Fibrinogen Genotyping in Peripheral Vascular Disease

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This thesis is dedicated to my parents,

Mr and Mrs Wood,

with all my love,

John

CONTENTS

ABBREVIATIONS

A	- Amp or Adenine
Arg	- Arginine
Asp	- Aspartic acid
bp	- Base pair
C	- Cytosine
c	- Centi
Cys	- Cysteine
°C	- Degrees Celsius
dATP	- 2' deoxy Adenosine 5' Tri-Phosphate
dCTP	- 2' deoxy Cytidine 5' Tri-Phosphate
dGTP	- 2' deoxy Guanidine 5' Tri-Phosphate
dTTP	- 2' deoxy Thymidine 5' Tri-Phosphate
dH2O	- Distilled water
DNA	- Deoxyribonucleic acid
cDNA	- Complementary deoxyribonucleic acid
EDTA	- Ethylene Diamine Tetra-Acetate
e.g.	- For example
et al	- et alia (and others)
G	- Guanine
g	- Gram
Gln	- Glutamic acid
Gly	- Glycine
HCL	- Hydrochloric acid
His	- Histidine
i.e.	- Therefore
i.u.	- International enzyme unit

Kb	- Kilo-base pairs
l	- Litre
M	- Molar or Morgan
m	- Milli
Met	- Methionine
u	- Micro
O.D.	- Optical density
RFLP	- Restriction Fragment Length Polymorphism
rpm	- Revolutions per minute
s	- Second
SDS	- Sodium Dodecyl Sulphate
SDW	- Sterile distilled water
Ser	- Serine
T	- Thymine
Thr	- Threonine
TRIS	- Tris (hydroxymethyl) amino-methane
TRIS-HCl	- HCl-neutralised (hydroxymethyl) amino-methane
Tyr	- Tyrosine
Val	- Valine
4q	- long arm of chromosome 4

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SUMMARY

Elevated plasma levels of fibrinogen have been associated with an increased risk of peripheral vascular disease and with elevated levels of blood pressure. Furthermore there is some evidence that plasma levels of fibrinogen may be determined at least in part by variation at the fibrinogen gene loci. This study was undertaken to investigate the contribution of variation at the fibrinogen loci with plasma fibrinogen levels and to seek association between fibrinogen gene variation and the determination of levels of blood pressure and the occurance of peripheral vascular disease.

Allele frequencies at the alpha, beta and gamma fibrinogen loci were determined in 235 cases and controls from the peripheral vascular disease study. The values were alpha/TaqI 0.75 (for A the 2.4Kb fragment), 0.25 (for a the 1.6Kb fragment); beta/BclI 0.84 (for B the 5.3Kb fragment), 0.16 (for b the 4.2Kb fragment) and gamma/KpnI/SacI 0.76 (for D the 14Kb fragment), 0.24 (for d the 11Kb fragment). Allele frequencies at the beta fibrinogen locus for the HaeIII polymorphism were determined using the polymerase chain reaction (PCR) in a further 170 of these individuals. The values were 0.81 (for H1 the 0.575Kb and 0.383Kb fragments) and 0.19 (for H2 the 0.958Kb fragment). The overall genotype frequencies were compared with those predicted from the Hardy-Weinberg equilibrium and no significant difference was noted. Allele frequencies were compared with previously published studies and a significant difference was noted for beta/BclI between this study on Scottish subjects and that of Humphries et al. (1987) on English subjects ($p<0.05$) but not between this study and

Berg and Kierulf (1989) on Norwegian subjects ($p>0.80$).

Strong linkage disequilibrium was found between the alpha/TaqI and gamma/KpnI/SacI markers and the beta/BclI and beta/HaeIII markers which was highly significant at any level ($p=1.1x10^{-27}$ and $2.24x10^{-8}$ respectively). A lesser association was found between the alpha/TaqI and beta/BclI loci ($p=6.6x10^{-4}$); beta/BclI and gamma/KpnI/SacI markers ($p=2.5x10^{-3}$); alpha/TaqI and beta/HaeIII ($p=1.3x10^{-4}$) markers and the gamma/KpnI/SacI and beta/HaeIII markers ($p=1.4x10^{-4}$). This is consistent with the known physical order of the loci and suggests a relative excess of recombination in the alpha/gamma-beta interval.

Fibrinogen levels were determined in the peripheral vascular disease case/control study by clotting or nephelometric assays and the relationship of fibrinogen level to genotype analysed. No statistically significant association was detected for any of the four markers TaqI, BclI, KpnI/SacI and HaeIII with respect to fibrinogen levels.

The 235 individuals in this study had one of 13 different haplotypes with respect to the TaqI, BclI and KpnI/SacI markers. No Statistically significant association was detected for any of these haplotypes in respect of fibrinogen levels. The percentage of phenotypic variability was calculated and varies from 2.4-4.9% in the different analyses.

155 individuals were genotyped with respect to the HaeIII-beta polymorphism. There was no association between plasma fibrinogen level and any genotype ($p=0.58$). Differences in genotype distribution between cases and controls were analysed using X^2. No significant

difference in distribution was observed (p=0.55). The contribution of genotype and smoking status to fibrinogen level was analysed. There was no relationship between the HaeIII RFLP, smoking and fibrinogen level (p=0.58).

The contribution of fibrinogen haplotype to the occurrence of peripheral vascular disease was assessed by multiple logistic regression using variables which represent the difference in the log odds of disease for each haplotype relative to haplotype 1. Haplotype was found to be a significant predictor of disease after accounting for other risk factors and there is some consistency in the results for haplotypes 3 (aa Bb DD) and 5 (Aa Bb Dd) suggesting that they are significant predictors of disease ($p<0.05$), over and above the level of fibrinogen. This effect was most marked for allele differences at the BclI (beta locus with a relative excess of the rarer b allele ($p<0.005$) in cases versus controls.

Allele frequencies at the beta fibrinogen locus were determined in 168 individuals in the Ladywell hypertension study. The values were .BclI/beta 0.85 (for B the 5.3Kb fragment) and 0.15 (for b the 0.15Kb fragment). The overall genotype frequencies were compared with those predicted from the Hardy-Weinberg equilibrium and no significant difference was noted. A comparison of the BclI genotype distribution was carried out between the four different groups in the Ladywell study. No statistically significant difference was recorded in the genotype distribution in the four groups.

This study was unable to demonstrate a relationship between fibrinogen genotype at the alpha, beta or gamma loci and plasma fibrinogen level in a large Scottish case and control study of peripheral vascular disease. Further no association could be detected between

predisposition to high or low blood pressure and variation at the fibrinogen locus. In contrast a statistically significant association was detected between fibrinogen haplotype and the occurrence of peripheral vascular disease. This suggests that the type of fibrinogen produced as a result of genetic variation rather than its level might be of importance in the causation of peripheral vascular disease. In view of the clinical importance of these observations other studies of this nature in peripheral vascular disease and other cardiovascular diseases are indicated.

1.0 INTRODUCTION

Atherosclerosis is the principal factor in the development of cardiovascular disease which is the most common cause of death in the UK. Well accepted risk factors in the development of atherosclerosis include cigarette smoking, hypercholesterolaemia and hypertension. There is also increasing evidence that haemostatic factors play an important role in thrombogenesis and atherogenesis. For example, increased plasma levels of fibrinogen have been shown in longitudinal studies to be associated with an increased risk of ischaemic heart disease and stroke (Meade et al., 1980, 1986, 1987; Wilhelmsen et al., 1984). Fibrinogen levels show a prognostic significance comparable to that of other major risk factors but to what extent levels of fibrinogen are environmentally or genetically influenced is not fully understood.

1.1 Fibrinogen Biochemistry

Fibrinogen (MW 340,000) is an abundant plasma glycoprotein with an overall length of 450-470 angstroms (Marder et al,. 1982) which consists of two sets of three different polypeptide chains. The chains are designated alpha, beta and gamma with molecular weights of 66000, 52000, and 46500, respectively (McKee et al., 1966). The alpha chain is the largest of the three chains and contains 625 amino acid residues; the beta chain contains 450 and the gamma chain 410 (Watt et al., 1978). The complete amino acid sequences of the three chains have been established (Henschen and Lottspeich, 1977; Lottspeich and Henschen, 1977; Doolittle et al., 1979; Henschen et al., 1979; Watt et al., 1979) and indicate considerable homology between the three genes.

The beta and gamma polypeptides, however, share a significantly higher degree of homology with each other than with the alpha chain, especially in the carboxy-terminal two thirds (fragment D) (Watt et al., 1978; Kant et al., 1983a).

This relationship between the three chains indicates that they probably arose by duplication and divergence from a common ancestral gene (Doolittle, 1973; Watt et al., 1978; Kant et al., 1983a; Chung et al., 1983). There are four carbohydrate clusters on the fibrinogen molecule, one on each of the two beta chains and one on each of the two gamma chains (Iwanager et al., 1968; Pizzo et al., 1972; Topfer-Petersen et al., 1976). No such cluster is found on the alpha chain. Each of the three chains bears valine at the carboxyl terminal (Oudke and Iwanaga, 1971). In total there are 29 disulphide bonds, three of which hold the dimeric halves of the molecule together and 13 occur twice per molecule (Marder et al., 1982; Bouma et al., 1978; Blomback et al., 1972).

All three chains are synthesised in the liver in hepatic parenchymal cells from three individual mRNA species (Foreman and Barnhart, 1964; Nickerson and Fuller, 1981; Chung et al., 1980; Crabtree et al., 1981; 1982). They are processed, glycosylated, assembled, and eventually secreted into the circulating plasma as a mature fibrinogen molecule at a concentration of approximately 3g/L in normal blood plasma (Shafer and Higgins, 1988). The rate limiting step in the production of fibrinogen is the synthesis of the beta chain (Roy et al.,1990). It therefore, follows that any change in the transcription of the beta gene will be reflected in a change in the rate of fibrinogen production. Figure 1.1 is a schematic

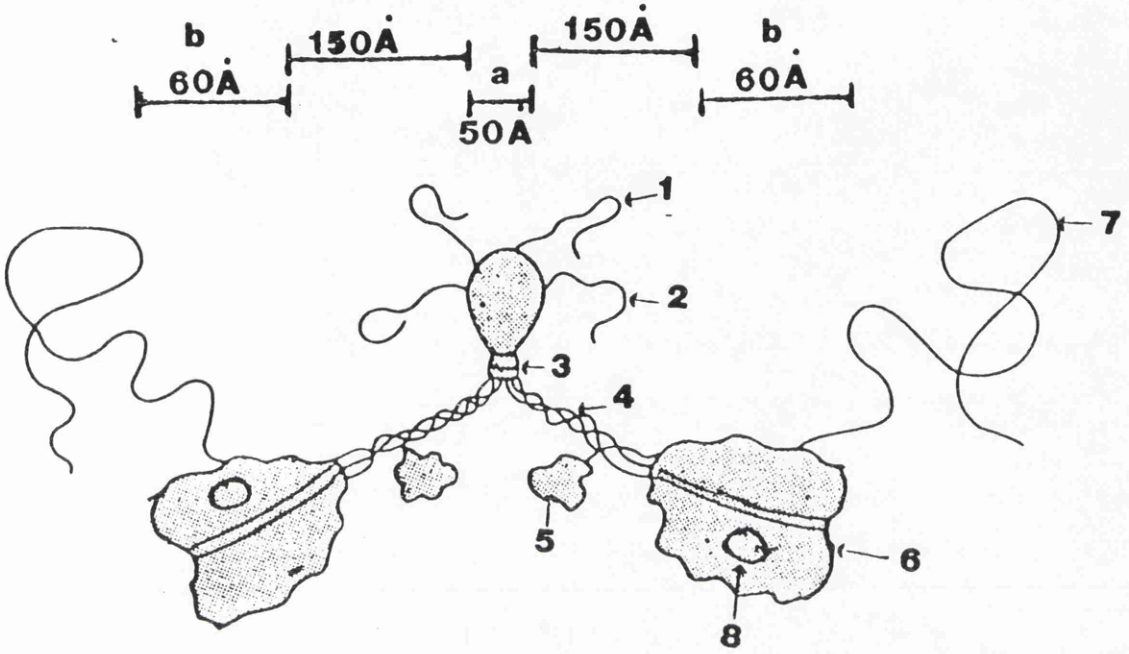

Figure 1.1. Schematic diagram of the Fibrinogen Molecule. (a) Central domain (MW 32,600); (b) Terminal domain (MW 67,200); (1) Thrombin cleavage site for the release of fibrinopeptide A; (2) Thrombin cleavage site for the release of fibrinopeptide B; (3) "disulphide girdle"; (4) thin connecting alpha helical coiled coils; (5) carbohydrate attached to gamma chain; (6) fragment D; (7) alpha polar appendage; (8) carbohydrate attached to the beta chain.

representation of the fibrinogen molecule modified from that of Doolittle (1975).

Fibrinogen participates in the final step of the coagulation pathway which can be initiated in one of two ways:

(i) The extrinsic system depends on the complexing of tissue factor (such as is exposed to blood in a ruptured atheromatous plaque, or those produced by the action of cytokines on endothelial cells) with factor VII.

(ii) The intrinsic system can be triggered by negatively charged lipoproteins such as chylomicrons or very low density lipoproteins which provides an appropriate surface for the activation of factor XII (Ganong, 1985). A summary of the coagulation pathway is presented in Figure 1.2.

During the coagulation process, thrombin removes fibrinopeptide A and B, from the alpha and beta chains respectively, by proteolysis, converting fibrinogen to fibrin monomers. After thrombin cleavage, the amino terminal residues of the alpha and beta chains are converted from alanine and glutamine, respectively, to glycine (Dayhoff, 1972). The fibrin monomers then polymerise allowing Factor XIII to catalyse the introduction of a small number of covalent bonds between certain side chains of adjacent molecules in the polymer, thereby stabilizing the fibrin clot (Doolittle, 1973, 1975). Factor XIII susceptible cross-link sites are present on the alpha and gamma chain, but not the beta chain (Marder et al., 1982; Chen and Doolittle, 1970).

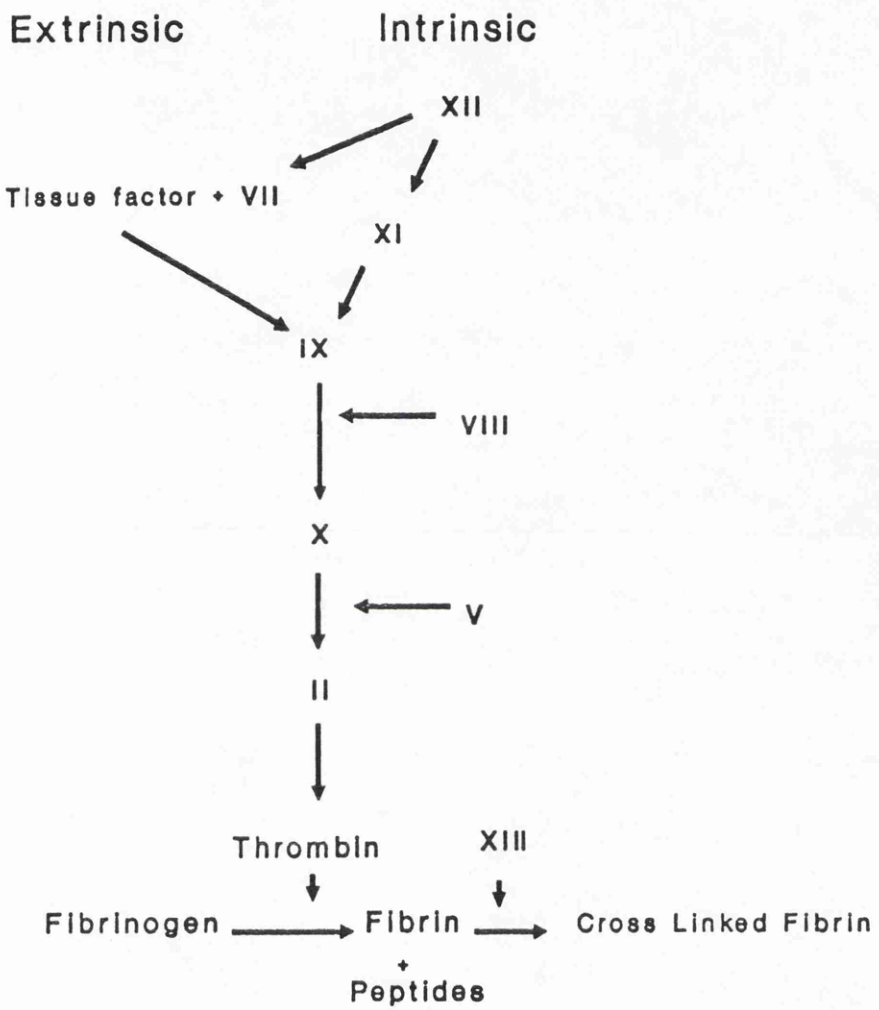

Figure 1.2. Summary of the coagulation cascade.

The dissolution of the fibrin clot, formed during the coagulation process, is achieved via the fibrinolytic system by proteolytic enzymes such as plasmin (Smith. 1986). The pattern of fibrinogen and fibrin breakdown was determined in part to elucidate the structure of the fibrinogen molecule. However, it was not until 1969 that Marder et al. demonstrated the structural relationship between the early products of plasmin digestion, fragments X and Y, and the late digestion products, fragments D and E. The degradation fragments (D and E) are important in stimulating macrophages to produce interleukin 6 and other hepatocyte stimulating factors, which feedback on the liver to increase fibrinogen synthesis (Cook and Ubben 1990). This mechanism is important both for the maintenance of steady state fibrinogen levels and for the acute phase reaction during an inflammatory response. Figure 1.3 represents the degradation of fibrinogen into its characteristic fragments.

1.2 Fibrinogen-Genetics

In man the alpha, beta and gamma genes are closely linked in a 50kb DNA segment (Kant et al., 1985) and have been mapped by various methods to the long arm of chromosome 4 (Olaisen et al., 1982; Henry et al., 1984; Humphries et al., 1984; Kant et al., 1983b, 1985 Marino et al., 1986). Aschbacher et al. (1985) used a series of restriction fragment length polymorphisms (RFLPs) at the fibrinogen locus to study linkage disequilibrium and concluded that the likely order is gamma, alpha, beta. This agrees with the order suggested by Kant et al. (1985). The alpha and gamma genes are transcribed in the 5' to 3' direction from one strand of DNA whereas the beta gene is transcribed in the 5' to 3' direction from the opposite strand of DNA (Kant et al., 1985). The alpha gene is 10Kb upstream from the gamma gene and

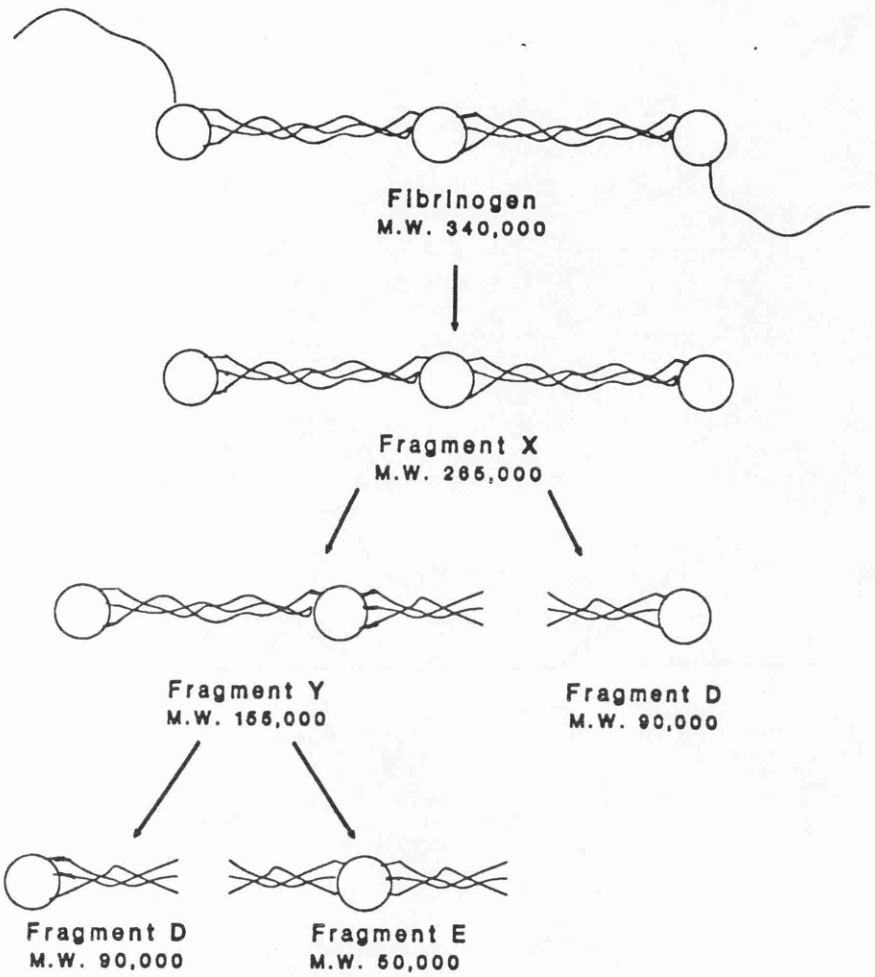

Figure 1.3. Schematic diagram of the degredation of fibrinogen by plasmin (After Marder et al., 1982). The conversion of fibrinogen to fragment X involves the removal of the alpha polar appendages. Fragment X is converted to fragments Y and D by the splitting of either of the three stranded ropes connecting the domains. Fragment Y is further degraded to another fragment D and fragment E.

13Kb downstream from the beta gene. All three genes are approximately 10Kb in length. The alpha gene consists of 5 exons and 4 introns; the beta gene of 8 exons and 7 introns and the gamma gene 8 exons and 7 introns (Chung et al., 1983, Kant et al., 1985).

The first fibrinogen gene to be mapped to chromosome 4 was the gamma gene. Family studies indicated close linkage between a gamma chain variant (FGG) and the MNSs blood group loci, which are known to map to 4q29-q31 (Olaisen et al., 1982). Henry et al. (1984) employed gene dosage analysis as well as cDNA clones and somatic cell hybrids to assign the fibrinogen genes to 4q21-q31. This region was further localised to 4q26-qter by Humphries et al. (1984) who showed that in somatic cell hybrids carrying a chromosome 4:12 translocation with a break point at 4q26, all three fibrinogen genes segregated with the 4q26-qter segment. This localisation was confirmed by in situ hybridization with the alpha fibrinogen probe which localized the distance to 4q29-4q31 (Humphries et al., 1984). Marino et al. (1986) further localized the fibrinogen gene cluster to band 4q31.

Several DNA polymorphisms of the fibrinogen genes have been reported (Humphries et al., 1984, 1987; Murray et al., 1986; Thomas et al.,1991)) and are presented in Table 1.1. A diagram of chromosome 4 indicating the location of the alpha, beta, and gamma genes and their corresponding RFLPs is presented in Figure 1.4.

Table 1.1. The RFLPs of the Fibrinogen Genes (Human Gene Mapping 10, 1990; Thomas et al., 1991)

Map Location	Locus Symbol	Probe	Enzyme	Constant Bands	Size Kb
4q31	FGA	pFA1	TaqI	0.7	2.4
4q31	FGB	pHIB2	HincII		16
			AvaII		4.3 4.1
			BclI	6.0	4.5 2.5
	FGB	pFB5	BclI	6.6	5.3 4.2
		FGFB	BglI		23 18
	FGB	PCR	HaeIII	0.34	0.95
					0.57
					0.38
4q31	FGG	pFGI	KpnI/SacI		11 14

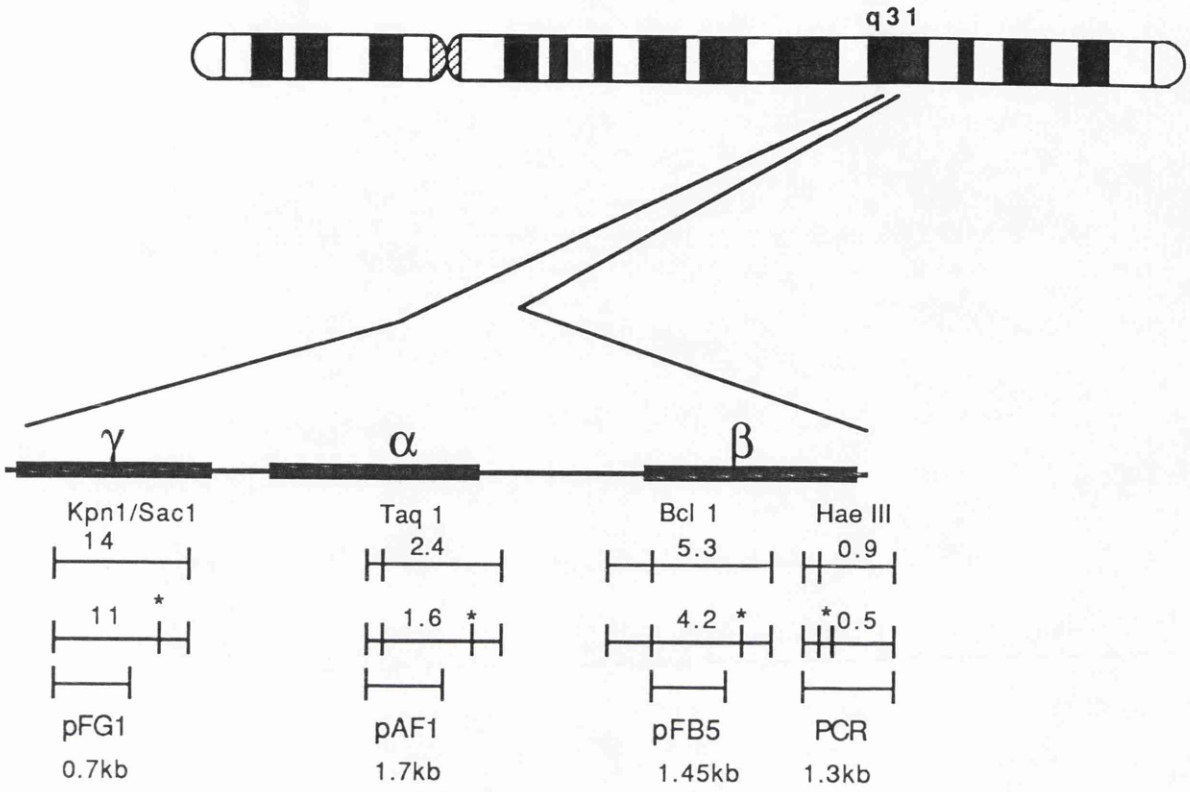

Figure 1.4. Organisation of the human fibrinogen gene locus. The above diagram shows the RFLPs produced by enzymes TaqI BclI, KpnI/SacI and HaeIII. The band size produced in the absence of the polymorphic site is that indicated on the upper band. The presence of a polymorphic site (*) will result in a reduced band size(s), the lower band. The DNA sequences that create the TaqI and the BclI polymorphisms map outside the gene coding region. The TaqI polymorphism is approximately 1000 bp from the 3' end of the alpha fibrinogen gene (Humphries et al, 1984). The BclI variable site occurs in intron 3 of the beta gene and lies closest to the gene promotor region (Humphries et al., 1987). The HaeIII variable site is 453bp upstream from the start of transaction of the beta gene (Thomas et al., 1991). The beta gene is on the opposite strand of DNA to that on the alpha and gamma gene and is therefore transcribed in the opposite orientation.

1.3 Fibrinogen Abnormalities

Abnormal fibrinogens can occur as an inherited abnormality (reviewed by Menache, 1973) or can occur in some pathological conditions such as chronic hepatitis when they are considered to be an acquired state (Soria et al., 1968). Dominant inheritance of dysfibrinogenemia and hypofibrinogenemia is indicated in most family pedigrees yet the presence of abnormal and normal molecules indicates "autosomal codominant" inheritance. The abnormal fibrinogens are named according to the cities where they were discovered (Table 1.2). Many of the fibrinogens have been shown to have an increased clotting time, some with pathological consequences. In the most severe circumstances the affected molecule may never form a clot. Some of the heritable disorders are associated with a decreased rate of fibrinopeptide cleavage. The majority, however, have the defect in the second step: aggregation of fibrin monomer to form a fibrin gel. Some defects can also be associated with the covalent crosslinking of fibrin to form an insoluble clot. (Menache, 1973; Ratnoff, 1973).

The first specific amino acid substitution was found in fibrinogen Detroit (Blomback et al., 1968) where serine is substituted for arginine. The location of the replacement is two residues away from the amino-terminal glycine of the alpha chain (Blomback and Blomback, 1970; Kudryk et al., 1976; Mammen, 1976). It has been suggested that this substitution is responsible for the virtual abolition of fibrin polymerization in the homozygous individual (Mammen et al., 1969, 1976; Menache, 1973). Other fibrinogens such as Cleveland, Nancy and Troyes have a normal rate of fibrinopeptide release but the second step, fibrin monomer aggregation, is abnormal (Forman et al. 1968; Streiff et al., 1971; Soria et al., 1972).

Fibrinogen Paris I is associated with an abnormality in the gamma chain. The affected molecules polymerize poorly and are unable to form cross links in the presence of factor XIII. The abnormal gamma chain is about 2500 daltons heavier than the normal gamma chain corresponding to an additional polypeptide chain of about 22 amino acid residues (Budzynski et al., 1974).

Comprehensive listings of the fibrinogen variants have been assembled by Menache (1973), Rupp and Beck (1984) and have recently been catalogued by Ebert (1990). There are now over 50 known variants. The majority of the alpha- and gamma-dysfibrinogenemias have been shown to result from missense mutations and the beta-dysfibrinogenemias from amino acid substitutions. A tabulation is presented in Table 1.2.

1.4 Fibrinogen-Association with Cardiovascular Disease

A number of studies have indicated a possible link between high fibrinogen levels and the onset of clinically manifest cardiovascular disease. (Stone and Thorp, 1985; O'Connor et al., 1984; Handa et al., 1989; Wilhelmsen et al., 1984; Kannel et al., 1987; Hampton et al., 1966; Meade et al., 1979). This hypothesis has been supported by the Northwick Park Heart Study (NPHS) which showed that high clotting factor levels are predictive for Ischaemic heart disease (IHD) (Meade et al., 1980, 1986). However, association does not prove causation. Atherosclerotic lesions present several characteristics of an inflammatory reponse and fibrinogen is an acute phase protein, therefore, the raised fibrinogen concentrations that are associated with an increased risk of cardiovascular disease may merely be an

Table 1.2 Abnormal Fibrinogens

Name	Molecular Pathology
Due to an Alpha Chain Defect	
AArhus-1	ARG-SER at position 19
Amiens-1	ARG-HIS at position 16
Amiens-2	ARG-HIS at position 16
Bergamo-1	ARG-CYS at position 16
Bergamo-3	ARG-HIS at position 16
Bern 2	ARG-HIS at position 16
Bicetne-1	ARG-HIS at position 16
Birmingham-1	ARG-HIS at position 16
Chapel Hill-2	ARG-HIS at position 16
Clermont-Ferrand-1	ARG-HIS at position 16
Detroit-1	ARG-SER at position 19
Giessen-1	ARG-HIS at position 16
Hershey-2	ARG-CYS at position 16
Hamburg-2	ARG-CYS at position 16
Hamburg-3	ARG-CYS at position 16
Kawaguchi-1	ARG-CYS at position 16
Leitchzield	ARG-HIS at position 19
Leogan	ARG-CYS at position 16
Lille-1	ASP-ASN at position 7
Long Beach-1	ARG-HIS at position 16
Louisville-1	ARG-HIS at position 16
Manachester-1	ARG-HIS at position 16
Metz-1	ARG-CYS at position 16
Munich-1	ARG-ASN at position 19
New Albany	ARG-CYS at position 16

Table 1.2 Continued Abnormal Fibrinogens

Name	Molecular Pathology
Osaka-1	ARG-CYS at position 16
Paris-6	ARG-HIS at position 16
Petosky-1	ARG-HIS at position 16
Rouen-1	GLY-VAL at position 12
Schwarzach-1	ARG-CYS at position 16
Seattle-2	ARG-HIS at position 16
Shezhield-2	ARG-HIS at position 16
Stony Brook-1	ARG-CYS at position 16
Sydney-1	ARG-HIS at position 16
Sydney-2	ARG-HIS at position 16
Tonno-1	ARG-CYS at position 16
White Marsh-1	ARG-HIS at position 16
Zurich-1	ARG-CYS at position 16
Due to a Beta Chain Defect	
Baltimore-2	ARG-LYS at position 448 Substitition is a neutral polymorphism
Christchurch-2	ARG-CYS at position 14
New York-1	De letion 9-27 exon 2 of Beta Gene
Pontoise-2	ALA-THR at position 337
Seattle-1	ARG-CYS at position 14
Due to a Gamma Chain Defect	
Asahi	MET-THR at position 310 Impaired polymerisation of rib monomer
Baltimore-1	GLY-VAL at position 296
Baltimore-3	ASN-ILE at position 308 defective fibrin monomer polymerisation
Baltimore-4	ARG-CYS at position 275

Table 1.2 Continued Abnormal Fibrinogens

Name	Molecular Pathology
Bergamo-2	ARG-HIS at position 275
Zssen-1	ARG-HIS at position 275
Haiza-1	ARG-HIS at position 275
Kyoto-1	ASN-LYS at position 3089
Kyoto-3	ASP-TYR at position 330
Milano-1	ASP-VAL at position 330
Morioka-1	ARG-LYS at position 275
Nagoya-1	GLN-ARG at position 329
Osaka-2	ARG-CYS at position 275
Oslo-3	Elongation of gamma chain at the L. terminal
Paris-1	Abnormally long gamma chains at L. terminal
Perugia-1	ARG-HIS at position 275
Saga-1	ARG-HIS at position 275
Tochigi-1	ARG-CYS at position 275
Tokyo-2	ARG-LYS at position 275
Vlissinger-1	6 base deletion give rise to absence of asparagine 319 and aspartic acid 320.

index of the severity of the manifest disease. To help overcome this problem it has been important to ensure that the measurement of fibrinogen precedes the clinical event, and to establish if the level of fibrinogen is of pathogenic significance, for example, are high levels found in groups at high risk of cardiovascular disease, and low levels in those at low risk.

1.4.1 High Risk Situations

The main environmental cause of high fibrinogen levels is cigarette smoking. A number of studies have established that fibrinogen levels are higher in smokers than non-smokers with a higher fibrinogen level in ex-smokers than those who have never smoked (Kannel et al., 1984, 1987; Meade et al., 1987; Balleisen et al., 1985; Lee et al., 1990). The NPHS showed that stopping/starting or resuming smoking was associated with a decrease or an increase in plasma fibrinogen levels of about 0.15 g/L. These changes would lower or raise the risk of IHD by approximately 20% (Meade et al., 1987).

Increasing age is probably the most powerful predictor of the risk of cardiovascular disease (CVD). Fibrinogen levels have been shown to increase with age in both men and women (Hampton et al., 1966; Ogston and Ogston, 1966 Lee et al., 1990) although in women there is evidence that the rise of fibrinogen with age may be correlated to menopausal status, with higher fibrinogen levels recorded in post- than pre-menopausal women (Meade et al., 1983).

Oral contraceptive users have been shown to have an increased risk of thromboembolic disease and the risk has been shown to rise with increasing oestogen dose (Inman et al., 1970) with a subsequent

elevation in fibrinogen concentration (Meade et al., 1977, 1979, 1980b, 1984).

Diabetes is associated with accelerated atherosclerosis. Levels of fibrinogen have been found to be significantly raised in insulin dependent diabetics. In addition, fibrinogen levels are increased in diabetics with microvascular disease in comparison to those without this complication (Wardle et al., 1973; Fuller et al., 1979).

There may be a psychological/social influence on fibrinogen levels. Mortality from coronary heart disease in civil servants in the lowest grade of employment (unskilled workers) has been found to be about three times that of men in the highest grade of employment (professional classes) with a significant difference in plasma fibrinogen concentration being recorded in the lower grade employment group (Markowe et al., 1985; Lee et al., 1990). Additionally Markowe et al. (1985) reported a relationship between fibrinogen levels and job satisfaction with the highest levels being recorded in those reporting dissatisfaction.

1.4.2 Low Risk Situations

Plasma fibrinogen levels appear very resistant to dietary influences (Meade et al., 1986). Vegetarians have a lower incidence of IHD compared to non-vegetarians although fibrinogen levels are reported to be similar in the two groups (Philips et al., 1978; Haines et al., 1980). There is, however, evidence that white fish consumption is associated with an increased fibrinogen level (Lee et al., 1990).

Moderate alcohol consumption has an apparently protective effect against ischaemic heart disease (IHD) and has been correlated with a lower fibrinogen level in drinkers compared to non-drinkers (Meade et al., 1979, 1984, 1987). This effect is maintained for drinkers in each of the three smoking groups, current, ex- and non-smokers (Lee et al., 1990).

These findings are confirmed in part by Balleisen et al. (1985) who also report a positive correlation between fibrinogen levels and age, smoking, oral contraception and body weight. No correlation was found with either menopausal status or alcohol consumption.

It is now widely accepted that increased fibrinogen levels play an important role in the development of CVD. It is also evident that environmental factors such as smoking, age, oral contraception and diet have a pronounced effect on fibrinogen levels and that these levels correlate positively with the risk of CVD. Even if raised fibrinogen levels are simply an indication of arterial damage an increase in fibrinogen concentration could still play a role in determining the thrombotic process preceeding the clinical event.

1.5 Fibrinogen Association with Peripheral Vascular Disease

Intermittent Claudication (IC) is the most common presentation of peripheral arterial disease (PAD). IC rarely causes tissue degeneration unless complicated by other factors such as advancing age, previous arterial obstruction, poor cardiac output or some metabolic or neurological complication, as in diabetes. The risk of coronary artery disease is much higher in patients with IC than in the general population (Hughson et al., 1978; Kannel and Shurtleff, 1971)

resulting in an increased risk of mortality (Kannel et al., 1984, 1985).

Smoking has been shown to be the strongest determinant in the development of IC (Hughson et al., 1978; Fowkes, 1989; Kannel et al., 1984, 1985) and claudicants who smoke run a much higher risk of dying during follow-up than non-smokers (Reunanen et al., 1982). Smoking is associated with severe atherosclerosis of the abdominal aorta which is associated with a much greater risk of aortic aneurysm and PVD (McGill, 1988). For example, Auerbach and Garfinkel, (1980) found that aortic aneurysms, involving the abdominal aorta, were eight times more common in smokers than in non smokers.

Fibrinogen has been shown to be a associated with IC (Hughson et al., 1978). Raised fibrinogen concentrations are associated with an increased blood viscosity, resulting in diminished tissue perfusion which could, therefore, contribute toward the symptoms of IC (Dintenfass et al., 1966). In 1973 Dormandy et al. studied 126 patients with IC and found that blood viscosity was significantly raised compared to the age-matched, normal controls, with raised plasma fibrinogen being the most common single biochemical abnormality. In this study 33 patients had definite evidence of IHD as well as peripheral arterial disease (PAD) and had significantly higher basal blood viscosity than those with PAD alone. 62 of the patients in this study were followed up over a period of 1-3 years to assess the prognostic significance of the clinical and laboratory findings (Dormandy et al., 1973b). A significant correlation was found between the initial plasma fibrinogen levels and progress. In the patients who improved the mean initial plasma fibrinogen was within normal limits

(<4g/L), whereas in patients who deteriorated the mean initia plasma fibrinogen was almost double the normal mean. Further evidence to support the role of fibrinogen as an important risk factor for PAD has been provided by Powell et al. (1987) and Baxter et al. (1988).

Powell et al. (1987) assessed asymptomatic arterial disease in 100 males and found that the mean plasma fibrinogen concentration in patients with asymptomatic arterial disease was similar to patients with symptomatic arterial disease. Amongst these patients the continuing smokers were found to have a poorer prognosis and an increased fibrinogen level. In 1988 Baxter et al. screened 100 men for asymptomatic peripheral arterial disease and found 15% to be affected. They all had an increased concentration of plasma fibrinogen compared with men having no evidence of arterial disease.

Smoking has been shown to be the major determining factor in the progression of PAD as well as the main enviromental cause for increasing fibrinogen levels. Fibrinogen levels have also been shown to rise depending upon the severity of the disease. This suggests that fibrinogen may be an important risk factor for the development and progression of PAD.

1.6 Fibrinogen Association with Hypertension

Increased plasma fibrinogen concentration and plasma viscosity in hypertensive patients are well known phenomena. Harris and McLoughlin (1930) recorded abnormally increased blood viscosity in untreated hypertensive patients and these results have since been confirmed by Tibblin et al. (1966), Sonkodi et al. (1979), Letcher et al. (1981) Kannel et al., 1987 and Koenig et al. (1989).

Fibrinogen, which is one determinant of plasma viscosity, has consistently been found to be elevated in hypertensive individuals. Sonkodi et al. (1979) were able to demonstrate that fibrinogen levels were significantly raised in 44 hypertensive patients compared to a control group. This has been confirmed by Letcher et al. (1981) who recorded abnormally high blood viscosity in untreated hypertensives and were able to demonstrate a direct relationship with fibrinogen levels, even in haematocrit-matched patients. Increased fibrinogen concentrations as well as fibrin degradation products have also been observed in patients with malignant hypertension (Garvas et al., 1975; Sonkodi et al., 1979). These observations have been substantiated by the work of Beatriz et al. (1989) who showed that the development of malignant hypertension was either directly or indirectly associated with the appearance of high levels of cross-linked forms of fibrinogen.

Other authors have shown that high fibrinogen levels in conjunction with high blood pressure may lead to an increased risk of CVD. The results of a study by Wilhelmsen et al. (1984) indicate an increased risk of stroke due to the synergistic interaction of blood pressure and fibrinogen and Stone et al. (1985) found that the incidence of heart attack in men with high systolic blood pressure was twelve times greater in those with high plasma fibrinogen levels than in those with low fibrinogen levels.

The two major factors which determine arterial blood pressure are cardiac output and resistance to blood flow (Pickering, 1936; Chien 1977; Guyton, 1980). The degree of resistance to blood flow is in turn determined by the diameter of the blood vessels and blood

viscosity (Scholz, 1975). The associations found between plasma fibrinogen levels, blood pressure and plasma viscosity indicate that increases in plasma fibrinogen concentration cause an increase in blood viscosity and, therefore, might play a key role in the early phase of the development of hypertension.

1.7 Fibrinogen Genotyping-Association Studies

Three recent studies have suggested that genetic factors are important in determining plasma fibrinogen levels. Hamsten et al. (1987) studied the relative importance of genetic and cultural factors in influencing fibrinogen levels in nuclear families. The study consisted of 85 families, identified by means of a proband with early myocardial infarction, and 85 families randomly selected from the general population. The results suggested that 51% of the variance of plasma fibrinogen levels is genetically determined and that cultural heritability had little if any effect.

Humphries et al. (1987) carried out an RFLP analysis on 91 healthy individuals from North-West London for the BclI polymorphism at the beta fibrinogen gene locus and the TaqI polymorphism at the alpha fibrinogen gene locus. From this study it was estimated that genetic variation associated with the TaqI and BclI polymorphisms contributed 4.2% and 9.0%, respectively, of the total phenotypic variance in fibrinogen levels. This work was extended in 1991 by Thomas et al. who discovered a new polymorphism, 453bp from the start of transcription of the beta gene, with the enzyme Hae III. They used this polymorphism to analyse a group of 292 healthy men aged 45-69 years. The group was divided into 120 smokers and 172 non-smokers. A significant association was found between fibrinogen levels and

genotype in both the smokers and non smokers. As a result of this study it was estimated that genetic variation associated with the HaeIII polymorphism accounted for 3.1% of the variance in fibrinogen levels.

The findings of Humphries et al.(1987) and Hamsten et al.(1987) were not confirmed in a Norwegian study. Berg and Kierulf (1989) looked at 178 healthy unrelated individuals recruited from monozygotic twins and their families. Gene frequencies and mean fibrinogen concentrations reported by Berg and Kierulf (1989) are similar to those reported by Humphries et al. (1987). There was, however, no association between plasma fibrinogen concentrations and any genotype in either of the two fibrinogen polymorphisms examined. Furthermore the heritability of fibrinogen levels, calculated using the intraclass correlation coefficient in monozygotic twin pairs, indicated a lower level of heritability than that estimated by Hamsten et al., (1987).

Thus at the time this study was initiated the role of genetic variation at the fibrinogen loci in relation to plasma fibrnogen levels was controversial.

1.8 AIMS

The present study was thus undertaken to address the controversy with regard to the genetic determination of fibrinogen levels by variation at the fibrinogen loci; to seek an association between the occurrence of intermittent claudication and fibrinogen genotype; and to seek an association between the predisposition to high or low blood pressure and the fibrinogen genotype. Specific aims within this framework were:

(1) to determine allele and haplotype frequencies at the alpha, beta and gamma fibrinogen loci in Scottish individuals.

(2) to seek linkage disequilibrium between the alpha, beta and gamma fibrinogen loci

(3) to correlate plasma levels of fibrinogen with various fibrinogen alleles/haplotypes

(4) to determine allele frequencies at the beta fibrinogen loci in individuals with contrasting predisposition to high blood pressure.

(5) to determine allele and haplotype frequencies at the fibrinogen loci in patients with intermittent claudication and matched controls.

2.0 PATIENTS AND METHODS

2.1 Materials

A list of materials and stock solutions is presented in Appendix 1. All chemicals used were Analar grade and purchased from BDH limited. Restriction endonucleases and the 1Kb ladder were purchased from BRL Life Technologies Inc. Random primed DNA labelling kits were purchased from Boehringer Mannheim Ltd; Hybond-N filters and P^{32} isotope from Amersham Ltd and reagents for the polymerase chain reaction from Perkin Elmer-Cetus.

2.2 Recruitment of Cases and Controls with Peripheral Vascular Disease

2.2.1 Study Population

In a cross-sectional study of peripheral arterial disease in Edinburgh, nearly 1600 subjects aged 55-74 years were selected by age and sex stratified sampling from registers of 10 general practitioners. Peripheral arterial disease has been measured in all subjects by means of a WHO questionaire for intermittent claudication (Rose, 1962), Doppler ankle pressure (Carter, 1968) and reactive hyperaemia test (Fowkes et al., 1988). In this study, cases and controls were selected from this community sample of 1600 subjects.

2.2.2 Sample Recruitment

Cases and controls were selected from the data base of 1600 subjects. After permission had been obtained from the subjects general practitioner those selected were invited by letter to attend the

Peripheral Vascular Clinic at Edinburgh Royal Infirmary. Non-responders were followed up by letter or telephone and 294 subjects were recruited for this study of which 152 were controls and 142 were patients.

Confirmation of the subject status as a case or control was obtained by repeat administration of the WHO questionaire on angina and intermittent claudication (Rose, 1962) and measurment of ankle and brachial systolic pressure using a Hawksley random zero sphygmomanometer and Doppler probe.

A questionaire on smoking was also repeatedly readministered although smoking histories were not matched. However, on the basis of interim results of a community study of 1000 subjects it would be expected that, of the cases recruited here, 37% will smoke cigarettes and 39% will be ex-smokers. Of the controls 23% will be smokers and 30% will be ex-smokers.

2.2.3 Case Selection

Case selection included all subjects with (i) intermittent claudication according to WHO questionaire. Specificity is 98% (Criqui et al., 1985) and/or (ii) ankle/brachial systolic ratio of <0.8 in at least one leg. Traditionally 0.9 is taken as the cut off point in hospital studies and is over 95% specific (Carter, 1968). Individuals were excluded from the study if they had previous arterial surgery, rest pain, ulcer or gangrene.

2.2.4 Control Selection

The controls were as closely age and sex matched to the cases as possible and were selected according to the following parameters (i) were shown to be free of all cardiovascular diseases (ii) to have an ankle/branchial systolic ratio of >1.0 in both legs (iii) to have a reactive hyperaemic ankle brachial ratio above the lowest 20% of the population distribution.

2.2.5 Laboratory Procedures

Fibrinogen genotyping was carried out by the author in the Duncan Guthrie Institute, Glasgow using gene probes provided by Dr. SE Humphries, Charing Cross Sunley Research Centre.

The following haemostatic factors were measured: fibrinogen by (three methods: nephelometric, clotting and immunological); cross-linked fibrin degradation products, Von Willebrand factor antigen, plasminogen activator inhibitor and B-Thromboglobulin. These assays were carried out by collegues at Edinburgh Royal Infirmary and the Royal Infirmary Glasgow.

2.3 Recruitment of Cases and Controls with Hypertension

2.3.1 Phase I

The aim of the Ladywell study is to identify those individuals most likely and those least likely to develop high blood pressure by the analysis of genetic, physiological and biochemical characteristics.

In 1979 6088 adults, aged 35-64yrs and representing 76% of all adults in this age group, were selected from 24000 individuals registered at the Ladywell medical centre, Edinburgh and screened in order to identify subjects for the MRC mild hypertension trial.

All participants were identified via systematic selection from age/ sex registers and patients on treatment for hypertension were included. 1809 husband/wife pairs were screened, with each person being given an age/sex adjusted blood pressure and parental blood pressures were combined to identify high/high and low/low families. Blood pressure was measured twice using a random zero sphygmomanometer and a standard adult cuff size. Diastolic blood pressures were recorded to the nearest 2mm Hg, after sitting for at least 10 minutes.

In 1986 the practice registers were reviewed. Of the original 1809 couples, 1473 were still resident in the Ladywell catchment area. Letters were sent to each couple asking for the dates of birth and current addresses of offspring. 2879 offspring were identified, of whom 1169 were natural offspring to both parents and were aged between 16-24 on 31-5-1986.

Offspring were invited to the medical centre where their blood pressure, pulse, height and weight were recorded. They were asked to complete questionnaires requesting information on age, sex, marital status, educational attainment, place of residence, employment status, personal medical history, current medication, menarche, contraception, pregnancy, parental medical history, physical activity, cigarette smoking, health knowledge, alcohol consumption and Bortner score. 864 individuals took part. This marked the end of Phase I, which was

completed in March 1987.

To enable a comparison to be made between the blood pressure of parents and offspring a Z score was calculated for each person as follows: the degree of deviation from the mean for each individual's age/sex group was calculated and divided by the standard deviation of blood pressures within the group. Individual scores and age-sex distributions were based on the arithmetic mean of all blood pressure data with two year age groups for the offspring and five year age groups for the parents. The scores for women were based on roughly equal numbers of those taking and not taking the oral contraceptive pill. Parents on treatment for hypertension were given Z scores corresponding to the mean score of the top 5% of the distribution of scores in their age and sex group. Using this approach 95% of scores are within the range from +/-2. Four groups of offspring were identified (Figure 2.1).

2.3.2 Phase II

Random samples were taken from the three largest groups in order to identify 4 groups of approximately 70 individuals. Statistical analysis allowed 238 offspring to be approached at the final stage, of which 201 participated. Height, weight, blood pressure were recorded again a non fasting blood sample was taken and a 24 hour urine sample was collected.

2.3.3 Phase III

One hundred of the 201 offspring took part in detailed biochemical and physiological studies.

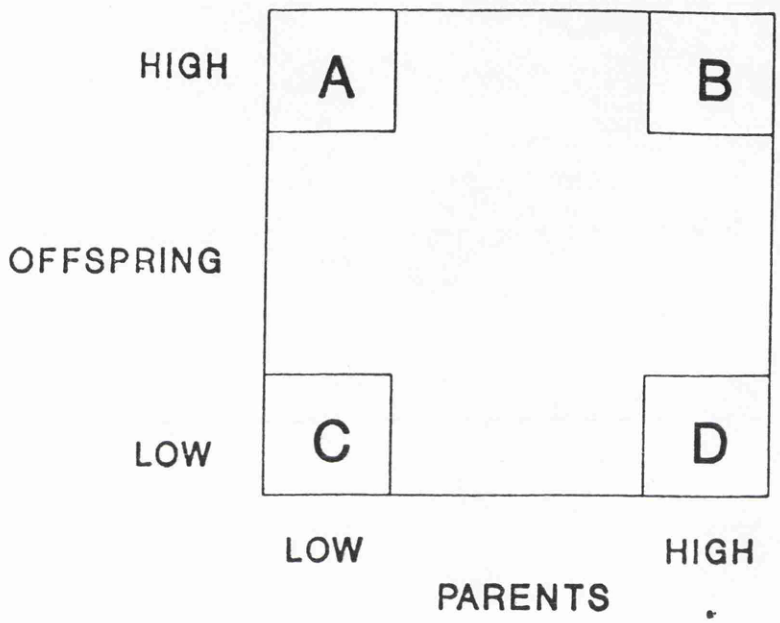

Figure 2.1. Offspring and parental Z scores identified by means of the four corners Approach. The offspring blood pressure scores are on the vertical axis with the parental on the horizontal axis. The scatter diagram was inspected to identify the score level which, when applied to both offspring and parental blood pressure would identify at least 70 offspring in each corner. It was found that a score +/- 0.35 identified the following number of offspring: A. Low parental, high offspring blood pressure 122; B. High parental, high offspring blood pressure 118; C. Low parental, low offspring blood pressure 164 and D. High parental, low offspring blood pressure 68.

2.3.4 Phase IV

Parents height, weight, blood pressure, pulse rate was measured and a blood sample was taken.

2.4 Preparation of Alpha, Beta and Gamma Fibrinogen Probes

The alpha, beta and gamma fibrinogen cDNAs were all cloned into the PstI site of pKT218 which is tetracycline resistant. Probes were grown up as follows:

2.4.1 Plasmid Preparation

10ml of L broth media containing 0.01mg/ml tetracycline was inoculated with a single bacterial colony and incubated at 37°C overnight with vigorous shaking. The following morning 25ml of L broth containing tetracycline was inoculated with 0.1ml of the overnight culture and incubated at 37°C with vigorous shaking until the culture reached its late log phase (OD at 600 nm approx 0.6). 500ml of L broth media with tetracycline was measured into a 2 litre flask and inoculated with the 25ml of late log culture. This was incubated for 2.5hrs at 37°C with vigorous shaking (OD at 600nm approx 0.4). 2.5ml of a solution of chloroamphenicol (34mg/ml in ethanol) was then added and incubation continued overnight.

The 500ml culture was split evenly into 2x250ml centrifuge tubes and spun down at 7K for 10min at 5°C. The supernatant was discarded and the cells resuspended in 25ml of 0.05M Tris-HCl pH8. The cell suspensions were then combined and transferred to a Borex tube and centrifuged at 9K for 10min at 5°C. The supernatant was discarded and the cell pellet resuspended in 25ml of lysis mix containing 0.125g

of freshly prepared lysosyme. This was placed on ice for 35min and stirred occasionally. The lysate was then transferred into a 250ml centrifuge tube to which 40ml of 0.2M NaOH/1% SDS was added and mixed until it became clear and then placed on ice for 5min. 20ml of 3M NaAc pH 4.8 was added and mixed and left on ice for 15min after which the solution was centrifuged at 10K for 10min at 5°C. The supernatant was transferred into a fresh bottle and the pellet discarded. 0.6 volume of ice cold isopropanol was added to the supernatant which was centrifuged at 10K for 10min at 5°C. The supernatant was discarded and 50mls of ice cold ethanol (70%) was added to the pellet and mixed. This was then centrifuged at 10K for 10min at 5°C. The ethanol was poured off and the DNA pellet allowed to air dry. Once dry the DNA was resuspended in 5ml of TE buffer.

2.4.2 Ethidium bromide-Caesium Chloride density Gradient Centrifugation

The resuspended DNA was removed to a universal containing 7.2g of ceasium chloride and made up to a final volume of 6.7ml with TE buffer. 700ul of ethidium bromide (5mg/ml) was then added and the mixture transferred to a Sorvall tube and overlayed with liquid paraffin. The tubes were sealed with an aluminium plug and screw in cover and any air bubbles removed by aspiration prior to sealing. These were then spun at 40,000rpm for a minimum of 40hrs at 20°C in a Sorvall Ultracentrifuge using a T.865.1 rotor.

DNA has a buoyant density of approximately 1.7g/cm^3. The density gradient produced by centrifugation results in the migration of the DNA to a point in the gradient where the CsCl density is also 1.7g/cm^3. With the addition of ethidium bromide (EtBr) super coiled

DNA can be separated from non super coiled molecules by its intercalation between adjacent base pairs, causing partial unwinding of the double helix. This unwinding results in a decrease in the buoyant density which causes super coiled molecules to band in an EtBr-CsCl gradient at a different position to linear and open circular DNA.

At the end of the spin the tubes were opened and the paraffin layer removed with a pipette. The super coiled DNA was collected with a Pasteur pipette and removed to a fresh tube. The EtBr bound to the plasmid DNA was extracted with isopropanol (500ul). This was done by gently mixing and removing the upper aqueous layer containing the EtBr. This process was repeated with the following exception: three drops of water were added before the addition of the isopropanol to prevent the ceasium chloride coming out of solution. Once the aqueous phase was seen to be clear i.e. all the ethidium bromide had been removed, the plasmid DNA was transferred to a freshly washed collodian bag. A needle was forced through the collodian bag about 8mm from the top. Dialysis was carried out overnight with de-ionised water to remove the ceasium chloride, and the plasmid stored at -20°C.

2.4.3 Releasing Insert from Plasmid.

62ul of plasmid was measured into a 0.5ml Eppendorf tube to which 8ul of React 2 buffer, 4ul of spermidine, and 48 units (6ul) of the restriction enzyme PstI were added. This was incubated overnight at 37°C. The released insert was resolved on a 1.2% low melting point (LMP) agarose gel (containing 3ul of EtBr) along with 20ul of 1Kb ladder at 120mA for 4hrs. The gel was then visualised over UV light. PstI digestion of pFAI (containing the alpha cDNA) gives a vector band

of 4.7Kb and a band of 1.7Kb which is used as the probe. pFB5 (beta) gives bands of 4.7Kb (vector) and 1.4Kb (probe). pFGI (gamma) gives bands of 4.7Kb (vector) and two bands of 700bp and 250bp, the larger of which is used as the probe. Figure 2.2 represents an LMP gel on which the released probes were resolved. The required band was excised and removed to a previously weighed Starstedt tube. The Starstedt tube containing the insert was weighed and the weight of the insert derived. A volume of SDW 3 times the weight of the insert was added and the insert boiled for 10min. This was frozen at -20°C until use.

2.5 Genomic DNA Extraction

Genomic DNA was extracted from Buffy coat samples (supplied by colleagues at Edinburgh University) using a method described by Millar et al. (1988).

30ml of ice cold lysis mix was added to 5ml of Buffy coat which was then centrifuged at 2.8k for 10min at 4°C in a IEC DPR-6000 centrifuge. The supernatant was discarded and the pellet resuspended in 3ml of nuclei lysis buffer to which 0.2ml of 10% SDS and 0.1ml of proteinase K were added. This was incubated at 37°C overnight.

After incubation, 1ml of 6M saturated NaCl was added and the mixture shaken vigorously for 15sec. An equal volume of phenol/chloroform mix was added and centrifuged at 2.5K for 15min at 20°C. The top aqueous layer was then removed to a universal and the DNA precipitated by the addition of 2 volumes of ethanol. The precipitated DNA was spooled out and resuspended in a suitable volume of TE buffer and stored at 4oC or frozen at -20°C until use.

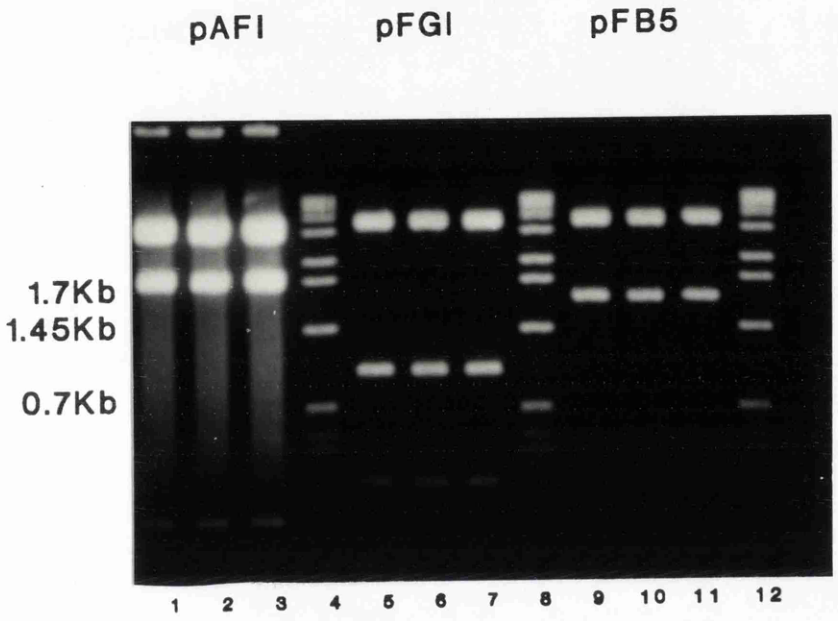

Figure 2.2. Resolution of alpha, beta and gamma fibrinogen probes. The alpha probe (1.7Kb tracks 1-3), gamma fibrinogen probe (0.7Kb tracks 5-7) and the beta fibrinogen probe 1.45Kb (tracks 9-11) were resolved on 1.2% agarose gel containing 3μl of EtBr at 120mA for 4 hours. A 1Kb ladder (tracks 4,8 and 12) was used to identify the required bands which were subsequently excised.

2.5.1 Digestion of Genomic DNA Using Restriction Enzymes

10ug of genomic DNA was used for each digestion and the concentrations of the extracted DNAs were calculated based on the O.D. of the samples at 260nm. The O.D. of a 1 in 100 dilution of each sample was measured in a dual beam spectrophotometer (LKB Biochem Ultrospec 4050) using a deuterium lamp.

Digests were set up using the appropriate restiction enzyme and incubated overnight at the recommended temperature (Table 2.1). Where a single restriction enzyme was used 4ul of the recommended reaction buffer, 2ul of 0.1M spermidine and 3ul of restriction enzyme were added to 10ug of DNA and made up to a final volume of 40ul with the appropriate volume of SDW. In the case of the KpnI/SacI digests 6ul of reaction buffer, 3ul of 0.1M spermidine and 4ul of each enzyme were added and made up to a final volume of 60ul with SDW.

2.5.2 Agarose Gel Electrophoresis of Digests

Digests were resolved on 0.8% agarose gels containing 3ul of EtBr (10mg/ml). Following addition of 6ul of loading mix, digests were loaded and run at 250mA for approximately 3hrs or at 70mA overnight. The gels were visualised on a U.V. transilluminator (316nm) and photographed with a Polaroid type 667 film in a Polaroid instant print camera fitted with a Kodak 22A Wratten filter.

Table 2.1. The international units required for the three enzymes, TaqI, BclI and KpnI/SacI, to cut 1μg of DNA at the stipulated incubation temperature.

Enzyme	Units/ul	Incubation Temp °C
Taql	10	65
Bcll	10	60
Kpnl/Sacl	5/11	37

2.6 Southern Blotting

Gels were treated for 15min with 0.25M HCl with gentle agitation followed by a 30min wash in denaturation solution and a 30min wash in neutralisation solution. The gels were blotted overnight according to the standard Southern blotting procedure (Southern, 1975). A tank was filled with 10xSSC. A wick was prepared by laying a double thickness of Whatman 3mm paper over a glass plate placed across the tank such that both ends of the 3mm paper were within the buffer. Once it was ensured that the wick was entirely wet and uniformly flat, the gel was placed on top of the wick. Hybond-N membrane, labelled and cut to size, was gently layed over the gel. Two sheets of 3mm paper (soaked in 2xSSC and cut to size) were layed on top of the membrane. Again care was taken to ensure that the membrane and 3mm paper were uniformly flat. The remaining area of wick was shrouded in plastic sheeting and a stack of paper hand towels, a glass plate and a small weight placed on top of the gel.

After overnight capillary action the blotting equipment was dismantled, the hybond filters were lifted off the gels and rinsed in 2xSSC. The filters were baked between 2 sheets of 3mm paper for 4-5hrs in a hot air oven set at 80°C.

2.7 Autoradiography

2.7.1 Prehybridisation of Filters

After baking, the filters were sealed in a polythene bag containing 8ml of prehybridisation solution and 80ul of sonicated denatured Salmon sperm DNA (10mg/ml; boiled for 10 min). The bags were incubated for at least 3hrs at 65°C (generally overnight)

2.7.2 Oligonucleotide Labelling Technique

The alpha, beta and gamma fibrinogen probes were labelled using random primed DNA labelling kits. 22ul of probe was measured into a starstedt tube, boiled for 10min and then incubated for 6min at 37°C. 2ul of the oligonucleotides dATP, dGTP and dTTP, 3ul of SDW, 4ul of reaction mix, 2ul of Klenow enzyme and 50uCi of P^{32} dCTP isotope (5ul) were then added to the denatured probe and the mixture incubated for 1-1.5hrs at 37°C. The reaction was terminated by the addition of 2ul of 0.5M EDTA.

2.7.3 Separation of Labelled from Unlabelled Probe

A column packed with Sephadex G50 equilibrated with 1xSSC/0.1% SDS was prepared and topped up with 1xSSC. The column was allowed to settle so that the Sephadex G50 was 4mm below the top. The 1xSSC was removed before addition of the probe. Once the probe had been added the bung sealing the base of the column was removed and the probe allowed to run into the column before the addition of more 1xSSC. The base of the column was monitored and the first peak (> 200cpm) corresponding to the labelled probe was collected in a Sarstedt tube (approximately 0.5ml). The column was then monitored for the second peak which corresponds to the unlabelled probe the presence of which indicates successful separation of the labelled from the unlabelled probe.

2.7.4 Hybridisation of Filters

The collected probe was boiled for 10min and placed on ice for 2min and then added directly to the filters in the polythene bag, ensuring that no air bubbles were trapped when resealing the bag. Hybridisation of the probe to the filters was carried out overnight at

65°C.

2.7.5 Washing Filters

Excess probe was removed by washing the hybridised filters in 2xSSC/0.1% SDS at room temperature for 20 minutes. Filters were washed sequentially in solutions of increasing stringency for 20-30 min each, as required; 1xSSC/0.1% SDS (room temperature), 1xSSC/0.1% SDS (65°C), 0.5xSSC/0.1% SDS (65°C), 0.1xSSC/0.1%SDS (65°C), 0.05xSSC/0.1% SDS (65°C). The strength of the signal was measured between washes with a Seris 900 mini monitor until the signal was below 10cps.

2.7.6 Producing an Autoradiograph

The filters were placed in a light-tight cassette containing intensifying screens with Kodak X-OMAT AR diagnostic film and put down at -70°C for an overnight exposure. The film was then developed in a Fuji RG-11 X-ray film processor. When the band was too faint, filters were put down with fresh film and exposure time increased.

2.7.7 Stripping of Filters

Some of the filters were stripped and reprobed. 0.4M NaOH was added to the filters and then incubated at 42°C for 30min. This was followed by treatment with neutralisation solution at room temperature for 30min. The filter was then rinsed in 2XSSC and blotted dry ready for prehybridisation and was stored until required.

2.8 DNA Amplification by the Polymerase Chain Reaction (PCR)

A specific 1.3Kb region of DNA at the beta fibrinogen locus, as defined by the oligonucleotide primers 766L (5'-CTC CTC ATT GTC GTT GAC ACC TTG GGA C-3') and 767L (5'-AAG AAT TTG GGA ATG CAA TCT CTG CTA CCT-3'), was amplified by PCR (Figure 2.3). A 50ul reaction was set up for each DNA sample. 5ul of dNTPs (2mM), 5ul of 10x reaction buffer, 250ng (0.5ul) of 766L primer, 250ng (0.5ul) of 767L primer, a previously determined volume of distilled water, 1ug of genomic DNA and 2.5iu (0.5ul) of Taq polymerase (Ampli-Taq) were measured into a 0.5ml Eppendorf tube. The primers were denatured at 93°C for 3.0min, annealed at 55°C for 1.0min and extended at 72°C for 2.0min for 1 cycle. The primers were then denatured at 93°C for 1.0min annealed at 55°C for 1.0min and extended at 72°C for 2.0min for 30 cycles in a Perkin Elmer-Cetus thermocycler 480.

2.8.1 PCR Control

2.8.1.1 Negative Control

An aliquot of reaction mixture containing everything except DNA was subjected to PCR with each batch of amplified DNA. Resolution of bands, in the negative control, by electrophoresis indicates the presence of contaminating bacterial or viral DNA.

2.8.2 Check Gel

To check the efficiency of the PCR reaction, 2ul of loading mix was added to 10ul of each of the PCR products which were then loaded and run with 20ul of a 1Kb ladder on a 1.4% SeaKem agarose gel (containing 3ul EtBr) in 0.5x TBE, at 100mA for approximately 30min.

2.9 Digestion of DNA with HaeIII

The PCR product of DNA samples which were proven to have been amplified, i.e. a 1.3Kb band was seen on the check gel, were incubated with the restriction endonuclease HaeIII. Eight iu. (3ul) of HaeIII and 4ul of React 2 buffer were added to 50ul of the PCR products which were then placed in a 37°C water bath for a minimum of 4hrs. HaeIII cuts the 1.3Kb PCR product to give polymorphic bands of 958 or 575 and 383bp. A constant band of 343bp is also detected.

2.9.1 Electrophoresis of the HaeIII Digests

The HaeIII digests were run on a 1.4% SeaKem agarose gel (containing 3ul EtBr) in 0.5xTBE buffer. 6ul of loading mix was added to 50ul of the digest products. The samples were loaded and run with 20ul of a 1Kb ladder at 100mA until the loading mix reached the bottom third of the gel.

2.9.2 Molecular Weight Marker

The 1.3Kb band, the 958bp band, the 575bp band, the 383bp band and the 343bp band were sized using the BRL 1Kb ladder which has approximate molecular weights of 0.5, 1, 1.6, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12Kb.

2.9.3 Visualising the Gels

The gels were visualised and photographed as stated in Section 2.5.2.

2.10 Statistical Methods

Gene frequencies were calculated by gene counting. Chi-square (X^2) analysis using Yates correction (Equation 1) was applied for comparison between allele frequencies reported here and those reported by Humphries et al. (1987) and Berg and Kierulf (1989); to compare alternative allele frequencies between cases and controls and for assesment of linkage disequilibrium.

Equation 1.

$$\Sigma X^2 = \frac{[(\text{Obs-Exp})-0.5]^2}{\text{Exp}}$$

Where: Obs = Observed numbers

Exp = Expected numbers

0.5 = Yates correction

Analysis was carried out to test for linkage disequilibrium between the alleles of the TaqI and BclI RFLPs; TaqI and KpnI/SacI RFLPs; and BclI and KpnI/SacI RFLPs using the standardised disequilibrium coefficient (delta value) (Equation 2). Delta values were calculated according to Chakravarti et al. (1984) and probability (p) values were calculated according to Fishers exact probabilities (Equation 3).

Equation 2.

$$\text{Delta} = \frac{g1g4 - g2g3}{\sqrt{(g_1+g_2)(g_3+g_4)(g_1+g_3)(g_2+g_4)}}$$

Where: $g_i = n_i/n (i=1...,4)$ and n_1, n_2, n_3 and n_4 are the numbers of ++, +-, -+, and -- haplotypes observed in a sample of n chromosomes. Delta is the correlation coefficient between the uniting gametes at the two loci and is independent of the true gene frequencies of the RFLPs.

Equation 3.

$$p = \frac{\Sigma(n_1+n_2)!(n_3+n_4)!(n_1+n_3)!(n_2+n_4)!}{n!n_1!n_2!n_3!n_4!}$$

Where:! denotes factorials and means successive multiplication by cardinal numbers in descending series.

3.0 RESULTS

3.1 The Alpha, Beta and Gamma Fibrinogen Gene RFLPs

The common and rare alleles detected by fibrinogen gene probes for the alpha, beta and gamma fibrinogen loci with the respective enzymes TaqI, BclI and KpnI/SacI are designated A and a, B and b and D and d and these are illustrated in Figure 3.1. The common and rare alleles detected by the restriction enzyme HaeIII at the beta fibrinogen locus are designated H1 and H2 and are illustrated in Figure 3.2.

A total of 235 genomic DNA samples (115 cases and 120 controls) in the peripheral vascular disease project were extracted and analysed with respect to the TaqI and KpnI/SacI gene rated RFLPs in the alpha and gamma fibrinogen loci. Of these individuals 114 cases and 119 controls were genotyped with respect to the BclI generated RFLP at the beta fibrinogen locus. These results are presented in Appendix 2. A further 170 of these samples were genotyped with respect to the HaeIII polymorphism at the beta fibrinogen locus the results of which are presented in Appendix 3.

3.1.1 Overall Genotype frequencies

Gene frequencies were calculated from inspection of the data in Appendices 2 and 3. For the TaqI/alpha cDNA fibrinogen polymorphism allele relative frequencies were 0.75 (for A the 2.4Kb fragment) and 0.25 (for a the 1.6Kb fragment). The BclI/beta cDNA fibrinogen polymorphism allele relative frequencies were 0.85 (for B the 5.3Kb fragment) and 0.15 (for b the 0.15Kb fragment). The KpnI/SacI cDNA

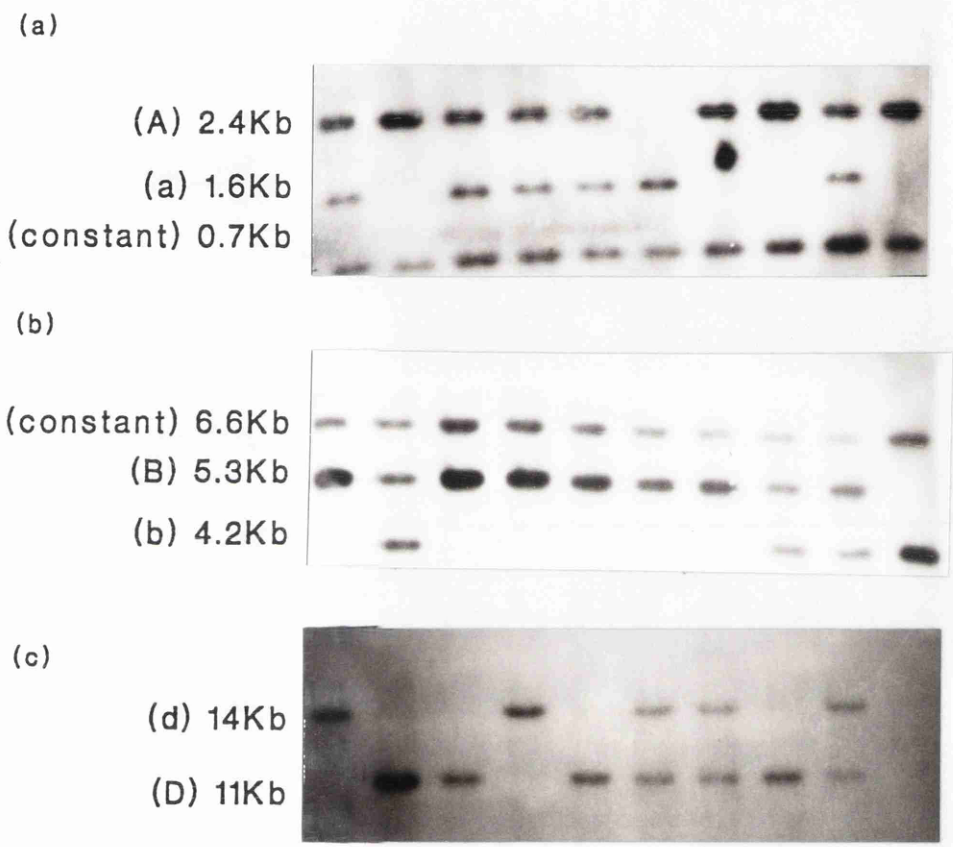

Figure 3.1 a) TaqI polymorphism detected by fibrinogen α cDNA probe. The polymorphic bands are 2.4Kb (A) and 1.6Kb (a) with a 0.7Kb constant band.

b) BclI polymorphism detected by fibringogen ß cDNA probe. The polymorphic bands are 5.3Kb (B) and 4.2Kb (b) with a 6.6Kb constant band.

c) KpnI/SacI polymorphism detected by fibrinogen γ cDNA probe. The polymorphic bands are 11Kb (D) and 14Kb (d).

Figure 3.2 Beta Fibrinogen Gene RFLPs Detected by the Restriction Enzyme HaeIII. The polymerase chain reacion (PCR) was used to amplify a specific 1.3Kb region of DNA upstream of the start of transcription of the beta fibrinogen gene. Restriction digests of the 1.3Kb PCR product produces the following band sizes; 575bp, 383bp (H1) and 958bp (H2) with a 343bp constant band.

fibrinogen polymorphism allele relative frequencies were 0.76 (for D the 11Kb fragment) and 0.24 (for d the 14Kb fragment) and the HaeIII/beta fibrinogen polymorphism allele frequencies were 0.82 (for H1 the 575bp and 383bp fragments) and 0.18 (for H2 the 958bp fragment).

The overall genotype frequencies were compared with those predicted from the Hardy-Weinberg equilibrium and no significant differences were noted (Table 3.1). The genotype frequencies for cases and controls were similarily treated and again no significant differences were observed (Table 3.2).

3.2 Linkage Disequilibrium

X^2 and the standardised disequilibrium coefficient (delta value) were calculated to test the null hypothesis that there was no association between the TaqI/alpha and BclI/beta, the BclI/beta and KpnI/SacI/gamma, the TaqI/alpha and KpnI/SacI/gamma and the BclI/beta and HaeIII/beta markers. The null hypothesis was rejected for all four combinations. X^2, delta and p-values are presented in Table 3.3 and indicate strong linkage disequilibrium between the TaqI/alpha and KpnI/SacI/gamma markers and the BclI/beta and HaeIII/beta markers ($p=1.1x10^{-27}$ and $2.24x10^{-8}$ respectively). A lesser association was found between the alpha/TaqI and beta/BclI loci ($p=6.6x10^{-4}$); beta/BclI and gamma/KpnI/SacI markers ($p=2.5x10^{-3}$); alpha/TaqI and beta/HaeIII ($p=1.3x10^{-4}$) markers and the gamma/KpnI/SacI and beta/HaeIII markers ($p=1.4x10^{-4}$).

Table 3.1 Comparison of observed and expected numbers of persons in the peripheral vascular disease study of different Fibrinogen genotypes for the TaqI, BclI, KpnI/SacI and HaeIII polymorphisms assuming Hardy-Weinberg equilibrium. Where 1=A, B, D or H1 and 2= a, b, d or H2.

Genotypes	TaqI		BclI		KpnI/SacI		HaeIII	
	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.
1-1	133	132.2	168	168.3	136	135.7	117	114
1-2	87	88.1	59	59.4	86	85.7	46	50
2-2	15	14.7	6	5.2	13	13.5	7	6
	X^2=0.025 (NS)		X^2=0.020 (NS)		X^2=0.126 (NS)		X^2 = 0.3 (NS)	

Table 3.2 A comparison of observed and expected numbers of persons with different genotypes for the case and control groups of the peripheral vascular disease study for the TaqI, BclI and KpnI/SacI polymorphisms at the alpha, beta and gamma fibrinogen loci assuming Hardy-Weinberg equilbrium.

Genotypes	Cases		Controls	
	Obs.No	Exp.No.	Obs.No.	Exp. No.
AA	64	66	71	68
Aa	47	42	38	45
aa	4	7	11	7
	X^2=1.41 (NS)		X^2=2.74 (NS)	
BB	74	73	97	96
Bb	35	36	21	22
bb	5	5	1	1
	X^2=0.06 (NS)		X^2=0.06 (NS)	
DD	68	70	71	69
Dd	43	39	40	44
dd	4	6	9	7
H_1H_1	52	52	61	61
H_1H_2	24	24	20	20
H_2H_2	3	3	2	2
	X^2 = 0.00 (NS)		X^2 = 0.00 (NS)	

Table 3.3. Chi-square (X^2), the standardised disequilibrium coefficient (Δ) and p-values from the analysis of linkage disequilibrium/equilibrium between the alpha, beta and gamma loci.

Comparison	X^2	Delta value	p value
β-BclI/α-TaqI	4.06 (NS)	-0.1423	6.6×10^{-4}
β-BclI/γ-KpnI/SacI	2.03 (NS)	-0.1450	2.5×10^{-3}
α-TaqI/γ-KpnI/SacI	358.08 (S)	0.6981	1.1×10^{-27}
β-HaeIII/β-BclI	106.61 (S)	0.462	2.2×10^{-8}
β-HaeIII/α-TaqI	4.49 (NS)	-0.174	1.3×10^{-4}
β-HaeIII/γ-TaqI	5.77 (NS)	-0.188	1.4×10^{-4}

NS = not significant
S = significant

3.3 Relationship of fibrinogen level to genotype

Fibrinogen levels were determined in the peripheral vascular disease case/control study by clotting (Clauss, 1957) or nephelometric assays (Stone et al., 1970) and the results are presented in Tables 3.4-3.7 for cases and controls separately/combined and with/without correction for age, sex and smoking history. None of the three DNA markers TaqI/alpha, BclI/beta, KpnI/SacI/gamma showed any statistically significant association with fibrinogen level in this study.

The 235 individuals in this study, analysed for the markers TaqI/alpha BclI/beta and KpnI/SacI/gamma had one of 13 different haplotypes. The 13 haplotypes are listed in Table 3.8 where the distribution of each haplotype between the cases and controls is indicated. An overall comparison of the difference in haplotype distribution between cases and controls was not statistically significant. Considered individually haplotypes 3, 4, 5 and 7 were more predominant in cases than controls with the difference in distribution of haplotye 5 reached statistical significance.

In Tables 3.9-3.12 the haplotypes for the TaqI/alpha, BclI/beta and KpnI/SacI/gamma markers recorded in this study are presented together with fibrinogen levels, using the clotting method or nepholometric methods with and without adjustment for age, sex and smoking habit.

No statistically significant association was detected for any of these haplotypes in respect of fibrinogen levels. The percentage of phenotypic variability in Tables 3.9-3.12 was calculated using the

Table 3.4. The mean fibrinogen levels and genotypes recorded in the peripheral vascular disease study. Fibrinogen levels were measured by the clotting method for cases and controls separately and combined. Values given here are unadjusted for age, sex and smoking.

	Mean Fibrinogen level (95% CI)		
Genotype	Controls	Cases	Total
AA	2.76 (2.63,2.90)	3.07 (2.91,3.24)	2.90 (2.79,3.01)
Aa	2.73 (2.50,2.98)	3.25 (3.03,3.49)	3.01 (2.84,3.19)
aa	2.72 (2.41,3.06)	3.48 (1.79,6.72)	2.86 (2.53,3.24)
p-value	0.95	0.30	0.48
BB	2.73 (2.61,2.85)	3.16 (2.99,3.34)	2.90 (2.80,3.01)
Bb	2.89 (2.60,3.22)	3.17 (2.94,3.43)	3.07 (2.88,3.26)
bb	2.19	2.97 (2.13,4.14)	2.82 (2.13,3.74)
p-value	0.33	0.82	0.29
dd	2.74 (2.39,3.14)	3.48 (1.80,6.72)	2.91 (2.53,3.34)
Dd	2.61 (2.44,2.79)	3.29 (3.06,3.53)	2.94 (2.79,3.11)
DD	2.83 (2.67,2.99)	3.06 (2.90,3.23)	2.94 (2.82,3.05)
p-value	0.21	0.20	0.99

Table 3.5. The mean fibrinogen levels and genotypes recorded in the peripheral vascular disease study. Fibrinogen levels were measured by the nephelometric method for cases controlled separately and combined. Values given are unadjusted for age, sex and smoking.

	Mean Fibrinogen level (95% CI)		
Genotype	Controls	Cases	Total
AA	3.90 (3.71,4.09)	4.27 (4.06,4.49)	4.07 (3.93,4.21)
Aa	3.84 (3.51,4.17)	4.74 (4.44,5.03)	4.33 (4.07,4.57)
aa	4.15 (3.33,4.78)	4.77 (3.70,5.84)	4.32 (3.70,4.94)
p-value	0.61	0.03	0.13
BB	3.84 (3.66,4.03)	4.51 (4.28,4.73)	4.12 (3.97,4.27)
Bb	4.21 (3.81,4.61)	4.42 (4.09,4.75)	4.34 (4.09,4.59)
bb	3.84	4.56 (3.78,5.34)	4.44 (3.77,5.11)
p-value	0.24	0.89	0.26
dd	4.26 (3.26,5.26)	3.01 (2.89,3.13)	4.42 (3.73,5.11)
Dd	3.69 (3.44,3.94)	2.95 (2.75,3.14)	4.28 (4.05,4.51)
DD	3.98 (3.77,4.19)	3.00 (2.63,3.37)	4.10 (3.95,4.25)
p-value	0.12	0.82	0.27

Table 3.6. The mean fibrinogen levels and genotypes recorded in the peripheral vascular disease study. Fibrinogen levels were measured by the clotting method for cases and controls separately and combined. Values presented here are adjusted for age, sex and smoking.

	Mean Fibrinogen level (95% CI)		
Genotype	Controls	Cases	Total
AA	2.86 (2.81,2.90)	3.03 (2.97,3.08)	2.94 (2.90,2.97)
Aa	2.82 (2.76,2.89)	3.03 (3.54,3.09)	2.94 (2.89,2.98)
aa	2.87 (2.76,2.99)	3.00 (2.59,3.49)	2.90 (2.80,3.00)
p-value	0.57	0.97	0.83
BB	2.86 (2.82,2.89)	3.04 (2.99,3.09)	2.94 (2.90,2.97)
Bb	2.78 (2.70,2.87)	3.00 (2.93,3.07)	2.91 (2.86,2.98)
bb	2.92	3.01 (2.80,3.24)	2.99 (2.83,3.17)
p-value	0.24	0.55	0.67
dd	2.89 (2.75,3.03	3.01 (2.59,3.49)	2.92 (2.80,3.04)
Dd	2.80 (2.74,2.86)	3.06 (3.01,3.11)	2.93 (2.88,2.78)
DD	2.87 (2.83,2.91)	3.01 (2.96,3.06)	2.94 (2.90,2.97)
p-value	0.14	0.44	0.94

Table 3.7. The mean fibrinogen levels and genotypes recorded in the peripheral vascular disease study. Fibrinogen levels were measured by the nephelometric method for cases and controls separately and combined. Values presented here are adjusted for age, sex and smoking.

	Mean Fibrinogen level (95% CI)		
Genotype	Controls	Cases	Total
AA	4.08 (4.01,4.15)	4.30 (4.22,4.38)	4.19 (4.13,4.24)
Aa	4.05 (3.95,4.15)	4.32 (4.23,4.40)	4.20 (4.13,4.27)
aa	4.12 (3.96,4.28)	4.30 (3.81,4.80)	4.17 (4.03,4.32)
p-value	0.72	0.98	0.95
BB	4.10 (4.04,4.15)	4.33 (4.26,4.41)	4.20 (4.15,4.25)
Bb	3.96 (3.82,4.08)	4.26 (4.16,4.36)	4.15 (4.06,4.23)
bb	4.19	4.26 (3.94,4.57)	4.25 (4.00,4.48)
p-value	0.10	0.44	0.48
dd	4.14 (3.95,4.340	4.30 (3.81,4.80)	4.19 (4.03,4.36)
Dd	4.01 (3.72,4.10)	4.35 (4.28,4.43)	4.19 (4.12,4.26)
DD	4.10 (4.04,4.17)	4.28 (4.20,4.37)	4.19 (4.14,4.25)
p-value	0.20	0.52	0.99

(three RFLP genotypes)

Table 3.8. The thirteen different haplotypes recorded in the peripheral vascular disease study and their distribution between the cases and controls.

Haplotype		Total	Controls	Cases	X^2 p-value
1	(aa BB DD)	86	49 (57%)	37 (43%)	0.18
2	(Aa BB Dd)	55	50 (55%)	25 (45%)	0.56
3	(aa Bb DD)	37	16 (43%)	21 (57%)	0.30
4	(Aa BB DD)	10	4 (40%)	6 (60%)	0.47
5	(Aa Bb Dd)	16	4 (25%)	12 (75%)	0.03
6	(AA BB dd)	12	8 (67%)	4 (33%)	0.27
7	(aa bb DD)	4	1 (25%)	3 (75%)	0.73
8	(aa BB Dd)	6	4 (67%)	2 (33%)	-
9	(AA Bb dd)	1	1 (100%)	0	-
10	(Aa Bb DD)	1	0	1 (100%)	-
11	(aa Bb Dd)	1	0	1 (100%)	-
12	(AA BB Dd)	2	2 (100%)	0	-
13	(Aa bb Dd)	2	0	2 (100%)	-

(thru RFLP gene hypm)

Table 3.9. The mean clottable fibrinogen levels and the 13 haplotypes in the peripheral vascular disease study. Values presented here are adjusted for age, sex and smoking. A one way analysis of variance (ANOVA) gave a p-value of 0.94. A between group variablity of 0% and a phenotypic variability of 2.4% were recorded.

Haplotype	Mean Fibrinogen Level (95% CI)	Total	Relative Frequency
1	2.94 (2.90, 2.99)	81	0.368
2	2.93 (2.87, 3.00)	52	0.236
3	2.91 (2.83, 2.99)	34	0.155
4	2.91 (2.80, 3.03)	10	0.045
5	2.93 (2.84, 3.02)	15	0.068
6	2.94 (2.82, 3.06)	11	0.05
7	2.98 (2.67, 3.33)	4	0.018
8	2.89 (2.66, 3.15)	6	0.027
9	2.67	1	0.0045
10	3.17	1	0.0045
11	3.06	1	0.0045
12	2.81 (2.18, 3.62)	2	0.009
13	3.01 (2.76, 3.30)	2	0.009
Total	2.93 (2.91, 2.96)	220	

(three RFLP genotypes)

Table 3.10 The mean clottable fibrinogen levels and the 13 haplotypes in the peripheral vascular disease study. Values presented here are unadjusted for age, sex and smoking. One way analysis of variance (ANOVA) gave a p-value of 0.31. A between group variability of 0.6% and phenotypic variance of 4.9% were recorded.

Haplotype	Mean Fibrinogen level (95% CI)	Total	Relative Frequency
1	2.89 (2.76, 3.02)	82	0.366
2	2.89 (2.69, 3.20)	53	0.237
3	2.99 (2.77, 3.22)	35	0.156
4	3.29 (2.57, 4.21)	10	0.045
5	3.21 (2.83, 3.63)	16	0.071
6-13	2.86 (2.63, 3.11)	28	0.125
Total	2.94 (2.85, 3.03)	224	

(three RFLP genotypes)

Table 3.11. The mean nephelometric fibrinogen levels and the 13 haplotypes for the peripheral vascular disease study. Values presented here are adjusted for age, sex and smoking. A one way analysis of variance (ANOVA) gave a p-value of 0.77. A between group variablity of 0% and a phenotypic variablity of 3.4% were recorded.

Haplotype	Mean Fibrinogen Level (95% CI)	Total	Relative Frequency
1	4.22 (4.15, 4.29)	85	0.371
2	4.19 (4.09, 4.28)	54	0.236
3	4.12 (4.00, 4.24)	36	0.157
4	4.16 (3.97, 4.36)	10	0.044
5	4.19 (4.07, 4.32)	15	0.066
6	4.23 (4.08, 4.38)	12	0.052
7	4.18 (3.76, 4.61)	4	0.017
8	4.07 (3.78, 4.37)	6	0.026
9	3.72	1	0.0044
10	4.47	1	0.0044
11	4.39	1	0.0044
12	4.04 (2.17, 5.89)	2	0.0088
13	4.37 (3.91, 4.82)	2	0.0088
Total	4.19 (4.15, 4.23)	229	

(three RFLP genotypes)

Table 3.12. The mean nephelometric fibrinogen levels and the 13 haplotypes for the peripheral vascular disease study. Values presented here are unadjusted for age, sex and smoking. A one way analysis of variance (ANOVA) gave a p-value of 0.22. A between group variablity of 1.2% and a phenotypic variablity of 4.1% were recorded.

Haplotype	Mean Fibrinogen Level (95% CI)	Total	Relative Frequency
1	4.01 (3.83, 4.20)	80	0.354
2	4.19 (3.89, 4.49)	55	0.234
3	4.19 (3.89, 4.49)	37	0.164
4	4.39 (3.42, 5.37)	9	0.039
5	4.65 (4.13, 5.19)	16	0.011
6-13	4.28 (3.94, 4.62)	29	0.128
Total	4.18 (4.06, 4.31)	226	

method of Sing and Davignon (1985) and as indicated varies from 2.4-4.9%.

155 individuals were genotyped with respect to the HaeIII/beta polymorphism. There was no association between plasma fibrinogen level and any genotype (p=0.58) (Table 3.13). Differences in genotype distribution between cases and controls was then analysed using X^2. No significant difference in distribution was observed (p=0.55) (Table 3.14). The contribution of genotype and smoking status to fibrinogen level was analysed. There was no relationship between the HaeIII genotypes, smoking and fibrinogen level (Table 3.15).

3.4 Fibrinogen genotyping in peripheral vascular disease

The contribution of fibrinogen haplotype to the occurence of peripheral vascular disease was assessed by multiple logistic regression using dummy variables which represent the difference in the log odds of disease for each haplotype (three RFLP genotype) relative to haplotype 1 (any haplotype could have been used as the reference group). The haplotype data was analysed to acertain whether haplotype was a factor associated with the presence of disease after accounting for a number of other possible risk factors. Eight models were used in the analysis and the data is presented in Tables 3.16-3.19. The first model was adjusted for the variables packyears, height, non high density lipoprotein (Non hdl), high density lipoprotein (HDL), triglyceride, current smokers (<5years), ex-smokers, age, sex, social class, and diabetic group (Table 3.16). Using this model haplotype 3 showed a significant association with disease (p<0.05) (Table 3.16). The second model was adjusted for smoking only (packyears, current smokers and ex-smokers). Using this model haplotypes 3 and 5 were found to have a

Table 3.13. The mean level of fibrinogen recorded in the peripheral vascular disease study for the HaeIII polymorphism (adjusted for age, sex and smoking).

NO Individuals	Genotype	Mean Fibrinogen Level g/L	95% CI
108	H1H1	2.92	(2.80 - 3.05)
42	H1H2	2.99	(2.76 - 3.24)
5	H2H2	2.68	(1.94 - 3.04)
		p = 0.58 NS	

NS = Not significant

Table 3.14 Comparison of Genotype distribution between cases and controls for the HaeIII polymorphism.

	H1H1	H1H2	H_2H_2
Control	61	20	2
Cases	52	24	3
	$X^2 = 1.18$	p = 0.55	

Table 3.15. HaeIII genotypes with fibrinogen levels (measured by the clotting method) and smoking status.

	Never Smoked		Ever Smoked		Total	
Genotype	Mean (95% C.I)	no.	Mean (95% C.I)	no.	Mean (95% C.I)	no
H1H1	2.85(2.65,3.07)	39	2.96(2.82,3.12)	69	2.92(2.81,3.05)	108
H1H2	2.77(2.44,3.15)	15	3.09(2.77,3.45)	24	2.99(2.76,3.24)	42
H2H2	2.79(0.15,52.3)	2	2.60(1.29,5.21)	3	2.68(1.94,3.69)	5
p-value	0.91		0.42		0.58	

Table 3.16. logistic regression for haplotypes 2-8 relative to genotype 1 with the log odds of disease adjusted for variables listed below. The odds ratio (OR) gives the relative increase in odds of disease of the genotype in question when compared with haplotype 1.

Haplotypes	Odds Ratio (95% CI) Adjusted for Fibrinogen, smoking, lipids etc	Odds Ratio (95% CI) Adjusted for age, sex & smoking
Haplotype 2: Haplotype 1	0.96 (0.32, 2.88)	0.95 (0.40, 2.27)
Haplotype 3: Haplotype 1	6.31 (1.55, 25.64)*	4.56 (1.55, 13.39)*
Haplotype 4: Haplotype 1	6.14 (0.37, 43.44)+	2.99 (0.67, 13.32)
Haplotype 5: Haplotype 1	6.87 (0.99, 47.23)+	9.17 (1.84, 45.72)*
Haplotype 6: Haplotype 1	0.13 (0.02, 1.02)+	0.41 (0.08, 1.99)
Haplotype 7: Haplotype 1	8.25 (0.17, 399.8)	3,39 (0.20, 56.41)
Haplotype 8: Haplotype 1	0.17 (0.01, 2.14)	0.24 (0.03, 1.96)
Included variables:		
(Fibc)	p=0.019	-
Packyears	p<0.001	p<0.001
Height	p=0.014	-
Nonhdl	p=0.001	-
Hdl	p=0.25	-
(Triglyceride)	p=0.74	-
Current Smoker	p=0.77	p=0.47
Ex-smoker	p=0.77	p=0.47
Age	p=0.18	p=0.32
Sex	p=0.18	p=0.04
Social class	p=0.77	-
Diabetic group	p=0.56	-

\+ p<0.1
* p<0.05
** p<0.01
*** p<0.001

Table 3.17 logistic regression for haplotyes 2-8 relative to haplotype 1 with the log odds of disease adjusted for variables listed. The odds ratio (OR) gives the relative increase in odds of disease of the haplotype in question when compared with haplotype 1.

Haplotypes	Odds ratio (95% CI) Adjusted for plasminogen activator/inhibitor, age, sex & smoking	Odds ratio (95% CI) Adjusted for lipid peroxide, age, sex & smoking
Haplotype 2: Haplotype 1	1.09 (0.45, 2.64)	1.00 (0.42, 2.41)
Haplotype 3: Haplotype 1	4.53 (1.44, 14.27)*	4.29 (1.41, 13.06)*
Haplotype 4: Haplotype 1	3.55 (0.80, 15.66)	2.42 (0.53, 11.05)
Haplotype 5: Haplotype 1	9.14 (1.77, 46.60)*	9.61 (1.89, 48.75)*
Haplotype 6: Haplotype 1	0.43 (0.08, 2.20)	0.45 (0.09, 2.16)
Haplotype 7: Haplotype 1	2.42 (0.11, 53.25)	3.42 (0.15, 80.27)
Haplotype 8: Haplotype 1	0.29 (0.03, 2.58)	0.22 (0.03, 1.92)
Included variables:		
Plasminogen Activator inhibitor	p=0.02	-
Packyears	p<0.001	p<0.001
Current Smoker	p=0.55	p=0.59
Ex-smoker (<5 years)	p=0.58	p=0.67
Age	p=0.26	p=0.33
Sex	p=0.08	p=0.05
Lipid peroxide	-	p=0.15

\+ p<0.1
* p<0.05
** p<0.01
*** p<0.001

Table 3.18. logistic regression for haplotypes 2-8 relative to haplotype 1 with the log odds of disease adjusted for the variables listed below. The odds ratio (OR) gives the relative increase in odds of disease of the haplotype in question when compared with haplotype 1.

Haplotype	Odds ratio (95% CI) Adjusted for Nephelometic Fibrinogen, age, sex & smoking	Odds ratio (95% CI) Adjusted Von Willebrand factor age, sex & smoking
Haplotype 2: Haplotype 1	0.95 (0.38, 2.36)	1.02 (0.42, 2.46)
Haplotype 3: Haplotype 1	4.05 (1.32, 12.44)*	4.43 (1.53, 12.92)**
Haplotype 4: Haplotype 1	2.75 (0.62, 12.23)	2.53 (0.55, 11.54)
Haplotype 5: Haplotype 1	7.59 (1.44, 40.11)*	10.22 (1.99, 52.47)**
Haplotype 6: Haplotype 1	0.28 (0.05, 1.56)	0.37 (0.07, 1.94)
Haplotype 7: Haplotype 1	3.20 (0.17, 60.05)	4.03 (0.19, 83.82)
Haplotype 8: Haplotype 1	0.22 (0.3, 1.84)	0.23 (0.03, 1.94)

Included variables:

Nephelometic Fibrinogen	p=0.10	-
Packyears	p<0.001	p<0.001
Current smoker	p=0.06	p=0.48
Ex smoker (<5 years)	p=0.59	p=0.88
Age	p=0.34	p=0.19
Sex	p=0.04	p=0.05
Von Willebrand factor	-	p=0.06

+	p<0.1
*	p<0.05
**	p<0.01
***	p<0.001

Table 3.19. logistic regression for haplotypes 2-8 relative to haplotype 1 with the log odds of disease adjusted for the variables listed below. The odds ratio (OR) gives the relative increase in odds of disease of the haplotype in question when compared with haplotype 1.

Haplotypes	Odds ratio (95% CI) Adjusted for β thromboglobulin, age & smoking	Odds ratio (95% CI) Adjusted for crosslinked fibrin degredation products, age & smoking
Haplotype 2: Haplotype 1	1.33 (0.52, 3.38)	0.98 (0.40, 2.40)
Haplotype 3: Haplotype 1	2.84 (0.87, 9.25)	3.75 (1.23, 11.45)*
Haplotype 4: Haplotype 1	2.75 (0.58, 13.01)	2.80 (0.59, 13.37)
Haplotype 5: Haplotype 1	6.59 (1.22, 35.62)*	7.58 (1.43, 40.16)*
Haplotype 6: Haplotype 1	0.44 (0.09, 2.21)	0.35 (0.07, 1.85)
Haplotype 7: Haplotype 1	2.47 (0.11, 54.23)	2.97 (0.18, 49.29)
Haplotype 8: Haplotype 1	0.23 (0.03, 2.06)	0.28 (0.03, 2.41)

Included variables:

ß Thromboglobulin	p=0.04	-
Packyears	p<0.001	p<0.0001
Current Smoker	p=0.81	p=0.80
Ex-smoker	p=0.50	p=0.47
Age	p=0.39	p=0.12
Cross-linked fibrin degredation products	-	p=0.02

+	p<0.1
*	p<0.05
**	p<0.01
***	p<0.001

Table 3.20 Comparison of allele frequencies between cases and controls for the Taq I (A,a), BclI (B,b) KpnI/SacI (D,d) and Hae III (H1,H2) polymorphic loci

	Allele frequencies		
	Cases	Controls	
A	0.761	0.776	chi-square = 0.146
a	0.239	0.224	(NS)
B	0.803	0.903	chi-square = 9.48
b	0.197	0.097	($P<0.005$)
D	0.778	0.758	chi-square = 0.262
d	0.222	0.242	(NS)
H1	0.810	0.855	chi-square = 1.196
H2	0.190	0.145	(NS)

significant association with disease (p=0.05) (Table 3.16). The remaining six models were adjusted for age, sex and smoking and one of the following: clottable fibrinogen (Table 3.16), plasminogen activator inhibitor (Table 3.17), lipid peroxide (Table 3.17), nephelometric fibrinogen (Table 3.18), Von Willebrand factor (Table 3.18) and cross-linked fibrin degredation products (Table 3.19). In these six models haplotypes 3 and 5 were again found to have a significant association ($p<0.05$) with disease over and above the level of fibrinogen and other risk factors. The log odds ratio of disease for haplotypes 2-13 relative to haplotype 1 are presented in Tables 3.16-3.19. From Tables 3.16-3.19 the log odds ratio of disease for haplotypes 3 and 5 are significantly higher than that of the reference haplotype 1. Table 3.20 compares allele frequencies in cases and controls and shows a highly significant ($p<0.005$) difference for the BclI polymorphism

3.5 Fibrinogen genotyping in the Ladywell study

168 genomic DNA samples were analysed in the hypertension study using the restriction enzyme BclI and the beta fibrinogen cDNA probe. Gene frequencies were calculated by gene counting and are presented in Appendix 4. The BclI/beta cDNA fibrinogen polymorphism allele frequencies were 0.85 (for B the 5.3Kb fragment) and 0.15 (for b the 0.15Kb fragment). The overall genotype frequencies were compared with those predicted from the Hardy-Weinberg equilibrium and no significant differences were noted (Table 3.21). A comparison of the BclI genotype distribution was carried out between the four different groups in the Ladywell study (Table 3.22). No statistically significant differences were recorded in the genotype distribution between the four groups.

Table 3.21 A comparison of observed and expected numbers of persons in the Ladywell hypertension study of different BclI/beta fibrinogen cDNA RFLP assuming Hardy-Weinberg equilibrium.

Genotype	BclI Polymorphism		X^2
BB	117	118.5	
Bb	47	45.0	0.13 (NS)
bb	4	4.3	

Table 3.22. Comparison of BclI/beta fibrinogen cDNA RFLP distribution between the four groups in the Ladywell hypertension Study. Group A is low parental/high offspring blood pressure, group B is high parental/high offspring blood pressure; group C is low parental/low offspring blood pressure and group D is high parental/low offspring blood pressure.

Genotype	Group A	Group B	Group C	Group D
Obs. No.				
BB	31	31	27	28
Bb	8	11	13	15
bb	2	1	1	0
Exp. No.				
BB	29.6	31.1	27.6	29.6
Bb	10.5	11	12.1	12.1
bb	0.9	1	1.3	1.2
X^2	0.74	0.0003	0.05	0.925
ΣX^2		1.75		

4.0 DISCUSSION

4.1 Fibrinogen genotype frequencies

A comparison of RFLP frequencies, at the alpha, beta and gamma fibrinogen loci, observed in this study with those reported in previous studies is presented in Table 4.1. From Table 4.1 it can be seen that the observed frequencies are broadly similar. The BclI polymorphism frequency recorded in this study did not show a statistically significant difference from that reported by Berg and Kierulf (1989) (X^2=1.28, p=>0.80) but was statistically different from that of Humphries et al. (1987) (X^2=7.28, p=<0.05). It was not possible to carry out a similar comparison between these results and those reported in an American study (Murray et al. 1987) for the KpnI/SacI polymorphism associated with the gamma fibrinogen gene as the number of individuals of each genotype was not reported. Observed differences might be chance or might represent a true population difference which further studies should clarify.

4.2 Linkage Disequilibrium

Linkage disequilibrium was predicted based on the calculation of the probability of association between the TaqI/alpha and BclI/beta, the TaqI/alpha and KpnI/SacI/gamma, the BclI/beta and KpnI/SacI/gamma and the BclI/beta and HaeIII/beta DNA markers (Table 3.3.). Association was found between all four combinations with the highest degree of association between the TaqI/alpha and KpnI/SacI/gamma markers and the BclI/beta and HaeIII/beta markers. The degrees of association seen here would be as expected according to physical mapping data for the three fibrinogen genes and suggest strong linkage disequilibrium between the alpha and gamma loci and

Table 4.1. Reported Fibrinogen gene frequencies for the Scottish, English, Norwegian and American populations.

Enzyme/ probe	Polymorph. bands (Kb)	PVD 1991 Scottish	PVD 1991 Scottish	Ladywell 1991 Scottish	Humphries 1987 English	Berg 1989 Norwegian	Murray 1987 American	Thomas 1991 English
TaqI/pAF	12.4 (A)	0.75			0.63	0.73		
	1.6 (a)	0.25			0.33	0.23		
BclI/pFB5	5.3 (B)	0.85		0.84	0.75	0.83		
	4.2 (b)	0.15		0.16	0.25	0.17		
KpnI/SacI/	11 (D)	0.76					0.82	
FFGI	14 (d)	0.24					0.18	
HaeIII	0.575 (H1)		0.81					0.82
	0.383							
	0.958 (H2)		0.19					0.18
No. individuals Analysed		235	170	168	91	55/118 α ß	~50	292

beta/BclI and beta/HaeIII markers with much weaker linkage disequilibrium between the alpha and beta and the beta and gamma loci. A lack of association between any of the four markers would be unexpected as all three genes are relatively close to each other. The high degree of association between the TaqI/alpha and the KpnI/SacI/gamma markers and the BclI/beta and HaeIII/beta markers would suggest that the physical distance between these markers is small resulting in a low rate of recombination in the interval and that the beta locus is either further away from the alpha and gamma loci or has a higher frequency of recombination in the interval (Figure 4.1). These results are consistant with those of other authors (Table 4.2). Physical mapping of the three genes gives a relative order of alpha, gamma, beta with similar distance between each. Hence relative weaker association of beta to alpha/gamma is not due to distance but may represent a relative hotspot for recombination which arose during the evolution of the three genes. It is believed that the ancestral genes duplicated giving rise to the alpha gene and pre-beta/gamma genes. Further duplication of the pre-beta/gamma genes resulted in individual beta and gamma genes. The present arrangement of the three fibrinogen genes may be explained in one of two ways. The first theory involves a three step mechanism where duplication of the ancestral gene and then of the pre-beta gamma genes is followed by inversion of the alpha/gamma region. The second theory is a two step mechanism in which duplication of the ancestral gene is followed by remote transposition and inversion (Kant et al., 1985). By either mechanism there must be at least one recombination in the alpha/gamma-beta interval.

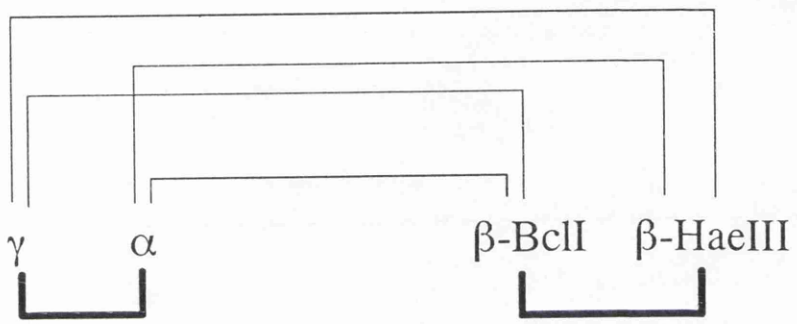

Figure 4.1. Organisation of the three fibrinogen genes according to physical mapping data and the linkage disequilibruim data of the four gene markers the TaqI, BclI, KpnI/SacI and HaeIII variable sites. The bold lines indicate strong association, all other lines indicate an association but to a lesser degree.

Table 4.2. The Standardised disequilibrium coefficient (Δ) and p-values for the α-TaqI/β-BclI, β-BclI/γ-KpnI/SacI α-TaqI/γ-KpnI/SacI and β-BclI/β-HaeIII loci as reported in this study and that of Aschbacher and Murray.

	No. of chromosomes studied	Δ Value	p-value
α-TaqI/β-BclI			
Aschbacher (1985)	-	0.01	0.59
Murray (1985)	~100	0.071	0.327
Present study (1991)	466	0.142	6.6×10^{-4}
β-BclI/γ-KpnI/SacI			
Aschbacher (1985)	-	0.10	0.33
Murray (1985)	~100	0.129	0.109
Present study (1991)	466	0.145	2.5×10^{-3}
α-TaqI/γ-KpnI/SacI			
Aschbacher (1985)	-	-0.88	<0.001
Murray (1985)	~100	-0.884	<0.0001
Present study (1991)	470	0.698	1.1×10^{-27}
β-BclI/β HaeIII			
Present Study 1991	340	0.462	2.2×10^{-8}

Association between the three loci was calculated based on a method by Chakravati using Fishers exact probability. Here the relationship between the delta value and the p-value depends upon the sample size (ie the larger the sample the smaller the p-value) and the more likely association between the points of interest. Thus when comparing the probability of association calculated for samples of greatly differing size it is important to take into account the effect of sample size on the resulting p-value. From table 4.2 the p-value decreases in proportion to the sample size thus using this method it is not possible to calculate the strength of linkage disequilibrium but only to predict the strength based on the degree of association observed and the size of sample used.

Although no association has been found between the TaqI/alpha and BclI/beta and the BclI/beta and KpnI/SacI/gamma markers by other authors it cannot be excluded given the size of these studies. An extremely large sample size would be necessary for the demonstration of negative linkage disequilibrium (Thompson et al. 1988). Based on this argument the apparent linkage equilibrium reported by Murray et al. (1985), calculated using data obtained from the analysis of 100 chromosomes, would need further investigation.

4.3 Relationship of fibrinogen level to genotype

Multiple statistical analyses of data recorded in this study failed to demonstrate any significant correlation of fibrinogen genotype with fibrinogen levels (Tables 3.4-3.7 and 3.9-3.13.) Three other studies have addressed this question. Humphries et al. (1987) carried out an RFLP analysis on 66 healthy men and 25 healthy women with a mean age of 46 years and of whom 15% were cigarette smokers.

Fibrinogen levels were adjusted for age, sex and smoking habits. The results showed that individuals homozygous for the less common BclI site at the beta fibrinogen locus had the highest mean fibrinogen level (3.69g/l) whereas individuals who were homozygous for the common allele had a value of 2.74g/l with heterozygotes having an intermediate value of 2.98g/l. A one way analysis of variance showed that these differences were statistically significant. Differences in fibrinogen level associated with the TaqI polymorphism at the alpha fibrinogen locus were not statistically significant. A comparison of fibrinogen level and genotype for the TaqI and BclI polymorphisms reported by Humphries et al. (1987) and Berg and Kierulf. (1989) and those recorded in this study is presented in Table 4.3. Humphries et al. (1987) report that the overall genetic variation associated with the TaqI and BclI polymorphisms contributes 13.2% of the total phenotypic variance in fibrinogen levels. In this and Berg and Kierulfs (1987) study a much lower contribution to phenotypic variance, 2.4-4.9%, was apparent (Tables 3.9-3.12).

The results of Humphries et al. (1987) were not confirmed by a Norwegian study. Berg and Kierulf (1989) studied 178 healthy unrelated individuals recruited from monozygotic twins and their families. The twins were aged between 38 and 57 years with a mean age of 44 years. One twin was chosen at random from each pair forming a group of unrelated people for use in association analysis. Of the 178 individuals recruited 118 were studied for the BclI polymorphism at the beta gene and 60 were studied for the TaqI polymorphism at the alpha gene. Quantitative fibrinogen levels were measured by the standard thrombin clotting time technique (Clauss, 1957) in each pair of twins for heritability analysis. Fibrinogen levels were adjusted

Table 4.3. The mean fibrinogen levels (adjusted for age, sex and smoking habit) and p-values for the TaqI and BclI polymorphisms in the present peripheral vascular disease study and those reported by Humphries et al. (1987) (on healthy subjects) and Berg and Kierulf (1989) (adjusted for age, and sex only using healthy subjects).

Genotype	Humphries No. individuals	Mean fibrinogen (g/L)	Present study No. individuals	Mean fibrinogen (g/L)	Berg & Kierulf No. individuals	Mean fibrinogen (g/L)
AA	47	3.00	133	2.94	35	2.90
Aa	37	2.74	87	2.94	18	2.91
aa	7	2.76	15	2.90	7	3.00
p-value		**<0.2**		**<0.83**		**p>0.05**
BB	50	2.74	168	2.94	79	2.79
Bb	37	2.98	59	2.91	37	3.00
bb	4	3.69	6	2.99	2	2.95
p-value		**<0.025**		**<0.67**		**p>0.05**

for age and sex. Gene frequencies and mean fibrinogen concentrations reported by Berg and Kierulf (1989) are similar to those reported by Humphries et al. (1987), however, no association was found between plasma fibrinogen concentrations and any genotype for either of the two fibrinogen polymorphisms examined (Table 4.3). Furthermore the heritability of fibrinogen levels, calculated using the intraclass correlation coefficient in monozygotic twin pairs, indicated a lower level of heritability (0.29) than that (0.51) estimated by Hamsten et al. (1987).

The number of individuals involved in this study was larger (235) than that (91) studied by Humphries et al. (1987). Humphries et al. (1987) reported a significant increase in fibrinogen level with genotype however the relative frequency of homozygotes for the rare allele for the BclI polymorphism was low (4 individuals) and calculation of fibrinogen concentration was based on a single measurement. In this study the sample population was Scottish and divided into patients and controls whereas the sample population studied by Humphries et al. (1987) was healthy and of English origin.

More recently Thomas et al. (1991) used a new polymorphism at the beta fibrinogen locus, detected by the enzyme HaeIII, to analyse a group of 292 healthy men aged 45-69 years. The group was divided into 120 smokers and 172 non-smokers. A significant association was found between fibrinogen levels and genotype in both and accounted for 3.1% of the variance in fibrinogen levels. The present study failed to show any association between the HaeIII polymorphism, smoking and fibrinogen level (Table 3.15). However, the population used here was a mixture of men and women, 50% of the sample had peripheral arterial

disease and the number of individuals of the genotype H2H2, the genotype Thomas et al. (1991) had previously associated with an increase in fibrinogen levels, was too low for any significant comparison to be made. Table 4.4 compares the results from this study and those reported by Thomas et al. (1991).

155 individuals in the PVD case and control study were genotyped with respect to the HaeIII polymorphism at the beta fibrinogen locus no statistically significant difference in mean fibrinogen level was recorded between the three groups (Table 3.13). The difference in genotype distribution between cases and controls was analysed using X^2 and no significant difference was recorded (p=0.55) (Table 3.14)

From this study there is no evidence of a statistically significant correlation between fibrinogen level and genotype for the polymorphisms studied. These findings differ from those of Humphries et al. (1987) and Thomas et al. (1991) but are in agreement with those of Berg and Kierulf (1989). However further studies are required.

4.4 Fibrinogen Genotyping in the Ladywell Study

The fibrinogen genotype analysis carried out on the Ladywell subjects detected no significant variation in the allelic frequencies of the four groups studied with the BclI polymorphism. This approach needs to be supported by sib pair analysis before concluding that the fibrinogen beta gene does not have a role in the determination of blood pressure.

Table 4.4 Comparison of HaeIII genotype, Mean fibrinogen level (measured by the clotting method) and smoking status reported in this study and that of Thomas et al. (1991).

	Thomas et al 1991	Mean (SD) Fib. (g/L)	Present Study	Mean (95% CI) Fib. (g/L)
Non Smokers	No. Individuals			
H1H1	115	2.58 (0.51)	39	2.85 (2.65, 3.07)
H1H2	49	2.81 (0.64)	15	2.77 (2.44, 3.15)
H2H2	8	2.91 (0.62)	2	2.79 (0.15, 52.3)
p-value				0.42
Smokers				
H1H1	75	2.90 (0.64)	69	2.96 (2.82, 3.12)
H1H2	42	3.18 (0.87)	24	3.09 (2.77, 3.45)
H2H2	3	3.32 (0.58)	3	2.60 (1.29, 5.21)
p-value				0.91
All				
H1H1	190	2.71 (0.59)*	108	2.92 (2.81, 3.05)
H1H2	91	2.98 (0.77)	42	2.99 (2.76, 3.24)
H2H2	11	3.02 (0.61)	5	2.68 (1.94, 3.69)
p-value				0.58

* Analysis of Variance: F=5.64, P<0.004

4.6 Fibrinogen Genotyping and Peripheral Vascular Disease

A comparison of haplotype distribution between cases and controls was carried out using X^2. The overall X^2 was not statistically significant. However, when individual haplotype distributions were considered haplotypes 3, 4, 5 and 7 were more predominant in the cases than controls, with the difference in distribution of haplotype 5 reaching statistical significance ($p<0.05$) (Table 3.8). It should be noted, however, that the probability of recording a significant result due to chance alone increases with the number of statistical tests applied.

The haplotype data was analysed by multiple logistic regression to acertain whether haplotype was associated with the presence of disease after accounting for a number of other possible risk factors (Tables 3.16-3.19). Haplotype 1 was used as the reference group for the analyses and the odds ratios of disease for haplotypes 2-13 relative to haplotype 1 are presented in Tables 3.16-3.19. Several models were used in the analysis. When the model included the variables packyears, height, non high density lipoprotein (Non hdl), high density lipoprotein (HDL), triglyceride, current smokers (<5years), ex-smokers, age, sex, social class, and diabetic group, haplotype 3 was found to be a significant predictor of disease ($p<0.05$). The second model was adjusted for age, sex and smoking (packyears, current smokers and ex-smokers). Using this model haplotypes 3 and 5 were found to be significant predictors of disease ($p=0.05$). The remaining six models were adjusted for age, sex, smoking and one of the following: plasminogen activator inhibitor, lipid peroxide, clottable fibrinogen, nephelometric fibrinogen, Von Willebrand factor and cross-linked fibrin degradation products. In

these six models haplotypes 3 and 5 were found to be significant predictors of disease over and above the level of fibrinogen and other risk factors. From the odds ratios haplotypes 3 and 5 are more susceptible to peripheral arterial disease than the other haplotypes irrespective of fibrinogen level. Thus individuals with these haplotypes may be genetically predisposed to peripheral vascular disease. This suggests that it may not be the level of fibrinogen but the molecular structure that is important. A genetic variant of fibrinogen has been reported in a 30 year old female presenting with arterial occlusion of the lower limbs (Brook et al., 1983). The woman was found to have an abnormal thrombin and reptilase time. Further investigation revealed an abnormal fibrinogen which has now been named fibrinogen 'Haifa'. The abnormality was localised to the carboxy terminal of the gamma chain resulting in abnormal fibrin monomer polymerization. The abnormal fibrinogen was found to be heritable with an identical abnormality being found in the probands mother and son (Brook et al., 1983).

In this study a statistically significant association was detected between fibrinogen haplotype and the occurence of peripheral vascular disease. There are no comparable studies on peripheral vascular disease in the medical literature and in view of the clinical importance of these ob servations other studies of this nature, in peripheral vascular disease and other cardiovascular diseases, are indicated. More haplotype studies would be necessary to confirm the results of this study and to acertain exactly which are 'high risk' haplotypes and to what degree individuals of a particular haplotype are at risk. Family studies would also have to be performed to clarify the phase at each fibrinogen locus within the combined haplotype.

The next step would then be to sequence the fibrinogen genes associated with the particular haplotype, to identify mutations and determine which ones are pathogenic with the eventual aim of predicting cardiovacular disease. An understanding of the disease mechanism at the molecular level will aid in counselling those known to be predisposed to CVD. The ability to characterise RFLPs and prove association with disease would also allow screening of individuals thought to be at risk. If a genetic predisposition to CVD can be proved, screening and genetic counselling are feasible propositions with the ultimate aim of targetted disease prevention.

APPENDIX 1

MATERIALS and SOLUTIONS

DNA Extraction

Lysis buffer:	0.32M Sucrose
	10mM Tris/HCl pH 7.5
	5mM MgCl2
	1% Triton X-100
Nuclei lysis buffer:	10mm Tris
	0.44M NaCl
	2mM EDTA pH 8.2
Proteinase K:	10mg/ml
10% SDS:	SDS 100g
	H2O 900ml
	adjusted pH to 7.2 with conc HCl, then water added to 1000ml
NaCl:	6M
Phenol/Chloroform mix:	1:1 ratio of water saturated
	phenol (equilibrated with tris to
	pH 7.5, 0.1% 8-hydroxy-quinoline)
	and chloroform
TE buffer:	10mM Tris(pH7.5)
	1mM EDTA(PH8.0)
Loading mix:	50% Glycerol
	2% Ficoll
	50Mm EDTA

1Kb Ladder marker:	25 ul DNA Ladder
	725 ul H_2O
	100 ul Loading mix

Southern Blotting

50 x EB:	Tris base 242g/l
	Glacial acetic acid 57.1ml/l
	0.5M EDTA (pH 8.0) 100ml
Electrophoresis buffer:	0.04M Tris
	0.0002M EDTA
Depurination solution:	0.25M HCl
Denaturation solution:	0.5M NaOH
	1.5M NaCl
Neutralisation solution:	3.0M NaCl
	0.5M Tris HCl(pH7.4)
20x SSC:	3.0M NaCl
	300mM Sodium citrate

Prehybridisation/Hybridisation

Cambridge prehyb:	2x Denhardts solution
	4.5x SSC
	0.1% SDS
	6% w/v PEG
100 x Denhardts solution:	2% Ficoll
	2% Bovine serum albumin
	2% Polyvinylpyrolidone
Ethidium bromide solution:	10ug/ml in H_2O
Sephadex G-50:	30g sephadex/500ml 1xSSC/0.1%SDS
Stripping solution:	0.4M NaOH

Plasmid preparation

L.Broth-per litre:	10g bacto-tryptone
	5g yeast extract
	10g NaCl
Tetracycline:	0.0125g/ml
	(0.01mg/ml tetracycline)
Lysis solution:	50mM glucose
	25mM Tris
	10mM EDTA pH 8
	Lysosyme 2mg/ml
Sodium hydroxide/	
Sodium dodecyl sulphate:	0.2M NaOH/1% SDS
Tris/HCl:	0.05M Tris adjust to pH 8 with conc
	HCl
Sodium Acetate:	3M NaAc adjust to pH 4.8 with
	glacial acetic acid
Isopropanol:	
70% Ethanol:	
Chloroamphenicol:	34mg/ml ~~of~~ ethanol
Ethidium bromide:	5mg/ml

PCR

0.5X TBE Buffer:	90mM Tris base
	90mM Boric acid
	1mM EDTA
10X Reaction Buffer:	100mM Tris-HCl pH 8.3 at 25°C
	500mM KCl
	15mM MgCl2
	0.1% gelatin

dNTPs (Stock 10uM):	Dilute to 2 uM
	Take 50ul of dATP
	dCTP
	dTTP
	dGTP
	and 50ul SDW
	Total Vol = 250ul of 2uM dNTP mix
	Use 10ul per reaction
	Gives final concentration of 200uM
	dNTP per reaction
PCR Reaction Mixture:	5 ul dNTPs 2 uM
	5 ul 10x buffer
	1 ul 766L primer
	1 ul 767L primer
	1 ug DNA
	0.5ul Taq Polymerase
	SDW (volume as required)
	Total volume = 50ul

A 1ml master mix may be prepared based on the above volumes and the required reaction volume removed as needed.

PCR Programmes

1 Cycle:-

Denaturation:	93°C for 3 min
Annealing:	55°C for 1 min
	72°C for 2 min

30 Cycles:-

Denaturation:	93°C 1 min
Annealing:	55°C 1 min
Extension:	72°C 2 min

HaeIII Digests

8 units (3ul) HaeIII

4ul react 2

43 ul PCR product

Total volume=50ul

APPENDIX 2

Fibrinogen genotype results in the peripheral vascular disease case-control study for the TaqI, BclI and KpnI/SacI polymorphism.

PATIENT NO.	LAB NO.	ENZYME	PROBE	RESULTS	GENOTYPE
0003X	R0329-89	TaqI	pAF1	2.4/0.7	AA
	R0329-89	BclI	pFB5	6.6/5.3/4.2	Bb
	R0329-89	KpnI/SacI	pFG1	11	DD
0004K	R0326-89	TaqI	pAF1	1.6/0.7	aa
	R0326-89	BclI	pFB5	6.6/5.3	BB
	R0326-89	KpnI/SacI	pFG1	14	dd
0005W	R0523-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0523-89	BclI	pFB5	6.6/5.3	BB
	R0523-89	KpnI/SacI	pFG1	14/11	Dd
0006H	R0322-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0322-89	BclI	pFB5	6.6/5.3	BB
	R0322-89	KpnI/SacI	pFG1	11	DD
0007V	R0328-89	TaqI	pAF1	2.4/0.7	AA
	R0328-89	BclI	pFB5	6.6/5.3/4.2	Bb
	R0328-89	KpnI/SacI	pFG1	11	DD
0008E	R0327-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0327-89	BclI	pFB5	6.6/5.3	BB
	R0327-89	KpnI/SacI	pFG1	14/11	Dd
0009R	R0325-89	TaqI	pAF1	2.4/0.7	AA
	R0325-89	BclI	pFB5	6.6/5.3/4.2	Bb
	R0325-89	KpnI/SacI	pFG1	11	DD
0010X	R0324-89	TaqI	pAF1	2.4/0.7	AA
	R0324-89	BclI	pFB5	6.6/5.3/4.2	Bb
	R0324-89	KpnI/SacI	pFG1	11	DD
0011K	R0612-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0612-89	BclI	pFB5	6.6/5.3	BB
	R0612-89	KpnI/SacI	pFG1	14/11	Dd
0012W	R0614-89	TaqI	pAF1	2.4/0.7	AA
	R0614-89	BclI	pFB5	6.6/5.3/4.2	Bb
	R0614-89	KpnI/SacI	pFG1	11	DD
0013H	R0617-89	TaqI	pAF1	2,4/1.6/0.7	Aa
	R0617-89	BclI	pFB5	6.6/5.3	BB
	R0617-89	KpnI/SacI	pFG1	14/11	Dd
0014V	R0616-89	TaqI	pAF1	2.4/0.7	AA
	R0616-89	BclI	pFB5	6.6/5.3	BB
	R0616-89	KpnI/SacI	pFG1	11	DD
0015E	R0599-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0599-89	BclI	pFB5	6.6/5.3	BB
	R0599-89	KpnI/SacI	pFG1	14/11	Dd
0016R	R0615-89	TaqI	pAF1	2.4/0.7	AA
	R0615-89	BclI	pFB5	6.6/5.3	BB
	R0615-89	KpnI/SacI	pFG1	11	DD

PATIENT NO.	LAB NO.	ENZYME	PROBE	RESULTS	GENOTYPE
0017B	R0597-89	TaqI	pAF1	2.4/0.7	AA
	R0597-89	BclI	pFB5	6.6/5.3/4.2	Bb
	R0597-89	KpnI/SacI	pFG1	11	DD
0020H	R0587-89	TaqI	pAF1	2.4/0.7	AA
	R0587-89	BclI	pFB5	6.6/5.3	BB
	R0587-89	KpnI/SacI	pFG1	11	DD
0021V	R0613-89	TaqI	pAF1	2.4/0.7	AA
	R0613-89	BclI	pFB5	6.6/5.3/4.2	Bb
	R0613-89	KpnI/SacI	pFG1	11	DD
0022E	R0594-89	TaqI	pAF1	2.4/0.7	AA
	R0594-89	BclI	pFB5	6.6/5.3	BB
	R0594-89	KpnI/SacI	pFG1	11	DD
0023R	R0600-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0600-89	BclI	pFB5	6.6/5.3/4.2	Bb
	R0600-89	KpnI/SacI	pFG1	14/11	Dd
0024B	R0598-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0598-89	BclI	pFB5	6.6/5.3	BB
	R0598-89	KpnI/SacI	pFG1	14/11	Dd
0025M	R0589-89	TaqI	pAF1	2.4/0.7	AA
	R0589-89	BclI	pFB5	6.6/5.3	BB
	R0589-89	KpnI/SacI	pFG1	11	DD
0026A	R0593-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0593-89	BclI	pFB5	6.6/5.3/4.2	Bb
	R0593-89	KpnI/SacI	pFG1	14/11	Dd
0027L	R0596-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0596-89	BclI	pFB5	6.6/5.3	BB
	R0596-89	KpnI/SacI	pFG1	14/11	Dd
0028X	R0604-89	TaqI	pAF1	2.4/0.7	AA
	R0604-89	BclI	pFB5	6.6/5.3	BB
	R0604-89	KpnI/SacI	pFG1	11	DD
0029K	R0610-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0610-89	BclI	pFB5	6.6/5.3	BB
	R0610-89	KpnI/SacI	pFG1	14/11	Dd
0030V	R0793-89	TaqI	pAF1	2.4/0.7	AA
	R0793-89	BclI	pFB5	6.6/4.2	bb
	R0793-89	KpnI/SacI	pFG1	11	DD
0032M	R0583-89	TaqI	pAF1	2.4/0.7	AA
	R0583-89	BclI	pFB5	6.6/5.3	BB
	R0583-89	KpnI/SacI	pFG1	11	DD
0033A	R0601-89	TaqI	pAF1	2.4/0.7	AA
	R0601-89	BclI	pFB5	6.6/5.3	BB
	R0601-89	KpnI/SacI	pFG1	11	DD
0034L	R0621-89	TaqI	pAF1	2.4/0.7	AA
	R0621-89	BclI	pFB5	6.6/5.3/4.2	Bb
	R0621-89	KpnI/SacI	pFG1	11	DD

PATIENT NO.	LAB NO.	ENZYME	PROBE	RESULTS	GENOTYPE
0035X	R0581-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0581-89	BclI	pFB5	6.6/5.3	BB
	R0581-89	KpnI/SacI	pFG1	11	DD
0036K	R0590-89	TaqI	pAF1	1.6/0.7	aa
	R0590-89	BclI	pFB5	6.6/5.3	BB
	R0590-89	KpnI/SacI	pFG1	14	dd
0037W	R0588-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0588-89	BclI	pFB5	6.6/5.3	BB
	R0588-89	KpnI/SacI	pFG1	14/11	Dd
0038H	R0609-89	TaqI	pAF1	2.4/0.7	AA
	R0609-89	BclI	pFB5	6.6/5.3	BB
	R0609-89	KpnI/SacI	pFG1	11	DD
0039V	R0586-89	TaqI	pAF1	2.4/0.7	AA
	R0586-89	BclI	pFB5	6.6/5.3/4.2	Bb
	R0586-89	KpnI/SacI	pFG1	11	DD
0040A	R0607-89	TaqI	pAF1	2.4/0.7	AA
	R0607-89	BclI	pFB5	6.6/5.3	BB
	R0607-89	KpnI/SacI	pFG1	11	DD
0041K	R0591-89	TaqI	pAF1	2.4/0.7	Aa
	R0591-89	BclI	pFB5	6.6/5.3/4.2	Bb
	R0591-89	KpnI/SacI	pFG1	11	DD
0042X	R0592-89	TaqI	pAF1	2.4/0.7	AA
	R0592-89	BclI	pFB5	6.6/5.3/4.2	Bb
	R0592-89	KpnI/SacI	pFG1	11	DD
0043K	R0606-89	TaqI	pAF1	2.4/0.7	AA
	R0606-89	BclI	pFB5	6.6/5.3	BB
	R0606-89	KpnI/SacI	pFG1	11	DD
0044W	R0603-89	TaqI	pAF1	2.4/0.7	AA
	R0603-89	BclI	pFB5	6.6/5.3	BB
	R0603-89	KpnI/SacI	pFG1	11	DD
0045H	R0595-89	TaqI	pAF1	2.4/0.7	AA
	R0595-89	BclI	pFB5	6.6/5.3	BB
	R0595-89	KpnI/SacI	pFG1	11	DD
0046V	R0605-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0605-89	BclI	pFB5	6.6/5.3	BB
	R0605-89	KpnI/SacI	pFG1	14/11	Dd
0047E	R0584-89	TaqI	pAF1	2.4/0.7	AA
	R0584-89	BclI	pFB5	6.6/5.3	BB
	R0584-89	KpnI/SacI	pFG1	11	DD
0048R	R0585-89	TaqI	pAF1	2.4/0.7	AA
	R0585-89	BclI	pFB5	6.6/5.3/4.2	Bb
	R0585-89	KpnI/SacI	pFG1	11	DD
0049B	R0608-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0608-89	BclI	pFB5	6.6/5.3	BB
	R0608-89	KpnI/SacI	pFG1	14/11	Dd

PATIENT NO.	LAB NO.	ENZYME	PROBE	RESULTS	GENOTYPE
0050K	R0631-89	TaqI	pAF1	2.4/0.7	AA
	R0631-89	BclI	pFB5	6.6/5.3	BB
	R0631-89	KpnI/SacI	pFG1	11	DD
0051W	R0634-89	TaqI	pAF1	2.4/0.7	AA
	R0634-89	BclI	pFB5	6.6/5.3	BB
	R0634-89	KpnI/SacI	pFG1	11	DD
0052H	R0643-89	TaqI	pAF1	2.4/0.7	AA
	R0643-89	BclI	pFB5	6.6/5.3	BB
	R0643-89	KpnI/SacI	pFG1	11	DD
0053W	R0618-89	TaqI	pAF1	2.4/0.7	AA
	R0618-89	BclI	pFB5	6.6/5.3	BB
	R0618-89	KpnI/SacI	pFG1	11	DD
0055R	R0626-89	TaqI	pAF1	2.4/0.7	AA
	R0626-89	BclI	pFB5	6.6/5.3	BB
	R0626-89	KpnI/SacI	pFG1	11	DD
0056B	R0655-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0655-89	BclI	pFB5	6.6/5.3/4.2	Bb
	R0655-89	KpnI/SacI	pFG1	14/11	Dd
0057M	R0632-89	TaqI	pAF1	2.4/0.7	AA
	R0632-89	BclI	pFB5	6.6/5.3/4.2	Bb
	R0632-89	KpnI/SacI	pFG1	11	DD
0058A	R0645-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0645-89	BclI	pFB5	6.6/5.3	BB
	R0645-89	KpnI/SacI	pFG1	14/11	Dd
0059L	R0637-89	TaqI	pAF1	2.4/0.7	AA
	R0637-89	BclI	pFB5	6.6/5.3	BB
	R0637-89	KpnI/SacI	pFG1	11	DD
0060V	R0622-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0622-89	BclI	pFB5	6.6/5.3	BB
	R0622-89	KpnI/SacI	pFG1	14/11	Dd
0061E	R0619-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0619-89	BclI	pFB5	6.6/5.3	Bb
	R0619-89	KpnI/SacI	pFG1	14/11	Dd
0062R	R0638-89	TaqI	pAF1	2.4/0.7	AA
	R0638-89	BclI	pFB5	6.6/5.3	BB
	R0638-89	KpnI/SacI	pFG1	11	DD
0063B	R0623-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0623-89	BclI	pFB5	6.6/5.3	BB
	R0623-89	KpnI/SacI	pFG1	14/11	Dd
0064M	R0627-89	TaqI	pAF1	2.4/0.7	AA
	R0627-89	BclI	pFB5	6.6/4.2	bb
	R0627-89	KpnI/SacI	pFG1	11	DD
0065A	R0635-89	TaqI	pAF1	2.4/0.7	AA
	R0635-89	BclI	pFB5	6.6/5.3	BB
	R0635-89	KpnI/SacI	pFG1	11	DD

PATIENT NO.	LAB NO.	ENZYME	PROBE	RESULTS	GENOTYPE
0066L	R0633-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0633-89	BclI	pFB5	6.6/5.3/4.2	Bb
	R0633-89	KpnI/SacI	pFG1	14/11	Dd
0067X	R0620-89	TaqI	pAF1	2.4/0.7	AA
	R0620-89	BclI	pFB5	6.6/5.3	BB
	R0620-89	KpnI/SacI	pFG1	14/11	Dd
0068K	R0629-89	TaqI	pAF1	2.4/0.7	AA
	R0629-89	BclI	pFB5	6.6/5.3	BB
	R0629-89	KpnI/SacI	pFG1	11	DD
0069W	R0624-89	TaqI	pAF1	2.4/0.7	AA
	R0624-89	BclI	pFB5	6.6/5.3	BB
	R0624-89	KpnI/SacI	pFG1	11	DD
0070B	R0653-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0653-89	BclI	pFB5	6.6/5.3/4.2	Bb
	R0653-89	KpnI/SacI	pFG1	14/11	Dd
0071M	R0630-89	TaqI	pAF1	2.4/0.7	AA
	R0630-89	BclI	pFB5	6.6/5.3	BB
	R0630-89	KpnI/SacI	pFG1	14/11	Dd
0072A	R0656-89	TaqI	pAF1	2.4/0.7	AA
	R0656-89	BclI	pFB5	6.6/4.2	bb
	R0656-89	KpnI/SacI	pFG1	11	DD
0073L	R0654-89	TaqI	pAF1	2.4/0.7	AA
	R0654-89	BclI	pFB5	6.6/5.3	BB
	R0654-89	KpnI/SacI	pFG1	11	DD
0074X	R0663-89	TaqI	pAF1	2.4/0.7	AA
	R0663-89	BclI	pFB5	6.6/5.3	BB
	R0663-89	KpnI/SacI	pFG1	11	DD
0075K	R0651-89	TaqI	pAF1	2.4/0.7	AA
	R0651-89	BclI	pFB5	6.6/5.3/4.2	Bb
	R0651-89	KpnI/SacI	pFG1	11	DD
0076W	R0646-89	TaqI	pAF1	2.4/0.7	AA
	R0646-89	BclI	pFB5	6.6/5.3	BB
	R0646-89	KpnI/SacI	pFG1	11	DD
0077H	R0649-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0649-89	BclI	pFB5	6.6/5.3	BB
	R0649-89	KpnI/SacI	pFG1	14/11	Dd
0078V	R0659-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0659-89	BclI	pFB5	6.6/5.3	BB
	R0659-89	KpnI/SacI	pFG1	14/11	Dd
0079E	R0659-89	TaqI	pAF1	2.4/0.7	AA
	R0650-89	BclI	pFB5	6.6/5.3	BB
	R0650-89	KpnI/SacI	pFG1	11	DD
0080L	R0657-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0657-89	BclI	pFB5	6.6/5.3/4.2	Bb
	R0657-89	KpnI/SacI	pFG1	14/11	Dd

PATIENT NO.	LAB NO.	ENZYME	PROBE	RESULTS	GENOTYPE
0081X	R0666-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0666-89	BclI	pFB5	6.6/5.3/4.2	Bb
	R0666-89	KpnI/SacI	pFG1	14/11	Dd
0082K	R0636-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0636-89	BclI	pFB5	6.6/5.3/4.2	Bb
	R0636-89	KpnI/SacI	pFG1	14/11	Dd
0083W	R0642-89	TaqI	pAF1	2.4/0.7	AA
	R0642-89	BclI	pFB5	6.6/5.3	BB
	R0642-89	KpnI/SacI	pFG1	11	DD
0084H	R0647-89	TaqI	pAF1	2.4/0.7	AA
	R0647-89	BclI	pFB5	6.6/5.3/4.2	Bb
	R0647-89	KpnI/SacI	pFG1	11	DD
0085V	R0625-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0625-89	BclI	pFB5	6.6/5.3	BB
	R0625-89	KpnII/Sac I	pFG1	14/11	Dd
0086E	R0652-89	TaqI	pAF1	2.4/0.7	AA
	R0652-89	BclI	pFB5	6.6/5.3	BB
	R0652-89	KpnI/SacI	pFG1	11	DD
0087R	R0670-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0670-89	BclI	pFB5	6.6/5.3/4.2	Bb
	R0670-89	KpnI/SacI	pFG1	11	DD
0088B	R0665-89	TaqI	pAF1	2.4/0.7	AA
	R0665-89	BclI	pFB5	6.6/5.3	BB
	R0665-89	KpnI/SacI	pFG1	11	DD
0089M	R0668-89	TaqI	pAF1	2.4/0.7	AA
	R0668-89	BclI	pFB5	6.6/5.3/4.2	Bb
	R0668-89	KpnI/SacI	pFG1	11	DD
0090W	R0661-89	TaqI	pAF1	2.4/0.7	AA
	R0661-89	BclI	pFB5	6.6/5.3/4.2	Bb
	R0661-89	KpnI/SacI	pFG1	11	DD
0091H	R0662-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0662-89	BclI	pFB5	6.6/5.3	BB
	R0662-89	KpnI/SacI	pFG1	14/11	Dd
0092V	R0669-89	TaqI	pAF1	2.4/0.7	AA
	R0669-89	BclI	pFB5	6.6/5.3	BB
	R0669-89	KpnI/SacI	pFG1	11	DD
0093E	R0664-89	TaqI	pAF1	2.4/0.7	AA
	R0664-89	BclI	pFB5	6.6/5.3/4.2	Bb
	R0664-89	KpnI/SacI	pFG1	11	DD
0094R	R0660-89	Taq	pAF1	1.6/0.7	aa
	R0660-89	BclI	pFB5	6.6/5.3/4.2	Bb
	R0660-89	KpnI/SacI	pFG1	14	dd
0095B	R0667-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0667-89	BclI	pFB5	6.6/5.3	BB
	R0667-89	KpnI/SacI	pFG1	14/11	Dd

PATIENT NO.	LAB NO.	ENZYME	PROBE	RESULTS	GENOTYPE
0096M	R0640-89	TaqI	pAF1	2.4/0.7	AA
	R0640-89	BclI	pFB5	6.6/5.3	BB
	R0-86409	KpnI/SacI	pFG1	11	Dd
0097A	R0641-89	TaqI	pAF1	2.4/0.7	AA
	R0641-89	BclI	pFB5	6.6/5.3	BB
	R0641-89	KpnI/SacI	pFG1	11	DD
0098L	R0628-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0628-89	BclI	pFB5	6.6/5.3	BB
	R0628-89	KpnI/SacI	pFG1	14/11	Dd
0099X	R0648-89	TaqI	pAF1	2.4/0.7	AA
	R0648-89	BclI	pFB5	6.6/5.3	BB
	R0648-89	KpnI/SacI	pFG1	11	DD
0100W	R0658-89	TaqI	pAF1	2.4/0.7	AA
	R0658-89	BclI	pFB5	6.6/5.3	BB
	R0658-89	KpnI/SacI	pFG1	11	DD
0101H	R0544-89	TaqI	pAF1	1.6/0.7	aa
	R0544-89	BclI	pFB5	6.6/5.3	BB
	R0544-89	KpnI/SacI	pFG1	14	dd
0102V	R0555-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0555-89	BclI	pFB5	6.6/5.3	BB
	R0555-89	KpnI/SacI	pFG1	14/11	Dd
0103E	R0568-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0568-89	BclI	pFB5	6.6/5.3	BB
	R0568-89	KpnI/SacI	pFG1	14/11	Dd
0104	R0558-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0558-89	BclI	pFB5	6.6/5.3	BB
	R0558-89	KpnI/SacI	pFG1	14/11	Dd
0105B	R0572-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0572-89	BclI	pFB5	6.6/5.3	BB
	R0572-89	KpnI/SacI	pFG1	14/11	Dd
0106M	R0576-89	TaqI	pAF1	2.4/0.7	AA
	R0576-89	BclI	pFB5	6.6/5.3	BB
	R0576-89	KpnI/SacI	pFG1	11	DD
0108L	R0574-89	TaqI	pAF1	2.4/0.7	AA
	R0574-89	BclI	pFB5	6.6/5.3	BB
	R0574-89	KpnI/SacI	pFG1	11	DD
0110E	R0570-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0570-89	BclI			
	R0570-89	KpnI/SacI	pFG1	14/11	Dd
0111R	R0563-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0563-89	BclI	pFB5	6.6/5.3/4.2	Bb
	R0563-89	KpnI/SacI	pFG1	14/11	Dd
0112B	R0566-89	TaqI	pAF1	2.4/0.7	AA
	R0566-89	BclI	pFB5	6.6/5.3	BB
	R0566-89	KpnI/SacI	pFG1	11	DD

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0113M	R0550-89	TaqI	pAF1	2.4/0.7	AA
	R0550-89	BclI	pFB5	6.6/5.3	BB
	R0550-89	KpnI/SacI	pFG1	11	DD
0114A	R0565-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0565-89	BclI	pFB5	6.6/5.3	BB
	R0565-89	KpnI/SacI	pFG1	14/11	Dd
0115L	R0561-89	TaqI	pAF1	2.4/0.7	AA
	R0561-89	BclI	pFB5	6.6/5.3	BB
	R0561-89	KpnI/SacI	pFG1	11	DD
0116X	R0573-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0573-89	BclI	pFB5	6.6/5.3	BB
	R0573-89	KpnI/SacI	pFG1	11	DD
0117K	R0553-89	TaqI	pAF1	2.4/0.7	AA
	R0553-89	BclI	pFB5	6.6/5.3/4.2	Bb
	R0553-89	KpnI/SacI	pFG1	11	DD
0118W	R0564-89	TaqI	pAF1	2.4/0.7	AA
	R0564-89	BclI	pFB5	6.6/5.3	BB
	R0564-89	KpnI/SacI	pFG1	11	DD
0119H	R0577-89	TaqI	pAF1	1.6/0.7	aa
	R0577-89	BclI	pFB5	6.6/5.3	BB
	R0577-89	KpnI/SacI	pFG1	14	dd
0120M	R0559-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0599-89	BclI	pFB5	6.6/5.3	BB
	R0599-89	KpnI/SacI	pFG1	14/11	Dd
0121A	R0547-89	TaqI	pAF1	2.4/0.7	AA
	R0547-89	BclI	pFB5	6.6/5.3	BB
	R0547-89	KpnI/SacI	pFG1	14/11	Dd
0122L	R0548-89	TaqI	pAF1	2.4/0.7	AA
	R0548-89	BclI	pFB5	6.6/5.3/4.2	Bb
	R0548-89	KpnI/SacI	pFG1	11	DD
0123X	R0546-89	TaqI	pAF1	2.4/0.7	AA
	R0546-89	BclI	pFB5	6.6/5.3/4.2	Bb
	R0546-89	KpnI/SacI	pFG1	11	DD
0124K	R0552-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0552-89	BclI	pFB5	6.6/5.3	BB
	R0552-89	KpnI/SacI	pFG1	14/11	Dd
0125W	R0545-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0545-89	BclI	pFB5	6.6/4.2	bb
	R0545-89	KpnI/SacI	pFG1	14/11	Dd
0126H	R0582-89	TaqI	pAF1	1.6/0.7	aa
	R0582-89	BclI	pFB5	6.6/5.3	BB
	R0582-89	KpnI/SacI	pFG1	14	dd
0127V	R0549-89	TaqI	pAF1	2.4/0.7	AA
	R0549-89	BclI	pFB5	6.6/5.3	BB
	R0549-89	KpnI/SacI	pFG1	11	DD

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0128E	R0554-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0554-89	BclI	pFB5	6.6/5.3	BB
	R0554-89	KpnI/SacI	pFG1	14/11	Dd
0130X	R0538-89	TaqI	pAF1	1.6/0.7	aa
	R0538-89	BclI	pFB5	6.6/5.3	BB
	R0538-89	KpnI/SacI	pFG1	14	dd
0131K	R0537-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0537-89	BclI	pFB5	6.6/5.3/4.2	Bb
	R0537-89	KpnI/SacI	pFG1	14/11	Dd
0132W	R0541-89	TaqI	pAF1	2.4/0.7	AA
	R0541-89	BclI	pFB5	6.6/5.3/4.2	Bb
	R0541-89	KpnI/SacI	pFG1	11	DD
0134V	R0535-89	TaqI	pAF1	2.4/0.7	AA
	R0535-89	BclI	pFB5	6.6/5.3	BB
	R0535-89	KpnI/SacI	pFG1	11	DD
0135E	R0542-89	TaqI	pAF1	2.4/0.7	AA
	R0542-89	BclI	pFB5	6.6/5.3/4.2	Bb
	R0542-89	KpnI/SacI	pFG1	11	DD
0136R	R0539-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0539-89	BclI	pFB5	6.6/5.3	BB
	R0539-89	KpnI/SacI	pFG1	14/11	DD
0138M	R0540-89	TaqI	pAF1	2.4/0.7	AA
	R0540-89	BclI	pFB5	6.6/5.3	BB
	R0540-89	KpnI/SacI	pFG1	11	DD
0139A	R0671-89	TaqI	pAF1	2.4/0.7	AA
	R0671-89	BclI	pFB5	6.6/5.3	BB
	R0671-89	KpnI/SacI	pFG1	11	DD
0140H	R0533-89	TaqI	pAF1	2.4/0.7	AA
	R0533-89	BclI	pFB5	6.6/5.3	BB
	R0533-89	KpnI/SacI	pFG1	11	DD
0143R	R0551-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0551-89	BclI	pFB5	6.6/5.3	BB
	R0551-89	KpnI/SacI	pFG1	14/11	Dd
0144B	R0562-89	TaqI	pAF1	2.4/0.7	AA
	R0562-89	BclI	pFB5	6.6/5.3/4.2	Bb
	R0562-89	KpnI/SacI	pFG1	11	DD
0145M	R0578-89	TaqI	pAF1	2.4/0.7	AA
	R0578-89	BclI	pFB5	6.6/4.2	bb
	R0578-89	KpnI/SacI	pFG1	11	DD
0146A	R0560-89	TaqI	pAF1	2.4/0.7	AA
	R0560-89	BclI	pFB5	6.6/5.3/4.2	Bb
	R0560-89	KpnI/SacI	pFG1	11	DD
0147L	R0580-89	TaqI	pAF1	2.4/0.7	AA
	R0580-89	BclI	pFB5	6.6/5.3/4.2	Bb
	R0580-89	KpnI/SacI	pFG1	11	DD

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0148X	R0571-89	TaqI	pAF1	2.4/0.7	AA
	R0571-89	BclI	pFB5	6.6/5.3	BB
	R0571-89	KpnI/SacI	pFG1	11	DD
0149K	R0567-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0567-89	BclI	pFB5	6.6/5.3	BB
	R0567-89	KpnI/SacI	pFG1	14/11	Dd
0150R	R0557-89	TaqI	pAF1	2.4/0.7	AA
	R0557-89	BclI	pFB5	6.6/5.3/4.2	Bb
	R0557-89	KpnI/SacI	pFG1	11	DD
0152M	R0714-89	TaqI	pAF1	2.4/0.7	AA
	R0714-89	BclI	pFB5	6.6/5.3	BB
	R0714-89	KpnI/SacI	pFG1	11	DD
0153A	R0724-89	TaqI	pAF1	2.4/0.7	AA
	R0724-89	BclI	pFB5	6.6/5.3/4.2	Bb
	R0724-89	KpnI/SacI	pFG1	14/11	Dd
0154L	R0794-89	TaqI	pAF1	2.4/0.7	AA
	R0794-89	BclI	pFB5	6.6/5.3/4.2	Bb
	R0794-89	KpnI/SacI	pFG1	11	DD
0155X	R0711-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0711-89	BclI	pFB5	6.6/5.3/4.2	Bb
	R0711-89	KpnI/SacI	pFG1	14/11	Dd
0156K	R0717-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0717-89	BclI	pFB5	6.6/5.3	BB
	R0717-89	KpnI/SacI	pFG1	14/11	Dd
0157W	R0575-89	TaqI	pAF1	2.4/0.7	AA
	R0575-89	BclI	pFB5	6.6/5.3	BB
	R0575-89	KpnI/SacI	pFG1	11	DD
0158H	R0719-89	TaqI	pAF1	2.4/1.6/0.	Aa
	R0719-89	BclI	pFB5	6.6/5.3	BB
	R0719-89	KpnI/SacI	pFG1	11	DD
0159V	R0698-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0698-89	BclI	pFB5	6.6/5.3	BB
	R0698-89	KpnI/SacI	pFG1	14/11	Dd
0160A	R0723-89	TaqI	pAF1	2.4/0.7	AA
	R0723-89	BclI	pFB5	6.6/5.3	BB
	R0723-89	KpnI/SacI	pFG1	11	DD
0161L	R0715-89	TaqI	pAF1	2.4/0.7	AA
	R0715-89	BclI	pFB5	6.6/5.3/4.2	Bb
	R0715-89	KpnI/SacI	pFG1	11	DD
0162X	R0695-89	TaqI	pAF1	2.4/0.7	AA
	R0695-89	BclI	pFB5	6.6/5.3	BB
	R0695-89	KpnI/SacI	pFG1	11	DD
0163K	R0718-89	TaqI	pAF1	2.4/0.7	AA
	R0718-89	BclI	pFB5	6.6/5.3	BB
	R0718-89	KpnI/SacI	pFG1	11	DD

PATIENT NO.	LAB NO.	ENZYME	PROBE	RESULTS	GENOTYPE
0164W	R0713-89	TaqI	pAF1	2.4/0.7	AA
	R0713-89	BclI	pFB5	6.6/5.3	BB
	R0713-89	KpnI/SacI	pFG1	11	DD
0165H	R0788-89	TaqI	pAF1	2.4/0.7	AA
	R0788-89	BclI	pFB5	6.6/5.3	BB
	R0788-89	KpnI/SacI	pFG1	11	DD
0167E	R0694-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0694-89	BclI	pFB5	6.6/5.3	BB
	R0694-89	KpnI/SacI	pFG1	14/11	Dd
0168R	R0710-89	TaqI	pAF1	2.4/07	AA
	R0710-89	BclI	pFB5	6.6/5.3	BB
	R0710-89	KpnI/SacI	pFG1	11	DD
0169B	R0689-89	TaqI	pAF1	2.4/0.7	AA
	R0689-89	BclI	pFB5	6.6/5.3/4.2	Bb
	R0689-89	KpnI/SacI	pFG1	11	DD
0170K	R0708-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0708-89	BclI	pFB5	6.6/5.3	BB
	R0708-89	KpnI/SacI	pFG1	14/11	Dd
0171W	R0703-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0703-89	BclI	pFB5	6.6/5.3	BB
	R0703-89	KpnI/SacI	pFG1	14/11	Dd
0173V	R0692-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0692-89	BclI	pFB5	6.6/5.3	BB
	R0692-89	KpnI/SacI	pFG1	14/11	Dd
0174E	R0683-89	TaqI	pAF1	2.4/0.7	AA
	R0683-89	BclI	pFB5	6.6/5.3	BB
	R0683-89	KpnI/SacI	pFG1	11	DD
0175R	R0700-89	TaqI	pAF1	2.4/0.7	AA
	R0700-89	BclI	pFB5	6.6/5.3/4.2	Bb
	R0700-89	KpnI/SacI	pFG1	11	DD
0176B	R0709-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0709-89	BclI	pFB5	6.6/5.3	BB
	R0709-89	KpnI/SacI	pFG1	14/11	Dd
0177M	R0716-89	TaqI	pAF1	2.4/0.7	AA
	R0716-89	BclI	pFB5	6.6/5.3	BB
	R0716-89	KpnI/SacI	pFG1	11	DD
0178A	R0720-89	TaqI	pAF1	1.6/0.7	aa
	R0720-89	BclI	pFB5	6.6/5.3	BB
	R0720-89	KpnI/SacI	pFG1	14	dd
0179L	R0696-89	TaqI	pAF1	2.4/0.7	AA
	R0696-89	BclI	pFB5	6.6/5.3	BB
	R0696-89	KpnI/SacI	pFG1	11	DD
0180V	R0679-89	TaqI	pAF1	2.4/0.7	AA
	R0679-89	BclI	pFB5	6.6/5.3	BB
	R0679-89	KpnI/SacI	pFG1	11	DD

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0181E	R0687-89	TaqI	pAF1	2.4/0.7	AA
	R0687-89	BclI	pFB5	6.6/5.3/4.2	Bb
	R0687-89	KpnI/SacI	pFG1	11	DD
0182R	R0697-89	TaqI	pAF1	1.6/0.7	aa
	R0697-89	BclI	pFB5	6.6/5.3	BB
	R0697-89	KpnI/SacI	pFG1	14	dd
0183B	R0681-89	TaqI	pAF1	2.4/0.7	AA
	R0681-89	BclI	pFB5	6.6/5.3	Bb
	R0681-89	KpnI/SacI	pFG1	11	DD
0184M	R0682-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0682-89	BclI	pFB5	6.6/5.3	BB
	R0682-89	KpnI/SacI	pFG1	14/11	Dd
0185A	R0706-89	TaqI	pAF1	2.4/0.7	AA
	R0706-89	BclI	pFB5	6.6/5.3	BB
	R0706-89	KpnI/Sac I	pFG1	11	DD
0186L	R0691-89	TaqI	pAF1	2.4/0.7	AA
	R0691-89	BclI	pFB5	6.6/5.3	BB
	R0691-89	KpnI/SacI	pFG1	11	DD
0187X	R0680-89	TaqI	pAF1	2.4/0.7	AA
	R0680-89	BclI	pFB5	6.6/5.3	BB
	R0680-89	KpnI/SacI	pFG1	11	DD
0188K	R0688-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0688-89	BclI	pFB5	6.6/5.3/4.2	Bb
	R0688-89	KpnI/SacI	pFG1	14/11	Dd
0189W	R0678-89	TaqI	pAF1	2.4/0.7	AA
	R0678-89	BclI	pFB5	6.6/5.3	BB
	R0678-89	KpnI/SacI	pFG1	11	DD
0190B	R0690-89	TaqI	pAF1	2.4/0.7	AA
	R0690-89	BclI	pFB5	6.6/5.3/4.2	Bb
	R0690-89	KpnI/SacI	pFG1	11	DD
0191M	R0693-89	TaqI	pAF1	2.4/0.7	AA
	R0693-89	BclI	pFB5		
	R0693-89	KpnI/SacI	pFG1	11	DD
0192E	R0722-89	TaqI	pAF1	2.4/0.7	AA
	R0722-89	BclI	pFB5	6.6/5.3/4.2	Bb
	R0722-89	KpnI/SacI	pFG1	11	DD
0193L	R0702-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0702-89	BclI	pFB5	6.6/5.3	BB
	R0702-89	KpnI/SacI	pFG1	11	DD
0194X	R0699-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0699-89	BclI	pFB5	6.6/5.3	BB
	R0699-89	KpnI/SacI	pFG1	11	DD
0195K	R0686-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0686-89	BclI	pFB5	6.6/5.3	BB
	R0686-89	KpnI/SacI	pFG1	11	DD

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0196W	R0707-89	TaqI	pAF1	2.4/0.7	AA
	R0707-89	BclI	pFB5	6.6/5.3/4.2	Bb
	R0707-89	KpnI/SacI	pFG1	11	DD
0197H	R0704-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0704-89	BclI	pFB5	6.6/5.3	BB
	R0704-89	KpnI/SacI	pFG1	14/11	Dd
0198V	R0684-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0684-89	BclI	pFB5	6.6/5.3/4.2	Bb
	R0684-89	KpnI/SacI	pFG1	14/11	Dd
0199E	R0685-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0685-89	BclI	pFB5	6.6/5.3	BB
	R0685-89	KpnI/SacI	pFG1	14/11	Dd
0201M	R0676-89	TaqI	pAF1	2.4/0.7	AA
	R0676-89	BclI	pFB5	6.6/5.3	BB
	R0676-89	KpnI/SacI	pFG1	11	DD
0202E	R0672-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0672-89	BclI	pFB5	6.6/5.3	BB
	R0672-89	KpnI/SacI	pFG1	11	DD
0203L	R0677-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0677-89	BclI	pFB5	6.6/5.3/4.2	Bb
	R0677-89	KpnI/SacI	pFG1	14/11	Dd
0204X	R0673-89	TaqI	pAF1	2.4/0.7	AA
	R0673-89	BclI	pFB5	6.6/5.3	BB
	R0673-89	KpnI/SacI	pFG1	11	DD
0205K	R0674-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0674-89	BclI	pFB5	6.6/5.3	BB
	R0674-89	KpnI/SacI	pFG1	14/11	Dd
0206W	R0801-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0801-89	BclI	pFB5	6.6/5.3	BB
	R0801-89	KpnI/SacI	pFG1	14/11	Dd
0207H	R0816-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0816-89	BclI	pFB5	6.6/5.3	BB
	R0816-89	KpnI/SacI	pFG1	11	DD
0210L	R0799-89	TaqI	pAF1	2.4/0.7	AA
	R0799-89	BclI	pFB5	6.6/5.3	BB
	R0799-89	KpnI/SacI	pFG1	11	DD
0211X	R0797-89	TaqI	pAF1	2.4/0.7	AA
	R0797-89	BclI	pFB5	6.6/5.3	BB
	R0797-89	KpnI/SacI	pFG1	11	DD
0212K	R0803-89	TaqI	pAF1	2.4/0.7	AA
	R0803-89	BclI	pFB5	6.6/5.3	BB
	R0803-89	KpnI/SacI	pFG1	11	DD
0213W	R0789-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0789-89	BclI	pFB5	6.6/5.3	BB
	R0789-89	KpnI/SacI	pFG1	11	DD

PATIENT NO.	LAB NO.	ENZYME	PROBE	RESULTS	GENOTYPE
0214H	R0812-89	TaqI	pAF1	1.6/0.7	aa
	R0812-89	BclI	pFB5	6.6/5.3	BB
	R0812-89	KpnI/SacI	pFG1	14	dd
0215V	R0819-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0819-89	BclI	pFB5	6.6/5.3	BB
	R0819-89	KpnI/SacI	pFG1	14/11	Dd
0217R	R0820-89	TaqI	pAF1	1.6/0.7	aa
	R0820-89	BclI	pFB5	6.6/5.3	BB
	R0820-89	KpnI/SacI	pFG1	14/11	Dd
0218B	R0811-89	TaqI	pAF1	2.4/0.7	AA
	R0811-89	BclI	pFB5	6.6/5.3	BB
	R0811-89	KpnI/SacI	pFG1	11	DD
0219M	R0798-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0798-89	BclI	pFB5	6.6/5.3	BB
	R0798-89	KpnI/SacI	pFG1	14/11	Dd
0221H	R0786-89	TaqI	pAF1	2.4/0.7	AA
	R0786-89	BclI	pFB5	6.6/5.3	BB
	R0786-89	KpnI/SacI	pFG1	11	DD
0223E	R0806-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0806-89	BclI	pFB5	6.6/5.3	BB
	R0806-89	KpnI/SacI	pFG1	14/11	Dd
0225B	R0810-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0810-89	BclI	pFB5	6.6/5.3	BB
	R0810-89	KpnI/SacI	pFG1	14/11	Dd
0227A	R0822-89	TaqI	pAF1	2.4/0.7	AA
	R0822-89	BclI	pFB5	6.6/5.3/4.2	Bb
	R0822-89	KpnI/SacI	pFG1	11	DD
0228L	R0817-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0817-89	BclI	pFB5	6.6/5.3	BB
	R0817-89	KpnI/SacI	pFG1	14/11	Dd
0229X	R0813-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0813-89	BclI	pFB5	6.6/5.3	BB
	R0813-89	KpnI/SacI	pFG1	14/11	Dd
0230E	R0800-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0800-89	BclI	pFB5	6.6/5.3/4.2	Bb
	R0800-89	KpnI/SacI	pFG1	14/11	Dd
0231R	R0823-89	TaqI	pAF1	2.4/0.7	AA
	R0823-89	BclI	pFB5	6.6/5.3	BB
	R0823-89	KpnI/SacI	pFG1	11	DD
0232B	R0825-89	TaqI	pAF1	2.4/0.7	AA
	R0825-89	BclI	pFB5	6.6/5.3/4.2	BB
	R0825-89	KpnI/SacI	pFG1	11	DD
0233M	R0826-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0826-89	BclI	pFB5	6.6/5.3/4.2	Bb
	R0826-89	KpnI/SacI	pFG1	14/11	Dd

PATIENT NO.	LAB NO.	ENZYME	PROBE	RESULTS	GENOTYPE
0234A	R0808-89	TaqI	pAF1	2.4/0.7	AA
	R0808-89	BclI	pFB5	6.6/5.3	BB
	R0808-89	KpnI/SacI	pFG1	11	DD
0235L	R0821-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0821-89	BclI	pFB5	6.6/5.3/4.2	Bb
	R0821-89	KpnI/SacI	pFG1	14/11	Dd
0236X	R0807-89	TaqI	pAF1	2.4/0.7	AA
	R0807-89	BclI	pFB5	6.6/5.3	BB
	R0807-89	KpnI/SacI	pFG1	11	DD
0237K	R0829-89	TaqI	pAF1	2.4/1.6/0.7	aa
	R0829-89	BclI	pFB5	6.6/5.3	BB
	R0829-89	KpnI/SacI	pFG1	14/11	Dd
0238W	R0828-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0828-89	BclI	pFB5	6.6/5.3	BB
	R0828-89	KpnI/SacI	pFG1	14/11	Dd
0239H	R0827-89	TaqI	pAF1	2.4/1.6/0.7	AA
	R0827-89	BclI	pFB5	6.6/5.3	BB
	R0827-89	KpnI/SacI	pFG1	14/11	Dd
0240M	R0802-89	TaqI	pAF1	2.4/0.7	AA
	R0802-89	BclI	pFB5	6.6/5.3	BB
	R0802-89	KpnI/SacI	pFG1	11	DD
0241A	R0818-89	TaqI	pAF1	2.4/0.7	AA
	R0818-89	BclI	pFB5	6.6/5.3	BB
	R0818-89	KpnI/SacI	pFG1	11	DD
0242L	R0785-89	TaqI	pAF1	1.6/0.7	aa
	R0785-89	BclI	pFB5	6.6/5.3	BB
	R0785-89	KpnI/SacI	pFG1	14	dd
0243X	R0815-89	TaqI	pAF1	2.4/0.7	AA
	R0815-89	BclI	pFB5	6.6/5.3	BB
	R0815-89	KpnI/SacI	pFG1	11	DD
0245W	R0809-89	TaqI	pAF1	2.4/0.7	AA
	R0809-89	BclI	pFB5	6.6/5.3	BB
	R0809-89	KpnI/SacI	pFG1	11	DD
0247V	R0805-89	TaqI	pAF1	2.4/0.7	AA
	R0805-89	BclI	pFB5	6.6/5.3	BB
	R0805-89	KpnI/SacI	pFG1	14/11	Dd
0249R	R0824-89	TaqI	pAF1	2.4/0.7	AA
	R0824-89	BclI	pFB5	6.6/5.3	BB
	R0824-89	KpnI/SacI	pFG1	14/11	Dd
0250X	R0784-89	TaqI	pAF1	2.4/0.7	AA
	R0784-89	BclI	pFB5	6.6/5.3	BB
	R0784-89	KpnI/SacI	pFG1	11	DD
0252W	R0790-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0790-89	BclI	pFB5	6.6/5.3	BB
	R0790-89	KpnI/SacI	pFG1	14/11	Dd

PATIENT NO.	LAB NO.	ENZYME	PROBE	RESULTS	GENOTYPE
0251K	R0791-89	TaqI	pAF1	1.6/0.7	aa
	R0791-89	BclI	pFB5	6.6/5.3	BB
	R0791-89	KpnI/SacI	pFG1	14	dd
0253H	R0134-89	TaqI	pAF1	1.6/0.7	aa
	R0134-89	BclI	pFB5	6.6/5.3	BB
	R0134-89	KpnI/SacI	pFG1	14	dd
0254V	R0139-89	TaqI	pAF1	2.4/0.7	AA
	R0139-89	BclI	pFB5	6.6/5.3	BB
	R0139-89	KpnI/SacI	pFG1	11	DD
0255E	R0130-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0130-89	BclI	pFB5	6.6/5.3	BB
	R0130-89	KpnI/SacI	pFG1	14/11	Dd
0257B	R0138-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0138-89	BclI	pFB5	6.6/4.2	bb
	R0138-89	KpnI/SacI	pFG1	14/11	Dd
0259A	R0141-89	TaqI	pAF1	2.4/0.7	AA
	R0141-89	BclI	pFB5	6.6/5.3	BB
	R0141-89	KpnI/SacI	pFG1	11	DD
0260H	R0140-89	TaqI	pAF1	2.4/0.7	AA
	R0140-89	BclI	pFB5	6.6/5.3	BB
	R0140-89	KpnI/SacI	pFG1	14/11	Dd
0261V	R0136-89	TaqI	pAF1	2.4/0.7	AA
	R0136-89	BclI	pFB5	6.6/5.3	BB
	R0136-89	KpnI/SacI	pFG1	11	DD
0262E	R0131-89	TaqI	pAF1	2.4/0.7	AA
	R0131-89	BclI	pFB5	6.6/5.3	BB
	R0131-89	KpnI/SacI	pFG1	11	DD
0263R	R0133-89	TaqI	pAF1	2.4/1.6/0.7	Aa
	R0133-89	BclI	pFB5	6.6/5.3	BB
	R0133-89	KpnI/SacI	pFG1	14/11	Dd

APPENDIX 3

Fibrinogen genotype results in the peripheral vascular disease case-control study for the HaeIII polymorphism using the polymerase chain reaction (PCR)

PATIENT NO.	LAB NO.	ENZYME	RESULTS
0003X	R0329-89	HaeIII	H1H2
0004K	R0326-89	HaeIII	H1H1
0005W	R0523-89	HaeIII	H1H1
0006H	R0322-89	HaeIII	
0007V	R0328-89	HaeIII	H1H2
0008E	R0327-89	HaeIII	
0009R	R0325-89	HaeIII	H1H2
0010X	R0324-89	HaeIII	H1H2
0011K	R0612-89	HaeIII	H1H1
0012W	R0614-89	HaeIII	H1H2
0013H	R0617-89	HaeIII	H1H1
0014V	R0616-89	HaeIII	H1H2
0015E	R0599-89	HaeIII	H1H1
0016R	R0615-89	HaeIII	H1H1
0017B	R0597-89	HaeIII	H1H2
0020H	R0587-89	HaeIII	H1H1
0021V	R0613-89	HaeIII	H1H2
0022E	R0594-89	HaeIII	
0023R	R0600-89	HaeIII	H1H2
0024B	R0598-89	HaeIII	
0025M	R0589-89	HaeIII	H1H1
0026A	R0593-89	HaeIII	H1H2
0027L	R0596-89	HaeIII	H1H1
0028X	R0604-89	HaeIII	H1H1
0029K	R0610-89	HaeIII	H1H1
0030V	R0793-89	HaeIII	H2H2
0032M	R0583-89	HaeIII	H1H1
0033A	R0601-89	HaeIII	
0034L	R0621-89	HaeIII	
0035X	R0581-89	HaeIII	H1H2
0036K	R0590-89	HaeIII	
0037W	R0588-89	HaeIII	H1H1
0038H	R0609-89	HaeIII	
0039V	R0586-89	HaeIII	H1H1
0040A	R0607-89	HaeIII	
0041K	R0591-89	HaeIII	H1H2
0042X	R0592-89	HaeIII	H1H2
0043K	R0606-89	HaeIII	
0044W	R0603-89	HaeIII	H1H1
0045H	R0595-89	HaeIII	
0046V	R0605-89	HaeIII	H1H1
0047E	R0584-89	HaeIII	
0048R	R0585-89	HaeIII	H1H2
0049B	R0608-89	HaeIII	H1H1
0050K	R0631-89	HaeIII	
0051W	R0634-89	HaeIII	H1H1
0052H	R0643-89	HaeIII	H1H1
0053W	R0618-89	HaeIII	H1H1
0055R	R0626-89	HaeIII	
0056B	R0655-89	HaeIII	H1H2
0057M	R0632-89	HaeIII	H1H2
0058A	R0645-89	HaeIII	H1H1
0059L	R0637-89	HaeIII	H1H1
0060V	R0622-89	HaeIII	H1H1

PATIENT NO.	LAB NO.	ENZYME	RESULTS
0061E	R0619-89	HaeIII	H1H1
0062R	R0638-89	HaeIII	H2H2
0063B	R0623-89	HaeIII	H1H1
0064M	R0627-89	HaeIII	H2H2
0065A	R0635-89	HaeIII	
0066L	R0633-89	HaeIII	H2H2
0067X	R0620-89	HaeIII	H1H1
0068K	R0629-89	HaeIII	H1H1
0069W	R0624-89	HaeIII	H1H1
0070B	R0653-89	HaeIII	H1H1
0071M	R0630-89	HaeIII	
0072A	R0656-89	HaeIII	
0073L	R0654-89	HaeIII	
0074X	R0663-89	HaeIII	
0075K	R0651-89	HaeIII	H1H2
0076W	R0646-89	HaeIII	
0077H	R0649-89	HaeIII	H1H1
0078V	R0659-89	HaeIII	H1H1
0079E	R0659-89	HaeIII	H1H1
0080L	R0657-89	HaeIII	H1H2
0081X	R0666-89	HaeIII	H1H2
0082K	R0636-89	HaeIII	
0083W	R0642-89	HaeIII	H1H1
0084H	R0647-89	HaeIII	
0085V	R0625-89	HaeIII	
0086E	R0652-89	HaeIII	H1H1
0087R	R0670-89	HaeIII	H1H2
0088B	R0665-89	HaeIII	
0089M	R0668-89	HaeIII	
0090W	R0661-89	HaeIII	H2H2
0091H	R0662-89	HaeIII	H1H1
0092V	R0669-89	HaeIII	
0093E	R0664-89	HaeIII	H1H2
0094R	R0660-89	HaeIII	H1H2
0095B	R0667-89	HaeIII	H1H1
0096M	R0640-89	HaeIII	
0097A	R0641-89	HaeIII	H1H1
0098L	R0628-89	HaeIII	
0099X	R0648-89	HaeIII	H1H1
0100W	R0658-89	HaeIII	
0101H	R0544-89	HaeIII	H1H1
0102V	R0555-89	HaeIII	H1H1
0103E	R0568-89	HaeIII	H1H1
0104R	R0558-89	HaeIII	H1H1
0105B	R0572-89	HaeIII	H1H1
0106M	R0576-89	HaeIII	H1H2
0108L	R0574-89	HaeIII	H1H1
0110E	R0570-89	HaeIII	
0111R	R0563-89	HaeIII	H1H2
0112B	R0566-89	HaeIII	
0113M	R0550-89	HaeIII	H1H1
0114A	R0565-89	HaeIII	H1H1
0115L	R0561-89	HaeIII	
0116X	R0573-89	HaeIII	H1H1
0117K	R0553-89	HaeIII	H1H2
0118W	R0564-89	HaeIII	H1H2
0119H	R0577-89	HaeIII	H1H1
0120M	R0559-89	HaeIII	H1H1
0121A	R0547-89	HaeIII	
0122L	R0548-89	HaeIII	H1H2

PATIENT NO.	LAB NO.	ENZYME	RESULTS
0123X	R0546-89	HaeIII	H1H2
0124K	R0552-89	HaeIII	H1H1
0125W	R0545-89	HaeIII	H1H2
0126H	R0582-89	HaeIII	H1H1
0127V	R0549-89	HaeIII	
0128E	R0554-89	HaeIII	H1H1
0130X	R0538-89	HaeIII	H1H1
0131K	R0537-89	HaeIII	
0132W	R0541-89	HaeIII	
0134V	R0535-89	HaeIII	H1H1
0135E	R0542-89	HaeIII	H1H2
0136R	R0539-89	HaeIII	
0138M	R0540-89	HaeIII	
0139A	R0671-89	HaeIII	H1H1
0140H	R0533-89	HaeIII	H1H1
0143R	R0551-89	HaeIII	H1H1
0144B	R0562-89	HaeIII	H1H1
0145M	R0578-89	HaeIII	H1H2
0146A	R0560-89	HaeIII	
0147L	R0580-89	HaeIII	
0148X	R0571-89	HaeIII	
0149K	R0567-89	HaeIII	H1H1
0150R	R0557-89	HaeIII	H1H2
0152M	R0714-89	HaeIII	
0153A	R0724-89	HaeIII	
0154L	R0794-89	HaeIII	H1H2
0155X	R0711-89	HaeIII	H1H2
0156K	R0717-89	HaeIII	
0157W	R0575-89	HaeIII	H1H1
0158H	R0719-89	HaeIII	
0159V	R0698-89	HaeIII	H1H1
0160A	R0723-89	HaeIII	H1H1
0161L	R0715-89	HaeIII	
0162X	R0695-89	HaeIII	
0163K	R0718-89	HaeIII	H1H1
0164W	R0713-89	HaeIII	H1H1
0165H	R0788-89	HaeIII	H1H1
0167E	R0694-89	HaeIII	H1H1
0168R	R0710-89	HaeIII	H1H2
0169B	R0689-89	HaeIII	H1H2
0170K	R0708-89	HaeIII	H1H1
0171W	R0703-89	HaeIII	H1H1
0173V	R0692-89	HaeIII	
0174E	R0683-89	HaeIII	H1H1
0175R	R0700-89	HaeIII	H1H1
0176B	R0709-89	HaeIII	H1H1
0177M	R0716-89	HaeIII	H1H1
0178A	R0720-89	HaeIII	H1H1
0179L	R0696-89	HaeIII	
0180V	R0679-89	HaeIII	H1H1
0181E	R0687-89	HaeIII	H1H2
0182R	R0697-89	HaeIII	
0183B	R0681-89	HaeIII	H1H1
0184M	R0682-89	HaeIII	H1H1
0185A	R0706-89	HaeIII	H1H1
0186L	R0691-89	HaeIII	
0187X	R0680-89	HaeIII	H1H1
0188K	R0688-89	HaeIII	H1H2
0189W	R0678-89	HaeIII	H1H1
0190B	R0690-89	HaeIII	H2H2

PATIENT NO.	LAB NO.	ENZYME	RESULTS
0191M	R0693-89	HaeIII	H1H1
0192E	R0722-89	HaeIII	H2H2
0193L	R0702-89	HaeIII	
0194X	R0699-89	HaeIII	
0195K	R0686-89	HaeIII	H1H1
0196W	R0707-89	HaeIII	
0197H	R0704-89	HaeIII	H1H1
0198V	R0684-89	HaeIII	H1H2
0199E	R0685-89	HaeIII	H1H1
0201M	R0676-89	HaeIII	H1H1
0202E	R0672-89	HaeIII	H1H1
0203L	R0677-89	HaeIII	H1H2
0204X	R0673-89	HaeIII	H1H1
0205K	R0674-89	HaeIII	H1H1
0206W	R0801-89	HaeIII	
0207H	R0816-89	HaeIII	H1H1
0210L	R0799-89	HaeIII	
0211X	R0797-89	HaeIII	
0212K	R0803-89	HaeIII	H1H1
0213W	R0789-89	HaeIII	H1H1
0214H	R0812-89	HaeIII	H1H1
0215V	R0819-89	HaeIII	H1H1
0217R	R0820-89	HaeIII	H1H1
0218B	R0811-89	HaeIII	H1H1
0219M	R0798-89	HaeIII	H1H1
0221H	R0786-89	HaeIII	H1H1
0223E	R0806-89	HaeIII	H1H1
0225B	R0810-89	HaeIII	H1H1
0227A	R0822-89	HaeIII	H1H2
0228L	R0817-89	HaeIII	H1H1
0229X	R0813-89	HaeIII	
0230E	R0800-89	HaeIII	H1H2
0231R	R0823-89	HaeIII	H1H1
0232B	R0825-89	HaeIII	H1H2
0233M	R0826-89	HaeIII	H1H2
0234A	R0808-89	HaeIII	
0235L	R0821-89	HaeIII	H1H2
0236X	R0807-89	HaeIII	H1H1
0237K	R0829-89	HaeIII	H1H1
0238W	R0828-89	HaeIII	H1H1
0239H	R0827-89	HaeIII	H1H1
0240M	R0802-89	HaeIII	H1H1
0241A	R0818-89	HaeIII	H1H1
0242L	R0785-89	HaeIII	H1H1
0243X	R0815-89	HaeIII	H1H2
0245W	R0809-89	HaeIII	H1H1
0247V	R0805-89	HaeIII	H1H1
0249R	R0824-89	HaeIII	H1H1
0250X	R0784-89	HaeIII	H1H1
0252W	R0790-89	HaeIII	H1H1
0253H	R0134-89	HaeIII	H1H1
0254V	R0139-89	HaeIII	
0255E	R0130-89	HaeIII	
0257B	R0138-89	HaeIII	
0259A	R0141-89	HaeIII	
0260H	R0140-89	HaeIII	H1H1
0261V	R0136-89	HaeIII	H1H1
0262E	R0131-89	HaeIII	H1H1
0263R	R0133-89	HaeIII	H1H1

APPENDIX 4

Fibrinogen genotype results in the Ladywell subjects with a polarised predisposition to high and low blood pressure.

PATIENT NUMBER	RESULT	GENOTYPE
10082	6.6/5.3	BB
10083	6.6/5.3	BB
10121	6.6/5.3/4.2	Bb
10173	6.6/5.3	BB
10252	6.6/5.3	BB
10371	6.6/5.3	BB
10502	6.6/5.3	BB
10531	6.6/5.3	BB
10592		
10621	6.6/5.3/4.2	Bb
10622	6.6/5.3/4.2	Bb
10892	6.6/5.3	BB
10921		
10943	6.6/5.3	BB
10952		
10953	6.6/5.3	BB
11001	6.6/5.3	BB
11002		
11062	6.6/5.3/4.2	Bb
11232		
11252	6.6/5.3/4.2	Bb
11291	6.6/5.3/4.2	Bb
11352	6.6/5.3	BB
11362	6.6/5.3	BB
11453	6.6/5.3	BB
11543	6.6/5.3	BB
11611	6.6/5.3	BB
11722		
11842	6.6/5.3	BB
11914		
11924	6.6/5.3	BB
11951	6.6/5.3/4.2	Bb
11952	6.6/5.3/4.2	Bb
12062	6.6/5.3	BB
12063	6.6/5.3	BB
12111	6.6/5.5/4.2	Bb
12112	6.6/5.3	BB
12132	6.6/5.3/4.2	Bb
12203	6.6/5.3/4.2	Bb
12251	6.6/5.3	BB
12252	6.6/5.3	BB
12281	6.6/5.3/4.2	Bb
12513	6.6/5.3	BB
12594	6.6/5.3	Bb
12792	6.6/5.3/4.2	Bb
12902		
12922	6.6/5.3	BB
12942		
12982	6.6/5.3	BB
12983	6.6/5.3	BB
13003	6.6/5.3/4.2	Bb
13101	6.6/5.3	BB
13102	6.6/5.3	BB
13122	6.6/5.3	BB
13222	6.6/5.3/4.2	Bb

PATIENT NUMBER	RESULT	GENOTYPE
13241	6.6/5.3	BB
13421	6.6/5.3	BB
13423	6.6/5.3	BB
13642		
13654		
13684	6.6/5.3	BB
13802	6.6/5.3	BB
13862	6.6/5.3	BB
13911	6.6/5.3	BB
14013	6.6/5.3	BB
14024	6.6/5.3	BB
14051	6.6/5.3/4.2	Bb
14241	6.6/5.3	BB
14242		
14323	6.6/5.3	BB
14332	6.6/5.3	BB
14542		
14582	6.6/4.2	bb
14622	6.6/5.3/4.2	Bb
14642	6.6/5.3/4.2	Bb
14672	6.6/5.3	BB
14763	6.6/5.3	BB
14792	6.6/5.3/4.2	Bb
14882	6.6/5.3/4.2	Bb
14911		
14974	6.6/5.3/4.2	Bb
12982	6.6/4.2	bb
15082	6.6/5.3/4.2	Bb
15282	6.6/5.3	BB
15502		
15601	6.6/5.3	BB
15691	6.6/5.3/4.2	Bb
15723	6.6/5.3	BB
15771	6.6/5.3/4.2	Bb
15833		
15932	6.6/5.3	BB
16111	6.6/5.3/4.2	Bb
16182	6.6/5.3	BB
16301		
16332	6.6/5.3/4.2	Bb
16382	6.6/5.3	BB
16531	6.6/5.3/4.2	Bb
16621		
16701	6.6/5.3	BB
16702	6.6/5.3/4.2	Bb
16771	6.6/5.3	BB
16841	6.6/5.3/4.2	Bb
17052	6.6/5.3/4.2	Bb
17161	6.6/5.3/4.2	Bb
17321	6.6/5.3	BB
17371	6.6/4.2	bb
17551	6.6/5.3	BB
17671	6.6/5.3	BB
17672	6.6/5.3	BB
17892	6.6/5.3	BB
20072	6.6/5.3/4.2	Bb
20091		
20131	6.6/5.3	BB
20142	6.6/5.3	BB
20152	6.6/5.3	BB

PATIENT NUMBER	RESULT	GENOTYPE
20252	6.6/5.3/4.2	Bb
20253	6.6/5.3	BB
20351	6.6/5.3	BB
20422	6.6/5.3	BB
20423	6.6/5.3	BB
20472		
20553	6.6/5.3	BB
20561	6.6/5.3	BB
20682	6.6/5.3	BB
20741	6.6/5.3	BB
21052	6.6/5.3	BB
21192	6.6/5.3	BB
21222	6.6/5.3	BB
21272		
21332	6.6/5.3/4.2	Bb
21451		
21522	6.6/5.3	BB
21702	6.6/5.3	BB
21714	6.6/5.3	BB
21722	6.6/5.3	BB
21843	6.6/5.3/4.2	Bb
22023		
22024	6.6/5.3	BB
22112		
22381	6.6/5.3	BB
22752	6.6/5.3	BB
22762	6.6/5.3	BB
22942	6.6/5.3/4.2	Bb
23061	6.6/5.3	BB
23082	6.6/5.3	BB
23256	6.6/5.3	BB
23293	6.6/5.3	BB
23393	6.6/4.2	bb
23403	6.6/5.3	BB
23452	6.6/5.3	BB
23523	6.6/5.3	BB
23583	6.6/5.3	BB
23662	6.6/5.3	BB
23691	6.6/5.3/4.2	Bb
23793	6.6/5.3/4.2	Bb
23892		
23952	6.6/5.3	BB
24011	6.6/5.3/4.2	Bb
24113	6.6/5.3	Bb
24261		
24262	6.6/5.3	BB
24301	6.6/5.3	BB
24302	6.6/5.3	BB
24363	6.6/5.3	BB
24372	6.6/5.3	BB
24492	6.6/5.3	BB
24516		
24521	6.6/5.3	BB
24531		
24682		
24721	6.6/5.3	BB
24751	6.6/5.3/4.2	Bb
24752	6.6/5.3	BB
24762	6.6/5.3/4.2	Bb
24821	6.6/5.3	BB

PATIENT NUMBER	RESULT	GENOTYPE
24931	6.6/5.3/4.2	Bb
25142	6.6/5.3	BB
25143	6.6/5.3/4.2	Bb
25202		
25303	6.6/5.3	BB
25311	6.6/5.3/4.2	Bb
25331	6.6/5.3	BB
25361	6.6/5.3	BB
25371	6.6/5.3	BB
25544	6.6/4.2	bb
25553	6.6/5.3	BB
25684	6.6/5.3	BB
25685	6.6/5.3	BB
25751	6.6/5.3/4.2	Bb
25814	6.6/5.3	BB
25852	6.6/5.3/4.2	Bb
25863	6.6/5.3	BB
25864	6.6/5.3	BB
25942	6.6/5.3	BB
25962	6.6/5.3	BB
26093	6.6/5.3	BB
26132		
26191	6.6/5.3	BB
26241	6.6/5.3	BB
26242		
26262	6.6/5.3	BB
26331		
26372	6.6/5.3	BB
26521		

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