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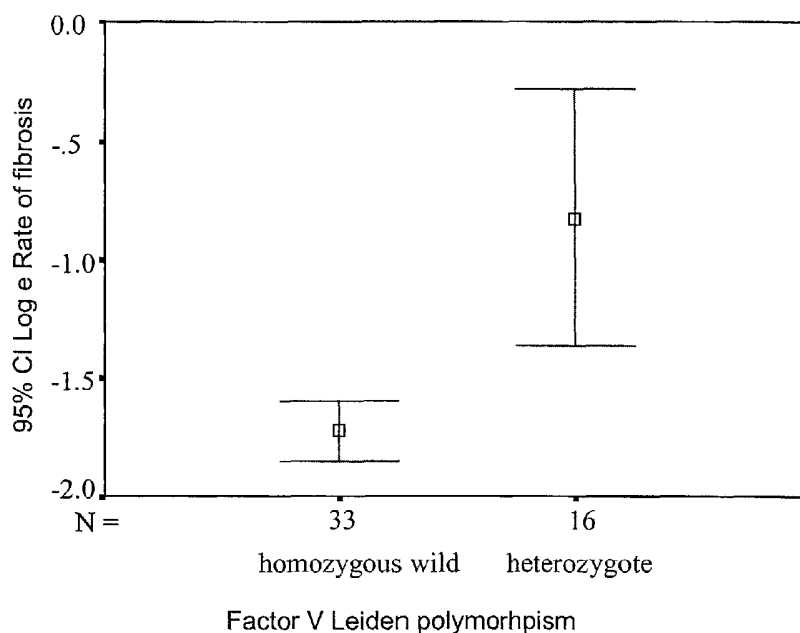
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(54) Title: METHODS



(57) Abstract: A method for assessing a patient's risk of development or progression of liver cirrhosis, comprising the step of determining the patient's genotype or phenotype for a coagulation factor. If the patient's genotype is a genotype associated with a procoagulant tendency or the patient's phenotype is a procoagulant tendency or indicative of a procoagulant tendency, then the patient is considered to be at a higher risk of developing liver cirrhosis or fibrosis and/or of rapid progression of liver cirrhosis or fibrosis. The coagulation factor may be Factor V. Patients with Factor V Leiden may be at a higher risk of developing liver cirrhosis or fibrosis and/or of rapid progression of liver cirrhosis or fibrosis.



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METHODS

The present invention relates to prognostic and treatment methods relating to fibrotic liver disease, including fibrotic liver disease arising from Hepatitis C virus (HCV) infection.

HCV infects 170 million people worldwide, and the majority of the morbidity and mortality associated with infection is due to the development of cirrhosis and its complications. The rate of progression to cirrhosis through the deposition of fibrous tissue varies among individuals. However, only a small proportion of the variability in the rate of fibrosis can be explained by the known demographic and environmental factors (18% in one large study(1)).

There are a number of both general and specific lines of evidence to suggest that host genetic factors play a key role. Host genotype has been demonstrated to determine outcome in a number of infectious diseases e.g. Malaria(2), HBV(3;4), HCV(5). The natural history of HCV has been shown to vary substantially even where age group, gender and viral variables are controlled as demonstrated in the cohort of Irish anti-D infected women(6). Identification of specific genetic factors may assist in prognosis, therapeutic decisions and even therapeutic discovery.

The coagulation pathways may be of importance in liver injury as elsewhere in the body with supporting evidence from carbon tetrachloride induced fibrosis in the rat demonstrating increased fibrin and fibrinogen deposition in the injured liver(7). Thrombin receptors on stellate cells are up regulated during liver damage(8) and Thrombin is also considered to be a stellate cell mitogen(9).

A number of coagulation factor polymorphisms have been shown to have functional effects on the clotting cascade and subsequent risk of thrombosis. For example, an Adenine for Guanine substitution at position 20210 in the 3'untranslated region of the thrombin gene is associated with excess thrombin generation(10) and an increased risk of both arterial and venous thrombosis. Factor V Lieden results from an amino acid substitution at position 506 of Gln for Arg and follows a single nucleotide substitution (Adenine for Guanine at position 1691). The polymorphism confers resistance to activated protein C, part of a negative feed back loop which inhibits activated factor V. Together with activated factor X, factor V converts prothrombin to thrombin. Thus, the polymorphism may lead to increased conversion of prothrombin to thrombin. The factor V Leiden mutation has been shown to increase the risk of venous thromboembolism(11). The Coagulation factor VII Arg353Gln polymorphism has been shown to affect plasma levels, with the highest in Gln/Gln homozygotes (12).

We demonstrate that a procoagulant tendency conferred by coagulation factor polymorphism is linked with more rapid progression towards cirrhosis. It is an object of the invention to provide methods useful in providing diagnoses and prognoses of liver disease, especially liver disease arising from infection with a hepatitis virus, particularly HCV, and for aiding the clinician in the management of liver disease or viral hepatitis or viral infection, for example HCV infection. In particular, an object of the invention is to provide a method of assessing the likely rate of liver fibrosis.

Further objects of the invention include the provision of methods of treatment of liver disease (including any chronic inflammatory disease of the liver which may develop cirrhosis) and infection with a hepatitis virus..

A first aspect of the invention provides a method for assessing a patient's risk of development or progression of liver cirrhosis, comprising the step of determining the patient's genotype or phenotype for a coagulation factor.

If the patient's genotype is a genotype associated with a procoagulant tendency (in particular Factor V Leiden), or the patient's phenotype is a procoagulant tendency or indicative of a procoagulant tendency, then the patient is considered to be at a higher risk of developing liver cirrhosis or fibrosis and/or of rapid progression of liver cirrhosis or fibrosis.

It will be appreciated that determining whether the patient has a procoagulant tendency (by genotype or phenotype assessment) may in itself be predictive of development of clinically significant liver disease, or it may be used by the clinician as an aid in reaching a diagnosis or prognosis.

The method may be used as an adjunct to known assessment or prognostic methods such as histopathological examination of biopsy tissue, or imaging or serum marker assays (Imbert-Bismut F *et al* (2001) Biochemical markers of liver fibrosis in patients with hepatitis C virus infection: a prospective study. *Lancet* **357**(9262):1069-1075; Rosenberg W *et al* (2001) Serum Markers Predict Liver Fibrosis. *British Association for the study of the Liver Meeting 2001, Book of Abstracts*, 23). The method may also be used in conjunction with consideration of other risk factors, for example sex (males may be at higher risk of rapid fibrosis) and age at infection (higher age at infection may be associated with a higher risk of rapid fibrosis).

Fibrosis and rate of fibrosis may be assessed as described in Example 1. For example, the degree of fibrosis may be scored using the modified histological activity index (HAI-Ishak) scoring system (Ishak *et al* (1995) *Hepatology* **22**(6), 696-699), which assigns a score of 0-6 for the degree of fibrosis and a score of 0-18 for necro-inflammatory activity. The rate of fibrosis in a patient may be estimated by assuming that the fibrosis stage at the time of infection is zero and may be expressed in terms of units per year. A rate of fibrosis of more than about 0.3, 0.4 or 0.5 units, still more 0.6, 0.7, 0.8, 0.9, 1 or 2 units per year may be considered to be fast, whilst a rate of fibrosis of less than about 0.25 or (more preferably) 0.2, for example about 0.18, 0.15, 0.13, 0.11, 0.1, 0.09, 0.08, 0.07, 0.06 or 0.05 units per year may be considered to be slow. A rate between about 0.2 or 0.25 and 0.3 units per year may be classified as intermediate rate. About 1/3 of patients may have such an intermediate rate of fibrosis. Alternatively the rate may be expressed in terms of predicted time to cirrhosis (from initiation of liver damage, for example from date of infection with HCV); predicted cirrhosis in less than 20 years may be considered to be fast, whilst predicted not to reach cirrhosis for more than 30 years may be considered to be slow. Cirrhosis corresponds to a score of 6 on the HAI-Ishak scoring system and to a score of 4 using the METAVIR System (Bedossa P, Poynard T. An algorithm for the grading of activity in chronic hepatitis C. The METAVIR Cooperative Study Group. *Hepatology* 1996 Aug; **24**(2):289-93; see also Brunt, EM (2000) Grading and staging the histopathological lesions of chronic hepatitis: the knodell histology activity index and beyond. *Hepatology* **31**, 241-246).

The presence of a procoagulant tendency (in particular presence of Factor V Leiden) indicates that the patient is likely to have a rate of fibrosis of > 0.3

following liver damage, for example caused by HCV infection. However, it will be appreciated that factors other than presence or absence of a procoagulant tendency (for example age and sex) may also influence the likely rate of fibrosis in a particular patient. The presence of a procoagulant tendency (in particular presence of Factor V Leiden) may increase the likely rate of fibrosis (in the absence of the procoagulant tendency) by a factor of between about 1.2 and 4, for example between about 1.3, 1.4 or 1.5 and 2.0, 2.5 and 3.

It will be appreciated that determination of the coagulation factor genotype or phenotype (and optionally other determinants of risk of rapid fibrosis) will be useful to the clinician in determining how to manage the patient's disease (for those with liver disease or infected with a hepatitis virus) or in determining what activities an individual may undertake or should not undertake (for example to reduce the risk of exposure to a hepatitis virus for an individual at risk of rapid fibrosis in view of a procoagulant tendency). For example, since a procoagulant tendency (in particular the presence of Factor V Leiden) is associated with rapid onset or progression of fibrosis, particularly in males and those with a late age of infection, the clinician may use the information concerning the coagulation factor genotype or phenotype to facilitate decision making regarding treatment of the patient. Thus, if the genotype or phenotype is indicative of the absence of a procoagulant tendency (in particular if Factor V Leiden is absent), it may be desirable to delay intervention with antiviral therapy (and thereby avoid adverse side effects and high costs). Similarly, if the genotype or phenotype is indicative of a procoagulant tendency, early antiviral intervention and/or anticoagulant treatment may be the preferred treatment.

It will be appreciated from the foregoing, and from the Examples below, that the determination of the coagulation factor genotype or phenotype (particularly determination of Factor V genotype or phenotype; still more particularly determination of the presence or absence of Factor V Leiden) may be exploited diagnostically to predict whether a given individual is likely to develop clinically significant liver fibrosis or cirrhosis, since the presence of a procoagulant tendency (in particular Factor V Leiden) is believed to correspond to possible future cirrhosis.

The coagulation factor may preferably be a coagulation factor involved in the regulation of thrombin activity, for example involved in (ie upstream of (in the intrinsic or extrinsic clotting pathway) or directly involved in) the cleavage of prothrombin to thrombin; or the inhibition or inactivation of thrombin; or thrombin itself.

It is particularly preferred that the coagulation factor is Factor V. It is still further preferred that the method comprises the step of determining whether the patient has a Factor V genotype or phenotype which confers resistance to activated protein C, for example the Factor V Leiden genotype. Factor V Leiden results from an amino acid substitution at position 506 of Gln for Arg and follows a nucleotide substitution (Adenine for Guanine at position 1691). Factor V Leiden may be detected by genotypic analysis, for example using a PCR-based method (for example as described in Example 1). Activated Protein C resistance (most frequently arising from Factor V Leiden) may be determined by known tests, for example the Activated Protein C (APC) sensitivity ratio, which is a partial thromboplastin time based clotting test in which results are compared in the presence and absence of APC. For example, the Coatest APC Resistance test supplied by Chromogenix, Sweden may be used in assessing APC resistance. Other

suitable assays may be described in *Thrombosis and Haemostasis* (1994) **72**, 880-886; Vogel *et al* (1996) *Blood* **88(10)**, Suppl1, part 1-2, 175a. A ratio of less than 2 may be indicative of APC resistance, though this value may vary slightly depending on the details of the test system used.

Alternatively (though less preferably) the coagulation factor is thrombin (including prothrombin). It is further preferred that the method comprises the step of determining whether the patient has an Adenine for Guanine substitution at position 20210 in the 3'untranslated region of the thrombin gene (ie a mutation associated with excess thrombin generation(10) and an increased risk of both arterial and venous thrombosis).

Alternatively, the coagulation factor may be Factor X, Factor VIII, Factor IX, Factor XI or Factor VII. As further alternatives, the coagulation factor may be Tissue Factor, HMW Kininogen or Prekallikrein. These factors may be involved in the activation of thrombin (including the modulation of such activation).

More preferably, the coagulation factor may be an antithrombin (for example antithrombin III), Protein C, Protein S, Protein C inhibitor, thrombomodulin or α_2 -Macroglobulin. These factors may be involved in the inactivation or inhibition of thrombin (including the modulation of such inactivation or inhibition). Deficiency states of antithrombin III, protein C and S are known and have a genetic basis (Bertina RM. Genetic approach to thrombophilia. *Thromb Haemost* 2001 Jul;**86(1)**:92-103).

PCR or other genetic tests have been used in investigating such deficiencies. Polymorphisms of Alpha 2 Macroglobulin also exist but it is unknown if they are procoagulant. Tests for Protein C and Protein S function are well

known in the art and include tests described in WO 96/42018 and references cited therein; US 5,169,786 and references cited therein; WO 91/01382 and references cited therein. In preferred methods, Protein S is measured using an ELISA kit. Protein C and antithrombin III are measured as activity levels using a chromogenic assays. These are conducted according to standard protocols in clinical haematology labs by those skilled in the art.

Although a significant association between thrombin (Factor II) or Factor VII genotype and rate of fibrosis was not detected in the study described in Example 1, it is considered that this may be due to the low frequency of thrombin and Factor VII procoagulant polymorphisms, which may make it difficult to detect an association (due to the small number of affected patients).

The method may comprise testing the genotype or phenotype of the patient in relation to more than one coagulation factor. For example, the method may comprise testing the patient's genotype in relation to Factor V and thrombin (and optionally also Factor VII). The method may comprise a multi-target PCR amplification step, for example as described in Example 1. The targets may be restricted to coagulation factors for convenience (ie need not include all the targets in the test kit used in Example 1).

In one preferred embodiment of the invention the patient genotype in relation to one or more coagulation factors is determined. Methods of genotypic analysis will be well known to those skilled in the art. The genotype may preferably be determined by testing a sample from the patient. Preferably, the sample contains nucleic acid, such as genomic DNA or mRNA (preferably genomic DNA), and the genotype is determined by a method which involves contacting said nucleic acid (or nucleic acid derived

from it, for example cDNA when the target nucleic acid is an RNA, for example a mRNA) with a nucleic acid which hybridises selectively to said coagulation factor nucleic acid.

For example, genotyping may be performed by any one of a large number of assays, for example Sequencing, RFLP, ARMS PCR, PCR and sequence-specific oligonucleotide hybridisation, SnapShot PCR, Ligase detection reaction, PCR and Maldi-TOF, Pyrosequencing. These and other suitable techniques will be well known to those skilled in the art.

The sample may conveniently be whole blood or genomic DNA extracted from whole blood, for example a 5 ml sample collected into EDTA and extracted using a commercial kit (for example as supplied by Nucleon II, Scotlabs, UK).

By “selectively hybridising” is meant that the nucleic acid has sufficient nucleotide sequence similarity with the said (human) nucleic acid that it can hybridise under moderately or highly stringent conditions. As is well known in the art, the stringency of nucleic acid hybridization depends on factors such as length of nucleic acid over which hybridisation occurs, degree of identity of the hybridizing sequences and on factors such as temperature, ionic strength and CG or AT content of the sequence. Thus, any nucleic acid which is capable of selectively hybridising as said is useful in the practice of the invention.

Nucleic acids which can selectively hybridise to the said (human) nucleic acid include nucleic acids which have >95% sequence identity, preferably those with >98%, more preferably those with >99% sequence identity, over at least a portion of the nucleic acid with the said human nucleic acid. As is

well known, human genes usually contain introns such that, for example, a mRNA or cDNA derived from a gene would not match perfectly along its entire length with the said human genomic DNA but would nevertheless be a nucleic acid capable of selectively hybridising to the said human DNA. Thus, the invention specifically includes nucleic acids which selectively hybridise to said coagulation factor mRNA or cDNA but may not hybridise to a said coagulation factor gene (and *vice versa*). For example, nucleic acids which span the intron-exon boundaries of the said coagulation factor gene may not be able to selectively hybridise to the said coagulation factor mRNA or cDNA.

Typical moderately or highly stringent hybridisation conditions which lead to selective hybridisation are known in the art, for example those described in *Molecular Cloning, a laboratory manual*, 2nd edition, Sambrook *et al* (eds), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA, incorporated herein by reference.

An example of a typical hybridisation solution when a nucleic acid is immobilised on a nylon membrane and the probe nucleic acid is ~ 500 bases or base pairs is:

6 x SSC (saline Na⁺ citrate)

0.5% Na⁺ dodecyl sulphate (SDS)

100 µg/ml denatured, fragmented salmon sperm DNA

The hybridisation is performed at 68°C. The nylon membrane, with the nucleic acid immobilised, may be washed at 68°C in 1 x SSC or, for high stringency, 0.1 x SSC.

20 x SSC may be prepared in the following way. Dissolve 175.3 g of NaCl and 88.2 g of Na⁺ citrate in 800 ml of H₂O. Adjust the pH to 7.0 with a few drops of a 10 N solution of NaOH. Adjust the volume to 1 litre with H₂O. Dispense into aliquots. Sterilize by autoclaving.

An example of a typical hybridisation solution when a nucleic acid is immobilised on a nylon membrane and the probe is an oligonucleotide of between 15 and 50 bases is:

3.0 M trimethylammonium chloride (TMACl)

0.01 M Na⁺ phosphate (pH 6.8)

1 mM EDTA (pH 7.6)

0.5% SDS

100 µg/ml denatured, fragmented salmon sperm DNA

0.1% nonfat dried milk

The optimal temperature for hybridization is usually chosen to be 5°C below the T_i for the given chain length. T_i is the irreversible melting temperature of the hybrid formed between the probe and its target sequence. Jacobs *et al* (1988) *Nucl. Acids Res.* **16**, 4637 discusses the determination of T_is. The recommended hybridization temperature for 17-mers in 3 M TMACl is 48-50°C; for 19-mers, it is 55-57°C; and for 20-mers, it is 58-66°C.

By “nucleic acid which selectively hybridises” is also included nucleic acids which will amplify DNA from the said coagulation factor genomic DNA or mRNA by any of the well known amplification systems such as those described in more detail below, in particular the polymerase chain reaction

(PCR). Suitable conditions for PCR amplification include amplification in a suitable 1 x amplification buffer:

10 x amplification buffer is 500 mM KCl; 100 mM Tris.Cl (pH 8.3 at room temperature); 15 mM MgCl₂; 0.1% gelatin.

A suitable denaturing agent or procedure (such as heating to 95°C) is used in order to separate the strands of double-stranded DNA.

Suitably, the annealing part of the amplification is between 37°C and 60°C, preferably 50°C.

Although the nucleic acid which is useful in the methods of the invention may be RNA or DNA, DNA is preferred. Although the nucleic acid which is useful in the methods of the invention may be double-stranded or single-stranded, single-stranded nucleic acid is preferred under some circumstances such as in nucleic acid amplification reactions.

The nucleic acid which is useful in the methods of the invention may be any suitable size. However, for certain diagnostic, probing or amplifying purposes, it is preferred if the nucleic acid has fewer than 10 000, more preferably fewer than 1000, more preferably still from 10 to 100, and in further preference from 15 to 30 base pairs (if the nucleic acid is double-stranded) or bases (if the nucleic acid is single stranded). As is described more fully below, single-stranded DNA primers, suitable for use in a polymerase chain reaction, are particularly preferred.

The nucleic acid for use in the methods of the invention is a nucleic acid capable of hybridising to the said coagulation factor genomic DNA or

mRNAs. Fragments of the said coagulation factor genes and cDNAs derivable from the mRNA encoded by the said coagulation factor genes are also preferred nucleic acids for use in the methods of the invention.

It is particularly preferred if the nucleic acid for use in the methods of the invention is an oligonucleotide primer which can be used to amplify a portion of the said coagulation factor (for example Factor V or Factor V Leiden) nucleic acid, particularly said coagulation factor genomic DNA (for example Factor V or Factor V Leiden genomic DNA).

Nucleic acids for use in the invention may hybridise to more than one allele of a coagulation factor or may hybridise only to one allele. Single-allele-specific and multi-allele-specific nucleic acids may be used in combination, for example in an amplification reaction. Preferred nucleic acids for use in the invention are those that selectively hybridise to a particular allele (ie a particular polymorphism) and do not hybridise to other alleles (polymorphisms) of the coagulation factor. Such selectively hybridising nucleic acids can be readily obtained, for example, by reference to whether or not they hybridise to the said coagulation factor allele (preferably genomic) nucleic acid and not to other allele (preferably genomic) nucleic acid.

Details of suitable primers are given in references in Example 1, for example Cheng *et al* (1999) *Genome Res* 9, 936-949. Methods and nucleic acids as described, for example, in Example 1, may be used.

Details of further polymorphisms (for example associated with Protein C and Protein S) which may be associated with a procoagulant tendency, and for which it may therefore be useful to determine the patient's genotype, are

indicated in, for example, Seligsohn & Lubetsky (2001) *New Engl J Med* **344(16)**, 1222-1231 and references cited therein. Suitable methods and nucleic acids for assessing such polymorphisms (if not already described in such references) may be designed or selected by methods well known to those skilled in the art, for example as indicated above and discussed further below.

The methods are suitable in respect of any fibrosing liver disease (which term excludes thrombotic conditions such as Budd Chiari syndrome and Portal vein thrombosis) but it is preferred if the patient has or is at risk of hepatitis virus infection, particularly HCV infection.

It is preferred that the patient is a patient with or at risk of viral hepatitis, for example with or at risk of infection with Hepatitis A virus (HAV), Hepatitis B virus (HBV) or Hepatitis C virus (HCV). Most preferably, the patient is infected with, or at risk of infection by, HCV. Infection may be judged by the presence of antibodies to the relevant virus and preferably also by the presence of RNA from the relevant virus. This may be determined by methods well known to those skilled in the art, and as noted in Example 1. The patient may have acute HCV infection or may have or be at risk of chronic HCV infection.

A patient may be at risk of viral hepatitis due to occupational exposure or an intravenous drug habit. The method may be useful in identifying persons whose exposure to hepatitis virus should be minimised due to a higher risk of rapid disease progression should infection occur.

It will be appreciated that the aforementioned methods may be used for presymptomatic screening of a patient who is in a risk group for liver

disease, particularly viral hepatitis, for example HCV infection. For example, healthcare workers may be at a greater risk of HCV infection (for example as a consequence of needle stick injuries) than other occupational groups. Intravenous drug users may also be at greater risk.

If testing coagulation factor phenotype (for example using a clotting assay as indicated above) it is preferred that the sample tested is blood or is derived from blood from the patient.

Although any sample containing nucleic acid derived from the patient is useful in the methods of the invention when determining genotype, it is preferred if the sample is readily obtainable from the patient, for example blood, urine, semen or skin cells (for example a sample of cells from the buccal cavity). Liver tissue taken at biopsy may be used. Most conveniently the sample is blood. Although it is preferred that the sample containing nucleic acid from the patient is, or is derived directly from, a cell of the patient, a sample indirectly derived from a patient, such as a cell grown in culture, is also included within the invention. Equally, although the nucleic acid derived from the patient may have been physically within the patient, it may alternatively have been copied from nucleic acid which was physically within the patient.

Conveniently, the nucleic acid capable of selectively hybridising to the said human nucleic acid such as genomic DNA and which is used in the methods of the invention may further comprise a detectable label.

By "detectable label" is included any convenient radioactive label such as ^{32}P , ^{33}P or ^{35}S which can readily be incorporated into a nucleic acid molecule using well known methods; any convenient fluorescent or

chemiluminescent label which can readily be incorporated into a nucleic acid is also included. In addition the term “detectable label” also includes a moiety which can be detected by virtue of binding to another moiety (such as biotin which can be detected by binding to streptavidin); and a moiety, such as an enzyme, which can be detected by virtue of its ability to convert a colourless compound into a coloured compound, or *vice versa* (for example, alkaline phosphatase can convert colourless *o*-nitrophenylphosphate into coloured *o*-nitrophenol). Conveniently, the nucleic acid probe may occupy a certain position in a fixed array and whether the nucleic acid hybridises to the said coagulation factor nucleic acid can be determined by reference to the position of hybridisation in the fixed array.

Primers which are suitable for use in a polymerase chain reaction (PCR; Saiki *et al* (1988) *Science* **239**, 487-491) are preferred. Suitable PCR primers may have the following properties:

It is well known that the sequence at the 5' end of the oligonucleotide need not match the target sequence to be amplified.

It is usual that the PCR primers do not contain any complementary structures with each other longer than 2 bases, especially at their 3' ends, as this feature may promote the formation of an artifactual product called “primer dimer”. When the 3' ends of the two primers hybridize, they form a “primed template” complex, and primer extension results in a short duplex product called “primer dimer”.

Internal secondary structure should be avoided in primers. For symmetric PCR, a 40-60% G+C content is often recommended for both primers, with

no long stretches of any one base. The classical melting temperature calculations used in conjunction with DNA probe hybridization studies often predict that a given primer should anneal at a specific temperature or that the 72°C extension temperature will dissociate the primer/template hybrid prematurely. In practice, the hybrids are more effective in the PCR process than generally predicted by simple T_m calculations.

Optimum annealing temperatures may be determined empirically and may be higher than predicted. *Taq* DNA polymerase does have activity in the 37-55°C region, so primer extension will occur during the annealing step and the hybrid will be stabilized. The concentrations of the primers are equal in conventional (symmetric) PCR and, typically, within 0.1- to 1µM range.

Any of the nucleic acid amplification protocols can be used in the method of the invention including the polymerase chain reaction, QB replicase and ligase chain reaction. Also, NASBA (nucleic acid sequence based amplification), also called 3SR, can be used as described in Compton (1991) *Nature* **350**, 91-92 and *AIDS* (1993), Vol 7 (Suppl 2), S108 or SDA (strand displacement amplification) can be used as described in Walker *et al* (1992) *Nucl. Acids Res.* **20**, 1691-1696. The polymerase chain reaction is particularly preferred because of its simplicity.

When a pair of suitable nucleic acids of the invention are used in a PCR it is possible to detect the product by gel electrophoresis and ethidium bromide staining. As an alternative to detecting the product of DNA amplification using agarose gel electrophoresis and ethidium bromide staining of the DNA, it is convenient to use a labelled oligonucleotide capable of hybridising to the amplified DNA as a probe. When the amplification is by

a PCR the oligonucleotide probe hybridises to the interprimer sequence as defined by the two primers. The oligonucleotide probe is preferably between 10 and 50 nucleotides long, more preferably between 15 and 30 nucleotides long. The probe may be labelled with a radionuclide such as ^{32}P , ^{33}P and ^{35}S using standard techniques, or may be labelled with a fluorescent dye. When the oligonucleotide probe is fluorescently labelled, the amplified DNA product may be detected in solution (see for example Balaguer *et al* (1991) "Quantification of DNA sequences obtained by polymerase chain reaction using a bioluminescence adsorbent" *Anal. Biochem.* **195**, 105-110 and Dilesare *et al* (1993) "A high-sensitivity electrochemiluminescence-based detection system for automated PCR product quantitation" *BioTechniques* **15**, 152-157).

PCR products can also be detected using a probe which may have a fluorophore-quencher pair or may be attached to a solid support or may have a biotin tag or they may be detected using a combination of a capture probe and a detector probe.

Fluorophore-quencher pairs are particularly suited to quantitative measurements of PCR reactions (eg RT-PCR). Fluorescence polarisation using a suitable probe may also be used to detect PCR products.

In a further preferred embodiment, the level or activity of a coagulation factor protein is measured. Preferably, the level of said protein is measured by contacting the protein with a molecule which selectively binds to said coagulation factor, or other known technique for quantifying the said coagulation factor. Manuals relating to assays for coagulation factors are well known to those skilled in the art, and include "Dacie and Lewis's

Practical Haematology”, 9th Edition (2001), Churchill Livingstone, Dacie, Lewis Eds.

The sample containing protein derived from the patient is conveniently blood.

It is particularly preferred if the molecule which selectively binds to the coagulation factor is an antibody. Antibodies which can selectively bind to a particular coagulation factor can be made using techniques well known to those skilled in the art, or may be obtained from, for example, Sigma (Sigma-Aldrich Company Limited, Fancy Road, Poole, Dorset, BH12 4QH, UK; see “Antibodies and Reagents for Coagulation Research section of catalogue).

The antibodies may be monoclonal or polyclonal. Suitable monoclonal antibodies may be prepared by known techniques, for example those disclosed in “Monoclonal Antibodies: A manual of techniques”, H Zola (CRC Press, 1988) and in “Monoclonal Hybridoma Antibodies: Techniques and applications”, J G R Hurrell (CRC Press, 1982), both of which are incorporated herein by reference.

The level of a coagulation factor which is indicative of a procoagulant tendency may be defined as the increased level (for procoagulant factors) or decreased level (for inhibitors of coagulation) present in known procoagulant patients over individuals with normal coagulation. The level may be, for example, at least 1.5 Standard deviations [SD] higher (or lower, for inhibitors of coagulation) in patients with a procoagulant tendency, or it may be at least 2 SD or 3 SD higher (or lower, as appropriate) when compared with a population of individuals with normal coagulation.

By "the relative amount of said coagulation factor protein" is meant the amount of said coagulation factor protein per unit mass (or volume) of sample tissue or per unit number of sample cells compared to the amount of said coagulation factor protein per unit mass of known normal tissue or per unit number of normal cells. The relative amount may be determined using any suitable protein quantitation method. In particular, it is preferred if antibodies are used and that the amount of said coagulation factor protein is determined using methods which include quantitative western blotting, enzyme-linked immunosorbent assays (ELISA) or quantitative immunohistochemistry. As noted above, it may be more convenient to quantify/determine some coagulation factors by activity rather than protein level.

It will be appreciated that other antibody-like molecules may be used in the method of the inventions including, for example, antibody fragments or derivatives which retain their antigen-binding sites, synthetic antibody-like molecules such as single-chain Fv fragments (ScFv) and domain antibodies (dAbs), and other molecules with antibody-like antigen binding motifs.

A further aspect of the invention provides the use of an agent which is capable of use in determining the genotype or phenotype of a patient for a coagulation factor (for example for characterising the patient's nucleic acid or coagulation process) in the manufacture of a reagent for assessing the patient's risk of development or progression of liver cirrhosis, for example following HCV infection. The agent may suitably be a nucleic acid which selectively hybridises to a coagulation factor nucleic acid (preferably Factor V nucleic acid, for example Factor V Leiden nucleic acid) or the agent may be a molecule which selectively binds to a coagulation factor protein or the agent may be an agent useful in selectively assaying the activity of the

coagulation factor or all or part of the patient's coagulation process. For example, reagents useful in measuring the APC sensitivity ratio test (partial thromboplastin time based clotting test) include platelet poor plasma, kaolin 5g/l in barbitone buffered saline pH 7.4, phospholipid, CaCl₂ and APC.

The agents as defined are therefore useful in a method of prognosing liver fibrosis or cirrhosis, particularly arising from HCV (or other hepatitis virus) infection.

A further aspect of the invention comprises a kit of parts useful for prognosis of liver fibrosis or cirrhosis, especially arising from HCV infection, comprising means for use in determining the genotype or phenotype of a patient for a coagulation factor (for example for characterising the patient's nucleic acid or coagulation process). The means may be or comprise an agent which may suitably be a nucleic acid which selectively hybridises to a coagulation factor nucleic acid, for example genomic DNA (preferably Factor V nucleic acid, for example Factor V Leiden nucleic acid). The agent may be a molecule which selectively binds to a coagulation factor protein or the agent may be an agent useful in selectively assaying the activity of the coagulation factor or all or part of the patient's coagulation process, for example as noted above.

Preferably, the kit further comprises a control sample containing coagulation factor nucleic acid or protein wherein the control sample may be a negative control (which contains a coagulation factor nucleic acid or protein which is not associated with a procoagulant tendency) or it may be a positive control (which contains a coagulation factor nucleic acid or protein which is associated with a procoagulant tendency). The kit may contain both negative and positive controls. The kit may usefully contain controls

of coagulation factor protein or nucleic acid which correspond to different amounts such that a calibration curve may be made.

The kit further comprises means suitable for use in determining whether the patient has been exposed to or infected with a hepatitis virus (preferably HCV) (for example by checking for antibodies to the virus) and/or whether the patient has continuing infection (for example by checking for viral RNA). Thus, the kit may preferably comprise (as an example) nucleic acids suitable for use as PCR primers for amplifying a hepatitis virus nucleic acid (for example following reverse transcription of viral RNA), preferably HCV nucleic acid.

A further aspect of the invention provides a method for treating a patient with or at risk of liver fibrosis or cirrhosis comprising the step of administering to the patient an anticoagulant agent.

A further aspect of the invention provides an anticoagulant agent for use in the treatment of a patient with or at risk of liver fibrosis or cirrhosis. A still further aspect of the invention provides an anticoagulant agent for use in the treatment of a patient with or at risk of hepatitis virus infection (for example HBV, HAV or HCV, preferably HCV). A further aspect of the invention provides the use of an anticoagulant in the manufacture of a medicament for the treatment of a patient with or at risk of liver fibrosis or cirrhosis, or with or at risk of hepatitis virus infection (for example HBV, HAV or HCV, preferably HCV).

A further aspect of the invention provides a method for treating a patient with or at risk of hepatitis virus infection (for example HBV, HAV or HCV,

preferably HCV) comprising the step of administering to the patient an anticoagulant agent. A further aspect of the invention provides an anticoagulant agent for use in the treatment of a patient with or at risk of hepatitis virus infection (for example HBV, HAV or HCV, preferably HCV).

A still further aspect of the invention provides a method for treating a patient with or at risk of liver fibrosis or cirrhosis, comprising the steps of (1) assessing the patient's risk of development or progression of liver cirrhosis, using a method comprising the step of determining the patient's genotype or phenotype for a coagulation factor; (2) if the patient's risk of development or progression of liver cirrhosis is high (ie the patient is at risk of rapid fibrosis), administering to the patient an anticoagulant agent and/or other therapeutic agent suitable for treating liver fibrosis. The therapeutic agent may be, for example, a steroid in autoimmune hepatitis or ursodeoxycholic acid in primary biliary cirrhosis.

A further aspect of the invention provides an anticoagulant agent and/or other therapeutic agent suitable for treating liver fibrosis for treatment of a patient with or at risk of liver fibrosis or cirrhosis, wherein the patient is one whose risk of development or progression of liver cirrhosis has been assessed using a method comprising the step of determining the patient's genotype or phenotype for a coagulation factor; and the patient's determined risk of development or progression of liver cirrhosis is high (ie whose predicted rate of fibrosis is rapid).

A still further aspect of the invention provides a method for treating a patient with or at risk of hepatitis virus infection (for example HBV, HAV or HCV, preferably HCV), comprising the steps of (1) assessing the

patient's risk of development or progression of liver cirrhosis, comprising the step of determining the patient's genotype or phenotype for a coagulation factor; (2) if the patient's risk of development or progression of liver cirrhosis is high (ie the patient is at risk of rapid fibrosis), administering to the patient an anticoagulant agent and/or other therapeutic agent suitable for treating viral hepatitis, for example an antiviral agent, for example α -interferon.

A further aspect of the invention provides an anticoagulant agent and/or other therapeutic agent suitable for treating viral hepatitis, for example an antiviral agent, for example α -interferon, for treatment of a patient with or at risk of hepatitis virus infection (for example HBV, HAV or HCV, preferably HCV), wherein the patient is one whose risk of development or progression of liver cirrhosis has been assessed, using a method comprising the step of determining the patient's genotype or phenotype for a coagulation factor; and the patient's determined risk of development or progression of liver cirrhosis is high (ie whose predicted rate of fibrosis is rapid).

In particular, the invention provides a method for treating a patient with or at risk of hepatitis virus infection (for example HBV, HAV or HCV, preferably HCV), comprising the steps of (1) determining the patient's genotype or phenotype for Factor V; (2) if the patient has Factor V Leiden, administering to the patient an anticoagulant agent and/or other therapeutic agent suitable for treating viral hepatitis, for example an antiviral agent, for example α -interferon.

A further aspect of the invention provides an anticoagulant agent and/or other therapeutic agent suitable for treating viral hepatitis, for example an

antiviral agent, for example α -interferon, for treatment of a patient with or at risk of hepatitis virus infection (for example HBV, HAV or HCV, preferably HCV), wherein the patient has Factor V Leiden.

The term "anticoagulant" will be well known to those skilled in the art and includes agents (or their prodrugs or precursors) which have the effect of reducing clotting tendency or extending the time taken for clotting to occur in response to an initiating event.

Particularly useful anticoagulants may be those useful in the treatment of patients with Factor V Leiden (homozygous or heterozygous) and thrombosis. These include (often after initial treatment with heparins) oral vitamin K antagonists (for example Warfarin, phenindione and acenocoumarol/nicoumalone).

Other particularly preferred anticoagulants are considered to include direct thrombin inhibitors (or their precursors), for example Hirudin, Bivalirudin, melagatran and napsagatran. Activated protein C (which has been administered therapeutically in meningococcal sepsis), or modified forms with longer half-lives and greater activity may also be useful.

It will be appreciated that it may be necessary to monitor the patient's response to the anticoagulant and make suitable adjustments to the dose of anticoagulant in order to avoid excessive bleeding. Methods suitable for monitoring different anticoagulants (where needed) are well known to those skilled in the art. For example, Hirudin and other direct thrombin inhibitors may be monitored using the Ecarin Clotting Time (ECT) test.

Other preferred agents suitable for treating viral hepatitis (and therefore suitable for use in relation to the present invention) may include agents discussed in the following patent applications (all incorporated herein by reference): PCT/GB01/03901 (relating to agents useful in inhibiting HCV replication in the CNS); PCT/GB01/04636 (relating to HCV prophylactic and therapeutic vaccines) and PCT/GB01/05722 (relating to recombinant bone marrow derived stem cells or cells derived therefrom capable of expressing therapeutic products).

In a particularly preferred embodiment, a patient with a procoagulant allele or polymorphism may be treated using recombinant bone marrow derived stem cells or cells derived therefrom (as described in PCT/GB01/05722) capable of expressing (for example when differentiated into liver cells) a "normal" (non-procoagulant) allele. For example, a patient with Factor V Leiden may be treated using recombinant cells capable of expressing (for example when differentiated into liver cells) a normal Factor V (ie not Factor V Leiden).

Thus, a further aspect of the invention provides a bone-marrow derived stem cell comprising a recombinant nucleic acid capable of expressing, in the bone-marrow derived stem cell or cell derived therefrom, normal Factor V. A still further aspect of the invention provides a recombinant polynucleotide suitable for expressing in a bone-marrow derived stem cell or cell derived therefrom normal Factor V. Preferably the recombinant polynucleotide is suitable for expressing the normal Factor V under control of a liver-specific promoter and/or an inducible promoter.

A further aspect of the invention provides a recombinant polynucleotide construct (chimaeroplasty construct) comprising RNA and DNA elements

suitable for correcting a Factor V Leiden genotype to a normal Factor V genotype in a bone-marrow derived stem cell or cell derived therefrom. Chimaeric RNA/DNA polynucleotide constructs and their use in gene therapy are described in, for example, Lai & Lien (2001) *Expert Opin Biol Ther* 1(1), 41-47 and Lai & Lien (1999) *Exp Nephrol* 7(1), 11-14. Chimaeric constructs have been shown to be useful in correcting point mutations in several genetic disease models, without using viral vectors. Chimaeric constructs may be used to correct the Factor V genotype of bone-marrow derived stem cells (or their progeny) obtained from the patient, and the corrected cells (or their progeny) returned to the patient.

Further aspects of the invention provide a gene therapy construct comprising such a recombinant polynucleotide. The construct may comprise a Moloney Leukaemia Virus (MLV) based retroviral vector or a lentiviral vector or adeno-associated vector (AAV), or a baculovirus based vector. Pieroni *et al* (2001) *Hum Gene Ther* 12(8), 871-881 and Pieroni & La Monica (2001) *Curr Opin Mol Ther* 3(5), 464-467 discuss the use of baculovirus vectors as gene therapy vectors. Baculovirus vectors may have the advantages that they do not replicate in mammalian cells, do not cause a cytopathic effect upon infection and are able to carry large DNA inserts. Although baculovirus vectors may not be suited to *in vivo* gene delivery by systemic administration, due to rapid removal by the complement system, they may be used in *ex-vivo* systems, in which cells are removed from the patient, treated with the virus and then returned to the patient (or progeny of the patient-derived cells treated or returned to the patient).

A further aspect of the invention provides a bone-marrow derived stem cell, recombinant polynucleotide or gene therapy construct according to the invention for use in medicine. A further aspect of the invention provides a

bone-marrow derived stem cell, recombinant polynucleotide or gene therapy construct according to the invention for use in treating a patient with or at risk of liver fibrosis and/or with or at risk of hepatitis virus infection (for example HBV, HAV or HCV, preferably HCV). The patient may be selected on the basis of coagulation factor genotype or phenotype; preferably the patient has Factor V Leiden.

A further aspect of the invention provides a pharmaceutical preparation comprising a bone-marrow derived stem cell, recombinant polynucleotide or gene therapy construct of the invention and a pharmaceutically acceptable carrier. The carrier(s) must be "acceptable" in the sense of being compatible with the bone-marrow derived stem cell, recombinant polynucleotide or gene therapy construct and not deleterious to the recipients thereof. Typically, the carriers will be water or saline which will be sterile and pyrogen free.

A further aspect of the invention provides a method for treating a patient with or at risk of liver fibrosis and/or with or at risk of hepatitis virus infection (for example HBV, HAV or HCV, preferably HCV) comprising the steps of (1) providing a bone-marrow derived stem cell according to the invention, or a recombinant nucleic acid of the invention (2) introducing the stem cell or recombinant nucleic acid into the patient. The bone-marrow derived stem cell may be provided by a method comprising the steps of (1) obtaining a bone-marrow derived stem cell from the patient, (2) introducing a recombinant nucleic acid encoding normal Factor V (ie not Factor V Leiden) into the bone-marrow derived stem cell. It is strongly preferred that the bone-marrow derived stem cells are derived from the patient being treated, but this not essential.

The recombinant nucleic acid may comprise an inducible promoter controlling expression of the Factor V and the method may comprise the step of administering to the patient a molecule that regulates expression from the inducible promoter.

The recombinant nucleic acid may further comprise an inducible promoter controlling expression of a cytotoxic product and the method may comprise the step of administering to the patient a molecule (cytotoxic inducer) that regulates expression from the inducible promoter controlling expression of the cytotoxic product.

It may be desirable to administer to the patient a composition (mobilising composition) that is capable of promoting mobilisation of bone-marrow derived stem cells into the peripheral circulation. It may further useful to administer to the patient a composition (colonisation composition) that is capable of promoting the colonisation of a tissue by, and/or differentiation of, and/or replication of bone-marrow derived stem cells or cells derived therefrom. However, because a relatively small number of cells may be able to produce sufficient normal Factor V to overcome the Factor V Leiden procoagulant tendency, it may not be necessary to use a mobilising and/or colonisation composition. The mobilising composition may comprise G-CSF (Granulocyte-colony stimulating factor). The colonisation composition may comprise T3, HGF and/or KGF.

A further aspect of the invention provides a kit of parts or composition comprising (1) (a) a bone-marrow derived stem cell of the invention or (b) a recombinant polynucleotide or gene therapy vector of the invention and an agent useful in promoting transfection of bone-marrow derived stem cells,

and (2) a mobilising composition or colonising composition as defined above and/or means for testing whether the patient has Factor V Leiden.

Further preferences and information regarding such a bone-marrow derived stem cell and constructs are indicated in PCT/GB01/05722.

A further aspect of the invention provides a method for identifying a compound for treating a patient with or at risk of liver fibrosis and/or hepatitis virus infection, the method comprising contacting a test compound with Factor V or Factor V Leiden (which term includes activated (thrombin-cleaved) Factor V or Factor V Leiden); determining whether said compound is capable of modulating the activity of Factor V or Factor V Leiden; and selecting a compound which has an activity which has an anticoagulant effect. For example, the compound may inhibit activated Factor V Leiden, or may render activated Factor V Leiden susceptible to inhibition by activated Protein C.

The method may comprise a step of contacting a test compound with Factor V or Factor V Leiden and determining whether the compound is capable of binding to Factor V or Factor V Leiden; compounds which are capable of binding may then be tested for modulation of the activity of Factor V or Factor V Leiden.

A further aspect of the invention provides a compound identified by the screening method of the invention for use in medicine. A further aspect of the invention provides such a compound for use in treating a patient with or at risk of liver fibrosis and/or with or at risk of hepatitis virus infection (for example HBV, HAV or HCV, preferably HCV). A further aspect of the invention provides a method for treating a patient with or at risk of liver

fibrosis and/or with or at risk of hepatitis virus infection (for example HBV, HAV or HCV, preferably HCV), comprising the step of administering to the patient such a compound. The patient may be selected for treatment on the basis of coagulation factor genotype or phenotype as described above; preferably the patient has Factor V Leiden.

All documents referred to herein are hereby incorporated by reference.

The invention will now be described by reference to the following, non-limiting Examples and Figures.

Figure 1: 95% confidence intervals for Log_e Rate of fibrosis by Factor V Leiden genotype. ANOVA $F(1,351)=8.63$, $p=0.004$.

Figure 2: The coagulation cascade

Figure 3: Role of Factor V

Figure 4: Potential mechanisms by which Factor V Leiden may lead to more rapid fibrosis

Example 1: Factor V Leiden polymorphism and the rate of fibrosis development in chronic hepatitis C virus infection.

The rate of progression to cirrhosis varies widely among individuals chronically infected with Hepatitis C Virus (HCV). Rapid progression may be considered as a specific phenotype and genetically as a complex trait, determined by a combination of host genetic, viral and environmental factors. We tested whether polymorphisms of the coagulation factors II, V

and VII might affect the rate of progression of HCV infected people to cirrhosis.

Disease association studies were performed to test for a relationship between possession of clotting factor polymorphisms and rate of fibrosis. The rate of fibrosis was calculated by dividing the number of fibrosis units (as graded by the Ishak modification of the Knodell scoring system) by the duration of infection. Genotyping was performed using reverse line blot hybridisation. A group of UK white Caucasian patients and a 2nd mainland European white Caucasian group were analysed first separately, and then as a pooled sample.

A significant association was seen in the pooled sample between rate of fibrosis and factor V Leiden genotype Arg560Gln (ANOVA $F(1,351)=8.63$, $p=0.004$, Fishers exact test $p=0.021$, odds Ratio=4 for fast vs. slow). This association was strongest in UK patients (ANOVA $F(1,153)=6.57$, $p=0.011$, Fishers exact test $p=0.03$ Odds Ratio=7.5 for fast vs. slow) with a trend in the main land European patients (Fishers exact test $p=0.278$, Odds Ratio=3, ANOVA $F(1,196)=3.6$, $p=0.058$). No associations were seen between factor II or factor VII genotypes and rate of fibrosis. However, the low incidence of mutant factor II or factor VII may mean that the number of affected individuals is insufficient for an association to be seen.

Possession of the Factor V Leiden polymorphism significantly increases the risk of rapid disease progression in HCV. This association suggests a role for the coagulation system in the pathogenesis of fibrotic liver disease.

Methods

Patients

Patients with chronic hepatitis C virus infection seen at St Mary's Hospital, London who had had at least one liver biopsy as part of routine clinical practice between 1st January 1990 and 30th June 2001 were identified. All patients included in this study were Caucasians known to be HCV antibody positive and all were known to be RNA positive. Patients were excluded if they had been given antiviral therapy prior to biopsy, if they had hepatocellular carcinoma, or evidence of other types of liver disease in addition to their hepatitis C, HIV co-infection or a non-interpretable biopsy. Data was collected with regard to patient demographic details (gender, date of birth, age at infection, alcohol intake, risk factor, ethnic origin), histological parameters and viral genotype. Date of infection was documented; in persons infected by blood products the date of transfusion was recorded. When the exact date was not known, but the year was, the estimated date of infection was recorded as the middle of that year. In persons infected by intravenous drug use the date of infection was estimated as the middle of the year of 1st drug use as elsewhere (1). A second cohort of patients collected as part of the HENCORE collaboration was also used in this study and has been described elsewhere(5).

Histopathology

Biopsies from all patients in both groups were stained with both haematoxylin & eosin and reticulin. These were scored by a single pathologist (RG) using the modified histological activity index (HAI-Ishak) scoring system(13). This scoring system assigns a score of 0-6 for the degree of fibrosis and a score of 0-18 for necro-inflammatory activity. Fibrosis stage was assumed to be zero at the time of infection.

DNA collection

All patients gave informed consent. The local regional ethics committee of St Mary's hospital gave approval for the study. Genomic DNA was extracted from a 5ml sample of whole blood collected into EDTA. Extraction was performed using a commercial kit (Nucleon II, Scotlabs, UK) according to the manufacturers instructions.

Genotyping

Genotyping was performed by a reverse line blot hybridisation linear array assay (Roche Molecular Systems, Alameda USA). Extracted genomic DNA was subjected to multi target PCR amplifications using a blend of primers. PCR products were then genotyped by hybridising to sequence specific oligonucleotides impregnated onto nylon strips and detected in a colorimetric reaction as described in(14). Strips were then examined for the presence of coloured bands indicating the presence of an allele.

Statistical analysis

All statistics were performed using SPSSv10 (SPSS inc. Chicago Illinois) and Microsoft Excel™. Rate of fibrosis was calculated as the ratio of the fibrosis score to duration of infection at biopsy. Demographic differences between fast (predicted cirrhosis in less than 20 years) and slow (not predicted to reach cirrhosis for more than 30 years) groups were assessed using independent samples t tests, analysis of variance (ANOVA) and Chi squared tests as appropriate.

Rate of fibrosis was logarithmically transformed to give a normal distribution. Comparison of rates between groups of individuals with different genotypes was performed using analysis of variance. Genotype

and allele frequency were compared between subsets of patients with fast or slow fibrosis using the Chi Squared or Fishers Exact test as appropriate.

Variations in rates of fibrosis with respect to genotype were sought in the St Mary's cohort. Reproducibility of the observed variations was then sought in the Hencore cohort. The two cohorts were then pooled to allow subgroup analysis. In order to account for demographic differences between the phenotypic groups, subgroup analysis was performed by gender and analysis of covariance (ANCOVA) to examine the effect of age at infection and gender.

Results

Demographic features

The demographic features for the groups of patients with fast and slow fibrosis are shown together with those for the group as a whole in table 1. As expected there was an excess of males in the fast fibrosis group ($p=0.001$) and age at infection was significantly higher in those with rapid fibrosis ($p<0.0001$). In this group, mode of acquisition of HCV, alcohol consumption and viral genotype were not related to rate of fibrosis progression. Fast fibrosers had a shorter duration of infection prior to biopsy and this correlated inversely with age at infection ($R=-0.485$ ($p<0.0001$)). The fast fibrosis group had a mean rate of fibrosis over ten fold greater than the slow fibrosis group (0.94 units/year vs. 0.09 units per year $p<0.0001$). Mean fibrosis score and necro-inflammatory score were both higher in the fast fibrosis group (3.67 and 5.18 units vs. 1.68 and 3.7 units $p<0.0001$) (Table 2).

Table 1. Demographic differences between Fast and Slow Fibrosis groups

	FAST (Cirrhosis in less than 20 years)	SLOW (More than 30 years to cirrhosis)	Comparisons	TOTAL (For ANOVA)
Male	80	87	P=0.001	198
Female	38	95		154
Age at infection	34.5	22.0	P<0.0001	27.06
Risk factor				
Blood	56	94	P=ns	175
IVDU	61	88		177
Unknown				
Alcohol				
Nil	64(54.7)	101(55.2)	P=ns	192(54.4)
Minimal (<20units/week)	20(17.1)	27(14.8)		54(15.3)
Moderate (20-40 units/week)	4(3.4)	6(3.3)		12(3.4)
Heavy (40+units/week)	12(10.3)	17(9.3)		34(9.5)
Unknown	17(14.5)	32(17.5)		60(17.2)
Viral Genotype				
1	40(34.2)	73(39.9)	P=ns	135(38.2)
Non 1	33(28.2)	37(20.2)		81(22.9)
Unknown	73(37.6)	73(39.9)		136(38.9)
Time infection to 1st biopsy (years)	6.35	19.8	P<0.0001	14.7

Table 2. Differences between fast and slow phenotypic groups.

(values are means)	FAST (Cirrhosis in less than 20 years)	SLOW (More than 30 years to cirrhosis)	Compariso ns	Group for ANOVA
Fibrosis Score (units)	3.67	1.68	P<0.0001	2.66
Necroinflammatory score (units)	5.18	3.7	P<0.0001	4.4
Rate of fibrosis (fibrosis units/year)	0.94	0.09	P<0.0001	0.4

Analysis of variance

The relationship between fibrosis rate and genotypes was examined using ANOVA. In the St Mary's cohort the median rate of fibrosis in patients who were heterozygous for the factor V Leiden mutation was 0.12 fibrosis units per year, compared to 0.33 fibrosis units per year in patients with wild type factor V (ANOVA (F (1,153)=6.57, p=0.011). A similar difference was observed in the Hencore cohort: 0.24 units/year in factor V Leiden heterozygotes v 0.42 units per year in wild type factor V (ANOVA (F (1,196)=3.6, p=0.058)).

In the sample as a whole a total of 352 genotyped individuals were included (table3). The association was significant ANOVA (F (1,351)=8.63, p=0.004) (Figure1).

Table 3. Summary of ANOVA results

Gene and polymorphism			ANOVA
Coagulation	Factor	V	F (1,351)=8.63, P=0.004
Arg506Gln			
Coagulation	Factor	VII	F (1,351)=0.891,
Arg353Gln			P=0.589
Thrombin G20210A			F (1,351)=0.913,
			P=0.421

ANOVAS: 352 patients Total

Disease associations

Disease associations were performed with DNA from the 182 individuals with slow fibrosis rates and 118 with fast rates (Table 4). A significant association was seen in the UK patients (Fishers exact test $p=0.03$ Odds Ratio=7.5 for fast vs. slow) with a trend in the HENCORE patients (Fishers exact test $p=0.278$, Odds Ratio=3) between the factor V polymorphic allele Arg560Gln (Factor V Leiden) and rate of fibrosis. In the sample as a whole there was a significant association (Fishers exact test $p=0.021$, Odds Ratio=4 for fast vs. slow).

Table 4. Summary of Disease association results

Gene and polymorphism	Combined populations			Genotype Frequency	Allele frequency
Coagulation Factor V Arg506Gln		Fast	Slow	P=0.011	P=0.017
	Arg/Arg	108	178		
	Arg/Gln	10	4		
	Gln/Gln	0	0		
Coagulation Factor VII Arg353Gln	Arg/Arg	91	140	P=0.98	P=0.919
	Arg/Gln	24	37		
	Gln/Gln	3	4		
Thrombin G20210A	G/G	112	176	P=0.576	P=0.886
	A/A	6	7		
	A/A	0	0		

Chi squared tests: 118 Fast, 182 Slow

Multivariate analysis

The effect of factor V genotype on the rate of fibrosis was most pronounced in males ($p=0.007$ Fishers exact test, odds ratio=10 for fast vs. slow fibrosis, ANOVA $F(1,197)=5.42$, $p=0.021$).

In order to take into account the influence of age at infection and gender on rate of fibrosis, analysis of covariance (ANCOVA) using a type 1 sum of squares model was performed. The significant effect of Factor V genotype on rate of fibrosis progression was sustained ($F(1,342)=7.33$, $p=0.007$) when age at infection ($F(1,342)=112.5$, $p<0.0001$) and gender ($F(1,342)=14$, $p<0.0001$) were taken into account.

Factor II and Factor VII

No associations were seen between factor II genotype ($p=0.6$ chi squared (fast v slow), $p=0.589$ ANOVA) and factor VII ($p=0.92$ chi squared fast v slow), $p=0.421$ ANOVA).

Discussion

This data provides evidence and confirmation that the factor V Leiden mutation leads to an increased rate of fibrosis in HCV infection. The functional significance of factor V Leiden is well described in that this mutation confers resistance to activated protein C which normally degrades factor V. Increased activity of factor V leads to increased thrombin activity and hence fibrin production. We hypothesise that those with the polymorphism have a procoagulant state in response to the liver inflammation resulting from HCV which gives rise to increased thrombin generation and increased fibrin deposition. Thrombin is a stellate cell mitogen(9). Increased thrombin levels affect stellate cell activation and hence may enhance fibrosis deposition. We have observed this association in 2 populations. The weaker association in the HENCORE patients from mainland Europe may reflect the greater genetic heterogeneity in this group(15). The procoagulant factor V Leiden is associated with rapid development of liver fibrosis and its effect appears to be predominant in males with only a trend observed in females. As has been previously reported by Poynard et al (1) age at infection and male gender were found to be associated with a rapid fibrosis rate in our study. Lack of association between alcohol and rate of fibrosis may relate to different drinking patterns between our pan European and Poynards(1) predominantly French patients. Genetic risk factors need to be examined in the context of environmental ones.

Factor V Leiden may influence the rate of fibrosis in hepatitis of other aetiologies. Establishing the influence of this genetic factor on rate of fibrosis opens the possibility of therapeutic intervention in the form of anticoagulation, for example for patients who do not respond to anti viral therapy.

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CLAIMS

1. A method for assessing a patient's risk of development or progression of liver cirrhosis, comprising the step of determining the patient's genotype or phenotype for a coagulation factor.
2. The method of claim 1 wherein if the patient's genotype is a genotype associated with a procoagulant tendency or the patient's phenotype is a procoagulant tendency or indicative of a procoagulant tendency, then the patient is considered to be at a higher risk of developing liver cirrhosis or fibrosis and/or of rapid progression of liver cirrhosis or fibrosis.
3. The method of claim 1 or 2 wherein the coagulation factor is Factor V.
4. The method of claim 3 wherein the presence or absence of Factor V Leiden is determined.
5. The method of any one of the preceding claims, wherein if the patient has Factor V Leiden, then the patient is considered to be at a higher risk of developing liver cirrhosis or fibrosis and/or of rapid progression of liver cirrhosis or fibrosis.
6. The method of claim 1 or 2 wherein the coagulation factor is thrombin.
7. The method of claim 1 or 2 wherein the coagulation factor is Factor X, Factor VIII, Factor IX, Factor XI, Factor VII, Tissue Factor, HMW Kininogen or Prekallikrein.

8. The method of claim 1 or 2 wherein the coagulation factor is an antithrombin (for example antithrombin III), Protein C, Protein S, Protein C inhibitor, thrombomodulin or α_2 -Macroglobulin.
9. The method of any of the preceding claims wherein the genotype or phenotype of the patient is determined in relation to more than one coagulation factor.
10. The method of any one of the preceding claims wherein a PCR-based method is used.
11. The method of any one of claims 1 to 9 wherein the level or activity of a coagulation factor is measured.
12. The method of any one of the preceding claims wherein the patient has or is at risk of hepatitis virus infection.
13. The method of claim 12 wherein the patient has or is at risk of Hepatitis C virus (HCV) infection.
14. Use of an agent which is capable of use in determining the genotype or phenotype of a patient for a coagulation factor in the manufacture of a reagent for assessing the patient's risk of development or progression of liver cirrhosis, for example following HCV infection.
15. The method of claim 14 wherein the agent is a nucleic acid which selectively hybridises to a coagulation factor nucleic acid.
16. The method of claim 15 wherein the coagulation factor is Factor V.

17. A kit of parts useful for prognosis of liver fibrosis or cirrhosis, comprising means for use in determining the genotype or phenotype of a patient for a coagulation factor and means for use in determining whether the patient has been exposed to a hepatitis virus and/or is infected with a hepatitis virus.

18. The kit of claim 17 wherein the coagulation factor is Factor V and the hepatitis virus is HCV.

19. A method for treating a patient with or at risk of liver fibrosis or cirrhosis comprising the step of administering to the patient an anticoagulant agent.

20. An anticoagulant agent for use in the treatment of a patient with or at risk of liver fibrosis or cirrhosis.

21. An anticoagulant agent for use in the treatment of a patient with or at risk of hepatitis virus infection (for example HBV, HAV or HCV, preferably HCV).

22. A method for treating a patient with or at risk of hepatitis virus infection (for example HBV, HAV or HCV, preferably HCV) comprising the step of administering to the patient an anticoagulant agent.

23. A method for treating a patient with or at risk of liver fibrosis or cirrhosis, comprising the steps of (1) assessing the patient's risk of development or progression of liver cirrhosis, comprising the step of determining the patient's genotype or phenotype for a coagulation factor; (2) if the patient's risk of development or progression of liver cirrhosis is

high (ie the patient is at risk of rapid fibrosis), administering to the patient an anticoagulant agent and/or other therapeutic agent suitable for treating liver fibrosis.

24. An anticoagulant agent and/or other therapeutic agent suitable for treating liver fibrosis for treatment of a patient with or at risk of liver fibrosis or cirrhosis, wherein the patient is one whose risk of development or progression of liver cirrhosis has been assessed by a method comprising the step of determining the patient's genotype or phenotype for a coagulation factor; and the patient's determined risk of development or progression of liver cirrhosis is high.

25. A method for treating a patient with or at risk of hepatitis virus infection (for example HBV, HAV or HCV, preferably HCV), comprising the steps of (1) assessing the patient's risk of development or progression of liver cirrhosis by a method comprising the step of determining the patient's genotype or phenotype for a coagulation factor; (2) if the patient's risk of development or progression of liver cirrhosis is high, administering to the patient an anticoagulant agent and/or other therapeutic agent suitable for treating viral hepatitis, for example an antiviral agent, for example α -interferon.

26. An anticoagulant agent and/or other therapeutic agent suitable for treating viral hepatitis, for example an antiviral agent, for example α -interferon, for treatment of a patient with or at risk of hepatitis virus infection (for example HBV, HAV or HCV, preferably HCV), wherein the patient is one whose risk of development or progression of liver cirrhosis has been assessed by a method comprising the step of determining the patient's genotype or phenotype for a coagulation factor; and the patient's

determined risk of development or progression of liver cirrhosis is high (ie whose predicted rate of fibrosis is rapid).

27. A method for treating a patient with or at risk of hepatitis virus infection (for example HBV, HAV or HCV, preferably HCV), comprising the steps of (1) determining the patient's genotype or phenotype for Factor V; (2) if the patient has Factor V Leiden, administering to the patient an anticoagulant agent and/or other therapeutic agent suitable for treating viral hepatitis, for example an antiviral agent, for example α -interferon.

28. An anticoagulant agent and/or other therapeutic agent suitable for treating viral hepatitis, for example an antiviral agent, for example α -interferon, for treatment of a patient with or at risk of hepatitis virus infection (for example HBV, HAV or HCV, preferably HCV), wherein the patient has Factor V Leiden.

29. The method or anticoagulant of any one of claims 19 to 28 wherein the anticoagulant is a direct thrombin inhibitor, for example Hirudin.

30. The method or therapeutic agent of any one of claims 19 to 28 wherein the therapeutic agent comprises a recombinant bone marrow derived stem cell or cell derived therefrom.

31. The method or therapeutic agent of claim 30 wherein the recombinant bone marrow derived stem cell or cell derived therefrom is capable of expressing normal Factor V.

32. A bone-marrow derived stem cell comprising a recombinant nucleic acid capable of expressing, in the bone-marrow derived stem cell or cell derived therefrom, normal Factor V.

33. A recombinant polynucleotide suitable for expressing in a bone-marrow derived stem cell or cell derived therefrom normal Factor V.

34. A recombinant polynucleotide construct comprising RNA and DNA elements suitable for correcting a Factor V Leiden genotype to a normal Factor V genotype in a bone-marrow derived stem cell or cell derived therefrom.

35. A gene therapy construct comprising a recombinant polynucleotide according to claim 33 or 34.

36. The gene therapy construct of claim 35 wherein the construct comprises a Moloney Leukaemia Virus (MLV) based retroviral vector or a lentiviral vector or adeno-associated vector (AAV) or a baculovirus based vector.

37. A bone-marrow derived stem cell, recombinant polynucleotide or gene therapy construct according to any one of claims 32 to 36 for use in medicine.

38. A bone-marrow derived stem cell, recombinant polynucleotide or gene therapy construct according according to any one of claims 32 to 36 for use in treating a patient with or at risk of liver fibrosis and/or with or at risk of hepatitis virus infection (for example HBV, HAV or HCV, preferably HCV).

39. The use as defined in claim 38 wherein the patient has Factor V Leiden.

40. A pharmaceutical preparation comprising a bone-marrow derived stem cell, recombinant polynucleotide or gene therapy construct as defined in any one of claims 32 to 36 and a pharmaceutically acceptable carrier.

41. A method for treating a patient with or at risk of liver fibrosis and/or with or at risk of hepatitis virus infection (for example HBV, HAV or HCV, preferably HCV) comprising the steps of (1) providing a bone-marrow derived stem cell according to the invention, or a recombinant nucleic acid of the invention (2) introducing the stem cell or recombinant nucleic acid into the patient. The bone-marrow derived stem cell may be provided by a method comprising the steps of (1) obtaining a bone-marrow derived stem cell from the patient, (2) introducing a recombinant nucleic acid encoding normal Factor V (ie not Factor V Leiden) into the bone-marrow derived stem cell.

42. A kit of parts or composition comprising (1) (a) a bone-marrow derived stem cell according to claim 31 or (b) a recombinant polynucleotide or gene therapy vector according to any one of claims 32 to 35 and an agent useful in promoting transfection of bone-marrow derived stem cells, and (2) a mobilising composition or colonising composition and/or means for testing whether the patient has Factor V Leiden.

43. A method for identifying a compound for treating a patient with or at risk of liver fibrosis and/or hepatitis virus infection, the method comprising contacting a test compound with Factor V or Factor V Leiden; determining whether said compound is capable of modulating the activity of Factor V or

Factor V Leiden; and selecting a compound which has an activity which has an anticoagulant effect.

44. The method of claim 43 wherein the selected compound inhibits activated Factor V Leiden, or render activated Factor V Leiden susceptible to inhibition by activated Protein C.

45. A compound identified by the screening method of claim 43 or 44 for use in medicine.

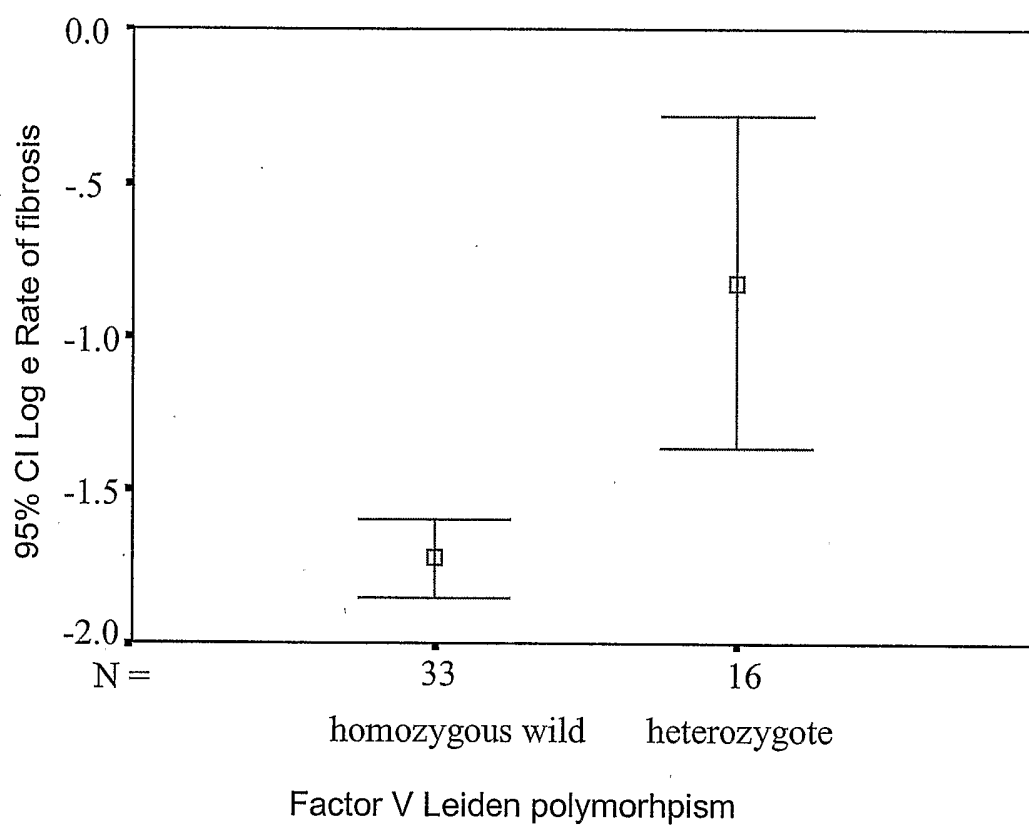
46. A compound as defined in claim 45 for use in treating a patient with or at risk of liver fibrosis and/or with or at risk of hepatitis virus infection (for example HBV, HAV or HCV, preferably HCV).

47. A method for treating a patient with or at risk of liver fibrosis and/or with or at risk of hepatitis virus infection (for example HBV, HAV or HCV, preferably HCV), comprising the step of administering to the patient a compound as defined in claim 45.

48. Any novel method of treatment or use of a therapeutic agent as herein disclosed.

49. Any novel screening method as herein disclosed.

50. Any novel cell, recombinant polynucleotide or gene therapy construct as herein disclosed.

Figure 1

The Coagulation cascade

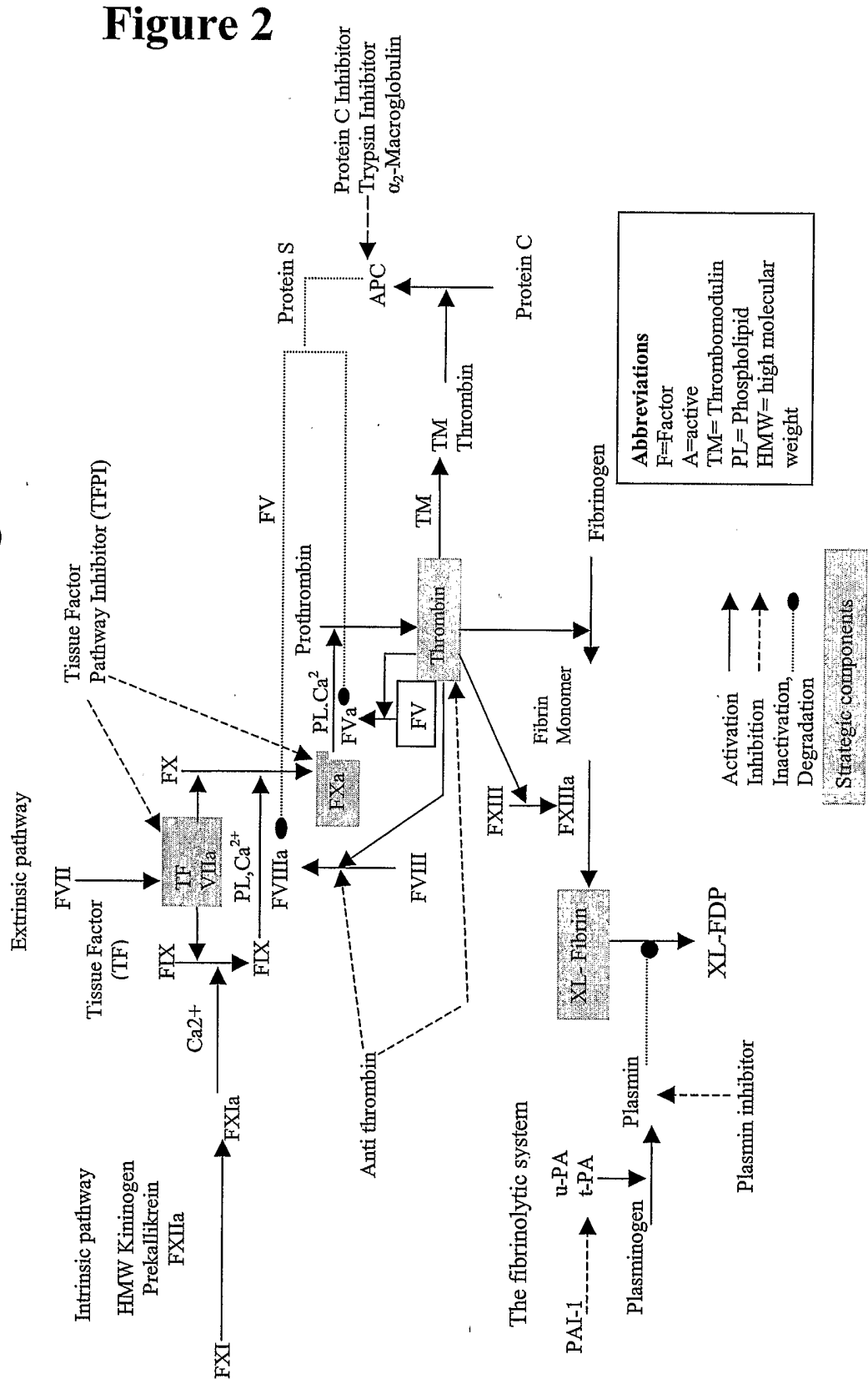
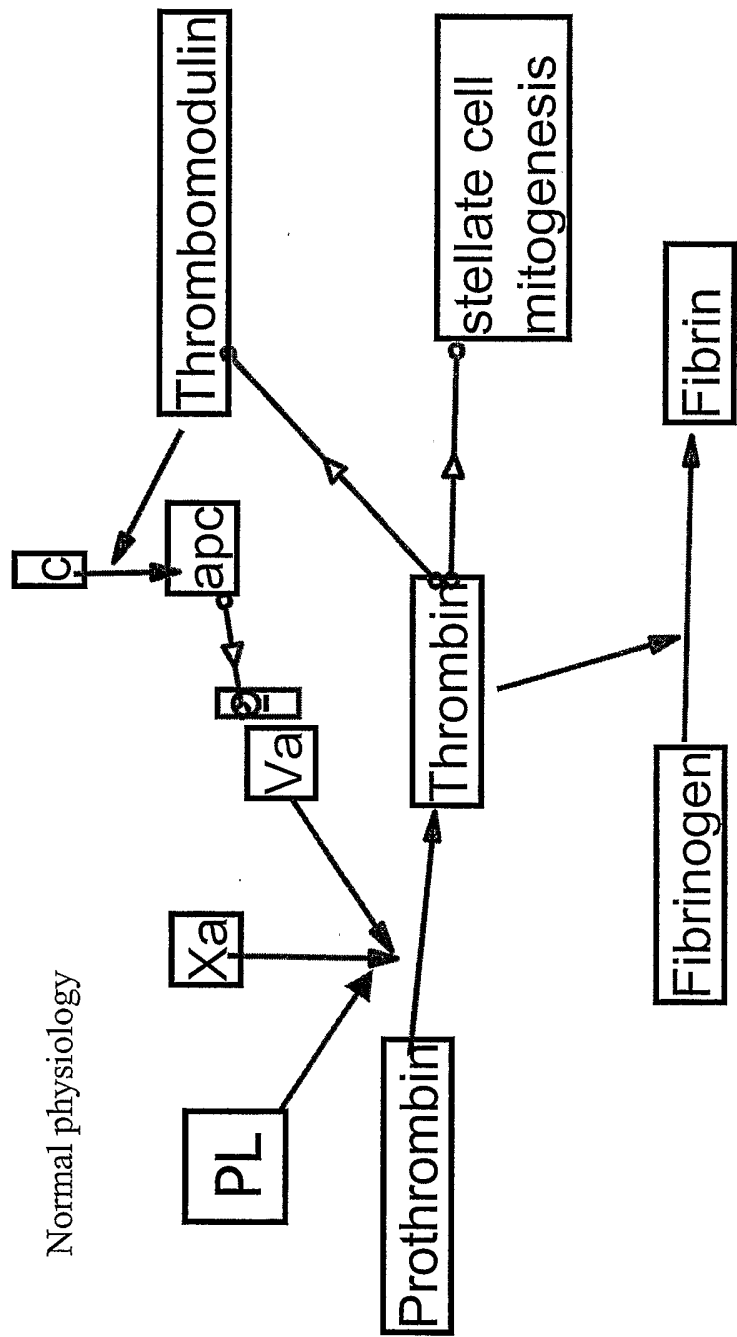


Figure 3

Factor V Polymorphism: G1691A (Arg560Gln)

Gln = Factor V Leiden (activated protein C Resistance)
4% UK Caucasian population



NB. Rat models, coagulation in healing, thrombin as a stellate cell mitogen

Figure 4