HAEMOPHILIA A: FROM MUTATION ANALYSIS TO NEW THERAPIES

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Abstract | Haemophilia is caused by hundreds of different mutations and manifests itself in clinical conditions of varying severity. Despite being inherited in monogenic form, the clinical features of haemophilia can be influenced by other genetic factors, thereby confounding the boundary between monogenic and multifactorial disease. Unlike sufferers of other genetic diseases, haemophiliacs can be treated successfully by intravenous substitution of coagulation factors. Haemophilia is also the most attractive model for developing gene-therapy protocols, as the normal life expectancy of haemophiliacs allows the side effects of gene therapy, as well as its efficiency, to be monitored over long periods.

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Haemophilia A is the most severe inherited bleeding disorder to affect humans and is among the most common disorders of this kind. Its effects were recognized even in ancient times: it was first described in the second century in the Talmud, where it was stated that male babies need not be circumcized if two brothers had already died from the procedure. In the twelfth century, an Arabian physician described a family in which males died of bleeding after minor injuries. The first family history of haemophilia, which spanned three generations, was described in 1803 (REF. 1), and the word 'haemophilia' was first used by Hopff in 1828 (REF. 2). In 1952, Aggeler *et al.*[3] and Biggs *et al.*[4] recognized and described the two currently accepted types of haemophilia: type A and type B (the latter is also referred to as Christmas disease; see REF. 5 for a detailed review on the early history of haemophilia). Both diseases are recessive and linked to the X chromosome. Haemophilia A and haemophilia B are caused by reduced levels of coagulation factors VIII and IX (F8 and F9), respectively, both of which are members of the blood-coagulation cascade.

Haemophilia A is also called the Royal disease, because Queen Victoria of England (1837–1901) was a carrier, and from her it spread to the royal families of Spain, Germany and Russia. Haemophilia A is considered to be one of the model disorders in the field of molecular human genetics, because several factors have made it a leading model in the field — these include its well-described phenotype and genetic basis, including the influence of other proteins on the penetrance and characteristics of the disease (for example, factor V (F5), prothrombin and von Willebrand factor (VWF)), its high frequency (1 in 5,000 males) and its successful treatment using isolated F8, which was first purified to homogeneity from blood in 1983 (REF. 6) (although see BOX 1 for the complications that are associated with this therapy).

Unfortunately, blood-derived F8 concentrates that were used for intravenous therapy in the 1980s were frequently contaminated with the human immunodeficiency virus (HIV), leading to AIDS in ~40% of the treated haemophiliacs[7] and causing death in most of them. As a result, the development of recombinant F8 concentrates was strongly encouraged. The sequence of the F8-encoding gene (*F8*) was reported in 1984, and was the largest gene (186 kb) to be described at that time[8]. Therapy using recombinant F8 started as early as 1993. Today, initial clinical trials have been undertaken for somatic gene therapy of haemophilia A. An overview of the principal features of the *F8* gene and its encoded protein is given in FIG. 1.

Owing to the rapid increase in the number of families that are being diagnosed with haemophilia

Box 1 | Alloantibodies against the coagulation factor VIII protein

One of the most severe complications to arise from the treatment of haemophiliacs with isolated coagulation factor VIII (F8) is the development of antibodies against F8 (alloantibodies, also called inhibitors), which neutralize the substituted F8. Three inhibitory mechanisms have been described so far:

- **Binding of anti-F8 antibodies to sites in F8 that interact with other coagulation factors[90];**
- **The effect of non-neutralizing antibodies on the half-life of transfused F8 protein[91];**
- **Cleavage of F8 by catalytic antibodies[92].**

This life-threatening situation can be overcome by so-called immune-tolerance therapy, which involves giving patients a large excess of F8 for 1–2 years[93].

Surprisingly, F8 antibodies have also been detected in healthy individuals and in haemophiliacs that do not have inhibitors. In these cases, inhibitors might be neutralized by ANTI-IDIOTYPIC ANTIBODIES. It is therefore possible that autoimmunity only arises when the equilibrium between F8-neutralizing antibodies and anti-idiotypic antibodies is disrupted[94,95]. It is also possible that the specific epitope repertoire recognized by CD4+ CELLS is crucial for the synthesis of IgG antibodies directed against F8. CD4+ T-cells have a central role in the humoral immune response to protein antigens and have also been described in normal subjects.

A, information about pathogenic alleles of *F8* and of interacting genes continues to increase. Today, more than 940 unique mutations of different types are collected in the worldwide haemophilia database (HAMSTeRS). Recently, Oldenburg *et al.*[9] described a further 845 mutations in the *F8* gene. The availability of such a high number of alleles has opened up a new avenue for understanding the function of the different domains of the large F8 protein in more detail. In addition, the haemophilia example supports the generally accepted view that a collection of many allelic variants facilitates the understanding of the genetic diversity that underlies a hereditary disorder. The study of haemophilia has also taught us that a monogenic disorder can be influenced by mutations and polymorphisms in other factors — in the case of haemophilia, by those involved in F8 processing and function. Given the wealth of information we have on haemophilia A, it is not surprising that this disorder has also stimulated pioneering approaches in the field of gene therapy.

Clinical features

Haemophilia A affects 1 in 5,000 males, an incidence that does not vary appreciably between populations. However, the worldwide incidence of the disease is probably higher than this, as not all affected patients are diagnosed in developing countries. This makes haemophilia A one of the most common inherited diseases. A familial history is known in about two thirds of cases, and it appears sporadically in one third of cases.

In healthy individuals, F8 circulates in the plasma in trace amounts (~100 to 200 ng ml^{-1}; REF. 10). A decrease in F8 activity caused by mutation leads to a loss of clotting activity and therefore to unchecked bleeding into joints, muscles or inner organs. Recurrent bleeding into joints leads to CHRONIC SYNOVITIS[11]. In developed countries, haemorrhage into the CNS is a leading cause of death in haemophiliacs, accounting for almost 20% of non-infective deaths.

Depending on the residual activity of mutant F8, the severity of the disorder in affected males ranges from mild (5–30% activity; individuals with values at the higher end of this range are said to suffer from subhaemophilia) to moderate (2–5% activity) to severe (<1% activity). The frequency of the mild, moderate and severe forms is 50%, 10%, and 40%, in corresponding order[12]. The residual activity of the F8 protein in carrier females is usually ~50%. However, in some cases, activity falls below this mark and reaches pathological levels, as is manifested by an extended period of menstruation. Homozygous females, although rare, also suffer from haemophilia A in a similar way to hemizygous male patients.

Factor VIII interacting proteins

Interactions in the cell. Biochemical data have provided strong evidence that F8 is one component of a functional network of other factors (consisting mainly of structural proteins and enzymes), and that it frequently interacts with them. These interactions affect *F8* expression, transport through the cell, activity, stability and function. Examples of the proteins involved in the transport of F8 are LMAN1 (lectin, mannose-binding 1; also known as ERGIC53, an endoplasmic reticulum (ER)–Golgi intermediate compartment 53 kDa protein) and MCFD2 (multiple coagulation factor deficiency 2 protein). Both proteins form stable complexes with F8 in liver cells. LMAN1 and MCFD2, both of which are intracellular, form a complex within the secretory pathway of the cell and probably transport newly synthesized F5 and F8 from the ER to the Golgi apparatus. Defects in LMAN1 (REFS 13,14) and MCFD2 (REF. 15) result in a combined deficiency for F5 and F8 (for a review see REF. 16).

Interactions outside the cell — VWF. Once F8 is secreted from the cell, several other proteins interact with this protein in the blood (for a summary and details see FIG. 2; BOX 2). The most important of these is VWF. Because of the strong interaction between F8 and VWF and the protective function of VWF for F8 against the proteolytic action of activated protein C (APC), it is not surprising that mutations which affect the binding sites of VWF and F8 for each other can cause clinically similar phenotypes[17]. Mutations in VWF that affect the F8-binding site (encoded by VWF exons 18–20) have been identified in patients with von Willebrand disease (VWD), type Normandy 2 (commonly referred to as 2N VWD), a phenotype that can be described as 'pseudohaemophilia'[18]. Unlike haemophilia A, the inheritance of 2N VWD is autosomal recessive. Depending on the mutations involved, F8 activity in patients with 2N VWD might be as low as that seen in severe haemophilia A (that is, ~1% of normal F8 activity; for example, in the rare VWD mutation Glu787Lys), in the range of moderate to mild haemophilia A (which is the case for the more common mutations Thr791Met, Tyr795Cys and Arg816Trp), or in the range of subhaemophilia (which is the case for Arg854Gln, the most common mutation seen in 2N VWD[19,20]).

ANTI-IDIOTYPIC ANTIBODIES
Antibodies that bind to the specific antigen-binding sites of other antibodies.

CD4+ CELLS
A subset of white blood cells that identify, attack and destroy infection and cancerous cells.

CHRONIC SYNOVITIS
The concurrent non-use of muscles around a joint that is subject to repeated bleeding leads to a periarticular muscular atrophy with loss of dynamic support, which predisposes the joint to further bleeding.

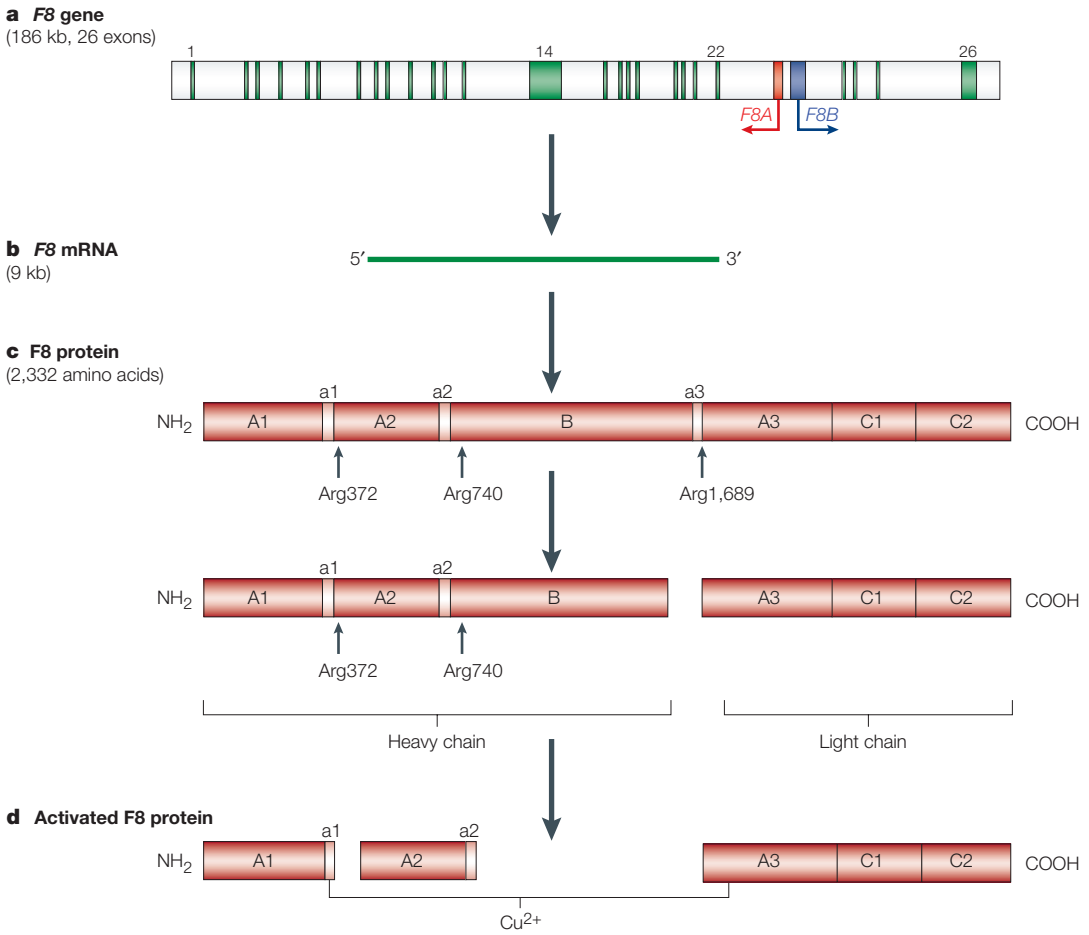


Figure 1 | **Principal features of the coagulation factor VIII gene and protein.** The diagram shows the salient features of the coagulation factor VIII (*F8*) gene, as well as the production and processing of the encoded protein. **a** | The *F8* gene spans 186 kb and consists of 26 exons, 24 of which vary in length from 69 to 262 bp; the remaining two exons, exons 14 and 26, are 3,106 and 1,958 bp in length, respectively, and are much larger than the others. Most of exon 26 consists of a 3′ untranslated sequence. Intron 22 contains two further genes (*F8A* and *F8B*); coloured arrows (red and blue, respectively) indicate the orientation of their transcription. **b** | The spliced *F8* mRNA is approximately 9kb in length. **c**,**d** | The *F8* mRNA is translated into a precursor protein of 2,351 amino acids (267 kDa), which is processed to the final product (shown in **c**, upper panel) that lacks only 19 amino acids from its N-terminal leader sequence. Further processing by thrombin (black arrows pointing upwards in **c**) leads to the activation of F8. First, cleavage at Arg1,689 (at the B–A3 junction) generates a variably-sized (90–210 kDa) heavy chain, consisting of domains A1 and A2 and heterogeneous fragments of the partially proteolysed B domain; during the process, a 40-amino-acid acidic peptide (a3) is released from the C-terminal product to form a 73-kDa light chain that consists of domains A3–C1–C2 (**c**, upper panel). Further cleavage by thrombin (**c**, lower panel) removes most of the B domain and cleaves the protein between the A1 and A2 domains: cleavage at Arg372 (between the A1 and A2 domains) and at Arg740 (between the A2 and B domains) generates the 54-kDa A1 and the 44-kDa A2 domains (**d**). The activated protein is held in a complex with Cu^{2+}. Homologues of *F8* in the mouse[105] and rat[106] are similar in sequence to the human, but are slightly shorter. The rat F8 sequence is 61 amino acids shorter than the mouse and 92 amino acids shorter than human F8, resulting in products that are 250 kDa and 266 kDa in the mouse and rat, respectively. The overall identity at the protein level between the mouse and human F8 is 74% and between rat and human only 61%.

Patients with 2N VWD cannot be distinguished from haemophilia A patients by conventional diagnostic tests. As a result, several individuals (in particular females) that were initially diagnosed with haemophilia A but that were subsequently found not to have mutations in *F8* were re-diagnosed with 2N VWD by means of an F8-binding assay and subsequent molecular testing[20,21]. As the VWF Arg854Gln variant, which affects VWF:F8 binding, is so common (the allele frequency is 1% in the Dutch population[22]), this mutation should also be regarded as a disease-modifying factor for haemophilia A. Mutations that cause 2N VWD are listed in the VWF database of the International Society on Thrombosis and Haemostasis web site.

The differential diagnosis of 2N VWD and haemophilia A has large implications for genetic counselling and the choice of adequate treatment. Prophylaxis and therapy of patients with 2N VWD using modern recombinant F8 concentrates will be inefficient owing to a lack of VWF. Therefore,

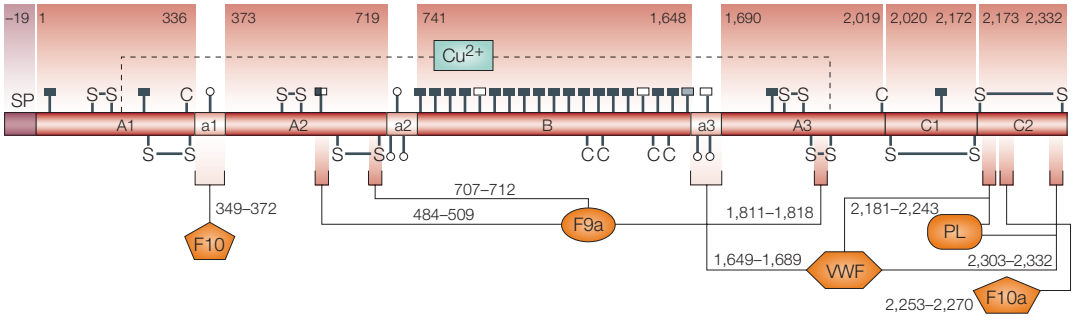


Figure 2 | **Properties and interaction sites of the coagulation factor VIII protein.** The entire coagulation factor VIII (F8) protein is shown (including the signal peptide (SP) at the N-terminus, the large domains A1, A2, B, A3, C1 and C2, and the intervening domains a1, a2 and a3; see also FIG. 1). The numbers indicate amino-acid positions. The sites of interaction of F8 with phospholipids (PL) and other coagulation factors (von Willebrand factor (VWF), activated coagulation factor IX (F9a), coagulation factor X (F10) and activated F10 (F10a)) are also indicated. The pattern of glycosylated Asn residues is important for the trafficking of F8 from the endoplasmic reticulum to the Golgi apparatus (black squares indicate glycosylated Asn residues, white squares indicate non-glycosylated Asn residues, black and white squares indicate partly glycosylated Asn residues, and grey squares indicate potentially glycosylated Asn residues). Sulphated Tyr residues are important for efficient thrombin cleavage (white circles). The correct formation of disulphide bridges (S-S), relative to the free Cys residues (C), is necessary for the proper folding of the entire protein. Apart from sites of interaction, data are taken from REFS 108,109.

treatment of patients with 2N VWD requires plasma-derived F8 concentrates that also contain sufficient amounts of functional VWF.

Interactions outside the cell — prothrombotic risk factors. Other factors that might influence the haemophilic phenotype are prothrombotic risk factors. One of these is a mutation in the *F5* gene (the F5 Leiden allele), which renders the activated F5 protein resistant to APC owing to the loss of an APC-cleavage site. This mutation predisposes the patient to thrombosis. The prevalence of F5 Leiden varies; however, heterozygosity for the F5 Leiden mutation is recognized as being the most common heritable THROMBOPHILIC defect in Caucasian populations[23]. Other mutations affect the thrombin gene (the Gly20,210Ala mutation, for example, is common among Caucasians[24]) and the *MTHFR* gene (which encodes 5,10-methylenetetrahydrofolate reductase[25]), as well as those that lower the plasma concentrations of antithrombin, protein C or protein S. How these mutations are connected to haemophilia is still controversial. A recent review of the literature[26] concludes that the F5 Leiden mutation is the factor that most consistently seems to decrease the severity of severe haemophilia, whereas the involvement of other thrombotic factors is inconclusive. For future research, it would be helpful to establish criteria that distinguish severe patients at the milder end of the spectrum from those with a more severe phenotype.

Mutant profiling in haemophilia A

The* F8 *gene. Human *F8* maps to the most distal band (Xq28) of the long arm of the X chromosome[27,28]. The gene is 186 kb in length and comprises 26 exons; it encodes a precursor protein of 2,351 amino acids, from which 19 N-terminal residues of the signal peptide are removed to generate the mature, functional product.

Intron 22 of the *F8* locus contains two nested genes, *F8A* (REF. 29) and *F8B* (REF. 30), which are controlled by a bidirectional promoter. *F8A* is transcribed, without introns, in the opposite direction to the *F8* gene. Recently, the *F8A* gene was shown to code for a 40-kDa huntingtin-associated protein (termed HAP40 (REF. 31)) and is thought to be involved in the aberrant nuclear localization of the huntingtin protein in Huntington disease. The function of the *F8B* gene is not known. As there is no *F8B* equivalent in the mouse genome, transgenic mice that express the wild-type human *F8B* under the control of a cytomegalovirus promoter have been used to understand its function. Surprisingly, these mice showed growth retardation, microcephaly and severe ocular defects, which should encourage further studies of this protein[32].

F8 *mutations.* Six introns of the human *F8* gene are exceptionally large (>14 kb); intron 1 and intron 22 are particularly noteworthy because of their frequent involvement in pathological inversions, which are caused by recombination with homologous regions outside the *F8* gene. The region that is homologous to intron 1 (*int1h2*) is located ~140 kb towards the telomere; this 1,041-bp repeat differs only at a single nucleotide from the intron-1 sequence (*int1h1*) and is in the opposite orientation. Intrachromatid or intrachromosome homologous recombination of these two regions causes an inversion that displaces exon 1 of the *F8* gene by ~140 kb towards the telomere[33,34] (FIG. 3a). This inversion causes 1–4% of all severe cases of haemophilia A[9,35].

However, the most frequent inversion to affect *F8* involves intron 22 (32 kb; see FIG. 3b). This inversion, which is responsible for almost half the cases of severe haemophilia A, occurs because there are two regions homologous to sequences in intron 22 that are positioned approximately 500 kb telomeric to the *F8* gene (these are termed *int22h2* and *int22h3*; *int22h1* is the sequence in intron 22 that also includes the *F8A* gene)[29,36]. The sequence identity of the three regions

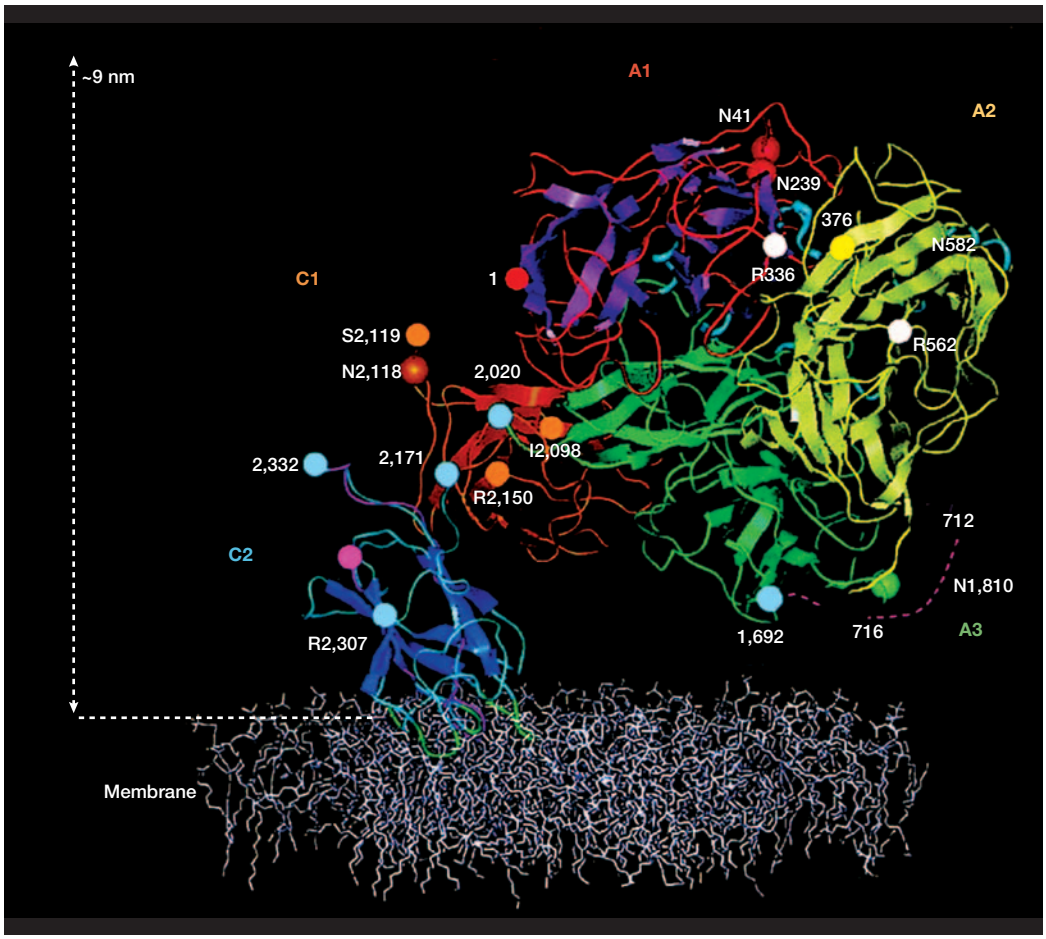
THROMBOPHILIA
Abnormal haemostasis parameters, as assessed in the laboratory, that are associated with an increased risk of blood clotting in veins or arteries.

Box 2 | **Crystal structure of mature coagulation factor VIII**

The three-dimensional structure of the membrane-bound form of the coagulation factor VIII (F8) protein is shown here (at 1.5-nm resolution)[68]. The three A domains (A1 (red), A2 (yellow) and A3 (green)) each comprise two highly conserved β-barrel core structures that are slightly tilted with respect to each other[63]. They bind Ca^{2+} and Cu^{+}, as well as coagulation factor X (F10) and activated coagulation factor IX (F9a), and are essential for catalytic co-factor activity. The central B domain contains cleavage sites for thrombin and is removed in the course of the transition from an inactive to an active stage of F8, and is therefore not shown. The two C-terminal C domains (C1 and C2 (both blue)) contribute to the binding of VWF and F10a, and, in the case of activated F8, also to the binding of phospholipids[63].

According to this model, the A3 domain is in close association with the C1 and C2 domains near the phospholipid membrane. The C2 domain is slightly inclined to the phospholipid surface, covering an area of 12 nm^2. Four C2 loops are embedded within the lipid monolayer. The C1 domain forms almost a right angle with C2, with its long axis nearly parallel to the membrane. Asn residues (N41; N239; N582; N1,810; and N2,118) that are known to be glycosylated are represented with large spheres; activated protein C (APC) cleavage sites at Arg336 and Arg562 are given as white spheres; the side chains of Arg2,307, Ile2,098, Ser2,119 and Arg2,150 are solvent-exposed and important for binding VWF.

Solving the structure of F8 in greater detail will no doubt improve our understanding of the structure–function relationship of this protein in health and disease. It can be used to confirm functional models that are based on biochemical and genetic data (such as known binding sites or mutational consequences), and it helps to generate an integrated model that accommodates our functional knowledge of each domain. The converse is also true — the effects of mutations can be predicted by the structure and, if necessary, confirmed by practical assays. Image reproduced, with permission, from REF. 68 © (2002) American Society of Hematology.



is 99.9%. Intrachromosomal homologous recombination between *int22h1* and one of its two telomeric copies leads to an inversion of the corresponding parts of the *F8* gene. Male germ cells show a >10-fold higher rate of this type of mutation than do females, in which this recombination event is inhibited during meiosis by homologous pairing of the X chromosomes[37].

The most recent update of the HAMSTeRS database (in November 2004) lists more than 120 large deletions (>50 bp) as being causative for haemophilia A

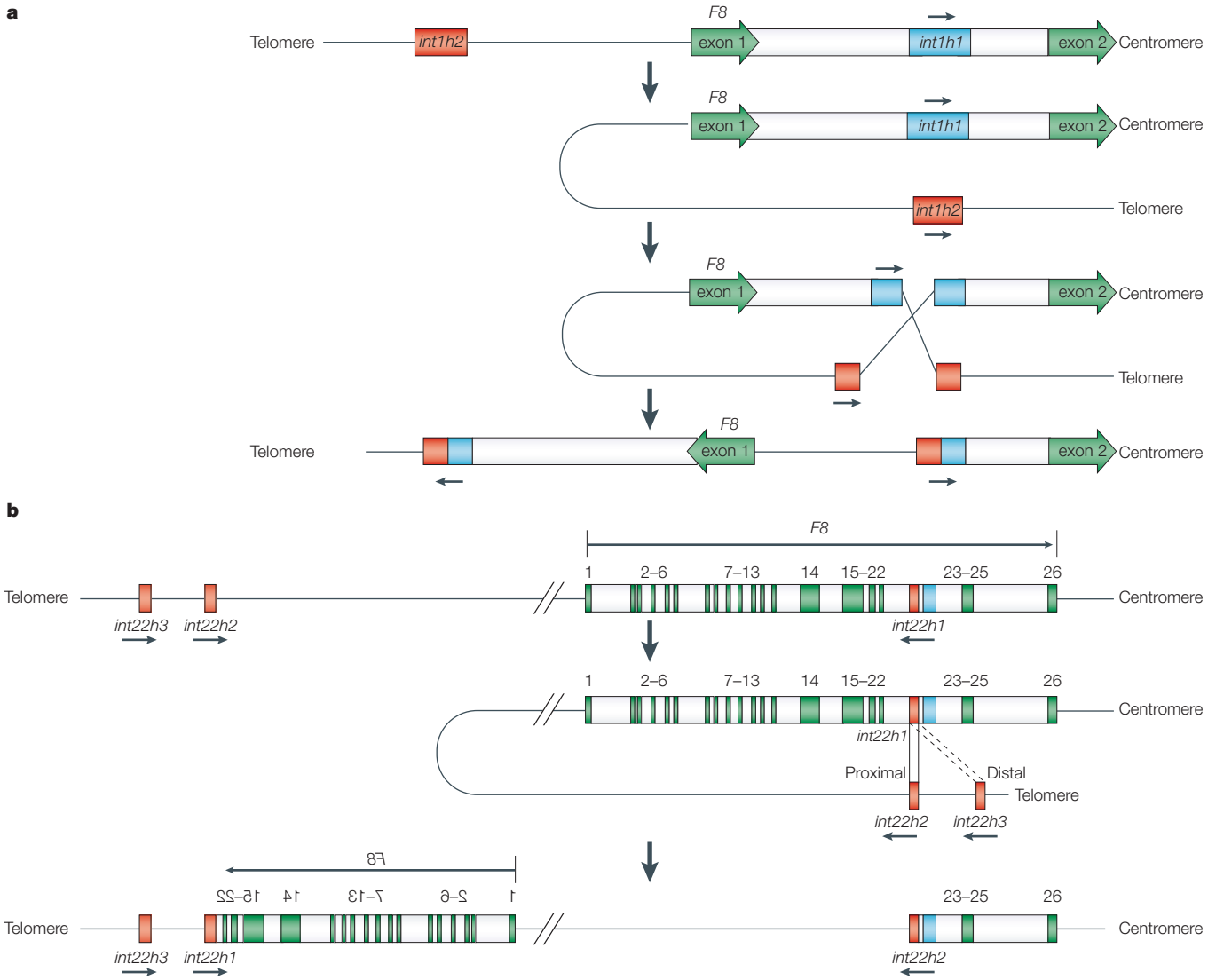


Figure 3 | **Pathological inversions of the coagulation factor VIII gene. a** | The figure shows coagulation factor VIII (*F8*) intron 1, which is flanked by exons (green; drawn to indicate the direction of transcription), and contains a repeated sequence *int1h1* (blue) flanked by unique sequences. The line represents DNA that lies outside the *F8* gene, and the repeated *int1h2* sequence is indicated as a red box. Arrowheads indicate the orientation of repeated sequences. The subsequent steps in the diagram illustrate how homologous recombination between the two *int1h* repeats explains the origin of inversion. This leads to severe haemophilia A in ~1% of all cases. Modified, with permission, from REF. 34 © (2002) American Society of Hematology. **b** | Within intron 22 of the *F8* gene, a fragment (referred to as *int22h1*) has sequence similarities to two fragments that are distal to the *F8* gene *(int22h2* and *int22h3*). Arrowheads indicate the orientation of the repeated sequences. By intrachromosomal homologous recombination, one of these outside regions forms a crossing-over structure with the corresponding element within intron 22, resulting in an inversion of exons 1–22 with respect to exons 23–26 of the *F8* gene. This leads to severe haemophilia A in ~35% (REF. 9) to 45% (REF. 36) of all cases. Although *int22h1* can recombine with either *int22h2* or *int22h3*, only the consequences of recombination with *int22h2* are shown. Modified, with permission, from *Nature Genetics* REF. 110 © (1993) Macmillan Magazines Ltd.

(comprising >90% of severe cases and a third of those associated with the formation of anti-F8 antibodies). Most of these deletions span several kilobases, but the exact breakpoints have been characterized only in a few cases. Some recent studies[38–40] indicate that recombination which is mediated by *Alu* elements can explain such large deletions and that this is a common mechanism of mutation in the *F8* gene. At least 49 regions related to the *Alu* core sequence have been identified in *F8* (REF. 39). By contrast, point mutations are the most common type of mutation in the *F9* gene[41]; in this case, spontaneous germ-line mutations that lead to haemophilia B occur mostly at CpG dinucleotides.

CpG dinucleotides also occur frequently in the *F8* gene. A high frequency of recurrent point mutations (stop and missense mutations) can be observed at these positions, which indicates that there are other hotspots for mutations in the *F8* gene. Four out of six Arg codons contain CpG dinucleotides, providing an explanation to why Arg is the most mutated

Table 1 | **Recurrent mutations in the coagulation factor VIII (*F8*) gene***

Exon	Nucleotide change	Amino acid [affected domain]	Amino-acid change	Proposed function	Disease severity	Frequency* (no. of cases)
4	GTG>ATG	162 [A1]	Val>Met	Unknown	Moderate–mild	26
8	CGA>TGA	336 [A1]	Arg>Stop	APC cleavage site	Severe	13
10	GGA>AGA	479 [A2]	Gly>Arg	Putative binding site for activated F9	Moderate–mild	14
11	CGG>TGG	527 [A2]	Arg>Trp	Unknown	Mild	22
11	CGC>TGC	531 [A2]	Arg>Cys	Incorrect disulphide bond?	Moderate–mild	19
11	CGC>CAC	531 [A2]	Arg>His	Unknown	Mild	12
12	CGC>TGC	593 [A2]	Arg>Cys	Incorrect disulphide bond?	Moderate–mild	35
14	TAT>TTT	1,680 [B]	Tyr>Phe	VWF binding	Moderate	24
14	CGC>TGC	1,689 [a3]	Arg>Cys	Thrombin activation site	Moderate	20
16	CGT>CAT	1,781 [A3]	Arg>His	Unknown	Moderate	11
18	CGA>TGA	1,941 [A3]	Arg>Stop	Unknown	Severe	15
18	CGA>CAA	1,941 [A3]	Arg>Gln	Unknown	Mild–moderate	10
18	CGA>TGA	1,966 [A3]	Arg>Stop	Unknown	Severe	13
18	CGA>CAA	1,966 [A3]	Arg>Gln	Unknown	Mild	12
19	CGG>TGG	1,997 [A3]	Arg>Trp	Unknown	Severe–moderate	28
19	GTG>GCG	2,016 [A3]	Val>Ala	Unknown	Moderate	11
22	CGA>TGA	2,116 [A3]	Arg>Stop	Unknown	Severe	13
23	CGA>TGA	2,147 [C1]	Arg>Stop	Unknown	Severe	17
23	CGT>CAT	2,150 [C1]	Arg>His	Impaired VWF binding	Mild–moderate	50
23	CGC>TGC	2,159 [C1]	Arg>Cys	Impaired VWF binding; incorrect disulphide bond?	Mild	27
23	CGC>CAC	2,163 [C1]	Arg>His	Unknown	Moderate	12
24	CGA>CAA	2,209 [C2]	Arg>Gln	Interaction with VWF and phospholipids	Severe–moderate	23
24	CGA>TGA	2,209 [C2]	Arg>Stop	Interaction with VWF and phospholipids; nonsense-mediated decay?	Severe	19
25	TGG>TGT	2,229 [C2]	Trp>Cys	Interaction with VWF; incorrect disulphide bond?	Mild–moderate	16
26	CGA>TGA	2,307 [C2]	Arg>Stop	Interaction with VWF; nonsense-mediated decay?	Severe	11
26	CGA>CAA	2,307 [C2]	Arg>Gln	Interaction with VWF	Mild	16

*Data according to the HAMSTeRS database; the mutation is mentioned in the table only if it was reported at least 10 times in the database. The total number of unique single-base mutations is 615. APC, activated protein C; F9, coagulation factor IX; VWF, von Willebrand factor.

amino acid in the F8 protein. The most frequent point mutation occurs at codon 2,150 in exon 23, where the CGT>CAT mutation (which converts the Arg to a His) has been reported 50 times (HAMSTeRS database; TABLE 1).

Other types of recurrent mutation consist of deletions or insertions of one A nucleotide in A stretches at different positions of the *F8* gene. The longest stretch consists of 9A nucleotides (at codons 1,191–1,194): an A deletion was reported 34 times at this position (HAMSTeRS database), whereas the insertion of an A is reported 12 times (HAMSTeRS database). In both cases, the mutations lead to a frameshift during translation and to a severe form of haemophilia A. The recurrent insertion or deletion of a single A has also been reported at shorter

Table 2 | **Mutation profile in 845 families with members who are haemophilia A patients***

Mutation type	Number of mutations observed	Percentage
Intron-22 inversion		
Distal	260	30.7
Proximal	37	4.4
Rare	5	0.6
Total	302	35.7
Intron-1 inversion		
Total	8	1.0
Point mutations		
Missense	323	38.2
Nonsense	79	9.3
Total	402	47.5
Small deletions or insertions		
Small deletions	63	7.5
Small insertions	22	2.6
Combination of insertions and deletions	1	0.1
Total	86	10.2
Large deletions (>50 bp)		
Total	25	3.0
Splice-site mutations		
Intronic	20	2.4
Cryptic	2	0.2
Total	22	2.6

*In this cohort, 25% of families had a mild or moderate haemophilia A phenotype in which almost exclusively missense mutations are found. In a cohort of severe haemophilia A patients the proportion of missense mutations would decrease to less than 15%, whereas the proportions of the other mutation types would increase by about 25% compared with the values given in the table[9].

stretches of A nucleotides. These short insertions or deletions are probably caused by slippage errors that are introduced by the DNA polymerase during DNA replication[42]. It has also been shown that such errors occur more frequently at A–T homo-oligomers than at C–G homo-oligomers[43]. Recently, Oldenburg *et al.*[9] have published the overall frequency of mutation types within 845 haemophilia A families; the corresponding overview is given in TABLE 2.

Mutation profiling in haemophilia A is routinely carried out on genomic DNA that is obtained from blood cells that still contain a nucleus (such as leucocytes, lymphocytes and monocytes). However, this approach usually cannot detect mutations that affect splicing. Even if the *F8* sequence reveals that highly conserved splice-donor or splice-acceptor sites are affected, the splicing product cannot be assessed; this is because the *F8* gene is expressed mainly in the liver, which is an inaccessible tissue. Therefore, efforts have been undertaken in the past few years to obtain *F8* mRNA from the 'leaky transcription' that takes place in leucocytes. In some cases, predicted splicing errors have been confirmed[44].

One of the remaining questions in haemophilia research is how expression of the *F8* gene is regulated. A few classical promoter studies[10,45,46] have reported that a short region from –279 to –64 contains all the necessary elements for maximum promoter activity. In particular, several transcription factors, such as the hepatocyte nuclear factors 1 and 4 (HNF1 (or TCF1) and HNF4), the CCAAT/enhancer-binding proteins (C/EBP) α and β (CEBPA and CEBPB), nuclear factor κB (NF-κB) and members of the nuclear transcription factor Y (NFY) family, interact with the proximal promoter. Surprisingly, no mutation in the promoter has been identified to be causative for decreased or increased F8 activity, and therefore to be responsible for haemophilia or the opposite condition, thrombophilia (although increased F8 activity is a clear risk factor for thrombophilia).

The principal functional domains of the F8 protein. The amino-acid sequence of human F8 was deduced from its cDNA sequence and is consistent with sequence analysis of tryptic peptides[47]. The domains of the mature protein belong to three classes, A, B and C: the three A domains (A1 to A3; see FIG. 1D and for more detail, FIG. 2) are homologous to those of another coagulation factor, F5, and to CERULOPLASMIN. Although the B domain has no substantial homology with other sequences, the C domain shares 20% amino-acid homology with the discoidin lectins from

CERULOPLASMIN
A plasma metalloprotein that binds most copper ions in the plasma. It is involved in the peroxidation of Fe^{2+} transferrin to form Fe^{3+} transferrin. Ceruloplasmin, F5 and F8 have a common evolutionary origin. Loss of ceruloplasmin activity leads to diabetes mellitus, retinal degeneration and dementia.

Dictyostelium discoideum. Three smaller domains (a1, a2 and a3) are located between the main domains A1–A2, A2–B and B–A3, in corresponding order. Much of the information on binding sites of reaction partners of the F8 protein have come from only a few missense mutations that lead to decreased F8 activity but do not affect the concentration of the F8 antigen (these cases are referred to as 'cross-reactive material positive' or CRM+). These are the only mutations for which we can assume that only the function of the F8 protein is destroyed, but not its structure.

Mutations that affect the A domains. Based on the HAMSTeRS database, approximately half the point mutations in the *F8* gene can be ascribed to domains A1 and A2, which demonstrates the importance of these domains for the activity of the F8 protein and/or that these regions are highly susceptible to mutations. Moreover, mutations in these regions alter, at least in part, the dynamics of the interaction of F8 with its reaction partners, and therefore lead to differences in the stability of the activated form of F8. These features can be detected by discrepancies in different assays for F8 activity (ONE-STAGE CLOTTING ASSAY versus the CHROMOGENIC ASSAY), each of which measures the activity of different aspects of F8 function within the coagulation cascade. Common missense mutations in the A domain include Glu321Lys, Tyr346Cys and Glu720Lys; all of the residues that are affected by these mutations lie either adjacent to or within the a1 or a2 acidic region[48–50]. Other mutations that have contributed to our understanding of the structure–function relationship of F8 occur at the thrombin cleavage sites at Arg372 (Arg372Cys[51] and Arg372His[52]) and Arg1,689 (Arg1,689Cys[53–55]). All of these mutations impair the activation of F8 by thrombin and show significantly higher F8 values in the chromogenic assay than in the one-stage assay. This is because small (normal) amounts of thrombin are present in the one-stage assay, whereas an excess amount of thrombin is present in the chromogenic assay. Because the chromogenic assay is often used for diagnosis of haemophilia A, this test might miss a haemophilic patient that has mutations affecting F8 activation by thrombin.

Patients with higher F8 activity values in the one-stage assay than in the chromogenic assay are more common and have mutations located within the A domains (examples are Ala284Glu, Arg527Trp, Arg531His, Ser289Leu, Asn694Ile, Arg698Trp, Arg698Leu, Leu1,932Phe and His1,954Leu[56–59]). Several mutations affect residues that are located at the interface of the A domains (Ala284Glu and Arg531His at the A1–A2 interface, Ser289Leu at the A1–A3 interface, Asn694Ile, Arg698Trp and Arg698Leu at the A2–A3 interface). These mutations facilitate the dissociation of the A2 subunit, which occurs naturally and inactivates the F8 protein[56].

Between the A1 and A2 domains, residues 351–365 are thought to include a binding site for coagulation factor X, F10 (REF. 60). Surprisingly, no mutation has been assigned yet to this region. Another loop on A2, including residues 484–508, is reported to interact with the activated F9 protease domain — most of the mutations affecting that area have mild consequences. For two further missense mutations within the A2 domain (Ile566Thr and Ser558Phe), it was possible to elucidate the mechanism through which they result in haemophilia A. Both F8 mutant molecules show a reduced specific activity, resulting from steric hindrance for interaction with activated F9; this study also confirmed that Ile566Thr creates a new N-linked glycosylation site[61]. Only one mutation leading to a severe form of haemophilia A has been characterized in this region (Ser488Stop). It might be suggested that mutations leading to stop codons give rise to nonsense-mediated decay, and therefore only a few mRNA molecules might be used for translation[62].

A few point mutations in the A3 domain lead to severe phenotypes, indicating the importance of this region (Ala1,779Pro, Arg1,781Cys, Arg1,781His, Tyr1,783Ser, Ser1,784Tyr and Phe1,785Ser; according to the HAMSTeRS database). Residues 1,778–1,823 of the A3 region seem to be located at the surface of the protein and to provide a secondary binding site for F9 (REFS 60,63).

Mutations that affect the B domain. Cleavage of F8 by thrombin at three sites produces the active form of the F8 protein. This activating cleavage leads to the removal of the B domain, which is no longer necessary for F8 activity. Therefore, missense mutations in the B domain are of clinical relevance only if they affect these cleavage sites or the thrombin-binding sites nearby. Nonsense mutations in the B domain, which lead to the premature termination of the protein, always affect the entire F8 protein and result in haemophilia A[9]. The Arg at position 372 is frequently affected by point mutations that lead either to Pro, His or Cys substitutions (17 mutations of this kind are listed in the HAMSTeRS database); position 1,689, which is important as a thrombin cleavage site, also has a high mutation frequency (TABLE 1; in addition to the Arg>Cys mutation mentioned in a previous section, an Arg>His exchange has also been reported several times). The exchange to His causes only mild forms of haemophilia A, whereas the exchange to Cys leads to moderate and sometimes even severe cases, probably owing to the incorrect formation of disulphide bonds (FIG. 2). Surprisingly, no point mutation has been reported to affect the N-terminal thrombin cleavage site at amino acid 740. Given that many mutations have now been analysed, it might be concluded that only a few sites in the B domain are of functional importance. The main part of this domain is used just as a 'spacer'; this might also explain its unique sequence, as mentioned earlier in this section[64].

Mutations that affect the interaction with VWF. As mentioned in the previous section on F8-interacting proteins, F8 interacts strongly with VWF. Many

ONE-STAGE CLOTTING ASSAY
Plasma from a patient is mixed with plasma that is known to be deficient in F8, and the time of formation of a fibrin clot is measured. The assay therefore compares the activity of the blood coagulation cascade in the patient's plasma with the normal activity.

CHROMOGENIC ASSAY
Diluted patient plasma is mixed with thrombin, F10 and activated F9 reagents. Activated F9 hydrolyses the chromogenic substrate S–2,765 and releases a chromophore. The colour intensity released by the chromophore is proportional to F8 activity in the sample.

mild–moderate haemophilia phenotypes are caused by missense mutations that alter the binding of F8 to VWF. These mutations are clustered within the C2 domain and surprisingly also within the C1 domain[65–67]. Moreover, one important VWF-binding site is between amino acids Glu1,649 and Arg1,689, with Tyr1,680 also being an important site. As outlined in TABLE 1, this last amino acid is frequently targeted by mutations.

Mutations that affect the interaction with phospholipids. The C2 domain of F8 (amino acids 2,173–2,332) is important for interactions with phospholipids, but also for binding to VWF. In particular, two pairs of adjacent, solvent-exposed hydrophobic residues (Met2,199 and Trp2,200, and Leu2,251 and Leu2,252) are involved[63]. Moreover, studies using electron crystallography[68] (BOX 2) indicate that F8 interacts with cell-membrane phospholipids through hydrophobic 'feet' that are formed by the side chains of Met2,199/Phe2,200, Val2,223, Leu2,251/Leu2,252 and Trp2,313–His2,315, which penetrate the membrane bilayer. This idea is supported in part by mutation analysis. However, missense mutations have been reported only for a few of these sites (at Val2,223Met and Trp2,313Arg; HAMSTeRS database). Several recurrent mutations at amino-acid positions 2,209, 2,229 and 2,307 have also been found (TABLE 1). At least one, at position 2,307, is part of a domain that is involved in membrane binding (amino acids 2,303–2,332 (REF. 69)). A recent paper reported that the region that includes amino acids 2,291–2,330 contains several universal epitopes for CD4+ cells. Complex formation of this part of the C2 domain of F8 with O-phospho-L-serine (the head group of phosphatidylserine in phosphatidylserine-rich vesicles) revealed not only decreased total inhibitory antibody concentrations, but also an increase in the physical stability of F8 (REF. 70). Here, a few mutations have been reported to be causative for severe haemophilia A (Pro2,300Ser, Gln2,311Pro, Gly2,325Ser and Cys2326Ser; HAMSTeRS database), which is consistent with the hypothesis that some of these amino acids have important functions.

Mutations that affect domains involved in alloantibody formation. Formation of ALLOANTIBODIES against endogenous F8 is largely restricted to severely affected haemophiliacs; about 30% of patients with severe haemophilia A are affected[71]. This number is even greater if only nonsense mutations (35%) or large deletions (40%) are considered. Nonsense and missense mutations in the light chain (FIG. 1) are associated with a higher risk of inhibitor formation than those occurring in the heavy chain. Splicing errors are the least likely mutation type to elicit inhibitor formation[72]. Unexpectedly, frameshift mutations within a stretch of A residues also run a small risk of generating inhibitory antibodies, although most of the mutations result in a premature stop codon. This is probably because the reading frame of some F8 molecules is restored by slippage errors of the RNA polymerase[71].

ALLOANTIBODY
Usually refers to an antibody that is raised naturally against foreign tissues from a member of the same species; in the case of haemophilia it describes the formation of antibodies against the therapeutic F8 protein. As the recombinant protein is of human origin it should be recognized as 'self', but it is not.

In contrast to patients with severe disease, inhibitor formation in patients with mild or moderate haemophilia A is rare. These patients typically have missense mutations that cause local conformational changes within the immunogenic domains of the F8 protein[73]. Important epitopes of these inhibitory antibodies have been identified in the A2 and C2 domain of the F8 protein. In particular, a common inhibitory epitope within the F8 protein was mapped to residues 484–509. Antibodies to this region seem to block a functional interaction of the A2 domain with F9. A second important epitope occurs within the C2 domain (residues 2,173–2,332). This region was discussed above as being involved in interaction with VWF and phospholipids. *In vitro* studies have shown that the residues at positions 2,199, 2,251 and 2,252 in particular are important for the formation of alloantibodies (see the overview by Fay and Jenkins[74] and references therein).

Treatment options for haemophilia A

Protein substitution therapy. Since the middle of the nineteenth century, haemophilia A and B were treated by blood transfusion. However, it took a century to understand the underlying mechanisms, and two further decades to develop the first cryoprecipitate of purified clotting factors for therapeutic purposes. Once plasma could be fractionated to produce lyophilized concentrates of clotting factors, prophylactic replacement therapy became possible. Two modes of intravenous replacement therapy then evolved — administration as soon as bleeding occurred (on-demand) or administration to prevent bleeding (prophylaxis). After infusion of F8 concentrates into haemophiliacs, the half-life of the protein usually ranges between 10 to 14 hours[63], which is in the same range as that of endogenous F8. At the end of the 1980s, highly purified F8 from plasma, as well as recombinant F8 products, were available. Recombinant F8 has become a suitable alternative to plasma-derived products as its development has significantly increased the availability of F8 for replacement therapy and has reduced the risk of transmission of blood-borne pathogens; this is principally because 'second-generation' recombinant F8 products have a markedly reduced albumin content (of human or bovine origin[75]). After at least four decades of using plasma-derived or recombinant concentrates, the situation for treated patients in developed countries has significantly improved. Whereas, in the past, men with haemophilia A were likely to die in their youth, through advances in diagnosis and the development of safe and effective treatment, affected individuals can now look forward to a normal life expectancy[76], with costs of approximately 100,000 euro per year for a severely affected patient.

Besides the high viral load that has been associated with the plasma-derived factors in the past (especially the presence of HIV and the hepatitis C virus), one of the most severe complications of replacement therapy is the development of inhibitory alloantibodies to the therapeutic F8 protein, which occurs in 30% of severely affected patients (see BOX 1 for more details)

Box 3 | **Gene therapy for haemophilia A**

***In vivo* therapy**

Using adenoviral systems, five trials have been reported to decrease the severity of haemophilia A in animal models. Among them, the best result was observed in knockout mice, where 150% of normal coagulation factor VIII (*F8*) expression was observed for more than 9 months[96]. The highest level of overexpression of F8 was reported in haemophilic dogs (800% of normal expression), although this increase was observed only for 1–2 weeks[97]. In both cases, an albumin promoter was used to drive *hF8ΔB*, a shorter version of the wild-type gene (see main text for details). A recent study that used a cytomegalovirus promoter and the entire *F8* cDNA reported 190% of normal *F8* expression for approximately 6 months in the canine model[98]. Similar results have been achieved with adeno-associated viral vectors in mice[99].

For the high-capacity adenoviral vector system, a phase I clinical study with one patient[100] led to a transient increase in F8 activity from almost zero to 1% for several months under the control of an albumin promoter. In a similar study, 13 patients suffering from severe haemophilia A were treated systemically *in vivo* with oncoretroviral vectors encoding *hF8ΔB*, the expression of which was driven by the promoter present in the Moloney murine sarcoma virus 5′ long-terminal repeat. Around 1% of normal F8 activity was transiently detected in 6 of the 13 patients[101]; 9 patients had F8 levels higher than 1% on at least 2 occasions, 5 or more days after infusion of exogeneous F8 (REF. 100). Due to the occurrence of transient unexpected side effects resulting from the high-capacity adenoviral protocol (fever, elevated TRANSAMINASE and a drop in platelet count), both clinical protocols involving patients with severe haemophilia A have been discontinued. Likewise, all clinical *in vivo* protocols with haemophilia B patients have been stopped.

***Ex vivo* therapy**

Two gene-transfer systems can be used — non-viral vectors (using physical or chemical methods to introduce the DNA into target cells) or retroviral systems. In a mouse model, ~500% of normal *F8* expression was observed for 4 weeks with a non-viral vector system[83]. A retrovirus system has also been tested in knockout mice to restore F8 deficiency. Under a Rous sarcoma virus promoter, the *hF8ΔB* remained active for more than 22 weeks and resulted in 74% of normal F8 activity[102].

In a clinical phase I trial, a plasmid-borne *hF8ΔB* in which the gene was controlled by a human fibronectin promoter was transferred to the skin-derived autologous fibroblasts of 9 patients with severe haemophilia A. Clonal cells were re-implanted into the OMENTUM of 12 patients. A transient rise in F8 activity (0.5–4% of normal) was detectable in some of the patients for a year, without any serious side effects[103,104]. However, as for *in vivo* studies, the use of these clinical protocols has been stopped owing to their inefficiency, as has also been the case for haemophilia B patients.

— a figure that is similar for all factor concentrates currently used in the treatment of haemophilia A. Today, the effects of these antibodies can be overcome by so-called immune-tolerance therapy (BOX 1).

Gene therapy. Most F8 is produced in the liver. However, for gene-therapy approaches, F8 can also be synthesized in several other tissues — including muscle, endothelia or fibroblasts — if an extra intact *F8* gene is transported into the nuclei of such cells. Despite the success of protein substitution treatment for haemophilia A patients, this disease is an ideal model to develop a gene-therapy protocol for several reasons. First, gene therapy would spare the patient the burden of regular prophylactic intravenous substitution (which must occur every 2–3 days owing to the short half-life of F8). Second, it would help to identify the side effects of gene-therapy protocols compared with alternative treatment with concentrates. Haemophilic animals (knockout mice and haemophilic dogs) are also available to test the efficacy of various gene-therapy strategies. A third reason for developing gene-therapy approaches is that this type of treatment has a high chance of succeeding, as even a small increase in F8 activity in the blood (~1–2%) can markedly decrease bleeding. Finally, the success of the therapy can easily be monitored using blood-coagulation tests that measure F8 activity.

However, the fact that the *F8* mRNA is long (~9 kb) has inhibited rapid progress in the field. This main disadvantage was partially overcome in animal studies, in which the gene to be transferred was significantly shortened by removing the large B domain (the shorter construct is referred to as *hF8ΔB*). In general, gene therapy can be carried out in two ways — *in vivo* or *ex vivo*. In the case of *in vivo* therapy, the modified viral vector is transferred directly into the animal or patient. Modified adenoviral and adeno-associated viral systems or retroviral systems are suitable because they infect cells that are either quiescent or that have a moderate division rate. In *ex vivo* therapy, cells have to be removed from the animal or patient and stimulated to divide in culture. Cells in which DNA has been integrated into the host genome are isolated, amplified and transferred back to the donor organism.

Despite some progress in developing effective gene-therapy protocols for haemophilia A (see BOX 3 for more details), at least three main issues remain to be addressed. First, it is necessary to increase the disappointingly low expression levels of *F8* that are currently being obtained in human patients. Second, it is absolutely necessary to avoid viral-vector-induced oncogenesis by vectors that are based on oncoretroviruses[77] (this requirement applies to gene therapy for any disease). Finally, stable integration of the therapeutic transgene into the genome is required, as transient expression for a few months only necessitates repeated clinical intervention.

TRANSAMINASE
A class of enzymes that causes transamination; that is, the transfer of an amino group. Elevated levels of transminase indicate various hepatic disorders.

OMENTUM
A sheet of fat that is covered by peritoneum. The greater omentum is attached to the bottom edge of the stomach and hangs down in front of the intestine. Its other edge is attached to the transverse colon. The lesser omentum is attached to the top edge of the stomach and extends to the lower surface of the liver.

New trends in bioengineering recombinant F8

Many lines of research have been directed at overcoming the various limitations associated with expressing recombinant F8 for therapeutic purposes; efforts are therefore underway to improve the inefficient expression of F8, the inefficient folding of the primary translation product within the ER and to facilitate the transport of F8 from the ER to the Golgi apparatus[75]. Moreover, it is necessary to improve the functional activity and half-life of F8 and to reduce its antigenicity and immunogenicity.

Increasing yield. The first improvement in this context was the increase in the yield of recombinant F8 when the *hF8ΔB* form of the *F8* gene is used. This region corresponds to ~38% of the coding sequence and, as discussed above, has been used to improve the delivery of the therapeutic gene. The therapeutic efficacy of full-length *F8* relative to *hF8ΔB* is still under discussion. Although a meta-analysis of 13 observational studies indicated that *hF8ΔB* is less efficient at preventing bleeding and that its product has a shorter half-life than full-length F8 (REFS 78,79), another study showed in a RANDOMIZED THREE-WAY CROSS-OVER STUDY that *hF8ΔB* is biologically equivalent to the full-length form[80]. The shorter product also seems to have similar antigenic properties to the full-length gene with respect to inhibitor formation[81].

Improving transport. The second approach to enhancing the production process involves improving the transport of F8 from the ER to the Golgi apparatus. This transport (secretion efficiency) is significantly enhanced (2.3-fold) if a conservative mutation is present in the A1 domain (for example, Phe309Ser): Phe309 is mainly involved in the high-affinity binding of F8 to the immunoglobulin-binding protein (BIP). This binding inhibits secretion; therefore, secretion requires high levels of ATP. Mutation of Phe at this position to Ser or Ala reduces the ATP-dependence of the F8 secretion and enhances this process[82]. Another way of increasing the efficiency of secretion is to introduce into the protein a small portion of the B domain (optimally 226 amino acids with 6 N-linked oligosaccharides), as well as a modified A1 domain. In this case, the secretory efficiency is increased 25-fold compared with the *hF8ΔB* form alone[83].

Extending the half-life of F8 The third line of investigation is concerned with extending the short half-life of F8, which is caused largely by the fact that the thrombin-activated heterotrimer is unstable and subject to spontaneous decay of its activity. This loss of activity can be attributed to the dissociation of the A2 subunit, which occurs at a physiological pH. Therefore, one approach for prolonging F8 activity involved inhibiting the dissociation of A2. An inactivation-resistant form of F8 was engineered in which the A2 domain is covalently linked to the light chain, to prevent its spontaneous dissociation[84]. Furthermore, missense mutations at inactivation cleavage sites for APC also provide resistance to further proteolysis of F8. Recent pre-clinical studies in the haemophilia A mouse model demonstrated that this inactivation-resistant form of F8 provided more efficient HAEMOSTASIS than recombinant F8 at a significantly lower protein dose[85]. There was also no indication that the model mice were at an increased risk of thrombophilia. Another system introduced Cys residues at positions 664 and 1,824 in the A2 and A3 domain, which resulted in the formation of disulphide bonds between the A2 and A3 domains, to stabilize the activated F8 (REF. 86). This particular form retains its activity five times longer than the wild-type form. These preclinical studies are promising approaches to significantly increase the activity of F8 and to prolong its half-life in patients; it will also help to reduce social costs and the frequency of intravenous infusions to patients.

Reducing antigenicity. Great efforts are underway to make recombinant F8 less antigenic. The most sophisticated constructions are human–porcine hybrid F8 molecules in which sequences within the A2, A3, C2, and ap (a 41-residue light chain activation peptide) regions of the human sequence have been replaced by porcine sequences[87]. As an alternative treatment, peptides that present or mimic epitopes could competitively bind to the F8 antibodies and therefore increase the level of F8 available in the coagulation cascade; preliminary results from experiments carried out in *F8*-knockout mice indicate that this is also a promising avenue[88,89].

Conclusions

Worldwide mutation analysis of haemophilia-A patients has revealed the underlying mutation in a large variety of cases; more than 940 are summarized in a public database. Approximately half the severe cases are caused by inversions between genes located within intron 22 of the *F8* gene and sequences outside the gene. For smaller deletions, insertions or point mutations, the degree of severity depends on the function of the affected domains. Surprisingly, in about 4% of the haemophiliacs no mutation could be found in the coding exons and their flanking sequences.

Therapy is based on blood-derived or recombinant F8 concentrates, which have a short half-life. Therefore, it might be helpful to look for the reason for elevated or prolonged F8 activity in thrombophilic patients to identify not only the underlying mutations, but also to learn from nature about how F8 activity can be enhanced. This will be helpful not only to improve significantly the use of recombinant F8 concentrates, but also to allow the rather optimistic hopes for gene therapy in haemophilia A to be realized.

Because of the tremendous knowledge of the physiology and biochemistry of the disorder and its molecular basis, and owing to the long-lasting experience in its successful treatment by recombinant replacement therapy and the first steps in gene therapy that are described above, haemophilia A is a leading model for many other monogenic disorders. Moreover, because it is a part of the complex network of homeostasis, it is also an excellent example how alterations in a single gene can be modulated by changes in its interaction partners.

RANDOMIZED THREE-WAY CROSS-OVER STUDY
Three groups of patients are formed by the random distribution of probands. Participants in one group receive one intervention for a period of time, then switch over to a second intervention and then to a third intervention (participants in the second group start with the second intervention and switch to the third, then the first, and so on). The result is that all groups will be treated with all three protocols (so, the term 'cross-over' is used).

HAEMOSTASIS
A balanced interaction of blood cells, vasculature, plasma proteins and low molecular weight substances. Perfect homeostasis means absence of bleeding and of thrombosis.

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The paper identifies the intron-22 inversion in the *F8* gene as being caused by homologous recombination of intron-22 sequences with repetitive sequences outside the *F8* gene.

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Competing interests statement

The authors declare no competing financial interests.

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