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From Genotype to Phenotype – When the Parents Ask the Question

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1. Introduction

Bleeding disorders often present with different degrees of severity, ranging from very mild to very severe. Clinical presentation is not always correlated with the grade of the molecular defect and the disruption it causes to the gene/s in question. This is especially true for circulating coagulation factor deficiencies where even 1-2 % difference between levels of the deficient factor may result in significant variations in the clinical presentation. It is often the case that the residual level of factor does not serve as a reliable predictor of the clinical course in the particular patient. This is related to a number of reasons, including timing and accuracy of level measurement, antigenic properties/activity ratio of the deficient factor, nature of the molecular defect, presence of factors augmenting or alleviating the bleeding tendency (such as antibodies to the deficient factor, co-inherited prothrombotic risk factors, etc.). Therefore, prognostication of disease course in young children and for the possibility of recurrence of the bleeding phenotype in the family could present quite a challenge to the genetic counselor. Nevertheless, this aspect of disease coping is very important to the family undergoing genetic counseling for haemophilia as it might influence their motivation for seeking adequate therapy and trying novel treatments, might induce overprotectiveness towards the affected child and most definitely could modify the family's future reproductive plans (having one ill child and a grim perspective for the disease course usually does not encourage having more children). The ability to answer the questions of the parents about the prospective course of the disease with an acceptable degree of reliability is a crucial component of genetic counseling for haemophilia and requires gathering and processing a lot of information in order to produce a reliable image of what could be expected and what could be avoided.

2. Factors that contribute to the haemophilic phenotype

The avalanche stopping in its tracks a few feet above the cowering village behaves not only unnaturally but unethically.

Vladimir Nabokov, Pnin (1957)

Haemophilia A is a common (affecting 1:8000-1:10000 males) bleeding disorder caused by a deficiency of Factor VIII, a cofactor for the activated blood clotting factor IXa (Kazazian et al., 1995). The latter is a serine protease, activating factor X in the coagulation cascade, which

ultimately leads to the conversion of prothrombin to thrombin and to formation of a fibrin clot. Deficiency of Factor IX produces haemophilia B, a more rarely encountered (approximately 1:40000 males) bleeding disorder which is practically identical to haemophilia A in terms of clinical presentation as both factors act in the same biochemical pathway.

It could be expected that the level of residual factor would be inversely proportional to the grade of clinical severity, i.e. the lower the level of residual factor, the more severe the bleeding tendency. The level of the deficient factor is calculated as a ratio of the coagulation capability of the patient's plasma compared to a plasma pool of healthy males (taken as a 100 % reference point). Generally, haemophilia in patients with levels of 0-1 % of the deficient factor is classified as severe, 2-5 % as moderate and 6-40 % as mild. Patients with undetectably low levels of the deficient factor or up to 1 % are at risk of bleeding after minor injury or even without apparent precipitating event (also called spontaneous bleeding).

Bleeding tendency in severe haemophilia is usually noted early in life (around the age when the baby starts walking) or even in the immediate neonatal period because of cephalhaematoma after assisted delivery by means of forceps or vacuum extraction. Patients with levels within the 2-5 % range exhibit a heterogeneous spectrum of bleeding severity, ranging from indistinguishable from severe cases to mild, which becomes apparent only after serious challenge (e.g. dental manipulations). Patients in the mild category (6 % and upwards) are usually identified as haemophiliacs later in life (up to the fourth and fifth decade) and will usually bleed profusely only following a significant precipitating event (e.g. surgery, serious injury, etc.).

Severe haemophilia accounts for about 50 % of all the cases that come to clinical attention, moderate cases are about 40 % and, respectively, mild haemophilia makes up for the remaining 10 %. It is believed that severe cases are overrepresented in the statistics at the expense of milder forms as patients with severe bleeding tendency are more likely to be entered into the relevant registries.

It is more often than not that the patient presents with signs and symptoms of bleeding tendency of certain grade and subsequent clotting factor level measurements do not support the clinical findings. There are a number of reasons that might explain the discrepancy and each and every one of these must be checked and verified before a reliable assessment of disease severity and prognostication of the course of the disease could be made.

2.1 Errors in determining the level of residual factor

The first and foremost of the sources of errors in determination of clinical severity is inadequate timing of the coagulation factor level measurement. Often, families are motivated to visit the genetic counseling unit because of newly discovered pregnancy or because they are planning a pregnancy. It is often the case that the family does not actually know whether it is haemophilia A or haemophilia B that presents in their family and any laboratory data of a correctly taken blood sample from the index patient might not be immediately available. Thus, a sample taken from a haemophilic male 'at random' might produce spurious results. As a rule, errors of this type are biased towards measuring higher level of the deficient coagulation factor because of recent transfusion of plasma or blood clotting factor preparations. In our practice we had one haemophilic boy who repeatedly measured over 10 % of factor VIII when blood was taken for genetic analysis and some of it was tested for Factor VIII activity by two-stage assay. Since the patient had several spontaneous bleeding incidents on record, further investigation of the boy's medical history was carried out. As it turned out, the boy liked competitive sports and his family regularly

requested a transfusion of factor VIII preparations at their local haemophilia center but chose not to share this information with the genetic counselor. Had the case not been thoroughly investigated, genetic analysis for most common defects causing severe haemophilia A would not have been chosen as first option in genetic analysis and the causative mutation would not have been discovered in time.

There is also the question of whether the circulating residual clotting factor has adequate biochemical activity *in vivo* or not. Back in 1968, the antigenic properties of the deficient factor were also included as a component in the laboratory testing panel for haemophilia A and B (Roberts et al., 1968; Zimmermann & Edgington, 1973), broadening the classification of haemophilia forms to CRM (cross-reacting material) positive or CRM - negative. Later, Denson (1973) introduced the concept of FVIII:C (the biochemical activity of residual Factor VIII) versus FVIII:Ag (the antigenic properties of residual Factor VIII). Basically, it is the concept of presence of Factor VIII as an immunochemically recognizable protein but not as a cofactor activity. This might be the case when the underlying genetic defect in the Factor VIII gene produces a truncated or incorrectly folded protein. The majority of patients with severe haemophilia A have no detectable level of factor VIII protein in plasma (CRM-negative) but there are about 5 % of all severe patients that are CRM-positive (though the amount of CRM is usually reduced). Therefore, the assessment of the residual factor VIII activity (FVIII: C) ought to be always coupled with measurement of the FVIII:Ag so as to avoid biased results because of detection of the FVIII protein in the plasma. It is very important that clear instructions are given about what measures should be taken to avoid errors in measurement of coagulation factor levels, as an incorrect result might cause delays in genetic analysis. The latter could be irreparable in cases when a high-risk pregnancy is involved.

2.2 Antibodies to factor VIII

About 10-50 % of patients with haemophilia who have been treated with plasma transfusions and/or preparations of Factor VIII or IX ultimately develop antibodies (also called inhibitors) to the deficient clotting factor (de Biasi et al., 1994; Oldenburg et al., 2000; Ghosh & Shetty, 2009). This results in decreased therapeutic efficiency (with potentially fatal consequences for the patient if a major bleeding could not be managed properly) and increases the costs of treatment (as more units of the clotting factor are required to achieve the desired effect). It is generally believed that recombinant Factor VIII is more immunogenic than Factor VIII derived from pooled plasma (Aledort, 2004; Goudemand et al., 2006), but the results of a large cohort study carried out in 2007 (Gouw et al.) did not show an association between type of Factor VIII preparation and rate of development of antibodies to the clotting factor.

Generally it is the patients with severe haemophilia that develop inhibitors to Factor VIII followed by patients with moderate disease (Schwaab et al., 1995; d'Oiron et al., 2008). This is only logical as the types of mutations which cause severe disease are more likely to produce CRM-negative haemophilia (hence, the exogenous Factor VIII protein is viewed as 'foreign' by the immune system) or CRM-positive haemophilia with misfolded protein exposing unusual immunogenic epitopes (Oldenburg et al., 2002; Goodeve & Peake, 2003; Ragni et al., 2009).

It is hard to predict whether the particular patient will develop antibodies to the deficient coagulation factor. The inhibitor phenotype is usually constituted via a fine interplay of genetic and non-genetic factors, though it is dependent on the type of the causative

mutation (Tuddenham & Oldenburg, 1995; Astermark, 2010). Generally, it is safe to advise parents of a patient with mild haemophilia that development of antibodies would be unlikely. This, however, would not be the parent's prime concern anyway, as their child most probably would not need regular transfusions of Factor VIII-containing preparations. In a case of severe or moderate haemophilia it is not acceptable to recite bluntly the risk percentages of antibody formation to the family as most often this will not register as a chance to have an uncomplicated therapy course but, rather, as a risk to develop an untreatable condition. It is more advisable to gently induce the parents to record the number of units transfused each time and the number of times when an unexpected bleeding has called for a transfusion out of schedule. This way a reliable estimate could be obtained of whether the same amount of units per kg body weight results in the same correction of the coagulation defect over time.

In our practice we have observed only patients with severe disease develop anti-Factor VIII antibodies. In one case the genetic background was particularly interesting as there were two maternal first cousins with severe haemophilia A, one of which had very severe disease complicated by haemophilic arthropathy of both knee and ankle joints at the age of 11, while his cousin, aged 13, who apparently shared the same Factor VIII-gene disrupting mutation, only used Factor VIII-containing preparations at 'on demand' basis, typically less often than once in two months and had no major joint injury. At the time it was only possible to rule out Factor VIII inversions with breakpoints within the repeated intron 22 unit so the nature of the mutation remained unknown. Since the patients were first cousins and came from an ethnic group with a long-standing tradition of consanguineous marriages, there was a high chance that the two patients also shared common polymorphisms in other genes that play a role in the risk of inhibitor development. It is possible that the one cousin developed antibodies to Factor VIII because of more frequent encounters with this protein than the other cousin but the reason why one had more severe phenotype than the other was not identified.

In cases when one haemophilic child with antibodies to the deficient clotting factor is already born, families are typically very reluctant to opt for another child as the presence of inhibitors seem to compromise the only possible therapy option. It is up to the genetic counselor to explain the risks of another case of antibody-complicated haemophilia to the family and to order additional tests if needed, such as HLA typing and/or IL-10 and TNF-alpha polymorphism typing (Hay et al., 1997; Pavlova et al., 2009; Chaves et al., 2010). In one of our cases, there was a 21 g. w. pregnancy with a male foetus in a woman who has had already one son with severe haemophilia A complicated by high-titer antibodies to Factor VIII. DNA from the index patient was unavailable, so the nature of the mutation causing haemophilia in the family was impossible to identify. The most common mutations causing haemophilia A (inversions with a breakpoint in intron 22) were not identified either in peripheral blood of the mother (so that germinative mosaicism could not be ruled out) or in the foetus. In the process of genetic counseling it became clear that the family was accepting of the fact that they might have another boy with haemophilia but the issue that worried them the most was the risk of developing inhibitors in the course of treatment. Since the index patient and the foetus from the present pregnancy had different biological fathers, the risk that they shared the same set of genes was less than the 25 % expected by pure chance in full siblings, therefore the risk that they had inherited the same HLA class II type that could contribute to the risk of inhibitor development was less than the norm for siblings. The mother did not carry the TNF-alpha G308A polymorphism. Therefore, since the causative mutation

could not be determined (at least not in the very condensed timeframe imposed upon us by the advanced pregnancy) the family was presented with the options of carrying the pregnancy to term and risking having another child with haemophilia A (about 30 % risk based on the premise that about 30 % of first cases of haemophilia A are born to noncarrier women) that might eventually develop inhibitors to Factor VIII, or, or selectively terminating the pregnancy and risking abortion of a healthy male foetus (70 %). After weighing the risks the family opted for carrying pregnancy to term and actually had a healthy boy.

The incidence of antibody development in Bulgarian patients seems to be lower than usual (less than 10 %). We presume that this may be related to the practice of 'on-demand' treatment which was prevalent in Bulgaria until several years ago. With coagulation factor preparations getting more available to the patients, it could only be expected that the proportion of patients developing inhibitors to Factor VIII and Factor IX will reach the level reported elsewhere.

2.3 Nature of the causative mutation

A reasonable prediction of haemophilia phenotype and the course of the disease could be made based on the characteristics of the molecular defect. One must bear in mind, however, that the phenotype of the particular patient is not a direct function of the type of mutation but that additional genetic, epigenetic and environmental factors may play a role.

The gene for Factor VIII is situated in the distal part of the long arm of the X chromosome, Xq28 (Gitschier et al., 1984). The gene spans 186 Mb genomic DNA and comprises 26 exons ranging from 64 to 3106 bp in length. The resulting Factor VIII protein has a complicated domain structure with numerous sites for interaction with other molecules. Therefore, almost every hit within the coding regions of the gene and sometimes in the noncoding sequences may result in haemophilia phenotype. Up to the present moment over 1000 different mutations in factor VIII gene have been reported (Schwaab et al., 1991; Tuddenham et al., 1994, The Haemophilia A Mutation Database,

available from: <http://hadb.org.uk/WebPages/PublicFiles/MutationSummary.htm>, retrieved 14 May 2011). The mutation spectrum is heterogeneous with over 95 % of all mutations apart from the large gene rearrangements being single-nucleotide substitutions.

As a rule, nonsense mutations result in severe, CRM-negative haemophilia. Missense mutations may produce a variable phenotype, depending on the site where the mutation has occurred. Generally, mutations in the sequence coding for the A2 domain of the Factor VIII gene result in CRM - positive haemophilia with varying severity (Wakabayashi & Fay, 2008) as the A2 domain is responsible for the stability of the protein. Deletions of exons usually produces severe CRM-negative haemophilia A because of reading frame disruption except for deletion of exon 22 which results in an in-frame loss of 156 bp coding sequence (Youssoffian et al., 1987). and moderate haemophilia A. Deletions in the factor VIII gene are reported to be associated with increased tendency for inhibitor development because of incorrect protein folding resulting in exposure of immunogenic epitopes that are usually buried within the protein core (Youssoffian et al., 1987; Gouw et al., 2007, 2011). Specific mutation hotspots are 5-methylated cytosine residues which are readily deaminated to thymine (Youssoffian, 1986). These mutations usually result in introduction of premature stop codon (transition CGA→TGA) and, ultimately, in a truncated protein. The severity may vary depending on the site where the transition occurred but usually results in severe haemophilia with or without cross-reacting material as the truncated protein might be unstable in plasma (Reiner & Thompson, 1992).

2.3.1 Inversions with breakpoints in the 9.5 Kb repeated sequence in intron 22 of the factor VIII gene

About 50 % of haemophilia A cases are severe. In about half of these 50 % (25 % of all cases) the causative mutation does not affect the coding sequences of the gene but, rather, rearranges the gene in a way that precludes normal splicing across the exon 22 – exon 23 boundary of the gene (Naylor et al., 1992). Namely, this is a large inversion of the genomic portion containing exons 1-22 together as a unit which ultimately places them at considerable distance from exons 23-26. This is a randomly occurring event resulting from homologous recombination between inverted repeats located within the Factor VIII gene (intron 22) and outside the gene (Levinson et al., 1990, 1992).

Intron 22 of the Factor VIII gene is remarkable in more than one aspect. It is a very large intron (32.4 Kb) and possesses a CpG island of its own. The latter serves as an origin of transcription for two transcripts internal to the Factor VIII gene, termed F8A and F8B, and orientated, respectively, one opposite (F8A) and one parallel (F8B) to the direction of transcription of Factor VIII (Levinson et al., 1990, 1992). The sequence coding for F8A is located within a 9.5 Kb fragment which constitutes the repeated unit. There may be three or, rarely, four or more repeated units per X chromosome, one of which is always located inside intron 22 of the factor VIII gene and the others are extragenic, at distances of approximately 300 and 400 Kb from the Factor VIII gene. The repeated units have a very high degree of homology (over 99.9 %). During meiosis, it is likely that homologous sequences may mispair with their homologues on the same chromosome and serve as breakpoints for recombination. As the repeats outside Factor VIII gene are orientated in the opposite direction to the intragenic copy, the resulting rearrangement is an inversion of the portion of the gene that contains exons 1-22 and relocation at considerable distance from the remaining part of the gene, namely, 300 Kb away for inversions that involve the proximal extragenic copy and 400 Kb away when recombination event involves the distal copy (Lakich et al., 1993; Naylor et al., 1995). Thus, transcription from the Factor VIII promoter is possible but a full-length transcript cannot be obtained.

These events occur exclusively during male meiosis, as the X chromosome does not pair with the Y chromosome except at the pseudoautosomal regions, providing ample opportunities for intrachromosomal homologous recombination. This means that the mutation occurs exclusively in the male germline (Becker et al., 1996; Arnheim & Calabrese, 2009), resulting in carrier females born to a healthy mother and a father who carries a germline inversion mutation. Consequently, in the majority of cases where inversion is ultimately found, the haemophilic male who presents at the genetic counseling office is the first case in a family without history for bleeding diathesis. Nevertheless, it is very likely (over 90 %) for a woman who has had one haemophilic son with inversion to be a carrier (Rossiter et al., 1994), therefore the risk of her having another boy affected by haemophilia is close to 50 %, which equals the risk for proven carriers. Explaining to the family the origin of causative mutation and the associated risks could be challenging, especially in the light of the popular notion that women are solely responsible for transmission of haemophilia (as they give birth to affected sons). Generally, about 20-25 % of the females who have had a son with haemophilia have inherited the defective X chromosome from their healthy fathers (Haldane, 1935). Having more than one affected son does not confirm the carrier status of female as the de novo mutation rate in the female germline is estimated to be about 4 times lower than in the male germline (Becker et al., 1996) but is still significant, about 5 %. Of course, for the purposes of prenatal diagnosis 95 % risk of carriership is as good (in the case

of risk assessment, as bad) as 100 %, but nevertheless the genetic counselor must attempt to relieve the psychological burden of female carriership of haemophilia by emphasizing that in terms of de novo mutation occurrence it affects males and females alike, and that inversions occur in males several times more often than in females.

When inversion is the case, the constitution of the disease phenotype could be fairly straightforward. As a rule, inversions of any type (involving distal, proximal and additional copies of the 9.5 Kb repeated unit) result in severe haemophilia with FVIII: C level below 1%, unless another genetic component plays a role (e.g. co-inherited prothrombotic mutations). There are exceptions to the rule but they are fairly infrequent.

It is very important to let parents know exactly what 'severe' means and what potential adverse outcomes there might be. A fairly common parental reaction is overprotectiveness. For chronically ill people of any age, and especially in children, overprotectiveness might actually be of a disadvantage as it might delay the development of various important skills. While parental concern is of prime importance in children with haemophilia, overprotectiveness may actually cause disregard for the major issue in severe haemophilia, namely, the proneness to spontaneous bleeding. Every day, dozens of small-scale bleeding events in the human body trigger the coagulation cascade. Some of them have the potential to develop into life-threatening internal bleedings if coagulation is defective. In haemophiliacs with very low levels of circulating factor bleeding might be provoked by seemingly minor causes or without identifiable reason. Therefore, while overly concerned parents do not let their boy participate in sporting activities or play with other children out of fear that it might spiral into a rough-and-tumble that might induce bleeding; they might overlook the importance of checking regularly for signs and symptoms of bleeding. In our practice we had two very caring and protective mothers who, nevertheless, lost their sons following spontaneous bleeding incidents which were not recognized and treated properly because the mothers thought that since there was no preceding traumatic event, there was no cause to worry. It is vital to instill into the family the idea that bleeding must be ruled out first as a reason for any unusual event or complaint in a haemophilic male.

There are other unhealthy psychological states in parents of chronically ill children, including children with haemophilia, that the genetic counselor must recognize and try to intervene. Among these, denial is prominent as it might lead to severe adverse consequences. Denial is often observed in families with young children that have just received diagnosis. Usually, denial in families with haemophilia manifests as unwillingness to accept the fact that the child would not 'outgrow' the disease and avoidance of routine therapy, such as refusing transfusion therapy with Factor VIII preparations and turning to purely symptomatic (cold pads, etc.) or alternative therapies, such as homeopathy, magnet therapy, etc. Unfortunately, parents in denial do not realize that they are paving their child's way for major health trouble. It is the genetic counselor's job to explain that the nature of the molecular defect causing the condition does not allow for the course of the disease to get any better so as to discourage any adverse health practices.

The question of whether immunological response would be launched against the exogenous Factor VIII is more difficult to answer. In cases of inversions with breakpoints within the intron 22 repeated unit, the inversion disrupts the gene structure, precludes generation of a contiguous full-length transcript and results in undetectably low levels of Factor VIII in the patient's plasma. Authors elsewhere have reported relatively high incidence of inhibitor formation in patients with inversion, up to 50 % (Oldenburg et al., 2002; Astermark et al., 2005; Salviato et al., 2007). This is not unusual, as since there is no endogenous synthesis of a

certain protein, any encounter with it might trigger an immune reaction. Surprisingly, however, in our cohort of patients, none of the 25 patients with inversion with breakpoints within the repeated unit in intron 22 developed inhibitors to Factor VIII. This could be explained, however, by the prevailing practice of 'on-demand' treatment, that is, Factor VIII is administered only if there is an evidence of bleeding. Patients and their families should be informed about alternative approaches to combating the immune response to exogenous clotting factors, such as Factor VIIa, as this may generate and/or maintain the motivation for getting adequate treatment.

2.3.2 Point mutations

Generally, nonsense mutations cause premature arrest in mRNA translation, resulting in production of truncated protein. Since factor VIII is a large molecule with a multitude of sites responsible for interaction with other molecules, it is logical to assume that truncated protein variants would have limited, if any, ability to fulfill its functions. Therefore, nonsense mutations usually produce severe haemophilia, unless modulated by additional factors. For such patients, the basic assumptions for counseling families with severe haemophilia stand in place.

Oftentimes non-inversion patients with factor VIII levels below 1 % develop inhibitors, which might be the phenotypic reflection of the immunogenicity of the truncated protein. We have had two patients with severe haemophilia and mutations in the 5' - regulatory region of the Factor VIII gene which supposedly prevented transcription of the gene. Interestingly, they didn't develop antibodies to exogenous Factor VIII, notwithstanding the fact that they have had over 20 years of treatment at the time of referral.

As for missense mutations, it is very much up to the nature of the mutation in question whether the disease would be severe, moderate or mild and it is exactly with missense mutations where other factors (including genetic factors) play the most important role, as the modified protein may lose sites for interaction with other proteins or may, alternatively, gain such sites. As a rule, missense mutations cause CRM-positive haemophilia A with varying severity, depending on the mutation in question and the parts of the gene it affects. Recently, Wakabayashi et al. demonstrated that Factor VIII devoid of its C2 domain remains functional, at least in vitro, but is less stable (Wakabayashi et al., 2010). In our practice, we have had two (related) patients with exon 24 missense mutation (the C2 domain) and with mild haemophilia A (40 %), supporting the notion of decreased stability of the protein.

In moderate haemophilia, prognostication about the course of the disease in the particular patient could be rather difficult and could require a lot of cooperation from the patients and their families so as to carry out all necessary tests that could prompt as to how the defect in the Factor VIII gene clicks in place with other genetic and/or immunological factors that constitute the patient's phenotype. For an example, we have had one adult patient with mild haemophilia according to Factor VIII levels (7 %) but prone since childhood to accidental haematuria without anaemia and/or haemolysis. Since haematuria is not very common even in severe haemophilia, further investigation was undertaken, resulting in identification of co-inherited Factor XI deficiency, as described in Berg et al., (1994). The patient visited the genetic counseling unit so as to get an estimate of risks for transmitting his bleeding tendency to his children (specifically, his newborn daughter) and was, on the one hand, very relieved to know that haemophilia rarely is symptomatic in females, but, on the other, rather discontented to know that there might actually be increased risk of bleeding diathesis.

It could be hard to predict whether the patient would develop antibodies to exogenous Factor VIII in moderate and mild haemophilia. The rule of thumb is, however, that mild haemophilia usually is uneventful in regard to antibody production (in which the less frequent encounter with the exogenous factor preparations may play a role). As for moderate haemophilia, it is advisable that the parents keep a log of the amounts of preparations of clotting factor their child have had, the frequency of infusions and of any adverse events that may occur during or after transfusion so as not to miss the early signs of an impending immune conflict.

2.4 Co-inheritance of prothrombotic mutations

Carriership of molecular defects in other genes coding for proteins acting in the coagulation cascade may produce the so-called “thrombotic phenotype”, that is, increased tendency for thrombus formation. Usually, heterozygous carriership of such mutations (also called prothrombotic factors) in healthy people increases the risk of thromboembolism about 2-10 times, depending on the mutation in question (Cumming et al., 1997; Hessner et al., 1999). Homozygous carriership may increase the risk for thrombotic incidents up to 50 - 100-fold (Souto et al., 1999; Ornstein & Cushman, 2003).

The most commonly encountered prothrombotic mutations are the C677T in the 5, 10-methylenetetrahydrofolate reductase (MTHFR) gene (very common, prevalence of heterozygous carriership about 30-45 % in the general population), the G1691A substitution in factor V (Factor V Leiden) (between 2 and 6 % in the general population) and the G20210A mutation in the prothrombin gene (2-3 % in the general population) (Cumming et al., 1997; Antoniadis et al., 1999; den Heijer et al., 2003). Generally, about half of the patients with thrombophilia carry either the Factor V Leiden mutation or the G20210 prothrombin mutation (Lillicrap, 1999). Carriership of both mutations in the heterozygous state additionally increases the risk of recurrent thrombosis about three times than the risk for carriers of each mutation alone (De Stefano et al., 1999).

It is believed that the bleeding phenotype in patients with haemophilia might be modulated by concurrent inheritance of prothrombotic risk factors. Indeed, clinical practice often sees patients with clinical course less severe than could be expected from the residual levels of the deficient factor (Escuriola Ettinghausen et al., 2001; Tizzano et al., 2002; Franchini & Lippi, 2010). In such patients the first symptomatic bleeding seems to occur at later age than in haemophilic counterparts without prothrombotic risk factors and the average amount of units per kg body weight used to manage the basic condition is lower. On the other hand, there are reports that co-inheritance of a mutation which causes increased tendency to bleed and a mutation that increases the proneness to thromboembolism may complicate the substitution therapy for haemophilia because of risk of deep venous thrombosis and/or central venous catheter (CVC)-thrombosis during transfusion of Factor VIII-containing preparations (Olcay et al., 1997; Kapur et al., 1997; Ettinghausen et al., 1999).

Therefore, information about the patient's status with regard to most common prothrombotic mutations may increase the degree of confidence when outlining the putative disease phenotype while, at the same time, advise caution when transfusing clotting factor preparations (especially concentrates) to a patient carrying a Factor VIII or factor IX mutation and a prothrombotic risk factor.

2.4.1 Factor V Leiden

The process of haemostasis is controlled at numerous levels so as to avoid unmanageable intravascular coagulation. Normally, one of the basic mechanisms to control the clotting

process is proteolytic deactivation of the activated Factor V by activated protein C (APC), a serine protease with anticoagulant properties (Esmon, 1992).

Factor V Leiden (FVL) is a common mutation in the factor V gene resulting in thrombophilia [Bertina et al., 1994]. The factor V protein product bears a high degree of homology to Factor VIII – both in domain structure and in function, as Factor V acts as a cofactor of factor Xa in the coagulation cascade (Kane & Davie, 1988). The Factor V gene, however, is an autosomal gene. In their homozygous state, mutation events disrupting the Factor V gene cause a rare bleeding diathesis called parahaemophilia or Owren's disease (Owren, 1947).

The Factor V Leiden mutation is a G→A transition at nucleotide 1691 in exon 10 of the Factor V gene. At protein level this produces an Arg-to-Gln substitution which renders Factor V molecule uncleavable by APC, therefore resistant to its anticoagulant action. In healthy people this usually increases the risk for deep venous thromboses (DVTs) - mainly in the veins of the legs, but also in the veins of the arms, the lungs, the abdomen, and the brain. In pregnant women, carriership of Factor V Leiden (and other prothrombotic mutations) is associated with multiplying (between 10 and 15-fold) the risk for of venous thromboembolism during pregnancy and late fetal loss (Koeleman et al., 1994; Gerhardt et al., 2000, Martinelli et al., 2000). When co-inherited with haemophilia, FVL may modulate the bleeding phenotype and/or complicate the therapy with coagulation factor preparations.

2.4.2 Prothrombin G20210A mutation

The prothrombin-thrombin conversion is a key step in the coagulation cascade. The prothrombin G20210A variant (PT20210) is the second most common genetic mutation enhancing blood clotting. Again, it is autosomally inherited and is a transition G→A but it has no effect on the coding sequence of the encoded protein, rather, it affects the polyadenylation site in the prothrombin gene (Poort et al., 1996), causing increased prothrombin levels in plasma. As with Factor V Leiden, the carriership of the PT20210 mutation results in increased risk for thrombotic events (Chamouard et al., 1999; Franco et al., 2009), associated with the same concerns in patients with haemophilia who are on replacement therapy.

2.4.3 MTHFR C677T mutation

The human gene for 5,10-methylenetetrahydrofolate reductase is an autosomal gene coding for an enzyme catalyzing the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate (Goyette et al., 1994), the latter being a substrate for methionine synthase catalyzing the homocysteine remethylation to methionine. The mutation is a C→T transition in the protein coding regions of the gene, causing an alanine to valine substitution in exon 4 and reduced enzyme activity (Frosst et al., 1995). The resulting phenotype is of homocysteinemia associated with increased risk of vascular disease and thrombosis.

The prevalence of the C677T mutation is very high in all ethnic groups. The heterozygous (CT) state is seen in 20 to 50 % in different populations while the TT homozygotes may amount up to 30 % (Wilcken et al., 2003), hence, it would certainly co-occur with haemophilia in a significant proportion of the patients.

Since the prothrombotic mutations predispose to increased tendency for blood clotting, presumably co-inheritance of prothrombotic mutations and a mutation in the Factor VIII or Factor IX genes causing haemophilia might result in amelioration of the disease phenotype. This could present as later age of onset (age at which first symptomatic

bleeding occurs – for severe haemophilia typically around the age of 9-12 months when the baby learns to walk and bumps and falls are frequent) and/or fewer serious bleeding episodes and/or less episodes of spontaneous bleeding and/or less frequent rebleeding (rebleeding is a fairly frequent occurrence in haemophilia patients, as the clot, when finally formed, is friable and prone to breaking apart) and/or use of less units of the deficient clotting factor (Lee et al., 2000).

The flip side of the coin in co-inheritance of common prothrombotic mutations and haemophilia is the risk for complications of therapy, related to the fact that the presence of the prothrombotic mutation often increases the rate of thrombin formation (van 't Veer et al., 1997). The several hundreds or thousands of coagulation factor units needed to stabilize the patient's haemostasis are often transfused rapidly, within one minute or even faster. The general recommendation is that any blood clotting factor preparation should be infused slowly with no more than 2 ml of solution infused per minute so as to avoid complications, but the unfortunate practice shows that in at-home treatment schemes and sometimes even in clinical settings the whole amount of 3-10 ml of reconstituted coagulation factor is infused too rapidly - within a couple of dozens of seconds, out of desire to achieve results faster. Thus, in haemophilic patients with prothrombotic mutations, the therapy with clotting factors may provoke thromboembolic events – with disastrous consequences for the patient. There have been reports of cerebral and cardiac thrombotic incidents associated with Factor V Leiden carriership in haemophilic patients (Olcay et al., 1997, Iannaccaro et al., 2005). Therefore, it is important to screen haemophilic patients for prothrombotic mutations – with regard to possible disease phenotype modulation as well as in relation to possible modifications of therapy, in order to prevent adverse events.

Our experience to date shows that the three most common prothrombotic mutations (Factor V Leiden, PT20210 and MTHFR C677T) seem not to modulate the disease phenotype in all patients with haemophilia. Apparently, Bulgarian patients with severe haemophilia gain no benefits from neither of the co-inherited prothrombotic mutations, as all identified carriers exhibit the classical 'severe' phenotype, with frequent spontaneous bleedings and age of onset before the first year of life. There was no difference between the number of haemorrhagic episodes and/or the units of Factor VIII used per kg body weight per year between patients carrying prothrombotic mutations and patients who did not have additional mutations affecting components of blood coagulation cascade. It seems that the carriership of prothrombotic mutations does not contribute to the phenotype constitution in the local haemophilic population.

It could be challenging to predict whether and when in the course of therapy a haemophilic patient might experience a thrombotic event, especially if the patient is very young at the time of referral. Considering the risks that are associated with replacement therapy, however, it is advisable that the families are warned about the possibility of thromboembolism during or after clotting factor application. Until proven otherwise, identification of a prothrombotic mutation in a patient with severe haemophilia is another factor that should be considered as an additional potential risk source when defining the risks associated with therapy. It is vital to teach the family members that in case of signs of impending haemorrhage quick action is advisable but that speed should be made in contacting the attending physician (if deemed necessary), obtaining the clotting factor preparation (if it is not immediately available) and preparing it for use but not in the process of infusion itself as it may lead to severe adverse events. Patients with non-00 AB0 blood group should constitute a group of special concern for such events as it has been repeatedly

demonstrated that blood group different from 00 additionally increases the risk for thrombosis about 5-fold (Fontcuberta et al., 2008; Jukic et al., 2009).

In our practice we have observed patients with Haemophilia A and unusually mild bleeding phenotype discordant with the level of residual factor - 0.1 % as determined by at least two independent tests. Namely, the patients were seldom treated with factor VIII - less than six times per year - usually as a preventative measure before surgery or dental procedures. Bleeding incidents were usually preceded by a precipitating event and managed effectively by bandaging, cold applications and rest. Only one patient (55 years of age at time of referral) had unilateral haemophilic arthropathy of the knee which did not interfere significantly with everyday activities. Interestingly, in about half of these patients the causative mutation of the factor VIII gene was the large inversion involving the repeated unit in intron 22, therefore, the milder phenotype could not be explained by Factor VIII-gene related reasons (in-frame deletions, 'leaky' mutations, etc). None of these patients had a Factor V Leiden or PTG20210 mutation and the prevalence of MTHFR C677T in this group did not differ significantly from the basic values for European Caucasoid populations (30 %). It is possible that the milder phenotype might be attributed to carriership of MTHFR C677T but, as of now, until proven otherwise, carriership of any prothrombotic mutation must be considered to be associated with increased risk for adverse effects of the replacement therapy. Families should be advised to keep a log of the infused units and the frequency of infusions of clotting factor-containing preparations so as to illuminate any positive connection between co-carriership of prothrombotic mutations and milder course in haemophilia.

Women carriers of prothrombotic mutations are at risk of preeclampsia and fetal loss. Female carriers of haemophilia and prothrombotic mutations who are pregnant or planning to become pregnant should be informed about the risks associated with the pregnancy (risks for having a haemophilic boy stemming from the carriership of haemophilia-producing mutation and risks for both the mother and the foetus because of the carriership of a prothrombotic mutation) and referred for intensive pregnancy monitoring so as to avoid complications. Again, contribution of other risk-modifying factors such as ABO blood group, smoking, etc. should be evaluated.

2.5 Infections transmitted by transfusion of blood or blood products

The risk of infection with any of the common blood-transmitted diseases is significant for patients receiving regular transfusions of plasma, blood and blood products. Even considering the improvements in the sensitivity of the methods screening for infectious disease transmitted by blood and blood products, there still remains a risk for infection which is proportional to the number of occasions in which the patient has been exposed to blood, plasma, cryoprecipitate, etc. Basically all Bulgarian patients over 20 years of age are chronically Hb_sAg - positive and a significant proportion of them test as HCV-positive. Thankfully, the rates of new infection with blood-transmitted diseases in Bulgarian haemophilic patients is experiencing a steady decline thanks to the introduction of recombinant Factor VIII, IX and VIIa preparations in routine practice.

It is, however, a common concern of families of patients with haemophilia that the replacement treatment may bring about adverse events associated with transmission of infectious agents, which may lead to adverse health practices. This might be especially true for parents with young, newly diagnosed children who are not yet acquainted with the risks of spontaneous bleeding and/or the long-term effects of untreated haemophilia on joints, internal organs and the brain, and also for parents who are in denial. Such parents may

choose not to treat their child or may revert to alternative treatments, believing that the consequences of blood-transmitted diseases might be graver than the consequences of repeated bleedings. Typically, however, refusal of replacement treatment by the parents is not associated with parental neglect but, rather, with overprotectiveness, the behaviour stemming from the erroneous belief that if parents are watchful enough so as not to allow physical trauma of the child, bleeding would not occur. Also, there might be significant parental opposition to the idea of using recombinant coagulation factor preparations out of fear that this might actually increase the risk of antibody production. It is vital that the attending clinician together with the genetic counselor review all the therapeutic options with the family and provide reliable information about the rates of infection with blood-transmissible agents when using different coagulation factor preparations. Misconceptions related to the idea that the replacement could actually make the child more ill should be carefully addressed and any associated practices should be actively discouraged.

3. Conclusion

*You have to make the good out of the bad
because that is all you have got to make it out of.*
Robert Penn Warren, *All King's Men* (1946)

Haemophilias produce a heterogeneous phenotype which may be modulated by a number of factors, endogenous or resulting from outside intervention. There is a significant inter-patient variety of disease phenotype even in related patients due to the fact that the clinical severity is not always correlated with the residual level of the clotting factor. Genetic counselling for haemophilia is a continuous process which aims at gathering maximal amount of genetic information pertaining to the nature of genetic mutation causing the disease in the particular family, the carriership of additional genetic variants that might modulate the phenotype and the potential risks associated with specific traits in the patient's genetic background. In genetic counselling, the patients, their families, the attending clinicians, the genetic counselling unit and the clinical laboratory specialists ought to work as a team in order to obtain and process information related to the genetic and immunological status of the patient and to come up with a management strategy tailored out specifically to the needs of the patient. Careful weighing of the risks is important when presenting the facts about the familial disease and needless adverse psychological impact related to the grave diagnosis and the risks of further transmission of the disease should be minimized.

The genetic counseling unit should be able to provide answers to the vital questions of the family associated with the well-being of the affected child/ren and the opportunities for family planning. Dealing with popular misconceptions such as the notion that having had one affected son means automatically that the mother is a carrier and the erroneous belief that haemophilia could be eventually outgrown must also be on the to-do list of the genetic counseling unit as the information they present could provide a convincing proof that the disease transmission is governed by strict rules and tends to have a chronic course. It is only through dialogue that the best possible outcome for the patient and for the family is attained; therefore the process of genetic counseling should include asking a lot of questions and giving a lot of answers in order to achieve the optimal results.

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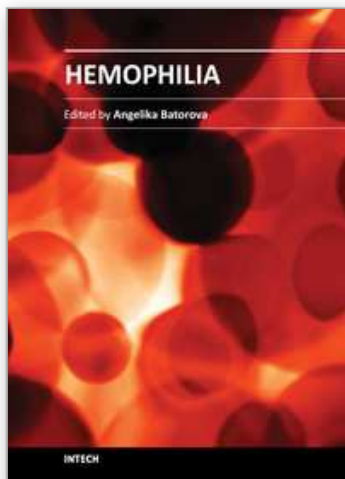
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This book demonstrates the great efforts aimed at further improving the care of the hemophilia, which may bring further improvement in the quality of life of hemophilia persons and their families.

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