Importance of immune response genes in hemophilia A

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Hemophilia A is a disease caused by a deficiency of coagulation factor VIII resulting from genetic inheritance linked to chromosome X. One treatment option is the administration of plasma or recombinant FVIII. However, some patients develop inhibitors or antibodies against this factor. Inhibitors are alloantibodies that bind to the epitope of factor VIII causing it to be recognized by the immune system as a foreign peptide. This is the most serious complication in hemophilia patients in respect to replacement therapy. Some studies have suggested that genetic factors influence the development of factor VIII inhibitors such as ethnicity, family history, mutations in the factor VIII gene and in genes of the immune system. The aim of this study was to conduct a literature review to assess the influence of genetic factors of immune response genes, especially genes of the major histocompatibility complex and cytokines, which may be related to the development of factor VIII inhibitors in hemophilia A patients. Understanding these risk factors will help to determine future differential treatment in the control and prevention of the development of inhibitors.

Keywords: *Hemophilia A; Blood coagulation; Factor VIII; Major histocompatibility complex; Cytokines; Human Leukocyte Antigen*

Introduction

Hemophilia A is a hereditary bleeding disorder caused by the deficiency or abnormality of factor VIII (FVIII) coagulant activity[1]. The deficiency or dysfunction of FVIII, a glycoprotein that operates as a cofactor in the activation of factor X (FX) via activated factor IX (FIX), does not allow the formation of a normal clot at the site of an injury[1-3]. In 2010, according to the register of the World Federation of Hemophilia, there were approximately 10,065 hemophilia patients in Brazil with 80% being hemophilia A[4].

The treatment of hemophilia A involves FVIII replacement therapy. However, some patients develop inhibitors, that are polyclonal antibodies against the administered FVIII.[5] This reduces the effectiveness of replacement therapy and is one of the main complications in the treatment of patients[5,6]. Various mechanisms are involved in the formation of inhibitors, for example, ethnicity, family history, FVIII gene mutations and processes that involve immune system genes. The study of genetic factors is essential to elucidate mechanisms that may influence the development of inhibitors as in the absence of a genetic predisposing factor there is much lower risk of developing inhibitors. On the other hand, the combined action of genetic and non-genetic factors can significantly contribute to the development of inhibitors. These factors may activate or inhibit the immune response depending on changes in immunological regulators and cytokine profiles. By predicting these factors in patients, it may be possible to avoid the formation of FVIII inhibitors and provide more effective treatment[7].

As the identification of factors related to the mechanisms involved in the development of inhibitors in hemophilia A is of paramount importance, the aim of this study was to conduct a review of the literature in order to assess the influence of genetic factors and immune system genes on the development of FVIII inhibitors in hemophilia A patients. This study may contribute to a greater understanding of the mechanisms involved in the susceptibility that some people have in producing alloantibodies against FVIII, as well as to develop new, more effective therapies for hemophilia patients with inhibitors.

Development of factor VIII inhibitors

Antibodies, also called inhibitors, are high-affinity immunoglobulin G (IgG) that are directed against infused FVIII; this reduces the effectiveness of treatment[8]. These inhibitors make the adequate correction of bleeding diathesis more difficult in cases of bleeding and in surgery, and also make regular prophylaxis with FVIII impossible[9]. Studies have shown that the overall prevalence of inhibitors in unselected hemophiliac populations is 5-7% and the prevalence amongst severe hemophilia patients is substantially greater, at between 12% and 13%[10].

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The FVIII inhibitors are classified as either high- or low-responding depending on how an individual's immune system is stimulated upon repeated exposure to FVIII. If the immune system reacts briskly and strongly, the amount of inhibitor directed against FVIII can rise quickly to very high levels with titers of at least 5 Bethesda units. This type of inhibitor is generally characterized as high-responding. On the other hand, the immune system may be stimulated in such a way that its response to exposure to FVIII is slower and weaker, and the inhibitor titer remains low, usually under 5 Bethesda units. When these characteristics are present, the inhibitor is generally termed low-responding[11].

It is noteworthy that during the lifetime of a person with hemophilia, the risk of developing an inhibitor is variable[11]. There are reports that the risk is at its highest soon after the first exposure to FVIII, with the cumulative risk of inhibitor development leveling off after 20 exposure days or at 6-10 years of age[10].

The treatment of hemophilia patients with inhibitors is a major challenge, and is based on the use of agents that generate thrombin, independent of the actions of FVIII or FIX, the so-called bypassing agents. The two most widely used bypassing agents are activated prothrombin complex concentrate (APCC) and recombinant activated factor VII (rFVIIa). Despite their efficacy in the control of most bleeding episodes, treatment with these agents present several limitations compared to the use of FVIII in non-inhibitor patients such as a less predictable hemostatic response, lack of laboratory monitoring tests and a higher cost. Furthermore, because of the lower efficacy and shorter half-life, regular prophylaxis is not feasible with these agents[11].

Mechanism of the formation of factor VIII inhibitors

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 (CAAs), such as macrophages, dendritic cells and B cells[12,13].

For the synthesis of antibodies against FVIII to occur, part of the FVIII administered to the hemophilia patient must be internalized by CAAs, degraded and presented to a class II Major Histocompatibility Complex (MHC) molecule[12,14]. A plasma membrane complex of CAAs, formed when FVIII peptides bind to MHC molecules, is then made available for recognition by $CD4^+$ T cells[15]. In addition to this, intracellular peptide fragments of FVIII, synthesized in small quantities by the patient, are presented via MHC Class I molecules to $CD8^+$ T cells[16].

The peptide-MHC complex on the surface of CAAs is recognized by antigen T cell receptors (TCRs). For the presentation of antigens to the TCRs to be efficient, a second signal occurs between the CAAs and T cells; the co-stimulating CD80/86 molecules expressed in CAAs bind to CD28 expressed in T cells[10,17]. Activation of T cells occurs when both signals are present; this can be either type-1 helper T cell (Th1) activation, responsible for the secretion of cytokines such as interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α) and interleukin-2 (IL-2), which are important in the cellular immunity system, or type-2 helper T cell (Th2) activation which secretes interleukin-4 (IL-4), interleukin-5 (IL-5), Interleukin-6 (IL-6) and interleukin-10 (IL-10), among others that are important in humoral immunity. Moreover, the expression of CD2, CD30, CD40L and CD28 is increased on the $CD4^+$ T cell surface[12,15,18,19]. When secreted by Th1 or Th2, these cytokines stimulate the differentiation of B cells, which change the isotype of immunoglobulin and produce specific antibodies against plasma FVIII. In addition, the B cells secrete the cytokine interleukin-12 (IL-12) that in turn stimulates the Th1-mediated production of IFN-γ[18]. The cytokines from Th1 stimulate the development of immunoglobulin G1 (IgG1) and immunoglobulin G2 (IgG2) subclasses, whereas Th2 cells stimulate the development of immunoglobulin G4 (IgG4). Studies in patients with hemophilia A have shown that the titers of high-response inhibitors are correlated to IgG4 levels, which suggests that the Th2-mediated immune response is strongly related to the synthesis of anti-FVIII antibodies[19].

Factors predisposing patients to the development of factor VIII inhibitors

Ethnicity and family history of factor VIII inhibitors

Ethnicity and family history have been associated with predisposition for the development of FVIII inhibitors. One study showed that Afro-Americans had a higher risk of developing inhibitors[20]. This association and family history of the development of inhibitors were also observed in The Malmö International Brother Study (MIBS). The results of this study show that the incidence of inhibitors is high in the subgroup of people of African descent when compared to Caucasians (55.6% vs. 27.4%)[21]. It is believed that the racial component is mainly based on genetic variants in immune response determinants, because the FVIII mutation spectrum does not differ between races[18].

Additionally, it was observed that the risk for the formation of inhibitors increases significantly in patients with a family history of inhibitors; the absolute risk for the development of inhibitors in patients with a family history of inhibitor development was 48% (95% confidence interval – 95% CI: 35-62%), while the risk in patients with no family history was 15% (95% CI: 11-21%)[21].

Another study by the same group assessed the possibility of genetic mutations influencing the formation of inhibitors in individuals of the same family. One hundred and thirteen mainly Caucasian families with two or more brothers with severe hemophilia were analyzed. All brothers in 59 of the families developed inhibitors and 25 (42.4%) of these had a family history of inhibitor development. It was found that the most common type of mutation in the FVIII gene was inversion of intron 22. Inhibitors were identified in 45 of 74 families (60.8%) with this type of mutation; and in 18 (40%) of these 45 families, all the brothers developed inhibitors[18].

In light of these data, the influence of genetic factors in the development of inhibitors is evident[21]. Non-genetic factors also appear to influence the immune response and consequently change the risk of developing inhibitors in each family. However, it is unlikely that these factors alone can explain the similarities reported. These observations suggest that there must be changes

in the immune response that may be based on both genetic markers and non-genetic factors[18].

Mutations in the factor VIII gene

The FVIII gene is located on the end of the long arm of chromosome X (Xq28). It comprises 186,000 base pairs distributed between 26 exons and 25 introns. The product of this gene is a polypeptide of 2332 amino acids (inactivated circulating pro-cofactor) and the activated polypeptide is formed of six arranged domains[2,22,23]. The A2, A3 and C2 domains are the regions in which anti-FVIII antibodies can react and impair the coagulation cascade[24,25]. Studies have shown that in patients with missense mutations clustered in the A2 and C2 domains, the risk of inhibitor formation is fourfold greater than in patients with mutations outside this region. This indicates that any changes in the three-dimensional structure of this part of the FVIII molecule may affect its immunogenicity[18]. The anti-A2 antibodies and in some cases, anti-C2 antibodies, for example, can interact with the A2 domain and C2 domain, respectively, and neutralize the procoagulant activity of FVIII[25]. The C2 domain however is affected by anti-C2 antibodies which prevent FVIII binding to phospholipids and von Willebrand factor[26], and anti-A3 antibodies target the A3 domain to prevent the interaction of FIX with activated FVIII[27].

By the year 2012, 5243 types of mutations associated with this disease had been reported according to the HAMSTeRS (Haemophilia A Mutation Test and Resource Search Site) electronic database[28]. The group with the highest risk of producing anti-FVIII antibodies is the one with the greatest changes in the gene[18,29]. One meta-analysis observed that the risk of inhibitor development in patients with large deletions and nonsense mutations is higher than in patients with intron 22 inversions (pooled OR = 3.6 and OR = 1.4, respectively). The risk of patients with intron 1 inversions and splice-site mutations is virtually equal (pooled OR = 0.9 and OR = 1.0, respectively), and the risk of patients with small deletions and insertions and missense mutations are lower (pooled OR = 0.5 and OR = 0.3, respectively)[9]. Inversions in intron 22 (30-50%) and intron 1 (0-5%) are usually associated with the severe hemophilia A phenotype and an intermediary risk for forming inhibitors[15,30,31].

In Brazil, a study of 86 Caucasian patients, investigated the occurrence of inversion mutations in 47 patients. Of these, 33 (70%) had severe hemophilia and 14 (30%) moderate or mild hemophilia. An increase in the frequency of inversion mutations (13/33; 39.4%) was observed in the subgroup classified as severe with the majority (11/13; 86.4%) being mutations in intron 22[32].

Class I and II human leukocyte antigen molecules

In humans, MHC genes, located on the short arm of chromosome 6, are named human leukocyte antigen (HLA) genes. The HLA system occupies a highly polymorphic DNA region of approximately 3600 kilobases (Kb). These loci contain most of the genetic information necessary for the development of antigen presentation activity[33].

There are two main types of HLA gene products, class I and class II molecules, which recognize different types of protein antigens, intracellular (cytosolic antigens) and extracellular. CD4 T cells recognize antigens presented by class II molecules, while CD8 cells recognize antigens that are together with class I molecules[34,35].

The class III region is found between the groups of class I and II genes, where genes that code for various components of the complement system and structurally related cytokines such as TNF-α, lymphotoxin-α and lymphotoxin-β are located[36].

The generation of antibodies against peptide antigens bound to FVIII involves cell cooperation that results in the presentation of antigens to T and B cells. The start of this process involves the processing of proteins by antigen-presenting cells and subsequent association of these peptides to HLA molecules in these cells. For extracellular proteins, such as the exogenous FVIII administered to hemophilia A patients, it is the HLA class II molecules that mediate the processing of antigenic peptides[14].

Recently, DNA systematic sequence analyses between haplotypes have yielded information on polymorphisms across the complete MHC. This information provides pools of sequence variants for disease association analysis[37]. Several studies are committed to identifying an association between haplotypes of HLA class I and II molecules and the risk for the development of inhibitors. Hay et al. studied 176 hemophilia patients in England and found a higher frequency of the HLA-DRB1*15:01/DQB1*06:02/DQA1*01:02 haplotype in patients with inhibitors, but only the frequency of the HLA-DQA1*01:02 allele was considered statically significant (OR = 2.7; 95% CI: 1.2-5.9). The HLA-DRB1*01, HLA-DQB1*05:01 and DRB1*01:01 alleles were also higher in the group of patients with inhibitors in the presence of the intron 22 inversion mutation, but the values were not statistically significant[38]. The HLA-DQA1*01:02, HLA-DQB1*06:02 and HLA-DRB1*15 alleles were found at higher frequencies in patients with inhibitors by Oldenburg et al. in Germany. Moreover, other HLA class I alleles (HLA-A*03, HLA-B*07 and HLA-C*07) were identified at higher frequencies[39].

Despite the weak haplotype association in these studies, another more recent investigation demonstrated a significant association for class II HLA molecules. This case-control study found a positive association of the DRB1*15:01/DQB1*06:02 haplotype with the formation of inhibitors in 260 severe hemophilia patients from Germany (p-value = 0.0423; OR = 1.9; 95% CI: 1.01-3.57)[40].

The studies that had the most statistically significant results were those that tested for associations of alleles of HLA genes in respect to susceptibility or resistance of patients in developing inhibitors. The main associations have been found for HLA class II molecules, perhaps because, from the point of view of immunity, these alleles are more important in presenting the peptides of FVIII to T cells, and so studies have focused more on genotyping these alleles. One study of 57 hemophilia patients (with and without inhibitors) and 36 blood donors without the disease from Thailand found a higher frequency of the DRB1*15 allele in patients with inhibitors (30.6%) than in patients without inhibitors (19.2%). However, statistical significance was obtained only when the frequency of this allele was compared between patients with inhibitors and controls (30.6% vs. 13.9%; p-value = 0.021; OR = 0.021; 95% CI: 1.16-6.47)[41].

In another study performed in Germany, the frequencies of the HLA-DRB1*16 (0.122 vs. 0.014; p-value = 0.0001) and DQB1*05:02 alleles (0.112 vs. 0.058; p-value = 0.0149) were higher in 57 patients with acquired hemophilia enrolled in the study compared to the normal European population. On the other hand, the HLA-DRB1*15 (0.087 vs. 0.172; p-value = 0.0260) and DQB1*06:02 alleles (0.078 vs. 0.142; p-value = 0.0149) were less frequent in the patients with acquired hemophilia. However, on comparing patients with acquired hemophilia but without inhibitors with those with inhibitors, the DRB1*16 and DQB1*05:02 alleles were correlated to lower risk (OR = 1.1 and 1.5, respectively) and the DRB1*15 and DQB1*06:02 alleles were considered high risk (OR = 2.2 and 3.7, respectively)[42].

Recently, an investigation conducted in southern Brazil evaluated the influence of HLA class I and class II alleles on the development of severe hemophilia in a group of 171 patients (50 patients with inhibitors and 131 without inhibitors). This study showed a high frequency of HLA-C*16 and HLA-DRB1*14 alleles in patients with inhibitors compared to those without inhibitors indicating a probable association between these alleles and the development of inhibitors. However, the difference in the frequency of the HLA-C*16 allele was not statistically significant compared to the healthy population in the region. The HLA-DRB1*14 allele is more common in white Brazilians than in Caucasians in general; this may be due to the great miscegenation of the population[43].

Most studies have investigated alleles in respect to susceptibility to develop inhibitors. However, resistance alleles are also of great importance in association studies. For example, the DQA1*01:03, DQB1*06:03 and HLA-C*02 alleles have been reported as having a role in resistance to the development of inhibitors in hemophilia patients[38,39].

HLA-C*05 is another allele that may provide protection against the formation of inhibitors. This was observed in an American population of 44 hemophilia patients, 28 of whom had no inhibitors and 16 were positive. HLA-C*05 was identified in 11 (39.29%) patients without inhibitors, whereas none in the group with inhibitors had this allele. Therefore, the presence of the HLA-C*05 acted as a protective factor in the development of inhibitors (p-value < 0.02)[44].

Furthermore, Ohta et al. demonstrated a protective role of the HLA-A*24 allele. The HLA-A, B and C antigens and HLA-DQA1, DQB1, DRB1 and DPB1 alleles were analyzed in 20 Japanese hemophilia A patients with inhibitors. The results showed that the absence of the HLA-A*24 allele is a risk factor for the formation of inhibitors compared to its presence (36.8% vs. 82.6%; p-value = 0.003; OR = 0.123). Additionally, according to this study, the HLA-DRB1*04, DQB1*04 and DQA*03:01 alleles may be associated with the development of inhibitors[45].

Although some studies have shown that the HLA system, in particular HLA class II molecules, may be of greater importance in the development of inhibitors, the association between HLA and the formation of FVIII inhibitors varies between different ethnic groups and depends on the geographic region. These data may be useful in the recognition of groups at high risk for the formation of inhibitors in different populations. Table 1 shows some association studies of HLA class I and class II alleles for risks or protection to the development of inhibitors in hemophilia patients.

Table 1 - Analysis of HLA alleles class I and II associated to the development of inhibitors

Alleles	Risk of the development of inhibitors	References
HLA Class I		
*C*05*	Decreased	Aly et al.[44]
*A*24*	Decreased	Otha et al.[45]
HLA Class II		
*DR4.1(DRB1*04:01)*	Increased	Otha et al.[45]
*DQB1*04*	Increased	Otha et al.[45]
*DQA1*03:01*	Increased	Otha et al.[45]
*DQB1*05:02*	Decreased	Pavlova et al.[42]
*DRB1*16*	Decreased	Pavlova et al.[42]
*DQB1*06:02*	Increased	Pavlova et al.[42]
*DQA1*01:02*	Increased	Hay et al.[38]
*DRB1*15*	Increased	Pavlova et al.[42]
*DRB1*15*	Increased	Nathalang et al.[41]

*The HLA alleles are presented in this table according to new nomenclature.

Polymorphisms of cytokine genes

Cytokines are a group of soluble proteins produced by different cells in response to antigens, which act regulating the innate and adaptive immune reactions. About 180 genes encode these proteins[46].

Single nucleotide polymorphisms (SNPs) in the regulatory regions of cytokine genes, common in many populations, can affect the transcription and influence the production of cytokines and consequently, change the profile of the immune response[47]. Thus, any difference in the gene frequency of these cytokines in different populations may have clinical relevance and be important to obtain more specific genetic markers for diagnosis and prognosis[47,48].

The SNPs in cytokine genes are known due to their association in several diseases[49,50], as well as their influence on the production of antibodies in autoimmune diseases[51]. Most of the SNPs studied are in promoter regions and in the exon itself, or in microsatellites of intron regions[48].

Several studies have reported associations of these SNPs with the development of inhibitors in hemophilia A patients (Table 2).

In a recent study of Chinese hemophilia patients, the -819T and -592A alleles of the *IL10* gene were more frequently found in individuals with FVIII inhibitors. In addition, some haplotypes of this gene (TA at -819 position and CA and CC at position -592) indicate predisposition of hemophilia patients for developing inhibitors[52].

Another cytokine, which also plays an important role in immune modulation in hemophilia patients, is the TNF. This cytokine has a potent pro-inflammatory action. The analysis of polymorphisms in four alleles of the *TNF* gene (-827C>T, -308G>A, -238A>G and 670A>G) of 164 hemophilia patients (124 severe, 26 moderate and 14 mild) identified an association between the -308A/A genotype and the formation of inhibitors. The -308A allele was identified in 46 (59.7%) of 77 patients with inhibitors and in 40 (46.0%) of 87 patients without inhibitors (p-value = 0.87; OR = 1.7). The association between the -308A/A genotype and the formation of inhibitors was also evident in the subgroup of patients (n = 124) with severe hemophilia (p-value < 0.001; OR = 19.2)[53].

Table 2 - Analysis of Single nucleotide polymorphisms in cytokine genes associated with the development of inhibitors in Hemophilia A

Cytokine gene	Genotype	Allele	Haplotype	Risk of the development of inhibitors	Reference
IL10^{-1082}	*IL10*$^{-1082}$ G/G	*IL10*$^{-1082}$*G	*IL10*$^{-1082,-819,-592}$ *GCC*	Increased	Pavlova et al.[40]
IL10$^{-1082,-819,-592}$	-	-	*IL10*$^{-1082,-819,-592}$ GCC/ATA	Decreased	Chaves et al.[56]
IL10$^{1082,-819,-592}$	-	-	*IL10*$^{-1082,-819,-592}$ GCC/ACC	Increased	Chaves et al.[56]
IL10^{-819}	*IL10*$^{-819}$ C/T	*IL10*$^{-819}$*T	*IL10*$^{-819}$ *TA*, *IL10*$^{-592}$ *CA*, *IL10*$^{-592}$ *CC*	Increased	Lu et al.[52]
IL10^{-592}	*IL10*$^{-592}$ A/C	*IL10*$^{-592}$*A	-	Increased	Lu et al.[52]
*IL10**	-	-	*IL10*$^{-1082,-819,-592}$ *ATA/ATA*	Decreased	Pinto et al.[57]
TNF^{-308}	*TNF*$^{-308}$ A/A	-	haplotype 2	Increased	Astermark et al.[53]
TNF^{-308}	*TNF*$^{-308}$ A/A	-	-	Increased	Zhang et al.[54]
TNF^{-857}	-	-	rs1799724C/T** GCC/ATA	Increased	Pinto et al.[57]

*IL10 promoter ´CA` dinucleotide microsatellite

**rs1799724C/T is the same as TNF^{-857} C/T

These findings were also observed in other patient groups. The polymorphism in the -308 region of the *TNF* gene was correlated with the development of inhibitors. Individuals homozygous for the allele A present a higher risk of developing inhibitors compared to heterozygotes (OR = 7519; 95% CI: 3168-17.844). This relationship is also valid on analyzing severe hemophilia patients (OR = 8163; 95% CI: 2521-26.434)[54].

Pavlova et al. also confirmed higher frequencies of the -308G>A polymorphism in the *TNF* gene of patients in Germany (0.22 vs. 0.13; OR = 1.80). The homozygous A/A genotype (OR = 4.7) was more pronounced in severe hemophilia patients with FVIII inhibitors. The same group of researchers found that the 1082G allele of the *IL10* gene was more common in these patients (0.55 vs. 0.43; p-value = 0.008)[40].

These and other association studies using genetic targets have focused on finding new markers to try to offer better treatment options to patients and avoid complications. Polymorphisms that influence the Th1/Th2 response may be instrumental to genotypically classify patients and check the risk of developing inhibitors[55]. Hence, it is evident that polymorphisms in the *TNF* and *IL10* genes are best characterized in respect to the development of inhibitors in hemophilia patients, and may be future candidate genes.

Conclusions

During the last few years, progress in the identification of determinants in the development of anti-FVIII alloantibodies has occurred. After analysis of different studies, several factors related to the appearance of hemophilia A were found that highlight immune response genes.

This review identifies several important points: the incidence of inhibitors is greater in individuals of African descendancy and in patients with a family history of the inhibitor. Furthermore, the risks of inhibitor development in patients with large deletions and nonsense mutations are higher than in patients with intron 22 inversions; these inversions are related to a higher risk of the development of inhibitors than in patients with intron 1 inversions and splice-site mutations. In addition, the risk of patients with small deletions and insertions and missense mutations is lower.

The majority of associations of immune response genes to the production of inhibitors in hemophilia patients are related to HLA class II alleles: HLA-DRB1*14, DRB1*15, HLA-DQB1*06:02, DQB1*06:03, HLA-DQA1*01:02 and DQA1*01:03 and the DRB1*15:01/DQB1*06:02 haplotype. Associations have also been found for some HLA class I alleles: HLA-A*24 and C*05.

In relation to cytokine genes, the *IL10* -1082G, -819T, -592A alleles are related to increased risk for the production of inhibitors in hemophilia patients. *TNF* is another cytokine gene associated with the formation of inhibitors, specifically the genotype -308A/A.

This review intends to assist in the development of more targeted genetic association studies of hemophilia patients and immune system genes, and also to assist in the understanding of the participation of these genes in the formation of inhibitors.

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