Haemophilia: strategies for carrier detection and prenatal diagnosis*

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In 1977 WHO published in the Bulletin *a Memorandum on Methods for the Detection of Haemophilia Carriers. This was produced following a WHO/WFH (World Federation of Haemophilia) Meeting of Investigators in Geneva in November 1976, and has served as a valuable reference article on the genetics of haemophilia. The analyses discussed were based on phenotypic assessment, which, at that time, was the only procedure available.*

The molecular biology revolution in genetics during the 1980s made enormous contributions to our understanding of the molecular basis of the haemophilias and now permits precise carrier detection and prenatal diagnosis. WHO and WFH held a joint meeting on this subject in February 1992 in Geneva. This article is the result of these discussions.

Assessment of the problem

The carrier state in haemophilia

Clinical and genetic considerations. Carriers of haemophilia usually inherit their abnormal factor VIII or factor IX gene from one of their parents. Since they have a second normal X-chromosomal gene from the other parent the clotting factor level is around 50% of normal, which is generally sufficient for normal haemostasis.

Symptoms of bleeding do occur, however, in carriers if their clotting factor level is in the range of mild haemophilia, below 40%. This may be due to homozygosity, Turner's syndrome, other chromosomal abnormalities, extreme lionization, or the co-inheritance of a variant von Willebrand factor allele (i.e., von Willebrand's disease Normandy). With the increasing success of patient associations, the chances of carriers marrying patients might be expected to rise, but until now, homozygosity has been distinctly rare, just as the unlikely coincidence of Turner's syndrome and carriership. Occasional true heterozygous carriers with low clotting factor levels due to extreme lionization, however, are known in all major haemophilia centres. Within the perspective of this article, this is relevant for invasive procedures for prenatal diagnosis and for the management of delivery.

Anxiety about the risk of haemophilia affecting their offspring is the reason why possible carriers seek advice. The first step in this genetic counselling procedure is to find out why the consultand thinks that she might carry the gene. This may lead to any of three conclusions: she is not a carrier, she is an obligatory carrier, she is a possible carrier.

Carriership is *excluded* if haemophilia occurs in the paternal family without her father himself being affected. Carriership is *obligatory* if her father has haemophilia or if she has maternal relatives with haemophilia as well as an affected child. In these situations each newborn son has a 50% chance of

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being affected. If the consultand has more than one son with haemophilia without other affected relatives, she may be either a true heterozygote, or a mosaic (*1–4*). In the case of somatic or germline mosaicism the recurrence risk of the disease in subsequent newborn sons depends on the proportion of ova carrying the abnormal gene, which is difficult to establish. Carriership is *possible* if the consultand has affected relatives on the maternal side and no affected children, or if she has one affected son and no other affected relatives. In the last case, there are four possibilities: the consultand may have inherited the gene through the silent maternal line, which makes her a true heterozygote; she may be a mosaic; the affected son may have received the abnormal gene from a mutation in the single ovum that he originated from; or, the affected son may himself be a somatic mosaic with a large proportion of factor VIII- or IX-producing cells carrying the mutation (*5*).

After this first step, the genetic counsellor proceeds with rigorous pedigree analysis, clotting factor assays, and DNA studies in order to minimize uncertainty and set the stage for decision-making about prenatal diagnosis. These phases are described below.

Psychological considerations. Every woman who considers carrier testing for haemophilia, has a multitude of sociological and psychological influences which might affect her perceptions of the personal implications of possible carrier status. Some influences may be created by the ethnic and/or religious background of the individual. A particular society's perception of a woman who carries the gene for haemophilia may certainly influence whether or not a woman at risk chooses to be tested. Decisions to undergo carrier testing may also be dependent upon the degree of anxiety about haemophilia and its complications, as well as the availability and safety of treatment. Women who live in countries where treatment for haemophilia is inadequate, may be very interested in carrier detection and prenatal diagnosis, and therefore a more agressive approach to testing may be successful.

Preconceived notions about the clinical aspects of the disease will be formed as a result of the degree of, and content of contact with male haemophilic relatives. Women who have male relatives with more clinically severe disease, with inhibitors, or AIDS, may be more inclined to seek out carrier testing. This is especially true if individuals have been able to closely observe the effects of the disease on themselves and their family over time. Women who are related to those with milder clinical disease, or who have more distantly related haemophilic relatives, may have different perceptions regarding carrier testing. In the case of mild clinical disease in the family, women may see no need for carrier testing because of perceptions that haemophilia will not have a large personal impact. Those with more distantly related haemophilic relatives may be eager to pursue testing because of a perceived low risk, but may be unprepared if positive results are obtained.

It is important that formal counselling be done before laboratory tests are even considered, in order to resolve conflicts that may exist between a woman's desire to learn of her carrier status and the implications of possible results. It should be emphasized that the results of carrier testing may also be that the woman is not a carrier of haemophilia. The anxieties a woman might have regarding genetic testing may also be complicated by the type of testing available and the requirements of such, as well as the reliability of the tests involved. The possibility of inconclusive test results, or results that are not highly accurate may deter some women from choosing to be tested.

Many women who choose to undergo carrier testing may perceive themselves as either carriers or non-carriers prior to actual laboratory testing. This perception may influence how an individual assimilates the results of her laboratory testing; it is therefore an important issue to discuss during counselling. Those who assume that they are non-carriers will be supported by negative results, but may feel shock and surprise if given positive results. Those who assume that they are carriers may be relieved when given negative results, but they may also feel guilty for "escaping" their family's genetic burden, and they may have difficulty coping with their new status if life decisions have been made based on their assumptions of carrier status. Women who receive positive results will need a great deal of support, as they then must deal with the reality of their carrier status within the context of their family and their society.

Prenatal diagnosis

Clinical and genetic considerations. In families at risk of having a child with haemophilia, assessment of carrier status and counselling regarding prenatal diagnosis should ideally be carried out before conception. Pregnant carriers requesting prenatal diagnosis should be counselled as to the available options, including the techniques for fetal tissue sampling, their limitations, and potential complications. If the fetus is affected, the options of (i) continuing with the pregnancy and either keeping or adopting their child, and (ii) terminating the pregnancy, are reviewed.

Diagnostic centres. Prenatal diagnosis should be undertaken in centres with full genetic, haemotological and obstetric expertise.

Diagnostic tests. In the early days of prenatal diagnosis of the haemophilias (until the mid 1980s) the policy was to perform amniocentesis at 16 weeks followed by fetal blood sampling at 20 weeks for phenotypic diagnosis in male fetuses. Subsequently, with the application of recombinant DNA techniques to the analysis of placental biopsy material, parents were offered the advantage of first trimester diagnosis, with the additional benefit that, in the presence of a male fetus, only one invasive test was required. However, fetal blood sampling for phenotypic diagnosis is still the preferred method in patients presenting in the second trimester of pregnancy, and it is necessary for those patients who are not informative for any of the available DNA probes, those requiring confirmation where normality is based on a linked probe, and those who have sporadic haemophilia or lack key relatives. Second trimester diagnosis may also be the preferred option for those patients wishing to avoid invasive testing for female fetuses because fetal sexing can now be performed by ultrasonography at 16–20 weeks.

Termination of pregnancy. Traditionally, termination of pregnancy in the first trimester was performed under general anaesthesia by dilatation of the cervix and evacuation of the uterine contents. In the second trimester, termination involved induction of labour and delivery of the fetus. Recently with the more widespread uptake of second trimester dilatation and evacuation, one of the potential advantages of first trimester diagnosis (less traumatic termination) may not be valid.

Psychological consideration. Prenatal diagnosis of the haemophilias holds a multitude of psychological considerations for the women and her partner as well as the wider family and the community as a whole. Parental anxieties arise from: (i) the risks of having an affected child with lifelong morbidity, (ii) attending a hospital and having a potentially painful invasive test, (iii) miscarrying as a result of invasive testing, (iv) receiving an abnormal result, (v) making a decision on whether to continue or terminate an affected pregnancy, and (vi) undergoing a termination, with its potential short-term and long-term complications. The parents are also subjected to further, either real or perceived, pressures. The wider family, especially affected members, close friends, and even their doctors may hold strong views concerning quality of life and attitudes to termination. Prevailing, cultural, religious and moral values within a society may impose additional stresses on the parents.

Genetic diagnosis

Assessment of carrier status

Family data: risk assessment. A pedigree of the family has to be carefully drawn with accurate information on the males being affected with haemophilia or not. If more than one haemophiliac exists or has existed in the family, the case is *familial.* If the haemophiliac is the only known case in the family it is considered as *isolated.* These two types of families have to be discussed separately.

Familial cases. Haemophilia A and B are X-linked, recessive disorders. The segregation probabilities are thus 50% for a carrier female to transmit the X-linked gene to each child, male or female, while the haemophilic male will have only normal sons and carrier daughters.

Study of the pedigree alone will identify some females as obligate carriers. An *obligate carrier* is defined as a woman who:

— has a father who is a haemophiliac (with the rare exception of him being a somatic mosaic);

— has more than one haemophilic son (identical twins excluded) or one haemophilic son and a daughter who has given birth to a haemophilic son;

— has a haemophilic son and a well documented haemophiliac on the maternal side of the family.

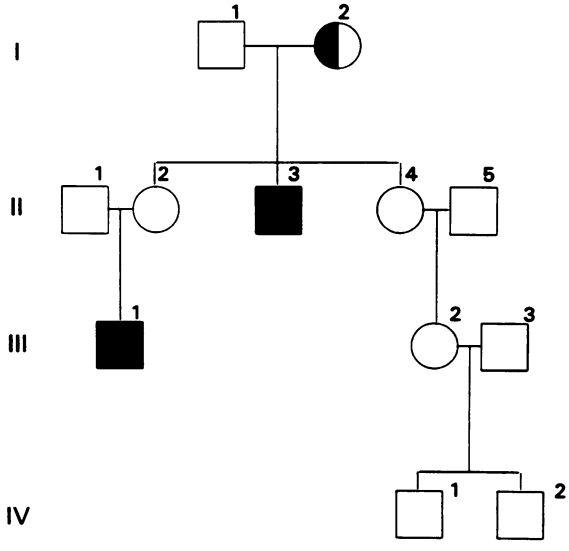
Obligate carriers have a probability of 1.0 for carriership and need no further investigation.

Females in the pedigree who are not obligate carriers are to be considered as possible carriers. If a consultand is a possible carrier her probability of carriership calculated from pedigree data should be done in two steps. In the first step only information from the pedigree anterior to her is used. One goes back in the pedigree from the consultand to the nearest maternal relative who is a haemophiliac or an obligate female carrier. For each step vertical or horizontal in the pedigree from this person, take 0.5 and multiply these factors together to arrive at the probability of the consultand being a carrier.

This is illustrated in the pedigree in Fig. 1. I:2 is an obligate carrier since she has given birth to a son with haemophilia and a daughter who has a son with haemophilia. II:4 is one step horizontal to the haemophiliac (or one step vertical to the obligate carrier) and thus has a probability of 0.5 for carriership. III:2 is one step horizontal and one step vertical to the haemophiliac (or two steps vertical to the obligate carrier) and thus has a probability for carriership of $0.5 \times 0.5 = 0.25$.

If the consultand has male descendants her probability for carriership has to be modified by

Fig. 1. **Example of a pedigree for calculating anterior probabilities of carriership.**



taking these into consideration. If the consultand has healthy male descendants her probability of being a carrier is diminished.

The probability for III:2 being a carrier according to the anterior family history is 0.25. She has given birth to two healthy sons. The next step is to calculate the likelihood that III:2 would have two normal sons, separately for the two possible cases that she is or is not a carrier. If she is a carrier the likelihood that her 2 sons would be healthy is $(0.5)^2 = 0.25$, since each son has a probability of 0.5 of not receiving the abnormal gene. If she is not a carrier the likelihood that her two sons would be normal is $1^2 = 1$. These two likelihoods expressed as odds for carriership gives $(0.5)^2:1^2$, i.e., 0.25:1.

In order to modify the probability of 0.25 obtained from the anterior pedigree by taking into consideration the odds obtained from the pedigree of the descendants, the former probability must be expressed as odds; 0.25:0.75 = 1:3. The odds in the anterior and descendant pedigree is multiplied to arrive at the final odds for carriership obtained from pedigree data; $0.25 \times 1:1 \times 3 = 0.25:3$. The odds for carriership can then be transformed again into a probability according to the formula; odds $a:b$ corresponds to the probability according to the formula; $P = a/(a+b)$; $0.25/(0.25 + 3) = 0.08$. Expressed in words, the fact that III:2 has given birth to two healthy sons reduces her probability of carriership from 0.25 to 0.08.

These calculations are illustrated in Table 1 with the general formula applying to a consultand having n sons. With one normal son (n=1), the final probability is reduced from 0.25 to 0.14; with two normal sons (n=2), it is reduced to 0.08, etc.

Isolated cases. An isolated case of haemophilia may result from transmission of the haemophilia gene through asymptomatic females in whom the gene has remained undetected; from a new mutation in the mother, resulting in her being a carrier, or a new mutation in the haemophiliac (= true *de novo* mutation). The existence of somatic mosaicism and germline mosaicism has also to be taken into consideration (*2, 3*).

The true proportion of *de novo* mutations will depend upon the mutation rate in males versus females (v/u). If it is higher in males, a high proportion of mothers of isolated cases will be carriers. If it is higher in females, many isolated haemophiliacs will be the result of true *de novo* mutations. The sex ratio of mutation frequencies in haemophilia has not been definitively established. Most studies show a higher mutation frequency in the male than in the female. In haemophilia A, v/u has been estimated as 5.0 (*6*), 9.6 (*7*) and 3.1 (*8*), respectively, in three recent studies. In haemophilia B it has been estimated as 11 (*9*). Even if these figures are cautiously interpreted, most mothers of isolated haemophiliacs are carriers. For practical purposes one can approximate the genetic probability to be 0.85 for carriership in mothers of an isolated case.

Phenotypic assessment. Haemophilia A and B are assessed by phenotype on the basis of the following.

(1) *Glossary of terms*

- Factor VIII:C (factor VIII coagulant activity) — the coagulant activity of factor VIII as assessed from the normalizing effect on the activated partial thromboplastin time (APTT) of plasma containing less than the 1% of the normal factor VIII:C concentration.

Table 1: **Calculating the probabilities for carriership**

Information	Probability or odds		
	Carrier-ship	Noncarrier-ship	Carrier:non-carrier
Anterior to III:2	0.25	0.75	1:3
Descendants of III:2			$(0.5)^n$:1
Anterior and descendants	$(0.5)^n/(0.5)^n+3$	$3/(0.5)^n+3$	$(0.5)^n$:3

- Factor VIII:Ag (factor VIII antigen) — the factor VIII protein as assessed by immunoassays.
- VWF:Ag (von Willebrand factor antigen) — the von Willebrand factor protein as assessed by immunoassays.
- Factor IX:C (factor IX coagulant activity) — the coagulant activity of factor IX as assessed from the normalizing effect on the APTT of plasma containing less than 1% of the normal factor IX:C concentration.
- Factor IX:Ag (factor IX antigen) — the factor IX protein as assessed by immunoassays.
- Substrate plasma is plasma devoid of either factor VIII:C or IX:C and used in the coagulation assay.
- Test plasma is the plasma sample taken from an individual to be tested.
- Local working standard plasma is a pool of plasma used as a "control" plasma; it should be calibrated in international units (IU).

(2) *Laboratory standards*

Factor VIII:C. All factor VIII:C estimations must be assayed by comparison with an international standard and expressed as international units (IU) of factor VIII coagulant activity. One IU of VIII:C is by definition the factor VIII coagulant activity in one millilitre dof "fresh normal human plasma". The IU is defined by the "International Standard for Blood Coagulation Factor VIII Plasma Human" (at present, 90/550), which is available from the National Institute for Biological Standards and Control, London, England.

In the local laboratory, pooled citrated plasma from at least 20 healthy donors, having an age and blood group distribution comparable to those of the local population from which test subjects are drawn, may be used as the working standard. Every new batch of this local standard plasma has to be calibrated against the international standard plasma or an "intermediate" standard which has been calibrated against the international standard. In calibration, the standard plasma should be tested three times in triplicate and a conversion factor should be calculated for local U VIII:C/ml to IU VIII:C/ml. A new working standard should be prepared every 2–3 months to minimize inaccuracy due to deterioration in storage. The standard must be stored at –70 °C.

Factor VIII:Ag. The local working standard calibrated against the international standard, as described above, is to be used as standard.

VWF:Ag. The local working standard should be prepared according to the guidelines above and calibrated against the international standard for VWF (at present, 5th British Standard for Blood Coagulation Factors, plasma human 91/516).

Factor IX:C. The local working standard should be prepared according to the guidelines above and calibrated against the "International Standard for Blood Coagulation Factor IX Human Plasma" (at present, the 5th British Standard for Blood Coagulation Factors, plasma human 91/516). This standard may also be used for Factor IX:Ag measurements.

(3) *Sampling of blood*

Both carriers and controls should be in good health at the time of sampling since inflammatory states, liver and other diseases, and certain drugs may influence the concentrations of the coagulation factors.

The syringes and tubes used should be of plastic or siliconized glass. Vacuum tubes may be used. The anticoagulant should be 0.11 or 0.13 mol sodium citrate, 1 volume to 9 volumes of blood. The tubes should be turned upside down immediately 2–3 times after sampling and centrifugation for at least 20 minutes (at 2000 *g*) should be performed as soon as possible. The plasma should not be haemolysed. If plasma is not analysed immediately it should be frozen at –70 °C and not stored for more than a few months.

The results of VIII:C and VWF determinations will not be confounded if carriers are pregnant until the 22nd week of gestation or are taking oral contraceptives at the time of blood sampling (*10–12*). The age (haemophilia A and B) and blood group (haemophilia A) have to be considered in both carriers and controls (*13, 14*). The data on the effect of age on IX:C concentrations are ambiguous; no effect was found by Graham et al. (*15*), whereas Orstavik et al. (*16*) in a population of twins found a significantly higher value in the senior twins. Oestrogen-containing drugs, like oral contraceptives, result in higher concentrations of both IX:C and IX:Ag (*17*).

(4) *Coagulant assays*

VIII:C should be measured by a one-stage clotting assay or chromogenic substrate method.

The one-stage method is based on the test sample's ability to correct the APPT in plasma which has a <1% VIII:C (*18*). The chromogenic substrate assay for measuring VIII:C has been shown to have a correlation of 0.92–0.98 to one-stage clotting assays (*19*). In a comparison between different VIII:C assays, the chromogenic substrate assay was found to have the highest precision (*20*).

VIII:Ag can be measured by various immunoradiometric (IRMA) or enzyme-linked immunosorbent (ELISA) assays (*21*). The local experience with the

different methods may favour one over the other, but there is no universal advantage of using VIII:Ag instead of VIII:C or vice versa.

VWF:Ag can be measured quantitatively with two different methods: electroimmunoassay (EIA) (*22*) and IRMA or ELISA (*23*). EIAs are performed by the "rocket technique" (*24*); they are as satisfactory a method as IRMA or ELISA in the detection of carriers of haemophilia A and the experience at the local laboratory should decide (*25*).

IX:C can be measured by one-stage or two-stage assays based on the same principles as VIII:C assays. The same precautions concerning sampling, working standard plasma, international standard plasma, and test plasma are applicable to IX:C assays. A chromogenic substrate method, which uses a factor Xa substrate, has been described based on the conversion of factor IX in plasma to IXa by addition of a semipure factor XIa in the presence of calcium (*26*).

IX:Ag can be measured by various immunological assays, the most reliable being IRMA and ELISA (*27, 28*). When these techniques were introduced it was found that haemophilia B could be classified into many subgroups according to the amount of IX:Ag present. CRM+ (cross-reacting material positive) had normal IX:Ag, CRM^R had reduced amounts, and CRM– undetectable IX:Ag.

The average concentrations of IX:C or IX:Ag are lower in haemophilia B– carriers than in non-carrier women. Both concentrations are influenced by the random inactivation of one of the X-chromosomes (Lyon-phenomenon) (*29*). Haemophilia B+ carriers also have lower concentrations of IX:C, though their IX:Ag may vary widely as a result of the Lyon-phenomenon (*30*). No consensus exists on the most effective way of classifying carriers of different types of haemophilia B. Measurement of IX:Ag offers only limited predictive improvement, mainly in haemophilia B+ families according to Kasper et al. (*31*) and Pechet et al. (*32*). Orstavik et al. (*30*), on the other hand, found that IX:Ag was of discriminant value in both haemophilia B– and B+ families. In another study the most efficient way of classifying haemophilia B– carriers was univariate discrimination based on IX:Ag. For haemophilia B+ carriers bivariate linear discriminant analysis using both IX:C and IX:Ag gave the best results (*15*). In this paper univariate linear discriminant analysis is advocated for haemophilia B, using IX:C measurements (see below).

(5) *Odds ratios based on laboratory data*

On average, carriers of haemophilia A or B have about 50% of the normal levels of factor VIII or IX. Due to considerable overlap between the levels in carriers and normal women it is usually not possible to establish carrier status on these laboratory data in an unambiguous way. Therefore, the laboratory data are used to calculate an odds ratio favouring carriership: an odds ratio "X" means that the laboratory findings in the consultand are X-times more likely to be found in a carrier than in a non-carrier. The subsequent use of Bayes' rule allows one to combine this odds ratio with the probability of carriership derived from the pedigree analysis and to obtain a "final" probability of carriership. In the 1977 WHO Memorandum (*33*) four ways were described to calculate odds from laboratory data. Currently, however, the preferred method for haemophilia A is bivariate linear discriminant analysis based on factor VIII:C and von Willebrand factor antigen measurements accommodating the effects of age and ABO blood group (*25*). For haemophilia B, univariate linear discriminant analysis is advocated using factor IX:C measurements and applying a correction for the use of oral contraceptives (*17, 34*). Although the estimation of factor IX antigen levels may be advantageous in some cases (*30*), the routine application of this assay does not seem to be justified (*31, 32, 34*).

Both in haemophilia A and B, laboratory data are obtained in reference groups of carriers and non-carriers using exactly the same methods, reagents and standards as used for prospective consultands. Also the subjects used for reference purposes should be as similar as possible in all respects to the consultands. This used to require the recruitment of about 30 obligatory carriers and an equal number of non-related but very similar women. Since DNA technology now allows one to definitely prove or disprove carriership one may use the laboratory data on previously diagnosed consultands for reference purposes. At all times, however, one should be aware that age, blood type, severity of haemophilia, pregnancy, use of oral contraceptives, and probably other factors may exert an influence on the outcome of the laboratory tests. Consequently it is important to note whether the reference subjects and prospective consultands are similar in these respects. Furthermore, the laboratory data usually need to be transformed, such that the distributions of the data for the reference groups are normal or at least non-skewed.

In Tables 2 and 3 we have provided one approach for haemophilia A using the universal discriminant (*25*), which obviates the need to study a reference group of carriers, and one approach for haemophilia B, applicable to both CRM-positive and CRM-negative forms (*17*). Considering the primary position of DNA analysis in carrier diagnosis we feel that these straightforward approaches should be sufficient in most cases. The calculations can be easily carried out in a spreadsheet type computer program.

Table 2: **Haemophilia A, bivariate universal discriminant analysis.** This method requires factor VIII:C and von Willebrand factor antigen reference data on non-carriers only. Data are transformed using the natural logarithm and the effects of age and ABO blood type are accommodated (*25*)

Enter for consultand:

α = age in years.

β = ABO-bloodtype: 0 for 0, 1 for non-0.

γ = VWF:Ag in IU/ml.

δ = VIII:C in IU/ml.

π = Genetic probability of carriership (fraction of 1).

Enter for normal reference group:

μ_x = mean of Ln transformed VWF:Ag levels in IU/ml.

μ_y = mean of Ln transformed VIII:C levels in IU/ml.

Compute for consultand:

$x = \ln(\gamma) - \mu_x$

$y = \ln(\delta) - \mu_y$

Compute the coefficients for the modified discriminants:

$a = -0.0955 - 0.0156\alpha + 0.000196\alpha^2 + 0.0298\beta$

$b = 0.649 - 0.00184\alpha + 0.0000314\alpha^2 + 0.117\beta$

Compute the predicted means of the discriminants for carriers and normals:

$c = -0.391 - 0.00571\alpha + 0.0001\alpha^2 - 0.0648\beta$

$d = -0.0347 - 0.00171\alpha + 0.0000473\alpha^2 + 0.0754\beta$

and compute the odds ratio:

$e = ax + by$

$f = 4.28\ (e - c)$

$g = 7.97\ (e - d)$

$h = 0.623 + 0.5\ (f + g)\ (f - g)$

$LR = \exp(-h)$, the odds ratio favouring carriership[a]

Compute the final probability of carriership:

$Pc = \pi LR\ /\ (\pi LR + 1 - \pi)$.

[a] Note: exp (–*h*) denotes '*e*', the base of natural logarithms, raised to the power of –*h*.

Table 3: **Haemophilia B, univariate linear discriminant analysis.** Factor IX:C levels are required for both the carrier and non-carrier reference groups assuming that the reference subjects do not use oral contraceptives. The factor IX levels of the consultand need to be corrected by a factor of 0.75 if she does. In this example the data are transformed by the square root to obtain symmetrical distributions

Enter for consultand:

α = Factor IX:C in IU/ml.

β = Oral contraceptive use: 0 for no, 1 for yes.

π = Genetic probability of carriership (fraction of 1).

Enter for the carrier and non-carrier reference groups:

μ_c = mean of square-root-transformed factor IX:C levels of the carriers in IU/ml.

σ_c = standard deviation of square-root-transformed factor IX:C levels of the carriers in IU/ml.

μ_n = mean of square-root-transformed factor IX:C levels of the non-carriers in IU/ml.

σ_n = standard deviation of square-root-transformed factor IX:C levels of the non-carriers in IU/ml.

Compute transformed and corrected factor IX:C for consultand

$a = [(1 - 0.25\beta)\alpha]^{1/2}$

Compute the odds ratio:

$b = 1/(2\sigma_c^2)$

$c = 1/(2\sigma_n^2)$

$d = (a - \mu_c)^2$

$e = (a - \mu_n)^2$

$f = bd - ce$

$g = \exp(f)$[a]

$LR = \sigma_n / (g\sigma_c)$, the odds ratio favouring carriership

Compute the final probability of carriership:

$Pc = \pi LR/(\pi LR + 1 - p)$.

[a] Note: exp (*f*) denotes "*e*", the base of natural logarithms, raised to the power of *f*.

(6) *Combining pedigree and laboratory data*

The pedigree probability of carriership (*P*) has to be transformed to the corresponding odds (*a*:*b*) according to the formula $a{:}b = P{:}(1\text{-}P)$. The probability $P = a/(a + b)$. In familial haemophilia the anterior pedigree itself supplies a probability of the consultand being a carrier. If descendants exist, one can modify the anterior probability with the odds of being a carrier derived from the descendant pedigree. In this method, the anterior probability is first transformed to odds before combining the two (Table 4).

The odds of being a carrier from the laboratory data, as calculated above, is multiplied with the pedigree odds to arrive at a combined odds. This is then

Table 4: **Combining pedigree and laboratory data**

Odds from pedigree	1 : 3
Odds from the laboratory data	10 : 1
Combined odds	1 × 10 : 3 × 1 = 10 : 3
Final probability	10/(10 + 3) = 0.77

again transformed into a probability by the formula $P = a/(a + b)$. The example in Table 4 illustrates the calculations.

Genotypic assessment. Haemophilia A and B can be assessed by genetic linkage using intragenic polymorphism analysis.

(1) *Genes encoding factor VIII and factor IX*

The factor VIII gene is situated at the telomeric end of the long arm of the X chromosome at band Xq28. The gene encompasses 186 kbp of genomic DNA (approximately 0.1% of the DNA sequence on the X chromosome) and comprises 26 exons ranging in size from 69 bp to 3.1 kbp (*35*). The factor IX gene is situated at band Xq27, approximately 40 megabases centromeric of the factor VIII locus on the X chromosome. The gene is approximately 33.5 kbp long, comprises 8 exons and encodes an mRNA of 1.4 kbp (*36*).

(2) *Genetic linkage*

The mutations that result in haemophilia A and B are heterogeneous and in most instances involve changes of single nucleotides (*37, 38*). These two factors in two large genes have made the direct detection of haemophilic mutations a difficult challenge. As a result, many laboratories continue to assess the genetic status of potential carriers of haemophilic mutations through the use of indirect genetic markers of the factor VIII and factor IX genes. These genetic polymorphisms represent natural variations of the genome sequence which occur in the general population and which can be used as convenient landmarks to track mutant genes through families (Fig. 2 and 3).

When the polymorphisms occur within the gene of interest (intragenic polymorphisms), the likelihood of the polymorphic marker becoming unlinked from the mutation through genetic recombination is related to the size of the gene. To date, there are no reports of intragenic recombination events in either haemophilia A or B and thus one can assume that genetic diagnoses based on the analysis of an intragenic polymorphism in these conditions is extremely accurate.

(3) *Types of sequence variation within the factor VIII and factor IX genes*

In order to be able to track individual copies of the factor VIII and factor IX genes, it is essential to have available polymorphic sequences which will differentiate between the two gene copies present in females. The presence of heterozygosity or informativeness for a polymorphism is a prerequisite which must be satisfied if genotypic studies are to be effective. In addition, as alluded to above, the polymorphic sequence should either be within the gene which the disease is segragating or close enough to ensure that the possibility for genetic recombination between the polymorphism and the disease mutation is minimal. The final point to re-emphasize is that although some polymorphisms may be in allelic association (linkage disequilibrium) with particular mutations, the polymorphisms themselves represent phenotypically neutral sequence variation that is found in the general population.

Two types of polymorphic sequence exist within the haemophilia genes (Tables 5 and 6). The most frequent and simple examples are the bi-allelic polymorphisms resulting from single nucleotide substitutions which either create or abolish restric-

Fig. 2. **The use of two polymorphisms in segregation analysis of a haemophilia A family.** The two markers shown are the multi-allelic intron 13 CA repeat and the extragenic DXS15 polymorphism which is located approximately 5 recombination units distant from the factor VIII gene. The haemophilia A mutation is segregating with the intron 13 "6" allele in this family. With this information in mind, the potential carrier female III.3 is identified as a non-carrier. The coincident analysis of the DXS15 marker shows that a recombination event has occurred between this locus and the factor VIII gene in the unaffected male III.2. In this individual, analysis with this extragenic marker alone would have incorrectly predicted that he had inherited the haemophilia A mutation.

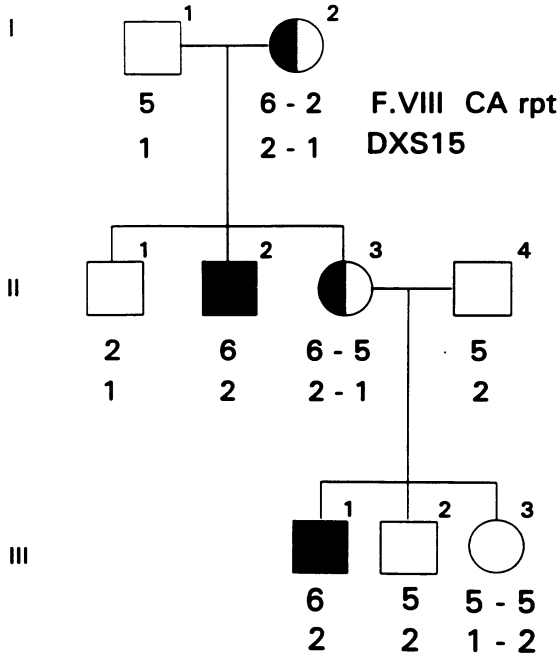
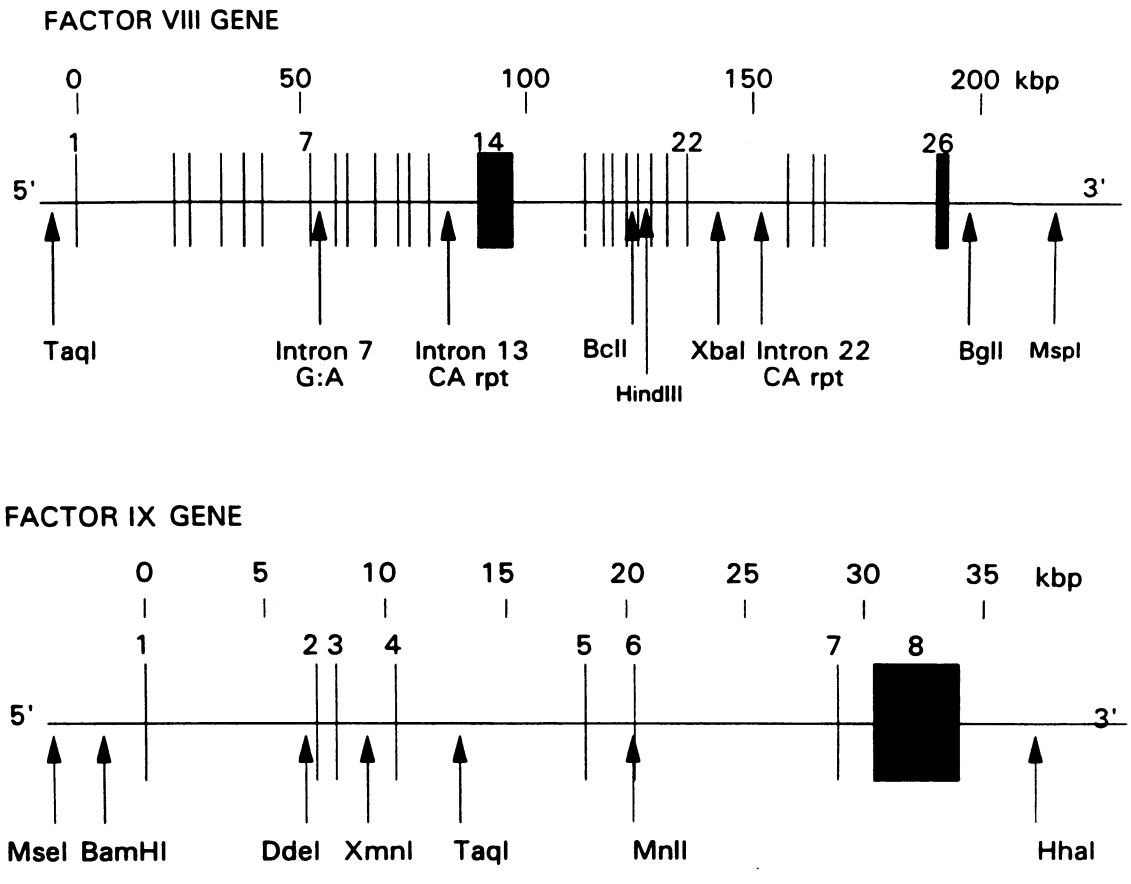


Fig. 3. **Line diagrams of the factor VIII and factor IX genes with the sites of common polymorphic sequences identified.**



tion endonuclease sites (restriction fragment length polymorphisms, RFLPs). The limitation of these bi-allelic systems is that the maximum level of heterozygosity or informativeness is 50%. One bi-allelic insertion/deletion polymorphism also exists in intron 1 of the factor IX gene.

Two multi-allelic intragenic polymorphisms have been identified to date in the factor VIII gene. These CA dinucleotide repeat sequences occur within introns 13 and 22 and the intron 13 repeat with eight alleles reported has a heterozygosity rating of approximately 80% (*39*).

(4) *Practical issues*

(*a*) *Extraction of DNA.* High molecular weight DNA is most often obtained from blood leukocytes by treatment with proteinase K followed by phenol:chloroform extraction. The preferred anticoagulant for blood collection is either EDTA or sodium citrate. In light of its interference with subsequent test procedures heparin is not recommended as an anticoagulant for these studies. The transport of samples from one centre to another can be accomplished in a number of ways. Ideally, DNA should be extracted at source and then sent through the regular postal system, either in aqueous solution (10 mmol Tris/1 mmol EDTA), precipitated in ethanol or following lyophilization. If DNA extraction at source is not feasible, the anticoagulated whole blood should be decanted into polypropylene tubes, indi-

Table 5: **Factor VIII intragenic DNA polymorphisms**

Site	Restriction enzyme	Testing method (PCR or Southern blot)	Heterozygosity (%)	Reference	Southern blot alleles	Allelic[a] frequencies (%)	PCR alleles
5' flanking region	TaqI	Blot Probe: p701.1	40	*98*	9.5 kb 4.0 kb	72 28	— —
Intron	—	PCR (G or A) Primers: 5' TGCAGAACATGAGCCAATTC 3' 5' TAATGTACCCAAGTTTTAGG 3' ASO Probes: 5' GCAAGACACTCTGACATTG 3' 5' GCAAGACACTCTAACATTG 3'	32	*99*	—	79 21	G A
Intron 13	—	PCR (CA repeat) Primers: 5' TGCATCACTGTACATATGTATCTT 3' 5' CCAAATTACATATGAAGCC 3'	80	*39*	—		CA repeat 8 alleles
Intron 18	Bcl I	PCR Primers: 5' TAAAAGCTTTAAATGGTCTAGGC 3' 5' TTCGAATTCTGAAATTATCTTGTTC 3'	42	*42*	1.1 kb 0.88 kb	29 71	142 bp 99+43 bp
Intron 19	HindIII	PCR Primers: 5' GGCGAGCATCTACATGCTGGGATGAGC 3' 5' GTCCAGAAGCCATTCCCAGGGGAGTCT 3'	42	*57, 100*	2.7 kb 2.6 kb	70 30	469+248 bp 469+167+81 bp
Intron 22	XbaI	PCR and blot Primers: 5' CACGAGCTCTCCATCTGAACATG 3' 5' GGGCTGCAGGGGGGGGGGACAACAG 3' Probe: p482.6	48	*42, 44*	6.2 kb 4.8+1.4 kb	41 59	96 bp 68+28 bp
Intron 22	—	PCR (CA repeat) Primers: 5' TTCTAAGAATGTAGTGTGTG 3' 5' TAATGCCCACATTATAGA 3'	44	*101*	25 repeats 26 repeats	33 67	CA repeat
3' of exon 26	Bgl I	Blot Probe: factor VIII cDNA-C	18	*102*	20 kb 5 kb	10 90	— —
3' of exon 26	MspI	Blot Probe: p625.3	44	*103*	7.5 kb 7.3 + 3.2 kb	68 32	— —

[a] Caucasian.

Table 6: **Factor IX intragenic DNA polymorphisms**

Site	Restriction enzyme	Testing method (PCR or Southern blot)	Heterozygosity (%)[a]	Reference	Southern blot alleles	Allelic[a] frequencies (%)	PCR alleles
5' flanking region	MseI	PCR 5' GATAGAGAAACTGGAAGTAGACCC 3' 5' TTAGGTCTTTCACAGAGTAGATTT 3'	44	*104*	—	33 67	83 bp 57+26 bp
5' flanking region	BamHI	PCR Primers: sequences not reported	4	*47, 105*	15 kb 13 kb	98 2	356 bp 216+140 bp
Intron 1	DdeI	PCR Primers: 5' GGGACCACTGTGGTATAATGTGG 3' 5' CTGGAGGATAGATGTCTCTATCTG 5'	36	*43, 56*	1.75 kb 1.70 kb	24 76	369 bp 317 bp
Intron 3	Xmn I	PCR Primers: 5' AATCAGAGACTGCTGATTGACTT 3 5' AAACAGCCAGATAAAGCCTCCA 3'	41	*43, 56*	11.5 kb 6.5 kb	71 29	222 bp 154+68 bp
Intron 4	TaqI	PCR Primers: 5' CTGGAGTATGACTGGCCAATTATCC 3' 5' GGTACACAAGGATTCTAAGGTTG 3'	45	*43, 106*	1.8 kb 1.3 kb	65 35	163 bp 124 + 39 bp
Intron 4	MspI	Southern blot	32	*107*	5.8 kb 3.4 kb	22 78	— —
Codon 148	MnI I	PCR Primers: 5' GATTTGAAAACTGTCCATGAAAATAAC 3 5' AAGTACCTGCCAAGGGAATTGACCTGG 3'	45	*108*	— —	33 67	126+279 bp 126+120+159 bp
3' flanking region	HhaI	PCR Primers: 5' ACAGGCACCTGCCATCACTT 3' 5' AAGTACCTGCCAAGGGAATTGACCTGG 3'	48	*53*	— —	61 39	230 bp 150+80 bp

[a] Caucasian

vidually packaged in plastic or polystyrene containers, and sent on dry ice via courier service to the testing laboratory. Whole blood samples can either be stored at –70 °C without additional manipulations or leukocyte pellets can be prepared prior to storage. In most instances using standard extraction protocols, between 200 and 500 μg of DNA will be obtained from a 10 ml blood sample. Abbreviated DNA extraction protocols have been proposed in the preparation of material for the polymerase chain reaction and these methods will result in material of adequate quality in most instances.

(*b*) *Analysis of polymorphic sequences.* The two molecular genetic techniques used to identify DNA polymorphisms are those of Southern blotting (*40*) and the polymerase chain reaction (PCR) (*41*). The former method has been in use for more than a decade and involves a relatively labour-intensive schedule comprising the capillary transfer of endonuclease-digested DNA fragments from an agarose gel to a membrane support and the subsequent probing of the membrane with a radiolabelled DNA fragment representing the sequence of interest. Studies utilizing Southern analysis require a minimum of 5 μg of high molecular weight DNA for testing and take at least five to seven days to produce results. The polymerase chain reaction has now replaced Southern blotting in many instances. This technique utilizes synthetic oligonucleotide primers to select for specific sequences of interest which are then amplified *in vitro* to produce a targeted product which is present in more than a millionfold its original concentration. The power of this method has resulted in several advantages including the ability to work with very small starting quantities of DNA (less than 1 μg), increased simplicity and biosafety (non-radioisotopic methods), and the completion of tests within 48 hours.

Most of the polymorphisms in the factor VIII and factor IX genes can now be studied through the analysis of DNA which has been amplified *in vitro* by PCR (*42, 43*). Assessment of the XbaI polymorphic genotype by PCR is complicated by the co-amplification of homologous sequences adjacent to, but outside of, the factor VIII gene. This complexity can be resolved by Southern analysis (*44*).

Following DNA extraction, the PCR amplification of these various sequences takes approximately three hours, after which the amplified products are digested with the appropriate restriction enzyme and analysed by polyacrylamide gel electrophoresis. As with all PCR studies, the complication of sample contamination with extraneous DNA must be guarded against. Most laboratories perform their PCR studies in a designated clean area and use a dedicated set of equipment (including positive displacement pipettes) and supplies to minimize the risk of DNA carry-over. In addition, the inclusion of a "no DNA template" blank tube in each experiment provides a further safeguard against this problem. Finally, although the endonuclease digestion of most PCR products will proceed to completion uneventfully, the inclusion of previously genotyped PCR amplified samples in each test run ensures that all components of the endonuclease reaction have been added and are functional. In addition, the inclusion of an invariant endonuclease site within the amplified fragment further assists in evaluating the digestion process.

(5) *Testing strategy*

(*a*) *Haemophilia A.* The strategy for polymorphism analysis in any particular family must take into account factors including the site of the polymorphism, the heterozygosity rating of the marker, and the ethnic origin of the family (see below).

The recently described intron 13 CA repeat polymorphism appears to be informative in approximately 80% of females and thus represents the logical starting point for analysis of factor VIII polymorphisms. This sequence can be amplified in a "multiplex" PCR with the other, Intron 22, CA repeat and the two sequences analysed simultaneously. The fact that these sequences require the use of radiolabelled amplification primer and electrophoretic separation of the products on a DNA sequencing gel may, however, result in some laboratories reserving the analysis of this marker for those cases in which the two other frequent BclI and XbaI polymorphisms are uninformative. Fortunately, these four markers are not in allelic association (linkage disequilibrium) and more than 95% of females will be informative for one or more of these polymorphisms. The remaining families should be tested with the BglI marker and with the intron 7 polymorphism which will be informative in approximately 10% of females who are homozygous for the absence of the BclI polymorphic site.

(*b*) *Haemophilia B.* The combined use of the TaqI, XmnI, DdeI, HhaI, MnlI and MseI polymorphisms will provide informative results in approximately 90% of females in haemophilia B genotype testing. Therefore, there will still be about 10% of families with haemophilia B in whom intragenic polymorphism analysis is uninformative and where either linked extragenic markers will have to be investigated or direct mutation detection will be necessary.

(6) *Advantages of genotype assignment with polymorphism testing*

Where a prior family history of haemophilia exists, and an intragenic polymorphism is informative,

diagnostic results with an error rate of less than 1% are attainable. The methods are straightforward, rapid and inexpensive to perform. Thus, in many families requesting genetic diagnosis of haemophilia, the use of intragenic polymorphism analysis represents the diagnostic strategy of choice.

(7) *Limitations to the use of polymorphism testing in haemophilia*

Although in many instances, the use of an informative intragenic polymorphism will provide highly accurate genetic diagnosis of haemophilia, there are, nonetheless, some limitations to this diagnostic strategy (*45*). All of these drawbacks relate to the fact that the haemophilic mutation itself is not identified by these methods.

(8) *Requirement for family sampling*

Polymorphism linkage analysis requires the participation of a minimum number of key individuals from a haemophilic family. At least one affected male should be available for testing to identify the polymorphic allele which is associated with the mutation in the family requesting diagnosis. With the recent catastrophe of HIV infection in the haemophilia population, this initial requirement may be compromised by early deaths of haemophilic males. However, even in the instances where all affected males in the family are deceased, the recovery of DNA from stored pathological samples for analysis by PCR still makes polymorphism testing feasible.

In addition to getting all appropriate family mem-bers to agree to participate in genetic testing, it is also vital that all stated family relationships (particularly paternity) are correct.

(9) *Families with an isolated affected haemophiliac*

Sporadic cases of haemophilia comprise 30–50% of the total haemophilic population. In these families, because polymorphism analysis does not identify the haemophilic mutation directly, it is not possible to ascertain at which level of the pedigree the mutation arose. In fact, past studies have indicated that approximately 85% of mothers of isolated haemophiliacs are carriers but in individual diagnostic cases, unless the coagulation studies of the mother are strongly suggestive of her being a carrier, it is probably unwise to attempt the diagnosis of a haemophilic allele by polymorphism testing. Therefore, in these families, one is often left with the option of using polymorphism studies to exclude transmission of the haemophilic mutation.

(10) *Requirement for heterozygosity and possibility of genetic recombination*

As detailed above, for polymorphism linkage studies to yield useful information, one must be able to differentiate between the two X chromosomes in key females through the presence of polymorphic heterozygosity. In the study of haemophilia A with intragenic markers, this requirement is now achieved in more than 95% of families due in large part to the intron 13 CA repeat polymorphism. In the analysis of the factor IX gene where a similarly multi-allelic sequence does not exist, some 10% of families will still require diagnostic studies with linked extragenic markers to achieve informative results. In these latter studies, the possibility of genetic recombination between the polymorphic site and the haemophilic mutation adds an additional uncertainty to the precision of genetic diagnosis.

Ethnic variation in frequency of polymorphisms, linked polymorphisms and linkage disequilibrium.

Factor IX polymorphisms. In Caucasians, the use of six intragenic RFLP sites allows linkage of the gene in approximately 80% of families (*46*). These sites are Taq I, Xmn I, Dde I, Msp I, BamH I as well as the residue 148 (Thr/Ala) base change (Mnl I). However, there is marked ethnic variation in the incidence of heterozygosity for these sites. In Blacks, both the 5' BamH I and the intron 3 BamH I sites are useful, with heterozygosity rates of 0.46 and 0.22 respectively (*47*). In comparison, informativity at these sites is rare in the Caucasian populations (*47, 48*). The incidence of heterozygosity for RFLP sites of different ethnic groups are given in Table 7. Orientals, such as Chinese (*49*), Japanese (*50*), Philipinos (*51*) as well as Malays (*52*), were found to have a low incidence or absence of the RFLPs listed above. The only RFLPs informative within these populations are those detected by HhaI and the recently described MseI RFLP (*104*), which has been shown to be informative in Thai populations.

Linked intergenic polymorphisms. An Sst I RFLP at locus DXS99 can be detected by probe pX58dIIIc (*54*). This polymorphism gives rise to two alleles of 5.9 kb and 8.8 kb respectively, with a frequency of 0.43 for the former. The polymorphic locus DXS99 is mapped to Xq26-q27 and tightly linked to the factor IX gene. The precise genetic distance between this locus and the factor IX gene has yet to be determined, but thus far, no recombination has been detected in 39 informative meiosis, giving a lod score of 9.79 at $\Theta = 0.0$, with 95% confidence limit of $\Theta = 0–0.06$. Since this marker and the FIX loci do not appear to be in linkage disequilibrium (*54*), the analysis of this Sst I RFLP at DXS99 in conjunction with the intragenic sites in the factor IX gene should increase the diagnostic efficiency to more than 90% of females at risk.

Table 7: **Frequencies of heterozygosity for polymorphisms associated with factor IX gene in different ethnic groups**

		Caucasian (*55*)[a]		Japanese (*50*)		Chinese (*49, 52*)		Malay (*52*)		American Blacks (*48*)		Asian (*52*)		Maori (*109*)		Polynesian (*109*)		Thai (*104*)	
Polymorphic sites	Alleles (kbp)	Allele freq.	Hetero-zygosity	Allele freq.	Hetero-zygosity	Allele freq.	Hetero-zygosity	Allele freq.	Hetero-zygosity	Allele freq.	Hetero-zygosity	Allele freq.	Hetero-zygosity	Allele freq.	Hetero-zygosity	Allele freq.	Hetero-zygosity	Allele freq.	Hetero-zygosity
5' BamH I	25 (–)	0.98	0.04 (*47*)	—	—	1.0	0	1.0	0	0.64	0.46	0.64	0.46	—	—	—	—	—	—
	23 (+)	0.02		—		0.0		0.0		0.36		0.36				—	—	—	
TaqI	1.8 (–)	0.65	0.45	1.0	0	0.99	0.02	0.99	0.02	0.86	0.24	0.96	0.07	0.93	0.13	0.99	0.02	—	—
	1.3 (+)	0.35		0.0		0.01		0.01		0.14		0.04		0.07		0.01		—	
Xmn I	11.5 (–)	0.71	0.41	1.0	0	0.99	0.02	1.0	0	0.88	0.21	0.96	0.07	0.94	0.11	—		—	—
	6.5 (+)	0.29		0.0		0.01		0.0		0.12		0.04		0.06		—		—	
DdeI	1.75	0.24	0.36	0.0	0	1.0	0	—	—	0.36	0.46	—	—	—	—	—	—	—	—
	1.7	0.76		1.0		0.0		—		0.64		—		—		—		—	
MspI	5.8 (–)	0.20	0.32	0.0	0	—	—	—	—	0.39	0.47	—	—	—	—	—	—	—	—
	3.4 (+)	0.80		1.0		—		—		0.61		—		—		—		—	—
BamHI (2)	25 (–)	0.94	0.11	—	—	0.98	0.04	—	—	0.87	0.22	—	—	—	—	—	—	—	—
	16 (+)	0.06		—		0.02		—		0.13		—		—		—			
Residue 148	Thre (ACT)	0.67	0.44	—	—	0.97	0.06	0.97	0.06	0.89	0.19 (*52*)	0.93	0.07	—	—	—	—	—	—
(MnI I)	Ala (GCT)	0.33		—		0.03		0.03		0.11		0.04				—		—	
3' Hha I	0.23 (–)	0.39	0.48	—	—	0.83	0.28	0.91	0.11	0.43	0.49 (*52*)	0.32	0.43	—	—	—	—	—	—
	0.15 (+)	0.61		—		0.17		0.06		0.57		0.68				—		—	
5' Mse I	(–)	0.67	0.44	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.79	0.33
	(+)	0.33		—		—		—		—		—		—		—		0.21	

[a] Figures within parentheses, in italics, are references.

Linkage disequilibrium of factor IX polymorphisms. In Caucasians, the Taq I, Xmn I, Msp I and Mnl I RFLPs show marked linkage disequilibrium (allelic association), thus the use of all four sites would only increase the diagnostic efficiency to 55% as opposed to 45% when using the Taq I site alone (*52, 55, 56*). However, Dde I and 3' Hha I polymorphisms showed much less disequilibrium and the combined use of these two sites and the Taq I site increases the heterozygosity rate to almost 76% (*53, 55*).

In the American Blacks, the linkage disequilibrium between Taq I and Msp I is less marked. While the intragenic BamH I+ alleles and Msp I– alleles showed disequilibrium, the 5' BamH I, Dde I and Xmn I sites appeared to be in equilibrium. The combined use of these latter sites showed an observed frequency of heterozygosity of 87% for Black females (versus c. 60% in Caucasians) (*48, 57*).

No linkage disequilibrium was observed between the Hha I locus at the 3' end of the factor IX gene and the other intragenic loci (*53*); thus it is likely that this polymorphic marker will be extremely useful for factor IX carrier testing. This has certainly been the case for Orientals (*49*), who lack heterozygosity for the common intragenic RFLP sites. The MseI polymorphism also shows minimal linkage disequilibrium with the other polymorphisms within the factor IX gene.

Factor VIII polymorphisms. As with factor IX, the incidence of factor VIII RFLPs differ significantly in various racial groups (*57, 58*); thus before a prenatal diagnosis programme can be instituted in a particular region, the RFLPs for that population should be studied, to decide the most suitable sites for use.

Table 8 summarizes the incidence of Bcl I, Xba I, Bgl I, Hind III and MspI polymorphisms in the factor VIII gene in various ethnic groups. The positive incidence of Bgl I polymorphism is higher in Chinese than in other races. Of particular interest is that in American Blacks, the rates of the (+) site for Bcl I and Hind III polymorphisms are the reverse of what is observed in other ethnic groups (*57*). Table 8 also shows the female heterozygosity rate. The Bcl I dimorphism is more informative in Mediterraneans, Indians and Japanese (42–47%) compared to Caucasian, American Blacks, Chinese and Malays (31–39%). The Hind III and Xba I RFLPs showed similar heterozygosity in the various groups tested, whilst the Bgl I RFLP is most useful in American Blacks (38%), but useless in Chinese (0%).

Linked intergenic polymorphisms. The physical mapping of the q28 region of the X chromosome has revealed that the loci DXS52 and DXS15 are 1–2 Mb centromeric to the factor VIII gene. The highly

Table 8: **Frequencies of heterozygosity for polymorphisms associated with factor VIII gene in different ethnic groups**

Polymorphic sites	Alleles (kb)	Caucasian		Japanese		Chinese		Malay		American Blacks		Asian		Maori		Polynesian	
		Allele freq.	Hetero-zygosity	Allele freq.	Hetero-zygosity	Allele freq.	Hetero-zygosity	Allele freq.	Hetero-zygosity	Allele freq.	Hetero-zygosity	Allele freq.	Hetero-zygosity	Allele freq.	Hetero-zygosity	Allele freq.	Hetero-zygosity
Bcl I	1.1 (–)	0.27	0.39	0.30	0.42	0.21	0.33	0.21	0.33	0.81	0.31	0.31	0.43	0.33	0.44	0.43	0.49
	0.88 (+)	0.73		0.70		0.79		0.79		0.19		0.69		0.67		0.57	
Hind III	2.7 (–)	0.74	0.38	—	—	0.76	0.37	0.77	0.35	0.22	0.34	0.71	0.41	—		—	
	2.6 (+)	0.26		—		0.24		0.23		0.78		0.29		—		—	
Xba I	6.2 (–)	0.44	0.49	0.41	0.48	0.42	0.49	—	—	—	—	—	—	—	0.49	—	0.5
	4.8+1.4 (+)	0.56		0.54		0.58		—		—		—		—		—	
Bgl I	20 (–)	0.15	0.25	0.09	0.16	0.0	0	—	—	0.26	0.38	0.06	0.11	—	0.11	—	0.02
	5 (+)	0.85		0.91		1.0		—		0.74		0.94		—		—	
Msp I (1)	7.5 (–)	0.68	0.43	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	4.3+3.2 (+)	0.32		—		—		—	—	—	—	—		—		—	
Msp I (2)	4.0 (–)	—	0.00	—	0.45	—	—	—	—	—	0.00	—	0.13	—	—	—	—
	3.8 (+)	—		—		—		—		—		—		—		—	

polymorphic St 14 probe detects a polymorphism within the cluster MN12, cpX67, and DX13 (*59*). The two extragenic RFLPs, Bgl II/DX13 (*60*) and Taq I/St 14 (*61*), are both closely linked to the haemophilia A locus. The Bgl II RFLP detected by the DX13 probe shows two alleles, 5.8 kb (allele 1) and 2.8 kb (allele 2) respectively. The heterozygosity rating for this polymorphism varies between ethnic groups from 0.30 (Japanese) to 0.5 (Caucasians), and the frequency of recombination with factor VIII is approximately 4.5%.

The Taq I RFLP detectable with the St 14.1 probe gives two independent systems of alleles (*61*). System I has eight alleles (1 to 8) ranging from 6.6 to 3.4 kb in length, and system II has two alleles, "A" (5.5 kb) and "B" (4.1 and 1.4 kb, respectively).

Caution should be exercised when using intergenic linked probes for diagnosis because of the possibility of meiotic recombination (*62, 63*). With the St 14.1 probe, reports from world literature suggested a genetic disease of 3 cM, and this should be taken into account during genetic counselling. The use of the DX13 probe would be even more prone to error, as the cross-over rate with the factor VIII gene is thought to be about 4.5% (*64*).

Linkage disequilibrium of factor VIII polymorphisms. There is a strong linkage disequilibrium between the intron 18 Bcl I, intron 19 Hind III and intron 25 Bgl I sites (*57, 63*). Thus little additional information will be gained in using more than one of these three sites. In contrast, the Xba I site in intron 22 is often informative in females who are homozygous for the Bcl I site (*65*). Even though the Bcl I and Xba I sites are not in complete linkage equilibrium, with a disequilibrium coefficient of 0.0722–0.1627 in various ethnic groups reported, the combined use of these two sites would significantly increase the informativeness of 79% in Japanese, 64–69% in Caucasians, and 52% in Chinese females respectively (*58, 65, 66*). In certain populations, multiple Xba I polymorphisms have been described, e.g., Chinese (*44*) and Canadian (*67*). Although these other polymorphisms may well be non-factor VIII sequences which are detected by the factor VIII intron 22 probe (p482.6), they are closely linked to the factor VIII gene and the combined use of all the Xba I and Bcl I RFLPs would increase the detection rate to 67% in Chinese.

Due to the highly polymorphic nature of the four intragenic (two CA repeat polymorphisms, Bcl I and Xba I) and one extragenic (Taq I/St 14.1) polymorphisms, carrier detection or prenatal diagnosis should be possible in 96–100% of females at risk (*58, 66, 68*).

Direct mutation detection in the haemophilias. Linkage analysis for carrier detection and prenatal diagnosis of haemophilia has widely appreciated benefits including rapidity, relative technical simplicity, wide availability and definitive diagnostic results in a high proportion of cases. However, it has certain inherent drawbacks and limitations which have been previously described.

In principle, all these defects of linkage analysis may be circumvented by identifying the specific mutation in a given kindred. It is then sufficient merely to check the putative carrier or fetus at risk for the relevant defect. In addition, identification of mutations provides scientifically interesting information that may give clues on structure and function of factor VIII and IX, on mechanisms of mutation, or on phenotype/genotype aspects such as the risk of inhibitor formation.

The obvious drawbacks of mutation analysis in haemophilia A and B are that the two genes involved are large and complex and the mutations therein extremely heterogenous, as expected for X-linked sublethal disorders. However, methods for rapid screening of large regions of DNA for small lesions have been developed in the past 5 years which now make this approach feasible in expert laboratories. These methods were first applied to the smaller gene, factor IX, with impressive results in several centres. For example, a project to identify all the mutations causing haemophilia B in the United Kingdom population is now well advanced with successful identification of a causative gene lesion in 160 out of 161 patient DNAs analysed so far (Giannelli, personal communication). The methods used for haemophilia B analysis comprise Southern blotting with a full length cDNA probe which detects deletions down to about 1.5 kb. This yields diagnostic information in about 1% of cases. For more detailed analysis, enzymatic amplification of each exon is performed using the polymerase chain reaction (PCR) followed by chemical cleavage mismatch detection (CCD) (*55*). A positive signal from CCD is confirmed by direct sequencing.

Mutation analysis in haemophilia A has to cope with the fact that the 26 exons of the factor VIII gene are distributed over 186 kbp of genomic DNA and exon 14 is over 3 kbp in length. Southern blotting after digestion of genomic DNA with the enzyme TaqI yields diagnostic information in c. 5% of cases due to the somewhat higher incidence of large deletions in haemophilia A and the occurrence of mutation hotspots in 5 TaqI sites. Currently three screening methods are being used to screen enzymatically amplified exons and flanking regions of the factor VIII gene. Higuchi and colleagues (*69, 70*) have applied denaturing gradient gel electrophor-

esis (DGGE) and reported on their results in 29 mild or moderate cases and 30 severe cases. Forty-five oligonucleotide primer sets were required to amplify 99% of the coding region and 41 of 50 splice junctions. The disease-producing mutation was found in 25 out of 29 mild or moderate cases (85%) but in only 16 of 30 severe cases (53%). Even allowing for incomplete coverage of splice junctions there is a clear implication that a high proportion of mutations causing severe haemophilia A lie with the previously accepted essential regions of the factor VIII gene. This highly interesting finding has been confirmed using an alternative approach (see "Developments in direct defect detection", below). Consequently until these unidentified mutations are located there is going to be a limitation on the effectiveness of even direct defect detection, underlining the continuing importance of polymorphism-based genotype assays and phenotype assays in carrier and fetal diagnosis.

Another powerful and technically simpler approach to mutation screening (compared to CCD and DGGE) is based on the property of single stranded DNA to form self-associated loop structures that are highly sequence dependent. The conformation adopted strongly influences the rate of migration of single-stranded DNA in non-denaturing polyacrylamide gel electrophoresis, enabling detection of sequence polymorphism (or mutation). The screening method exploiting these phenomena is called Single Stranded Conformational Polymorphism (SSCP) analysis. A comparison of SSCP with CCD for mutation detection in factor VIII exons 1 to 14 has yielded essentially identical sensitivity (Tuddenham, unpublished observations), so this method may become more widely utilized. Finally, direct sequencing of amplified DNA is used to identify any specific mutation.

Data — haemophilia A. A compilation of mutations in the factor VIII gene updated to August 1991 has been published in *Nucleic acids research* (*37*); 81 different point mutations, 6 insertions, 7 small deletions, and 60 large deletions are catalogued. Information where available is also provided for F.VIII coagulant and antigen level, clinical severity and inhibitor status. A unique number has been assigned to each patient for future identification. Thirty-eight percent of point mutations are located in CpG dinucleotides. This frequency is biased by screening with TaqI which identifies five such sites, but an unbiased estimate from Higuchi's data (*69*) is similar at 32%. Recurrent mutation in CpG dinucleotides has occurred in at least 16 sites, where identity by descent can be excluded on RFLP haplotype or extreme geographical separation. Recurrence at non-CpG sites also occurs, for example at Arg 2307Leu, found in American, German and Japanese patients.

This international database will be updated annually and published in *Nucleic acids research.* New cases for inclusion should be submitted to Dr S. Antonarakis, The Johns Hopkins University, Baltimore, USA or Dr E. Tuddenham, MRC, Harrow, England. The haemophilia A database has been submitted to the Genome Database (Welch Medical Library, 1830 East Monument Street, Baltimore, MD 21205, USA) and will be accessible from there by electronic means. In future as the number of patients successfully analysed increases, the only practical means of updating and retrieval will be via computerized databases, organized nationally and internationally. Appropriate security of information access will be built into these systems.

Data—haemophilia B. A compilation of point mutations, short deletions and insertions in the factor IX gene has been produced by Giannelli et al. (3rd edition, 1992) published in *Nucleic acids research* (*38*).

The data tabulated includes levels of factor IX clotting activity and antigen, inhibitor status but not clinical severity; 574 mutants are listed, representing 278 different molecular alterations. These are distributed across the entire coding region from the signal peptide to codon 412, only four residues before the Stop codon. Eleven examples of mutations in the promoter region giving rise to the Leyden phenotype are recorded. Thirty examples of mutations affecting RNA processing have been observed. This information has now been incorporated into the EMBL database, providing on-line access to updates.

Application of polymorphism analysis and direct defect detection in developed and developing countries. *Developed countries.* Developments in molecular genetics mean that it is now possible to identify practically all the functional mutations in the factor IX gene in patients with haemophilia B and a significant number within the factor VIII gene in patients with haemophilia A (see above). The techniques used are specialized and require staff with molecular biology experience, and several groups within Europe and North America have established laboratories where these analyses are performed upon request. They also provide a follow-up service for subsequent carrier status assessment and prenatal diagnosis.

In these countries the identification of the specific defect will increasingly replace family studies based on gene tracking of polymorphisms (see above). Although polymorphism analysis, particularly when based on PCR technology is extremely simple to perform, the problems related to non-informative family members, non-paternity and sporadic

disease mean that where the technology is available, defect detection techniques are preferable. The recently reported cases of germline mosaicism in haemophilia present problems for family studies based on either approach. It is also expected that increased technological advances will make defect detection easier and more applicable to the routine laboratory. However, without these advances it is probable that polymorphism-based gene tracking will still remain important in those laboratories where molecular biology experience for defect detection is not available.

Developing countries. It is unlikely that direct defect detection will be available in the foreseeable future in many countries where the identification and treatment of the disease itself is only in its infancy. However, it should be pointed out that genetic analysis of families with patients with haemophilia, even though the treatment of the haemophiliac may not be optimal, can still be of assistance to the family by identifying carriers and preventing the birth of affected individuals. Where resources are scarce it is probable that the least expensive techniques of gene tracking which will involve, at the moment, polymorphism analysis will be the only ones applicable. It is, therefore, important that such techniques should be made as simple as possible and their application in developing countries is something that may well advance quite rapidly in the next decade under the guidance of the World Federation of Haemophilia.

Current situation

Prenatal diagnosis

Obstetric techniques. *Ultrasonography.* All pregnant women should be offered a detailed ultrasound examination for (i) confirmation of fetal viability and gestational age, (ii) the diagnosis of multiple pregnancies, and (iii) exclusion of both major malformations but also smaller defects, which may lead to the diagnosis of an underlying chromosomal abnormality or genetic haemophilia. Ultrasonography at 16–20 weeks will reliably diagnose the fetal sex. In the presence of a female fetus, invasive fetal testing can be avoided.

Fetal blood sampling. In the 1970s and early 1980s the method of fetal blood sampling was fetoscopy, which involved the introduction into the amniotic cavity of an endoscope (3 mm in diameter). Blood vessels in the chorionic plate or umbilical cord were visualized and punctured to provide pure fetal blood (*71*). The patients were usually hospitalized and the procedure was carried out under heavy sedation. The technique was confined to very few centres and under heavy sedation. The technique was confined to very few centres and in the best hands the procedure-related risk of fetal death was 2–5%. More recently, improvements in imaging by ultrasonography have made fetoscopic guidance unnecessary and fetal blood can be obtained by ultrasound-guided puncture of an umbilical cord vessel (cordocentesis) or the fetal heart (cardiocentesis). The preferred method is cordocentesis, which involves the ultrasound-guided insertion of a 20 or 22 gauge needle through the maternal abdomen and into an umbilical cord vessel (*72, 73*). The procedure is carried out on an outpatient basis and no maternal sedation or anaesthesia is required. Several centres throughout the world have now developed considerable expertise in this technique. Maternal complications are negligible. The risk of fetal death following cordocentesis is approximately 1–2%. The risks are higher when the mother is obese, the placenta is posterior, and the gestation at the time of sampling 16–19 weeks rather than 20–21 weeks.

Chorion villus sampling (CVS). Placenta tissue can be successfully obtained from as early as six weeks' gestation by the transabdominal or transcervical entry of aspiration cannulas, biopsy forceps, or needles of variable sizes (*74, 75*). More than 200 000 procedures have now been performed throughout the world and the technique is carried out in many centres. However, the recent report on the possible association between CVS at less than 10 weeks and fetal limb abnormalities is likely to confine its application to pregnancies beyond this gestation (*76*). Furthermore, the European MRC trial has demonstrated a significantly higher risk of fetal death after CVS than after second trimester amniocentesis (*77*). Despite these limitations, CVS has the advantage of providing prenatal diagnosis in the first trimester rather than at 18–20 weeks as with traditional amniocentesis.

Amniocentesis. In the context of prenatal diagnosis of haemophilia, amniocentesis is used for fetal sexing and DNA analysis. When amniocentesis was first performed, it was limited to 16 weeks onwards, because at earlier gestations there was a high failure rate in obtaining amniotic fluid. However, during the last five years several studies have established the feasibility of early amniocentesis at 10–14 weeks (*78*). Furthermore, the original apprehension about the smaller cellular content of amniotic fluid has not been substantiated; although the number of amniotic fluid cells at 10 weeks is smaller than at 16 weeks, the number of viable cells is the same. At amniocentesis, a 20 or 22 gauge needle is guided by ultrasound into the amniotic cavity and 10 ml of fluid is

aspirated. Cell culture and cytogenetic analysis are successful in 98% of the cases and results can be available within 2–3 weeks (*78*). For diagnosis of haemophilia, DNA can be extracted from the cells either directly after separation or after culture.

Collection, preparation and storage of prenatal samples. *Fetal blood.* Meticulous care, consistency and speed are critical in collecting the diagnostic blood samples. All the necessary tubes are prepared before cordocentesis. Three successive fetal blood samples are aspirated into 1-ml plastic syringes. The first sample is collected into a heparin tube for (i) immediate cell size analysis and (ii) subsequent Kleihauer-Betke testing, to confirm its fetal origin.

The subsequent two samples are delivered into six polystyrene precipitin tubes, exactly to the 500 µl mark without airlocks or bubbling. The polystyrene tubes contain accurate volumes of buffered-citrate anticoagulant; in two tubes the citrate to fetal blood ratio is 1:1 and in three it is 1:9. The tubes are held sideways between the forefinger and thumb and jerked vigorously 2–3 times to mix. The rest of each sample is collected into heparin tubes for (i) cell size analysis and determination of haematocrit, which are compared with the result of the first sample, to ensure that all samples are fetal and not contaminated by amniotic fluid, and (ii) immunoradiometric assay of VIII:Ag or IX:Ag in the supernatant plasma. All tubes containing fetal samples are capped, placed in ice, and taken to the laboratory for assay, where they are left undisturbed for one hour.

Amniotic fluid. Amniotic fluid (10 ml) is collected into a sterile container, and kept at 4 °C until further analysis. The sample is divided into two halves, one to be used for direct DNA analysis and the second for cell culture to confirm the initial result. The amniocytes are separated by centrifugation in Eppendorf tubes and PCR is performed on the DNA, which is extracted using proteinase K and phenol, followed by ethanol precipitation.

Chorionic villi. Samples are aspirated into culture medium and examined under a dissecting microscope for any contamination with decidua, in which case the tissue must be separated immediately after collection. The chorionic villi are noted for a central vascular core and budding cytotrophoblast, unlike decidua which are more sheet-like and less vascular. The chorionic villi are weighed in a pre-weighed Eppendorf tube; the minimum required is 6 mg, although 60–80 mg of wet tissue is usually obtained.

Measurements on fetal blood. Both factors VIII and IX are measured in all cases to provide an added check on the validity of the samples. In addition, factor V is measured as an indicator of possible consumption or activation of VIII (*79*). When the fetus is at risk of haemophilia B, factor X is also measured.

Prenatal diagnosis of haemophilia A and B by DNA analysis. The effective use of DNA analysis for prenatal diagnosis of haemophilia involves prior planning and coordination between the testing laboratory and an obstetrician specialist in fetal medicine.

The use of PCR-based genetic testing has greatly simplified the prenatal diagnostic strategies used in screening for haemophilia (*42*). The ability to perform PCR studies on less than 1 µg of DNA makes this technique especially useful in prenatal analysis where the yield of fetal tissue for testing may be limited. There has now been considerable experience with the use of PCR-based prenatal testing for both polymorphism linkage analysis and more recently for direct mutation detection in the haemophilias.

Analysis of chorionic villus (CV) tissue. The extraction of DNA from both CV material and amniocytes is best carried out by alternative, "gentler" methods to those used for blood extractions. All routine precautions to avoid sample contamination with extraneous DNA must be strictly adhered to and the possibility of maternal contamination of the CV tissue must also be kept in mind. Most laboratories will perform two sets of tests on the CV DNA. The first involves sexing of the fetus through the use of amplification primers corresponding to the regions ZFX and ZFY on the X and Y chromosome respectively. The DNA is then amplified for haemophilia genotyping using either one of the previously detailed intragenic polymorphisms or one of the methods used for direct mutation analysis. Diagnostic studies are achievable within 48 hours of receipt of the CV tissue.

Amniocyte DNA analysis. Two options are available in testing cells obtained at amniocentesis. Direct analysis of DNA extracted from amniocytes spun down from 10 ml of amniotic fluid is now feasible and avoids the two-week hiatus before sufficient quantities of cultured amniocytes are available for analysis. Once again, in light of the small quantities of fetal cells available for amplification, PCR contamination must be guarded against using techniques previously alluded to in this document. The strategy for DNA testing is identical to that outlined for CVS-based diagnoses.

Genetic counselling services for haemophilia

The individual or individuals involved in genetic counselling for haemophilia should have proficiency

in the following areas: haemophilia and haemophilia care, human genetics, prenatal diagnosis, molecular genetics, and interpersonal communication and counselling. Proficiency may be gained through specific medical/clinical genetics or genetic counselling training programmes, coursework or laboratory experience. If all of the aforementioned aspects cannot be covered in the training of one individual, then a team approach to counselling is recommended so that expertise may be available in all areas. When a team approach is utilized, one individual should coordinate activities of the team, and act as an interface between the patient and team members. Liaison and communication between professional colleagues is highly recommended, especially between those who perform and interpret laboratory tests and those who provide clinical information to the patients and their families.

The aims of genetic counselling services should be (1) to provide patients with sufficient information to make informed choices regarding carrier testing and prenatal diagnosis, and (2) to provide psychosocial support to the patient throughout the process of testing. Information should be given in a non-directive manner prior to the time of actual testing, so that patients and their families can make choices regarding their medical care.

All potential carriers should be tested early in life to determine factor VIII or IX activity levels. This should be done for safety reasons alone, to detect individuals who may have bleeding problems in certain situations. Then, at the age of informed consent, carrier testing should be offered to all female relatives of haemophilic males. The individual undergoing testing, should be aware of the purposes of the test and its implications. It is preferable that carrier testing be completed before a potential carrier becomes pregnant. Contact can be made with females at risk directly, or through their male relatives. An accurate family history should be obtained, and the specific clinical diagnosis of haemophilic males within the family should be ascertained. Knowledge of the severity of disease is extremely important when counselling family members regarding reproductive risks. If a potential carrier is not familiar with the clinical presentation of haemophilia, then various methods of introduction to the disease should be considered, such as audiovisual materials, selected reading, and/or personal interviews with affected individuals and their families.

Technical aspects of both phenotypic and genotypic carrier testing should be discussed with each potential carrier, as well as the accuracy and limitations of the tests. Individuals at risk should be informed that the most accurate type of testing available at this time is genotypic testing, and that direct defect detection is preferred if it is available. If genotypic testing is to be done, family counselling should be considered so that all family members may be informed of the nature of the test, and the possible knowledge that will be gained upon completion of testing. All females with negative carrier test results should carefully be advised of the accuracy of the diagnosis, especially in the case of phenotypic testing, or when using linked polymorphic probes.

Women who have positive carrier test results should be advised of all of their reproductive options, including adoption and *in vitro* fertilization where applicable. They should also be aware of the current treatment options and prognosis for an individual with haemophilia in their area. If they consider having a child of their own, all prenatal testing options should be discussed, as well as the appropriate methodology for termination of an affected pregnancy. Risks for each testing procedure should be given as accurately as possible, taking into consideration the testing facilities which would be available to the patient. Counselling and psychological support should be available to the patient during all aspects of prenatal testing.

Present situation in developed and developing countries

Only recently have methods for prenatal diagnosis of haemophilia moved from the research laboratory to the clinical setting. Because of this, they are at present only available in developed countries, usually in academic settings.

Developed countries. There is very little information internationally on the availability, organization and proficiency of centres that provide these diagnostic services. The information available stems from publications or communications at meetings. As examples, the situation in Italy and the USA, two countries where the provision of prenatal diagnosis of haemophilia appears to be arranged with different strategies will be described.

Italy, a country with a population of 56 million, has registered 2036 patients with severe and moderate haemophilia A and B, i.e., those that are most likely to be concerned with prenatal diagnosis. The distribution of the diagnostic services is regional, with units established in Milan and Genoa (Northern Italy), Rome (Central Italy), Naples (Southern Italy) and Cagliari (located on Sardinia). With the exception of the latter, these diagnostic units are establish-

ed within comprehensive haemophilia centres and enlist the integrated cooperation of a coagulation laboratory that provides phenotypic diagnosis, an obstetric service that performs techniques such as ultrasound, amniocentesis and chorionic villus sampling, and a laboratory of molecular biology that performs DNA analysis. Delivery to the patient of diagnostic information and genetic counselling are provided by genetic counsellors belonging to the staff of the haemophilia centres. This situation is particularly advantageous for the families that are regularly followed at such centres, because the stressful situation of prenatal diagnosis is dealt with by the same staff who attend to haemophiliacs in their day-to-day care. This regional organization has developed spontaneously in Italy, around haemophilia centres which chose to develop DNA techniques for research purposes. There is no state or regional recognition of the diagnostic services, which are funded with research money provided by the hospitals or universities where the haemophilia centres are established.

The regional organization of Italy, based on conveniently sized and located units providing the coordinated services needed for prenatal diagnosis, contrasts with the organization of other countries such as the *USA*. In this country with a population of 250 million, and approximately 20 000 haemophiliacs, very few haemophilia centres (for instance, in San Francisco and Chapel Hill, NC) have established their own services of prenatal diagnosis, spanning from counselling and obstetric procedures to DNA diagnosis. Most centres enlist the help of obstetric units (not necessarily located in the same hospital) to perform chorionic villus sampling or amniocentesis and then send DNA samples to institutions (such as the Mayo Clinic, Johns Hopkins University and the University of North Carolina at Chapel Hill) that have a large experience in molecular biology of congenital coagulation disorders. This system has some appeal, because these large units acquire considerable proficiency by analysing a large number of DNA samples. On the other hand, it has the disadvantage of being somewhat fragmented, in that the consultands are referred from a comprehensive haemophilia centre (not always the centre where regular care is provided) to an obstetric unit for chorionic villus sampling or amniocentesis (not always in the same State), with the actual DNA diagnosis eventually carried out in a totally different institution.

Developing countries. Recent initiatives in primary health care are reducing infant mortality, so that congenital disorders are inevitably beginning to be recognized in developing countries. The burden of handicapping genetic diseases such as haemophilia is heavier in developing countries, because the infrastructure and services needed to assist the handicapped are usually primitive or non-existent. Because of the lack or deficiency of the social support system, the heavy burden of chronic disease for the individual and the family further emphasizes the desirability of prevention. This is particularly evident for conditions such as haemophilia, which requires expensive therapeutic measures. Hence, there is a considerable demand for prenatal diagnosis of haemophilia and allied disorders in developing countries. As a result of this demand, there are many families at risk that seek genetic diagnostic testing in developed countries, but obviously these services can only be afforded by the richest. Local diagnostic facilities are practically non-existent, with the exception of a few academic centres where research interest in molecular biology of coagulation defects has prompted the application of DNA techniques to carrier detection and prenatal diagnosis of haemophilia. The current availability of comprehensive haemophilia centres in developing countries is too scanty to envisage the rapid development of a network of genetic services as feasible and reasonable. On the other hand, it must be realized that knowledge spreads faster than technology, which is expensive. This will create significant problems in developing countries, because knowledge about haemophilia and the corresponding expectations are fast outdistancing the ability to provide modern therapy.

Role of the World Federation of Haemophilia (WFH)

Despite the fact that the WFH is the only international organization that has the institutional goal of striving for the improvement of the well-being of haemophilic patients, the Federation has as yet no information on the availability and organization of units for prenatal diagnosis in member countries (National Member Organization, NMO). It is, therefore, recommend that the medical advisory board or the medical secretaries of the WFH, in collaboration with WHO, take action to gather this important information, by questionnaires or other methods of survey. This input is essential to prepare a registry of diagnostic units and plan further action. The ongoing designation of a few International Haemophilia Training Centres of the WFH as WHO Collaborating Centres on Haemophilia, should provide a network where staff from developing countries that choose to develop genetic services for prenatal diagnosis of haemophilia can be trained.

Future considerations

Genetic diagnosis and management of haemophilia

The post-HIV situation. Although the impact of HIV-I infection on the haemophilic population differs widely from country, this threatening infection is an important cause of death among haemophiliacs. The death of a large number of haemophiliacs from AIDS has important implications on the feasibility of carrier detection or prenatal diagnosis in haemophilic families at risk. The currently available diagnostic methods based on recombinant DNA technology rely heavily on the availability of the DNA from at least one affected member of the haemophilic families. This need materializes not only when RFLPs are employed to track the abnormal allele, as occurs most often in the diagnosis of haemophilia A, but also when the disease-causing DNA defect is searched for directly, as occurs more and more frequently in haemophilia B.

Unfortunately, because of HIV infection, these studies cannot be carried out in some families at risk because the critically affected family members have died, so that their DNA is not available for examination. On the other hand, blood for DNA extraction, and the DNA itself, is stable if stored under proper conditions and may be used for studies many years later. To avoid the aforementioned pitfalls that make carrier detection and prenatal diagnosis often impossible, the World Federation of Haemophilia has recently recommended that samples of blood suitable for DNA extraction be obtained from all persons with haemophilia A and B, particularly from those affected by HIV infection or other potentially lethal conditions. A technique suitable for processing and storing DNA samples has also been recommended (*80*). These samples, stored at the haemophilia centres locally or nationally, will provide valuable material for future studies and represent a resource that will be increasingly important as DNA analysis provides a better understanding of the molecular basis of haemophilia.

It is therefore recommended that WHO should also suggest the organization of the storage of DNA samples from patients with haemophilia and allied congenital coagulation disorders to all member countries.

Developments of RFLP detection techniques. Genetic diagnosis of haemophilia A and B can be performed most effectively by direct detection of the mutation itself. However, as mentioned above, there are technical and other reasons which limit the application of this form of diagnosis. Thus, diagnosis at the DNA level often relies on the familial segregation of linked restriction fragment length polymorphisms (RFLPs). With the development of *in-vitro* DNA amplification using the polymerase chain reaction (PCR) convenient, non-radioactive detection methods have become available. As alluded to above, this form of testing is now in widespread use for both factor VIII and factor IX intragenic and extragenic RFLPs (*42, 52, 53, 57*). Another approach that would obviate the need for restriction enzyme digestion is PCR followed by differential hybridization with sequence-specific oligonucleotide probes. These probes can be linked to horse-radish peroxidase and a colour detection system used instead of radioisotopes (*81*).

A more recent development is the use of immobilized sequence-specific oligonucleotide probes for genetic analysis of PCR-amplified DNA (*82*). Unlike the former method of immobilized DNA, where each probe requires a separate hybridization, this method would enable simultaneous screening of a sample for a number of RFLPs at an amplified locus.

Developments in direct defect detection. Analysis of large multiexonic genes on an exon-by-exon basis becomes increasingly laborious and time-consuming as the number of exons increases. Although the factor IX gene with 8 exons is tractable by present screening methods, the 26 exons of factor VIII stretch resources of time, personnel and laboratory equipment to the limits so that only a few laboratories have taken up the challenge of mutation analysis in haemophilia A. The recent discovery that small amounts of processed mRNA for many if not all genes are present in tissues that do not normally express those genes promises to greatly speed up the analysis of genes whose normal tissue of specific expression is inaccessible.

"Ectopically transcribed" mRNA can be isolated from peripheral blood lymphocytes, reverse transcribed to produce cDNA, and then enzymatically amplified with specific primers. The PCR products representing regions of processed mRNA can then be screened with chemical cleavage mismatch detection, and sequenced to identify mutations (*83*). Preliminary experience with this approach suggests that mutations, including those affecting mRNA processing, can be successfully identified in most cases of moderate or mild haemophilia A and in up to 60% of cases of severe haemophilia A (Giannelli, personal communication).

The speed and power of this method are not in doubt but the degree to which the technology can be transferred from its originator (highly expert) laboratory to other groups remains to be established. Also

the problem of unidentified mutations in severe cases of haemophilia A remains, although a preliminary report from this same group suggests that a defect in the processing of intron 22 from the factor VIII mRNA may be responsible for some of these additional mutations (*84*).

Advances in prenatal diagnostic techniques. Advances in molecular genetics, such as DNA amplification of a single cell by PCR, have stimulated research into the feasibility of genetic diagnosis in the pre-implantation embryo and in fetal cells in the maternal circulation.

(i) *Pre-implantation genetic diagnosis*

The potential advantages of pre-implantation diagnosis are (1) avoidance of repeated terminations in couples at high risk for affected offspring, and (2) correction of the disease by such measures as gene therapy. Possible approaches for pre-implantation diagnosis include biopsy from the polar body, the 6–8-cell embryo at day 3, or the blastocyst at days 5–6.

Polar body biopsy. Demonstration of the affected allele in the sample implies that the primary oocyte carries the normal allele. However, the possibility of recombination should be considered, which is up to 50% for genes near the telomeres. Furthermore, PCR on single cells can fail to produce sufficient DNA for diagnosis in approximately 20% of cases. Of the 83 cases in which this method was attempted (including 28 at risk of haemophilia), only one successful pregnancy was achieved, and in this case diagnosis by CVS demonstrated the fetus to be affected (*85, 86*).

Early cell embryo biopsy. This procedure which involves either the aspiration or herniation of one cell from the 6–8-cell embryo does not result in obvious abnormal development of fetuses in cattle and mice, although, in mice, where like humans, the pre-implantation period is relatively short, the viability of the embryos is reduced. In one human study, 38 embryos underwent biopsy; 30 had two pronuclei (normal), and 8 had either 3 or no pronuclei. In 27 of the 30 normal embryos, morphological development was assessed and 10 (37%) developed into blastocysts; this percent is similar to that of unmanipulated embryos (*87, 88*).

Therefore, it appears that removal of one cell from an eight-cell embryo does not affect embryo development or the success rate of establishing a viable pregnancy. However, as with polar body biopsy, material from one cell is not always sufficient for successful PCR amplification. As an alternative to PCR, fluorescence *in situ* hybridization may be applied. However the latter technique is less suitable for diagnosis of unique sequence DNA mutations.

Blastocyst biopsy. This technique involves slitting the zona and allowing 10–30 cells to extrude. The herniated cells are cut off and can be used for replicate assays. Although the frequency of hatching is apparently not affected by the biopsy, no blastocysts have yet been transferred back to the mother (*89*).

(ii) *Recovery of fetal cells from maternal blood*

The possibility of recovering fetal cells from maternal blood was first raised by Walknowska et al. (*110*) who found male metaphases in the blood of pregnant women. More recently, the presence of fetal cells in the maternal circulation has been confirmed by the combined use of (1) monoclonal antibodies against fetal specific antigens on syncytiotrophoblasts or HLA-A2 antigen-positive lymphocytes from HLA-A2 antigen-negative women or transferrin receptor and glycophorin-A on erythroblasts, (2) flow cytometry, to isolate or enrich fetal cells, and (3) PCR amplification of Y chromosome specific sequences (*90–93*).

Gene therapy—a reality? Haemophilias A and B are considered suitable targets for development of gene therapy on the following grounds:

- The relevant genes have been cloned.
- Tight regulation of expression may not be essential.
- A modest increase in circulating levels of factor VIII or IX would greatly improve the clinical bleeding tendency, e.g., 0% to 10% convert from severe to mild bleeding.
- Haemophilia is a lifelong condition with severe effects on the sufferer.
- Treatment by replacement of deficient factor is not entirely satisfactory, since it involves frequent injections of highly expensive replacement products that are not free of all risks.

However haemophilia is not amongst the very first genetic disorders to be targeted for gene therapy as it is not invariably fatal and the best present treatment is successful in controlling the major clinical symptoms, and in achieving a relatively normal life for sufferers.

The basic concept of gene therapy is straightforward. It is proposed that a normally functioning gene be transferred into somatic cells of the recipient, such that those cells make and continue to make sufficient gene product to correct the inherited defect. In the case of haemophilia A or B, either factor VIII or IX must find its way into the circulation from whichever cell type is chosen for gene transfer, in a correctly modified form, at a rate sufficient to raise the circulating level of clotting factor. The prob-

lem may therefore be conveniently considered under several headings.

(i) *Somatic cell type targeted for gene transfer*

In vivo factor VIII is synthesized by hepatocytes and by some as yet unidentified cell type in the lymphoid system, particularly the spleen. However, ectopic production might be satisfactory, e.g., in haematopoietic cells. Thus, target cells under consideration include endothelial cells, hepatocytes, fibroblasts, myoblasts, and bone marrow stem cells.

(ii) *Vector for DNA transfer*

Since a highly efficient transfer system is essential to deliver DNA to a large number of cells into which the DNA should be stably integrated, retroviruses have attracted most attention. The retrovirus is modified so that essential coding sequences required for packaging and therefore infectivity are replaced by CDNA inserts representing a therapeutic gene.

Packaged virus is obtained using a cell line which contains defective viral DNA producing the deleted packaging proteins but no infectious viral RNA. Vector design is undergoing continuous development at the present time, but a limitation of retrovirus is that only about 7 kb of inserted DNA can be effectively packaged. Therefore a modified factor VIII gene must be used, lacking the B domain, which is dispensable for normal function. The retroviruses' life-cycle leads to stable integration of viral DNA but only into actively replicating cells, which precludes the use of hepatocytes as target host cells. Therefore, alternative vectors have been developed including adenoviruses. The maximum size of foreign gene insert in these viruses is 5 kb. Non-virus-based transfer methods are also being developed.

(iii) *Animal models*

Relevant animal models for stable DNA transfer, integration and expression are needed. As regards haemophilia, dog models of haemophilia A and B are available (*94, 95*), and at least one colony of haemophilic cats is being maintained. No mouse model of haemophilia exists but it is now feasible to produce mouse lines defective in any specified gene sequence by targeted homologous recombination in embryonic stem cells. Subsequent recovery of fertile chimeric adults with the modified genotype present among their gametes is obtained by injecting targeted ES cells into morula-stage embryos. Further breeding produces mice with the targeted gene disrupted in all their cells, provided that the phenotype is compatible with embryonic development. By this means many new strains of mice representing human hereditary defects have been produced.

(iv) *Gene transfer experiments involving factors VIII or IX*

Factor IX. Modified retrovirus containing human factor IX coding sequence has been transferred into hepatoma cell lines, and murine rat and human fibroblasts. The majority of the factor IX protein expressed by these cells was correctly gamma-carboxylated and functional *in vitro*. Canine factor IX has been expressed in dog fibroblasts and bovine endothelial cells. When fibroblasts secreting human factor IX were transplanted into syngeneic mice, human factor IX could be detected in their circulation for two weeks until an immunological response occurred. However, the vector was inactivated by an epigenetic mechanism regardless of the immune response to foreign protein (*96*).

Factor VIII. Functional B-domainless human factor VIII has been secreted from human fibroblasts transfected with a retroviral vector containing B-domain-deleted factor VIII CDNA. When transferred to normal mice these cells could be recovered after two months and shown to still synthesize factor VIII. However, no factor VIII was detected in the recipient mice, probably due to the short half-life of human factor VIII in this animal (*97*).

(v) *General conclusions*

Although considerable progress has been made towards developing gene therapy, at present (September 1992) no satisfactory experiments in animal models have been described. Clearly there is a need to use conspecific factor in a relevant animal model before the success or otherwise of gene therapy can be judged.

The nearest approach has been reached with dog factor IX in dog fibroblasts *in vitro*. Presumably these will be tested in haemophilic dogs in the near future. It is certain that extensive efforts to validate gene therapy in haemophilia will be undertaken over the next 10 to 20 years with some considerable chance of ultimate success. Should such procedures be shown to be safe, efficacious and reasonably cheap, then gene therapy might become the therapy of choice—especially in developing countries where other means of support are too expensive to be widely available. Conversely, if the price of recombinant factor VIII were to fall very considerably and especially if the oral route became feasible, then gene therapy would be less attractive.

Conclusions and recommendations

Recent advances in molecular genetic procedures have resulted in a realization that accurate DNA-based carrier and prenatal diagnosis in haemophilia

can be successfully achieved in many countries. The provision of these accurate methods combined with the availability of professional counselling and obstetric procedures performed by specialists in fetal medicine is seen as the ultimate goal for the effective genetic control of haemophilia. The role of WHO and WFH in encouraging the provision of such facilities was recognized.

Techniques for specific mutation detection are now available, particularly in relation to haemophilia B, and developments in this area should be actively encouraged in order to make such procedures simpler and more applicable to routine analysis. It was however accepted that at present in most cases diagnoses will be based on DNA polymorphism analysis.

Differences between the cultural, social and economic situations in different countries will significantly affect the application of genetic procedures and the ethnic variation in frequency in many of the polymorphic markers will also result in varying efficiency of such procedures. Services for carrier detection and prenatal diagnosis of haemophilia should be established in the places where they will be used. This will take into account local ethnic and other influences.

The participants agreed that the present situation with regard to pre-implantation diagnosis of haemophilia and gene therapy, while offering exciting possibilities, was unlikely to affect significantly the requirements for genetic analysis procedures in the foreseeable future.

Future research into the feasibility of non-invasive fetal testing and gene therapy for haemophilia should be encouraged.

Recommendations

1. There should be a multidisciplinary approach to the prenatal diagnosis of haemophilia involving experts in the fields of fetal medicine, genetic counselling, haemophilia care and molecular genetics.

2. Potential carriers of haemophilia should be tested for clotting factor deficiencies early in childhood to assess the potential for clinical bleeding problems. These same potential haemophilia carriers should subsequently be offered carrier diagnosis after the age of consent and preferably prior to pregnancy.

3. Initial risk assessment for carriership should be based on pedigree analysis. Subsequent carrier testing by phenotypic analysis should include, where practical, the calibration of all reference material used in assays against approved international standards, and the final probability of carriership should be based on the combined likelihoods obtained from pedigree and laboratory analysis.

4. Genotypic analysis offers the most accurate method of carrier detection. In haemophilia, this will most often involve the use of linked polymorphic markers to follow the inheritance of a haemophilic gene within a pedigree. This assessement should initially involve the study of the most informative intragenic markers with reference to the ethnic origin of the family being tested. Where circumstances permit, the genetic diagnosis of haemophilia should be based on the direct identification of the disease-causing mutation in the factor VIII or factor IX gene.

5. DNA should be acquired and stored locally or nationally from all haemophiliacs to facilitate future genetic diagnoses. National Haemophilia Societies should be actively involved in this process.

6. Family members undergoing counselling should be made familiar with the limitations of laboratory testing and thus be able to provide informed consent. The counselling process should be coordinated by a single well-trained individual, who should be familiar with the concepts of haemophilia care and clinical/molecular genetics. The genetic counselling process must also include a critical evaluation of the type of haemophilia in male relatives of the consultand (haemophilia A or B, its severity, CRM status, etc.).

7. All professionals involved in providing genetic services to haemophiliacs should attain appropriate levels of training. In this regard an international registry of potential training centres should be established and maintained by WFH. Some of these centres may be considered, after due process, as WHO Collaborating Centres.

8. WHO/WFH should conduct an international survey to assess the facilities available for prenatal diagnosis of haemophilia, and subsequently an international registry of such facilities should be established.

9. WHO/WFH should support the existing international data bases for specific haemophilia mutations and encourage the development of new technologies to identify such mutations. Where possible, all genetic information should be added to National Haemophilia Registries.

Résumé

Hémophilie: stratégies de dépistage des conductrices et de diagnostic prénatal

En 1977, l'OMS a publié dans le *Bulletin* un mémorandum sur les méthodes de dépistage des conductrices de l'hémophilie. Ce mémorandum

avait été préparé à l'occasion d'une réunion OMS/FMH (Fédération mondiale de l'Hémophilie) qui s'est tenue à Genève en novembre 1976, et constitue une référence en matière de génétique de l'hémophilie. Les analyses examinées étaient basées sur l'évaluation phénotypique qui, à l'époque, était la seule méthode disponible.

Grâce aux récents progrès de la génétique moléculaire, on sait maintenant qu'il est possible, dans de nombreux pays, d'obtenir avec précision, par analyse de l'ADN, un diagnostic de l'état de conductrice de la maladie et un diagnostic prénatal de l'hémophilie. On estime que la possibilité de disposer de ces méthodes, associées à un conseil dispensé par des professionnels qualifiés et à des méthodes obstétricales appliquées par des spécialistes en médecine fœtale, est le but à atteindre si on désire un contrôle génétique efficace de l'hémophilie. Le rôle d'encouragement de l'OMS et de la FMH dans ce domaine a été reconnu.

Il existe maintenant des techniques de dépistage spécifique des mutations, notamment pour l'hémophilie B, et les progrès dans ce domaine doivent être activement encouragés de façon à simplifier ces méthodes et les rendre davantage applicables en routine. Il est toutefois reconnu que, actuellement, dans la plupart des cas les diagnostics seront encore basés sur l'analyse du polymorphisme de l'ADN.

Les différences culturelles, sociales et économiques d'un pays à l'autre modifient de façon sensible l'application des méthodes génétiques, et les variations ethniques de la fréquence des marqueurs du polymorphisme entraîneront également des différences d'efficacité des méthodes d'analyse. Les services de dépistage des conductrices et de diagnostic prénatal de l'hémophilie devront être créés sur les lieux mêmes où ils seront utilisés. On tiendra pour cela compte des facteurs ethniques locaux et d'autres facteurs pertinents.

Les participants sont convenus que la situation actuelle en ce qui concerne le diagnostic de l'hémophilie avant implantation de l'œuf et la thérapie génique, bien qu'offrant des perspectives extrêmement intéressantes, n'est pas assez avancée pour modifier sensiblement les besoins en analyses génétiques dans un avenir prévisible.

Les recherches sur la faisabilité d'un dépistage fœtal non invasif et d'une thérapie génique de l'hémophilie devront être encouragées.

Recommandations

1. Le diagnostic prénatal de l'hémophilie devrait faire l'objet d'une approche multidisciplinaire impliquant des experts en médecine fœtale, conseil génétique, soins aux hémophiles et génétique moléculaire.

2. Les conductrices potentielles de l'hémophilie devront faire l'objet d'un examen des facteurs de coagulation dès l'enfance afin d'évaluer le risque de problèmes hémorragiques d'ordre clinique. Ces mêmes femmes devront par la suite se voir proposer un diagnostic lorsqu'elles auront atteint l'âge du consentement et de préférence avant d'entamer une grossesse.

3. L'évaluation initiale du risque d'être conductrice de la maladie devra être basée sur l'analyse généalogique. Les tests ultérieurs par analyse phénotypique devront comporter, lorsque ces techniques sont disponibles, l'étalonnage de toutes les substances de référence utilisées par rapport aux étalons internationaux approuvés, et la probabilité finale d'être conductrice devra être fondée sur les probabilités groupées obtenues par l'analyse généalogique et par les examens de laboratoire.

4. L'analyse génotypique constitue la méthode la plus exacte de détection de l'état de conductrice. Dans l'hémophilie, cette technique fera souvent appel à des marqueurs liés du polymorphisme afin de suivre la transmission d'un gène de l'hémophilie au sein d'une généalogie. Cette évaluation commencera par l'étude des marqueurs intragéniques les plus informatifs compte tenu de l'origine ethnique de la famille. Lorsque les circonstances le permettent, le diagnostic génétique de l'hémophilie sera basé sur l'identification directe de la mutation provoquant la maladie dans le gène codant pour le facteur VIII ou le facteur IX.

5. On devra se procurer, et conserver à l'échelon local ou national, de l'ADN de tous les hémophiles, afin de faciliter les diagnostics génétiques futurs. Les sociétés nationales de l'hémophilie devront participer activement à ce processus.

6. Les membres de la famille qui reçoivent un conseil génétique devront être informés des limites des analyses de laboratoire et, par conséquent, être en mesure de fournir un consentement éclairé. Le processus de conseil devra être coordonné par un professionnel dûment formé, qui devra être familiarisé avec les principes des soins aux hémophiles et avec la génétique clinique et moléculaire. Le processus de conseil génétique doit également comprendre une évaluation critique du type d'hémophilie chez les sujets de sexe masculin appartenant à la famille du consultant

(hémophilie A ou B, degré de gravité, présence ou absence du CRM, etc.).

7. Tous les spécialistes participant aux activités de conseil génétique aux hémophiles devront avoir un niveau de formation approprié. Pour cela, il conviendra de créer un registre international des centres de formation potentiels, registre qui sera tenu à jour par la FMH. Certains de ces centres pourront, après avoir été dûment évalués, être pressentis comme centres collaborateurs de l'OMS.

8. L'OMS et la FMH devront procéder à une enquête internationale pour évaluer les laboratoires qui pratiquent le diagnostic de l'hémophilie, et par la suite créer un registre international de ces laboratoires.

9. L'OMS et la FMH devront soutenir les bases de données internationales consacrées aux mutations spécifiques de l'hémophilie et encourager le développement de nouvelles technologies permettant d'identifier ces mutations. Si possible, toutes les données génétiques obtenues devront être incorporées dans les registres nationaux de l'hémophilie.

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