100  
Revision: Q  
Registration Semen/Testicular Tissue  
Release Date: 12/15/2010  
Effective Date: 12/15/2010
Appendix 4: Memorial Blood Centers requisition for infectious disease testing

<table>
<thead>
<tr>
<th><strong>Memorial Blood Centers Donor Testing Laboratory</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Viral Screening, Red Cell Typing, PCR Testing</td>
</tr>
<tr>
<td>737 Pelham Blvd., St. Paul, MN 55114-1739</td>
</tr>
<tr>
<td>Phone - 651-332-7111  Fax - 651-332-7005</td>
</tr>
<tr>
<td>CLIA # 24D0663800</td>
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<table>
<thead>
<tr>
<th><strong>Required Information</strong></th>
<th><strong>Additional Information</strong></th>
</tr>
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<tbody>
<tr>
<td><em>Source ID</em> (and/or) [CustomerId/Unique Patient ID]</td>
<td>DOB</td>
</tr>
<tr>
<td><em>Patient Last Name</em></td>
<td>SSN (only)</td>
</tr>
<tr>
<td><em>Patient First Name</em></td>
<td>Patient ID</td>
</tr>
<tr>
<td><em>Date Drawn</em></td>
<td><em>Date Frozen</em></td>
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<tr>
<td></td>
<td>Physician</td>
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<tr>
<th><strong>Test(s) Requested:</strong></th>
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</thead>
<tbody>
<tr>
<td>□ 1 DM Panel (HBsAg, Anti-HBc, Anti-HCV, Anti-HIV-1,2,2,0, MPX (HIV/HCV/HBV PCR), Anti-HTLV, syphilis TP, ABO Rh, Capture CMV Total)</td>
</tr>
<tr>
<td>□ Female HCT/P Panel (HBsAg, Anti-HBc, Anti-HCV, Anti-HIV-1,2,2,0, MPX (HIV/HCV/HBV PCR), syphilis TP, Chlamydia, Gonorrhea)</td>
</tr>
<tr>
<td>□ Male HCT/P Panel (HBsAg, HBc, HCV, HIV-1,2,2,0, HTLV, syphilis TP, Chlamydia, Gonorrhea)</td>
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<thead>
<tr>
<th><strong>Hepatitis B Virus</strong></th>
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<tr>
<td>□ HBsAg (Neutralization performed on reactive samples)</td>
</tr>
<tr>
<td>□ HBsAg Neutralization</td>
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<td>□ Anti-HBc Total</td>
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<tr>
<th><strong>Hepatitis C Virus</strong></th>
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<tr>
<td>□ Anti-HCV (RIBA performed on reactive samples)</td>
</tr>
<tr>
<td>□ Anti-HCV – no reflex (No RIBA performed if sample is reactive)</td>
</tr>
<tr>
<td>□ RIBA</td>
</tr>
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<thead>
<tr>
<th><strong>HIV Virus</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>□ Anti-HIV-1,2 plus O (Western Blot performed on reactive samples)</td>
</tr>
<tr>
<td>□ Anti-HIV-2</td>
</tr>
<tr>
<td>□ Anti-HIV-2 – reflex (Sent for HIV-2 Immunoblot on reactive samples)</td>
</tr>
<tr>
<td>□ HIV-1 Western Blot</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Miscellaneous</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>□ ABO Rh</td>
</tr>
<tr>
<td>□ Red Cell Antibody Screen</td>
</tr>
<tr>
<td>□ Sickle Cell Screen</td>
</tr>
<tr>
<td>□ Capture CMV Total – no reflex</td>
</tr>
<tr>
<td>□ Capture CMV Total – IgM reflex (Sent to ViroMed for IgM if reactive)</td>
</tr>
<tr>
<td>□ Capture CMV Total – IgM/IgG reflex (Sent to ViroMed for IgM and IgG if reactive)</td>
</tr>
<tr>
<td>□ T. Crass (Chagas) (RIPA performed on reactive samples)</td>
</tr>
<tr>
<td>□ RIPA (Performed at Quest)</td>
</tr>
<tr>
<td>□ HLA Class I Antibody</td>
</tr>
<tr>
<td>□ HLA Class II Antibody</td>
</tr>
<tr>
<td>□ Syphilis RPR</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Nucleic Acid Testing</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>□ MPX (HIV/HCV/HBV PCR)</td>
</tr>
<tr>
<td>(These 3 tests are done together, they cannot be separated out)</td>
</tr>
<tr>
<td>□ WNVV PCR</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Serologic Test for Syphilis</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>□ Syphilis TP (Syphilis TP sent out for FTA if reactive)</td>
</tr>
</tbody>
</table>

*Client Code/Physician ID: MW

[rules and instructions]

To be completed by person submitting:
1. # of Serum Tubes (Red) |
2. # of Plasma Tubes (Purple) |
3. # of Unknown Tube Type |
4. # of Urine |
5. # of Swab

Accession /Patient ID: [redacted]
Appendix 5: Application for financial assistance with Reprotech

ReproTech Financial Assistance
Criteria & Application

PROGRAM OVERVIEW

Overview

The ReproTech Financial Assistance program is designed to provide discounted long-term storage for clients with sperm already in storage (either at ReproTech or other facilities) facing financial hardship. ReproTech and its network of Freezing Centers participate with Fertile Hope to provide services to patients who have yet to bank.

What is covered?

Discounts for annual storage services and shipping of specimens from another facility to ReproTech for long-term storage. Currently a discount of $200.00 is offered to eligible applicants, resulting in an annual fee of $75.00 for the first year. Subsequent storage fees will be dependent on participant’s continued eligibility and ReproTech’s then current storage fees.

What is not covered?

Prior to shipping sperm to ReproTech, all patients are required to have infectious disease blood tests. If the test results are not received, the participant may be charged additional quarantine fees.

HOW TO APPLY

Eligibility Criteria

ReproTech selects participants for the Financial Assistance program based on the following criteria. Only participants who meet ALL of the following criteria will be accepted.

• US Citizen or Permanent Resident
• Annual household income less than $50,000 (single) or $75,000 (married)
• Diagnosis of cancer or other potentially fertility damaging disease
• Prescribed treatments present the risk of infertility as determined by an oncologist or Reproductive Endocrinologist
• No contraindication to fertility preservation and/or fertility treatments as determined by both an oncologist and reproductive endocrinologist

Application Requirements

Please complete the following forms with the help of your medical team and make a copy for your records. Please print clearly and submit your completed application to ReproTech via mail or fax to:

ReproTech
550 Village Oaks Dr, Suite 300
St. Paul, MN 55127
FAX: 651-489-0442

Please note your application will not be processed if you do not meet the above criteria or if any of the following information has not been received:

- Completed Patient Authorization and Consent Form
- Completed Oncologist Referral
- Copy of your Federal Tax Returns from the most recent year (Form 1040)
- If you did not file taxes, call the IRS at (800) 829-1040 and request a Tax Return Transcript.

Version 4/18/2018
Appendix 6: Study follow-up telephone script and questionnaire

Testicular Tissue Cryopreservation Study - Follow-up Call Script

Subject Name: ________________________________ Date of Birth: __________________
Research ID Number: ________________________ Date of Interview: __________________

*Note for patients under the age of 18:
○ Survey is completed with the patient’s parent/legal guardian
○ If the patient has not yet reached puberty, mark question as “N/A”

If Applicable, name of patient’s parent/legal guardian: ________________________________
If the patient is over 18, you must speak with the patient, and not the parent/guardian.

Before any phone calls are made, please check with the patient’s primary treating team to avoid calling a family/patient that is deceased. Please also check the patient’s vital status and last progress note in Epic to help determine the patient’s current status.

Interviewer: “Hi (patient’s/parent’s name). This is (your name) from (your institution). I am calling to ask you about the clinical tissue that you/your child had frozen at the University of Pittsburgh. I would like to ask you to complete a 5-10 minute telephone survey to update your contact and health information and to ask you for some extra information for our research. Your participation in this survey is completely voluntary. This means that you do not have to participate in this survey unless you want to. You may end the phone conversation at any point in time. There is a small chance that some of the questions may make you feel uncomfortable. You do not have to answer those questions if you do not want to. All the information I receive from you by phone will be strictly confidential. Would you be willing to participate?”*

Participant: “Yes.”
Interviewer: “Thank you. I’d like to start by updating your contact information.”
   1. “Is there another phone number that you prefer we call?”

   ____________________________________________________________

   2. “Can you verify your home address?”

   ____________________________________________________________
   ____________________________________________________________

   ____________________________

   3. “Is there an email address that we can have on file?”

   ____________________________________________________________
Interviewer: “Thank you for updating your contact information. May I proceed with the survey now?”

4. “Do you have any questions?”

________________________________________________________________________

________________________________________________________________________

________________________________________________________________________

Go to Question 5.

OR

Participant: “No.”
Interviewer: “Is there a better time that I can call back?”

________________________________________________________________________

NOTE: Answers to questions 1-4 should be stored separately from the answers to the questions below to protect subject confidentiality. Please store pages 1-2 separately from those that follow.

*If the patient is deceased, please begin with Question 5 on the next page.
5. **Participant:** “The patient is deceased.”

**Interviewer:** “If you recall, was the patient’s tissue designated for research? How was the patient’s tissue allocated? If you do not know, that’s fine too.”
(Place N/A if the parent is not able to recall the disposition of the tissue.)

**Interviewer:** Go to Question 19.

6. “What is your [son’s] diagnosis and scheduled treatment?”

**Diagnosis:** __________________________________________

**Treatment:**
- [ ] Chemotherapy ONLY
- [ ] Chemotherapy + radiation
- [ ] Radiation ONLY
- [ ] Surgery ONLY
- [ ] Surgery + chemotherapy
- [ ] Surgery + chemotherapy + radiation
- [ ] Bone marrow transplant
- [ ] Stem cell transplant
- [ ] Other (specify): ______________________________________

7. “Have you [Has your son] finished your [his] treatment yet?”

- [ ] Yes
- [ ] No

8. Have you [has your son] been diagnosed with any other disease or condition since you [he] stored tissue here?

- [ ] Yes
- [ ] No

**If no, Interviewer** go to question 10.

9. “What is your [son’s] diagnosis and scheduled treatment?”

**Diagnosis:** __________________________________________

**Dates of diagnosis and treatment:** __________________________________________

**Treatment:**
- [ ] Chemotherapy ONLY
- [ ] Chemotherapy + radiation
- [ ] Radiation ONLY
- [ ] Surgery ONLY
- [ ] Surgery + chemotherapy
- [ ] Surgery + chemotherapy + radiation
- [ ] Bone marrow transplant
10. “How would you describe your [son’s] current health?”
   - [ ] Excellent
   - [ ] Very good
   - [ ] Good
   - [ ] Fair
   - [ ] Poor

[ ] Stem cell transplant
[ ] Other (specify):

11. “Has your son started puberty?”
   - [ ] Yes
   - [ ] No
   - [ ] N/A

12. “Has your son’s pediatrician told you anything about his growth and development?”
   - [ ] Yes
   - [ ] No
   - [ ] N/A

   a. “If so, what were you told about your son’s growth and development?”

13. “Have you [Has your son] tried to get your [his] partner pregnant since treatment stopped?”
   - [ ] Yes
   - [ ] No
   - [ ] N/A

14. “Are you [Is your son] actively trying to get your [his] partner pregnant now?”
   - [ ] Yes
   - [ ] No
   - [ ] N/A

15. “Is your [Is your son’s] partner currently pregnant?”
   - [ ] Yes
   - [ ] No
   - [ ] N/A

16. “Has your [Has your son’s] partner been pregnant since you [he] started treatment?”
   - [ ] Yes
   - [ ] No
   - [ ] N/A

17. “Do you [Does your son] anticipate using your [his] stored tissue in the future?”
   - [ ] Yes
   - [ ] No
   - [ ] N/A

   a. If NO, “why not?”

18. “Do you [Does your son] know how to use/access your [his] tissue?”
   - [ ] Yes
   - [ ] No
   - [ ] N/A
a. “If you [your son] wanted to access your [his] tissue, how would you [he] proceed?”

____________________________________________________________________
____________________________________________________________________
____________________________________________________________________
____________________________________________________________________

19. Your [your son’s] tissue was initially shipped to ReproTech, Ltd for long-term storage.
   a. “Is your [your son’s] tissue still stored at ReproTech?”
      □ Yes       □ No       □ N/A
   b. “How has your interaction been with ReproTech?”
      __________________________________________
      __________________________________________
      __________________________________________

20. “Although I cannot give you specific information on your [son’s] tissue, would you like to have information about the research?”
      □ Yes → Give website or email website to participant
      □ No

21. “Now that you’ve had some time to think about your decision, how are you feeling about the decision to store tissue?”

      __________________________________________
      __________________________________________
      __________________________________________

22. “What would you recommend to a friend who was diagnosed with cancer and concerned about preserving his fertility?”
      □ Store tissue
      □ Do not store tissue
      □ Don’t know

23. “Is there anything else I can help you with?”

      __________________________________________
      __________________________________________
      __________________________________________

**Interviewer:** Thank you for completing this survey. I appreciate you taking the time to answer my questions. I would like to contact you in one year, and annually after that, to repeat this survey. Is that acceptable to you?
      □ Yes       □ No
Appendix 7: American Society for Clinical Oncology recommendations for fertility preservation in cancer patients

Appendix 8: American Society for Reproductive Medicine Ethics Committee Position
Appendix 9: Summed Alkylating Agent dose score (Green et al., 2009)

<table>
<thead>
<tr>
<th>Alkylating Agent</th>
<th>Cumulative Dose by Tertile</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>First</td>
</tr>
<tr>
<td>BCNU, mg/m²</td>
<td>1300</td>
</tr>
<tr>
<td>Busulphan, mg/m²</td>
<td>1317</td>
</tr>
<tr>
<td>CNU, mg/m²</td>
<td>1381</td>
</tr>
<tr>
<td>Chlorambucil, mg/m²</td>
<td>1165</td>
</tr>
<tr>
<td>Pirenperone cyclophosphamide, mg/m²</td>
<td>13,704</td>
</tr>
<tr>
<td>Oral cyclophosphamide, mg/m²</td>
<td>14,722</td>
</tr>
<tr>
<td>Ifosfamide, mg/m²</td>
<td>116,771</td>
</tr>
<tr>
<td>Melphalan, mg/m²</td>
<td>139</td>
</tr>
<tr>
<td>Nitrogen mustard, mg/m²</td>
<td>144</td>
</tr>
<tr>
<td>Procarbazine, mg/m²</td>
<td>14,200</td>
</tr>
<tr>
<td>Intrathecal thiopeta, mg</td>
<td>180</td>
</tr>
<tr>
<td>Thiopeta, mg/m²</td>
<td>177</td>
</tr>
</tbody>
</table>

**NOTE.** First tertile score is 1; second is 2; and third is 3. Abbreviations: BCNU, carmustine; CNU, lomustine.
Appendix 10: Cyclophosphamide equivalent dose calculation (Green et al., 2014)

CED (mg/m²) = 1.0 (cumulative cyclophosphamide dose (mg/m²))
    + 0.244 (cumulative ifosfamide dose (mg/m²))
    + 0.857 (cumulative procarbazine dose (mg/m²))
    + 14.286 (cumulative chlorambucil dose (mg/m²))
    + 15.0 (cumulative BCNU dose (mg/m²))
    + 16.0 (cumulative CCNU dose (mg/m²))
    + 40 (cumulative melphalan dose (mg/m²))
    + 50 (cumulative Thio-TEPA dose (mg/m²))
    + 100 (cumulative nitrogen mustard dose (mg/m²))
    + 8.823 (cumulative busulfan dose (mg/m²)).
Appendix 11. Estimated Costs associated with Testicular Tissue Cryopreservation (per patient)

Consultation with surgeon (CPT code 99241-45: range $100-$800)
Outpatient Orchiectomy/Wedge Resection (OR use, surgeon and anesthesiologist): $18,000

    Surgeon’s fee: $3,000 (CPT code 54520 for orchiectomy or 54522 for partial orchiectomy)
    Anesthesia fee: $2,000 (CPT code 00928).
    Hospital fee (OR time, equipment fees): $13,000

Infectious Disease testing: $240
Shipping of tissue to Reprotech: $215
Annual storage: $275; Discount based on financial need to $75/year.

Costs may change based on changes on the Charge Master.