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Full Length Article

The role of variant alleles of the mannose-binding lectin in the inhibitor development in severe hemophilia A☆

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ABSTRACT

Introduction: The administration of FVIII leads to inhibitors in up to 30% of patients with hemophilia A (HA), the most severe treatment complication. FVIII-mannosylation fosters the presentation of FVIII to $CD4^+$-T-lymphocytes. Mannose as primary ligand for the mannose-binding lectin (MBL) activates the lectin pathway of complement. *MBL2* single nucleotide polymorphisms (SNPs) lead to low peripheral MBL concentrations that may hamper the removal of mannosylated FVIII.

Objective: Investigation of the association between the inhibitor development in hemophilia A and *MBL2*-SNPs.

Methods: In a case-control study the *MBL2*-SNPs in exon 1 at codons 52, 54 and 57 (C, B, D-Alleles respectively) were determined in 237 patients with severe hemophilia A with and without inhibitors to FVIII (119 vs 118). The association of *MBL2*-SNPs and the -308 G > A *TNF-α*-polymorphism with the presence of inhibitors were determined.

Results: In the inhibitor group higher frequencies of the B allele (codon 54) (OR: 1.77, $P < 0.05$) were present. Summarising the *MBL2* SNPs (alleles B, C and D) as 0, the 0/0 type occurred only in the inhibitor group (frequencies: 0.08 vs 0, $P = 0.003$). Based on the genetic background a functional immune response phenotype was determined. 11.8% of patients with inhibitors were of the low MBL/high TNF-α phenotype vs 0.03% of the non-inhibitor patients (OR: 3.71).

Conclusion: Data suggest an association of *MBL2*-SNPs alone or combined with the 308-*TNF-α* polymorphism in the inhibitor development. Investigations of components of all three complement pathways are required to comprehend their individual and overall contribution to the inhibitor development in HA.

1. Introduction

It is well known that the administration of therapeutic FVIII induces anti-FVIII- antibodies (inhibitors) in up to 30% of patients suffering from severe hemophilia (HA) and in up to 5% of patients with mild to moderate forms of the disease [1–6]. There is a high interest to identify genetic, treatment related and immunological factors to help minimise the risk of inhibitor formation and to improve treatment results also in the context of the newly arising CAR- (chimeric antigen receptor) and BAR- (B-cell targeting antibody receptors) immunotherapies that aim at the suppression of FVIII specific antibody responses [2]. The glycosylation of FVIII appears to be such a potential factor.

Factor VIII itself is a heterodimeric glycoprotein composed of a heavy and a light chain. N-glycosylation sites are included in the "B-region" of the heavy chain [7,8]. The glycans terminate with either galactose, *N*-acetyl neuramic acid (Neu5AC2), fucose or mannose residues [7–10]. The terminal galactose residues are commonly sialated whereas mannose-terminating residues appear to be not sialated. However they form the second most prevalent glycans on FVIII [7,10]. Mannosylation of non-self-antigens was shown to enhance their

Abbreviations: BAR, B-cell targeting antibody receptors; CAR, chimeric antigen receptor; CI, confidence interval; DC, dendritic cells; FVIII, Factor VIII; HA, Hemophilia; MBL, Mannose-binding lectin; OD, Odd ratio; vWF, von Willebrand factor; SNP, single nucleotide polymorphism; TNF-α, Tumor-necrosis factor α

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endocytosis by dendritic cells (DC) and the subsequent presentation to antigen specific T lymphocytes [9–12]. Dasgupta et al. [9,10] investigated the relevance of exposed mannosylation on FVIII as a ligand for the FVIII entry into DC as part of the induction of a specific immune response. They could indeed demonstrate the entry of FVIII into DC and identified the macrophage mannose receptor (CD206) as a candidate endocytotic receptor for FVIII on DC. The interaction between FVIII and CD206 was blocked by the von Willebrand Factor (vWF), suggesting that under physiological conditions the mannose dependent immunogenicity of FVIII is quenched by endogenous immunochaperones [9,10].

As part of the soluble complement system the mannose binding lectin (MBL) recognises patterns of neutral carbohydrates such as mannose and *N*-acetylglucosamine (GlcNAc) on usually non-self-structures. Like FVIII, MBL is synthesised in the liver. It can directly opsonise microorganisms and enhance the activity of phagocytes [13,14]. Four functions have been attributed to MBL [15]: 1) complement activation 2) promotion of complement-independent opsonophagocytosis 3) clearance of apoptotic cells and 4) modulation of inflammation [15]. MBL-binding is followed by triggering the lectin pathway of complement through MBL-associated serine proteases (MASP) 1 and 2-activation. Terminal sialic acid or galactose chains are no targets for MBL. The serum concentrations of MBL vary substantially due to single nucleotide polymorphisms (SNPs) in the *MBL2* gene [16–18]. The genes for MBL are coded on chromosome 10 (10q11.2-q21) and distributed on four exons (E1–E4) [19,20]. 87 polymorphic sites have been identified within the human MBL2 gene [21]. But the SNPs with the major impact on the serum levels, respectively MBL deficiencies, are present in exon 1 on the codons 52, 54 and 57 [22]. The SNP (G > A) in codon 54 is termed B allele, the SNP (G > A) in codon 57 is termed C allele, the SNP C > T in Codon 52 is termed D allele or (A/B, A/C, A/D) with A standing for the wildtype gene. The variant alleles B, C and D are often collectively designated “O” [21–29] although there is no link between the three SNPs.

The SNPs generate amino acid substitutions in the collagen-like region preventing the oligomerisation of the basic triplet polypeptide subunit (and therefore a normal interaction with MASPs), resulting in diminished complement activation and opsonic activity [14,16,21,29–33].

The genotyping of *MBL2* is used frequently as surrogate for MBL serum levels since SNPs in exon 1 are associated with relative or absolute serum deficiencies [22,28,29,34–37]. Low MBL concentrations have been associated with an increased susceptibility to various infectious diseases [16,17,34–36,39,40], but also with certain autoimmune diseases like Lupus erythematosus [18].

We hypothesised that the *MBL2*-SNPs which are linked to a functional MBL-deficiency are associated with a higher risk of inhibitor development among patients with hemophilia A due to a less efficient clearance of FVIII with exposed mannosylation sites, thereby circumventing a specific immune response. We further investigated the *TNF-α* promotor polymorphism (G to A) at position −308 in these patients group since this polymorphism was already shown to promote the inhibitor formation [41,42] and is associated with a high production of TNF-α in the presence of inflammatory stimuli.

2. Patients, Material and methods

2.1. Patients

The case-controlled study was carried out with 237 patients of hemophilia A. The same cohort has been described earlier [41]. In summary, unrelated white German patients suffered from severe HA (FVIII activity < 1%). In 119 patients inhibitors were present whereas in 118 patients no inhibitors were present. Both patient groups were matched according to their mutation type. The mutation spectrum for both groups included only F8 null mutations. Patients with a non-null mutation were excluded because of the possible presence of endogenous FVIII protein [41]. All non-inhibitor patients had > 150 exposure days. Informed patient consent was obtained according to the Declaration of Helsinki.

2.2. Methods

2.2.1. DNA-analysis and factor VIII gene analysis

Genomic DNA was isolated from EDTA-treated blood cells as described earlier [41,42].

Standard methods for the analyses of the FVIII gene were used and included Southern blot and long-range polymerase chain reaction (PCR) for inversion analysis and direct DNA sequencing as described [41,42].

2.2.2. Clotting activity and inhibitor quantification

FVIII activity was quantified by an in-house-modified one-stage activated partial thomboplastin time-based assay as described earlier. Factor VIII inhibitor activity was determined by the modified Nijmegen Bethesda Assay [42].

2.2.3. Determination of the mannose-binding lectin- and the −308 TNF-polymorphisms

The *MBL2* SNPs were determined by polymerase chain reaction (PCR) and subsequent restriction fragment length polymorphism analysis (RFLP) as described [43]. The −308 *TNF*-α-polymorphism was determined as described [42].

2.3. Statistical methods

Gene frequencies were calculated as described earlier [22] by gene counting and expressed as a ratio of the number of each allele observed within a locus to the total number of alleles observed in that locus. A conventional chi-square test was applied to compare the frequencies in between the HA patients with and without inhibitors. Fisher's exact test was used if sample sizes were < 5. All tests were two-sided and a P-value below 0.05 was considered to indicate statistical significance. To correct the level of significance in the chi-square test and to eliminate significance by chance, odds ratios (ORs) were calculated and were considered to be significant if the lower value of the 95% confidence interval (CI) was larger than 1.0.

3. Results

Allele frequencies of *MBL2* and the *TNF-α* polymorphism were analysed for their association with the inhibitor development in 237 patients suffering from severe HA.

3.1. MBL2 allele frequencies

The allele frequencies for the *MBL*-SNPs in the codons 52 (allele D), 54 (allele B) and 57 (allele C) are shown in Table 1.

The B allele occurred in the inhibitor group with a statistically higher frequency compared to the non-inhibitor group (0.17 vs 0.097) (P < 0.05, OR: 1.8). 4% (5/119) of the inhibitor patients were even homozygous for the B-allele compared to none in the non-inhibitor group. There was no statistical difference with respect to the C-allele in both groups. The D-allele, however, was present in 5.04% (6/119) of the inhibitor patients but in < 1% (1/118) of the patients with no-inhibitor (Table 1). In the inhibitor group two patients were carrying the B and the C-allele and one patient was carrying the B and the D-allele and one patient was homozygous for the C-Allele.

Besides analysing the frequencies of the individual alleles in both groups, we also looked for the combined effect of the SNPs in exon 1 in both groups. The wild-type allele is designated with “A”, whereas the common designation for the *MBL2*-SNPs (alleles B, C and D) is “0”. Table 2 shows the distribution of these alleles in both groups. Here the

Table 1
Frequencies of *mannose binding lectin* (*MBL2*) *gene* polymorphisms in patients with severe hemophilia A with and without inhibitors.

	Inhibitor patients (n = 119)		Non-inhibitor patients (n = 118)				
MBL2	n	Frequency	n	Frequency	P	OR	CI
Allele B							
A/A (wildtype)	83	0.697	95	0.805	0.47	0.866	0.587–1.287
B/A	31	0.261	23	0.195	0.34	1.337	0.736–2.427
B/B	**5**	**0.042**	**–**		**0.034**	Fisher Exact	
Allele frequency							
A	197	0.83	213	0.903		0.917	0.704–1.195
B	**41**	**0.17**	**23**	**0.097**	**0.037**	**1.767**	**1.029–3.037**
Allele C							
A/A (wildtype)	114	0.958	116	0.983	1	0.975	0.680–1.401
C/A	4	0.034	2	0.017	0.35	1.983	0.356–11.03
C/C	1	0.008	–		1		
Allele frequency							
A	232	0.966	234	0.992		0.983	0.761–1.27
C	6	0.023	2	0.008	0.285	2.975	0.594–14.889
Allele D							
A/A (wildtype)	113	0.95	117	0.991	0.444	0.958	0.666–1.38
A/D	6	0.05	1	0.009	0.068	5.95	0.71–50.18
Allele frequency							
A	232	0.975	235	0.996		0.979	0.758–1.264
D	6	0.025	1	0.004	0.066	5.95	0.711–49.8

CI: confidence interval; OR: odds ratio. Statistically significant associations are shown in bold. "A" generally stands for wildtype. The following SNPs are termed B, C, D: Allele B (G > A), allele C (G > A), allele D (C > T).

0/0 type was present in 7.6% (9/119) of the inhibitor patients but not present in the non-inhibitor patients (P = 0.003). In the total of both groups together (n = 237) variant *MBL2*-alleles occurred in 73 patients (30.8%).

3.1.1. Impact of the combination of the MBL2-SNPs and the −308 TNF-α polymorphism on the inhibitor presence

The impact of the different combinations of the *MBL2*-SNPs and the TNF-α-SNP is shown in Table 3. Patients with a homozygous or heterozygous SNP in exon 1 (B, C, D alleles) were considered low MBL-phenotypes whereas patients with a homozygous or heterozygous G > A polymorphism at position −308 were considered high TNF-α producers. An association of the low *MBL*/high *TNF-α* haplotype with the inhibitor formation (0.12 vs 0.03; P < 0.02; OR: 3.47) was observed. Conversely the high *MBL*/low *TNF-α* producers (43 vs 73) were more prominent in the non-inhibitor group (P < 0.03). Table 3 includes the major combinations (*MBL* low/*TNF-α* high; *MBL* low/*TNF-α* normal) (*MBL* normal/*TNF-α* high; *MBL* low/*TNF-α* normal).

4. Discussion

In our total group of HA patients 30.8% (73/237) showed *MBL2*-SNPs which are associated with MBL serum deficiencies. This percentage is high, but in agreement with the incidence of low MBL levels < 500 ng/ml in the human population (30%) [17]. The linkage of *MBL2*-SNPs to low MBL serum concentrations is summarised in Fig. 1.

The presence of structural gene mutations in the MBL2 gene is regarded as the most important determinant of MBL-serum levels [32]. Therefore studies in adults usually define MBL deficiency according to the MBL2 genotype [44,45]. As a guide, the median serum concentrations of MBL for British Caucasoids were found to be for the wildtype (A/A) 1630 ng/ml, heterozygous for codon 54 (A/B): 358 ng/ml and homozygous for codon 54 (B/B): ~10 ng/ml [37]. The link between the MBL2 genotype and MBL deficiency has been studied intensively in different populations and is elaborated in several reviews [21–40,44,45]. The present gist related to "our SNPs" may be summarised as follows: the presence of SNPs (0/0; A/0) in the MBL2 gene, as determined by us, are good predictors for low serum levels, however the estimation of the serum levels does not allow a conclusion regarding the genotype.

Our data demonstrate an association of the *MBL2*-SNPs to the FVIII-inhibitor development in HA-patients for *MBL2*-SNPs alone or in combination with the −308 *TNF-α*-SNP.

FVIII can bind in vitro to receptors of the lectin family including the mannose receptor (CD206) [10] or the asiloglycoprotein receptor (ASGPR) [46] and additionally also to siglec-5, galectin-1, galectin 3 [47,48]. Whether these receptors are involved in the internalisation of FVIII in vivo has been questioned [10,47–49]. Our finding that HA-patients, deficient of the lectin-pathway, have a higher inhibitor development, indirectly supports the hypothesis of the involvement of the lectin pathway in the internalisation processes in vivo. *MBL2*-SNPS could influence the inhibitor formation via a lack of clearance of mannosylated FVIII with subsequent presentation of mannosylated/glycosylated FVIII to APCs and thereby fostering an inhibitor

Table 2
Frequencies of the overall *mannose-binding lectin* (*MBL2*) polymorphisms in patients with severe hemophilia A with and without inhibitors.

	Inhibitor patients (n = 119)		Non-inhibitor patients (n = 118)				
Genotype A/0[a]	n	Frequency	n	Frequency	P	OR	CI
0/0	**9**	**0.076**	**–**	**–**	**0.003**	**Fishers Exact**	
A/0	38	0.319	26	0.22	0.192	1.449	0.828–2.537
A/A	72	0.605	92	0.78	0.213	0.776	0.520–1.158

[a] "A" refers to the wildtype allele. "O" collectively refers to the mutated B, C and D alleles. Thus 0/0 stands for either B/B, B/C, B/D, C/C, C/D or D/D. A/0 can represent A/B, A/C or A/D. A/A represents the wildtype. Statistically significant associations are shown in bold. CI: confidence interval; OR: odds ratio. For Alleles B, C and D see also Fig. 1.

Table 3
Frequencies of mannose binding lectin (MBL) low producer and tumor necrosis factor-α (TNF-α) high producer predicted phenotypes in patients with severe hemophilia A with and without inhibitors.

	Inhibitor patients (n = 119)		Non-inhibitor patients (n = 118)				
Combinations	n	Frequency	n	Frequency	P	OR	CI
MBL (A/0, 0/0) + TNF-α (A/G; A/A)	**14**	**0.118**	**4**	**0.034**	**0.020**	**3.47**	**1.11–10.85**
MBL (A/A) + TNF-α (GG)	**46**	**0.387**	**73**	**0.619**	**0.031**	**0.61**	**0.39–0.96**
MBL (A/A) + TNF-α (A/G; A/A)	23	0.19	19	0.161	0.583	1.20	0.62–2.23
MBL (A/0;0/0) + TNF-α (G/G)	36	0.302	22	0.186	0.105	1.62	0.90–2.92

MBL (A/0, 0/0): low producer, common designation for wild-type A, and 0 for the variant alleles B, C, D. TNF-α (A/G) or (A/A): high producer. Please note A/G in brackets behind TNF-α stand for the nucleotide bases Adenine, Guanine whereas A in the brackets behind MBL generally stands for wildtype for the Codons 52, 54 and 57. Statistically significant associations are shown in bold. CI: confidence interval; OR: odds ratio.

development.

In autoimmune diseases like RA or SLE the observed increases in the severity in relation to MBL have been explained by the failure of clearance of immune complexes and apoptotic materials and the impairment of normal humoral responses [18,45].

However, MBL is clinically relevant for its role as third pathway of complement activation. A decreased activity of MBL could reduce the ability to activate the complement system and one might even hypothesise that this could actually decrease the likelihood of an immune response to FVIII. Recent research of Rayes et al. [50] on the possible role of the complement system in inhibitor development indeed showed that complement activation can enhance the immune response to FVIII. Authors demonstrated that the complement component C3 and its cleavage product C3a enhance FVIII endocytosis by dendritic cells and thereby the presentation to a FVIII-specific CD4-T-cell hybridoma in vitro. In a mouse model of severe hemophilia a transient C3 depletion induced by humanised cobra venom factor significantly reduced the primary anti-FVIII response, but did not affects anti-FVIII recall immune responses [50].

An MBl/MASP activation could lead to C3 releases with an enhanced FVIII endocytosis by dendritic cells, resulting in an immune response with a higher number of inhibitors. However we observed the opposite. Several explanations for this observation can be discussed: The C3 convertase of the classical and the lectin pathway (C4b2a) is molecularly distinct from that of the alternative pathway (C3bBb). This C3bBb is special in the complement activation, because by producing C3b it can generate more of itself. This means that once some C3b has been formed by whichever pathway, the alternative pathway can act as an amplification loop to increase C3b production rapidly [51].

One could speculate that a lack of MBL may be compensated by a higher activity of the alternative pathway. This would agree with the proposal of Rayes et al. [50] that a higher spontaneous tick-over of C3 to form C3(H2O) and the fluid phase C3 convertase - C3(H2O)Bb, could lead to a permanent C3b generation, sufficient to facilitate FVIII uptake by professional APCs, resulting in an anti-FVIII immune response.

Indeed, compensatory measures within and among the complement pathways have been observed e.g. in the atypical hemolytic uremic syndrome (aHUS), or in the more common infectious form of this disease (D (+) HUS, EHEC-HUS). The total complement function (e.g. CH50, AH50) and C3 levels were reduced, but went along with increased plasma concentrations of the complement activation products C3a/C3d, Bb, and SC5b-9 [45,52]. Therefore, as suggested by Rayes et al. [50], simultaneous measurements of activation products of the classical, the lectin and the alternative pathway in blood would be required to elucidate the role of the different complement pathways in the inhibitor development, their interplay and potential compensatory

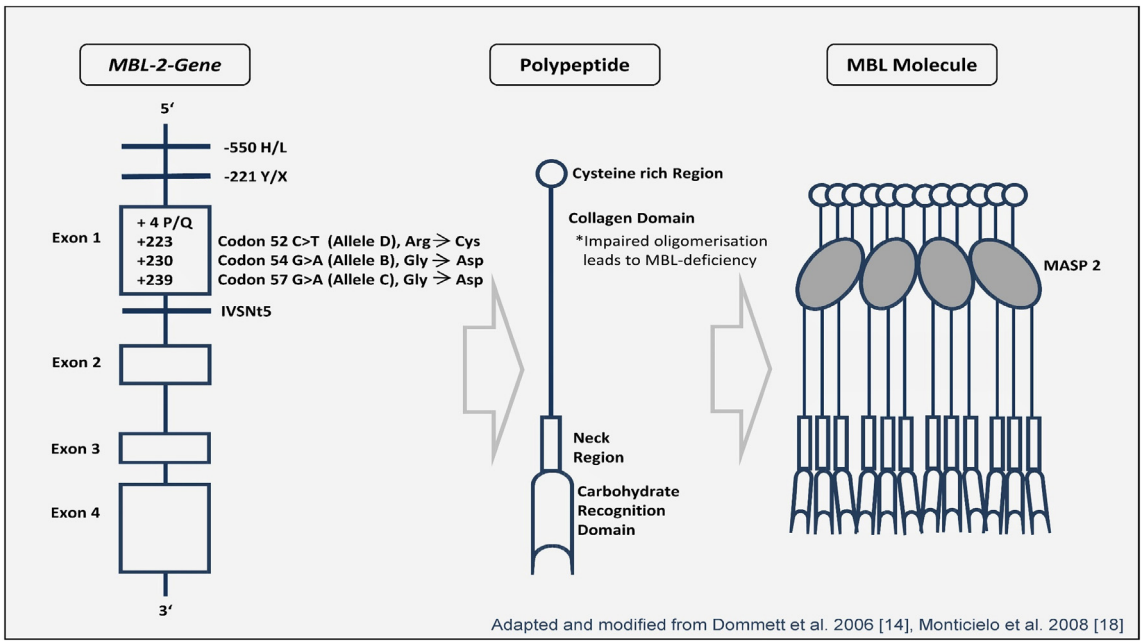

Fig. 1. The organisation of the Exons 1 to 4 of *the MBL2* gene coding for different parts of the MBL-polypeptide are shown. Major impacts on the structural organisation of the MBL molecule have single nucleotide polymorphisms in Exon 1 (Alleles D, B, C). A common designation for the variant alleles D, B and C is 0. They generate amino acid substitutions in the collagen domain. This leads to an impaired oligomerisation and thus a non-functional MBL. MASP: MBL-associated serine proteinases, MBL: mannose binding lectin. Arg: Arginin, Cys: Cystin, Asp: Asparagin. Gly: Glycin.

reactions of the innate immune system.

The immunogenicity of FVIII is intensively investigated [3,53,54]. The carbohydrate structures of FVIII have been identified as relevant for its immunogenicity [9,10,55–58]. Changes in glycosylation sites which participate in packing and stabilisation [55] are likely to lead to changes in the 3D structure and thereby could yield a more immunogenic FVIII. N-glycosylation sites are assumed for the A1 (N42 and N239), C1 (N2118) and A3 (N1810) sites. N239 and N2118 are proposed to be located in the domain interface between A1 and A2 for N239 and between A3 and C1 for N2118. Identified inhibitors are directed towards epitopes in the C2, A3 and A2-domains of the FVIII molecule [56] and thus would match one of the proposed glycosylation sites (A3/N1810). Kosloski et al. [58] demonstrated that a complete deglycosylation of rFVIII resulted in a 40% decrease in cofactor activity, impaired binding to phosphatidylserine and in a lower immune response [58]. Our data support this role of the FVIII glycosylation towards its immunogenicity in patients. Also the ongoing discussions about the immunogenicity of plasma derived (pd) and recombinant FVIII consider differences in the glycosylation of FVIII as highly relevant [57]. A recent analysis of the glycosylation of pdvWF proposed > 300 unique N-linked glycans at 12 sites and 18 O-linked glycans at 10 sites of FVIII. Authors suggest that there might be an optimal glycoform of FVIII which provides stable co-factor activity with less immunogenicity [57].

In this context it is important to note that the C1/C2 domains are proposed to be critical for the immunogenicity of FVIII because of their role in binding to vWF [41,42]. C1 carries the glycosylation site N-2118. Also the A3 domain with its oligosaccharide group at N1810 is believed to be involved in the binding of vWF [55]. VWF was shown to block the mannose dependent immunogenicity of FVIII in vitro [10]. The vWF shielded the uptake of FVIII by antigen-presenting cells [9]. A shielding of immunogenic FVIII glycosylation sites by vWF is also in agreement with recent clinical findings. The first prospective study in which 48 hemophilia A patients with inhibitors and poor prognosis for the immune tolerance induction (ITI) were treated with a combined concentrate of pdFVIII/vWF resulted in a high ITI success [59]. However, it may be noted that conclusions on potential differences in the immunogenicity of rFVIII or pdFVIII [60] cannot be drawn from these results. A shielding of potential immunogenic sites by vWF may take place irrespective of the source of FVIII products.

Another mechanism by which *MBL2*-SNPs could contribute to a higher FVIII inhibitor development arises from recent findings on the involvement of the MBL-pathway in the coagulation process [61–64]. Native MBL and MASP-1/3 together were required for a thrombin-like activity that initiated coagulation [61], leading to the suggestion of a cross-talk between the MBL/MASP complement pathway and coagulation pathway. MASP-1 knock out mice showed prolonged bleeding times [61,65] and significantly decreased ferric chloride-induced occlusive arterial thrombogenesis [65,66]. A higher number of bleeding events is a known risk factor for inhibitor development in HA. However, data regarding an association of an additional bleeding tendency due to an MBL deficiency in HA are not available so far. A study investigating the potential influence of MBL deficiencies and the half-life of therapeutic FVIII could further substantiate links between the inhibitor development and MBL as well as the cross-talk between the MBL complement and the coagulation pathway. It may in this context be mentioned that MBL deficiency in combination with a high activation of the complement pathway has very recently been linked to unprovoked venous thromboembolism. The underlying mechanism for this possible association is not yet known [67].

The kinetics of MASP-1 and -2-mediated cleavage of coagulation factors are slow compared to coagulation proteases and therefore the function of MASP-1 as coagulation proteases might be disputed [68]. Nevertheless the presented data support the suggestion that complement and coagulation may be considered as inseparable network [68].

The *MBL2*-SNPs are also associated with an increased susceptibility to infections in neonates, in young children with recurrent serious infections and in immunocompromised individuals [17,69]. Thus a higher rate of infections could lead to a higher rate of inhibitors.

In HA a higher rate of infections is discussed as part of the danger theory [3,70]. Surgery, severe bleeds, vaccinations and infections may be episodes initiating danger signaling in patients, resulting in immune reactions to administered FVIII. In particular, severe joint bleeds in HA could stimulate a local pro-inflammatory response and antigen presenting cells (APCs) could take up FVIII with subsequent antigen presentation to CD4+ T cells [3,71] followed by an antiFVIII immune response. But clear conclusions regarding the role of infections on inhibitor development are still difficult to draw due to discordant results in different studies [3,72].

It has further been suggested that microbial components trigger the toll-like receptors (TLRs) and thereby preformed FVIII-specific memory B-cells are restimulated. They foster the inhibitor formation by high doses of FVIII during IT- therapy [73]. A higher rate of infections due to the lack of MBL could generally trigger TRLs and stimulate the innate immune system in such a way that it promotes inhibitor formation and in case of an ITI interferes with the progression of immune tolerance therapies.

An increased inhibitor formation by the −308 *TNF-α* polymorphism has been demonstrated earlier [42]. A combination with the *MBL*-SNPs increases the inhibitor presence in the patient group to substantial 11.8% which could indicate a relevance of MBL-SNPs as genetic risk factors for inhibitor development in HA. However, our study in patients with severe hemophilia has some limitations. The statistically significant association between MBL2 alone and the inhibitor development occurs in the 0/0 genotype which is present in 9 out of 237 patients. Thus the impact of MBL-SNPs alone on the total number of inhibitor occurrences is limited (9/119). Further experimental and mechanistic data are required to understand whether and how the lectin pathway of the innate immune system interferes with the inhibitor development and how a removal of mannosylated FVIII via MBL could provide a protection.

5. Conclusion

We can demonstrate an association of *MBL2*-SNPs alone or combined with the −308 *TNF-α* SNP with the inhibitor development. Simultaneous investigations of components of all three complement pathways are essential to understand their individual and overall contribution to the inhibitor development in HA.

Declaration of Competing Interest

The authors stated that they had no interests which might be perceived as posing a conflict or bias.

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Authors' contributions

GUM, AP, AH and GG performed the research. GUM, AP and JO designed the research study. GUM, HA and AP analysed the data. GUM wrote the manuscript with support of AP and all authors providing critical input and editing.

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Appendix A. Supplementary data

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