


BASIC SCIENCE ARTICLE



Can T-cell and B-cell excision circles predict development of inhibitors in pediatric hemophilia A?

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BACKGROUND: Hemophilia A (HA) therapy requires intravenous replacement infusions of factor (F) VIII concentrate. Inhibitors are high-affinity immunoglobulin G that are directed against FVIII and thereby render replacement therapy ineffective. This complication has significant prognostic implications. We aimed to examine the immune system involvement in inhibitor formation specifically T-cell excision circles (TRECs) and B-cell excision circles (KRECs), markers of new T and B cells, respectively, and examine them as surrogate markers for inhibitor formation.

METHODS: Blood samples were collected from 35 children with severe HA. Children were divided into two groups: with FVIII inhibitors and without FVIII inhibitors. TRECs and KRECs were measured in peripheral blood.

RESULTS: A total of 11 patients with inhibitors and 24 without were evaluated. Children with inhibitors had higher levels of TRECs however not statistically significant ($p = 0.085$). CjKREC levels were higher in the inhibitor patients ($p = 0.003$). Moreover, the sj/cjKREC ratio was lower in the inhibitor patients ($p = 0.015$).

CONCLUSIONS: Our findings may add to the notion that inhibitor formation is attributed to humoral immunity due to peripheral B-cell expansion and loss of peripheral tolerance. Improved knowledge regarding the involvement of the immune system in the formation of FVIII inhibitors will enable better therapy tailoring in the era of non-replacement therapies.

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IMPACT:

- The etiology of FVIII inhibitor formation is multifactorial, in which the immune system plays a pivotal role.
- Our findings may add to the notion that inhibitor formation is attributed to humoral immunity due to peripheral B-cell expansion and production of antibodies against FVIII.
- Improved knowledge regarding the involvement of the immune system in the development of FVIII inhibitors will enable the identification of patients prone to inhibitor development and better therapy tailoring in the new era of non-replacement therapies.

INTRODUCTION

Hemophilia A (HA) is a common inherited bleeding disorder caused by mutations in the *F8* gene and is characterized as a functional or quantitative deficiency of coagulation factor VIII (FVIII). Its severity is classified according to the patient's FVIII clotting activity in plasma (FVIII:C): severe if <1%, moderate if between 1 and 5%, and mild if >5 and <40% of normal.¹ Replacement therapy requires frequent, repeated intravenous lifelong infusions of FVIII concentrate. The major complication of this therapy is the development of neutralizing alloantibodies which inhibit FVIII activity (inhibitors). Inhibitors are high-affinity immunoglobulin G (IgG) antibodies directed against FVIII, thereby rendering replacement therapy ineffective. The incidence of

inhibitor formation is 20–30% in patients with severe HA.¹ This complication has significant prognostic implications.

The etiology of FVIII inhibitor formation is multifactorial.² We aimed to examine the immune system involvement in inhibitor formation and search for immune surrogate markers for inhibitor formation prior to its appearance. Notably, the thymus is known for its protective role against autoimmunity. This function is maintained by eliminating self-reactive T lymphocytes and generating FOXP3⁺ regulatory T cells.³ T-cell receptor excision circles (TRECs) are produced during T lymphocyte maturation in the thymus. TREC is a by-product of VDJ rearrangement process that occurs in the thymus, and can be used as a marker for thymic output. These circular segments of DNA are unable to replicate

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during mitosis, being diluted in peripheral blood after cell division. TRECs are found in high copy numbers in peripheral blood during the first two decades of life and provide information about the naive T lymphocytes that emigrate from the thymus.⁴ A similar process occurs in B cells; however, this elimination of self-reactive cells occurs in the bone marrow. Kappa receptor excision circle (KREC) is a marker of new B cells originating from the bone marrow.⁵ There are two subtypes of KREC that include sjkKREC (signal joint), which is only present in developing cells undergoing V(D)J recombination, while an intronic rearrangement termed cjkKREC (coding joint) is present in all mature B cells. Thus, B-cell replication history can be deduced by dividing sjkKREC (new B cells) by cjkKREC (all B cells), providing an estimation of new B cells.

One of the mechanisms that may explain the formation of inhibitors is the breakdown of immune tolerance. Accordingly, a defect during the development and maturation of lymphocytes can lead to autoimmunity against FVIII. This defect may be noted at an early stage in the development of the thymus and bone marrow (central tolerance)^{6–8} or later in mature lymphocytes as a result of antigenic stimulation (peripheral tolerance).

Defects in this early tolerance induction have been observed in subjects with rheumatoid arthritis, systemic lupus erythematosus, and type 1 diabetes.^{9–12} It has been reported that T- and B-cell markers (TREC and KREC, respectively) may play a role in autoimmune diseases. Patients with non-chronic ITP, an autoimmune disease, have higher levels of KRECs and this may explain the underlying difference between chronic and non-chronic patients.⁵

It has been previously reported that TRECs can be used as markers in alloimmunity as well.¹³ The distinction of inhibitors in patients with hemophilia as alloimmune is not always accurate. It should be noted that inhibitors may develop also in mild hemophilia patients therefore autoimmunity may also be involved in the pathogenesis.

Our aim was to investigate whether inhibitor formation in HA may be related to loss of central tolerance or loss of peripheral tolerance, as well as examine if TREC or KREC can be used as a surrogate marker for inhibitor formation prior to its development.

We performed a pilot study examining the differences in TREC and KREC levels in children with severe HA with and without inhibitors. In addition, in a subgroup of children who developed inhibitors, we examined the differences in TREC and KREC before and after inhibitor formation. By understanding the mechanism underlying the development of an inhibitor, we may be able to identify the patients at risk and individually tailor the most beneficial therapy for them.

MATERIAL AND METHODS

Patients

Between the period 2010 and 2020, blood samples were collected from 35 children with the diagnosis of severe HA followed and treated in the national hemophilia center at Sheba Medical Center. All patients had a level of FVIII of less than 1% and the majority had a genetic diagnosis as well. Children's parents gave consent for their child's participation in the study. The study was approved by the Ethics Committee of Sheba Medical Center.

Clinical data

Clinical data were collected from medical records and the children were divided into two groups: with FVIII inhibitors and without FVIII inhibitors (following at least 50 exposures to FVIII concentrate). A comparison was made between the two groups regarding the demographic and clinical data (Table 1).

Quantifying KREC and TREC

Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood. Extraction of DNA from PBMCs was done using kit G-DEX IIb

(iNtRON) according to the manufacturer's instructions. TREC quantification was determined by quantitative real-time PCR (qRT-PCR). PCR reactions contained TaqMan universal PCR master mix (Applied Biosystems), specific primers (900 nM), and probes labeled with FAM-TAMRA (250 nM), and 0.5 mcg of genomic DNA. RT-PCR was performed using StepOne Plus device (Applied Biosystems). The calibration curve was prepared by decimal dilution (10^6 – 10^3 copies) of plasmid known signal-TREC. TREC copy number in a particular sample was automatically calculated by comparing the Ct value of that sample to the calibration curve using an absolute quantification algorithm. The amounts of sjkKREC and cjkKREC were determined by real-time PCR as described for TREC, using appropriate primers and probes. All the reactions were performed in triplicates. Amplification of RNaseP (TaqMan assay, Applied Biosystems) was used for quality control, to ensure that equal amounts of genomic DNA were used for PCR analyses. The numbers of TREC and KREC copies are copies/500 ng of DNA.

Statistical analysis

Statistical analysis was performed with IBM SPSS Statistics (version 23.0; Armonk, NY: IBM Corp.). Continuous variables were presented as median (range or interquartile range, as indicated). Categorical variables were presented as counts or proportions. For continuous variables, the Mann–Whitney *U* test was used to compare two independent patient subgroups (without vs. with inhibitors), and the Wilcoxon signed-rank test was used to compare two dependent patient subgroups (before vs. after inhibitor development). For categorical variables, the Fisher exact test was used to compare two independent patient subgroups (without vs. with inhibitors). Two-tailed *p* values of less than 0.05 were considered statistically significant.

RESULTS

Patients

The study population included 35 male children with severe HA, followed and treated routinely in our comprehensive hemophilia treatment center. Table 1 shows patients' demographic data. All samples were taken after at least 50 days of exposure to recombinant FVIII replacement therapy, 11 patients had active FVIII inhibitors and 24 patients did not develop inhibitors. Patients developed an inhibitor after 5–20 exposure days (median 12 days) (Table 1). The rate of family history of inhibitor formation was significantly higher in the inhibitor patients ($p < 0.001$). We further evaluated samples available from three patients with inhibitors that were checked prior to inhibitor formation and compared them to samples of the same patients reevaluated following inhibitor formation.

The three patients for whom we had levels before and after inhibitor formation had blood samples at the age of 1 month, 1 week, and 3 months. Plasma samples following inhibitor formation were obtained at 9, 11, and 4 months, respectively. Interestingly, two of these patients harbored an inversion 22 mutation and one had a deletion of exons 8–9. All had a family history of inhibitor formation.

TREC and KREC levels

TREC copies. TREC levels were determined in all 35 patients. In 3 of 35 patients, we had blood samples before and after inhibitor development. When analyzing TREC levels, children with inhibitors had a higher median level of TREC; however, the difference was not statistically significant ($p = 0.085$; Fig. 1). When we compared only the three patients for whom we have samples before and after inhibitor formation, the difference compared to the non-inhibitor patients was not significant either ($p = 0.17$). When comparing the three children (Fig. 2) who had samples before and after inhibitor formation, we found numerically higher levels of TREC copies after inhibitor formation; however, the difference was not statistically significant ($p = 0.250$), which can be explained by the small number of patients in the subgroup. We also compared if patients with low responding inhibitors BU <5 had differences in comparison to patients with high responding inhibitors >5 BU and found no significant difference ($p = 0.733$).

Table 1. Genetic and clinical parameters in our hemophilia patients.

Parameter	With FVIII inhibitor (n = 11)	Without FVIII inhibitors (n = 24)	p value
Family history of inhibitor (n)			<0.001
Yes	7	1	
No	4	23	
Type of FVIII prophylaxis (n)			0.092
Plasma-derived FVIII	2	0	
Recombinant FVIII	9	24	
Type of FVIII gene mutation (n)			0.0164
Inversion 22	7	14	
Deletion: exons 8–10	0	1	
Deletion: exons 8–9	1	0	
Missense: c.1171C>T p.Arg391Cys	0	1	
Nonsense: c.3439C>T p.Gln1147	1	0	
Nonsense: c.6976C>T p. Arg2326	1	0	
Deletion: exon 1–14	1	0	
Unknown	0	8	

FVIII coagulation factor VIII, n number of patients. p values are calculated by the Fisher exact test.

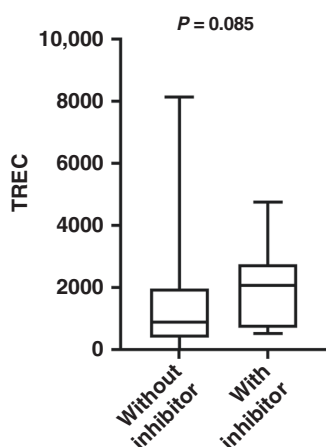


Fig. 1 TREC levels in hemophilia A patients with and without inhibitors. The data are presented as box-and-whiskers plots. The boxes span the 25th to the 75th percentile, the line inside each box denotes the median, and the whiskers span the lowest to the highest observations. The Mann–Whitney *U* test was used to compare the patient groups.

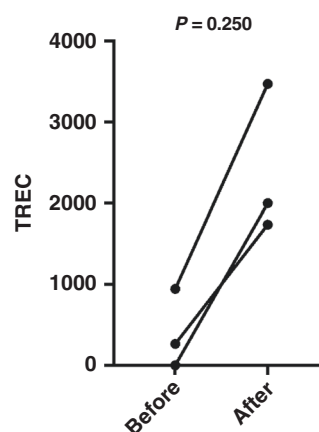


Fig. 2 TREC levels in three hemophilia A patients with inhibitors before and after inhibitor formation. Each dot represents a single measurement, and each line connects two dots that refer to the same patient, demonstrating the individual changes in the TREC level. The Wilcoxon signed-rank test was used to compare the TREC level before and after inhibitor formation.

KREC levels. sjKREC levels, assessed in 32 patients, were not significantly different between the patients with and without inhibitors ($p = 0.651$, Fig. 3a); in contrast, cjKREC levels were significantly higher in patients with inhibitors ($p = 0.003$, Fig. 3b). Accordingly, the sj/cjKREC ratio was significantly lower in the inhibitor patients, as compared to non-inhibitor patients ($p = 0.015$, Fig. 3c). When comparing the three children who had samples before and after inhibitor formation, we found numerically higher sjKREC (in 2/3 patients) and cjKREC (in 3/3 patients) levels following inhibitor formation (Fig. 4a, b, respectively). In addition, a lower sj/cjKREC ratio was observed following inhibitor formation; however, the difference was not statistically significant.

We did not find a statistically significant difference between the three patients with samples prior to inhibitor formation compared to the patients without inhibitor formation. sjKREC ($p = 0.2$). We also compared inhibitor patients with low responding inhibitors BU <5 to patients with high responding inhibitors >5 BU and

found no significant difference (sjKREC, $p = 0.48$; cjKREC, $p = 0.146$; sj/cjKREC, $p = 0.7$).

DISCUSSION

The development of inhibitors against FVIII represents the most severe complication of therapy in HA patients, as inhibitors seriously affect patients' morbidity and mortality.¹⁴

The reason why inhibitors develop in only a fraction of patients has puzzled clinicians and scientists for years, but there is still no unequivocal explanation. Risk factors for the development of inhibitors are classified into two main groups: genetic (non-modifiable) and environmental (modifiable).¹⁵ Genetic factors include, among others, F8 mutation, family history, ethnicity, and polymorphisms in immune-modulating genes.^{16–18} Environmental factors include “danger signals” such as intensive exposure to FVIII¹⁹ and presence of pro-inflammatory signals (might occur

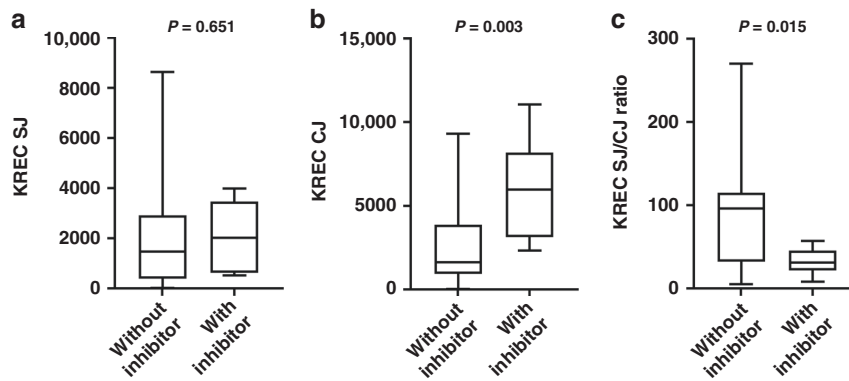


Fig. 3 KREC levels in hemophilia A patients with and without inhibitors. **a** sjKREC levels in hemophilia A patients with (before) and without inhibitors. **b** cjKREC levels in hemophilia A patients with (before) and without inhibitors. **c** sj/cjKREC ratio levels in hemophilia A patients with (before) and without inhibitors. The data are presented as box-and-whiskers plots. The boxes span the 25th to the 75th percentile, the line inside each box denotes the median, and the whiskers span the lowest to the highest observations. The Mann–Whitney *U* test was used to compare the patient groups.

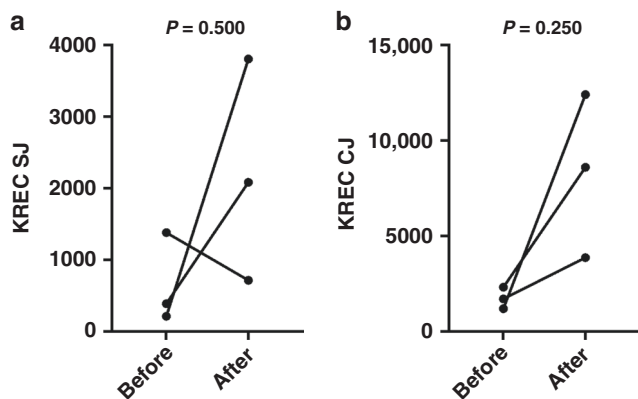


Fig. 4 KREC levels in three hemophilia A patients prior to and after inhibitor formation. **a** sjKREC levels in hemophilia A patients before and after inhibitor formation. **b** cjKREC levels in hemophilia A patients before and after inhibitor formation. Each dot represents a single measurement, and each line connects two dots that refer to the same patient, demonstrating the individual changes in the measured parameters. The Wilcoxon signed-rank test was used to compare the values before and after inhibitor formation.

during treatment of large bleeds, infections, surgery, vaccines). Risk factors, as well as their complex inter-relationships with the immune system, increase the hazard of inhibitor formation, particularly in the previously untreated patient population.²⁰

We found that the majority of patients who developed an inhibitor had a family history of inhibitor formation and high-risk F8 gene mutations (e.g., F8 intron 22 inversion, see Table 1), in accordance with previous reports.^{21–23} As the majority of patients were treated with recombinant FVIII products, it is impossible to assess differences in inhibitor formation with respect to the type of FVIII product used in our cohort.

The role of the immune system in the formation of inhibitors has been previously investigated however this is the first study assessing the importance of TRECs and KRECs in these immune mechanisms. The combination of genetic and non-genetic factors can significantly activate or inhibit the immune response as a result of changes in immunological regulators and cytokine profiles.^{24–27} Several studies indicate that the immune response triggered by the presence of exogenous FVIII is a T helper cell-mediated event that depends on antigen-presenting cells (APCs).²² For the synthesis of antibodies against FVIII to occur, FVIII must be presented to an MHC class II molecule to be recognized by CD4+ T cells. Along with this, FVIII fragments are

presented via MHC class I to CD8+ T cells. This peptide-MHC complex on the surface of APCs is recognized by antigen T-cell receptors. To improve the effectiveness of this course, a second signal occurs between APCs and T cells, the binding of CD80/86 on APCs and CD28 on T cells.²⁴ Activation of T cells can be type 1 helper T-cell activation that is important for cellular immunity, or type-2 helper T-cell activation resulting in humoral immunity. The cytokines released from Th1 cells stimulate the development of IgG1 and IgG2 subclass whereas Th2 cells stimulate the development of IgG4. Studies in hemophilia have shown that titers of high-response inhibitors are correlated with IgG4, which suggests that Th2-mediated immune response is strongly related to the synthesis of anti-FVIII antibodies.²⁸

TREC and KREC have been used as a biomarker of thymus and bone marrow function in several pathological processes, as well as in newborn screening tests for primary immunodeficiencies; evaluation of immune system recovery during AIDS treatment with antiretroviral therapy or after bone marrow transplantation; and clarification of autoimmune disease pathogenesis.⁶ If central tolerance is involved in inhibitor pathogenesis, we expect the TREC and sjKREC levels to be increased or unchanged, whereas if peripheral tolerance occurs, we expect the TREC levels to be decreased, as well as the ratio of cj/sjKREC levels due to dilution, attributed to T- and B-cell replication in the periphery. When there is peripheral tolerance, the proliferation of B cells yields a decrease in sjKREC levels; however, cjKREC levels increase, and therefore the ratio of sj/cjKREC decreases.

We found a higher level of TRECs in patients who developed inhibitors; however, this was not statistically significant (Fig. 1). With regard to KREC, we found a higher level of cjKREC and lower sj/cjKREC ratio in children with inhibitors (see Fig. 3). Among the three patients who were tested before and after inhibitor detection, no significant difference was found (Figs. 2 and 4); however, this may be attributed to the small sample size.

Our findings may add to the notion that inhibitor formation is attributed to humoral immunity mediated by Th2 and production of IgG4, due to peripheral B-cell expansion and production of antibodies against FVIII (loss of peripheral tolerance). This is in agreement with previous studies that have shown a similar pattern in autoimmune diseases.^{29, 30} Due to the small group of patients we cannot rule out that T cells (evidenced by TREC) play a role in the pathogenesis of inhibitor formation. This pilot study has some limitations. The number of patients was small, which limited the statistical significance of our findings. Furthermore, ideally, we should have samples of all patients prior to and after the development of FVIII inhibitor, which would potentially enable us to predict when and who will develop an inhibitor. We cannot

know if TRECs and KRECs represent FVIII-specific cells and not third-party antigen reactive cells.

Future studies with larger cohorts are required to elucidate this issue, along with newer and more accurate tests such as next-generation sequencing for a better understanding of the immune aspects of inhibitor formation.

Improved knowledge regarding the involvement of the immune system in the formation of FVIII inhibitors will enable better therapy tailoring in the new era of non-replacement therapies.

DATA AVAILABILITY

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

REFERENCES

- Blanchette, V. S. et al. Definitions in hemophilia: communication from the SSC of the ISTH. *J. Thromb. Haemost.* **12**, 1935–1939 (2014).
- Oldenburg, J. & Pavlova, A. Genetic risk factors for inhibitors to factors VIII and IX. *Haemophilia* **6**(12 Sup), 15–22 (2006).
- Hazenbergh, M. D. et al. Increased cell division but not thymic dysfunction rapidly affects the T-cell receptor excision circle content of the naive T cell population in HIV-1 infection. *Nat. Med.* **6**, 1036–1042 (2000).
- Serana, F. et al. Thymic and bone marrow output in patients with common variable immunodeficiency. *J. Clin. Immunol.* **31**, 540–549 (2011).
- Levy-Mendelovich, S. et al. Quantification of specific T and B cells immunological markers in children with chronic and transient ITP. *Pediatr. Blood Cancer* **64**, 12 (2017).
- Machnes-Maayan, D. et al. Insight into normal thymic activity by assessment of peripheral blood samples. *Immunol. Res.* **61**, 198–205 (2014).
- Lev, A. et al. The kinetics of early T and B cell immune recovery after bone marrow transplantation in RAG-2-deficient SCID patients. *PLoS One* **7**, e30494 (2012).
- Somech, R. T-cell receptor excision circles in primary immunodeficiencies and other T-cell disorders. *Curr. Opin. Allergy Clin. Immunol.* **11**, 517–524 (2011).
- Hampe, C. S. B cells in autoimmune diseases. *Scientifica* **2012**, 215308 (2012).
- Amariglio, N. et al. T-cell compartment in synovial fluid of pediatric patients with JIA correlates with disease phenotype. *J. Clin. Immunol.* **31**, 1021–1028 (2011).
- Yurasov, S. et al. Defective B cell tolerance checkpoints in systemic lupus erythematosus. *J. Exp. Med.* **201**, 703–711 (2005).
- Samuels, J., Ng, Y. S., Coupillaud, C., Paget, D. & Meffre, E. Impaired early B cell tolerance in patients with rheumatoid arthritis. *J. Exp. Med.* **201**, 1659–1667 (2005).
- Lev, A. et al. Characterizing T cells in SCID patients presenting with reactive or residual T lymphocytes. *Clin. Dev. Immunol.* **2012**, 261470 (2012).
- Astermark, J. Why do inhibitors develop? Principles of and factors influencing the risk for inhibitor development in haemophilia. *Haemophilia* **3**(12 Suppl), 52–60 (2006).
- Ljung, R., Petrini, P., Lindgren, A. C., Tengborn, L. & Nilsson, I. M. Factor VIII and factor IX inhibitors in haemophiliacs. *Lancet* **339**, 1550 (1992).
- Luna-Záizar, H. et al. F8 inversions of introns 22 and 1 confer a moderate risk of inhibitors in Mexican patients with severe hemophilia A. Concordance analysis and literature review. *Blood Cells Mol. Dis.* **71**, 45–52 (2018).
- Ghosh, K. & Shetty, S. Immune response to FVIII in hemophilia A: an overview of risk factors. *Clin. Rev. Allergy Immunol.* **37**, 58–66 (2009).
- Doshi, B. S. et al. B cell-activating factor modulates the factor VIII immune response in hemophilia A. *J. Clin. Invest.* **131**, e142906 (2021).
- Peyvandi, F. et al. Randomized TRIAL OF Factor VIII and neutralizing antibodies in hemophilia A. *N. Engl. J. Med.* **374**, 2054–2064 (2016).
- Carcao, M., Re, W. & Ewenstein, B. The role of previously untreated patient studies in understanding the development of FVIII inhibitors. *Haemophilia* **22**, 22–31 (2016).
- White, G. C. II, Kempton, C. L., Grimsley, A., Nielsen, B. & Roberts, H. R. Cellular immune responses in hemophilia: why do inhibitors develop in some, but not all hemophiliacs? *J. Thromb. Haemost.* **3**, 1676–1681 (2005).

- Rammensee, H. G., Friede, T. & Stevanovic, S. MHC ligands and peptide motifs: first listing. *Immunogenetics* **41**, 178–228 (1995).
- Schwaab, R. et al. Haemophilia A: mutation type determines risk of inhibitor formation. *Thromb. Haemost.* **74**, 1402–1406 (1995).
- Hay, C. R. et al. HLA class II profile: a weak determinant of factor VIII inhibitor development in severe hemophilia A. A UKHCO Inhibitor Working Party. *Thromb. Haemost.* **77**, 234–237 (1997).
- Abdulqader, A. M. R., Mohammed, A. I. & Rachid, S. Polymorphisms in the cytotoxic T lymphocyte-associated protein-4 immune regulatory gene and their impact on inhibitor development in patients with hemophilia A. *J. Int. Med. Res.* **47**, 4981–4992 (2019).
- Chaves, D., Belisário, A., Castro, G., Santoro, M. & Rodrigues, C. Analysis of cytokine genes polymorphism as markers for inhibitor development in haemophilia A. *Int. J. Immunogenet* **37**, 79–82 (2010).
- Hoyer, L. W. The incidence of factor VIII inhibitors in patients with severe hemophilia A. *Adv. Exp. Med. Biol.* **386**, 35–45 (1995).
- Pratt, K. P. & Thompson, A. R. B-cell and T-cell epitopes in anti-factor VIII immune responses. *Rev. Bras. Hematol. Hemoter.* **37**, 80–95 (2009).
- de Alencar, J. B. et al. Importance of immune response genes in hemophilia A. *Rev. Bras. Hematol. Hemoter.* **35**, 280–286 (2013).
- Thewissen, M. et al. Analyses of immunosenescent markers in patients with autoimmune disease. *Clin. Immunol.* **123**, 209–218 (2007).

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AUTHOR CONTRIBUTIONS

S.L.-M., G.K., R.S., A.L., and I.B. made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data. E.A., R.D., and A.A.B. contributed to drafting the article or revising it critically for important intellectual content. All authors approved the final version of the manuscript to be published.

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COMPETING INTERESTS

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Children's parents gave informed consent for their child's participation in the study. The study was approved by the Ethics Committee of Sheba Medical Center.

ADDITIONAL INFORMATION

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