VALIDATION OF THE SELF-BAT (SELF-ADMINISTERED BLEEDING ASSESSMENT TOOL) AND EVALUATION OF DETERMINANTS OF HEALTH IN HEMOPHILIA CARRIER

by

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Abstract

Hemophilia A and B are X-linked deficiencies of coagulation factor VIII and IX respectively. Males are affected, while females are carriers of the disease. Recently, it has become clear that more than a third of hemophilia carriers (HC) are symptomatic. Carriers may experience prolonged bleeding postpartum, following trauma and surgery as well as menorrhagia. This is the first research to incorporate both a bleeding assessment tool, to assist in objectively quantifying severity of bleeding symptoms, as well as a validated quality of life (QoL) questionnaire to facilitate an understanding of the relationship between bleeding symptoms and QoL.

The objectives for this study are to validate a self-administered bleeding assessment tool (Self-BAT) as a screening tool to accurately identify HC with low factor levels (FL) and abnormal bleeding symptoms, to measure QoL of HC using the SF-36v2 and describe its relationship to bleeding severity and FL, and to investigate variability in FL.

To date, 59 HC have been enrolled. A positive or abnormal Self-BAT bleeding score (≥6) has a sensitivity of 83%, specificity of 42%, a positive predictive value (PPV) of 0.48 and a negative predictive value (NPV) of 0.79 for the identification of HC with low FL. Analysis of QoL data shows an inverse correlation between QoL and BS (r=-0.52, p<0.0001). No significant relationship is observed between QoL and FL. This study found skewed X-inactivation ratio (XIR) to be significantly more prevalent in this population than has been observed in the general population. This suggests that determination of XIR may be useful in predicting risk of deficient FL.
This study validates the Self-BAT as an effective tool to identify HC with low FL and/or abnormal bleeding symptoms. For HC, Self-BAT BS is a more accurate measure of bleeding severity and resultant QoL than FL. HC will benefit from treatment strategies aimed at controlling bleeding symptoms which will facilitate improvements in the management of these patients overall.
Co-Authorship

Julie Grabell assisted in recruiting subjects and organizing visits to Kingston General Hospital in addition to accessing data from patient files.

Angie Tuttle assisted in the FVIII, FIX and VWF analyses.

Wilma Hopman assisted with statistical analyses and sample size calculations.

Dr. David Good assisted in the editing at various stages of the project.

Dr. Natalia Rydz was responsible for all subject recruitment and data collection in Calgary, AB, Canada.

Dr. Johnny Mahlangu was responsible for all subject recruitment and data collection in Johannesburg, South Africa.

Dr. Paula James oversaw the study, wrote the grant to obtain operating funds and assisted in the design of the study, REB application and focus group. In addition, Dr. James assisted in the writing and editing of this thesis.

All other work is my own.
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<td>BAT</td>
<td>bleeding assessment tool</td>
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<tr>
<td>BS</td>
<td>bleeding score</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic</td>
</tr>
<tr>
<td>HC</td>
<td>hemophilia carrier</td>
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<tr>
<td>FVIII</td>
<td>Factor VIII</td>
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<td>FIX</td>
<td>Factor IX</td>
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<tr>
<td>FL</td>
<td>Factor Level</td>
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<tr>
<td>HRQoL</td>
<td>Health Related Quality of Life</td>
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<tr>
<td>ISTH</td>
<td>International Society on Thrombosis and Haemostasis</td>
</tr>
<tr>
<td>KGH</td>
<td>Kingston General Hospital</td>
</tr>
<tr>
<td>PTT</td>
<td>Partial Thromboplastin Time</td>
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<td>QoL</td>
<td>Quality of Life</td>
</tr>
<tr>
<td>RCo:Ag</td>
<td>VWF ristocetin cofactor activity: VWF antigen ratio</td>
</tr>
<tr>
<td>SPSS</td>
<td>Statistical Product and Service Solutions</td>
</tr>
<tr>
<td>VWD</td>
<td>von Willebrand disease</td>
</tr>
<tr>
<td>VWF</td>
<td>von Willebrand factor</td>
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<tr>
<td>VWF:Ag</td>
<td>von Willebrand factor antigen</td>
</tr>
<tr>
<td>VWF:RCo</td>
<td>von Willebrand factor ristocetin cofactor activity</td>
</tr>
<tr>
<td>XCI</td>
<td>X-chromosome inactivation</td>
</tr>
<tr>
<td>XIC</td>
<td>X-inactivation centre</td>
</tr>
<tr>
<td>Xist</td>
<td>X-inactivation specific transcript</td>
</tr>
<tr>
<td>XIR</td>
<td>X-inactivation ratio</td>
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Chapter 1
Introduction

1.1 Hemophilia

Hemophilia is an X-linked recessive bleeding disorder caused by a deficiency in coagulation factor VIII (hemophilia A) or factor IX (hemophilia B). Factors VIII and IX are encoded by the $F8$ and $F9$ genes on the X-chromosome. A mutation in the gene can lead to the absence of the protein, or production of an inadequately functioning protein $^1$. Due to the inheritance pattern, mostly males are affected. Hemophilia A affects approximately 1 in 5,000 males while hemophilia B affects 1 in 20,000 males $^{2,3}$. This discrepancy in prevalence is attributed to location and length of the genes on the X chromosome. The $F8$ gene is located very near the end of the long arm of the X chromosome and shares a region of homology with an extragenic area positioned closer to the telomere $^4$. This can result in an inversion mutation at intron 1 or 22 which are responsible for about half of all cases of hemophilia A $^4$. In addition, $F8$ includes 186 kb and 26 exons whereas $F9$ has only 34 kb and 8 exons $^5$. The increased size of $F8$ means increased chance of spontaneous mutation. Factors VIII and IX are produced in endothelial cells and hepatocytes respectively, and circulate in the bloodstream in an inactive form. Factor VIII is carried by von Willebrand factor (VWF) until it is activated in response to injury and separates from VWF to interact with factor IX. Factor VIII and IX are involved in the intrinsic pathway of the coagulation cascade (Fig.1).
When a blood vessel is injured, sub-endothelial tissue factor and collagen are exposed and bind factor XII which then activates factor XI and pre-kallikrein. These proteins are anchored to the sub-endothelium. Activated factor XI then activates factor IX in the presence of calcium. Activated factor IX then proceeds to form the tenase complex with activated factor VIII and calcium. The tenase complex binds to the phospholipid rich surface of platelets where it activates factor X. This process comprises the intrinsic pathway of the coagulation cascade.

The common pathway involves activated factor X complexing with its cofactor, factor V in the presence of calcium. This complex then converts prothrombin (II) to thrombin (IIa).

This pathway is the primary means of thrombin generation, which leads to the conversion of fibrinogen to fibrin, and formation of a thrombus. Continued activation of FVIII and FIX by
thrombin maintains the system in a prothrombotic state. Another model of hemostasis is the cell-based model of coagulation in which coagulation is regulated by properties of cell surfaces, emphasizing the role of specific receptors for coagulation proteins. This model theorizes that, rather than a cascade, the process occurs in three overlapping phases which occur on different cell surfaces: initiation on the tissue factor-bearing cell, (ex. Fibroblast); amplification on the platelet as it becomes activated; and propagation on the activated platelet surface \(^7\). (Fig. 2)

Deficiency of either FVIII or IX can lead to excessive bleeding \(^8\). The normal range for FVIII/FIX in peripheral blood is 0.50 to 1.50 IU/mL \(^8\). Due to numerous different mutations, which can cause hemophilia, patients with hemophilia can have varying factor levels. Individuals with less than 0.01 IU/mL are classified as having severe hemophilia, those with 0.01-0.05 IU/mL have moderate hemophilia, and those with mild hemophilia have between 0.05-0.50IU/mL \(^9\).
The mainstay of treatment is clotting factor replacement therapy, which is the infusion of FVIII or FIX derived from either human or recombinant plasma\textsuperscript{10}. Depending on the severity of the condition, replacement therapy may be carried out on an as-needed basis (on demand) or on a regular basis to prevent bleeding episodes (prophylactic)\textsuperscript{10}. Coagulation factor concentrates are administered intravenously and the infusions can be done at home with proper instruction and training. Mild cases of hemophilia A can be treated with desmopressin (DDAVP) which stimulates the release of endogenous VWF from endothelial cells and can be given intravenously or as a nasal spray\textsuperscript{11}.

Figure 1-2 Cell-Based Model of Hemostasis (Adapted from Hoffman et al. 7)
1.2 Hemophilia Carriers

Due to the X-linked recessive inheritance pattern of hemophilia, mostly men are affected while females may be heterozygous for the mutation, referred to as carriers of hemophilia. Female carriers of hemophilia (HC) have one unaffected allele, so it might be expected that they have a plasma concentration of factor VIII or IX corresponding to at least 50% of the concentration found in healthy individuals. However, approximately one third of HC manifest low FVIII or FIX levels and can have abnormal bleeding symptoms. Abnormal bleeding symptoms have even been reported at levels as high as 0.60 IU/mL. Carriers may experience prolonged bleeding postpartum, following trauma and surgery as well as menorrhagia. Historically, HC were widely considered to be asymptomatic and their bleeding symptoms were assumed to be ‘normal’. Since the development of bleeding assessment questionnaires that can objectively measure these symptoms, health care professionals have been able to quantify ‘normal bleeding’ and therefore, identify HC with symptoms that are excessive or abnormal. Once a HC has been identified, either by appropriate family history or gene sequencing, clotting factor activity can be measured. Carriers can have FL ranging from <1% to >250% and individual treatment will be prescribed based on degree of deficiency as well as type and severity of bleeding symptoms. Treatment may include factor replacement therapy (for HC with FL <5%), tranexamic acid, desmopressin, hormone therapy to minimize menorrhagia, and iron replacement therapy for anemia. Although there is a relationship between factor
level (FL) and bleeding symptoms, there is a high degree of variation in FL among HC and it is not always representative of their bleeding symptoms. More recently, we understand that HC can experience pathological bleeding with FL well within the normal range. Therefore, this is a population of individuals who are at risk of going unrecognized or improperly managed by the health care system.

1.3 Bleeding Assessment Tools (BATS)

It is important to assess the risk of bleeding in carriers in order to enable physicians to plan individual carrier management. Family history, clotting factor levels, and FVIII or FIX genotype are useful in the recognition and diagnosis of carriers. However, these methods have well-recognized pitfalls which include their poor availability/accuracy, costliness, laboriousness and limited accessibility. There is therefore a distinct need for alternative methods of evaluation of HC. Currently, there is no standardized tool for the assessment of bleeding symptoms in HC. Since 2005, many Bleeding Assessment Tools (BATs) have been developed, studied and validated primarily in the setting of von Willebrand disease (VWD) in an attempt to objectively quantify bleeding symptoms and as a result evaluate abnormal bleeding. In order to consolidate the knowledge obtained from these studies and to develop a consensus BAT, a Working Party sponsored by the VWF and Perinatal/Pediatric Hemostasis Subcommittees of the ISTH SSC (International Society on Thrombosis and Haemostasis Scientific and Standardization Committee) was established in 2008. This group, with input from the Women’s Health Issues in Thrombosis and Haemostasis SSC, published the ISTH-BAT in 2010.
The ISTH-BAT is an expert administered questionnaire, which uses a 0 to 4 scoring system for each bleeding symptom. The overall bleeding score is summative, and scores of $\geq 4$ for men and $\geq 6$, for women, are considered positive or abnormal $^{32}$. The questionnaire covers both mucocutaneous and musculoskeletal bleeding. The intention of the working party was for the ISTH-BAT to become the universal tool by creating a “common language” for the reporting of bleeding in all inherited bleeding disorders. Although the ISTH-BAT is currently being validated in HC, we have chosen it as the expert administered BAT for this study because of its endorsement by the International Society on Thrombosis and Haemostasis and its anticipated widespread use.

The Self-BAT is a self-administered bleeding questionnaire that was generated by converting the ISTH-BAT into lay language, to ensure all adults could read it $^{33}$. Healthy adult controls and adults affected with Type 1 VWD were then recruited to participate in a study ensuring optimization, and measuring validity and reliability of the Self-BAT in comparison to the ISTH-BAT. Intraclass Correlation Coefficient (ICC) was used to determine the correlation between scores on the Self-BAT and ISTH-BAT. It was found that the scores were highly correlated with an intraclass correlation coefficient of 0.87. Test re-test reliability of the Self-BAT was also measured with the group of participants and found to be highly consistent with an ICC of 0.95. When the normal range was measured for this tool, it was found to be $<6$ for women, and $<4$ for men, consistent with the ISTH-BAT. In the female VWD population, it had a sensitivity of 100%, specificity of 21%, PPV of 0.17 and NPV of 1.0 $^{33}$. This research indicates that the Self-BAT is a promising screening tool for mild inherited bleeding disorders and warrants testing in the HC population.
1.4 Quality of Life (QoL)

Health-related quality of life (HRQoL) assessment is recognized as an important outcome in the evaluation of different therapeutic regimens for individuals with hemophilia\(^\text{10}\). Heterogeneity in the clinical phenotype of hemophilia, including severity of bleeding symptoms, indicates that a 'one-size-fits-all' approach is unlikely to achieve optimal therapy.

This applies to carriers of hemophilia as well, who are at an increased risk for complications due to menstruation, pregnancy, and childbirth. Such complications may include anemia, post-partum hemorrhage and those concerned with hysterectomy and other uterine surgeries that carry inherent risk\(^\text{17,20,25,26,34}\). In addition, their QoL may be affected by limited participation in education, employment and social activities. Studies have shown that complications can be minimized or prevented and QoL can be improved by early diagnosis and appropriate management\(^\text{25,26,35}\). Therefore, it would be informative to measure QoL in HC using a validated questionnaire to compare to normative Canadian values. This would assist in guiding treatment regime and measuring its effectiveness.

An available tool for QoL assessment is the SF-36v2\(^\text{36}\). The SF-36v2 is a multi-purpose, short-form health survey consisting of 36 questions. It yields an 8-scale profile of functional health and well-being scores as well as psychometrically-based physical and mental health summary measures. It is a generic measure, as opposed to one that targets a specific age, disease, or treatment group. The SF-36v2 has proven useful in surveys of general and specific populations, comparing the relative burden of diseases, and in
differentiating the health benefits produced by a wide range of different treatments \textsuperscript{37-42}. Although there are disease specific QoL tools that have been developed for hemophilia \textsuperscript{43}, understandably they focus primarily on the symptoms of importance to men, such as joint and muscle bleeds and not enough on symptoms that are relevant for HC including menorrhagia and post-partum hemorrhage.

1.5 Variation in Factor Levels

Coagulation factor levels range between 0.5 and 1.5 IU/mL in the normal population, and are even more variable in the HC population, ranging from <0.01 to >2.50 IU/mL \textsuperscript{25}. The literature suggests this could be due to a number of influences such as lyonization (skewed X-chromosome inactivation), ABO blood type, type of mutation, age, VWF levels, and even time of day (diurnal variation) \textsuperscript{12,17,20,21,25,44-47}. It is critical to understand the mechanisms driving this variation and the degree to which they contribute to resulting bleeding symptoms in order to predict and prevent excessive blood loss.

Skewed X-chromosome inactivation (XCI) has long been considered the primary cause of low factor levels among HC \textsuperscript{12,16,17,20,44,46,48}. Mammalian females have two X-chromosomes in each cell, whereas males have only one. In the female, early in development, when approximately 8-16 progenitor cells are present, each cell inactivates one of the X-chromosomes \textsuperscript{49}. This process is initiated by the X-inactivation centre (XIC), which includes the gene for a long non-coding RNA known as X-inactive-specific-transcript (Xist) which is expressed by the inactive chromosome. The Xist transcript coats the future inactive X-chromosome and triggers gene silencing. This is thought to be a
random process, and each daughter cell in the lineage will have the same X-chromosome (either paternal or maternal) inactivated. If this inactivation process were skewed to favour the X-chromosome carrying the FVIII or FIX mutation, it would result in lower factor levels. Although a number of studies have shown an association between X-inactivation ratio (XIR) and coagulation factor level, others have not\textsuperscript{21,50}. Some carriers with levels outside of the normal range have a non-skewed XIR, and some with skewed XIR have normal levels. Further investigation is warranted. The Xist transcript also induces a cascade of changes including DNA methylation. It has been shown that DNA methylation decreases with age and may lead to an increasingly skewed XIR especially in women over the age of \textsuperscript{55}\textsuperscript{51,52}. Theoretically, this could result in either increasing or decreasing factor levels. If the chromosome carrying the wild-type allele became increasingly methylated, it would result in a lower FL. Alternatively, if the mutated allele was increasingly methylated, a higher FL may result. However clinically, carrier levels tend only to increase with age\textsuperscript{53,54}.

There are hundreds of mutations on the \textit{F8} and \textit{F9} genes that cause hemophilia\textsuperscript{5}. Some mutations, for example intron 22 inversion, are associated with lower FL and a more severe bleeding phenotype than others. It is reasonable to assume that bleeding in carriers would also be influenced by mutation type and by extension, related to severity of bleeding in affected male relatives. However, multiple studies suggest that there is no significant relationship between mutation type, FL, and severity of hemophilia within the family\textsuperscript{12,13}.

VWF and ABO blood type have been shown to affect the level of FVIII in plasma, but not specifically in the carrier population. VWF is a large multimeric glycoprotein that is
found in blood plasma and is produced constitutively by endothelium (stored in the Weibel-Palade bodies) and megakaryocytes (stored in α-granules of platelets). VWF carries inactive FVIII in the circulation. When FVIII is activated by thrombin, it is released from VWF. When FVIII is not complexed with VWF it is quickly degraded and cleared from plasma. VWF and FVIII normally exist in a 1:1 ratio in peripheral blood. VWF levels are significantly lower among individuals with blood type O, and 30% of variation in VWF levels is attributable to the effect of the ABO blood group. Blood type may be acting indirectly via VWF to alter FVIII concentration in plasma. This is a key variable to consider in the prediction of bleeding risk in HC.

1.6 Conclusion

A self-administered screening tool with a high degree of sensitivity for the identification of HC with low coagulation FL could facilitate the appropriate management of the individual by their health care provider resulting in a higher quality of care.

Male hemophiliacs have been reported to have a decreased QoL, and menorrhagia has been associated with a decreased QoL. It is reasonable to assume that HC also suffer a decreased QoL, although this has only been evaluated in a few studies and none have included quantitative assessment of bleeding. It is important to investigate the QoL of carriers compared to normative values as an important measure of the impact of carrier status on both physical and mental health. Pre- and post- treatment QoL scores could help guide individual treatment plans in addition to providing focus for innovative treatments.
Although the association between coagulation FL and bleeding severity has been documented, the cause of variability in FVIII/FIX levels in plasma has not been thoroughly elucidated. The most popular theories involve skewed X-inactivation, mutation type, age, VWF levels, and ABO blood type. An understanding of these influences will provide a more comprehensive view of HC health and well-being allowing health care professionals to better understand and treat those suffering abnormal bleeding symptoms.

1.7 Objectives

**Overall Study Aim:** To investigate clinical tools to improve the diagnosis and management of HC.

**Objective 1: Validation of Self-BAT**

1.1 To evaluate agreement between Self-BAT and ISTH-BAT bleeding scores when these tools are used in HC.

1.2 To assess the diagnostic accuracy of the Self-BAT as a screening tool to distinguish HC with low coagulation factor levels from those with normal coagulation factor levels.

1.3 To verify reliability of the Self-BAT (test-retest).

**Hypothesis:** The Self-BAT will prove to be a reliable tool, its bleeding
scores will be in agreement with the ISTH-BAT bleeding scores and it will be shown to be a sensitive tool to distinguish HC with low coagulation factor levels from those with normal levels.

**Objective 2: Assessment of QoL**

2.1 To assess QoL in HC.

2.2 To correlate the QoL to FVIII/IX levels and Self-BAT bleeding scores.

**Hypothesis:** The QoL of HC will be decreased compared to the general population and will inversely correlate with bleeding severity.

**Objective 3: Investigation of physiologic determinants of factor levels**

3.1 To measure and assess the relative contributions of age, mutation, ABO blood type and X-chromosome inactivation ratio (XIR) on resulting coagulation factor levels.

**Hypothesis:** Skewed XIR favouring the mutant allele as well as mutations associated with a more severe phenotype among hemophiliacs will contribute to lower factor levels among carriers. For hemophilia A carriers, blood type O will be associated with lower levels of FVIII, and age will be positively correlated to plasma levels of FVIII.
Chapter 2

Methods

2.1 Subject Recruitment

2.1.1 Participants

Hemophilia A or B Carriers were defined by known FVIII or FIX mutation, or appropriate family history (the daughter of a man with hemophilia or the mother of two sons with hemophilia or the mother of one son with hemophilia plus one other affected male relative).

Subjects were enrolled whether they exhibited bleeding symptoms or not. All study participants gave informed consent and study approval was obtained from the Research Ethics Board of Queen’s University, Kingston, Canada and from each of the participating institutions.

2.1.2 Carrier Recruitment

On June 25th, 2013 a dinner and information session was held at the Holiday Inn in Kingston, Ontario. All known HC in the region were invited to attend along with their family members. At the event HCs were asked to participate in this study. Remaining participants were HC who were recruited from Dr. Paula James’ bleeding disorders clinic at Kingston General Hospital and Hotel Dieu Hospital and were approached and invited to participate in the study during clinic. In addition, mothers of hemophiliacs who visited Dr. Mariana Silva’s pediatric clinic were invited to participate. Two other sites also recruited subjects - in Calgary
and Johannesburg, HC who attended Dr. Natalia Rydz’s, or Dr. Johnny Mahlangu’s clinic respectively, were invited to participate in the study.

2.3.1 Inclusion Criteria

Participants were included in the study if they were: HC known by either appropriate family history or mutation analysis, willing and able to give informed consent, 18 years or older, willing and able to complete the Self-BAT independently and the ISTH-BAT with assistance. Participants were excluded if they were diagnosed with non-hemophilia bleeding disorders with hepatic or renal disease, or thrombocytopenia. Participants were also excluded if they were on medications that might contribute to bleeding, were unable to understand the Self-BAT, or were not able to give informed consent.

2.2 Data Collection

All enrolled HC were asked to complete either the ISTH-BAT or Self-BAT, given in random order, at least 2 weeks apart. The order was determined by an online random number generator. The ISTH-BAT was administered by either Julie Grabell or myself. Both questionnaires were scored according to the ISTH-BAT 0 to +4 scoring key. A subset of consecutively consenting participants (n=24) completed the Self-BAT a second time at least 2 weeks after the first, allowing for calculation of test re-test reliability. The QoL questionnaire, SF-36v2 was administered to evaluate QoL of HC compared to normative Canadian values. Since there are additional concerns for carriers of an X-linked disease including feelings of guilt and responsibility that are associated with having a hemophiliac
child, nine additional questions specifically designed to target carriers who are mothers of hemophiliacs, were added to the SF-36v2 using the same format (Appendix E).

2.3 Laboratory Analysis

Four tubes of whole blood were collected from each subject on the day of study enrolment, of these, two were collected in 3.2% sodium citrate (at a ratio of 9:1) and two in EDTA (ethylenediaminetetraacetic acid). The blood stored in sodium citrate was separated into platelet-poor plasma (methods below, section 2.1.6). With the plasma, FVIII/FIX, VWF:Ag, and VWF:RCo activity levels of each subject were measured (methods below, section 2.1.7-9). From the blood stored in EDTA, DNA was extracted for F8 or F9 gene amplification and sequencing, ABO blood group, and X-inactivation analysis (methods below, section 2.1.10-13). No central lab was used, all assays were performed as described, in the lab where the samples were drawn.

2.3.1 Separation of Platelet-Poor Plasma

Whole blood collected in sodium citrate was spun in the centrifuge (Beckman) for 10 minutes at 2200g in order to separate the plasma from the red blood cells. The plasma was then pipetted into 2x 500μl aliquots into 1.5mL tubes and frozen at -80°C.
2.3.2 FVIII/IX Analysis

Normal and abnormal control FVIII/IX (Precision Biologic) along with the STAGO reagents were used to create a standard curve by performing serial dilutions (1/5, 1/10, 1/20, 1/40, 1/80, 1/200, 1/400) of the standard reference (i.e. normal) plasma and performing APTTs. The clotting times for the APTT were then plotted against dilution and a best fit line drawn through them (dilutions were plotted on the logarithmic X-axis and clotting times on the linear Y axis). That line was then used to determine the dilution of normal plasma required to produce any given APTT. Hence, a reference range was established (1/10 =100%, 1/20 = 50%, etc). Thawed subject plasma samples were then loaded in the STAGO Compact machine to obtain a FVIII/IX level by measuring the ability of subject FVIII/IX to correct the PTT of FVIII/IX-deficient plasma 62.

2.3.3 VWF Antigen (VWF:Ag) ELISA

VWF:Ag levels were measured by an in-house ELISA performed by coating an Immulon 4 HBX plate (Dynex) with 100µl of Rabbit Anti-Human VWF (DAKO), diluted to 10µg/ml. The plate was incubated at 4°C overnight. Plasma samples were diluted with wash buffer (10 mM sodium phosphate, 145 mM NaCl, pH 7.2) using titer tubes (Biorad). A standard curve was prepared using reference plasma with dilutions beginning at 1:20. The plate was washed with 200µl wash buffer (PBS, 10 mM NaH2PO4, 500 mM NaCl, and 1% Tween 20, pH 7.2) 3 times using the plate washer before 100µl of sample was added to each well. The plate was incubated at room temperature for 2 hours and then washed as above.
Detecting antibody (Rabbit Anti-Human VWF HRP, DAKO) was added to the plate at a concentration of 1.1µg/µl before a 1-hour incubation. The plate was washed as above and then the colour reagent (15 mL 0.1M Citric Acid-Phosphate Buffer, 2x 5 mg of dissolved o-phenylenediamine (OPD) and 6.2 µl of 30% peroxide) was added to the plate until the standard curve was apparent. The reaction was stopped with 1M H2SO4 and the results were read at 492 nm on the plate reader (VERSAmax). Protocol based on manufacturer’s recommendations.

2.3.4 VWF Ristocetin Cofactor (VWF:RCo) Assay

The VWF:RCo assay is used to measure the ability of VWF to induce platelet aggregation. The aggregometer (Chrono-log) was first calibrated with standard curves using both normal and abnormal human plasma (quick-thawed, Precision Biologic). Lyophilized platelets (Helena Laboratories) were reconstituted with TRIS-buffered saline and was added to cuvettes with a stir bar, placed into the aggregometer and allowed to incubate for 4 minutes. Once the incubation was over, 50µL [10mg/mL] ristocetin (American Biochemical and Pharmaceutical Corporation) was added to each cuvette for two minutes before 50µL thawed plasma sample was added. After the addition of the plasma, the aggregometer measured the ability of the subject VWF to induce platelet aggregation using measurements of light transmission.

2.3.5 DNA Extraction

To isolate the patient DNA from peripheral blood collected in EDTA, the salt
extraction method was used. Briefly, this involved salting out the cellular proteins by dehydration and precipitation with a saturated sodium chloride solution. The precipitated DNA strands were stored in a microcentrifuge tube containing 100µL TE Buffer at -20°C.

2.3.6 F8/F9 Gene Sequencing

All exons were amplified by PCR with forward and reverse primers as per protocol by DK Lahiri et al. See primer sequences and conditions (Appendix H). Amplified products were sent for Sanger DNA sequencing by McGill University and Genome Quebec (http://gqinnovationcenter.com).

2.3.7 ABO Blood Group Analysis

ABO blood groups were determined using the mutagenically separated polymerase chain reaction (PCR). Two sets of PCR reactions were performed, each amplifying nucleotides at position 261 and 703 from cDNA at the ABO locus (chromosome 9). Two forward primers and one reverse primer of different lengths were designed specifically for each reaction (Figure 2-1), producing a 216 bp, 195 bp, 126 bp, and 106 bp fragment. PCR products were obtained using 10 ng of DNA in a 50 µl reaction mixture and electrophoresed on a 3% agarose gel. The 216 bp fragment of the PCR products for the 261th nucleotide was A or B allele-specific and the 195 bp fragment was O allele-specific. The 126 bp fragment of the PCR products for the 703th nucleotide was B allele-specific and the 106 bp fragment was A or O allele-specific. The ABO
genotypes were determined by the intersection of the predicted alleles from these two PCR reactions. Figure 2-2 shows bands representing the various fragments.

<table>
<thead>
<tr>
<th>Sequences of primers used in MS-PCR for ABO genotyping.</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR reaction</td>
<td>Allele specificity</td>
</tr>
<tr>
<td>MS-PCR I</td>
<td>A/B</td>
</tr>
<tr>
<td>for the 26th nucleotide</td>
<td>O</td>
</tr>
<tr>
<td></td>
<td>261C</td>
</tr>
<tr>
<td>MS-PCR II</td>
<td>B</td>
</tr>
<tr>
<td>for the 70th nucleotide</td>
<td>A/O</td>
</tr>
<tr>
<td></td>
<td>701C</td>
</tr>
</tbody>
</table>

Primer 261C and 701C are common primers used to pair with the allele-specific primers. Mutagenic bases are those underlined.

**Figure 2-1 Primer Sequences for ABO Genotyping (Lee et al. 66)**

![Figure 2-1 Primer Sequences for ABO Genotyping](image)

**Figure 2-2 ABO Bands (Lee et al. 66)**

![Figure 2-2 ABO Bands](image)

### 2.3.8 X-Inactivation Ratio (XIR) Analysis

The X-chromosome inactivation (XCI) status was established by analysis of DNA methylation at the human androgen receptor (HUMARA) locus 67. As per the protocol provided by The Hospital for Sick Children, Toronto, Canada, (see appendix F), two aliquots of DNA from each sample were used: the first was directly amplified by PCR, and the second was digested with three methylation sensitive restriction enzymes (BamHI, HapII, and HhaI),
before amplification. DNA was then purified using Agencout AMPure XP beads and an Agencourt SPRIPlate 96 Super Plate to separate beads from solution. Digested and undigested DNA samples were then amplified using PCR protocol (see Appendix F). Figure 5 shows the X-inactivation assay.

Figure 2-3 X-Inactivation Assay

2.4 Statistical Analysis

Descriptive statistics of the study participants were tabulated and presented using means, medians and ranges for continuous values and percentages for discrete variables. The Self-BAT bleeding score was calculated according to the same 0 to +4 scoring system as is used for the ISTH-BAT. Sensitivity, specificity, positive and negative predictive values were calculated based on the number of subjects with positive vs. negative bleeding scores and those with normal vs. low coagulation factor levels. Positive likelihood ratios were also
calculated across the range of bleeding scores, and a receiver operator curve (ROC) curve was generated.

Correlation analysis (Pearson r or Spearman rho) was performed to identify relationships between; coagulation factor level and bleeding score, mutation and bleeding score, blood type and bleeding score, and XIR and factor level. Correlation analysis (Spearman rho) was also performed in order to calculate test-retest reliability as well as inter-test reliability using SPSS.

2.5 Sample Size

A target sample size of n=137 HC was calculated based on recommendations for diagnostic studies. The following criteria were included in the calculation: based on previous publications, the expected prevalence of symptomatic carriers is 30% in the overall group, and the expected sensitivity of the Self-BAT is 95% (with a lower confidence limit of 87%) \(^6^9\). To address Specific Objective 1.3, a subset of 24 from the original 137 (approximately 10 from each center) participated in test-retest.
Chapter 3

Results

3.1 Participant Data

In total, there were fifty-nine participants. Tables 3.1 and 3.2 include information on all carriers enrolled in the study presently from the three clinics, (Kingston n=19, Calgary n=25, Johannesburg n= 15)

<table>
<thead>
<tr>
<th></th>
<th>Hemophilia A n=50</th>
<th>Hemophilia B n=9</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (range)</td>
<td>39 (21-65)</td>
<td>46 (27-57)</td>
</tr>
<tr>
<td><strong>Factor Level (IU/mL)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (range)</td>
<td>FVIII 0.60 (0.12-1.25)</td>
<td>FIX 0.57 (0.12-0.92)</td>
</tr>
<tr>
<td><strong>Self-BAT BS</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (range)</td>
<td>9 (0-28)</td>
<td>4 (0-18)</td>
</tr>
<tr>
<td><strong>ISTH-BAT BS</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (range)</td>
<td>5 (2-11)</td>
<td>9 (2-12)</td>
</tr>
</tbody>
</table>

Table 3.1 Baseline Age, Factor Level, Self-BAT & ISTH-BAT Bleeding Scores for HC enrolled
<table>
<thead>
<tr>
<th></th>
<th>Kingston</th>
<th>Calgary</th>
<th>Johannesburg</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Median Age</strong></td>
<td>41</td>
<td>37</td>
<td>38</td>
<td>n.s</td>
</tr>
<tr>
<td><strong>Median Self-BAT BS</strong></td>
<td>10</td>
<td>8</td>
<td>7</td>
<td>n.s</td>
</tr>
<tr>
<td><strong>Median FL</strong></td>
<td>0.55</td>
<td>0.53</td>
<td>0.87*</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Table 3.2 Comparison of HC populations between each participating centre.

*FL from Johannesburg were significantly higher than both Kingston and Calgary

At the time of blood sampling a small number of hemophilia A carriers were pregnant or taking OCP or HRT (four in Kingston, four in Calgary, six in Johannesburg). Estrogen has been shown to influence VWF levels and potentially FVIII \(^{70,71}\). Therefore, a recent historical baseline level was used in place of the current value for these 14 participants.

### 3.2 Agreement Analysis

Preliminary analysis shows that the ISTH-BAT and Self-BAT bleeding scores are well correlated, with an Intraclass Correlation Coefficient of 0.80 (p<0.05) (Figure 3.1).
Figure 3-1 Bleeding scores using the ISTH-BAT and Self-BAT (n=55) with line of equality (dotted line). Overall ICC = 0.80.

3.3 Test re-test Reliability

Twenty-four subjects were asked to complete the Self-BAT a second time, at least 2 weeks after completing their first questionnaire. The test re-test reliability, calculated with the ICC found to be 0.95 (p<0.0001) (Figure 3.2).
Figure 3-2 Test re-test reliability (n=24). The ICC of the Self-BAT # 1 scores and Self-BAT # 2 scores was found to be 0.95.

3.4 Predictive Value

Among all participants, a positive or abnormal Self-BAT BS (≥ 6) has a sensitivity\(^1\) of 83%, specificity of 42%, a positive predictive value (PPV) of 0.48 and a negative predictive value (NPV) of 0.79 for the identification of those with low FVIII or FIX levels.
Self-BAT BS is inversely correlated with FL ($r=-0.37$, $p<0.0001$) (Figure 3.3).

![Figure 3-3 The Spearman Rho is -0.37, indicating a negative association between the Self-BAT bleeding score and coagulation factor level ($p<0.00001$). The yellow rectangle encompasses 20 HC who have positive BS and normal FL.](image)

3.5 Hemophilia Carrier Quality of Life

Fifty-four HCs have completed the SF-36v2+9 (45 Hemophilia A, 9 Hemophilia B) with mean age 41 years (range 21 – 64), mean FL 0.60 (0.12-1.25), median BS 9 (0-28). Preliminary analysis shows no significant differences between HC and Canadian normative values. However, three domains are approaching significance including Social Functioning ($p=0.079$), with HC scoring six points lower than normal, Mental
Health (p=0.076), HC score five points lower than normal, and Mental component Summary (p=0.060), HC score three points lower than normal.

There is no significant correlation between QoL and FL. However, a significant negative correlation is observed between QoL and Self-BAT BS in multiple domains including Physical Functioning (r=-0.38, p=0.005), Role Physical (r=-0.34, p=0.013), Bodily Pain (r=-0.43, p=0.001), General Health (r=-0.42, p=0.002), and Physical Component Summary (r=-0.52, p<0.0001). In summary, these data suggest that the QoL of HC is significantly associated with Self-BAT BS and corresponding severity of bleeding symptoms. Several QoL domain scores differed significantly between Canada (CA) and South Africa (SA). Median factor level was higher in SA (CA 0.54, SA 0.87, p<0.0001). Interestingly, there was no significant difference in bleeding scores between countries. Carriers in SA had decreased QoL scores for Social Functioning (p=0.014) and Mental Component Summary (p=0.018) compared to HC in CA. When assigning agreement to the following statements from the additional +9 category, HC in SA were significantly more likely to agree with the following 4 statements: “I feel I am responsible for my child’s bleeding disorder” (p=0.002), “I feel guilty about my child’s state of health” (p=0.001), “I sometimes feel my child’s medical condition is a burden” (p=0.018), “I wish there was a cure for my child’s bleeding disorder” (p=0.020).
3.6 Physiologic Determinants of FVIII and/or FIX

3.6.1 Age

There does not appear to be a relationship between age and FVIII. Among the 52 carriers analysed, the analysis revealed no association.

3.6.2 VWF

VWF antigen levels are significantly correlated with FVIII levels among HC (r=0.49, p<0.001). None of the carriers show expected ratios of 1:1 (VWF Ag:FVIII). The ratio ranged from 1.2 (VWF = 0.68 IU/mL, FVIII = 0.58 IU/mL), to 8.7 (VWF = 1.3 IU/mL, FVIII = 0.15 IU/mL). A significant correlation is seen between VWF Ag:FVIII ratio and both Self-BAT BS (r=-0.25, p<0.001), and % wild type active X (r=0.27, p<0.001).

3.6.3 ABO

For ABO blood typing, 13 hemophilia A carriers have been analysed, including 8 type O, 4 type A and 1 type B. No participants have blood type AB. The mean FVIII among carriers with type O was 0.45 IU/mL. For carriers with blood type A mean FVIII was 0.51 IU/mL and the carrier with blood type B had a FVIII of 0.15 IU/mL.

3.6.4 Mutation Analysis

Mutation analysis of the F8 or F9 genes of all carriers from Kingston and Calgary has been completed. Table 3.3 shows the identified mutations. Sequencing is not available to the HC in Johannesburg.

29
<table>
<thead>
<tr>
<th>Gene</th>
<th>Centre</th>
<th>Mutation</th>
<th>FVIII:VWF</th>
<th>Cases</th>
<th>Severity</th>
</tr>
</thead>
<tbody>
<tr>
<td>F8</td>
<td>Kingston</td>
<td>Thr154Ala-het (Exon 4)</td>
<td>0.79</td>
<td>n=1</td>
<td>mild</td>
</tr>
<tr>
<td>F8</td>
<td>Kingston</td>
<td>Val162Met-het (Exon 4)</td>
<td>0.25, 0.40</td>
<td>n=2</td>
<td>mild</td>
</tr>
<tr>
<td>F8</td>
<td>Kingston</td>
<td>Pro151Ser-het (Exon 4)</td>
<td>0.60</td>
<td>n=1</td>
<td>mild</td>
</tr>
<tr>
<td>F8</td>
<td>Calgary</td>
<td>Thr295Ala –het (Exon 7)</td>
<td>nd</td>
<td>n=2</td>
<td>mild</td>
</tr>
<tr>
<td>F8</td>
<td>Calgary</td>
<td>Glu272Lys-het (Exon 7)</td>
<td>nd</td>
<td>n=1</td>
<td>nd</td>
</tr>
<tr>
<td>F8</td>
<td>Kingston</td>
<td>Tyr346Cys-het (Exon 8)</td>
<td>0.57</td>
<td>n=1</td>
<td>mild</td>
</tr>
<tr>
<td>F8</td>
<td>Kingston</td>
<td>Arg372Cys-het (Exon 8)</td>
<td>0.25</td>
<td>n=1</td>
<td>severe</td>
</tr>
<tr>
<td>F8</td>
<td>Calgary</td>
<td>Asp392Gly –het (Exon 8)</td>
<td>nd</td>
<td>n=2</td>
<td>mild</td>
</tr>
<tr>
<td>F8</td>
<td>Calgary</td>
<td>Asn474Asp –het (Exon 10)</td>
<td>nd</td>
<td>n=1</td>
<td>severe</td>
</tr>
<tr>
<td>F8</td>
<td>Kingston</td>
<td>insertion of AAACC causing frameshift –het (Exon 14) (c357303577eup.pLeu1193GFnfxX27)</td>
<td>0.80, 0.73</td>
<td>n=2</td>
<td>severe</td>
</tr>
<tr>
<td>F8</td>
<td>Calgary</td>
<td>deletion at nt 3629-3637 (norm. repeat 9As) causing frameshift-het (Exon 14)</td>
<td>nd</td>
<td>n=1</td>
<td>severe</td>
</tr>
<tr>
<td>F8</td>
<td>Calgary</td>
<td>Asn1922Ser –het (Exon 18)</td>
<td>nd</td>
<td>n=1</td>
<td>severe</td>
</tr>
<tr>
<td>F8</td>
<td>Kingston</td>
<td>Val2005Glu-het (Exon 19)</td>
<td>0.47</td>
<td>n=1</td>
<td>unknown</td>
</tr>
<tr>
<td>F8</td>
<td>Kingston</td>
<td>Ser2011Asn –het (Exon 19)</td>
<td>0.51</td>
<td>n=2</td>
<td>mild</td>
</tr>
<tr>
<td>F8</td>
<td>Kingston, Calgary</td>
<td>Intron 22 inversion</td>
<td>0.42, 0.71</td>
<td>n=4</td>
<td>severe</td>
</tr>
<tr>
<td>F8</td>
<td>Kingston</td>
<td>Arg2159Cys-het (Exon 23)</td>
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<td>n=1</td>
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<tr>
<td>F8</td>
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<td>Arg2307Gln –het (Exon 26)</td>
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<tr>
<td>F8</td>
<td>Kingston</td>
<td>No Mutations Found (2N-)</td>
<td>0.35</td>
<td>n=1</td>
<td>nd</td>
</tr>
<tr>
<td>F9</td>
<td>Kingston</td>
<td>Cys350Tyr-het (Exon H)</td>
<td>nd</td>
<td>n=2</td>
<td>severe</td>
</tr>
<tr>
<td>F9</td>
<td>Kingston</td>
<td>ASN to ASP -het (Exon B)</td>
<td>nd</td>
<td>n=1</td>
<td>mild</td>
</tr>
<tr>
<td>F9</td>
<td>Calgary</td>
<td>Arg248Gln –het (Exon H)</td>
<td>nd</td>
<td>n=1</td>
<td>severe</td>
</tr>
<tr>
<td>F9</td>
<td>Calgary</td>
<td>Arg180Gln –het (Exon F)</td>
<td>nd</td>
<td>n=1</td>
<td>severe</td>
</tr>
<tr>
<td>F9</td>
<td>Calgary</td>
<td>T to C at nt 31311 –het (Exon H)</td>
<td>nd</td>
<td>n=1</td>
<td>severe</td>
</tr>
<tr>
<td>F9</td>
<td>Calgary</td>
<td>Arg338STOP –het (Exon H)</td>
<td>nd</td>
<td>n=1</td>
<td>severe</td>
</tr>
</tbody>
</table>

Table 3.3 Mutations Identified
Mutations were separated into two categories based on the reported FLs published in the Hemophilia A database *Hamsters* (www.hadb.ca) and Hemophilia B database *FIX* (www.factorix.org). Those mutations most commonly associated with a FL below 0.05 IU/mL in affected males were categorised as severe, and >0.05IU/mL were categorised as mild. The median FL and BS of the two groups (severe vs. mild) were compared using Mann-Whitney (Figure 3.4). The analysis showed no significant difference in FL or BS based on severity of mutation.

**Figure 3-4** Factor levels and Bleeding Scores from HC with mutations associated with a mild or severe phenotype in Hemophiliac males are not significantly different. Severe group (red): n=19, median FL=0.43, BS=10. Mild group (orange): n=15, median FL=0.60, BS=9
3.6.5 X-Inactivation Ratio (XIR)

The X-inactivation assay has been performed on the DNA samples from 20 subjects to date. Of those, one showed an almost entirely skewed XIR of 2:98, three subjects had dramatically skewed XIRs of <20:80 or >80:20, six were mildly skewed with XIRs of <40:60 or >60:40, and six had an unskewed XIR between 40:60 and 60:40.

![Graph showing inverse correlation between XIR and coagulation factor level](image)

**Figure 3-5 Inverse correlation of 0.69 between XIR and coagulation factor level (p<0.001).**

Four samples had maternal and paternal HUMARA CAG repeats that were too close in length to discriminate the peak heights and will be checked by running them on a Southern blot. By assuming a normal FL is evidence of the presence of wild-type allele on the predominantly active X-chromosome, results were analysed in relation to FL. There is an inverse correlation of -0.69 (P<0.001). As skewedness increases, FL decreases.

(Figure 3.5 FL, Figure 3-6 BS)
Figure 3-6 Positive correlation of 0.43 between XIR and Self-BAT bleeding score (p<0.001).
Chapter 4

Discussion

4.1 Discussion

The purpose of this study was to validate the Self-BAT for use as a novel screening tool to identify HC with low coagulation factor levels, investigate the QoL of HC, and elucidate physiologic mechanisms influencing variation in coagulation factor levels in this population.

Analysis of our data shows the Self-BAT and ISTH BAT are in agreement (ICC=0.80, p<0.05). The Self-BAT BS is inversely correlated with coagulation factor level (r=-0.39, p<0.001), and shows promise as a screening tool with a higher sensitivity of 83%, specificity of 42%, PPV of 0.48 and a NPV of 0.79 for the identification of HC with deficient FL. In addition, the Self-BAT is highly reliable with strong agreement on test-retest analysis (r=0.95, p<0.0001).

Recruitment of carriers may have been a potential source of bias if only those seen in clinic for bleeding symptoms were recruited. Although participants were recruited from clinic, not all were symptomatic. A number were identified due to a father, son or brother who is a haemophiliac, irrespective of bleeding symptoms. In this study, the prevalence of HC with deficient FL is 39%, which is consistent with previously published data 20.

For 12 carriers, there was a larger than expected discrepancy between ISTH-BAT BS and Self-BAT BS. Eight of these were from carriers in Johannesburg. In each case, the Self-BAT BS was higher by 5-11 points (mean=7). Possible explanations for these
discrepancies include: 1) the expert administrator is under scoring true bleeding symptoms, 2) the participants over-reported bleeding symptoms.

Nineteen subjects had a positive Self-BAT BS with a normal FL. It is possible that these individuals may have another undiagnosed bleeding disorder. Research shows that 12% of individuals with mild inherited bleeding disorders have a concomitant disease, however it can be difficult to diagnose \(^{72}\). In fact, approximately half of all bleeding disorders fail to be diagnosed in clinic \(^{72}\). Another explanation is over reporting of treatment. Occasionally, a participant reported receiving treatment for a bleed in several categories instead of only the episode for which it was given. Additionally, some carriers might be overly sensitized to the presence of bleeding symptoms because of their family history. This could be investigated by comparing Self-BAT questionnaires of HC without hemophiliac relations to those with. More research is needed to investigate other potential causes of bleeding in HC due to the increasing awareness that HC can be symptomatic with FL in the normal range \(^{18,20,23,24}\).

In contrast, four participants with normal bleeding scores had low clotting FL. In one case, FL was 0.48 U/mL, which is very near normal and may simply be a product of the assay’s inherent margin of error. There are a few possible explanations for the other three cases (ages 26, 31 and 37 with BS 2, 1, 1 and FL 0.34, 0.41, 0.39U/mL respectively). Many bleeding symptoms experienced by HC are as a result of challenges such as tooth extraction, trauma or surgery. A carrier who has not had these experiences would score 0 in these categories, but may in fact have abnormal bleeding if faced with these challenges in the future. This is more likely in younger women who are less likely to have
experienced pregnancy, surgery, and trauma. Additionally, a concomitant thrombophilic disorder such as factor V Leiden could act as a disease modifier resulting in a normal BS in someone with low FVIII. Perhaps for some carriers, the reverse of the comment above is possible; that the focus on male relatives with severe bleeding symptoms including musculoskeletal bleeds might result in a decreased recognition of their symptoms as abnormal. Other limitations include lab testing in different facilities. We attempted to control for this by using the same assays and comparing reference ranges and protocols between centres to maximize consistency.

The QoL data of HC is not significantly different from Canadian normative values. This could be because these HC are receiving proper health care to better manage their symptoms so are not suffering as an untreated symptomatic carrier might. There is however, a negative correlation between BS and multiple SF-36v2 categorical scores, indicating that as BS increases, QoL decreases. Surprisingly, a significant correlation was not found relating FL to QoL. Correspondingly, when Plug et al. used logistic regression to assess the relation of carrier status and FL with the occurrence of hemorrhagic events among 274 HC, they found that those with FL between 0.41 and 0.60 IU/mL were at greater risk of bleeding than those with levels below 0.41 IU/mL. In fact, in their study 7% of HC with FL in the normal range were experiencing joint bleeds (hemarthrosis). In the present study, 10% of HC reported having experienced hemarthrosis with FL ranging from 0.19-1.25 IU/mL. Of these, 20% were spontaneous and 80% were the result of injury. This is a concern because recurrent intra-articular hemorrhage (even sub-clinical) can lead to hemophilic arthropathy, characterized by chronic proliferative synovitis and
cartilage destruction. Acute hemarthroses must be treated appropriately and typically involves the administration of coagulation factor in order to avoid these long-term consequences. Recognition that this is occurring in HC is an important first step in the prevention of hemophilic arthropathy.

A more recent study involving 126 HC concluded that FL was only weakly correlated to bleeding score and might not be sufficient to predict the bleeding tendency. These results suggest that BS is a more accurate predictor of bleeding tendency and related decreases in QoL than FL. The absence of an adequate association between FL and bleeding tendency could explain the 20 carriers who have positive BS and normal FL.

In this study, age was not significantly associated with FL. This could be due to the fact that this is a cross-sectional sampling so factor levels were measured at a single time point. Within this context of the current study, it would be very difficult to measure the degree to which a particular individual’s coagulation factor level changes with age, given that significant age related changes take place over years.

This study indicates that the Self-BAT BS is an important tool for assessment of health in HC. Additional data is currently being collected in Calgary and Johannesburg, which will contribute to a larger sample size as well as allow for comparison between centres. The SF-36v2+9 will be useful in measuring the change in QoL between initial diagnosis and post-treatment to determine the efficacy and quality of care received.

It is difficult to assess to what degree X-inactivation may contribute to FL at this point. By assigning mutation to the predominantly active allele in HC with low FL, the potential relationships between XIR and both BS and FL were analysed. Using this assumption,
analysis revealed a negative correlation between FL and % activation of mutant X (r=-0.69) and a positive correlation between BS and % activation of mutant X (r=0.43), however accurate identification of the mutated allele as maternal or paternal and assignment to either the predominantly active or inactive X-chromosome is necessary.

It is interesting that the prevalence of dramatically skewed XIR in this population is far greater than in a normal population (25%, 8% respectively). The results are quite different than what would be expected in both the normal population (8% skewed) and based on a model of 16 progenitor cells assuming random inactivation, which suggests 1.65% of the population would be dramatically skewed \(^{21}\). This suggests that perhaps the process is not random in HC, but that the hemophilia mutation is somehow influencing the XIR. In 2007, Renault et al. also found unexpectedly high numbers of females with dramatically skewed XIR (27%) in a family carrying hemophilia \(^{21}\). One possible explanation is a genetic influence leading to skewed X-inactivation is linked to the haemophilia-causing mutation on the X chromosome.

4.2 Conclusion

This study addresses challenges faced by HC that may be obstacles impeding their access to high quality health care. By using a self-administered BAT and QoL questionnaire, a health care professional can access a wealth of information regarding the bleeding symptoms and overall health of their patient. This, along with a better understanding of the mechanisms influencing FL deficiency and bleeding in carriers will lead to treatment plans that are more tailored to the individual and are likely to be more successful in preventing abnormal bleeding in this population.
Chapter 5

Future Research

5.1 Objective 1…Self-BAT

Self-BAT, ISTH-BAT and FL data are currently being collected in Calgary and Johannesburg and will be added to this study. The BATs will be scored and the data analysed as a complete set when they are available. Results of the BATs will then be compared to those of type 1 VWD patients who previously participated in research to validate the Self-BAT in the VWD population.

5.2 Objective 2…Quality of Life

SF-36v2+9 data is currently being collected in Calgary and Johannesburg to allow an increased sample size for analysis of HC QoL compared to Canadian normative values. All data will be reanalysed as a whole group as well as compared to each other by region (Kingston, Calgary, and Johannesburg), and country (Canada, South Africa). Findings from the last page, (referred to as +9), of the questionnaire will continue to be analysed for significance between regions and summarized qualitatively when all data are collected.

5.3 Objective 3…Coagulation Factor Variation

In Calgary, Dr. Rydz is also contributing data about HC VWF antigen levels and activity (VWF:RCo), ABO blood type, and mutation analysis to this study. This data will be analysed with data from Kingston.
To draw meaningful conclusions from XIRs of carriers, samples of HC relatives will be obtained from the National Program for Hemophilia Mutation Testing, Kingston, and investigated for highly polymorphic HUMARA gene [CAG] repeat to determine if carriers’ predominantly active allele carries the hemophilia mutation.


45. Iversen PO, Groot PDE, Hjeltnes N, Andersen TO, Mowinckel MC, Sandset PM. Impaired circadian variations of haemostatic and fibrinolytic parameters in tetraplegia. *Br*


PATIENT INFORMATION AND CONSENT FORM

Title of Study: Validation of Self-BAT in Hemophilia Carriers

Principal Investigator: Dr. Paula James, MD, FRCPC
Associate Professor, Department of Medicine
Queen’s University, Kingston, ON
Tel: (613) 533-2946
FAX: (613) 533-6855
Email: jamesp@queensu.ca

Master’s Student: Jane Young, BScH, MSc.Candidate
Department of Pathology and Molecular Medicine
Queen’s University, Kingston, ON
Email: 11jey2@queensu.ca

We invite you to participate in a research study to test how well a bleeding questionnaire works as a screening tool for hemophilia carriers with low coagulation factor levels. You are being invited to participate because you are known to be a carrier of hemophilia. The study has been reviewed for ethical compliance by the Queen’s University Health Sciences and Affiliated Teaching Hospitals Research Ethics Board.

Background Information:

It is important to assess the risk of bleeding in hemophilia carriers to enable physicians to improve care for these patients. Recently, we are becoming aware of the presence of symptomatic carriers. Family history, clotting factor levels, and genotyping can be used to recognize carriers, but these methods may be inaccurate and/or costly. The Self-BAT is a self-administered bleeding questionnaire that takes no more than 20 minutes to complete. A screening tool with a high degree of sensitivity for the identification of carriers with abnormal bleeding would facilitate the
appropriate management for the individual from their health care provider resulting in a higher quality of care.

Purpose of the Study:
We hope to validate the Self-BAT in hemophilia carriers for use as a screening tool to identify those with low coagulation factor levels, and to describe severity of bleeding symptoms.

Description of the Research
If you are interested in participating, you will be asked to complete 2 Bleeding Questionnaires at two points in time (approximately 2 weeks apart), which are standardized forms that ask a number of questions about a variety of bleeding and bruising symptoms. We will then ask you to provide us with one extra tube of blood that will be drawn at the same time as the questionnaire is filled out. The extra blood will be used to extract your DNA for genetic testing, identification of blood type and coagulation factor levels. The sample will be kept in a secure freezer for the duration of the study. At your next clinic visit you will be asked to complete a third Bleeding Questionnaire in addition to a short questionnaire about your quality of life. At the end of this consent form, we will ask you a series of questions about your wishes regarding your DNA.

Risks
There is a risk of pain associated with the blood tests, and a small risk of developing a bruise or infection at the needle site.

If you are a First Nations person, or an indigenous person and you have contact with spiritual “elders” you may want to talk with them before you agree to participate in this study. Elders have reservations about genetic research.

Benefits
It is unlikely that you will benefit directly from this study. However, an improvement of our knowledge of bleeding in hemophilia carriers and a screening tool to identify those with low coagulation factor levels may help other patients in the future.

Inclusion/Exclusion
You are eligible for this study if you are ≥ 18 years old and have been identified as a hemophilia carrier. You are not eligible if you have another inherited bleeding disorder, or if you take anticoagulation medication.

Confidentiality
All information obtained during the course of this study is strictly confidential and your anonymity will be protected at all times. All documents containing your name will be kept in locked files and will only be available to study personnel. This includes Drs. James, Jane Young, the graduate student who will help you complete the Bleeding Questionnaire, and the technologists who will be performing the blood tests. You will be assigned a unique identifying code that will be used in the Excel spreadsheet where we will maintain the study data. Study data will also be maintained in hard copy form in locked files. You will not be identified in any publications resulting from this study.

Voluntary Nature of Study
Your participation in this study is voluntary. You may withdraw at any time and your withdrawal will not affect your future medical care with your Hematologist or at this hospital.

Consent
I have read and understand the consent form for this study. I acknowledge that the study has been explained to me and that any questions that I have asked have been answered to my satisfaction. I am voluntarily signing this form. I will receive a copy of this consent form for my information.

By signing this consent form, I do not waive my legal rights nor release the investigator(s) and sponsors from their legal and professional responsibilities.

________________________________________________________
Name of Patient (print)                                   Name of person obtaining consent (print)

________________________________________________________
Signature                                                  Signature

________________________________________________________
Date
Additional Research Questions:
My medical record (including health card number) may be accessed in order to confirm details of my medical history (if necessary) and to obtain the results of the tests ordered by my Hematologist today.

YES    NO

__________________ / ________________
Initials          Date

Given that this study involves your DNA, there are several more options for you to consider. You can choose all, some, or none of them. Please provide an answer for each option.

I agree that the study investigators can share my DNA and relevant health records with other researchers for studies on **inherited bleeding disorders**, while maintaining my confidentiality.

YES    NO

__________________ / ________________
Initials          Date

I agree that the study investigators can share my DNA and relevant health records with other researchers for studies on **disorders other than inherited bleeding disorders**, while maintaining my confidentiality.

YES    NO

__________________ / ________________
Initials          Date

I agree to be contacted in the future for additional research purposes directly related to the present project.

YES    NO

__________________ / ________________
Initials          Date

If research with your DNA reveals some other medical condition relating to you (when possible):

Do you wish to be informed?
Do you wish your family doctor to be informed?

YES  NO

____________/______________

Initials  Date
If you have any questions about the research you may contact:

Dr. Paula James  
Department of Medicine  
Queen’s University  
Kingston, ON K7L 2V6  
Tel: (613) 533-2946

If you have any questions about your rights as a research subject, you may contact:

Dr. Albert Clark  
Chair, Research Ethics Board  
Queen’s University Health Sciences and Affiliated Teaching Hospitals  
Office of Research Services  
Fleming Hall, Jemmett Wing  
Queen’s University  
Kingston, ON K7L 3N6  
Tel: (613) 533-6081
Appendix B
Case Report Form (CRF)
Validation of the Self-BAT (Self-Administered Bleeding Assessment Tool) in Hemophilia Carriers

Notes to person completing this CRF:

1. **Inclusion criteria are:**
   - Hemophilia A or B carriers as defined by either:
     - Known FVIII or FIX mutation
     - Appropriate family history (mother of a son with hemophilia or daughter of a man with hemophilia)
   - Willing and able to give consent
   - 18 years or older
   - Willing and able to complete the Self-BAT and ISTH-BAT with assistance
   - Carriers with or without bleeding symptoms
   - Documented FVIII or FIX level (either historic or at the time of study enrollment)

2. **Exclusion criteria are:**
   - Patients with other inherited bleeding disorders (von Willebrand disease, platelet function disorders or other coagulation factor deficiencies (ie: FXI deficiency)
   - Patients with hepatic or renal disease (defined as ALT >4 ULN or creatinine > 2ULN respectively) or thrombocytopenia (defined as platelet < 100)
   - Patients on antiplatelet drugs, anticoagulants or herbal medicines that might contribute to bleeding
3. Complete **one CRF for each** research subject except for page 3, which only needs to be completed once for each center.

**GENERAL INFORMATION**

Subject ID: ___ ___ ___ -- ___ ___ ___  
(Institution Code*)  (Subject number “001,” etc.)

*see below for three-digit code

PLEASE enter the subject ID in the upper right-hand corner of each page of this form.

**RECORD REVIEW**

1. Date record review and CRF completed: ___ ___/___ ___/___ ___  
   M M D D Y Y Y Y

2. Initials of person completing form: ___ ___ ___
# CASE REPORT FORM

**PATIENT DEMOGRAPHICS AND DIAGNOSIS AS A HEMOPHILIA CARRIER**

<table>
<thead>
<tr>
<th>3. Year of birth:</th>
<th>__ __ __ __</th>
<th>Current Age _____________</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Y Y Y Y</td>
<td></td>
</tr>
</tbody>
</table>

| 4. How is this patient known to be a Hemophilia carrier? (check all that apply) |
|----------------------------------|---------------------------------------------------------------|
| ❏ Family history (if checked, indicate relationship) | ☐ Mother of son with hemophilia | ☐ Daughter of man with hemophilia |
| ❏ Genetic testing – mutation ________________________ |

<table>
<thead>
<tr>
<th>5. What is the baseline factor level?</th>
</tr>
</thead>
<tbody>
<tr>
<td>FVIII ______IU/mL (lab reference range ____________)</td>
</tr>
<tr>
<td>FIX ______IU/mL (lab reference range ____________)</td>
</tr>
<tr>
<td><strong>BLEEDING SCORE SUMMARY DATA</strong></td>
</tr>
<tr>
<td>--------------------------------</td>
</tr>
<tr>
<td>6. Self-BAT/ISTH-BAT Bleeding Score at enrollment __________________</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>7. For inter-test reliability, what is the date of the administration of the Self-BAT/ISTH-BAT for the second time?</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>___ ___ / ___ / ___ ___</td>
</tr>
<tr>
<td>M M D D Y Y Y Y</td>
</tr>
<tr>
<td>Self-BAT/ISTH-BAT Bleeding Score #2 __________________</td>
</tr>
<tr>
<td>Initials of observer (ISTH-BAT) __________</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>8. For test retest, what is the date of administration of the Self-BAT for the second time?</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>___ ___ / ___ / ___ ___</td>
</tr>
<tr>
<td>M M D D Y Y Y Y</td>
</tr>
<tr>
<td>Self-BAT Bleeding Score #2 __________________</td>
</tr>
</tbody>
</table>
Appendix C
Self-BAT
SELF-BLEEDING ASSESSMENT TOOL:

Patient Information

Name __________________________________________________________

Address _______________________________________________________

_________________________________________________________________

Phone Number ___________________ Email __________________________

Gender  Male ☐  Female ☐

Age _____________  Date of Birth _______________ (DD/MO/YY)

Ethnic Background ____________________________________________

Presenting complaint of bleeding or bruising today  Yes ☐  No ☐

Personal history of bleeding or bruising  Yes ☐  No ☐

Ever been diagnosed with a bleeding disorder? Yes ☐  No ☐

Diagnosis: ____________________________________________________

Immediate or extended family history of bleeding? Yes ☐  No/ Unsure ☐

Relation of family member with bleeding: ____________________________

What was the diagnosis? __________________________________________

Please describe any other diagnosed medical conditions, past or present:

________________________________________________________________

________________________________________________________________

Are you currently on birth control? ☐ Yes  ☐ No

If yes, please list the type and brand name (ex. IUD, Mirena):

________________________________________________________________

________________________________________________________________

Are you pregnant? ___________  Gestation time ___________

Specify any herbals and/or medications that you have taken in the past 30 days:

Name:  Last time taken:

________________________________________________________________

________________________________________________________________

________________________________________________________________

________________________________________________________________

________________________________________________________________
1. **Have you ever experienced nosebleeds?**

   [ ] Yes  [ ] No (skip to 2)

1.1 Please check all of the reasons that have caused your nosebleeds.

   - an injury  [ ]
   - picking your nose  [ ]
   - dry air  [ ]
   - a stuffy nose (cold, allergy)  [ ]
   - taking an aspirin  [ ]
   - no reason, my nosebleeds just start on their own  [ ]

1.2 How long do your nosebleeds usually last?

   [ ] 10 minutes or less  [ ] more than 10 minutes

1.3 How often do you have nosebleeds?

   [ ] 5 times per year or less  [ ] more than 5 times per year

1.4 Have you ever made an appointment to talk to a doctor about your nosebleeds?

   [ ] Yes  [ ] No (skip to 2)

1.5 Have you ever been given medical treatment for your nosebleeds?

   [ ] Yes  [ ] No (skip to 2)

   If yes, please check all of the treatments that you have had.

   - my nose was cauterized or packed at least once  [ ]
   - I was on a medication (liquid or pills) at least once  [ ]
   - I was given a medication intravenously (IV), or with a needle under the skin at least once  [ ]
   - I was given a medication in a nose spray at least once  [ ]
   - I was given a blood transfusion at least once  [ ]
   - I was given a treatment, but don't know what it was  [ ]

Comments:

______________________________________________________________________
______________________________________________________________________
2. Have you ever had unexplained bruises or bruises that are bruises that are larger than you think they should be?  [ ] Yes  [ ] No (skip to 3)

2.1 Please check all the types of bruising you have had.
   - petechiae, i.e. small (1-2 mm) red or purple spots on the skin  [ ]
   - a bruise  [ ]
   - a hematoma, i.e. a bruise that has a hard lump  [ ]
   - I don’t know  [ ]

2.2 How large are your bruises usually?
   - the size of a pea or smaller  [ ]
   - between the size of a pea and an orange  [ ]
   - the size of an orange or larger  [ ]

2.3 How often do you get bruises?  [ ] 5 times per year or less  [ ] more than 5 times per year

2.4 Where do you usually get bruises?
   - on the arms and legs only  [ ]
   - on the chest, back and stomach only  [ ]
   - all over your body  [ ]

If you get small red-purple spots (petechiae), where do you usually see them?
   - on the legs only  [ ]
   - on your face only  [ ]
   - all over your body  [ ]
2.5 Have you ever made an appointment to talk to a doctor about your bruising? [ ] Yes [ ] No (skip to 3)

2.6 Have you ever been given medical treatment for your bruising? [ ] Yes [ ] No (skip to 3)

If yes, please check all of the treatments that you have had.

- I was treated with medications at least once [ ]
- I was given a blood transfusion at least once [ ]
- I was given a treatment but don't know what it was [ ]
<table>
<thead>
<tr>
<th>Question</th>
<th>Yes</th>
<th>No (skip to 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3. Have you ever had bleeding from a small cut?</td>
<td>[ ] Yes</td>
<td>[ ] No (skip to 4)</td>
</tr>
<tr>
<td>3.1 How long do you usually bleed after a small cut?</td>
<td>[ ] 10 minutes or less</td>
<td>[ ] more than 10 minutes</td>
</tr>
<tr>
<td>3.2 How often do you have bleeding from a small cut?</td>
<td>[ ] 5 times per year or less</td>
<td>[ ] more than 5 times per year</td>
</tr>
<tr>
<td>3.3 Have you ever made an appointment to talk to a doctor about bleeding from small cuts?</td>
<td>[ ] Yes</td>
<td>[ ] No (skip to 4)</td>
</tr>
<tr>
<td>3.4 Have you ever been given medical treatment for a small cut?</td>
<td>[ ] Yes</td>
<td>[ ] No (skip to 4)</td>
</tr>
<tr>
<td>If yes, please check all of the treatments that you have had.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- I had stitches at least once</td>
<td>[ ]</td>
<td></td>
</tr>
<tr>
<td>- I was given a medication intravenously (IV) or with a needle under the skin at least once</td>
<td>[ ]</td>
<td></td>
</tr>
<tr>
<td>- I was given medication orally at least once</td>
<td>[ ]</td>
<td></td>
</tr>
<tr>
<td>- I was given a blood transfusion at least once</td>
<td>[ ]</td>
<td></td>
</tr>
<tr>
<td>- I was given a treatment, but don’t know what it was</td>
<td>[ ]</td>
<td></td>
</tr>
</tbody>
</table>
4. Have you ever seen blood in your urine? (If you are a female, this does NOT include when you have had your period.)

[ ] Yes  [ ] No (skip to 5)

4.1 Please check all of the causes of blood in the urine that you have had.

- kidney stones [ ]
- infection [ ]
- another kidney or bladder disease [ ]
- no reason that I know [ ]

4.2 Have you ever made an appointment to talk to a doctor about unexplained blood in your urine?

[ ] Yes  [ ] No (skip to 5)

4.3 Have you ever been given medical treatment for unexplained blood in your urine?

[ ] Yes  [ ] No (skip to 5)

If yes, please check all of the treatments that you have had.

- I had surgery at least once to stop the bleeding [ ]
- I was on treatment with iron at least once [ ]
- I was given a medication intravenously (IV), or with a needle under the skin at least once [ ]
- I was given a blood transfusion at least once [ ]
- I was given antibiotics at least once [ ]
- I was given a treatment but don’t know what it was [ ]
5. Have you ever had bleeding inside your intestines, stomach or bowel? [ ] Yes [ ] No (skip to 6)

5.1 Have you ever:
- vomited red blood, or what looked like coffee grounds [ ]
- passed black, tarry stools while you were not taking iron supplements [ ]
- passed red blood in or with your stools [ ]

5.2 Please check all of the causes of this bleeding that you have had
- an ulcer [ ]
- liver disease [ ]
- abnormal and fragile blood vessels in the bowel (angiodysplasia) [ ]
- hemorrhoids, ‘piles’ or anal fissures [ ]
- another identifiable cause [ ]
- for no reason [ ]

5.3 Have you ever made an appointment to talk to a doctor about unexplained bleeding from your stomach or bowel? [ ] Yes [ ] No (skip to 6)

5.4 Have you ever been given medical treatment for unexplained bleeding from your stomach or bowel? [ ] Yes [ ] No (skip to 6)

If yes, please check all of the treatments that you have had.
- I had surgery to stop the bleeding at least once [ ]
- I was on a medication (liquid or pills) at least once [ ]
- I was given a medication intravenously (IV), or with a needle under the skin at least once [ ]
- I was given a blood transfusion at least once [ ]
- I was given a treatment but don’t know what it was [ ]
6. Have you ever noticed bleeding from the mouth? (This does NOT include tooth extraction at the dentist.)

[ ] Yes    [ ] No (skip to 7)

6.1 Please check all of the causes of bleeding from the mouth that you have had.

- new teeth coming in or tooth loss [ ]
- brushing/flossing [ ]
- bite on lip, tongue or cheek [ ]
- cleaning at the dentist’s [ ]
- another cause [ ]

Please specify:

6.2 How long does this bleeding usually last?

[ ] 10 minutes or less
[ ] more than 10 minutes

6.3 Have you ever made an appointment to talk to a doctor or dentist about bleeding from the mouth?

[ ] Yes    [ ] No (skip to 7)

6.4 Have you ever been given medical treatment for bleeding from the mouth?

[ ] Yes    [ ] No (skip to 7)

If yes, please check all of the treatments that you have had.

- I had dental packing, cauterization or had stitches to stop the bleeding at least once [ ]
- I was on a medication (liquid or pills) at least once [ ]
- I was given a medication intravenously (IV), or with a needle under the skin at least once [ ]
- I was given a blood transfusion at least once [ ]
- I was given a treatment but don’t know what it was [ ]
7. Have you ever had a tooth/teeth taken out at the dentist?  [ ] Yes  [ ] No (skip to 8)

7.1 Please check what kind of tooth was taken out and note how many of each
- baby tooth  [ ] _______
- adult tooth  [ ] _______
- wisdom tooth  [ ] _______

7.2 Did you experience any abnormal bleeding after any of these extractions?  [ ] Yes  [ ] No (skip to 8)

7.3 Have you ever made an appointment to talk to a doctor or dentist about this bleeding?  [ ] Yes  [ ] No (skip to 8)

7.4 Have you ever been given medical treatment for bleeding after a tooth was taken out?
If yes, please check all of the treatments that you have had.
- I had dental packing or had stitches to stop the bleeding, at least once  [ ]
- I was on a medication (liquid or pills) at least once  [ ]
- I was given a medication intravenously (IV), or with a needle under the skin at least once  [ ]
- I was given a blood transfusion at least once  [ ]
- I was given a treatment but don't know what it was  [ ]
8. Have you ever had surgery or a major trauma (e.g. car accident)?

[ ] Yes [ ] No (skip to 9)

8.1 Please check what kind of surgery/trauma you had

- tonsils/adenoids taken out [ ]
- other surgery of the nose or throat [ ]
- surgery of the chest [ ]
- surgery of the womb or ovaries, including caesarian section, removal of the womb [ ]
- other surgery of the stomach or belly [ ]
- other surgeries [ ]
- trauma [ ]

Please specify: __________________

Please specify: __________________

8.2 Did you experience any abnormal bleeding during or after any of these surgeries?

[ ] Yes [ ] No (skip to 9)

8.3 Have you ever made an appointment to a doctor about the bleeding during or after you had surgery?

[ ] Yes [ ] No (skip to 9)

8.4 Have you ever been given medical treatment for bleeding during or after surgery?

[ ] Yes [ ] No (skip to 9)

If yes, please check all of the treatments that you have had.

- I had packing or stitches to stop the bleeding, at least once [ ]
- I was on a medication (liquid or pills) at least once [ ]
- I was given a medication intravenously (IV), with a needle under the skin, at least once [ ]
- I was given a blood transfusion at least once [ ]
- I was given a treatment but don’t know what it was [ ]
If you are a male, please skip to 11 now.

9. Have you ever had a period?  
[ ] Yes  [ ] No (skip to 10)

Are you:

☐ Pre-menopausal  ☐ Post-menopausal

*If you are post-menopausal, please answer the following questions to the best of your ability

9.1 Were/are your periods regular?  
[ ] Yes  [ ] No

Please check all that applies to the heaviest period you ever had:

- I had to change my pad/tampon more often than every 2 hours  [ ]
- the period lasted for more than 7 days  [ ]
- I passed clots and had flooding  [ ]
- Spotting mid-cycle  [ ]

9.2 Have you stayed at home from work/school more than twice a year because of heavy bleeding?  
[ ] Yes  [ ] No

9.3 Have your periods been heavy from the get-go?  
[ ] Yes  [ ] No

9.4 How long have you had a problem with heavy periods?  
[ ] 1 year or less  [ ] more than 1 year

9.5 Have you ever made an appointment to talk to a doctor about your heavy periods?  
[ ] Yes  [ ] No
9.7 Have you ever been given medical treatment for heavy periods?

[ ] Yes [ ] No (skip to 10).

If yes, please check all of the treatments that you have had.

- I was on iron or on other medications (liquid or pills) at least once
- I was given the birth control pill because of heavy periods
- I was given the birth control pill as well as on other pills
- I had surgery to stop the bleeding at least once (e.g. removal of the womb, burning (ablation) or scraping (curettage) of the lining of the womb)
- I was given a medication intravenously (IV), or with a needle under the skin at least once
- I was given a blood transfusion at least once
- I was admitted to hospital at least once
- I was given a treatment but don't know what it was
- I was given medication for pain associated with cramping

Comments: ________________________________
<table>
<thead>
<tr>
<th></th>
<th><strong>10. Have you ever been pregnant?</strong></th>
<th>[ ] Yes</th>
<th>[ ] No (skip to 11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.1</td>
<td>Have you ever been pregnant but not carried the baby to term?</td>
<td>[ ] Yes</td>
<td>[ ] No (skip to 10.5)</td>
</tr>
<tr>
<td></td>
<td>If so, how many times?</td>
<td>____</td>
<td></td>
</tr>
<tr>
<td>10.2</td>
<td>Was it associated with excessive bleeding?</td>
<td>[ ] Yes</td>
<td>[ ] No</td>
</tr>
<tr>
<td>10.3</td>
<td>Did you seek medical attention?</td>
<td>[ ] Yes</td>
<td>[ ] No</td>
</tr>
<tr>
<td></td>
<td>If yes, please check all of the treatments you have had:</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>- I was given a medication intravenously (IV) to induce contraction of the womb at least once</td>
<td>☐</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- I was put on iron or other pills at least once</td>
<td>☐</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- I was given a medication intravenously (IV), or with a needle under the skin at least once</td>
<td>☐</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- I was given a blood transfusion at least once</td>
<td>☐</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- I had an examination and/or packing of the womb while I was put asleep at least once</td>
<td>☐</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- I had surgery (eg. removing the womb, tying off the bleeding vessels,…) at least once</td>
<td>☐</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- I was in the intensive care unit (ICU) at least once</td>
<td>☐</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- I was given a treatment but don’t know what it was</td>
<td>☐</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Other or non-applicable</td>
<td>☐</td>
<td></td>
</tr>
</tbody>
</table>
10.5 Have you ever given birth by vaginal delivery? (If no, skip to 11) [ ] Yes [ ] No (skip to 11)

How many times? ______

Did you experience problems with bleeding during the pregnancy or after the birth? (If no, skip to 11) [ ] Yes [ ] No

10.6 When did the problems with vaginal bleeding occur? [ ] within the first 24 hours after delivery
[ ] between 24 hours and 6 weeks after delivery
[ ] all of the above

10.8 How long did the vaginal discharge last? [ ] less than 6 weeks
[ ] more than 6 weeks

10.9 Did you have to stay in the hospital longer because of this bleeding? [ ] Yes [ ] No

10.10 Have you ever talked to a doctor about this bleeding? [ ] Yes [ ] No (skip to 11)
10.7 Have you ever been given medical treatment for bleeding after having a baby?

[ ] Yes  [ ] No (skip to 11)

10.8 If yes, please check all of the treatments that you have had.

- I was given a medication intravenously (IV) to induce contraction of the womb at least once

- I was put on iron or other pills at least once

- I was given a medication intravenously (IV), or with a needle under the skin at least once

- I was given a blood transfusion at least once

- I had an examination and/or packing of the womb while I was put asleep at least once

- I had surgery (eg. removing the womb, tying off the bleeding vessels,...) at least once

- I was in the intensive care unit (ICU) at least once

- I was given a treatment but don’t know what it was
11. Have you ever had bleeding into a muscle? (This would look like a bruise on the skin, but it would be hard and hurt a lot more.)

[ ] Yes  [ ] No (skip to 12)

<table>
<thead>
<tr>
<th>Question</th>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>11.1 Was this bleeding caused by an injury?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11.2 Have you ever made an appointment to a doctor about your bleeding into a muscle?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11.3 Have you ever been given medical treatment for bleeding into a muscle?</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

If yes, please check all of the treatments that you have had.

- I had surgery to take away the blood at least once [ ]
- I was given a medication intravenously (IV), or with a needle under the skin at least once [ ]
- I was given clotting factors at least once [ ]
- I was given a blood transfusion at least once [ ]
- I was given a treatment but don’t know what it was [ ]
<table>
<thead>
<tr>
<th></th>
<th><strong>12. Have you ever had bleeding into a joint?</strong></th>
<th>[ ] Yes</th>
<th>[ ] No (skip to 13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.1</td>
<td>Was the bleeding caused by an injury?</td>
<td>[ ] Yes</td>
<td>[ ] No</td>
</tr>
<tr>
<td>12.2</td>
<td>Have you ever made an appointment to a doctor about bleeding into a joint?</td>
<td>[ ] Yes</td>
<td>[ ] No (skip to 13)</td>
</tr>
<tr>
<td>12.3</td>
<td>Have you ever been given medical treatment for bleeding into a joint?</td>
<td>[ ] Yes</td>
<td>[ ] No (skip to 13)</td>
</tr>
</tbody>
</table>

If yes, please check all of the treatments that you have had.

- I had surgery to take away the blood at least once
- I was given a medication intravenously (IV), or with a needle under the skin at least once
- I was given clotting factors at least once
- I was given a blood transfusion at least once
- I was given a treatment but don’t know what it was
13. Have you ever had bleeding into or out of the head, brain or spine?  
[ ] Yes  [ ] No (skip to 14)

13.1 Where was the bleeding?
- Scalp  [ ]
- Under the skull and around the brain  [ ]
- Within the brain tissue  [ ]
- I don’t know  [ ]

13.2 Please check all of the treatments that you have had.
- I had surgery to take away the blood  [ ]
- I had surgery to have a shunt put in  [ ]
- I was given a blood transfusion at least once  [ ]
- I was given a treatment but don’t know what it was  [ ]

Comments:
______________________________________________________________________
______________________________________________________________________
14 Other types of bleeding. Some of these other types of bleeding would have happened shortly after birth.

14.1 Have you ever had any of the following?

- A problem with bleeding from the umbilical stump at birth

- cephalohematoma, i.e. a collection of blood under the scalp as a newborn, presenting as a soft swelling at the back of the head

- bleeding upon suctioning of the mouth and nose at birth

- bleeding into your cheek, caused by sucking during bottle or breastfeeding

- a problem with bleeding during or after the surgery to remove the foreskin of the penis (circumcision)

- a problem with bleeding from a needle poke when blood was drawn

- bleeding in the white of your eye

- bleeding after sexual intercourse

[ ] Yes  [ ] No  [ ] Unsure

14.2 Have you ever made an appointment to a doctor about any of those bleeding symptoms?  [ ] Yes  [ ] No
Have you ever been given medical treatment for any of these bleeding symptoms? [ ] Yes [ ] No

For each of these symptoms separately, please check the treatments that you have had.

- I was on a medication (liquid or pills) at least once [ ]
- I had surgery to stop the bleeding or had stitches at least once [ ]
- I was given a medication intravenously (IV), with a needle under the skin, at least once [ ]
- I was given a blood transfusion at least once [ ]
- I was given a treatment but don’t know what it was [ ]

If you have had problems with any other bleeding symptoms that were not included in this questionnaire, please comment on these here.

______________________________________________________________________
______________________________________________________________________
Appendix D

ISTH BAT
Our BAT

Only symptoms and treatment BEFORE and AT diagnosis should be considered

1. Epistaxis

1.1 Have you ever had spontaneous epistaxis?  ☐ Yes  ☐ No or trivial (skip to 2)

1.2 Has the symptom ever required medical attention?  ☐ Yes  ☐ No (resolve spontaneously; skip to 1.5)

1.3 If answer to 1.2 is yes, please specify

☐ Consultation
☐ Cauterization
☐ Packing
☐ Antifibrinolytics
☐ Iron therapy
☐ Treatment with desmopressin
☐ Treatment with plasma
☐ Treatment with platelet concentrate
☐ Treatment with factor concentrates
☐ Blood (RBC) transfusion

1.4 How many times in your life did you receive any of the above treatments (# 1.3)?

☐ 1 - 2
☐ 3 to 5
☐ 6 to 10
☐ more than 10

1.5 At what age did you first have symptoms?

☐ Before 1 year
☐ Between 1-5 years of age
☐ Between 6-12 years of age
☐ Between 13-25 years of age
☐ After 25 years of age

1.6 Approximate number of episodes NOT requiring medical attention

☐ <1 per year
☐ 1 per year
☐ 2-5 per year
☐ >5 per year

1.7 Duration of average single episode (min.) NOT requiring medical attention

☐ 1 minute or less
☐ 1 - 10 minutes
☐ > 10 minutes
2. Cutaneous bleeding (Bruising, ecchymoses, purpura, subcutaneous hematomas)

2.1 Have you ever had any of the above cutaneous bleeding?
   ☐ Yes   ☐ No or trivial skip to 3

2.2 Has the symptom ever required medical attention?
   ☐ Yes   ☐ No skip to 2.5

2.3 If answer to 2.2 is yes, please specify
   ☐ Consultation
   ☐ Treatment with desmopressin
   ☐ Treatment with plasma
   ☐ Treatment with platelet concentrate
   ☐ Treatment with factor concentrates
   ☐ Blood (RBC) transfusion

2.4 How many times in your life did you receive any of the above treatments (#2.3)?
   ☐ 1 to 2
   ☐ 3 to 5
   ☐ 6 to 10
   ☐ more than 10

2.5 At what age did you first have symptoms?
   ☐ Before 1 year
   ☐ Between 1-5 years of age
   ☐ Between 6-12 years of age
   ☐ Between 13-25 years of age
   ☐ After 25 years of age

2.6 Approximate number of episodes NOT requiring medical attention
   ☐ <1 per year
   ☐ 1 per year
   ☐ 2-5 per year
   ☐ >5 per year

2.7 Type of bleeding
   ☐ Petechiae
   ☐ Bruises
   ☐ Hematomas

2.8 Location
   ☐ Exposed sites
   ☐ Unexposed sites
   ☐ Both

2.9 Common size
   ☐ ≤1 cm
   ☐ >1 cm
   ☐ Extensive (palm sized or larger)

2.10 Minimal or no trauma
   ☐ Yes   ☐ No

2.11 How many bruises >1 cm in exposed areas in the most severe manifestation?
   ☐ ≤5
   ☐ >5

2.12 Location of petechiae
   ☐ Limited to lower limbs
   ☐ Diffuse
### 3. Bleeding from minor wounds (not requiring stitches in the average patient)

<table>
<thead>
<tr>
<th>Section</th>
<th>Question</th>
<th>Yes</th>
<th>No or trivial skip to 4</th>
<th>No skip to 3.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Have you ever had prolonged bleeding from minor wounds?</td>
<td></td>
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</tr>
<tr>
<td>3.2</td>
<td>Has the symptom ever required medical attention?</td>
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<td></td>
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<tr>
<td>3.3</td>
<td>If answer to 3.2 is yes, please specify</td>
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<td></td>
<td></td>
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<tr>
<td>3.4</td>
<td>How many times in your life did you received any of the above treatments (# 3.3)?</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>3.5</td>
<td>At what age did you first have symptoms?</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.6</td>
<td>Approximate number of episodes NOT requiring medical attention</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.7</td>
<td>Duration of average single episode (min.)</td>
<td></td>
<td></td>
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</tbody>
</table>

- Consultation
- Surgical hemostasis
- Treatment with desmopressin
- Treatment with plasma
- Treatment with platelet concentrate
- Treatment with factor concentrates
- Blood (RBC) transfusion

**3.1**
- Yes
- No or trivial skip to 4

**3.2**
- Yes
- No skip to 3.5

**3.3**
- Consultation
- Surgical hemostasis
- Treatment with desmopressin
- Treatment with plasma
- Treatment with platelet concentrate
- Treatment with factor concentrates
- Blood (RBC) transfusion

**3.4**
- 1 - 2
- 3 to 5
- 6 to 10
- more than 10

**3.5**
- Before 1 year
- Between 1-5 years of age
- Between 6-12 years of age
- Between 13-25 years of age
- After 25 years of age

**3.6**
- <1 per year
- 1 per year
- 2-5 every year
- >5 per year

**3.7**
- 1 to 5 minutes
- 5 to 10 minutes
- >10 minutes
4. Oral cavity bleeding (Tooth eruption, spontaneous or after brushing/flossing, gum bleeding, bleeding after bites to lip & tongue)

4.1 Have you ever had oral cavity bleeding?  
❑ Yes  ❑ No or trivial skip to 5

4.2 Has the symptom ever required medical attention?  
❑ Yes  ❑ No (skip to 4.5)

4.3 If answer to 4.2 is yes, please specify  
❑ Consultation only  ❑ Surgical hemostasis (dental packing, suture, cauterization)  
❑ Antifibrinolytics  ❑ Treatment with desmopressin  
❑ Treatment with plasma  ❑ Treatment with platelet concentrate  
❑ Treatment with factor concentrates  ❑ Blood (RBC) transfusion

4.4 How many times in your life did you receive any of the above treatments (#4.3)?  
❑ 1 - 2  ❑ 3 to 5  ❑ 6 to 10  ❑ more than 10

4.5 At what age did you first have symptoms?  
❑ Before 1 year  ❑ Between 1-5 years of age  
❑ Between 6-12 years of age  ❑ Between 13-25 years of age  
❑ After 25 years of age

4.6 Approximate number of episodes NOT requiring medical attention  
❑ <1 per year  ❑ 1 per year  ❑ 2-5 per year  ❑ >5 per year

4.7 Duration of average single episode (min.)  
❑ 1 to 10 minutes  ❑ > 10 minutes
5. Gastrointestinal bleeding (Hematemesis, Melena, Hematochezia)

5.1 Have you ever had gastrointestinal bleeding?
   - Yes
   - No skip to 6

5.2 If answer to 5.1 is yes, please specify
   - Type of bleeding
     - Hematemesis
     - Melena
     - Hematochezia
   - Presence of associated GI disease
     - Yes
     - No
     - Specify:
       - Ulcer
       - Portal hypertension
       - Angiodysplasia

5.3 Has the symptom ever required medical attention?
   - Yes
   - No
   - skip to 5.6

5.4 If answer to 5.3 is yes, please specify
   - Consultation only
   - Surgical hemostasis
   - Antifibrinolytics
   - Treatment with desmopressin
   - Treatment with plasma
   - Treatment with platelet concentrate
   - Treatment with factor concentrates
   - Blood (RBC) transfusion

5.5 How many times in your life did you receive any of the above treatments (#5.4)?
   - 1-2
   - 3-5
   - 6-10
   - > 10
   - Before 1 year
   - Between 1-5 years of age
   - Between 6-12 years of age
   - Between 13-25 years of age
   - After 25 years of age

5.6 At what age did you first have symptoms?
   - <1 per year
   - 1 per year

5.7 Approximate number of episodes NOT requiring medical attention
   - 2-5 every year
   - >5 per year
### 6. Hematuria

<table>
<thead>
<tr>
<th>Question</th>
<th>Options</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.1 Have you ever had hematuria?</td>
<td>☐ Yes ☐ No skip to 7</td>
</tr>
<tr>
<td>6.2 If the answer to 6.1 is yes, please specify:</td>
<td>☐ Yes ☐ No</td>
</tr>
<tr>
<td>Presence of associated urologic disease?</td>
<td></td>
</tr>
<tr>
<td>6.3 Has the symptom ever required medical attention?</td>
<td>☐ Yes ☐ No skip to 6.6</td>
</tr>
<tr>
<td>6.4 If the answer to 6.3 is yes, please specify</td>
<td>☐ Consultation only</td>
</tr>
<tr>
<td>☐ Surgery</td>
<td></td>
</tr>
<tr>
<td>☐ Iron therapy</td>
<td></td>
</tr>
<tr>
<td>☐ Treatment with desmopressin</td>
<td></td>
</tr>
<tr>
<td>☐ Treatment with plasma</td>
<td></td>
</tr>
<tr>
<td>☐ Treatment with platelet concentrate</td>
<td></td>
</tr>
<tr>
<td>☐ Treatment with factor concentrates</td>
<td></td>
</tr>
<tr>
<td>☐ Blood (RBC) transfusion</td>
<td></td>
</tr>
<tr>
<td>6.5 How many times in your life did you receive any of the above treatments (#6.4)?</td>
<td>☐ 1-2 ☐ 3-5 ☐ 6-10 ☐ &gt;10</td>
</tr>
<tr>
<td>6.6 At what age did you first have symptoms?</td>
<td>☐ Before 1 year</td>
</tr>
<tr>
<td>☐ Between 1-5 years of age</td>
<td>☐ Between 6-12 years of age</td>
</tr>
<tr>
<td>☐ Between 13-25 years of age</td>
<td>☐ After 25 years of age</td>
</tr>
<tr>
<td>6.7 Approximate number of episodes NOT requiring medical attention</td>
<td>☐ &lt;1 per year</td>
</tr>
<tr>
<td>☐ 1 per year</td>
<td>☐ 2-5 per year</td>
</tr>
<tr>
<td>☐ &gt;5 per year</td>
<td></td>
</tr>
</tbody>
</table>
7. **Bleeding after Tooth/Teeth extraction**

7.1 Please specify number of extractions

If no extractions, skip to section 8

7.2 Have you ever had bleeding after tooth (teeth) extraction?

- Yes
- No

7.3 After how many extractions did you have bleeding?

----

*Please fill in the following for the tooth extraction with the worst bleeding*

<table>
<thead>
<tr>
<th>Type of extraction</th>
<th>Age at extraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Deciduous</td>
</tr>
<tr>
<td></td>
<td>Permanent</td>
</tr>
<tr>
<td></td>
<td>Molar</td>
</tr>
</tbody>
</table>

Actions taken to prevent bleeding

- None
- Antifibrinolytics
  - Desmopressin
  - Plasma or clotting factor concentrates
  - Platelet infusion

Bleeding after extraction?

- Yes
- No

Actions taken to control bleeding

- None
- Resuturing
  - Packing
  - Antifibrinolytics
  - Desmopressin
  - Plasma or clotting factor concentrates
  - Platelet infusion
  - Blood (RBC) transfusion
8. Bleeding after Surgery or Major Trauma

8.1 Please specify number of surgeries/major traumas

If no Surgery or Trauma skip to section 9

8.2 Have you ever had bleeding after surgery or major trauma?

Yes ☐ No ☐

8.3 After how many surgeries or major traumas have you experienced bleeding?

Please fill in the following for the surgery or major trauma episode with the worst bleeding.

Age at intervention/trauma ☐ Major-abdominal ☐ Major-toracic ☐ Major-thoracic

Type of surgery ☐ Tonsillectomy/Adenoids ☐ Major-gynecology ☐ Pharynx/Nose ☐ Other

Actions taken to prevent bleeding

None ☐ Antifibrinolytics ☐ Desmopressin ☐ Plasma or clotting factor concentrates ☐ Platelet infusion

Bleeding after intervention? ☐ Yes ☐ No

Actions taken to control bleeding

None ☐ Surgical hemostasis ☐ Antifibrinolytics ☐ Desmopressin ☐ Plasma or clotting factor concentrates ☐ Platelet infusion ☐ Blood (RBC) transfusion
9. **Menorrhagia**

9.1 Have you ever had very heavy menstrual bleeding (menorrhagia)?
- Yes
- No or trivial skip to 10

   If answer to 9.1 is yes, please specify
   - Changing pads/tampons more frequently than every 2 hours
   - Bleeding more than 7 days
   - Clot and flooding
   - Pictorial Bleeding Score Assessment

9.2 Has the symptom ever required medical attention?
- Yes
- No skip to 9.5

9.3 If answer to 9.2 is yes, please specify
- Consultation only
- Antifibrinolytic therapy
- Iron therapy
- Hormonal therapy/birth control pill use
- Combined antifibrinolytics & Hormonal therapy
- IUD (levonorgestrel-releasing intrauterine system)
- D & C
- Endometrial ablation
- Hysterectomy
- Treatment with desmopressin
- Treatment with plasma
- Treatment with platelet concentrate
- Treatment with factor concentrate
- Blood (RBC) transfusion
- Hospital admission and emergency treatment

9.4 How many times in your life did you received any of the above treatments (# 9.3)?
- 1 - 2
- 3 to 5
- 6 to 10
- more than 10

9.5 At what age did you first have symptoms?
- At menarche
- Between 14-25 year of age
- After 25 years of age

9.6 Have you had time off work/school for menorrhagia?
- <twice a year
- >twice a year

9.7 Duration of menorrhagia
- Since menarche
- > 12 months
- < 12 months
<table>
<thead>
<tr>
<th>Section</th>
<th>Question</th>
<th>Options</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.1</td>
<td>Number of successful pregnancies (live births)</td>
<td>☐☐</td>
</tr>
<tr>
<td>10.2</td>
<td>Have you ever had post-partum haemorrhage?</td>
<td>☐ Yes ☐ No or trivial skip to 11</td>
</tr>
<tr>
<td>10.3</td>
<td>Did it occur</td>
<td>☐ In the first 24 hours after delivery (Primary) ☐ Between 24 hours and 6 weeks postpartum (Secondary) ☐ Both Primary and Secondary</td>
</tr>
<tr>
<td>10.4</td>
<td>How long did vaginal discharge (lochia) last?</td>
<td>☐ &lt; 6 weeks ☐ &gt; 6 weeks</td>
</tr>
<tr>
<td>10.5</td>
<td>Did it require changing pads/tampons more frequently than every 2 hours?</td>
<td>☐ Yes ☐ No</td>
</tr>
<tr>
<td>10.6</td>
<td>Did this bleeding cause delay of hospital discharge/readmission to hospital?</td>
<td>☐ Yes ☐ No</td>
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<tr>
<td>10.7</td>
<td>Has the symptom ever required medical treatment?</td>
<td>☐ Yes ☐ No</td>
</tr>
<tr>
<td>10.8</td>
<td>If answer to 10.7 is yes, please specify</td>
<td>☐ Consultation only /oxytocin i.v.infusion ☐ Additional uterotonic medications ☐ Iron therapy ☐ Antifibrinolytic therapy ☐ Treatment with desmopressin ☐ Treatment with plasma ☐ Treatment with platelet concentrate ☐ Treatment with factor concentrates ☐ Blood (RBC) transfusion ☐ Any procedure requiring examination under anaesthesia ☐ Uterine balloon/package to tamponade the uterus ☐ Any procedure requiring critical care or surgical intervention (includes: hysterectomy, internal iliac artery legation, uterine artery embolization, uterine brace sutures)</td>
</tr>
<tr>
<td>10.9</td>
<td>Number of deliveries that required any of the above treatments (# 10.8)?</td>
<td>☐☐</td>
</tr>
<tr>
<td>11.1</td>
<td>Have you ever had muscle hematomas?</td>
<td></td>
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<td>-------</td>
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<td></td>
<td>If yes, was it spontaneous or after trauma?</td>
<td></td>
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<tr>
<td>11.2</td>
<td>Has the symptom ever required medical attention?</td>
<td></td>
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<tr>
<td>11.3</td>
<td>If answer to 11.2 is yes, please specify</td>
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<tr>
<td>11.4</td>
<td>How many times in your life did you receive any of the above treatments (# 11.3)?</td>
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<tr>
<td>11.5</td>
<td>At what age did you first have symptoms?</td>
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<tr>
<td>11.6</td>
<td>Approximate number of episodes NOT requiring medical attention</td>
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<tr>
<td>12. Hemarthrosis</td>
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<td>-----------------</td>
<td></td>
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<tr>
<td><strong>12.1</strong> Have you ever had hemarthrosis?</td>
<td>☐ Yes ☐ No or trivial skip to 13</td>
<td></td>
</tr>
<tr>
<td>If yes, was it spontaneous or after trauma?</td>
<td>☐ spontaneous ☐ trauma-related</td>
<td></td>
</tr>
<tr>
<td><strong>12.2</strong> Has the symptom ever required medical attention?</td>
<td>☐ Yes ☐ No skip to 12.5</td>
<td></td>
</tr>
<tr>
<td><strong>12.3</strong> If answer to 12.2 is yes, please specify</td>
<td>☐ Consultation</td>
<td></td>
</tr>
<tr>
<td>☐ Surgical draining</td>
<td></td>
<td></td>
</tr>
<tr>
<td>☐ Treatment with desmopressin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>☐ Treatment with plasma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>☐ Treatment with platelet concentrate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>☐ Treatment with factor concentrates</td>
<td></td>
<td></td>
</tr>
<tr>
<td>☐ Blood transfusion</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>12.4</strong> How many times in your life did you receive any of the above treatments (# 12.3)?</td>
<td>☐ 1 - 2</td>
<td></td>
</tr>
<tr>
<td>☐ 3 to 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>☐ 6 to 10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>☐ more than 10</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>12.5</strong> At what age did you first have symptoms?</td>
<td>☐ Before 1 year</td>
<td></td>
</tr>
<tr>
<td>☐ Between 1-5 years of age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>☐ Between 6-12 years of age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>☐ Between 13-25 years of age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>☐ After 25 years of age</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>12.6</strong> Approximate number of episodes NOT requiring medical attention</td>
<td>☐ &lt;1 per year</td>
<td></td>
</tr>
<tr>
<td>☐ 1 per year</td>
<td></td>
<td></td>
</tr>
<tr>
<td>☐ 2-5 per year</td>
<td></td>
<td></td>
</tr>
<tr>
<td>☐ &gt;5 per year</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### 13. CNS bleeding (spontaneous)

<table>
<thead>
<tr>
<th>13.1</th>
<th>Have you ever had cranial or spinal bleeding?</th>
<th>Yes</th>
<th>No or trivial skip to 14</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>If yes, was it spontaneous or after trauma?</td>
<td>spontaneous</td>
<td>trauma-related</td>
</tr>
<tr>
<td>13.2</td>
<td>If answer to 13.1 is yes, please specify</td>
<td>☐ Subdural</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Type of bleeding</td>
<td>☐ Intracerebral</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>☐ Subarachnoid</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Was the diagnosis made by</td>
<td>☐ CT scan</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>☐ MNR</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>☐ Angiography</td>
<td></td>
</tr>
<tr>
<td>13.3</td>
<td>Type of treatment</td>
<td>☐ Consultation</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>☐ Surgical draining</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>☐ Treatment with plasma, platelet or factor concentrates</td>
<td></td>
</tr>
</tbody>
</table>

### 13.4 At what age did you have CNS bleeding?

- ☐ Before 1 year
- ☐ Between 1-5 years of age
- ☐ Between 6-12 years of age
- ☐ Between 13-25 years of age
- ☐ After 25 years of age
14.1 Have you ever had one of the following?

- Excessive umbilical stump bleeding
  - [ ] Yes
  - [ ] No

- Cephalohematoma
  - [ ] Yes
  - [ ] No

- Bleeding at circumcision
  - [ ] Yes
  - [ ] No

- Venipuncture bleeding
  - [ ] Yes
  - [ ] No

- Suction Bleeding
  - [ ] Yes
  - [ ] No

- Ovulation bleeding (in women)
  - [ ] Yes
  - [ ] No

14.2 Has one of these symptoms ever required medical attention?

- [ ] Yes
- [ ] No

14.3 If answer to 14.2 is yes, please specify

- [ ] Consultation
- [ ] Antifibrinolytics
- [ ] Surgery
- [ ] Treatment with desmopressin
- [ ] Treatment with plasma
- [ ] Treatment with platelet concentrate
- [ ] Treatment with factor concentrates
- [ ] Blood (RBC) transfusion

14.4 How many times in your life did you receive any of the above treatments (# 14.3) for this symptom?

- [ ] 1 - 2
- [ ] 3 to 5
- [ ] 6 to 10
- [ ] more than 10
<table>
<thead>
<tr>
<th>SYMPTOMS</th>
<th>0§</th>
<th>1§</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Epistaxis</strong></td>
<td>No/trivial</td>
<td>&gt;5/year or &gt;10 minutes</td>
<td>Consultation only*</td>
<td>Packing or cauterization or antifibrinolytic</td>
<td>Blood transfusion or replacement therapy (use of hemostatic blood components and rFVIIa) or desmopressin</td>
</tr>
<tr>
<td><strong>Cutaneous</strong></td>
<td>No/trivial</td>
<td>For bruises 5 or more (&gt;1cm) in exposed areas</td>
<td>Consultation only*</td>
<td></td>
<td>Extensive Spontaneous hematoma requiring Blood transfusion</td>
</tr>
<tr>
<td><strong>Bleeding from minor wounds</strong></td>
<td>No/trivial</td>
<td>&gt;5/year or &gt;10 minutes</td>
<td>Consultation only*</td>
<td>Surgical hemostasis</td>
<td>Blood transfusion or replacement therapy or Desmopressin</td>
</tr>
<tr>
<td><strong>Oral Cavity</strong></td>
<td>No/trivial</td>
<td>Present</td>
<td>Consultation only*</td>
<td>Surgical hemostasis or antifibrinolytic</td>
<td>Blood transfusion or replacement therapy or Desmopressin</td>
</tr>
<tr>
<td><strong>GI bleeding</strong></td>
<td>No/trivial</td>
<td>Present (not associated with ulcer, portal hypertension, hemorrhoids, angiodysplasia)</td>
<td>Consultation only*</td>
<td>Surgical hemostasis or antifibrinolytic</td>
<td>Blood transfusion or replacement therapy or Desmopressin</td>
</tr>
<tr>
<td><strong>Hematuria</strong></td>
<td>No/trivial</td>
<td>Present (macroscopic)</td>
<td>Consultation only*</td>
<td>Surgical hemostasis, iron therapy</td>
<td>Blood transfusion or replacement therapy or Desmopressin</td>
</tr>
<tr>
<td><strong>Tooth Extraction</strong></td>
<td>No/trivial or none done</td>
<td>Reported in &lt;25% of all procedures, no intervention**</td>
<td>Reported in &gt;25% of all procedures, no intervention**</td>
<td>Resutting or packing</td>
<td>Blood transfusion or replacement therapy or Desmopressin</td>
</tr>
<tr>
<td><strong>Surgery</strong></td>
<td>No/Trivial or none done</td>
<td>Reported in &lt;25% of all procedures, no intervention**</td>
<td>Reported in &gt;25% of all procedures, no intervention**</td>
<td>Surgical hemostasis or antifibrinolytic</td>
<td>Blood transfusion or replacement therapy or Desmopressin</td>
</tr>
<tr>
<td><strong>Menorrhagia</strong></td>
<td>No/Trivial</td>
<td>Consultation only* or changing pads more frequently than every 2 hours or clot and flooding or PBAC score &gt;100</td>
<td>Time off work/school &gt;2/year or requiring antifibrinolytics or hormonal or iron therapy</td>
<td>Requiring combined treatment with antifibrinolytics and hormonal therapy or IUD or Present since menarche and &gt;12 months</td>
<td>Acute menorrhagia requiring hospital admission and emergency treatment or Blood transfusion, replacement therapy, desmopressin or Dilatation &amp; curettage or endometrial ablation or hysterectomy</td>
</tr>
<tr>
<td><strong>Post-partum hemorrhage</strong></td>
<td>No/Trivial or no deliveries</td>
<td>Consultation only* or use of syntocin or lochia &gt;6 weeks</td>
<td>Iron therapy or antifibrinolytics</td>
<td>Requiring blood transfusion, replacement therapy, desmopressin or requiring examination under anaesthesia and/or the use of uterine balloon/package to tamponade the uterus</td>
<td>Any procedure requiring critical care or surgical intervention (eg hysterectomy, internal iliac artery ligation, uterine artery embolization, uterine brace sutures)</td>
</tr>
<tr>
<td><strong>Muscle hematomas</strong></td>
<td>Never</td>
<td>Post trauma, no therapy</td>
<td>Spontaneous, no therapy</td>
<td>Spontaneous or traumatic, requiring desmopressin or replacement therapy</td>
<td>Spontaneous or traumatic, requiring surgical intervention or blood transfusion</td>
</tr>
<tr>
<td><strong>Hemarthrosis</strong></td>
<td>Never or post trauma, no therapy</td>
<td>Spontaneous, no therapy</td>
<td>Spontaneous, no therapy</td>
<td>Spontaneous or traumatic, requiring desmopressin or replacement therapy</td>
<td>Spontaneous or traumatic, requiring desmopressin or replacement therapy</td>
</tr>
</tbody>
</table>
### Table: Cardiovascular System (CNS) Bleeding

<table>
<thead>
<tr>
<th>CNS bleeding</th>
<th>Never</th>
<th>-</th>
<th>-</th>
<th>Subdural, any intervention</th>
<th>Intracerebral, any intervention</th>
</tr>
</thead>
<tbody>
<tr>
<td>Other bleedings^</td>
<td>No/Trivial</td>
<td>Present</td>
<td>Consultation only*</td>
<td>Surgical hemostasis or antifibrinolytics</td>
<td>Blood transfusion or replacement therapy or desmopressin</td>
</tr>
</tbody>
</table>

In addition to the guidance offered by the table, it is mandatory to refer to the text for more detailed instructions.

§ Distinction between 0 and 1 is of critical importance. Score 1 means that the symptom is judged as present in the patient’s history by the interviewer but does not qualify for a score 2 or more

* Consultation only: the patient sought medical evaluation and was either referred to a specialist or offered detailed laboratory investigation

** Example: Extraction/surgery resulting in bleeding (100%): the score to be assigned is 2; 2 extractions/surgeries, 1 resulting in bleeding (25%): the score to be assigned is 1

# If already available at the time of collection

^ Include: umbilical stump bleeding, cephalohematoma, cheek hematoma caused by sucking during breast/bottle feeding, conjunctival hemorrhage or excessive bleeding following circumcision or venipuncture. Their presence in infancy requires detailed investigation independently from the overall score
Minimal criteria defining what is considered significant or trivial bleeding

For each specific bleeding symptom, the ISTH/SSC joint working group proposed minimal criteria in order to classify a symptom as significant and thus receive a score of 1 or more (see also Table 1):

1. Epistaxis: Any nosebleed, especially occurring after puberty, that causes patient concern (e.g., interference or distress with daily or social activities) is considered significant. In general, epistaxis should not be considered significant when it lasts less than 10 minutes, has a frequency of < 5 episodes/year, has a seasonal occurrence, or is associated with infections of the upper respiratory tract or other identifiable cause (e.g., dusty dry air).

2. Cutaneous bleeding: Bruises are considered significant when 5 or more (> 1cm) in exposed areas; petechiae when adequately described by the patient or relatives; or hematomas when occurring without trauma.

3. Minor cutaneous wound: Any bleeding episode caused by superficial cuts (e.g., by shaving razor, knife, or scissors) or that requires frequent bandage changes is considered significant. Insignificant bleeding from wounds includes those of duration < 10 minutes and lesions that usually require stitches in normal subjects (e.g., under the chin). Symptoms should also be manifest on more than one occasion to be considered significant.

4. Oral cavity bleeding: Gum bleeding should be considered significant when it causes frankly bloody sputum and lasts for 10 minutes or longer on more than one occasion. Tooth eruption or spontaneous tooth loss bleeding should be considered significant when it requires assistance or supervision by a physician, or lasts at least 10 minutes (bleeding associated with tooth extraction is considered separately). Bleeding occurring after bites to lips, cheek, and tongue should be considered significant when it lasts at least 10 minutes or causes a swollen tongue or mouth.

5. Hematemesis, melena, and hematochezia: Any gastrointestinal bleeding that is not explained by the presence of a specific disease should be considered significant.
6. Hematuria: Only macroscopic hematuria (from red to pale-pink urine) that is not explained by the presence of a specific urologic disease should be considered significant.

7. Tooth extraction: Any bleeding occurring after leaving the dentist’s office and requiring a new, unscheduled visit or prolonged bleeding at the dentist’s office causing a delay in the procedure or discharge should be considered significant.

8. Surgical bleeding: Any bleeding judged by the surgeon to be abnormally prolonged, that causes a delay in discharge, or requires some supportive treatment is considered significant.

9. Menorrhagia: Any bleeding that interferes with daily activities such as work, housework, exercise or social activities during most menstrual periods should be considered significant. Criteria for significant bleeding may include any of the following: changing pads more frequently than every 2 hours; menstrual bleeding lasting 7 or more days; and the presence of clots > 1 cm combined with a history of flooding. If a patient has previously made a record of her menstrual loss using a pictorial blood loss assessment chart (PBAC), a PBAC score higher than 100 also qualifies for a score of 1.

10. Post-partum bleeding. Vaginal bleeding or uterine discharge (lochia) that lasts for more than 6 weeks. Any bleeding of lesser duration that is judged by the obstetrician as abnormally heavy or prolonged, that causes a delay in discharge, requires some supportive treatment, requires changing pads or tampons more frequently than every 2 hours, or causes progressive anemia is also considered significant.

11. Muscle hematomas or hemarthrosis. Any spontaneous joint / muscle bleeding (not related to traumatic injuries) is considered significant.

12. CNS bleeding. Any subdural or intracerebral hemorrhage requiring diagnostic or therapeutic intervention is scored 3 or 4, respectively.
Other bleeding symptoms. When these bleeding symptoms occur during infancy, they are scored 1 or more. Their presence when reported by either the patient or a family member should always prompt detailed laboratory investigation.
Appendix E
SF-36v2 +9
SF-36v2 Standard Self-Report for Your Health and Well Being

Your Health in General

Please answer every question. Some questions may look like others, but each one is different. Please take the time to read and answer each question carefully, and mark an □ in the one box that best describes your answer. Thank you for completing this survey!

1. In general, would you say your health is:

<table>
<thead>
<tr>
<th>Excellent</th>
<th>Very good</th>
<th>Good</th>
<th>Fair</th>
<th>Poor</th>
</tr>
</thead>
<tbody>
<tr>
<td>▼</td>
<td>▼</td>
<td>▼</td>
<td>▼</td>
<td>▼</td>
</tr>
<tr>
<td>□1</td>
<td>□2</td>
<td>□3</td>
<td>□4</td>
<td>□5</td>
</tr>
</tbody>
</table>

2. Compared to one year ago, how would you rate your health in general now?

<table>
<thead>
<tr>
<th>Much better now than one year ago</th>
<th>Somewhat better now than one year ago</th>
<th>About the same as one year ago</th>
<th>Somewhat worse now than one year ago</th>
<th>Much worse now than one year ago</th>
</tr>
</thead>
<tbody>
<tr>
<td>▼</td>
<td>▼</td>
<td>▼</td>
<td>▼</td>
<td>▼</td>
</tr>
<tr>
<td>□1</td>
<td>□2</td>
<td>□3</td>
<td>□4</td>
<td>□5</td>
</tr>
</tbody>
</table>

3. The following questions are about activities you might do during a typical day. Does your health now limit you in these activities? If so, how much?

<table>
<thead>
<tr>
<th>Yes, limited a lot</th>
<th>Yes, limited a little</th>
<th>No, not limited at all</th>
</tr>
</thead>
<tbody>
<tr>
<td>▼</td>
<td>▼</td>
<td>▼</td>
</tr>
</tbody>
</table>

a. Vigorous activities, such as running, lifting heavy objects, participating in strenuous sports…….

b. Moderate activities, such as moving a table, pushing a vacuum cleaner, bowling or playing golf…

c. Lifting or carrying groceries ……………………

d. Climbing several flights of stairs ……………

e. Climbing one flight of stairs …………………
Yes, limited a lot ▼ ▼ ▼ ▼ ▼
Yes, limited a little ▼ ▼ ▼ ▼ ▼
No, not limited at all ▼ ▼ ▼ ▼ ▼

f. Bending, kneeling or stooping
1 2 3

g. Walking more than a mile
1 2 3

h. Walking several hundred yards
1 2 3

i. Walking one hundred yards
1 2 3

j. Bathing or dressing yourself
1 2 3

4. During the past week, how much of the time have you had any of the following problems with your work or other regular daily activities as a result of your physical health?

All of the time ▼ ▼ ▼ ▼ ▼
Most of the time ▼ ▼ ▼ ▼ ▼
Some of the time ▼ ▼ ▼ ▼ ▼
A little of the time ▼ ▼ ▼ ▼ ▼
None of the time ▼ ▼ ▼ ▼ ▼

a. Cut down on the amount of time you spent on work or other activities 
1 2 3 4 5

b. Accomplished less than you would like 
1 2 3 4 5

c. Were limited in the kind of work or other activities 
1 2 3 4 5

d. Had difficulty performing the work or other activities (for example, it took extra effort) .. 
1 2 3 4 5

5. During the past week, how much of the time have you had any of the following problems with your work or other regular daily activities as a result of any emotional problems (such as feeling depressed or anxious)?

All of the time ▼ ▼ ▼ ▼ ▼
Most of the time ▼ ▼ ▼ ▼ ▼
Some of the time ▼ ▼ ▼ ▼ ▼
A little of the time ▼ ▼ ▼ ▼ ▼
None of the time ▼ ▼ ▼ ▼ ▼

a. Cut down on the amount of time you spent on work or other activities 
1 2 3 4 5
6. During the past week, to what extent has your physical health or emotional problems interfered with your normal social activities with family, friends, neighbours or groups?

<table>
<thead>
<tr>
<th>Not at all</th>
<th>Slightly</th>
<th>Moderately</th>
<th>Quite a bit</th>
<th>Extremely</th>
</tr>
</thead>
<tbody>
<tr>
<td>▼</td>
<td>▼</td>
<td>▼</td>
<td>▼</td>
<td>▼</td>
</tr>
<tr>
<td>□1</td>
<td>□2</td>
<td>□3</td>
<td>□4</td>
<td>□5</td>
</tr>
</tbody>
</table>

7. How much bodily pain have you had during the past week?

<table>
<thead>
<tr>
<th>None</th>
<th>Very mild</th>
<th>Mild</th>
<th>Moderate</th>
<th>Severe</th>
<th>Very severe</th>
</tr>
</thead>
<tbody>
<tr>
<td>▼</td>
<td>▼</td>
<td>▼</td>
<td>▼</td>
<td>▼</td>
<td>▼</td>
</tr>
<tr>
<td>□1</td>
<td>□2</td>
<td>□3</td>
<td>□4</td>
<td>□5</td>
<td>□6</td>
</tr>
</tbody>
</table>

8. During the past week, how much did pain interfere with your normal work (including both work outside and inside the home and housework)?

<table>
<thead>
<tr>
<th>Not at all</th>
<th>A little bit</th>
<th>Moderately</th>
<th>Quite a bit</th>
<th>Extremely</th>
</tr>
</thead>
<tbody>
<tr>
<td>▼</td>
<td>▼</td>
<td>▼</td>
<td>▼</td>
<td>▼</td>
</tr>
<tr>
<td>□1</td>
<td>□2</td>
<td>□3</td>
<td>□4</td>
<td>□5</td>
</tr>
</tbody>
</table>

9. These questions are about how you feel and how things have been with you during the past week. For each question, please give the one answer that comes closest to the way you have been feeling. How much of the time during the past week…

<table>
<thead>
<tr>
<th>All of the time</th>
<th>Most of the time</th>
<th>Some of the time</th>
<th>A little of the time</th>
<th>None of the time</th>
</tr>
</thead>
<tbody>
<tr>
<td>▼</td>
<td>▼</td>
<td>▼</td>
<td>▼</td>
<td>▼</td>
</tr>
<tr>
<td>□1</td>
<td>□2</td>
<td>□3</td>
<td>□4</td>
<td>□5</td>
</tr>
</tbody>
</table>
c. Have you felt so down in the dumps that nothing could cheer you up? .................. □ 1  □ 2  □ 3  □ 4  □ 5

d. Have you felt calm and peaceful? .............. □ 1  □ 2  □ 3  □ 4  □ 5

e. Did you have a lot of energy? ..................... □ 1  □ 2  □ 3  □ 4  □ 5

f. Have you felt downhearted and depressed? .... □ 1  □ 2  □ 3  □ 4  □ 5

g. Did you feel worn out? ........................... □ 1  □ 2  □ 3  □ 4  □ 5

h. Have you been happy? ............................. □ 1  □ 2  □ 3  □ 4  □ 5

i. Did you feel tired? ................................. □ 1  □ 2  □ 3  □ 4  □ 5

10. During the past week, how much of the time has your physical health or emotional problems interfered with your social activities (like visiting with friends, relatives, etc.)?

<table>
<thead>
<tr>
<th>All of the time</th>
<th>Most of the time</th>
<th>Some of the time</th>
<th>A little of the time</th>
<th>None of the time</th>
</tr>
</thead>
<tbody>
<tr>
<td>□ 1</td>
<td>□ 2</td>
<td>□ 3</td>
<td>□ 4</td>
<td>□ 5</td>
</tr>
</tbody>
</table>

11. How TRUE or FALSE is each of the following statements for you?

<table>
<thead>
<tr>
<th>Definitely true</th>
<th>Mostly true</th>
<th>Don’t know</th>
<th>Mostly false</th>
<th>Definitely false</th>
</tr>
</thead>
<tbody>
<tr>
<td>□ 1</td>
<td>□ 2</td>
<td>□ 3</td>
<td>□ 4</td>
<td>□ 5</td>
</tr>
</tbody>
</table>

a. I seem to get sick a little easier than other people .................. □ 1  □ 2  □ 3  □ 4  □ 5

b. I am as healthy as anyone I know .............. □ 1  □ 2  □ 3  □ 4  □ 5

c. I expect my health to get worse .............. □ 1  □ 2  □ 3  □ 4  □ 5

d. My health is excellent .......................... □ 1  □ 2  □ 3  □ 4  □ 5
12. Please indicate to what extent you agree/disagree with each of the following statements.

<table>
<thead>
<tr>
<th>Statement</th>
<th>Strongly Disagree</th>
<th>Disagree</th>
<th>Neither agree nor disagree</th>
<th>Agree</th>
<th>Strongly agree</th>
</tr>
</thead>
<tbody>
<tr>
<td>a. I feel I am responsible for my child’s bleeding disorder</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>b. I feel guilty about my child’s state of health</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>c. I sometimes feel my child’s medical condition is burden</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>d. I wish there was a cure for my child’s bleeding disorder</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>e. I have access to carrier testing facilities</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>f. I have access to genetic counselling services</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>g. I have access to prenatal diagnosis services</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>h. I have access to a multidisciplinary team</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>i. I seek medical attention whenever I have a bleeding episode</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
</tbody>
</table>

Thank-you!
Appendix F

X-Inactivation Protocol
X inactivation

Purpose:
To provide instructions for the study of X inactivation.

Procedure:

Restriction Digest

Equipment & Supplies:

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Supplies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pipetman (p2, p10, p20, p200, p1000)</td>
<td>1.5 mL Eppendorf tubes</td>
</tr>
<tr>
<td>Vortex</td>
<td>Hap II (10 U/µL) (Invitrogen)</td>
</tr>
<tr>
<td>Microfuge</td>
<td>Hha I (10 U/µL) (Invitrogen)</td>
</tr>
<tr>
<td>37°C waterbath</td>
<td>BamHI (15 U/µL) (Invitrogen)</td>
</tr>
<tr>
<td>1.5 mL Eppendorf tubes</td>
<td>React 5 Digest Buffer (10 mM Tris-HCl pH 8.2; 8 mM MgCl₂) (&quot;Buffer 5&quot;)</td>
</tr>
<tr>
<td>1.0 µL BamHI (15 U/µL)</td>
<td>Autoclaved ddH₂O (deionized distilled)</td>
</tr>
<tr>
<td>24 µL 1 µg DNA + autoclaved ddH₂O (deionized distilled)</td>
<td>Sarstedt 0.2 mL 8-strip tubes</td>
</tr>
<tr>
<td>1.0 µL</td>
<td>Agencourt Ampure XP beads</td>
</tr>
<tr>
<td></td>
<td>Agencourt SPRIPlate 96 Super plate</td>
</tr>
</tbody>
</table>

Procedure:

1. Determine the required number of restriction digests as follows:
   
   Total # of restriction digest tubes for Set-up = (# of patient specimens) + (controls) + 1.

2. Label the required number of 1.5 mL Eppendorf tubes, using the same numbering as on your SMS worksheet. Be sure to digest appropriate controls. Refer to Control Binder for most recent controls (1 normal female, 1 normal male (not mixed or from cell line), skewed female). See control binder.

3. Use the following table to make a "Mastermix" (i.e. solution containing all reagents for the total # of digest reactions except DNA and autoclaved ddH₂O (deionized distilled). To do this, multiply the volume required for each reagent by the "total # of PCR tubes for set-up". Combine all reagents, in the order listed, in a 1.5 mL Eppendorf tube.

   24 µL 1 µg DNA + autoclaved ddH₂O (deionized distilled) 
   1.0 µL BamHI (15 U/µL)
1.0 µL Hap II (10 U/µL)  
1.0 µL Hha I (10 U/µL)  
3.0 µL React 5 Digest Buffer (10 mM Tris-HCl pH 8.2; 8 mM MgCl₂)  
30.0 µL Total volume per reaction (including DNA)

Note:
- To make 2 mL of React 5 Digest Buffer (10 mM Tris-HCl pH 8.2; 8 mM MgCl₂)

  - 20 µL 1 M Tris-HCl pH 8.2  
  - 640 µL 25 mM MgCl₂  
  - 1340 µL ddH₂O  
  - 2000 µL total volume

- Filter sterilize.

4. Aliquot 1 µg DNA of each sample to be tested (including controls) and appropriate volume of water into your prelabeled 1.5 mL tubes (total DNA + ddH₂O = 40.5 µL). Distribute your mastermix into each of your tubes (in this case the volume of mastermix to add to each tube should be 9.5 µL/tube). Vortex briefly and quick spin in a microfuge.

5. Place tubes in a 37°C waterbath for 5 hours. Do not digest overnight.

**DNA Purification**

1. Add 54 uL Agencout AMPure XP beads to 30 uL digested DNA. Mix by pipetting up and down 10 times. Transfer the mixture to 8-strip reaction tubes. Incubate at room temperature for 10 minutes (mix by pipetting up and down midway through incubation).

2. Place the 8-strip reaction tubes onto an Agencourt SPRIPlate 96 Super Plate for 5-10 minutes to separate beads from solution.

3. Keep the 8-strip reaction tubes on Agencourt SPRIPlate 96 Super Plate. Insert p200 tip straight into the bottom of the tubes without disturbing the ring of separated magnetic beads on tube walls. Aspirate the cleared solution from the reaction plate and discard.

4. Dispense 200 uL fresh prepared 70% ethanol to each reaction tube. Mix by pipetting up and down 5 times. Incubate for 2-5 minutes at room temperature on Agencourt SPRIPlate 96 Super Plate until clear. Aspirate out all the 70% ethanol and discard. Repeat 2 more times.
5. After final ethanol wash, leave the reaction tubes open to air dry for 15 minutes on the Agencourt SPRIPlate 96 Super Plate. Make sure the tubes are dried completely.

6. Add 30 µL TE pH 8.0 to dried reaction tubes. Close tubes and vortex for 20 seconds at maximum speed twice. Quick spin down. Mix by pipetting up and down 10 times. Ensure that the solution is at bottom of tube.

7. Place the reaction tubes onto the Agencourt SPRIPlate 96 Super Plate for 5 minutes at room temperature.

8. Use a p20 to aspirate 20 µL cleared eluent and transfer into a new tube for PCR amplification step.

**DNA Amplification**

**Equipment & Supplies:**

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Supplies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pipetman (p10, p20, p200, p1000)</td>
<td>1.5 mL Eppendorf tubes</td>
</tr>
<tr>
<td>Vortex</td>
<td>0.2 mL thin walled PCR Tubes</td>
</tr>
<tr>
<td>Microfuge</td>
<td>1.5 mM MgCl₂ AR buffer, -20°C</td>
</tr>
<tr>
<td>Perkin Elmer 9700 or Bio-Rad Tetrad</td>
<td>Forward primer (AR-a) (5 pmol/µL) (5’FAM labelled) ACCAGGTAGCCTGTGGGGCCTCTACGATGGGC</td>
</tr>
<tr>
<td></td>
<td>Reverse primer (AR-b) (5 pmol/µL) CCAGAGCGTGCGCGAAGTGATCCAGAACCC</td>
</tr>
<tr>
<td></td>
<td>AmpliTaq® DNA Polymerase</td>
</tr>
<tr>
<td></td>
<td>7-deaza dGTP, -20°C (Pharmacia #27-2090-01)</td>
</tr>
<tr>
<td></td>
<td>DMSO, RT (Fisher Scientific # D128-500)</td>
</tr>
<tr>
<td></td>
<td>DNA, 200 ng/µl concentration, 4°C</td>
</tr>
<tr>
<td></td>
<td>dNTP’s, -20°C (Pharmacia #27-2038-01)</td>
</tr>
<tr>
<td></td>
<td>Autoclaved ddH₂O (deionized distilled)</td>
</tr>
</tbody>
</table>
Note:

- Each sample must have 2 PCR reactions set up: one with digested DNA and one with undigested DNA.

Procedure:

1. Determine the required number of PCR reactions as follows:

   Total # of samples for Set-up = 2 X (# of patient specimens + (controls) + 1.

2. Label the required number of 0.2 mL thin-walled PCR tubes, using the same numbering as on your SMS worksheet. Be sure to amplify controls and water blank. Refer to Control Binder for most recent controls.

3. Use the following table to make a "Mastermix" (i.e. solution containing all reagents for the total # of PCR reactions except DNA+ddH\textsubscript{2}O). To do this, multiply the volume required for each reagent by the "total # of PCR tubes for set-up". Combine all reagents, in the order listed, in a 1.5 mL Eppendorf tube.

   \[
   \begin{align*}
   x \mu L & \quad 100 \text{ ng DNA uncut (or } 3.0 \mu L \text{ cut and purified DNA)} + \text{ autoclaved ddH}_2\text{O (deionized distilled)} \\
   10.0 \mu L & \quad \text{AR buffer (see attached)} \\
   1.0 \mu L & \quad \text{forward primer (AR-a FAM) (5 pmol/\mu L)} \\
   1.0 \mu L & \quad \text{reverse primer (AR-b) (5 pmol/\mu L)} \\
   0.25 \mu L & \quad \text{AmpliTaq\textregistered DNA Polymerase} \\
   15.0 \mu L & \quad \text{Total volume per reaction (including DNA)}
   \end{align*}
   \]

4. Aliquot 100 ng DNA (undigested) or 3.0 µL digested and purified DNA + autoclaved ddH\textsubscript{2}O (deionized distilled) of each sample to be tested (including controls) into your prelabeled tubes. Distribute your mastermix into each of your PCR tubes (in this case the volume of mastermix to add to each tube should be 12.25 µL/tube). Vortex briefly and quick spin in a microfuge.

5. Immediately place your PCR reactions into a PCR machine and run the following conditions:

<table>
<thead>
<tr>
<th>Undigested</th>
<th>Machine: MJ-AR</th>
</tr>
</thead>
</table>
| Step Cycle | 95°C - 5 minutes (Ramp 0.6°C/sec)  
55°C - 25 seconds (1 cycles) (Ramp 0.3°C/sec)  
72°C - 25 seconds |
### Preparation and Analysis

#### Equipment & Supplies:

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Supplies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pipetman (p 2, p10, p20, p200)</td>
<td>ROX 500, 2-8°C (LIZ)</td>
</tr>
<tr>
<td>Vortex</td>
<td>HiDi Formamide</td>
</tr>
<tr>
<td>Microfuge</td>
<td>1.5 mL Eppendorf tubes</td>
</tr>
<tr>
<td>ABI PRISM 3130 Sequencer</td>
<td></td>
</tr>
</tbody>
</table>

#### Procedure:

1. Prepare PCR products for electrophoresis on ABI 3130 Capillary Sequencer.
2. Prepare your PCR reactions for analysis by making a "mastermix" of the following reagents. To do this, multiply the volume required for each reagent by the total number of PCR reactions +3. Add each reagent to a 1.5 mL Eppendorf tube.

   - 8.7 µL HiDi Formamide
   - 0.3 µL ROX 500 (LIZ)
   - 9.0 µL total (reagent) volume per reaction
3. Fill out a plate loading sheet indicating the plate location of each sample.
4. Pipette 9.0 µL of mastermix into each sample. Pipette 10.0 µL of HiDi formamide into any of the unused wells.
5. Add **1.5 µL** of your undigested PCR product or **3.0 µL** digested PCR product to ABI Micro-Amp plate containing mastermix. Vortex briefly and quick spin. Store products in the dark until ready to run.
6. Cover with thermaseal foil and quick spin the plate in a centrifuge to force all liquid to the bottom of the well.
7. Prior to running the plate remove the thermaseal foil and replace with 96-well plate septa. Wipe the bottom of the plate to remove any moisture before transferring to the plate holder and retainer.
8. Prepare to run the 3130 Capillary sequencer. Refer to "Operating the ABI PRISM 3130 XL Genetic Analyzer", OMG4015 ==>  mù. Set up the following specific conditions:

<table>
<thead>
<tr>
<th>Running Conditions</th>
<th>3130</th>
</tr>
</thead>
<tbody>
<tr>
<td>Instrument Protocol</td>
<td>D_POP-4_36_10s</td>
</tr>
<tr>
<td>Results Group</td>
<td>Results Group</td>
</tr>
<tr>
<td>Analysis Method</td>
<td>X-inactivation-25 window</td>
</tr>
<tr>
<td>Panel</td>
<td>None</td>
</tr>
<tr>
<td>Size Standard</td>
<td>GS 500(-250)</td>
</tr>
<tr>
<td>Sample Type</td>
<td>Sample</td>
</tr>
</tbody>
</table>

Run Module is “FragmentAnalysis36_POP4_10sec”
The run voltage is 15
Run Time is 1500
Injection Time is 10sec
Dye Set is D

Gene Mapper settings for Analysis Method:
Min Peak Half Width is 2
Polynomial is 3
Peak Window width is 29 pts
Baseline Window is 51

> Results Capture and Interpretation
1. Ensure that DNA is completely digested by checking male control (no amplification after digestion) and that skewed heterozygous female control matches previous expected results.

2. Amplified fragments are in the ~250-300 bp range. The non-repeat size is 178 bp.

   \[ \# \text{ repeats} = \frac{\text{fragment size} - 178\text{bp}}{3} \]

3. Use the X-Inactivation Excel Spreadsheet (OMG1430B) to do the following calculation for X inactivation:

   **For uncut PCR product:**

   i) Correct for preferential PCR amplification in the **uncut PCR product** (two peaks have different peak heights).

   1 = allele with greater uncut peak height

   2 = allele with smaller uncut peak height

   Correction value (correction for smaller peak height (2)) = greater uncut peak height (1)
   
   smaller uncut peak height (2)

   **For Cut product:**

   i) Calculate the percentage X inactivation of the **allele with greater uncut peak height, ‘1’**, by normalizing the sum of alleles to 100% (cut PCR product).

   \[
   \frac{\text{Cut peak height of ‘1’} \times 100}{\text{Cut peak height of ‘1’} + (\text{Cut peak height of ‘2’} \times \text{Correction value})}
   \]

   ii) Calculate the percentage X inactivation of the **allele with smaller uncut peak height, ‘2’** by normalizing the sum of alleles to 100% (cut PCR product).

   \[
   \frac{\text{Cut peak height of ‘2’} \times \text{Correction value} \times 100}{\text{Cut peak height of ‘1’} + (\text{Cut peak height of ‘2’} \times \text{Correction value})}
   \]

**Quality Control**

1. If the uncut controls and cut female controls do not amplify, the analysis must be repeated.
2. If the cut male control is amplified, the analysis must be repeated (digestion did not work).
3. If amplification product is seen in the blank, discard the H₂O (or buffer, primers) and repeat the analysis.

**Recording of Results**

1. The results of the assay are recorded on the worksheet and entered into SMS database:

Example:

Choose from pull down list for specific allele sizes.

<table>
<thead>
<tr>
<th>Sex</th>
<th>X inactivation</th>
<th>% of inactive X chromosome (Allele 1 / Allele 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>X chromosome active</td>
<td>23 (0%)</td>
</tr>
<tr>
<td>Female</td>
<td>Random (heterozygous)</td>
<td>23 (52%) / 25 (48%)</td>
</tr>
<tr>
<td>Female</td>
<td>Skewed (heterozygous)</td>
<td>23 (88%) / 25 (12%)</td>
</tr>
<tr>
<td>Female</td>
<td>Homozygous</td>
<td>22 (N/A%) / 22 (N/A%)</td>
</tr>
</tbody>
</table>

**Preparation of AR Buffer (also X-Inactivation PCR Buffer)**

**Equipment and Supplies:**

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Supplies</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Pipetman (p1000, p200, p20)</td>
<td>• Applied Biosystems 10 X Buffer II (does not contain MgCl₂)</td>
</tr>
<tr>
<td>• Vortex</td>
<td>• Applied Biosystems 25 mM MgCl₂</td>
</tr>
<tr>
<td>• Microfuge</td>
<td>• 100 mM dATP, dCTP, dTTP stocks</td>
</tr>
<tr>
<td>• Electronic pipet aid</td>
<td>• 5 mM 7-deaza dGTP</td>
</tr>
<tr>
<td></td>
<td>• DMSO</td>
</tr>
<tr>
<td></td>
<td>• Autoclaved ddH₂O</td>
</tr>
<tr>
<td></td>
<td>• 6 mL snap cap polypropylene tubes</td>
</tr>
<tr>
<td></td>
<td>• 1.5 mL Eppendorf tubes</td>
</tr>
<tr>
<td></td>
<td>• Filter tips (1000, 200, 20 µL)</td>
</tr>
</tbody>
</table>
**Preparation Procedure:**

<table>
<thead>
<tr>
<th>Step</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Thaw Applied Biosystems 10 X PCR Buffer II, 100 mM dATP, dCTP, dTTP, 5 mM 7-deaza dGTP, Applied Biosystems 25 mM MgCl₂ and a new aliquot of autoclaved ddH₂O from freezer. Once thawed keep all reagents on ice.</td>
</tr>
<tr>
<td>2.</td>
<td>While solutions are thawing, record lot numbers, date made, and your initials on &quot;AR Buffer Form&quot;, OMG1543A ==&gt; [INSERT IMAGE]. Once buffer is tested, the SMS number and date should also be recorded on this form.</td>
</tr>
<tr>
<td>3.</td>
<td>Vortex all solutions for 1 minute. Quick spin in microfuge.</td>
</tr>
<tr>
<td>4.</td>
<td>Measure out 1456 µL ddH₂O using a p1000 pipetman and put in 6 mL snap cap polypropylene tube. Place on ice.</td>
</tr>
<tr>
<td>5.</td>
<td>Add 400 µL Applied Biosystems 10 X PCR Buffer II using a p1000 pipetman.</td>
</tr>
<tr>
<td>6.</td>
<td>Add 240 µL Applied Biosystems 25 mM MgCl₂ using a p200 pipetman.</td>
</tr>
<tr>
<td>7.</td>
<td>Add 8 µL each of 100 mM dATP, dCTP, dTTP stocks (i.e. do not add dGTP) using a p20 pipetman.</td>
</tr>
<tr>
<td>8.</td>
<td>Add 160 µL 5 mM 7-deaza dGTP (stored in PrePCR FraX box) using a p200 pipetman.</td>
</tr>
<tr>
<td>9.</td>
<td>Add 400 µL DMSO using a p1000 pipetman.</td>
</tr>
<tr>
<td>11.</td>
<td>Mix well using vortex.</td>
</tr>
<tr>
<td>12.</td>
<td>Aliquot into 1.5 mL Eppendorf tubes.</td>
</tr>
<tr>
<td>13.</td>
<td>Follow &quot;Reagent Preparation&quot;, OMG1500 ==&gt; [INSERT IMAGE] and label each tube with date, &quot;AR Buffer&quot;, and number each tube sequentially so they can be used in order. Fill out &quot;AR Buffer Form&quot;, OMG1543A ==&gt; [INSERT IMAGE] appropriately.</td>
</tr>
</tbody>
</table>
Appendix G

Research Ethics Board (REB) Approval
QUEEN'S UNIVERSITY HEALTH SCIENCES AND AFFILIATED TEACHING HOSPITALS
RESEARCH ETHICS BOARD ANNUAL RENEWAL

Queen's University, in accordance with the "Tri-Council Policy Statement 2, 2010" prepared by the
Interagency Advisory Panel on Research Ethics for the Canadian Institutes of Health Research, Natural
Sciences and Engineering Research Council of Canada and Social Sciences and Humanities Research Council
of Canada requires that research projects involving human participants be reviewed annually to determine their
acceptability on ethical grounds.

A Research Ethics Board composed of:

Dr. A.F. Clark, Emeritus Professor, Department of Biomedical and Molecular Sciences, Queen's University
(Chair)
Dr. H. Abdollah, Professor, Department of Medicine, Queen's University
Dr. C. Cline, Assistant Professor, Department of Medicine, Director, Office of Bioethics, Queen's University,
Clinical Ethicist, Kingston General Hospital
Dr. R. Brison, Professor, Department of Emergency Medicine, Queen's University
Dr. M. Evans, Community Member
Ms. J. Hudacin, Community Member
Mr. D. McNaughton, Community Member
Ms. S. Rohland, Privacy Officer, ICES-Queen's Health Services Research Facility, Research Associate,
Division of Cancer Care and Epidemiology, Queen's Cancer Research Institute
Dr. M. Sawhney, Assistant Professor, School of Nursing, Queen's University
Dr. A. Singh, Professor, Department of Psychiatry, Queen's University
Dr. J. Walia, Assistant Professor and Clinical Geneticist, Department of Paediatrics, Queen's University and
Kingston General Hospital
Ms. K. Weisbaum, LL.B. and Adjunct Instructor, Department of Family Medicine (Bioethics)

has reviewed the request for renewal of Research Ethics Board approval for the project “Validation of Self-
BAT (Self-administered Bleeding Assessment Tool) in Hemophilia Carriers.” as proposed by
Dr. P. James of the Department of Medicine, at Queen's University. The approval is renewed for one year,
effective June 07, 2014. If there are any further amendments or changes to the protocol affecting the
participants in this study, it is the responsibility of the principal investigator to notify the Research Ethics
Board. Any unexpected serious adverse event occurring locally must be reported within 2 working days or
earlier if required by the study sponsor. All other adverse events must be reported within 15 days after
becoming aware of the information.

Date: May 23, 2014
Chair, Health Sciences Research Ethics Board
Renewal 1[x] Renewal 2 [ ] Extension [ ] Code# DMED-1612-13 Romeo file# 6009925
Appendix H
F8 & F9 Gene Sequencing Primers and PCR Conditions
## F8/F9 Primer Sequences

<table>
<thead>
<tr>
<th>Exon</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Annealing Temp °C</th>
<th>Mg (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F8-Exon 1</td>
<td>5’-ACT GCT CTC AGA AGT GAA TG-3’</td>
<td>5’-GTA GCA TCA CAA CCA TCC TA-3’</td>
<td>55</td>
<td>1.5</td>
</tr>
<tr>
<td>F8-Exon 2</td>
<td>5’-CCT CCT TGC TAA TAG TAG AA-3’</td>
<td>5’-ATT CTC TTT GGC AGC TGC AC-3’</td>
<td>55</td>
<td>1.0</td>
</tr>
<tr>
<td>F8-Exon 3</td>
<td>5’-ACT GTG ACC TTG ACT CTA A -3’</td>
<td>5’-ATC TAG TAA ATG TTA AGA AAT-3’</td>
<td>55</td>
<td>1.5</td>
</tr>
<tr>
<td>F8-Exon 4</td>
<td>5’-CAG AGT GAG ACT CCG TCT CAA-3’</td>
<td>5’-GAT TCA GTT GTT TGT ACT TCT C-3’</td>
<td>56</td>
<td>1.5</td>
</tr>
<tr>
<td>F8-Exon 5</td>
<td>5’-TTC TTA CTG TCA AGT AAC TG-3’</td>
<td>5’-CTG AAG AGT AGT AAT GTA ATT TA-3’</td>
<td>55</td>
<td>2.0</td>
</tr>
<tr>
<td>F8-Exon 6</td>
<td>5’-TCC CAC TTA TTG TCA TGG AC-3’</td>
<td>5’-TAC AGA ACT CTG GTG CTG AA-3’</td>
<td>55</td>
<td>1.5</td>
</tr>
<tr>
<td>F8-Exon 7</td>
<td>5’-GGC AAG AGC TGT TGG TTT G-3’</td>
<td>5’-ATT AAA AGT AGG ACT GGA TAT-3’</td>
<td>55</td>
<td>1.5</td>
</tr>
<tr>
<td>F8-Exon 8</td>
<td>5’-CCA TAT AGC CTG CAG AAC AT-3’</td>
<td>5’-CTG ATG CTC AGC TAT GTT AG-3’</td>
<td>55</td>
<td>1.5</td>
</tr>
<tr>
<td>F8-Exon 9</td>
<td>5’-TGA ATC ATA TAA GCT GTT TTA G-3’</td>
<td>5’-AGA TAT GTC CAT TGG AGA CAA-3’</td>
<td>55</td>
<td>1.5</td>
</tr>
<tr>
<td>F8-Exon 10</td>
<td>5’-CTA GCC TCA AAT TAC TAT AAT-3’</td>
<td>5’-ACT TTA GAC TGG AGC TTG AG-3’</td>
<td>55</td>
<td>1.0</td>
</tr>
<tr>
<td>F8-Exon 11</td>
<td>5’-TCG GAC TTT AGC TTC CAC TT-3’</td>
<td>5’-GGA CAT ACA CTG AGA ATG-3’</td>
<td>50</td>
<td>1.5</td>
</tr>
<tr>
<td>F8-Exon 12</td>
<td>5’-TCG CAT CGC TTT CAT CAT AG-3’</td>
<td>5’-CAT TCA TTA TCT GGA CAT CAC-3’</td>
<td>56</td>
<td>1.5</td>
</tr>
<tr>
<td>F8-Exon 13</td>
<td>5’-GGC ATA ACA TAT AAT TCC T-3’</td>
<td>5’-CCT CAA GCA AGA GAA TGC TA-3’</td>
<td>50</td>
<td>1.5</td>
</tr>
<tr>
<td>F8-Exon 14-1</td>
<td>5’-GAC CTG TGA TAT AAT GAT ACT G-3’</td>
<td>5’-TGT GGC CTG AAG TGT GTC AT-3’</td>
<td>60</td>
<td>1.5</td>
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<tr>
<td>F8-Exon 14-2</td>
<td>5’-TGC CAC CAC AAT TCC AGA AAA-3’</td>
<td>5’-TAC CAC TCT CTG TTG ACG ATA-3’</td>
<td>60</td>
<td>1.5</td>
</tr>
<tr>
<td>F8-Exon 14-3</td>
<td>5’-GCA GCA GGT ACT GAT AAT AC-3’</td>
<td>5’-AGA GTT CTT TCC ATG AGT CC-3’</td>
<td>60</td>
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<tr>
<td>F8-Exon 14-4</td>
<td>5’-GAA TAG TCC ATC AGT CTG GC-3’</td>
<td>5’-GAA CCT TCT ACA TTT TGC CTA G-3’</td>
<td>60</td>
<td>1.5</td>
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<tr>
<td>F8-Exon 14-5</td>
<td>5’-TAC AAA GGA CGT AGG ACT CA-3’</td>
<td>5’-TCT CAT TGT AGT CTA TCT GTG-3’</td>
<td>60</td>
<td>1.5</td>
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<tr>
<td>F8-Exon 14-6</td>
<td>5’-ATA TGC ATG CAC CAC AAG GAT-3’</td>
<td>5’-CAT TAG TGC TTT CCG TAC GGT-3’</td>
<td>60</td>
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<tr>
<td>F8-Exon 14-7</td>
<td>5’-TTA GCC ATT CTA ACC TTG GAG-3’</td>
<td>5’-CCT CTT GAT CTG ACT GAA GA-3’</td>
<td>60</td>
<td>1.5</td>
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<tr>
<td>F8-Exon 14-8</td>
<td>5’-ACT CAG ATA CCA AAA GAA GAG-3’</td>
<td>5’-TAA GAG TTT CAA GAC ACC TTG-3’</td>
<td>60</td>
<td>1.5</td>
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<tr>
<td>F8-Exon 15</td>
<td>5’-AGA TGA AGT GGT TAA CTA TGC-3’</td>
<td>5’-GTG GGA ATA CAT TAT AGT CAG-3’</td>
<td>55</td>
<td>1.5</td>
</tr>
<tr>
<td>Exon</td>
<td>5' Sequence</td>
<td>3' Sequence</td>
<td>Temperature</td>
<td>GC Ratio</td>
</tr>
<tr>
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<tr>
<td>F8-Exon 16</td>
<td>5'-GTC GTT ATT GTT CTA CAG-3'</td>
<td>5'-TCA GTA GAT TCC AGA ATG ACA-3'</td>
<td>58</td>
<td>1.5</td>
</tr>
<tr>
<td>F8-Exon 17</td>
<td>5'-TGT CAT TCT GGA ATC TAC TGA-3'</td>
<td>5'-CAC TCC CAC AGA TAT ACT CT-3'</td>
<td>55</td>
<td>1.5</td>
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<tr>
<td>F8-Exon 18</td>
<td>5'-AGA GTA TAT CTG TGG GAG TG-3'</td>
<td>5'-CTT AAG AGC ATG GAG CTT GT-3'</td>
<td>56</td>
<td>1.5</td>
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<tr>
<td>F8-Exon 19</td>
<td>5'-GCA AGC ACT TTG CAT TTG AG-3'</td>
<td>5'-AGC AAC CAT TCC AGA AAG GC-3'</td>
<td>55</td>
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<tr>
<td>F8-Exon 20</td>
<td>5'-TGA ATC ATT CTA GCA CCT GT-3'</td>
<td>5'-GAG AGG CAC TTA TGG AAT AGA-3'</td>
<td>59</td>
<td>1.5</td>
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<tr>
<td>F8-Exon 21</td>
<td>5'-GAC AAC AAG GAT AAG CAA TAT C-3'</td>
<td>5'-GAG TGA ATG TGA TAC ATT TCC-3'</td>
<td>55</td>
<td>1.5</td>
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<tr>
<td>F8-Exon 22</td>
<td>5'-AAA TAG GTT AAA ATA AAG TGT TAT-3'</td>
<td>5'-TGG AAG CTA AGA GTG TTG TC-3'</td>
<td>55</td>
<td>1.5</td>
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<tr>
<td>F8-Exon 23</td>
<td>5'-ATC GTA ACA TCC ATC TAA CCA-3'</td>
<td>5'-AGT CTC AGG ATA ACT AGA ACA-3'</td>
<td>55</td>
<td>1.5</td>
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<td>F8-Exon 24</td>
<td>5'-CAG TGG AAG CTG CTC AGT AT-3'</td>
<td>5'-CCC ATA ACC AAA CTT CCT TG-3'</td>
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<td>1.5</td>
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<tr>
<td>F8-Exon 25</td>
<td>5'-AGT GCT GTG GTA TGG TTA AG-3'</td>
<td>5'-TTG CTC TGA AAA TTT GGT CAT A-3'</td>
<td>59</td>
<td>1.5</td>
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<tr>
<td>F8-Exon 26</td>
<td>5'-GGT TTA ATC CGT GAC TAC TG-3'</td>
<td>5'-GGC AGT GTC TGC TAG GAT TTA-3'</td>
<td>56</td>
<td>1.5</td>
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<tr>
<td>F9-Exon A</td>
<td>5'-CCA CTC ATA CAT TGC TGA TGG A-3'</td>
<td>5'-CCT AGC TAA CAA AGA ACC AGT-3'</td>
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<tr>
<td>F9-Exon B+C</td>
<td>5'-TCA CAG ATT TTT GGC TCC ATG-3'</td>
<td>5'-GCA GAG AAA CCC ACA TAAT-3'</td>
<td>58</td>
<td>2.0</td>
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<tr>
<td>F9-Exon D</td>
<td>5'-GCT AGT AGT TTT GCT CTG AC-3'</td>
<td>5'-GTT TAT AAG CAT CAA AGG TAT-3'</td>
<td>58</td>
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<tr>
<td>F9-Exon E</td>
<td>5'-ATG AGT CAG TAG TIC CAT GTA C-3'</td>
<td>5'-TAG GTT TGT TAA AAT GCT GAA G-3'</td>
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<td>F9-Exon F</td>
<td>5'-AGG ATG GGC CTC AAT CTC AAT-3'</td>
<td>5'-CTT CTC ACA TCC CAA TAG GTC-3'</td>
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<td>F9-Exon G</td>
<td>5'-AAG CTC ACA TTT CCA GAA AC-3'</td>
<td>5'-TGG GTT CTG AAA TTA TGA C-3'</td>
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<td>1.5</td>
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<tr>
<td>F9-Exon H</td>
<td>5'-TAA GAA TGA GAT CTT TAA CA-3'</td>
<td>5'-AAG ATG GGA AAG TGA TTA GTT A-3'</td>
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<td>2.0</td>
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