https://theses.gla.ac.uk/

Theses Digitisation:

https://www.gla.ac.uk/myglasgow/research/enlighten/theses/digitisation/

This is a digitised version of the original print thesis.

Copyright and moral rights for this work are retained by the author

A copy can be downloaded for personal non-commercial research or study, without prior permission or charge

This work cannot be reproduced or quoted extensively from without first obtaining permission in writing from the author

The content must not be changed in any way or sold commercially in any format or medium without the formal permission of the author

When referring to this work, full bibliographic details including the author, title, awarding institution and date of the thesis must be given

Enlighten: Theses
https://theses.gla.ac.uk/
research-enlighten@glasgow.ac.uk

Genetic polymorphisms, platelet activation and plasma homocysteine concentrations in atherothrombotic stroke.

David J Meiklejohn BSc MBChB

Thesis submitted for the degree of MD to the University of Glasgow, June 2000.

ProQuest Number: 10656445

All rights reserved

INFORMATION TO ALL USERS
The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript
and there are missing pages, these will be noted. Also, if material had to be removed,
a note will indicate the deletion.

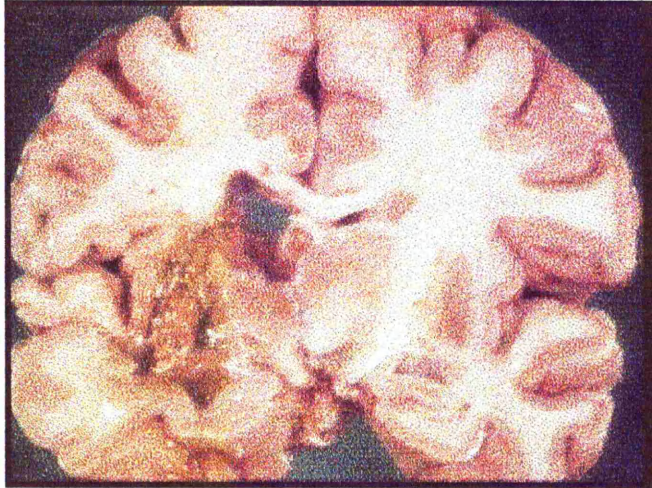
ProQuest 10656445

Published by ProQuest LLC (2017). Copyright of the Dissertation is held by the Author.

All rights reserved.
This work is protected against unauthorized copying under Title 17, United States Code
Microform Edition © ProQuest LLC.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

GLASGOW
UNIVERSITY
LIBRARY:
12444
COPY 2



Coronal section, cerebral infarction

// Acknowledgements

There are many people I wish to thank for their help in performing the work for and writing this thesis. I am grateful to Isobel Ford in the Haematology research laboratory at the University of Aberdeen, and David Stevenson, Irene Moore and Nicholas Robinson at the Immunology Lab at the Blood Transfusion Service for their advice and help with FACS analysis and PCR techniques. I thank the staff of the Medical Wards at Aberdeen Royal Infirmary for their cooperation with patient recruitment. I am grateful to Mark Vickers for continuing recruitment to ensure as few patients as possible were missed if I was on leave, and for his very helpful suggestions regarding the manuscript.

Special thanks are due to Professor Mike Greaves, who enabled me to carry out this work, and who has been a source of great encouragement and inspiration throughout.

This work was performed thanks to research grants from Chest, Heart and Stroke (Scotland), and the Aberdeen Royal Hospitals Endowment Fund.

Dedication

This thesis is dedicated to Linda and Laura.

Contents

Section	Page
Declaration	i
Summary	ii
Chapter one. Introduction.	**1-27**
1.1 Definitions and epidemiology	2
1.2 Pathophysiology of atherothrombotic stroke	3
1.3 Platelet activation in acute arterial thrombosis	4
1.4 Platelet membrane glycoproteins	8
1.5 Risk factors for atherothrombotic stroke	10
1.6 Candidate genetic polymorphisms	11
1.7 Outline of studies undertaken	17
Chapter two. Patient and volunteer recruitment, sample handling and characteristics.	**28-40**
2.1 Platelet donor study	29
2.2 Stroke study	30
2.3 Results	33
Chapter three. An investigation of the effect of HPA 1a/1b genotype on platelet fibrinogen binding in healthy subjects.	**41-55**
3.1 Background	42
3.2 Aims	43
3.3 Methods	44
3.4 Results	48
3.5 Discussion	49
Chapter four. In vivo platelet activation in atherothrombotic stroke: relationship to polymorphisms of platelet GPIIIa and GPIb.	**56-88**
4.1 Background	57
4.2 Aims	58
4.3 Methods	59
4.4 Results	71
4.5 Discussion	75
Chapter five. Plasma Homocysteine concentrations in the acute and convalescent phases of atherothrombotic stroke.	**89-112**
5.1 Background	90
5.2 Aims	93
5.3 Methods	94
5.4 Results	101
5.5 Discussion	103

Chapter six. The contribution of Factor VII gene polymorphisms to longevity 113-126
6.1 Background 114
6.2 Methods 115
6.3 Results 119
6.4 Discussion 120

Chapter seven. Conclusions . 127-144

7.1 Platelet polymorphism studies 129
7.2 Functional studies of platelet polymorphisms 135
7.3 Homocysteine as a causative risk factor 136
7.4 Conclusions 138

References 145-180

Appendices 181-197
I Reagents 182
II Patient and control information sheets 190
III Publications arising from this thesis. 196

Declaration

I declare that I am the author of this thesis, and that all references cited have been consulted by myself. I personally performed the following: recruitment and sampling of all subjects described in this thesis; flow cytometric analysis of the donors described in chapter two, and of the patients and controls in chapter three; HPA 1a/1b genotyping of patients and controls; R353Q genotyping of 60 subjects described in chapter four; plasma homocysteine assays by HPLC described in chapter five; and for all data analyses. I also had the following help: Miss Irene Moore of the North East of Scotland Blood Transfusion Service performed the HPA 1a/1b genotyping of the donors described in chapter two and the HPA 2a/2b genotyping of patients and controls described in chapter three; Dr Rona Morrison was of invaluable help in preparing samples for DNA extraction, plasma separation and flow cytometric analysis of a substantial number of patients and controls; Mr Neil Youngson and Ms Zoe Riches performed the FVII gene polymorphism analyses in chapter four. I am very grateful to each for their assistance. This work was carried out between June 1997 and May 1999 while I was a Research Registrar in Haematology at Aberdeen Royal Infirmary. No part of this work has previously been accepted for a higher degree.

David J Meiklejohn. June 2000

Summary

Atherothrombotic stroke arises following rupture of an atheromatous plaque, and occlusion occurs directly due to thrombosis in small arteries, or indirectly by embolisation if a large vessel plaque ruptures. Three risk factors that are claimed to influence these process were investigated.

- The influence of platelet activation and genetic polymorphisms of platelet membrane glycoproteins on the risk of thrombotic stroke was assessed. Following plaque rupture, platelets have a pivotal role in arterial thrombus formation, and platelet membrane glycoproteins (GP) IIIa (the fibrinogen receptor) and Ib (which binds von Willebrand factor) are crucial in this process. The 1b allele of the HPA 1a/1b GPIIIa polymorphism and the 2b allele of the HPA 2a/2b GPIb polymorphism are claimed to be risk factors for stroke and myocardial infarction (MI), but reports are conflicting and consistent functional evidence of enhanced thrombogenicity is lacking.

- Increased factor VII activity (VIIc) has been claimed to be a risk factor for MI and stroke, but the data are conflicting. VIIc is dependent on both environmental and genetic influences, and recently two polymorphisms of the factor VII gene associated with lower VIIc have been claimed to be protective against MI.

- A raised plasma homocysteine concentration has been proposed as a cause for atherosclerosis. However the role of homocysteine in stroke aetiology remains

controversial, since prospective studies have reported a weaker association than those conducted retrospectively. Furthermore there are few reports of plasma homocysteine concentrations both before and after the event.

The following studies were conducted to address these issues:

An investigation of the effect of HPA 1a/1b genotype on platelet fibrinogen binding by whole blood flow cytometry in healthy subjects.

The effect of the 1b allele on platelet fibrinogen binding was investigated in healthy subjects by whole blood flow cytometry. 35 platelet or plasma donors (34 HPA 1a/1b and one HPA 1b/1b) possessing the 1b allele were compared with 35 donors homozygous for the 1a allele. There was no allele dependent difference in the percentage of platelets binding fibrinogen at baseline (p=0.14, Mann Whitney U test) or following stimulation with ADP (p=0.72, Student's *t*-test). An paradoxical increase in the density of fibrinogen binding sites was observed in 1a platelets after ADP stimulation (p=0.05, Mann Whitney U test). 1b platelets tended to exhibit greater activation as assessed by the percentage of platelets expressing P-Selectin, but this did not reach statistical significance (p=0.08, Mann-Whitney U test). These data do not identify a functional mechanism by which the 1b allele might mediate an increased risk of arterial thrombosis.

In vivo platelet activation in atherothrombotic stroke: relationship to polymorphisms of human platelet GPIIIa and GPIb.

The expression of platelet activation markers in 150 consecutive patients (98 male/52 female; mean age 58.3) with acute atherothrombotic stroke was measured usually within 24 hours of the onset of symptoms. Evidence of persisting platelet activation was sought in 77 patients who reattended in the convalescent period (mean 100 days). The 1b and 2b allele frequencies were determined, and the influence of genotype on platelet activation was assessed. Results were compared with 150 healthy age and sex matched controls recruited from a local general practice. Neither the 1b allele (allele frequency 0.11 vs 0.13, OR{CI} 0.8{0.5-1.3} nor the 2b allele (0.09 vs 0.07, OR{CI} 1.4{0.8-2.4} was significantly overrepresented in patients. Increased numbers of activated platelets were found following stroke (geometric mean acute P-Selectin expression 0.64% vs control 0.35%, $p<0.001$, Student's *t*-test; acute mean fibrinogen binding 1.6% vs control 0.9%, $p<0.001$, Student's *t*-test). This persisted in the convalescent phase (P-Selectin 0.62%, fibrinogen binding 1.4%, $p<0.001$ and $p=0.005$ vs controls respectively). Expression of P-Selectin and fibrinogen was influenced by neither HPA 1a/1b genotype ($p>0.95$ for each marker, Scheffe's test), nor 2a/2b genotype ($p>0.95$ for each). Thus *in vivo* platelet activation and the relationship with platelet genotype was studied in acute thrombosis, and although persisting platelet activation was observed in atherothrombotic stroke, it remained independent of HPA 1a/1b and 2a/2b genotype. These data suggest an underlying prothrombotic tendency, but do not support the polymorphisms studied as risk factors for thrombotic stroke in this population.

The influence of factor VII gene polymorphisms on longevity in the nonagenarians.

The hypothesis tested was that these polymorphisms are overrepresented in a group of nonagenerians from a region with high cardiovascular mortality, since reaching extreme old age should be favoured by avoiding stroke and MI. The frequencies of the Q allele of the R353Q polymorphism and H7 allele of the IVS7 polymorphism were determined in 113 subjects (86 female/ 27 male) over 90 years of age (median 96; range 90-103) and 100 (80 female/ 20 male) younger controls (median 32 years; range 19-44). 15.6% enrichment of the Q (0.111 vs 0.096, p=0.61) and 9.7% enrichment of the H7 (0.395 vs 0.360, p=0.45) alleles in long-lived individuals was found. Although the frequencies of the 'protective' RQ and H7H7 genotypes were over-represented in the study population, these differences were not statistically significant (p=0.36 and 0.3 respectively). These data help to quantify any possible effect of FVII polymorphisms on longevity and, if the allele frequencies observed reflect the true magnitude of any effect, it is calculated that between 750 (for the H7 allele) and 1600 (for the Q allele) subjects are required to attain statistical significance.

Plasma Homocysteine concentrations in the acute and convalescent phases of atherothrombotic stroke.

Fasting total plasma homocysteine (tHcy) concentrations were measured immediately after acute atherothrombotic stroke by HPLC with fluorescence detection. 106 patients (59 men/ 47 women, mean age 57.2 {25-70}and 56.5 {26-69} years respectively) were recruited within 24 (up to 96) hours of onset, and 82 patients were resampled in the convalescent phase. There was no significant difference in acute median tHcy between patients and matched controls (Men; 9.2μmol/l{range 4.4-22.8} vs 8.7μmol/l{(4.9-20}, p=0.09, Mann Whitney U test;

Women 8.1μmol/l{(4.8-32.3} vs 7.6μmol/l {(3.3-14.4}, p=0.58). Median plasma concentrations increased significantly in the convalescent period (from 8.5μmol/l{4.8-19.2}to 10.1μmol/l{4.3-31.5}, p<0.001, Wilcoxon ranked signs test). Median convalescent Hcy concentrations were greater in both men and women than in controls (p=0.03 and 0.05 respectively, Mann Whitney U test). This did not appear to be explained by a reduction in red cell folate or serum B12 concentration, despite these factors being significant covariates on multiple regression analysis. Although mean convalescent creatinine concentrations increased by 4μmol/l, it is calculated (given a Beta weight of 0.42) that this predicts a mean increase of only 0.3μmol/l, compared to the observed rise of 1.6μmol/l, and is insufficient to fully explain the rise in convalescent tHcy. These data do not support the hypothesis that raised plasma homocysteine concentrations predate atherothrombotic stroke. Instead they offer explanation for the discrepancy between prospective and retrospective studies, and suggest that tHcy levels may be caused by the disease process itself.

Chapter One

Introduction

1.1 Definitions and epidemiology

Stroke or cerebrovascular accident (CVA) is defined as a sudden loss of global or focal cerebral function due to sudden interruption of the blood supply to the brain, and is the third most common cause of mortality in the developed world after ischaemic heart disease and cancer (Warlow, 1996). The annual incidence of first stroke in the United Kingdom is two per thousand, but this increases with age, 50% of all events occurring in patients over the age of 75 (Warlow, 1996). In 1997 cerebrovascular disease accounted for 11.7% of all deaths in the Scottish population, and varied with increasing age from 5.8% of deaths in those under 65 to 13.4% in those aged 65 and older (Scottish Health Statistics, 2000).

Stroke can be classified pathologically (*figure 1.1*). Haemorrhage may arise in the subdural or subarachnoid spaces, or may be intracerebral. Infarction results from thrombotic occlusion of a brain supplying artery, and symptoms reflect ischaemia in the territory it supplies. Thrombus may arise from local atheromatous disease, or may form proximally and dislodge, occluding a vessel distally. Half of such emboli arise from the heart, e.g. due to atrial fibrillation, valvular heart disease or mural thrombus, and the other half from proximal arterial disease, especially of the carotid vessels. When considering risk factors for stroke it is important to recognise that any risk factor may influence only one of these very different processes. This thesis therefore limits investigation to stroke arising from the single pathophysiological process of atherothrombosis.

1.2 Pathophysiology of atherothrombotic stroke

The dominant arterial lesion responsible for atherothrombotic stroke and myocardial infarction is the atherosclerotic plaque. The 'response to injury hypothesis' has been proposed to explain the development of atheroma (Basha & Sowers, 1996). An inflammatory endothelial response is thought to be the initiating process in atheroma formation, with ongoing inflammation contributing to lesion development (Ross, 1999). This is supported by the observation that C-reactive protein (CRP) concentration is an independent risk factor for myocardial infarction (MI) and stroke (Ridker *et al*, 1997a). The earliest lesion is the fatty streak, which is present from childhood. Monocytes, T lymphocytes and smooth muscle cells accumulate in the arterial intima. These secrete chemokines which stimulate smooth muscle proliferation and collagen deposition, contributing to arterial luminal narrowing (Basha & Sowers, 1996). Monocytes mature to macrophages which accumulate intracellular lipid and express tissue factor (TF). Ongoing inflammation and endothelial damage leads to accumulation of these foam cells, and plaques are formed when extracellular lipid accumulates. The lipid core is surrounded by a fibrous cap, of collagen, elastin and proteoglycans.

There are two distinct types of plaque (Taubman *et al*, 1997). "Stable" plaques are predominantly fibrotic and heavily calcified. They progressively narrow and are associated with chronic stable arterial disease. "Unstable" plaques are lipid rich and prone to fissuring and rupture, leading to exposure of TF and thrombus propagation. The thrombus may either become organised and incorporated into the developing atheroma, or at a critical point cause abrupt vessel occlusion. This precipitates an acute event such as stroke, MI or unstable angina (Burke *et al*,

1997). Coagulation and platelet activation following plaque rupture are therefore crucial events in the pathogenesis of atherothrombotic stroke.

1.3 Platelet activation in acute arterial thrombosis.

The processes of coagulation and platelet activation are inextricably linked, since coagulation activation stimulates recruitment and activation of platelets to the thrombus and *vice versa*. A natural anticoagulant system predominates in the resting physiological state, in order to ensure that blood circulates in the fluid phase. In arterial thrombosis homeostatic balance is altered in favour of thrombosis when a plaque ruptures, exposing the plasma and platelets to TF and subendothelial proteins.

Coagulation activation

Coagulation is mediated by a cascade of proteolytic reactions which begins when exposed plaque TF interacts with activated plasma factor VII (VIIa) to activate factors IX and X. This results ultimately in the generation of thrombin, which is required to cleave fibrinogen and produce a fibrin clot. Many of these reactions require Ca^{2+} ions and phospholipid as cofactors.

Platelet recruitment and activation

Platelets provide this phospholipid template for fibrin generation, as well as forming a platelet plug. The action of platelets in thrombus formation can be summarised thus (del Zoppo, 1998):

- *Platelet adhesion.* Exposure of subendothelial collagen leads to platelet adhesion. This is mediated by surface membrane glycoprotein (GP) receptors.

In the conditions of high shear stress seen in narrowed arteries this predominantly occurs via binding of collagen to high molecular weight multimers of von Willebrand Factor, which in turn bind to the GPIb portion of the GPIb-GPIX complex (Nurden, 1995). Platelets may also bind directly with collagen through the GPIa ligand.

- *Platelet spreading.* Ca^{2+} dependent intracellular signal pathways resulting in actin polymerisation and pseudopodia formation are activated following platelet adhesion. These result in spreading and elongation, and the movement of the dense body and alpha-granule organelles to the centre of the platelet.

- *Platelet activation.* Simultaneously activation occurs via a number of specific agonist-receptor interactions. GPIb and GPIa ligand binding initiates intracellular activation signalling upon platelet adhesion. There are specific receptors and signalling pathways for agonists involved in platelet activation. The major agonists to activate platelets are collagen and thrombin, but positive feedback occurs via the actions of adenosine diphosphate (ADP), adrenaline, serotonin (5HT) and arachidonic acid which are produced by platelet activation and granule release. The thrombin receptor is distinguished by the fact that thrombin generated from coagulation activation and from platelet degranulation cleaves it to mediate activation (Brass *et al*, 1994). Initiation of these pathways results in platelet aggregation, and in degranulation of dense bodies and alpha-granules. These contain a variety of compounds that promote platelet and coagulation activation and recruit circulating platelets to the developing thrombus. These include fibrinogen, collagen, vWF, platelet factor

4 (PF4), betathromboglobulin (ß-TG) and P-Selectin from alpha-granules, and agonists such as ADP and 5HT from dense bodies. Phospholipid rich membrane, previously internalised in the platelet canalicular system, is exposed on the surface and increases the area upon which coagulation activation can occur.

There are three main intracellular signalling pathways (Wu, 1996).

1. *The phospholipase C pathway* is activated following G protein receptor signal transduction. This enzyme catalyses the hydrolysis of phosphatidylinositol biphosphate to produce two second messengers: Firstly inositol triphosphate (IP_3) which causes an increase in cytoplasmic Ca^{2+} by release from the dense tubular system. Calcium is involved in numerous processes involved in platelet activation, including cell contraction via actin and myosin and production of arachidonic acid. Secondly diacylglycerol induces protein phosphorylation necessary for platelet activation by activation of protein kinase C.
2. *Adenylate cyclase activity* is inhibited by an inhibitory G protein (Gi) that is coupled to the thrombin receptor. This leads to a reduction in intracellular cyclical Adenosine Monophosphate (cAMP) levels, which results in a further increase in intracellular Ca^{2+} and potentiates platelet activation.
3. *Phospholipase A_2* activity is stimulated by increased cytosolar Ca^{2+} and activation of protein kinase C. This enzyme cleaves arachidonic acid (AA) from platelet membrane phospholipid. This is converted to

prostaglandin H_2 by the action of cyclo-oygenase which in turn is converted to thromboxane A_2 (TXA_2) by thromboxane synthetase. TXA_2 enhances platelet activation by reducing cAMP levels. It is rapidly metabolised in serum to TXB_2, which is excreted in the urine.

- *Platelet aggregation.* Conformational change in surface membrane GPIIIa occurs on platelet activation, leading to increased surface density of these ligands and exposure of the fibrinogen binding site. Aggregates are formed when neighbouring platelet GPIIbIIIa complexes are crosslinked by fibrinogen and vWF (Nurden, 1995).

Assessment of platelet activation

There are therefore numerous ways in which platelet activation in patients can be assessed. There are advantages and disadvantages associated with each method, which will be discussed in chapters two and three. These are summarised in *table 1.1.* Plasma markers of platelet activation are relatively easy to assay by enzyme linked immunoassay (ELISA), but may be difficult to interpret since they are prone to artefact, given that *ex vivo* platelet activation might arise at the time of sampling or after centrifugation. Similar difficulties are encountered when preparing platelet rich plasma for aggregometry (Michelson 1996). This can be overcome by measuring urinary metabolites of TXA_2, but although a 24 hour urine sample may yield information regarding *in vivo* platelet activation, it is a more indirect assessment of platelet function. Whole blood flow cytometry directly assesses platelet activation by P-Selectin expression and GPIIbIIIa

fibrinogen binding and avoids these problems, but has the disadvantage that samples must be processed and analysed immediately.

1.4 Platelet membrane glycoproteins.

As discussed above platelet glycoproteins are transmembrane molecules that are important in the process of clot formation. They exist in non-covalent association with other GPs to form heterodimeric complexes, and have two distinct roles in platelet activation and aggregation:

1. *'Inside-out' ligand binding.* GP complexes bind to specific proteins which mediate interactions on the platelet membrane surface which promote clot formation. For example GPIb binds vWF, which is important in facilitating the adhesion of platelets to the vessel wall under conditions of high shear stress, as are found in arteries narrowed by atherosclerosis.
2. *'Outside-in' receptor signalling.* GPs function as receptors for agonists, and via signal transduction by G-proteins on the internal membrane surface, generate second messengers which mediate intracellular events leading to activation.

Specific GP complexes are discussed overleaf (summarised in Nurden, 1995):

1.4.1 GPIbIX

This complex mediates initial platelet tethering to the vessels via vWF. Glycoprotein Ib consists of two chains, a large α-chain of 154kDa and a shorter 22kDa ß-chain which are linked by a disulphide bond. These chains are rich in

leucine residues, a feature shared with GPIX. The vWF binding region is located on the outermost extracellular part of GPIbα, and lies somewhere between position 235 and 279. A high affinity receptor for thrombin has also been identified on Ibα, although its function is unclear (Katagiri *et al*, 1990). Although the binding sequences are found on the α chain, it is speculated that interaction with the ß chain and GPIX occurs via leucine rich regions, and that these influence the avidity of vWF binding.

1.4.2 GPIaIIa

This complex mediates further platelet tethering by binding to type I and type III collagen, and promotes platelet activation by outside-in receptor signalling via the pathways described above. It has been demonstrated that the ability of platelets to bind collagen is dependent on surface receptor density (Kunicki *et al*, 1993).

1.4.3 GPIIbIIIa

The GPIIbIIIa complex in the resting platelet is mainly internalised in the internal canalicular system, but on platelet activation is translocated to the surface and undergoes conformational change which mediates platelet aggregation by fibrinogen binding. The genes for both GPIIb and GPIIIa are found very close to each other in the long arm of chromosome 17, and the two proteins are packaged together in the endoplasmic reticulum prior to transport of the heterodimer to the surface membrane (Nurden, 1995).

GPIIb consists of a heavy (115kDa) and a light (22kDa) chain linked by a disulphide bridge. The latter is tethered to the platelet membrane, while the

former protrudes form the platelet surface and interacts with GPIIIa. This occurs via a series of dodecapeptide repeat sequences which contain Ca^{2+} binding sites, and are important in divalent IIbIIIa bonding. GPIIIa is a short (90kDa) single chain polypeptide that is characterised by numerous cysteine residues, which confer a tight globular structure by disulphide linking. Binding of fibrinogen and other ligands such as fibronectin and vitronectin is mediated by RGD peptides (arg-gly-asp), the principal site being located between residues 109-171 of GPIIIa, and aggregation is mediated by the association of a dodecapeptide sequence the fibrinogen with the Ca^{2+} binding region of GPIIb on an adjacent platelet.

1.5 Risk factors for atherothrombotic stroke.

Arterial thrombosis has a multifactorial aetiology, and arises due to a complex interaction between genetic and environmental risk factors (Ridker & Stampfer, 1999a). Conditions or behaviours which influence the processes of atherogenesis or thrombosis might therefore increase the risk of stroke. Standard risk factors for stroke are summarised in *table 1.2* (Rosendaal, 1997a). In most cases an effect on atherogenesis or thrombosis can be inferred. For example, cigarette smoking is associated with endothelial dysfunction and atherogenesis, as well as platelet activation and thrombosis (Powell, 1998). Similarly raised plasma cholesterol can be envisioned to contribute to lipid accumulation in the plaque lesion, and LDL cholesterol is involved in numerous stages of atherogenesis following ingestion and modification by macrophages (Berliner *et al*, 1997).

1.6 Polymorphisms of factors involved in thrombosis as candidate risk factors for stroke and myocardial infarction.

A family history of atheromatous disease is associated with an increased risk of stroke (Kiely *et al*, 1993) and although there may be common shared environmental risk factors within families, twin studies indicate that there is also a genetic component (Brass *et al*, 1992). CADASIL (*c*erebral *a*utosomal *d*ominant *a*rteriopathy with *s*ubcortical *i*nfarcts and *l*eucoencephalopathy) is a condition arising from a gene defect on chromosome 19q12 which results in a Mendelian inheritance of stroke, but this is very rare (Pullicino *et al*, 1997). Polymorphisms are gene mutations more commonly found in the population. These are usually the result of single nucleotide substitutions, or variation in the number of tandem repeat sequences (VNTRs) in a non-coding region (intron) of the gene. The effect of a polymorphism on gene function may be subtle or neutral, but some may contribute to disease pathogenesis.

Numerous polymorphisms have been studied, and those potentially involved in thrombosis are listed in *table 1.3*. Case control studies have attempted to associate these with disease by demonstrating greater allele frequencies in survivors of myocardial infarction and stroke than healthy controls, but consistent associations are often not apparent.

Factor V Leiden

With this mutation the substitution of adenine for guanine at position 1691 leads to the substitution of arginine by glutamine on position 506 of the amino acid chain. This mediates thrombosis by rendering Factor Va resistant to cleavage by activated Protein C (Bertina *et al*, 1994). This mutation might be viewed as the 'gold standard' in studies of genetic polymorphisms and thrombosis, since there is both epidemiological evidence of risk in venous thrombosis and a convincing functional explanation for how this is mediated.

Despite these observations, the role of Factor V Leiden in thrombotic stroke and MI is less clear. Studies are summarised in *table 1.4.* Only two have found an association with MI, and none with stroke. Rosendaal *et al* (1997b) observed a doubling of risk of MI in those possessing the 1691A allele, in a small study of 84 young women. The relative risk rose to 3.6 in the 69 who also smoked, suggesting that genetic and environmental factors might interact. However caution was advocated in interpreting these data since the small sample size did not provide adequate statistical power to fully investigate such relationships. These preliminary observations were supported by a larger study of 560 survivors of MI, where a substantial increase in risk was also reported in those who smoked (Doggen *et al*, 1998a). However in a cohort of 2210 patients these findings were not reproduced in neither patients with stable coronary artery disease nor acute MI (Gardemann *et al*, 1999). No association was observed in 386 older patients with acute stroke, nor was the G allele associated with enhanced thrombogenicity (Catto *et al*, 1995). This study included patients with all subtypes of stroke, including haemorrhage, and it could be speculated that an effect might be

restricted to those with thrombosis. Several other studies demonstrated the mutation was not a risk factor for stroke or myocardial infarction in any subgroup (Ridker *et al*, 1995; Cushman *et al*, 1998; Longstreth *et al*, 1998; Ardissino *et al*, 1999; Gowda *et al*, 2000).

G20210A prothrombin gene mutation

This single base substitution arises in the 3' end of the prothrombin gene and the A allele is associated with enhanced thrombogenicity and increased risk of venous thrombosis, possibly as a result of the observed increase in plasma prothrombin concentrations (Poort *et al*, 1996). Clinical studies of arterial disease are summarised in *table 1.5,* and are again conflicting despite the mutation producing a plausible prothrombotic phenotype. Rosendaal *et al* (1997c) reported a fourfold increased risk of MI in 79 young women, with a relative risk of 43 in smokers. A similar risk was observed in young patients with ischaemic stroke (De Stefano *et al*, 1998). The size of these studies necessitates caution when interpreting their significance, and others have yielded conflicting results (Corral *et al*, 1997a; Ferraresi *et al*, 1997; Eikelboom *et al*, 1998; Longstreth *et al*, 1998; Arruda *et al*, 1998; Ardissino *et al*, 1999; Franco *et al*, 1999; Ridker *et al*, 1999b). A study of 263 young patients with ischaemic heart disease demonstrated that, despite the A allele being associated with greater prothrombin concentrations, their was no statistically significant association of the polymorphism with arterial disease (Franco *et al*, 1999).

The dissociation between prothrombotic phenotype and MI or stroke has led some to suggest that the influence of a single gene on stroke pathogenesis is small, and

only results in a clinically significant effect when there is interaction with environmental or other genetic risk factors. This view is supported by a large study of patients with MI, where an interaction between the prothrombin gene mutation and smoking was observed (Doggen *et al*, 1999a). Gardemann *et al* (1999) reported no association with MI or stable coronary disease, but noted that the extent of stable disease (determined angiographically) was influenced by genotype in those who possessed other risk factors. However there was no evidence of such an interaction influencing the risk of acute coronary thrombosis.

Thus despite evidence of a plausible prothrombotic effect of a polymorphism and clear evidence of risk in venous thrombosis, neither factor V Leiden nor the prothrombin mutation are consistent independent risk factors for stroke .

Fibrinogen gene polymorphisms

Similar inconsistencies have been observed in studies of fibrinogen gene polymorphisms (*table 1.6*). Possession of the A allele of the -455 G/A polymorphism in the promoter region of the ß gene is associated with higher basal plasma fibrinogen concentrations in most studies. Despite this, and although a higher plasma fibrinogen concentration is itself an independent risk factor, associations of the A allele with IHD and MI are inconsistent (Behague *et al*, 1996; Yu *et al*, 1996; Carter *et al*, 1996; Wang *et al*, 1997; Gardemann *et al*, 1997; Tybjaerg-Hansen *et al*, 1997; de Maat *et al*, 1998). The Bcl 1 ß-gene polymorphism does not increase baseline risk, but was observed to amplify the acute phase response and increase the risk of myocardial infarction in subjects with systemic infection (Zito *et al*, 1999). This was a small study and possible

interactions between gene and environment require further exploration in larger studies.

There are fewer data regarding the risk of stroke. The -455A allele conferred a doubling of risk in a small study of Japanese patients (Nishiuma et al, 1998), but did not influence plasma fibrinogen in this cohort. Others have observed an association with stroke, but the study included all stroke subtypes and the association was only found in women (Carter *et al*, 1997a). Carter *et al* (1999) did not observe an association between stroke and the Thr312Ala alpha gene polymorphism, but reported shortened survival in the 101 patients who also had atrial fibrillation. Caution in interpreting these observations is necessary since subgroup analyses of modest numbers of patients were performed, and reproduction of these data in other populations is desirable. The Austrian Stroke Prevention Study identified homozygosity for the T allele of the C148T polymorphism within the interleukin-6 responsive element of the ß gene as an independent risk factor for carotid atherosclerosis, despite there being no correlation between plasma fibrinogen and genotype (Schmidt *et al*, 1998).

The lack of association in most studies between genotype and arterial thrombosis, despite an association with fibrinogen level, weakens the proposal that fibrinogen is a causative risk factor for stroke, and. it could be suggested that levels are merely a marker of the systemic inflammatory response observed in individuals with atherosclerosis.

Other polymorphisms

Platelet glycoprotein, MTHFR and factor VII polymorphisms are discussed in subsequent chapters. Polymorphisms of the plasminogen activator inhibitor-1 (PAI-1) gene have been studied (*table 1.7*), since elevated plasma levels are associated with MI, and might promote a prothrombotic state by inhibiting fibrinolysis. Despite demonstration of the influence of genotype on plasma PAI-1 concentrations, reports are again conflicting and associations are only found in certain subgroups of the patient cohort in some studies (Dawson *et al,* 1991; Mansfield *et al*, 1995; Eriksson *et al*, 1995; Ye *et al*, 1995; Ridker *et al*, 1997; Catto *et al*, 1997; Ossei-Gerning *et al*, 1997; Iwai *et al* ,1998; Doggen *et al*, 1999; Gardemann *et al,* 1999; Iacoviello *et al*, 1998a).

There are few reports of the Val34Leu polymorphism of Factor XIII, but the allele coding for Leu was found to be underrepresented in subjects with coronary artery disease who subsequently developed acute MI (Kohler *et al*, 1998), and it was suggested that the mutation might be protective against MI. One other study supports these observations (Wartiovaara et al, 1999). Confirmation in other populations is required, but these preliminary findings are supported by the obervation that the 'protective' allele increases the risk of primary intracerebral haemorrhage in a study of all subtypes of stroke (Catto *et al*, 1998), possibly as a result of impaired fibrin clot stability.

1.7 Conclusions and outline of studies undertaken in this thesis.

The role of haemostatic gene polymorphisms is clearly more difficult to elucidate in arterial disease than in venous thrombosis due to the more complex pathogenesis, and associations are less easy to demonstrate. The main conclusions drawn from this literature review, and the studies undertaken to further investigate these points are listed below:

1) Candidate genes should have a plausible effect on haemostatic function to support a role as a risk factor.

Polymorphisms of platelet glycoprotein receptors are such candidates. As will be discussed, and in common with the candidate genes discussed above, epidemiological data are conflicting and there are no consistent functional data to support these polymorphisms as risk factors for stroke. In Chapter three the HPA 1a/1b polymorphism of GPIIIa was studied in healthy subjects, and a correlation between platelet genotype and function was sought. In chapter four this approach was extended to the setting of an acute thrombotic event. Platelet activation status was measured in patients in the acute and convalescent periods of atherothrombotic stroke. This was related to HPA 1a/1b and HPA 2a/2b (polymorphism of GPIb) genotypes in order to provide a functional assessment of these risk factors.

2) Even in instances where a plausible mechanism is demonstrated, many epidemiological studies fail to confirm the candidate gene as a risk factor for stroke or MI.

This was observed in the case of ß-fibrinogen polymorphisms. Despite demonstration of increased plasma fibrinogen levels, the -455A allele was not an independent risk factor for arterial disease, and the causative role of a raised fibrinogen concentration could therefore be challenged. Similarly, as will be discussed in chapter five, the MTHFR polymorphism results in an increases in plasma homocysteine concentration and, although increased levels independently increase the risk of MI and stroke, MTHFR genotype itself does not. The role of mild hyperhomocysteinaemia as a cause of atherothrombotic stroke is therefore unclear. Fasting concentrations were measured in the study cohort immediately after stroke, and in those who reattended at least three months later. These were compared with age and sex matched controls, and the influence of factors known to increase homocysteine concentrations was investigated.

3) Some polymorphisms might be protective against stroke or MI.

The suggestion that some polymorphisms reduce the risk of stroke (and MI) was tested in chapter six. Alleles of factor VII gene polymorphisms that are associated with reduced factor VII concentrations and are claimed to be protective against MI were elucidated in a population of extremely elderly people. The hypothesis that these alleles contribute to longevity by facilitating the avoidance of arterial events was tested, by comparing allele frequencies with a younger control group.

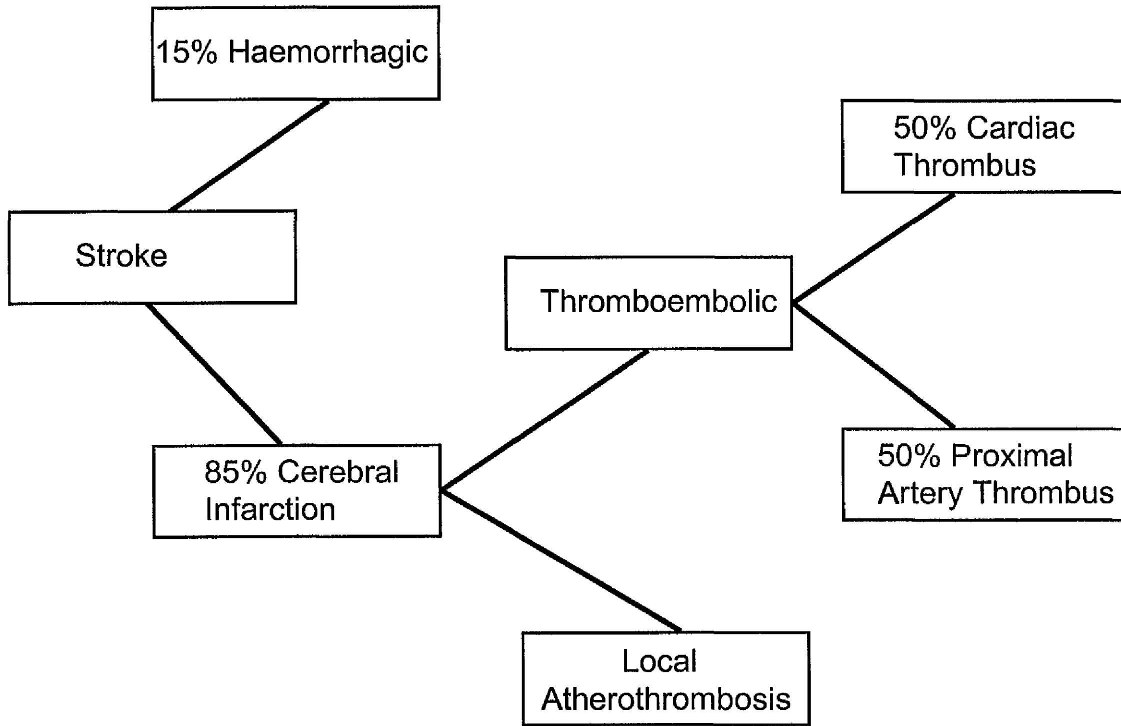


figure 1.1. Pathophysiology of stroke.

Method	Advantage	Disadvantage
Plasma P-Selectin	Simple	*Ex vivo* activation Indirect method
Plasma ß-TG	Simple	*Ex vivo* activation Indirect
Plasma TXA_2	Simple	Unstable in plasma
Urinary TXB or ß-TG	Stable, not subject to *ex vivo* influences	Indirect assessment of platelet function
Aggregometry	Direct assessment of platelets	*Ex vivo* activation
Flow cytometry of platelet rich plasma.	Direct assessment of platelets	*Ex vivo* activation
Whole blood flow cytometry	Direct method No *ex vivo* activation	Labour intensive Must be done immediately

Table 1.1. Methods available for assessment of platelet activation.

Risk factors for cerebral infarction
Hypertension
Smoking
Diabetes Mellitus
Hypercholesterolaemia
Family History
Obesity
Oral Contraceptives
Hyperhomocysteinaemia
Antiphospholipid syndrome
High plasma fibrinogen concentration

Table 1.2. Risk factors for thrombotic stroke.

Candidate polymorphisms
Factor V Leiden
Factor II G20210A
Fibrinogen gene polymorphisms
Polymorphisms of platelet membrane GPIb, IIIa and IaIIa
Factor VII polymorphisms
MTHFR C677T polymorphism
PAI-1 4G/5G polymorphism
Factor XIII polymorphisms

Table 1.3. Polymorphisms of factors involved in thrombosis investigated in stroke and myocardial infarction.

Study	Number of patients	Mean age (range)	Association Found?	OR (95% CI) or P value	Comments
Catto et al 1995	386 with acute stroke	74 (65-80)	NO	P=0.41	All stroke subtypes, including haemorrhage. Factor V genotype did not influence thrombin generation markers.
Ridker et al 1995	374 MI 209 stroke	59.5 for MI 62.9 for stroke	NO	P=0.9 for MI P=0.4 for stroke	Cohort of large prospective study. No association on subgroup analysis.
Rosendaal et al 1997	84 women with MI	18-44	YES	OR 2.4 (1-5.9)	Risk greater in subgroup analysis of 62 smokers OR 3.6 (0.9-14.4).
Cushman et al 1998	373 with MI, CVA or TIA	>65	NO	0.83 (0.5-1.4) overall	OR 0.5 (0.2-1.2)for MI 0.8 (0.5-1.4) for stroke
Martinelli et al 1998	873 relations of patients with thrombophilia.	42 (SD 20)	NO	Not given	Only 17 arterial events recorded.
Ardissino et al 1999	200 MI survivors	<45	NO	1.1 (0.4-3.3)	
Doggen et al 1998	560 MI survivors	56.2 (SD 9.0)	YES only in subgroup analysis	1.4 (0.8-2.2)	Statistically significant increased risk only in subgroup of 339 who also smoked. OR 6.1 (3-12.5).
Longstreth et al 1998.	106 women with stroke.	18-44	NO	0.2 (0.03-1.7)	
Gardemann et al 1999	2210 patients with coronary artery disease on angiography	62.2(SD 9.9)	NO		No association in subgroups with other risk factors. No relationship with extent of coronary artery disease.
Gowda et al 2000	109 with MI	25-91	NO	P=0.42	

Table 1.4. Studies of Factor V Leiden and stroke or myocardial infarction.

Study	Number of patients	Age (range)	?Association found	OR(95% CI) or P value	Comments
Rosendaal et al 1997	79 women with MI	18-44	YES	4.0 (1.1-15.1)	Greater risk in subgroup of 59 smokers. OR 43.3(6.7-281)
Corral et al 1997a	101 with MI/unstable angina and 104 with stroke/TIA	62.9(24-88)	NO	P=0.41 for IHD, 0.56 for CVD	
Ferraresi et al 1997	195 with IHD and CVD	Not given	NO	0.77 (0.1-4.3)	
Eikelboom et al 1998	644 with IHD (402 with MI)	<50	NO	0.8 (0.4-1.8)	No association in those with acute MI (OR 0.7 {0.3-2.0}
De Stefano et al 1998	72 with ischaemic stroke	<50	YES	5.1 (1.6-16.3)	Patients with other risk factors excluded
Doggen et al 1999	560 men with MI	<70	YES- only in subgroups	Overall 1.5 (0.6-3.8)	Risk confined to those with additional risk factors and G20210A **or** V Leiden eg in smokers with either polymorphism OR 6.1 {3-12.5)
Longstreth et al 1998	106 women with stroke	18-44	NO	1.5 (0.2-9.2)	
Arruda et al 1998	220 with MI	63 'young' :38 (23-45) 157 older; 59.9(46-82)	YES	P=0.003	No age related influence on association
Ardissino et al 1999	200 with MI	<45	NO	1.4 (0.5-3.9)	
Gardemann et al 1999	2210 with IHD	62.2 (SD 9.9)	YES- only in subgroups		Coronary heart disease score was greater in those with A allele plus other risk factors, but no association with MI.
Franco et al 1999	263 with IHD or CVD	40 (24-50)	NO	2.7 (0.8-9.4)	A allele associated with higher prothrombin concentrations and markers of thrombin generation.
Ridker at al 1999	663 with stroke or MI	59.1 (SD 8.6)	NO	1.05 (0.7-1.60) 0.8 for MI (P=0.4) 1.1 for stroke (P=0.8)	Nested case control study. Subjects and controls matched for smoking status.

Table 1.5. Summary of studies of PT G20210A polymorphism and arterial thrombosis.

Study	Polymorphism	Number of subjects	Mean age (range)	?Association found	Odds ratio (95% CI) or P value	Comments
Behague et al, 1996	Ten poly-morphisms of ß-chain	565 men with MI	25-64	NO.		-854 polymorphism associated with severity of coronary artery disease.
Yu et al, 1996	-455 G/A on ß-chain	undergoing angioplasty.		YES	2.1(1.7-2.8) for CAD 2.0(1.3-3.3) for MI	
Carter et al, 1996	-455 G/A	187 patients with NIDDM	63 (55.7-69.6)	YES	1.8(1.1-2.9)	Comparison between 38 subjects with IHD and 149 without.
Wang et al, 1997	-455 G/A	545 patients with IHD	Men 55.5 Women 57.4	NO	P>0.05	Fibrinogen related to severity of coronary disease, but not genotype.
Gardemann et al, 1997	-455 G/A	923 undergoing coronary angiography	63 (SD 0.9)	NO	Not significant for MI nor stable coronary disease.	Genotype influenced basal fibrinogen and acute phase response.
Tybjaerg-Hansen et al, 1997	-455 G/A	9127 population. 470 with IHD	Men 56.8 Women 58.3	NO	P>0.05	Genotype influenced plasma fibrinogen.
De Maat et al, 1998	-455 G/A	679 with IHD	55.8 (SD 8.2)	YES	P=0.024	A allele associated with disease progression.
Nishiuma et al, 1998	-455 G/A	85 with stroke	70 (SD 9.5)	YES	2.05	No influence of genotype on plasma fibrinogen.
Carter et al, 1997	448 G/A on ß-chain	305 with stroke	Women 74(68-82) Men 72(62-78)	YES-in women only	P=0.008	Haemorrhagic and thrombotic strokes.
Schmidt et al 1998	C148T on ß-chain	399 healthy volunteers	60 (45-75)	YES	6.3 (1.9-20.7)	TT genotype associated with asymptomatic carotid atherosclerosis
Carter et al, 1999	Thr312Ala of alpha chain	519 with stroke	>70	NO	P>0.05	A allele associated with shortened survival in 101 with atrial fibrillation.
Zito et al, 1999	Bcl1 of ß-chain	101 with MI	57 (SD 7)	NO	P=0.1	Additive risk with helicobacter pylori infection: OR 7.6 (1.8-31.6)

Table 1.6. Summary of fibrinogen gene polymorphism studies.

Study	Number of subjects	Mean age (range or SD)	?Association found	Odds Ratio (95% CI) or P value	Comments
Dawson et al, 1991	145 with MI	<45	NO	P>0.05	4G/4G associated with higher PAI-1 levels.
Ye et al 1995	565 with MI	24-64	NO	P>0.05	Genotype influenced PAI-1 levels.
Mansfield et al, 1995	160 with NIDDM 38 had MI	65 (61-70)	YES	P<0.05	
Eriksson et al, 1995	94 with MI	<45	YES	2.2 (1.2-4.0)	
Catto et al, 1997	558 with stroke	73 (64-80)	NO	P=0.32	PAI-1 level but not genotype associated with early mortality after stroke.
Ridker et al, 1997	374 with MI	59.5 (8.7)	NO	1.0 (0.8-1.3)	Nested case control study from a large prospective study.
Ossei-Gerning et al, 1997	453 undergoing angiography, of which 127 had MI	men 57.5 (9.4) women 60.8(8.5)	YES	2.0 (1.1-3.7)	No influence of genotype on extent of coronary disease.
Iwai et al, 1998	301 with IHD	58.9	YES	2.7 (1.3-5.6)	4G/4G genotype associated with risk of acute coronary event.
Doggen et al, 1999	331 men with MI	56.1 (9.0)	NO	1.0 (0.6-1.5)	No association between genotype and PAI-1 levels in controls.
Gardemann et al 1999	2565 undergoing angiography	62.7 99.3)	YES- associated with the presence of coronary disease	P=0.02	4G/4G also associated with extent of coronary disease in those with other risk factors. No association with MI.

Table 1.7. Studies of the association of the PAI-1 4G/5G VNTR polymorphism and arterial disease.

Chapter Two

Patient and volunteer recruitment, sample handling and characteristics.

2.1Platelet donor study (Chapter 3).

Platelet and plasma donors attending the North East of Scotland Blood Transfusion Service apheresis unit of known HPA 1a/1b genotype were approached. This had previously been determined by the polymerase chain reaction with sequence-specific priming (Cavanagh *et al*, 1997) in a study investigating the incidence of neonatal alloimmune thrombocytopenia (NAITP) in the Grampian region of Scotland. This PCR-SSP technique is described in chapter four. Prior to donation written informed consent was obtained, and blood samples taken from resting subjects who had been supine for at least five minutes. Donors had a large (16G) apheresis needle inserted into an antecubital vein with a cuff applied to the upper arm. Care was taken to avoid artefactual platelet activation: venepuncture was performed in supine subjects following at least ten minutes rest; minimal stasis was applied and the tourniquet removed following the free flow of blood into a syringe. The first five ml of blood was discarded, and the whole sample was rejected if resistance to flow or air bubbles in the syringe were encountered. At the bedside 9ml whole blood were added to 1ml of 3.2 % trisodium citrate in a universal container. A 50µl aliquot was immediately mixed with 450µl of HEPES-magnesium buffer. All samples were subsequently processed within two hours of sampling.

Thirty five subjects possessing the 1b allele (34 heterozygotes and 1 homozygote) were compared with 35 subjects homozygous for the 1a allele. All subjects were healthy, with no history of symptomatic CVA, ischaemic heart disease, or use of aspirin or other drugs affecting platelet function.

2.2 Stroke Study patients and controls (Chapters 4 and 5)

2.2.1 Recruitment

Consecutive patients admitted to Aberdeen Royal Infirmary within 24 hours (up to 96 hours) of the onset of symptoms of stroke were assessed for eligibility between June 1997 and December 1998. This is the sole primary referral centre for the Grampian region of Scotland (population approximately 600 000); patients are therefore representative of the general stroke population. The study was approved by the Grampian Regional Ethical Committee. In an attempt to study a single pathological cause of stroke patients with a history of atrial fibrillation or valvular heart disease were not approached. Stroke was defined as a sudden loss of global or focal cerebral function which persisted for more than 24 hours. Patients were approached on admission and formal written consent obtained. A computerised tomographic (CT) brain scan was performed on all patients, and those with evidence of intracranial haemorrhage or of alternative intracranial pathology were excluded. In order to study only probable atherothrombotic stroke, a subsequent clinical assessment was made to exclude a previously undetected cardioembolic pathogenesis. Patients with evidence of valvular heart disease or thrombus on echocardiography, and those in, or who subsequently developed, atrial fibrillation were excluded. Those considered to have suffered a transient ischaemic attack (TIA, symptoms resolving within 24 hours) were included, provided that a cardiac source of embolus was considered unlikely based on the clinical assessment. Additional diagnoses and drug therapies were recorded, and patients with a past history of a connective tissue disease or those taking oral anticoagulants were not recruited. Duplex ultrasound examinations were performed to identify evidence of carotid atheroma. Age and sex matched controls were obtained from a local general practice, which cares for a population from a large area

of the City of Aberdeen, and consists of a similar racial and social class mix to the patient cohort. Those born in the same year as subjects with no history of stroke, TIA, or peripheral vascular or ischaemic heart disease were recruited after written informed consent.

The distribution of clinical risk factors, fasting lipid measurements and haematological variables in patients and controls were recorded. A diagnosis of hypertension, diabetes mellitus or hyperlipidaemia was defined as receiving current treatment for, or a past history of the condition. A family history was defined as an arterial thrombotic event in a first degree relative before the age of 55 years.

It was calculated that 150 subjects and 150 controls were required to detect a twofold difference in HPA 1b allele frequencies between cases and controls with 80% statistical power, assuming a control frequency of 19% based on published frequencies (Weiss *et al*, 1996). It was intended to select as young a cohort as possible to amplify any genetic effect on stroke risk, and a study of admissions to hospital with stroke in the preceding five months was performed. The number of admissions for the following 18 month period was estimated, and it appeared that selection of patients younger than 60 years would allow recruitment of the desired cohort size in this time (*figure2.1*). However in January 1998 the arbitrary cut-off age had to be increased to 70 years to permit recruitment the desired number of subjects in the given time, since the initial estimate did not account for the exclusion criteria described above.

2.2.2 Sampling for platelet flow cytometry and genotyping

A sample was obtained from patients and controls using a large (21G) butterfly needle. The method of venepuncture to ensure minimal *ex vivo* platelet activation was as described above for donors. Additional blood was obtained for leucocyte DNA extraction, full blood count, plasma fibrinogen concentration, and fasting cholesterol concentrations.

Patients were invited to reattend by letter for repeat sampling in the convalescent period, at least three months after the acute event. In those who agreed repeat measurements of platelet activation status and plasma fibrinogen concentration were obtained, and the use of antiplatelet drugs was recorded. In order to avoid selection of those with mild stroke only (who were able to attend the clinic), patients in residential care or in long-term rehabilitation were visited at their place of residence for sampling.

2.2.3 Sampling for plasma homocysteine measurement.

4.5 ml blood samples were taken between 7am and 9am following an overnight fast, into Vacutainer tubes containing EDTA (Becton Dickinson, Cedex, France). EDTA is preferable to citrate as it inhibits reoxidation of samples following the reduction step of preparation (Ueland *et al*, 1993). These were taken immediately to the laboratory and the plasma separated. In order to avoid release of Hcy from cellular elements all samples were centrifuged within one hour of collection, as this is associated with minimal spurious increases in plasma Hcy (Ueland *et al*, 1993). Plasma was stored at -70 °C until analysis. Additional samples were obtained for red cell folate and serum B12 assays, and for estimation of serum creatinine and fasting

cholesterol concentrations. The use of medications associated with an increased plasma homocysteine was recorded.

Patients who reattended after three months also provided a fasting blood sample for convalescent plasma homocysteine measurement. A subgroup of patients provided samples for convalescent phase red cell folate, and serum B12 and creatinine estimations.

2.3 Results

2.3.1 Subjects

The outcome of patient recruitment is summarised in *figures 2.2.*and *2.3.* 179 consecutive patients with CVA or TIA and no previous history of valvular heart disease, atrial fibrillation or vasculitis consented to participate. 29 patients were subsequently excluded: ten with intracranial haemorrhage; seven with atrial fibrillation; four with cardiac thrombi or valvular heart disease; four with intracranial tumours; and four who were considered to have an alternative diagnosis to stroke. The mean age (range) of patients and controls was 58.3 years (25-70) and 56.9 (24-72) respectively. 77 (51.3%) patients were recruited within 24 hours of stroke onset, a further 53 (35.3%) within 48 hours, 15 (10%) within 72 hours, and the remaining 5 (3.3%) within 96 hours. 140 patients (93.3%) were considered to have suffered a cerebral infarction and 10 (6.7%) a TIA. 109/150 patients (72.7%) had a carotid scan. Of these 42 (38.5%) had evidence of carotid atheroma. The mean time to follow up was 100.3 days (range 68-270), and by May 2000 15 patients had died.

HPA 1a/1b and HPA 2a/2b genotypes of 150 cases (52 male/98 female) and 150 controls (52 male/98 female) were determined. Platelet activation was assessed in all patients and in 112 controls (52 male/60 female). 77 (48 male/29 female) patients provided a repeat sample for platelet activation markers in the convalescent period.

Measurements of fasting tHcy concentrations were obtained in 106 age and sex matched controls. Since tHcy is age and sex dependent (Selhub *et al*, 1996), the 44 patients for whom a control was unavailable were also excluded to ensure the cohorts were closely matched, and males and females were analysed separately. The mean age (range) of male patients and controls was 57.2 (25-70) years and 56.7 (24-72) respectively, and in females 56.5 (26-69) and 56.5 (30-71) respectively. Fifty six (52.8%) patients were recruited within 24 hours, 33 (31.1%) within 48 hours, 13 (12.3%) within 72 hours and the remaining 4 (3.8%) within 96 hours of the onset of symptoms. 96 (90.6%) patients were considered to have suffered a cerebral infarction, and 10 (9.4%) a TIA. 83/106 (78.3%) had a carotid scan performed. Of these, 28 (34%) had evidence of carotid atheroma. 82 patients provided a fasting sample in the convalescent period. Data were not available on the remaining 24 patients, since they either refused to provide a convalescent sample or had died.

The mortality rate in the study population studied is low, which might reflect that the majority of patients were admitted to an acute stroke unit with resultant improved survival (Diez-Tejedor and Fuentes, 2001), or this may be a chance observation. The possibility of recruiting less severely affected patients is unlikely given that consecutive patients admitted to hospital were included. This potential bias was also

corrected for in the follow up phase, by resampling those unable to attend hospital as described above.

2.3.2 Conventional risk factors

Personal risk factors for ischaemic stroke and routine laboratory variables in the study of platelet function are summarised in *table 2.1*. Comparisons between patients and controls matched for the study of homocysteine yielded very similar observations (data not shown). Current smoking and hypertension were confirmed as risk factors for atherothrombotic stroke, but the odds ratios for diabetes mellitus and a positive family history did not reach statistical significance. Neither a history of treatment for hyperlipidaemia, nor fasting total, LDL cholesterol, nor triglyceride concentrations were associated with stroke. However an apparent protective effect of HDL cholesterol was detected, the mean concentration being significantly greater in controls ($p<0.001$). The mean acute phase plasma fibrinogen concentration {SD} was significantly higher than in controls (4.3{1.1}g/l vs 3.3{0.9}, $p<0.001$, Student's *t*-test), and this difference persisted into the convalescent phase (4.1{1.1} vs 3.3{0.9}, $p<0.001$). In patients who attended for follow up the mean fibrinogen concentration was higher immediately after stroke than in the convalescent period, but this difference did not reach statistical significance (4.3{1.0}g/l vs 4.1{1.0}g/l, $p=0.06$ paired *t*- test). Acute phase fibrinogen concentration correlated with that on follow up ($r^2=0.493$, $p<0.001$). There was no statistically significant difference in mean platelet count nor mean platelet volumes in acute and convalescent phases, nor between patients and controls (*table 2.1*).

The lack of an association between serum total cholesterol or LDL and stroke may reflect a fall in concentrations following the acute event, possibly as a result of poor nutrition in this period (Butterworth *et al,* 1997). This observation also supports the suggestion that the study included patients with severe stroke, since a reduced cholesterol concentration measured within 24 hours of ictus is been associated with reduced survival (Dyker *et al,* 1997).

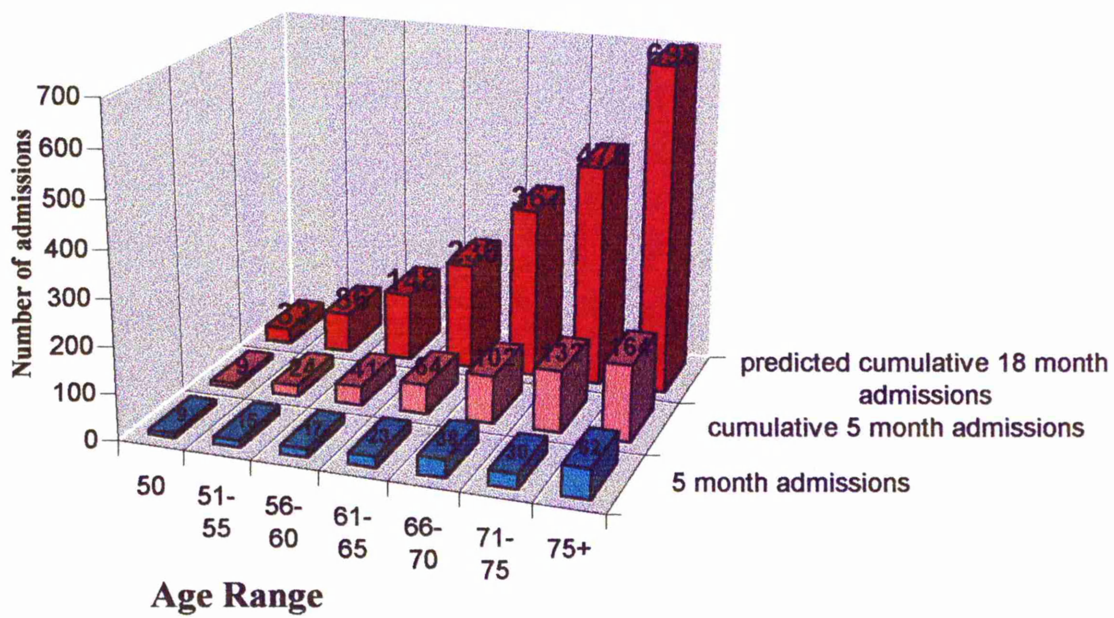


Figure 2.1 Admissions with stroke to Aberdeen Royal Infirmary 11/01/97-12/06/97.

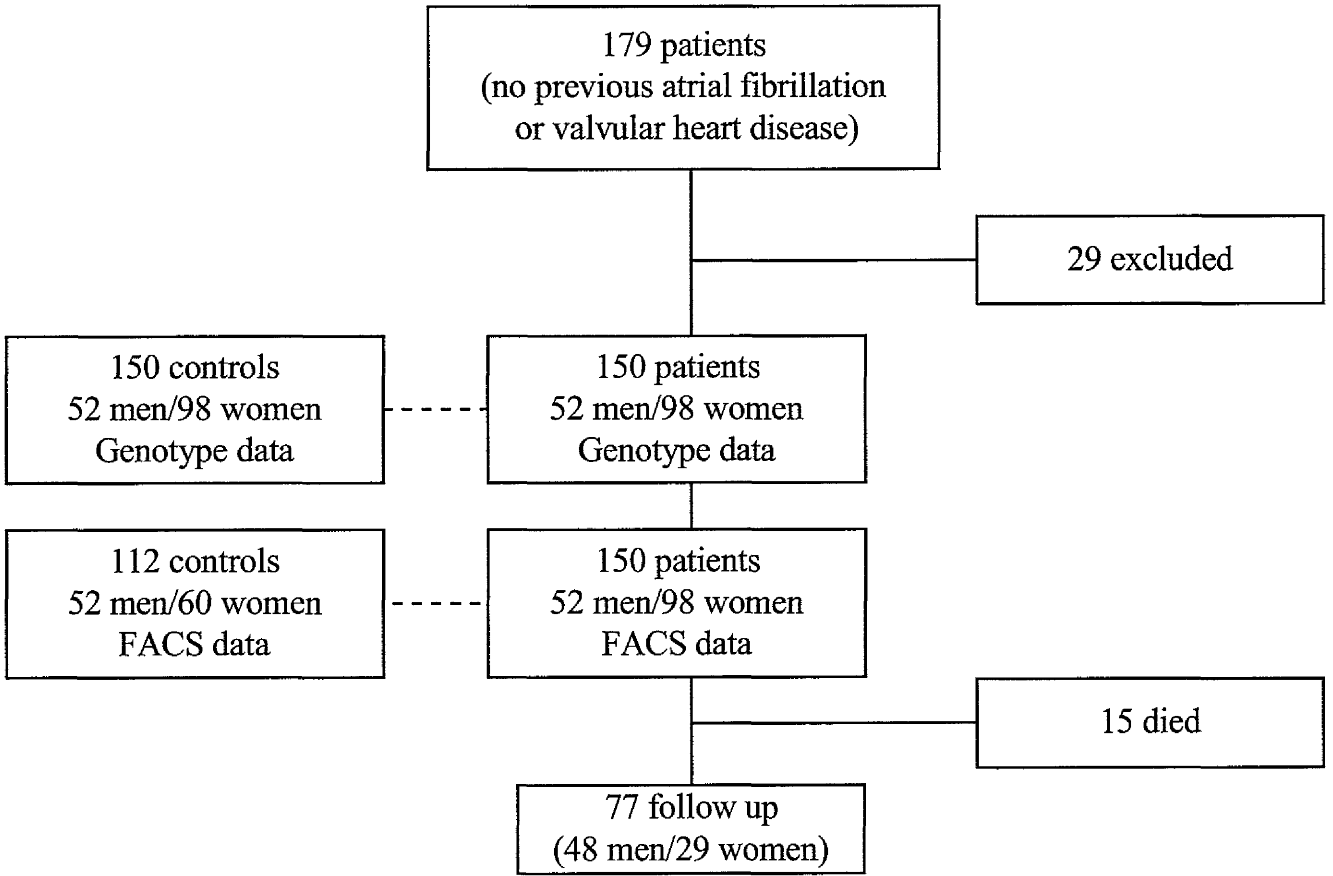


Figure 2.2. Platelet Study recruitment

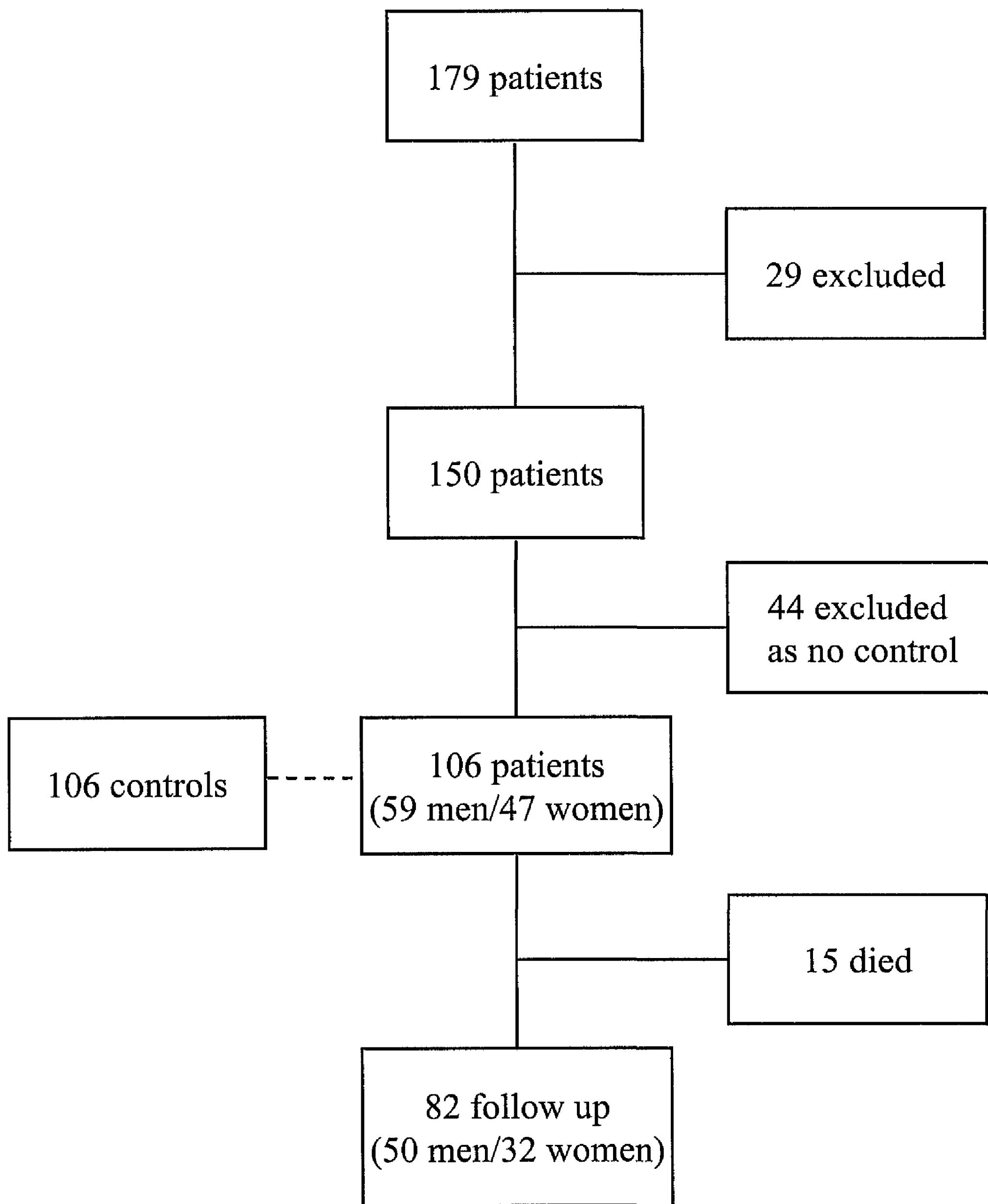


Figure 2.3. Outcome of homocysteine study recruitment. Complete data were available from 106 patients with atherothrombotic stroke or TIA and 106 healthy age and sex matched controls.

Risk factor	150 subjects n(%)	109 controls n(%)	Odds Ratio (95%CI)	p value
Current smoker	77 (51.3)	26 (23.8)	3.4 (2.0-5.8)	………
Hypertension	65 (43.3)	16 (14.7)	4.4 (2.4-8.3)	………
Family history	45 (30)	27 (24.8)	1.3 (0.7-2.3)	………
Diabetes mellitus	12 (8)	4 (3.6)	2.3 (0.7-7.2)	………
Hyperlipidaemia	18 (12)	26 (23.8)	0.4 (0.2-0.8)	………
No risk factor	11 (7.3)	25 (22.9)	0.2 (0.1-0.6)	………
Total serum cholesterol (mmol/l) mean (SD)	5.8 (1.3)	6.0 (1.1)	……………	0.22[1]
LDL cholesterol (mmol/l) mean (SD)	3.8 (1.2)	3.8 (1.0)	……………	0.80[1]
HDL cholesterol (mmol/l) mean (SD)	1.2 (0.4)	1.5 (0.3)	……………	<0.001[1]
Triglyceride (mmol/l) median (range)	1.4 (0.3-8.2)	1.3 (0.4-4.2)	……………	0.12[3]
Plasma fibrinogen (g/l) mean (SD)	4.3 (1.1)	3.3 (0.9)	……………	<0.001[1]
Mean platelet volume (fl) mean (SD)	acute 9.1 (0.8) convalescent 9.1 (1.3)	9.2 (0.9)	……………	acute vs control p=0.42[1] convalescent vs control p=0.72[1] acute vs convalescent p=0.87[2]
Platelet count ($x10^9/l$) mean (SD)	acute 252 (77.0) convalescent 266 (59.3)	252 (66.4)	……………	acute vs control p=0.93[1] convalescent vs control p=0.12[1] acute vs convalescent p=0.23[2]

Table 2.1. Established risk factors. [1] Student's *t*-test, [2] Paired *t*-test, [3] Mann Whitney U test.

Chapter Three

An investigation of the effect of HPA 1a/1b genotype on platelet fibrinogen binding by whole blood flow cytometry in healthy subjects.

3.1 Background

As discussed in chapter one, genetic factors might contribute to the risk of stroke, and it has been postulated that polymorphisms of platelet surface membrane glycoproteins (GP), by an effect on platelet function might be involved. GPIIIa, in heterodimeric complex with GPIIb, is central in the process of platelet aggregation which is mediated mainly by fibrinogen binding (Nurden, 1995). The HPA 1b (PlA_2) allele of the 1a/1b (PlA_1/A_2) polymorphism arises due to a substitution of thymine by cytosine at position 1565 in exon 2 of the human GPIIIa gene. This results in the replacement of leucine by proline at amino acid 33 of the protein (Newman *et al*, 1989). HPA 1b is common, being present in around 25% of Northern Europeans (Bray, 1999), and has been widely investigated as a candidate risk factor for arterial thrombosis. Studies have failed to consistently link the 1b allele with ischaemic stroke (Ridker *et al*, 1997c; Carter *et al*, 1998), but an association in younger patients has been proposed (Carter *et al*, 1998; Wagner *et al*, 1998). Some case control studies (Weiss *et al*, 1996; Carter *et al*, 1997b; Gardemann *et al*, 1998; Garcia-Ribes *et al*, 1998; Mikkelsson *et al*, 1999), but not others (Marian *et al*, 1996; Osborn *et al*, 1996; Herrmann *et al*, 1997; Ridker *et al*, 1997c; Samani & Lodwick, 1997; Durante-Mangoni *et al*, 1998) have associated the 1b allele with increased risk of MI.

The plausibility of any relationship between this candidate gene and thrombosis is dependent upon evidence of altered platelet reactivity associated with individual alleles, and interpretation of these conflicting epidemiological data is hindered by a lack of corresponding functional data in most studies. Although the polymorphism is

some distance from the fibrinogen binding sites on GPIIIa (positions 109-171 and 211-222), it has been proposed that the 1b allele might alter fibrinogen binding, which in turn might influence platelet aggregation (Bray, 1999). In addition to its function as a ligand ('inside-out' signalling), GPIIIa is also involved in 'outside-in' signalling, where platelet activation by receptor mediated intracellular signalling follows the binding of fibrinogen and vWF to GPIIIa (Shattil, 1999). Thus thrombotic risk might be postulated to arise as a result of enhanced platelet activation mediated by the 1b allele, but at the time of this study a plausible prothrombotic mechanism had not been described to support the 1b allele as a risk factor for thrombosis.

3.2 Aims

An effect of the HPA 1b allele on platelet function was sought in healthy subjects. This was explored by whole blood flow cytometry to investigate 'inside-out' fibrinogen binding directly, since this permits the direct study of the interaction of fibrinogen with its receptor, and obviates the artefactual effects encountered in aggregometry (Michelson, 1996). The influence of genotype on fibrinogen binding at rest and following stimulation with the weak agonist adenosine diphosphate (ADP), was determined. In addition, platelet P-Selectin expression was investigated as a reflection of 'outside-in' receptor signalling, and an allele dependent effect was sought.

3.3 Methods

2.3.2 Detection of platelet activation markers by whole blood fluorescence activated cell sorting (FACS)

Detection of platelet activation markers by FACS

Platelet surface binding of fibrinogen was assessed by single colour technique using FITC conjugated polyclonal rabbit antihuman fibrinogen antibody, which is not affected by non-specific platelet fibrinogen binding or plasma fibrinogen concentration (Warkentin *et al*, 1990).

The technique can be summarised thus:

- Platelets are identified by light scatter characteristics and by FITC conjugated antiCD61 (GPIIIa).
- Fibrinogen binding is analysed using single colour analysis with FITC conjugated antifibrinogen.
- Basal activation status is assessed in resting platelets (no added agonist).
- Platelet reactivity is assessed by analysis of samples preincubated with ADP (a weak agonist).
- P-Selectin expression is analysed by dual staining with FITC-antiGPIIIa and PE conjugated anti-P Selectin (CD62P).

Sample preparation

Subsequent reactions were performed in a standardised manner. 40µl aliquots of whole blood in HEPES were incubated in polypropylene tubes for 30 minutes at room temperature in the dark with 10 µl monoclonal antibody. Antibodies were

diluted to yield a final optimal concentration of 2ng/µl in each reaction. This was determined by individual manufacturer's recommendations and had been previously validated in the laboratory. Aliquots from each subject were subject to seven separate reactions as described below:

Tube 1: FITC-mouse IgG isotype control (Becton Dickinson Ltd, California USA).

Tube 2: PE-mouse IgG isotype control (Becton Dickinson Ltd, California, USA).

Tube 3: FITC- murine antihuman GPIIIA (CD61) (Dako Ltd, Glostrup, Denmark) to identify platelets.

Tube 4: FITC-murine antiCD61 (Dako) and PE-murine antiP-Selectin (CD62P) dual staining to assess basal P-Selectin expression.

Tube 5: As tube 4, preincubated for 5 minutes with 5 µl PMA (final concentration 1µg/ml, Sigma, St Louis, USA) as positive control.

Tube 6: FITC-rabbit polyclonal antihuman fibrinogen (Dako Ltd, Glostrup, Denmark) to assess basal fibrinogen binding.

Tube 7: As tube 6, preincubated with 5µl ADP (Sigma Chemical Co, St Louis, USA), at a final concentration of 10^{-5}M to assess fibrinogen binding after platelet activation.

Reactions were stopped by the addition of 2ml phosphate buffered saline (PBS), and samples stored at 4°C until FACS analysis. Samples were analysed within three hours of processing.

FACS analysis

The study was performed using a Coulter XL-MCL flow cytometer (Coulter Electronics, Luton, UK). Numbered tubes were placed in an autoanalyser carousel.

The sample was drawn into the analyser at the 'low' flow rate setting. Listmode data were stored and processed using a personal computer and System II software version 1.0 (Coulter Electronics Ltd, Luton, UK), and converted to scatterplots and histograms. Cells were separated by their forward and side light scatter characteristics (determined by cell size and granularity respectively). Particles corresponding to platelets in the scatterplot *(figure 3.1)* were enclosed in an electronic bitmap and analysed for fluorescence following laser excitation at 488nm, FITC conjugated antibody emitting a 530nm signal (green) and PE a 585nm signal (red). Sampling was terminated automatically following accumulation of 10000 events, and the percentage of positive platelets and mean cell fluorescence recorded.

Fibrinogen binding was assessed indirectly by first identifying the percentage of the events analysed which were platelets as defined by FITC-antiCD61 positivity in tube 3. The percentage of events positive for FITC-antifibrinogen at baseline in tube 6 and following ADP stimulation in tube 7 *(figure 3.2)* was then adjusted for this value. This method has been previously validated (Janes *et al*, 1993). P-Selectin expression was recorded from events dual staining for anti-CD61 and anti-CD62P *(figure 3.3)*.

Statistical analysis and sample size

Calculations were performed using SPSS for Windows version 8.0 statistical software. Allele dependent differences in means were assessed by Student's t-test for

normally distributed data and medians were compared by Mann Whitney U-tests when data were skewed. The sex distribution of the two groups was compared by Fisher's exact test. It was calculated that 35 subjects homozygous for HPA 1a/1a and 35 possessing the 1b allele were required to detect with 80 % statistical power a 10% difference in fibrinogen binding after incubation with ADP. A two tailed p value of <0.05 was considered significant.

Quality assurance

A positive control sample (*figure 3.4)* was included to ensure the presence of reactive platelets, by assessing CD62P expression upon incubation with phorbol myristate (PMA, Sigma Chemical Co, St Louis, USA), which induces activation via the protein kinase C intracellular pathway (Michelson, 1996). Correction for non-specific binding of antibody to platelets was facilitated by the use of idiotype antibodies (Dako, Glostrup, Denmark). Since almost all antibodies used were of murine origin these were conjugated mouse IgG_1 antibodies with specificity for an antigen absent in humans.

The flow cytometer was aligned daily with 'Flowcheck^{TM}' and 'Immunobrite^{TM}' beads (Coulter Electronics, Luton, UK) to calibrate light scatter and fluorescence parameters respectively. A laboratory log of these steps was recorded daily to ensure regular calibration. The flow cell was cleaned thoroughly between individual subject samples to exclude carry over of platelets to subsequent analysis using Coulter Clenz^{TM} cleansing solution (Coulter Ltd, Luton, UK). Solutions were filtered prior to use to remove small particles of a similar size to platelets, and were renewed weekly.

Aliquots of ADP and PMA were stored at -20 °C prior to use, and it was determined that thawed aliquots of ADP should be used immediately, since use more than one hour after thawing resulted in a 10% reduction in stimulated fibrinogen binding (data not shown). PMA could be repeatedly thawed and refrozen, as this had no effect on the extent of CD62P expression.

The optimal dilutions of each new batch of antibody were established by comparison with the working concentration of antibody from the previous batch. The isotype controls were used to correct for non specific platelet protein binding and the negative cut-off was set at 0% positivity. To avoid the influence of artefactual platelet activation, data were normalised and samples yielding a % fibrinogen binding or CD62P value greater than three standard deviations from the mean were discarded, and the subject was resampled at the next donation visit.

3.4 Results

Results are summarised in *table 3.1*. There was no allele dependent difference in the median percentage of platelets binding fibrinogen at baseline (p= 0.14, Mann Whitney U-test) nor in means following incubation with ADP (P=0.72, Student's *t*-test, see *figure 3.5*). The density of fibrinogen binding sites, as measured by mean cell fluorescence, was greater in 1a than 1b platelets following stimulation with ADP (p=0.05 Mann Whitney U test). This is illustrated in *figure 3.6*. There was a tendency for more 1b platelets to express P-Selectin, but this did not achieve statistical significance (p=0.08, Mann Whitney U-test). No difference in CD62P mean cell fluorescence was observed (p=0.54, Mann-Whitney U test).

There was no significant age difference between the two groups (mean age {range} 42.2 years {27-60} in 1a/1a group, and 41 {25-56} in those possessing the 1b allele, p=0.6, Student's *t*-test), and no difference in sex distribution between the two groups was observed (26 male/9 female in 1a/1a versus 22 male/13 female in 1a/1b and 1b/1b group, p=0.44, Fisher's exact test). 5/70 subjects initially had baseline increased fibrinogen and/or P-Selectin expression suggestive of *ex vivo* activation. In each case repeat sample collection yielded values within the normal range (not shown), and initial data were excluded from analysis.

Genotype	% Fibrinogen median (range)	%Fibrinogen post ADP mean (SD)	Fibrinogen mean X post ADP median (range)	% CD62P median (range)	CD62P mean X median (range)
1a/1a	0.7 (0.1-3.1)	59.9 (13.8)	9.4 (4.7-20.3)	0.1 (0-0.7)	1.4 (0.95-2.16)
1a/1b and 1b/1b	0.5 (0.2-2.4)	58.7 (14.2)	7.2 (3.4-16.0)	0.25 (0-1.3)	1.4 (0.94-2.18)
Significance	0.14[1]	0.72[2]	0.05[1]	0.08[1]	0.54[1]

Table 3.1 Results. The effect genotype on the percentage of platelets (%) binding fibrinogen and expressing P-Selectin (CD62P). Mean X = mean cell fluorescence in arbitrary fluorescence units. Fibrinogen binding was measured in resting platelets and following incubation with ADP, a weak agonist.

[1]Mann Whitney U test
[2]Student's *t*-test

3.5 Discussion

In this study platelets were analysed by whole blood flow cytometry to avoid the potential for *in vitro* activation, which can occur when preparing platelet rich plasma. Furthermore, it was possible to exclude activated samples by measuring the expression of baseline fibrinogen binding and P-Selectin. Since the resting level of

binding in normal individuals is low, as illustrated in *figure 3.2* and previously reported (Janes *et al*, 1993) samples were stimulated with ADP to expose the active fibrinogen binding sites on GPIIIa and amplify any possible allele dependent difference.

These data do not reveal a genetically determined difference in platelet fibrinogen binding patterns to plausibly support the 1b allele as a risk factor for thrombosis. Indeed, it was found that the density of fibrinogen binding sites in stimulated platelets was less in 1b platelets than those from subjects homozygous for HPA 1a. A greater increase in the percentage of platelets binding fibrinogen after ADP stimulation in 1b subjects was not observed, in contrast to observations in patients with ischaemic heart disease studied by the same method (Goodall *et al*, 1999). This might reflect differences in studying platelet function in healthy subjects and in those with arterial disease. Given that the current study is of comparable size to that of Goodall *et al*, a similar allele dependent difference in fibrinogen binding should have been apparent, but a type 2 statistical error of 20% renders it possible that a smaller effect below the limit of detection may be present. However it is unclear whether such an effect would be of biological significance.

There was a tendency for more platelets from HPA 1b subjects to express P-Selectin than those homozygous for 1a, but this did not reach statistical significance. It is acknowledged that this study may be underpowered to detect a significant difference in P-Selectin expression, since the sample size was primarily determined to detect an effect on fibrinogen binding patterns. If the observed differences are truly

representative it is calculated that (given an approximate standard deviation of 0.25% calculated from the transformed data) 44 subjects with HPA 1b and 44 1a/1a homozygotes would be required for significance at the 5% level to be detected with 80% statistical power.

Following completion of this study it could be suggested that any allele dependent differences may be small if present at all, or may not be apparent in a relatively small study of healthy subjects. Such differences may be unmasked in disease states when platelet activity might be increased, and on recruitment of a larger sample size. Thus in order to plausibly demonstrate the 1b allele as a risk factor it is desirable to observe an effect in patients with acute arterial thrombosis. These points are addressed in chapter four, when the role of platelet activation markers and genotype was studied in patients following acute stroke.

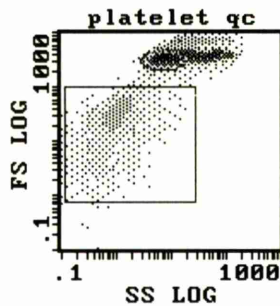


Figure 3.1. Scatterplot obtained on flow cytometry of whole blood. Platelets are identified by their size and granularity, and gated (small rectangle). SS=side scatter (granularity). FS=forward scatter (size).

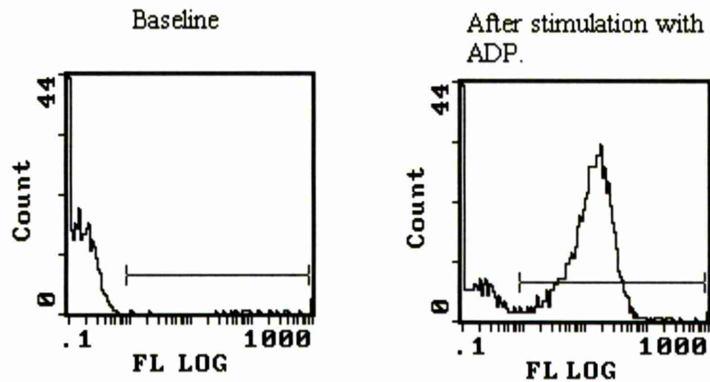


Figure 3.2. Histograms depicting platelet fibrinogen binding, before and after stimulation with ADP. Events with fluorescence falling within the horizontal bar are considered positive. This was adjusted for non-specific binding by moving the bar until a value of 0% was obtained with the FITC isotype control. FL=fluorescence.

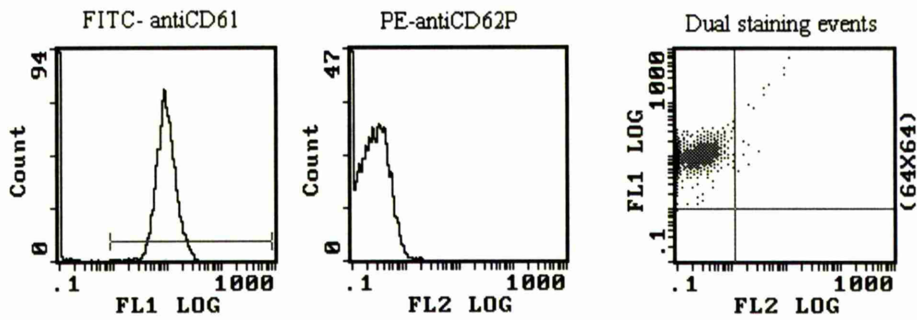


Figure 3.3. Histograms and scatterplots depicting platelet P-Selectin expression, as determined by dual binding with anti-GPIIIa and anti-CD62P. FL1=FITC. FL2=PE.

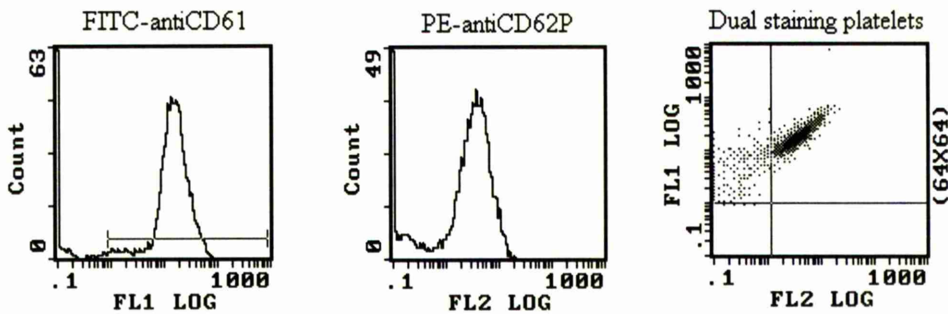


Figure 3.4. Stimulation of P-Selectin expression in the same subject as *figure 3.3* following incubation with PMA.

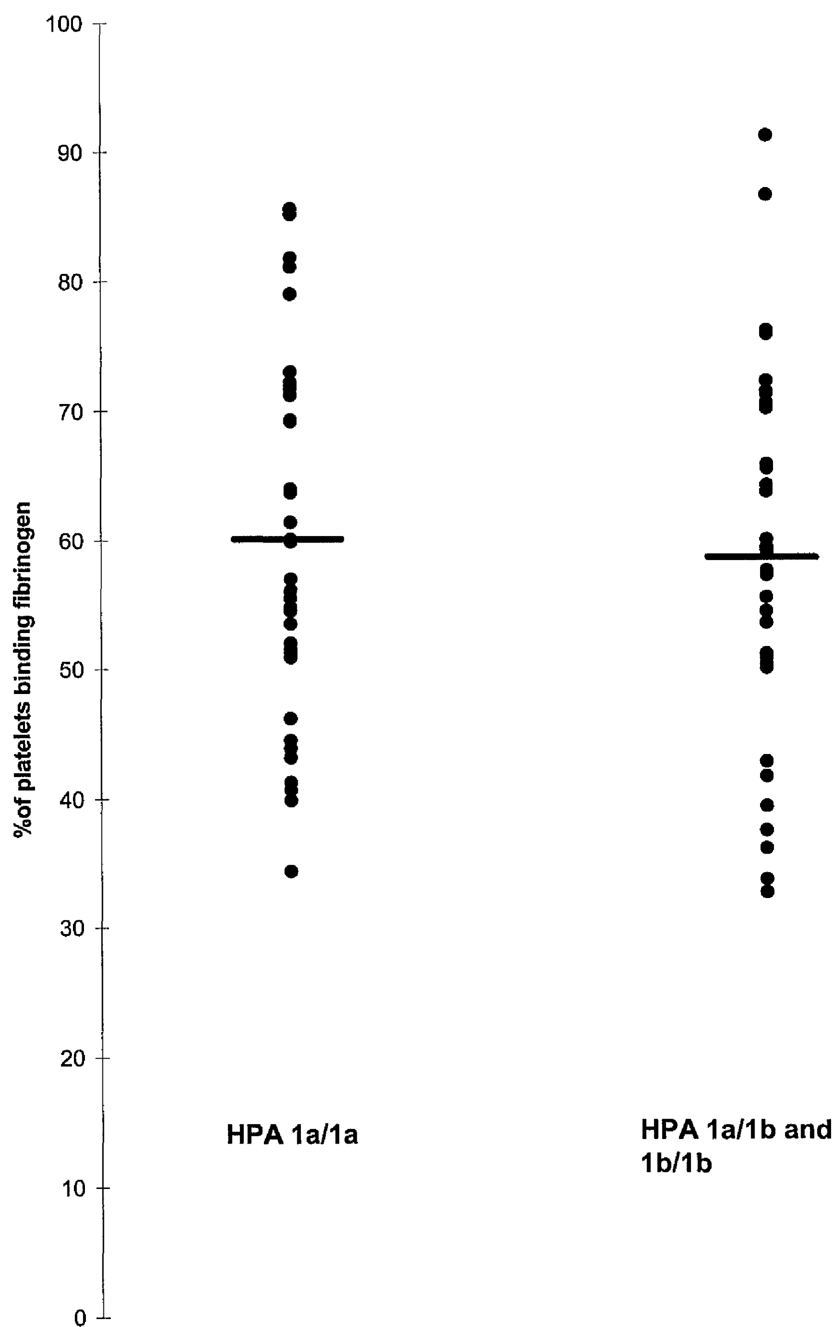


Figure 3.5. Percentage of platelets binding fibrinogen after stimulation with ADP. Mean values are denoted by horizontal bars.

HPA 1a/1a

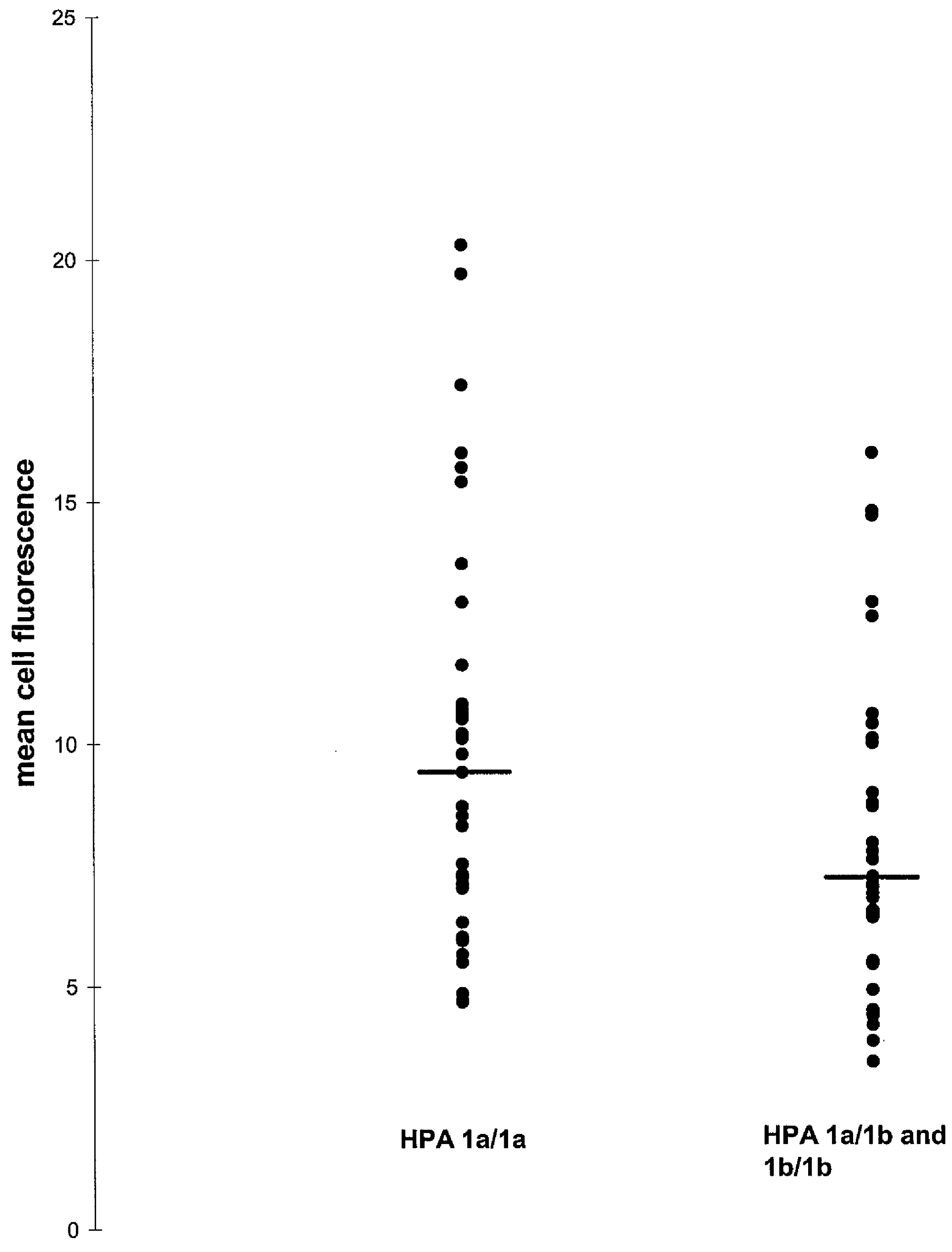


Figure 3. 6 Fibrinogen mean cell fluorescence following stimulation with ADP. Horizontal lines denote median values.

Chapter Four.

In vivo platelet activation in atherothrombotic stroke: relationship to polymorphisms of human platelet GPIIIa and GPIb.

4.1 Background

In chapter three a plausible effect of the HPA 1b allele on platelet function to support a role in thrombosis was not apparent in healthy subjects, and it was suggested that allele dependent effects might be revealed in patients with atherothrombotic stroke where platelet activity might be enhanced.

As discussed in chapter one, platelets have a pivotal role in thrombus formation following atheromatous plaque rupture (del Zoppo, 1998). Given the efficacy of antiplatelet therapy with aspirin in secondary prevention of acute arterial thrombosis (Antilatelet Trialists' Collaboration, 1994), and that enhanced platelet aggregation has been shown prospectively to predict mortality following myocardial infarction (MI) (Trip *et al*, 1990), enhanced platelet activation might be a risk factor for arterial thrombosis. However, prospective data on platelet activitation as a risk factor for future stroke development are lacking. In case control studies platelet function has been measured either immediately following acute stroke (van Kooten *et al*, 1997; Zeller *et al*, 1999) or in the stable convalescent phase (Couch & Hassanein, 1976; Shah *et al*, 1985; van Kooten *et al*, 1999), but only one study has measured activation at both timepoints (Konstantopoulos *et al*, 1995), and reported persisting increased shear-induced platelet aggregation in fifteen patients with non-lacunar stroke.

As well as GPIIIa, platelet membrane glycoprotein GPIb (as part of the Ib-V-IX complex) mediates platelet adhesion via vWF binding, and therefore is also important in mediating arterial thrombosis (Nurden, 1995). The HPA 2a/2b (Ko^a/Ko^b, threonine at position 145 in 2a and methionine in 2b) and variable

number of tandem repeats (VNTR) polymorphisms of the GPIb gene are in linkage disequilibrium, and have been studied as candidate risk factors for arterial thrombosis, since they might alter vWF binding. As was the case in studies of the HPA 1a/1b polymorphism, some reports (Murata *et al*, 1997; Gonzalez-Conejero *et al*, 1998; Sonoda *et al*, 2000) but not others (Carter *et al*, 1998; Corral *et al*, 2000) have linked the 2b allele of HPA 2a/2b and the C/B VNTR genotype with coronary artery and cerebrovascular disease. It has been suggested that the variability in the distance of the vWF binding site from the platelet surface seen with the VNTR polymorphism might influence platelet adhesion (Nurden, 1995), but there are currently no functional data to support these polymorphisms as thrombotic risk factors. The HPA 2a/2b and VNTR GP1b polymorphisms are not associated with density of surface receptor expression (Corral *et al*, 2000).

4.2 Aims

A case control study was performed to address these issues. There were three main objectives in this study:

i) Markers of platelet activation were measured by flow cytometry immediately following atherothrombotic stroke and compared with healthy controls. Evidence of persisting platelet activation was sought by resampling patients in the convalescent period (at least three months later).

ii) HPA 1a/1b and 2a/2b allele frequencies were determined in patients and controls to investigate epidemiologically whether these polymorphisms are risk factors for stroke.

iii) A correlation between polymorphisms and platelet function was sought by investigating the influence of genotype on the expression of platelet activation markers.

4.3 Methods

Patient and control selection, recruitment and sampling is described in chapter two.

4.3.1 Platelet flow cytometry

Samples were treated in a standardised manner within one hour of collection. Sample preparation and analysis was performed as described in chapter three. Platelets were analysed within 4 hours of preparation, although antibody binding was found to be stable for up to 6 hours if stored at 4 °C (data not shown). The percentage of platelets positive for P-Selectin and binding fibrinogen, and mean cell fluorescence as a measure of the average density of antibody binding per platelet, were recorded for each subject. Basal activation status was assessed in resting samples, and platelet reactivity following incubation with Adenosine Diphosphate (ADP) at a final concentration of 10^{-5}M for 5 minutes (Sigma, St. Louis, USA).

4.3.2 DNA extraction

Leucocyte DNA was extracted using a proprietary kit. (Nucleon BACC 2 Extraction Kit, Nucleon Biosciences, Coatbridge, UK). The following steps were taken:

- Whole blood was centrifuged at 1300g for 15 minutes (MSE Chilspin centrifuge, Fisons, UK) and plasma removed.
- Buffy coats were transferred to a universal container, and approximately 5ml of Nucleon Reagent A (red cell lysis solution) were added.
- The mixture was shaken at room temperature for five minutes, and then centrifuged at 1300g for five minutes to precipitate white cells.
- The supernatant was carefully removed and 2ml Nucleon Reagent B (white cell lysis buffer) added. This was mixed until viscous, and left overnight at 37 °C.
- The solution was transferred to a polypropylene tube and mixed with 500μl sodium perchlorate (supplied with kit).
- An equal volume (2.5ml) of 100% chloroform (BDH Laboratory Supplies, Poole, UK) was added.
- 250μl Nucleon resin were added and the mixture was centrifuged at 1300g for five minutes.
- The upper layer was transferred to a clean tube and two volumes of cold 100% Ethanol (4ml) added to precipitate DNA.
- DNA was removed with a blue pipette tip, placed in an eppendorf tube, and washed with 70% Ethanol.
- The pellet was centrifuged at 5000g for five minutes (ALC Microcentrifuge, ALC International, Italy), allowed to dry then resuspended in 10mM Tris/EDTA.

The DNA concentration was assessed by spectrophotometry (PTP-6 Spectrophotometer, Perkin Elmer). 5μl were mixed with 245μl of dH_2O and the absorbance (A) at 260nm against a blank was recorded.. Since an A of 1.0 corresponds to 50μg/ml of DNA, the final concentration was calculated by multiplying by 2500. The ratio of A280: A260 was recorded as an indicator of protein contamination. Samples with a high value underwent repurification by repeating the extraction steps above. Working dilutions of DNA at 100ng/μl were made by dilution with dH_2O, which were stored at -70 °C until use.

4.3.3 HPA 1a/1b genotyping by PCR-SSP.

Principle

This method has been described previously (Cavanagh *et al*, 1997). Given that they differ from each other by a single nucleotide base at position 1565 in exon 2 of the human GPIIIa (thymidine in 1a, cytosine in 1b, Newman *et al*, 1989), alleles are distinguished by amplifying DNA by the polymerase chain reaction (PCR) using primers with this difference reflected at the 3' end. In the absence of the allele no product is formed, since DNA polymerase incorporates nucleotides from the 3' end of a strand. This technique is known as PCR with sequence-specific priming (PCR-SSP) or Amplification of Refractory Mutation System (ARMS).

Each patient sample is therefore subject to two PCR reactions, with 1a and 1b primers separately. A common primer is included to generate a 90 base pair (bp) product:

1a primer: 5'TCACAGCGAGGTGAGGCC**A**3'

1b primer: 5'TCACAGCGAGGTGAGGCC**G**3'

Common primer: 5'GGAGGTAGAGAGTCGCCATAG3'

The presence of an allele is therefore indicated by the presence of a 90 bp product and its absence of by the absence of a product. Internal control primers for Human Growth Hormone (HGH) are added to the primer mix and generate a 429 bp product, confirming success of the amplification reaction:

5'GCCTTCCCAACCATTCCCTTA3'

5'TCACGGATTTCTGTTGTGTTTC3'

Materials and methods

10µl reactions were performed in thin walled tubes. Each contained:

- 200ng (2µl) patient or control DNA in dH_2O.
- 1µl NW3 PCR reaction Buffer.
- 0.35 units Taq polymerase.
- 0.2µl of deoxynucleotide triphosphates (dNTP).
- 0.1µl of Cresol Red 10mg/ml to assist visualisation of product on electrophoresis.
- 5µl of primer mix.
- dH_20 to make a total volume of 10µl per reaction.

2µl of DNA were placed in a reaction tube containing 3µl of a master mix consisting of nucleotides, buffer and *Taq* polymerase, plus 5µl of primers. 24 samples were analysed in each experiment, a total of 48 PCR reactions per microtitre plate. Therefore the following mastermix (50 reactions) was used:

- 10µl dNTP's.
- 50µl NW3 buffer.
- 4µl Taq polymerase.
- 5µl Cresol red.
- 81µl dH_2O.

Using a PTC-200 Pelter Thermal Cycler (Genetic Research Instrumentation Limited, Dunmow, Essex, UK), the following temperature cycling conditions were applied :

96°C initial denaturation for 1 min; 5 cycles of 96°C for 25s then 68°C for 45s then 72°C for 30s; 28 cycles of 96°C for 25s then 61°C for 45s then 72°C for 30s; 72°C for 3 min. These had previously been determined to be the optimal cycling conditions using the above instrument at SNBTS Aberdeen, and differ from those described (Cavanagh *et al*, 1997).

10μl of the product was electrophoresed on 1.5% Agarose gel at 200V for approximately 15 minutes in 0.5% Tris-Borate EDTA. The gel was stained with Ethidium Bromide, to enable visualisation by Ultra Violet fluorescence, and a photographic record obtained. A 100bp ladder was included in one lane of each gel to indicate the size of DNA bands.

In all experiments performed using this method the 1b/1b homozygote control cross-reacted with the 1a primer mix, yielding a faint band following gel electrophoresis. Attempts were made to eradicate this mispriming (as suggested by Bottema & Sommer, 1993) by:

- Increasing the annealing temperature.
- Reducing Mg concentration.
- Reducing DNA concentration.
- Reducing primer concentration.
- Reducing dNTP concentration.
- Reducing *Taq* polymerase concentration.

These manoeuvres were unsuccessful and resulted in either persistence of a band or failure of amplification altogether. Since HPA 1b/1b homozygotes were not unequivocally identifiable using this method, it was decided to abandon genotyping by SSP-PCR and use a restriction enzyme based method instead.

4.3.4 HPA 1a/1b genotyping by PCR with restriction enzyme digest.

Principle

Substitution of cytosine for thymidine at position 1565 in exon 2 of the GPIIIa gene results in the sequence CCGG, a cleavage site for the restriction endonuclease *msp 1*. Using the following primers (Jin *et al*, 1993) amplification of genomic DNA by PCR yields a 266bp product:

sense: 5'TTCTGATTGCTGGACTTCTCTT3'

antisense: 5'TCTCTCCCCATGGCAAAGAGT3'

The a allele contains a single *msp1* site, yielding 221 and 45 bp fragments. The presence of a b allele is indicated by the presence of a second cleavage site, resulting in a 177 bp fragment. Alleles can therefore be separated electrophoretically (Weiss *et al*, 1996).

Optimisation of PCR conditions

Optimal PCR conditions were established by Mg titration. 4μl of 50ng/μl of genomic DNA were added to a 50μl thin walled PCR tube. To each tube the following were added:

- 200ng genomic DNA.
- 20pmol of each primer.
- 200μmol of dNTPs.
- 2 units *Taq* DNA polymerase.
- $MgCl_2$, varying between 0.5-5mM.
- 20mM Tris HCl.
- 25mM KCl.
- dH_2O to a final volume of 50μl.

Each reaction was subject to the following conditions: 38 cycles of: denaturation at 95 °C for 60s; annealing at 60 °C for 45s; and extension at 72 °C for 75s in a Techne Progene Thermal Cycler (Cambridge, UK). 15 μl of product and 2 μl of loading dye were pipetted onto a 2% agarose gel, electrophoresed at 75V for two hours, and stained with ethidium bromide. The product band stained weakly over the range of Mg concentrations, indicating an inefficient PCR reaction. It was felt that the digested product would be difficult to visualise following further dilution in enzyme reaction buffer, and the thermal cycling conditions were altered by increasing the duration of each temperature cycle as indicated below:

- Denaturation at 94 °C for two minutes, 1 cycle;
- Denaturation at 94 °C for one minute, annealing at 60 °C for two minutes, extension at 72 °C for 2.5 minutes, 35 cycles;
- Extension at 72 °C for ten minutes, one cycle.

This produced a satisfactory product, and it was then determined that optimal $MgCl_2$ concentration was 2mM per reaction. In subsequent reactions 4μl of

genomic DNA was added to 16μl of a mastermix solution and 30μl of dH_2O. The mastermix contained (per reaction):

- 5μl of 10X Reaction Buffer.
- 1μl of dNTP's.
- 2μl sense primer (10pmol/μl).
- 2μl antisense primer (10pmol/μl).
- 4μl 25mM $MgCl_2$.
- 0.4μl Taq polymerase.
- 1.6μl dH_2O.

The thermal cycler processed 20 samples in a single run, consisting of a blank, 16 subject samples and three controls of known genotype.

Restriction enzyme digest

Conditions were optimised using subjects of known genotype (kindly supplied by Dr G Cavanagh, Newcastle Blood Transfusion Service). The PCR product was incubated overnight at 37 °C with 20 units of *mspl*. 15μl of product were added to 15μl of a mastermix consisting of (per reaction):

- 3μl Buffer Y.
- 2μl *mspl* (10units/μl).
- 10μl dH_2O.

13μl of the product plus 2μl of loading dye were electrophoresed on 4% (4g in 100 ml of TBE) agarose gel for two hours at 90 volts. An example of the bands obtained to discriminate individual genotypes is given in *figure 4.1*.

Quality assurance

Each experiment included a blank (no DNA) to exclude reagent DNA contamination. Samples of known genotype were included in every experiment. Analyses were repeated at random to confirm the reproducibility of the method; three samples (one HPA 1a/1a and two 1a/1b) were genotyped three times each and the same result was obtained every time. Occasionally difficulty discriminating homozygous 1b/1b from heterozygous 1a/1b samples was encountered, as evidenced by the presence of both 177 and 221 bp bands in the enzyme digest of the HPA 1b/1b control. When this occurred the experiment was repeated. This occurred on three occasions and coincided with use of the last aliquot of a batch of enzyme. This difficulty was overcome by increasing the concentration of enzyme used to 30 units per reaction.

4.3.5 HPA 2a/2b genotyping by PCR-SSP

This was determined using the reaction conditions described in *4.3.3* using the following primers to obtain a 258bp product:

2a primer: 5'GCCCCCAGGGCTCCTGA**C**3'

2b primer: 5'GCCCCCAGGGCTCCTGA**T**3'

common primer: 5'TCAGCATTGTCCTGCAGCCA3'

Unlike the HPA 1a/1b genotyping no mispriming was encountered with this method. These experiments were performed by Miss Irene Moore in SNBTS

Aberdeen, in a laboratory which is accredited by the Clinical Pathology Association of the UK (CPA) and which performs satisfactorily in a national external quality assurance scheme (NEQAS, Sheffield, UK).

4.3.6 Plasma fibrinogen concentration

Assays were performed in the Haematology laboratory at Aberdeen Royal Infirmary. 4.5ml of whole blood anticoagulated in 3.9% trisodium citrate were collected in a Vacutainer® sample tube (Becton Dickinson, Cedex, France), and analysed in an ACL 3000 coagulometer (Instrumentation Laboratory, Warrington, UK). This is a fully automated technique, and plasma fibrinogen concentration was derived from a prothrombin time. The sample was placed on an autoanalyser after centrifugation at 1000g for ten minutes to separate plasma from cells. Plasma was mixed in the analyser automatically with the PT reagent, consisting of calcium and rabbit thromboplastin (IL TEST™ PT-Fibrinogen, Instrumentation Laboratory, Warrington, UK) to induce clot formation. This was detected nephelometrically, as the analyser detects the change in light scatter produced by thrombus formation. Following coagulometer calibration with standards of known concentration (IL Calibration Plasma, IL, Warrington, UK), a plasma fibrinogen concentration was calculated.

Quality Assurance

The laboratory performing this assay is CPA accredited. Internal quality control is assessed by the analysis of control samples of known fibrinogen concentration (Lyophilised normal and abnormal pooled plasma, Baxter Immuno, Wien

Germany). The intra- and interassay coefficients of variation are consistently <5%. The laboratory also participates in NEQAS, and performance to date has been satisfactory.

4.3.7 Platelet count and mean platelet volume (MPV)

These were performed in the Haematology laboratory at Aberdeen Royal Infirmary, using a Bayer Technicon H3 autoanalyser (Bayer, Cedex, France). 4.5 ml of tripotassium EDTA anticoagulated whole blood were collected in a Vacutainer® sample tube (Becton Dickinson, Cedex, France) for full blood count (FBC) analysis. Cells are identified by flow cytometry, and platelet number and size are assessed by forward (size) and side (granularity) light scatter characteristics.

The laboratory is CPA accredited, and also participates in the NEQAS scheme for FBC analysis. Three control samples (Bayer, Cedex, France) are analysed daily to assess interassay precision, and consist of:

i) Testpoint™ Control normal; Platelets 225 ± 30 x10^9/l, MPV 8.7 ± 1.1fl.

ii) Testpoint™ Control low; Platelets 80 ± 15x10^9/l, MPV 8.2 ± 1.2fl.

iii) Testpoint™ Control high; Platelets 480 ± 60 x10^9/l, MPV 8.3 ± 1.1fl.

Results are accepted if the control sample results fall within these ranges.

In addition a sample received in the laboratory with values within the normal range is analysed thrice daily. A log of all quality assurance procedures is kept and signed daily to ensure adherence.

4.3.8 Fasting total cholesterol, and LDL, HDL and triglyceride quantitations.

These assays were performed in the Biochemistry laboratory at Aberdeen Royal Infirmary using a DAX-72 autoanalyser (Bayer, Basingstoke, UK). This is a CPA accredited laboratory which participates satisfactorily in a NEQAS scheme.

4.3.9 Statistical analysis

Calculations were performed using SPSS for Windows version 8.0 statistical software. The distributions of genotypes, allele frequencies and clinical risk factors for arterial disease in cases and controls were compared by Chi-square tests. Odds ratios (OR) and 95% confidence intervals (CI) were calculated using standard formulae.

Platelet activation data were visualised in boxplots (*figures 4.2-4.4*). Boxes display the median and the 25th and 75th centiles, and whiskers represent extremes of the data. Outliers are arbritrarily defined as lying >1.5 boxlengths from the median, and are not plotted. Skewed continuous variables were normalised by $\log_{10}$ transformation, and geometric means calculated. In order to avoid the influence of artefactual platelet activation, values greater than three standard deviations from the mean were excluded. Numbers quoted in individual groups are therefore less than the total number analysed. Results were not altered by the inclusion of these outliers in analysis (data not shown). Mean differences between patients and controls were analysed by Student's *t*-test, and between acute and convalescent patient variables by paired *t*-tests of transformed data.

Unpaired skewed continuous variable medians were compared with the Mann-Whitney U test. A two-tailed p value of <0.05 was considered significant. The effect of genotype on expression of activation markers was assessed by analysis of variance (ANOVA) of transformed data and by Scheffe's *post hoc* analysis for multiple comparisons.

4.4 Results

The outcome of study recruitment is described in chapter 2.

4.4.1 Platelet genotpe distributions

The distributions of platelet genotypes and allele frequencies are summarised in *table 4.1.* The 1b allele was not overrepresented in patients, and there was no difference in distribution of the HPA 1a/1b or HPA 1b/1b genotypes between cases and controls. There was no significant difference in 1b allele distribution {allele frequency} when analysis was restricted to subjects under the age of 60 (16/79 {0.20} vs 22/90 {0.24}, OR (CI) 0.8(0.4-1.6). There was no overrepresentation of the HPA 2b allele in the entire study cohort, nor in those under 60 (n=80, 16/160 {0.10} vs 16/184 {0.09}, OR 1.01(0.5-2.1). The number of patients heterozygous or homozygous for the HPA 2b allele did not differ statistically significantly from controls, and there were no differences when those under 60 were studied.

4.4.2 Platelet activation

Data on platelet expression of P-Selectin and fibrinogen binding are summarised in *table 4.2.* There were significantly more platelets expressing P-Selectin in

acute stroke than in control samples ($p<0.001$, Student's *t*-test), and this difference persisted into the convalescent period ($p<0.001$, Student's *t*-test). This is illustrated in *figure 4.2.* The proportion of platelets binding fibrinogen was also greater in acute and convalescent stroke patients compared to controls ($p<0.001$ and $p=0.002$ respectively, Student's *t*-test). We found no statistical difference in mean percentage P-Selectin expression between the acute and convalescent periods in patients who provided a sample at both times ($p=0.67$, paired *t*-test), but the number of platelets binding fibrinogen decreased from a geometric mean of 2.1% in the acute period to 1.4% in the convalescent period ($p=0.02$, paired *t*-test, *figure 4.3*). Values obtained immediately after stroke correlated with follow up values ($r^2=0.431$, $p<0.001$ for $\log_{10}$%CD62P expression and $r^2=0.231$, $p=0.048$ for $\log_{10}$fibrinogen binding). There was also evidence of a persisting greater density of anti GPIIIa binding sites in stroke patients, as evidenced by greater mean cell fluorescence in acute and convalescent patients compared to controls ($p<0.001$ and $p=0.008$ respectively, Student's *t*-tests, *figure 4.4*). There was no alteration in mean cell fluorescence between the acute and convalescent periods ($p=0.26$, paired *t*-test). No difference in mean cell fluorescence for P-Selectin expression or fibrinogen binding between acute, convalescent and control samples was detected (data not shown). There were no differences in results when analyses were restricted to the 112 patients for whom a matched control was available (data not shown). Neither CD62P expression ($r^2=-0.09$, $p=0.12$) nor fibrinogen binding ($r^2=-0.08$, $p=0.58$) correlated with plasma fibrinogen concentration.

Evidence of reduced platelet reactivity immediately after stroke was detected (*table 4.2*), since the percentage of platelets binding fibrinogen following stimulation with ADP was lower in the acute phase than in controls ($p<0.001$). The density of fibrinogen binding sites after ADP stimulation did not significantly differ between the three groups (acute geometric mean{range} mean cell fluorescence 8.45{2.0-39.8} units vs 7.66{2.3-37.2} in controls, p=0.15, Student's *t*-test; convalescent mean 7.69 (2.1-46.8) p=0.1 vs controls, p>0.95 vs acute, paired *t*-test).

Of 146 patients with evaluable data 48 (33%) were taking aspirin at the time of their stroke. Those not taking aspirin had significantly greater numbers of platelets binding fibrinogen than those who did not (median{range} 1.5%{0.2-5.5} vs 1.2{0.2-4.4}, p=0.05, Mann-Whitney U test). There was also a tendency for those not on aspirin to have more platelets expressing CD62P, but this was not statistically significant (0.70%{0.1-1.85} vs 0.53{0.1-1.81}, p=0.1, Mann-Whitney U test). In a subset of the study cohort (n=35) the effect of commencing aspirin following stroke on platelet activation was assessed. No significant difference in the expression of CD62P (median{range} 0.7%{0.1-1.85} before aspirin cf 0.5%{0.1-1.81} after, p=0.2, Wilcoxon ranked signs test) nor in fibrinogen binding (1.4%{0.4-5.3} before aspirin vs 1.2%{0.2-4.3} after, p>0.95, Wilcoxon ranked signs test) was found.

4.4.3 Platelet genotype and activation

There was no statistically significant difference in the median number of platelets expressing P-Selectin in patients and controls homozygous or heterozygous for

HPA 1b (n=57) compared with those (n=201) with the 1a/1a genotype (geometric mean {range} 0.49{0.1-4.37}% vs 0.51{0.1-6.46}% respectively, p=0.83, Student's *t*-test). There was no HPA 1a/1b genotype dependent difference in platelet fibrinogen binding (geometric mean 0.86{0.1-13.5}% in those with the 1b allele vs 0.9{0.1-18.2}% in individuals with the 1a/1a genotype, p=0.78, Student's *t*-test). No genotype dependent difference in antiGPIIIa binding density was observed (1a mean{SD} 9.1{2.1} fluorescence units vs 1b 9.0{1.7}, p=0.88, Student's *t*-test). No difference in platelet reactivity based on genotype as assessed by fibrinogen binding patterns following stimulation with ADP was identified (1a mean {SD} 59.1{17.5}% vs 1b 56.6{16.9}%, p=0.53, Student's *t*-test).

Platelet activation was not dependent on HPA 2 genotype, since those possessing the 2b allele (n=43) did not have greater numbers of platelets binding fibrinogen or expressing P-Selectin than those homozygous for HPA 2a (n=214): Platelet fibrinogen binding 2a geometric mean{range} 0.9{0.1-18.2}% vs 2b 0.9{0.1-13.5}%, p=0.78, Student's *t*-test; P-Selectin 2a geometric mean{range} 0.9{0.1-18.2}% vs 2b 0.9{0.1-10}%, p=0.90). Mean cell fluorescence for antiGPIIIa binding was not dependent on genotype (2a mean{SD} 8.0{3.5} vs 2b 7.8{3.5}, p=0.65, Student's *t*-test). No difference in platelet reactivity was identified (2a mean{SD}fibrinogen binding after ADP 61.1{18.1}% vs 2b 64.6{14.8}, p=0.18, Student's *t*-test).

The effect of the 1b and 2b alleles in the acute phase, the convalescent phase and in controls were investigated separately by ANOVA and Scheffe's test for

multiple comparisons. Results are summarised in *table 4.3*. No subgroup of 1b or 2b subjects exhibited greater fibrinogen binding or P-Selectin expression than their 1a/1a or 2a/2a counterparts. Subgroup analysis did not identify differences in fibrinogen binding following stimulation with ADP.

It was determined whether allele dependent altered sensitivity to aspirin was a potential confounding factor in the study. Results are summarised in *table 4.4*. There was no difference between 1a and 1b platelets nor 2a and 2b containing platelets in expression of activation markers in those who were taking aspirin at the time of sampling and those who were not.

4.5 Discussion

Difficulties in interpreting conflicting data from epidemiological studies of platelet polymorphisms has given rise to the view that genetic influences are modified by environmental factors, so that only certain patients may be at increased risk (eg smokers, those of young age or female gender), and subgroup analysis may be necessary to identify these risk groups (Carter *et al*, 1998; Wagner *et al*, 1998; Bray, 1999). However, epidemiological studies of modest size which conduct subgroup analyses are likely to overestimate the risk, especially given the publication bias associated with positive studies (Ridker & Stampfer, 1999a). Thus it has been suggested only large population based studies should be undertaken to study the association of genetic polymorphisms with arterial disease (Ridker & Stampfer, 1999a; Keavney *et al*, 2000). In this modestly sized study epidemiological data was combined with an investigation of

the influence of polymorphisms on platelet function. Evidence of platelet activation in the acute and convalescent phases of stroke was found, but there was no association between the HPA 1a/1b and 2a/2b polymorphisms of GPIIIa and GP1b respectively and expression of these markers in neither healthy controls nor patients in the acute and convalescent periods of stroke. There was no significant epidemiological association with genotype and atherothrombotic stroke in the study cohort, even when analysis was restricted to younger patients (less than 60 years).

It has been suggested that HPA 1b, as a polymorphism of the platelet fibrinogen receptor might mediate increased risk of thrombosis by enhancing platelet fibrinogen binding (Bray, 1999). In chapter three no such relationship was demonstrated in platelets from healthy subjects, and these findings are replicated in this study, despite evidence of increased numbers of platelets binding fibrinogen following stroke. It is acknowledged that platelet genotype may influence function by another mechanism, and it has been suggested the HPA 2a/2b polymorphism may result in altered vWF binding in the conditions of high shear stress seen in arteries narrowed by atheroma (Bray, 1999), but data to support this are lacking. However, both P-Selectin expression and fibrinogen binding occur in the later stages of platelet activation (del Zoppo, 1998), and although GPIIIa and GPIb polymorphisms may not influence these directly, any effect on platelet activity should ultimately be reflected in these endpoints. The only study conducted prior to this one to demonstrate enhanced 1b platelet activity compared to 1a recruited a much larger cohort than other functional studies (Feng *et al*, 1999), and did not study *in vivo* activation during an acute thrombotic event.

It is recognised this study may be underpowered to detect a more modest effect, but it is unclear whether a smaller *in vitro* effect would be sufficient to influence a clinical event like thrombotic stroke.

The study of stroke pathophysiology is associated with particular difficulties. Stroke arises from numerous processes, including intracranial haemorrhage, cardiac embolisation, atherothrombosis (rupture of either large vessel atheroma with cerebral embolism or of small vessel atheroma with thrombotic occlusion), and vasculitis. Most studies have failed to distinguish between these diverse stroke types (Couch & Hassanein, 1976; Dougherty *et al*, 1977; Hoogendijk *et al*, 1979; Shah *et al*, 1985; van Kooten *et al*, 1997; van Kooten *et al*, 1999) and any individual risk factor might influence only one of these processes. Subjects with a single underlying pathophysiological process were therefore studied. An association of atherothrombotic stroke with smoking, hypertension and plasma fibrinogen concentration was confirmed, but there was no association with diabetes mellitus, hyperlipidaemia nor family history of arterial events. This lack of association between family history of thrombosis and stroke, and the observation of no association between platelet genotype and stroke in our cohort further support the view that the influence of a single gene on cardiovascular disease is weak (Ridker & Stampfer, 1999a).

In vitro measurements of platelet function may not necessarily reflect *in vivo* activity. In particular, assays of plasma beta thromboglobulin, platelet factor 4 (Shah *et al*, 1985) and platelet aggregometry (Couch & Hassanein, 1976; Dougherty *et al*, 1977; Konstantopoulos *et al*, 1995) are susceptible to artefactual

platelet stimulation as a result of centrifugation and stirring procedures (Michelson, 1996). Platelet function was studied directly by whole blood flow cytometry to avoid this effect, and was related to polymorphisms of receptors that have a major role in platelet recruitment in acute thrombus formation (Nurden, 1995). Artefact was further minimised by careful blood sampling and by excluding values greater than three standard deviations from the mean from statistical analysis. However, it should be acknowledged that this study assessed function in circulating platelets. This may not necessarily reflect localised platelet activity within the thrombus in the acute phase of stroke, and as such remains an indirect assessment of *in vivo* platelet function in arterial thrombosis. This point is illustrated by the observation that the absolute numbers of platelets expressing activation markers is a fraction of the total platelet count in patients (*table 4.2)*.

Prospective data on platelet activity as a risk factor for future stroke development are lacking, but this study found evidence of persisting platelet activation in stroke, indicated by circulating platelets expressing P-Selectin and binding fibrinogen, and by greater density of GPIIIa binding in both the acute and convalescent phases. Although platelet activity immediately after stroke may reflect a secondary response to tissue injury (Robertson *et al*, 1980; Dougherty *et al*, 1979), the observation of persisting platelet activation may indicate an underlying prothrombotic tendency. Reduced platelet responsiveness in acute stroke was observed, indicated by reduced numbers of platelets binding fibrinogen following stimulation with ADP. This is consistent with circulation of activated platelets which are no longer susceptible to further stimulation. These changes

are clearly subtle, since they are not reflected in altered numbers of circulating platelets nor in their mean volume.

Although an association between raised mean platelet volume and increased mortality following MI has been suggested (Martin *et al*, 1991), there was no association between MPV and stroke in this study. It is acknowledged that the timing of full blood count analysis was not identical for every sample, and despite the fact that all were analysed within two hours of collection, *in vitro* increases in MPV in EDTA are measurable within this period (Trowbridge *et al*, 1985).

Potential confounding by aspirin use was investigated, as it has been suggested that sensitivity to aspirin may be influenced by platelet HPA 1a/1b genotype (Cooke *et al*, 1998). There was no difference in expression of activation markers between 1a and 1b platelets nor 2a and 2b platelets in either subjects who were taking aspirin and those who were not. The observation of greater platelet activation in those subjects not taking aspirin on admission, and the subsequent failure to reduce this when aspirin was commenced after stroke, suggests that a subgroup of patients might benefit from additional antiplatelet therapy. This was performed in a subgroup of the study cohort, and it is possible that a small effect was not detected as a result of type 2 statistical error. A larger study is therefore required to investigate this specifically.

In conclusion there is evidence of platelet activation in acute atherothrombotic stroke, which persists months after the acute event. This suggests that platelet activation is a marker of a prothrombotic state, and may be involved in the

pathogenesis of acute stroke. However, neither the HPA 1b nor HPA 2b allele is associated with stroke, and they do not appear to influence *in vivo* platelet activation. These data do not support these polymorphisms of platelet membrane GPIb and GPIIIa as risk factors for atherothrombotic stroke in the Grampian population.

PCR with *MSP 1* digest.

300 bp
200 bp

ladder
Uncut
1b/1b
1a/1b
1a/1a

Figure 4.1. HPA 1a/1b genotypes following *msp I* digest.

Figure 4.2. Boxplot of P-Selectin expression

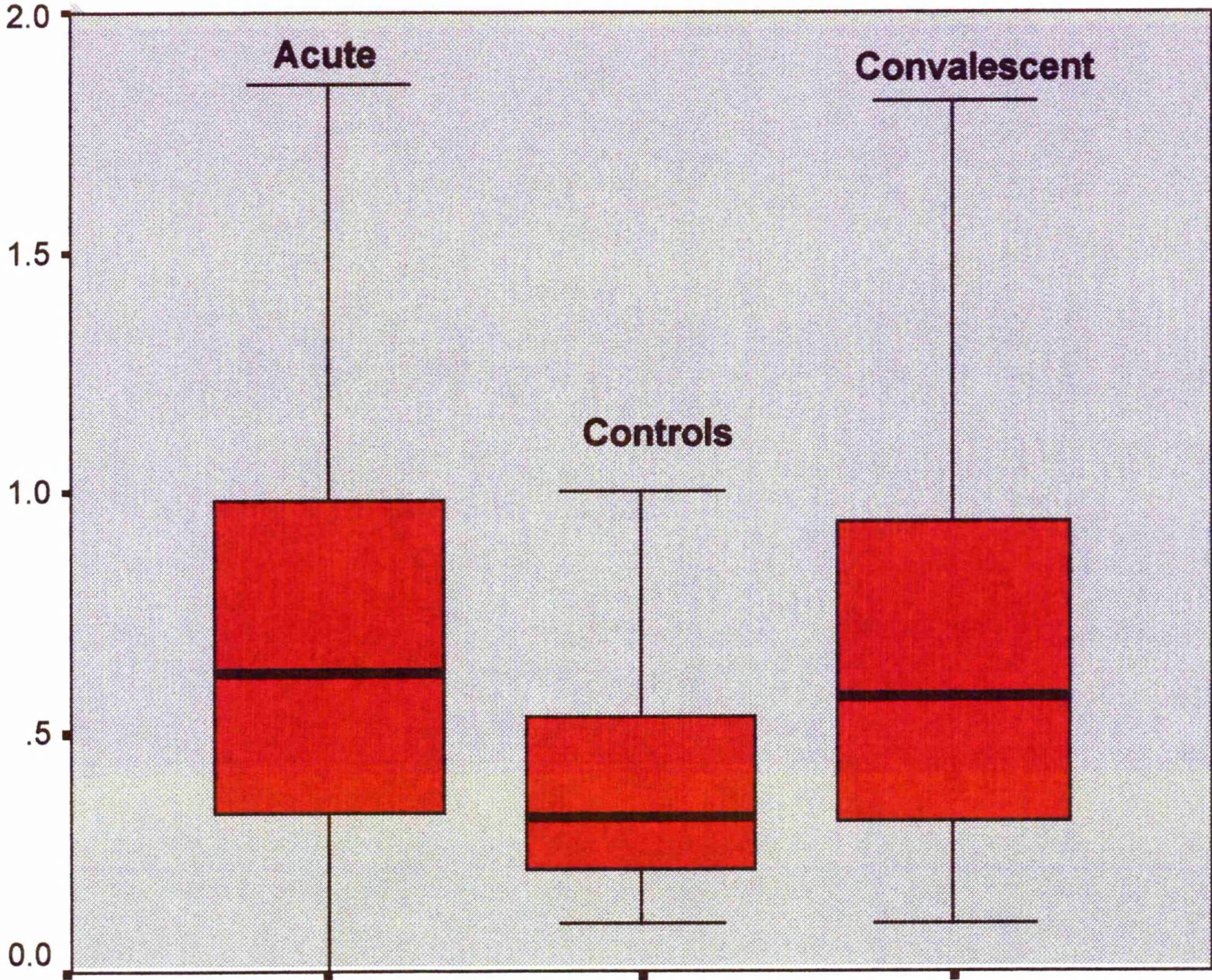
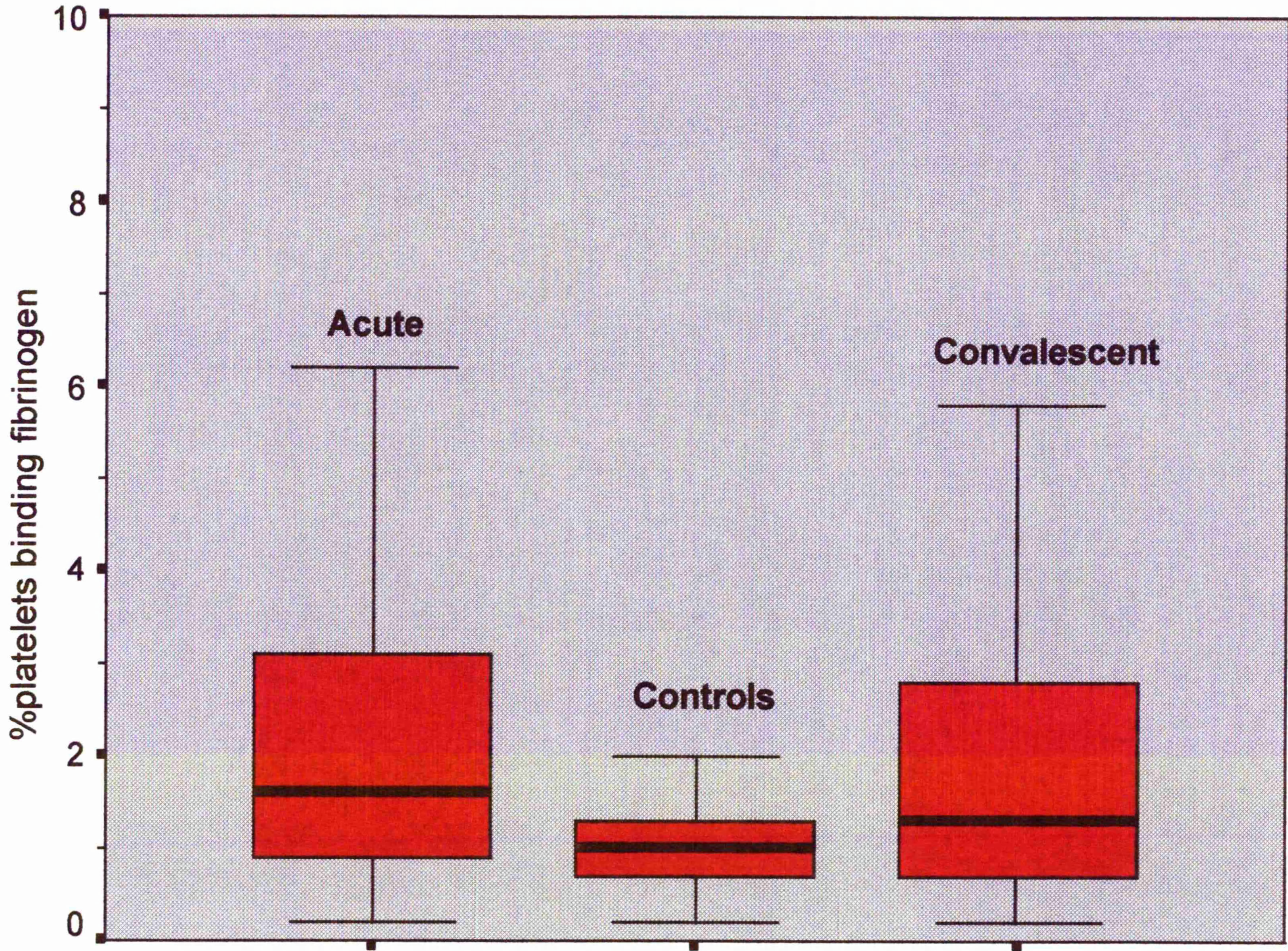


Figure 4.3. platelet fibrinogen binding



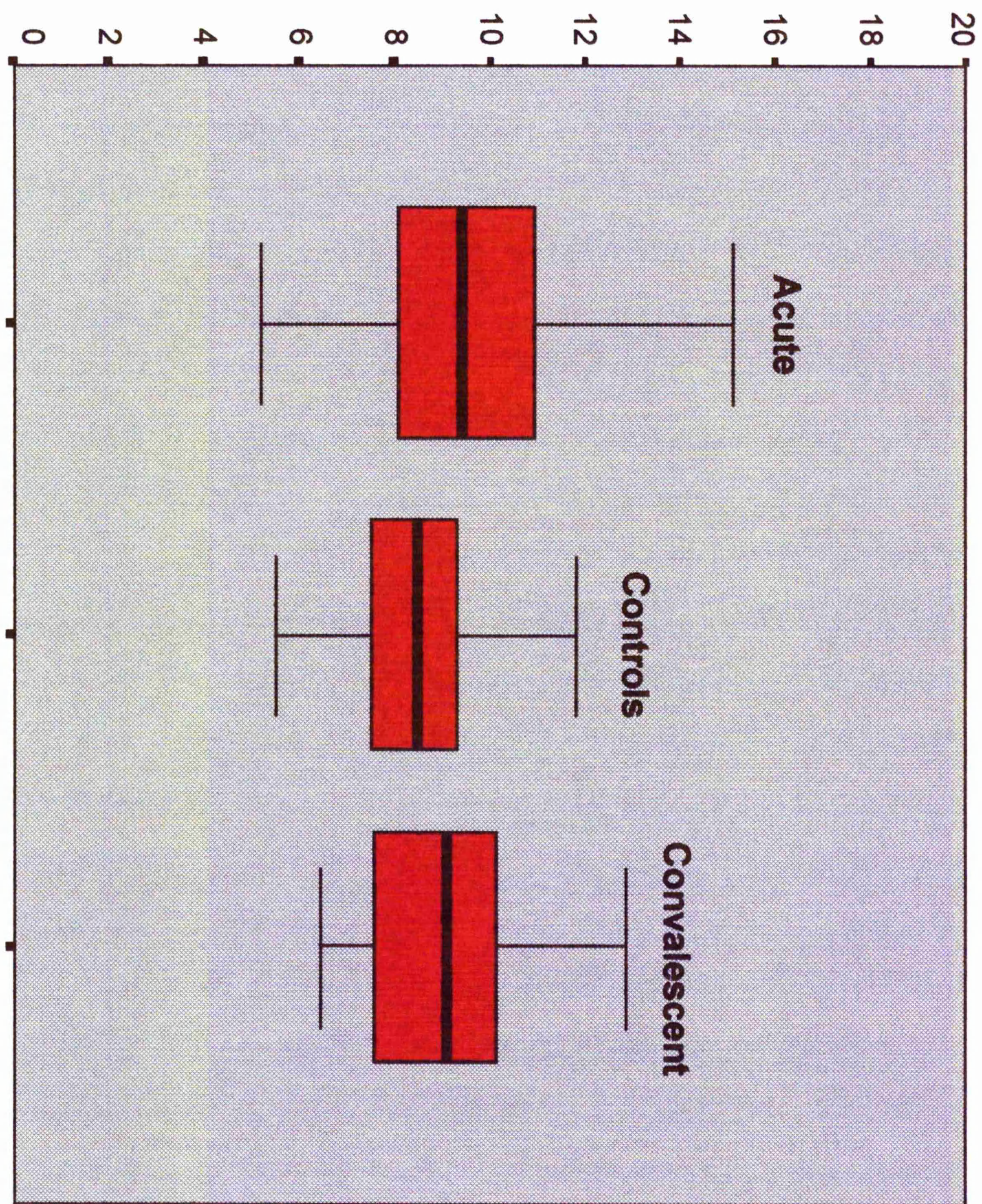


Figure 4.4. GPIIIa mean cell fluorescence

Genotype	Patients (n=150)	Controls (n=150)	Significance
1a/1a 1a/1b 1b/1b	118 31 1	112 36 2	X^2=0.67, p=0.41 1 df
2a/2a 2a/2b 2b/2b	122 27 1	128 22 0	X^2=0.86, p=0.35 1 df
Allele Frequency 1a 1b	 267/300 (0.89) 33/300 (0.11)	 260/300 (0.87) 40/300 (0.13)	Odds Ratio (CI) 0.8 (0.5-1.3)
2a 2b	271/300 (0.91) 29/300 (0.09)	278/300 (0.93) 22/300 (0.07)	1.4 (0.8-2.4)

Table 4.1. Genotype distributions and allele frequencies. Heterozygous and homozygous carriers of HPA1b or 2b were combined for chi square analysis. df=degrees of freedom. CI=95% confidence intervals.

Group	Acute n=147	Control n=110	Convalescent n=74	Significance
% CD62P expression geometric mean (range)	0.64 (0-4.4)	0.35 (0.1-6.5)	0.62 (0.1-4.3)	acute vs control $p<0.001$[1] convalescent vs control $p<0.001$[1] acute, n=74, geometric mean (range) 0.62 (0.1-4.4) vs convalescent $p=0.67$[2]
% fibrinogen binding geometric mean (range)	1.6 (0.2-11.0)	0.9 (0.2-10.2)	1.4 (0.2-8.7)	acute vs control $p<0.001$[1] convalescent vs control $p=0.005$[1] acute, n=74, geometric mean (range) 2.08 (0.2-4.4) vs convalescent $p=0.02$[2]
Anti-CD61 mean cell fluorescence units mean (SD)	9.5 (2.3)	8.4 (1.5)	9.1(1.6)	acute vs control $p<0.001$[1] convalescent vs control $p=0.008$[1] acute, n=76, mean (SD) 8.7 (1.8) vs convalescent, $p=0.26$[2]
% fibrinogen binding post ADP Mean (SD)	55.2 (17.2)	64.2 (16.5)	60.9 (17.4)	acute vs controls $p<0.001$[1] convalescent vs control $p=0.43$[1] acute, n=71 mean (SD) 60.8 (18.4) vs convalescent, $p>0.95$[2]

Table 4.2. Platelet activation markers in atherothrombotic stroke.

[1] Student's *t*-test.

[2] Paired *t*-test.

Group (number)	% expressing CD62P Geometric mean (range)	p	% binding fibrinogen Geometric mean (range)	p	% binding fibrinogen post ADP Mean (SD)	p	Fibrinogen binding Mean cell fluorescence post ADP Geometric mean (range)	p
1a acute (108)	0.63(0.1-4.17)	>0.95	1.59(0.2-10.9)	>0.95	54.5(16.7)	0.88	8.2(2.3-39.8)	0.77
1b acute (30)	0.69(0.2-4.37)		1.65(0.4-10.0)		59.1(18.3)		9.7(3.0-24.0)	
1a control (86)	0.36(0.1-6.46)	>0.95	1.01(0.2-10.2)	>0.95	64.8(16.9)	>0.95	8.0(3.0-37.1)	0.72
1b control (26)	0.34(0.1-1.2)		0.92(0.4-2.4)		59.7(17.4		6.6(2.3-13.2)	
1a convalescent (62)	0.58(0.1-4.27)	0.82	0.83(0.2-8.3)	0.10	58.4(17.1)	0.18	7.2(2.1-46.8)	0.33
1b convalescent n (13)	0.98(0.4-1.82)		2.57(0.5-8.7)		72.6(14.2)		10.7(5.8-21.9)	
2a acute (121)	0.63(0.1-4.37)	>0.95	1.77(0.2-18.2)	>0.95	60.1(19.2)	>0.95	8.5(2.0-39.8)	>0.95
2b acute (28)	0.74(0.2-2.57)		1.49(0.3-10.0)		61.5(15.8)		8.1(4.6-23.4)	
2a control (93)	0.37(0.1-6.46)	>0.95	1.06(0.2-15.49)	>0.95	63.0(16.9)	0.69	7.5(2.3-37.2)	>0.95
2b control (15)	0.33(0.1-1.2)		1.04(0.4-2.29)		71.6(11.7)		8.5(3.7-14.8)	
2a convalescent (58)	0.68(0.1-7.76)	>0.95	1.56(0.2-25.7)	0.94	60.0(18.1)	>0.95	7.6(2.1-46.8)	>0.95
2b convalescent (14)	0.83(0.3-7.59)		1.17(0.4-3.98)		64.3(14.6)		8.0(3.3-18.6)	

Table 4.3. Subgroup analyses of the influence of HPA 1a/1b and 2a/2b genotypes on the expression of markers of platelet activation by ANOVA and Scheffe's test.'

Group (n)	GPIIIa mean fluorescence mean (SD)	P value	% P-Selectin expression Geometric mean (range)	P value	% fibrinogen binding Geometric mean (range)	P value
On aspirin						
HPA 1a (39)	9.07 (1.77)	0.55	0.48 (0.1-4.37)	0.42	1.4 (0.2-18.2)	0.50
HPA 1b (11)	9.47 (2.00)		0.58 (0.2-1.78)		1.8 (0.4-17.0)	
HPA 2a (39)	9.08 (1.98)	0.90	0.48 (0.1-4.37)	0.42	1.6 (0.2-18.2)	0.58
HPA 2b (12)	9.16 (1.42)		0.60 (0.2-1.78)		1.3 (0.3-6.0)	
No aspirin						
HPA 1a (156)	8.80 (2.07)	>0.95	0.49 (0.1-6.46)	0.80	1.3 (0.2-15.8)	>0.95
HPA 1b (43)	8.78 (1.59)		0.47 (0.1-2.34)		1.2 (0.4-12.3)	
HPA 2a (169)	8.79 (2.05)	>0.95	0.48 (0.1-6.46)	0.91	1.3 (0.2-15.8)	>0.95
HPA 2b (28)	8.77 (1.59)		0.49 (0.1-2.57)		1.3 (0.4-10.6)	

Table 4.4. The influence of aspirin on genotype dependent expression of platelet activation.

Chapter Five

Plasma Homocysteine concentrations in the acute and convalescent phases of atherothrombotic stroke.

5.1 Background

Homocysteine is a thiol containing amino acid which is derived solely from the metabolism of dietary methionine. It circulates in plasma in three forms: as a single free amino acid homocystine (1%); as homocysteine or cysteine-homocysteine disulphides (20-30%); or bound to plasma proteins (70-80%). Together these account for total plasma homocysteine (tHcy). There are two main routes of metabolism (summarized in *figure 5.1*): remethylation to methionine using either folate or (in the liver) betaine as a methyl donor, or transulphuration ultimately to cysteine. These reactions are dependent on cofactors, including vitamin B12 and folate for remethylation, and vitamin B6 for transulphuration. When plasma methionine is raised (for example following a protein rich meal) transulphuration is the main route of metabolism, while in fasting conditions remethylation predominates.

Homocystinuria is a rare inborn error of metabolism (approximately 1 in 100 000 live births) arising from homozygous deficiency of Hcy metabolizing enzymes, usually cystathionine ß synthase, which results in extremely high tHcy concentrations (>100μmol/l, severe hyperhomocysteinaemia). This is associated with mental handicap; skeletal and ocular lens abnormalities; and premature arterial and venous thrombosis in 50% of untreated patients by the age of thirty years, possibly as a result of oxidative damage mediated by the sulphydryl group of free single chain homocystine (Mudd *et al*, 1985).

It is less clear whether smaller elevations of tHcy are associated with thrombosis. Heterozygotes for cystathionine ß synthetase deficiency have normal or raised tHcy concentrations, but studies of an association with thrombosis are conflicting (Boers *et al*, 1985; de Valk *et al*, 1996). Mild hyperhomocysteinaemia (HHC, concentration >95th centile of the normal range, which is approximately 5-15μmol/l) results from nutritional and more subtle genetic influences. Subclinical deficiencies of folate, vitamins B12 and vitamin B6 (Selhub *et al*, 1993) are associated with these mild elevations. Other conditions including renal impairment, hypothyroidism and drug therapy (e.g. folate antimetabolites, theophylline, smoking or oral contraceptives) are also associated with mild hyperhomocysteinaemia (Hankey & Eikelboom, 1999). Whether this can cause premature arterial thrombosis is a contentious issue. Retrospective case control studies have associated HHC with both arterial and venous thrombosis (Graham *et al*, 1997; den Heijer *et al*, 1996). In contrast, results from prospective studies have been inconsistent, both supporting (Stampfer *et al*, 1992; Wald *et al*, 1998; Arnesen *et al*, 1995; Nygard *et al*, 1995) and refuting (Chasan-Taber *et al*, 1996; Evans *et al*, 1997; Folsom *et al*, 1998) hyperhomocysteinaemia as a risk factor for myocardial infarction (MI).

Consideration of reports of plasma tHcy and stroke identifies similar difficulties. Case control studies reported stronger associations (Graham *et al*, 1997; Yoo *et al*, 1998) than prospective studies, where some (Perry *et al*, 1995; Giles *et al*, 1998) but not others (Alfthan *et al*, 1994; Verhoef *et al*, 1994) claimed that raised plasma homocysteine concentration is a risk factor for future stroke development.

A genetic influence on Hcy concentration has been investigated. The thermolabile variant of methylenetetrahydrofolate reductase (MTHFR) arising due to homozygous substitution of cytosine by thymine at position 677 is common, found in approximately 12% of Western populations (Brattstrom *et al*, 1998), and is associated with defective remethylation due to reduced enzyme activity (Frosst *et al*, 1995). Subjects with this genotype have raised fasting tHcy concentrations, especially when folate is low (Malinow *et al*, 1997). Despite this most studies have failed to identify the TT genotype as an independent risk factor for arterial thrombosis (Brattstrom *et al*, 1998). This lack of association between genotype and a clinical endpoint has raised further doubt about the role of homocysteine in vascular disease.

Two further issues are relevant:

1) As discussed in chapter one stroke arises from numerous pathophysiological processes including haemorrhage, cardiac embolisation, atherothrombosis and vasculitis. Most studies have failed to distinguish between these diverse stroke types, and any individual risk factor might influence only one of these processes. Recent studies in humans have shown that acute hyperhomocysteinaemia after methionine loading causes endothelial dysfunction, which might promote atheroma development (Chambers *et al*, 1999; Nappo *et al*, 1999). Furthermore raised homocysteine concentrations are associated with asymptomatic carotid artery wall thickening and stenosis (Malinow *et al*, 1993; Selhub *et al*, 1995), and correlate with the severity of

cerebral artery stenosis (Yoo *et al,* 1998). It could therefore be postulated that elevated tHcy is a risk factor for atherothrombotic stroke in particular.

2) Very few data are available regarding tHcy concentration immediately after acute stroke. There is currently debate about whether tHcy is a causative risk factor in stroke and MI, or is merely a secondary marker of risk in survivors (Kuller & Evans, 1998; Dudman, 1999). Such data would help to resolve this question, since the observation of a raised tHcy immediately following acute stroke would be more suggestive of a causal association than the occurrence of hyperhomocysteinaemia in survivors sampled at a time distant from the event.

5.2 Aims

A case control study was designed to address these issues. This was restricted to subjects with atherothrombotic stroke by exclusion of cases of intracranial haemorrhage and likely cardioembolic or vasculitic aetiology as described in chapter two. Subjects were recruited between June 1997 and December 1998. Fasting tHcy concentrations were measured in both the acute and convalescent periods of stroke, and any change in tHcy between these times assessed. Changes in factors known to affect Hcy metabolism, such as B12 and folate concentrations, smoking habit and drug history, were also assessed to determine whether these were responsible for any observed change in tHcy concentration observed between the acute and convalescent period.

5.3 Methods

Patient and control recruitment and sampling is described in chapter two.

5.3.1 Total plasma homocysteine assay by reverse phase HPLC and fluorescent detection.

Principle of HPLC

High performance liquid chromatography (HPLC) utilizes the principle that the passage of a substance in solution over a column is delayed if it interacts with that column. In this way conditions can be manipulated to separate a given substance from a mixture, since it will be released from the column by elution at a specific time compared to other substances. (In this case thiol amino acids can be separated from each other and other constituents of plasma). The conditions of the column are manipulated to enhance the separation. There are two main constituents in HPLC:

1) The *mobile phase* is the solvent used to transport the sample.
2) The *solid phase* is the column which the sample interacts with. The main constituent is usually silica gel.

These constituents are manipulated to enhance the separation of the molecule under study, and are therefore dependent on the chemical and physical properties of the substance assayed. There are therefore four types of HPLC (Bird, 1989):

i) *Normal phase chromatography* is used to separate hydrophilic molecules by enhancement of hydrophilic binding on the column.

ii) *Reverse phase chromatography:* hydrophobic molecules are adsorbed onto the silica column.

iii) *Ion exchange chromatography:* positively or negatively charged molecules are attracted to their opposite in the silica molecules of the column.

iv) *Size exclusion chromatography;* pores in the silica hamper the passage of larger molecules.

Reverse phase HPLC is used in Hcy analysis since it is an organic hydrophobic molecule. The optimal solid and mobile phase conditions for its separation from other thiol containing amino acids have been determined (Ubbink *et al*, 1991).

Sample preparation

Samples were analysed between February and May 1999. They were thawed and 80μl of plasma and 20μl dH_2O mixed in an eppendorf. The following steps were then performed in a standardised manner:

- **Reduction**: *Protein-bound homocystine and disulphide forms are converted to single thiols by mixing with a reducing agent.* Samples were reduced by the addition of 10μl of 10% tri-n-butylphosphine (Sigma Chemicals, St Louis, USA) in a fume hood. These were mixed thoroughly and placed at 4°C for 30 minutes to ensure complete reduction. Protein was precipitated by the addition of 110μl of

10% Trichloroacetic acid (BDH Laboratory Supplies). This was mixed thoroughly, stored at 4 °C for 10 minutes and centrifuged at 14,000 rpm for 10 minutes.

- **Derivatisation**: *Homocysteine is conjugated with a derivatising agent. This is a compound with no inherent fluorescent properties in the free state, which when bound will fluoresce on laser excitation, enabling detection.* 100μl of clear supernatant were then added to a second eppendorf containing the derivatisation compound: 100μl SBD-F (ammonium-7 fluoro-2,1,3-benzoxadiazole sulphonate, Fluka, New-Ulm, Switzerland) 0.5mg/ml in borate buffer; 50μl 3M NaOH (BDH Laboratory Supplies, Poole, UK); and 200μl 0.1M borate buffer. Samples were incubated for one hour at 60 °C on a heating block to allow full derivatisation, and the reaction then stopped by placing on ice for 5 minutes. 18.5μl of orthophosphoric acid were added to adjust pH to 2.2.

- **Reverse phase HPLC and data processing**:. *Reduced and derivatised homocysteine is passed over the column in mobile phase, and detected by spectrophotometry, and a real time record of detected fluorescence is produced.* Samples were transferred to autoanalyser tubes (Gilson Medical Electronics, Villiers-Le-Bel, France). These were mounted on a Gilson 715 HPLC and fluorometer apparatus (Gilson Medical Electronics, Villiers-Le-Bel, France). The following components were utilised to optimise separation of thiol amino acids;

1) Mobile phase consisted of 7% actetonitrile (ACN) in 25mM Potassium dihydrogen phosphate buffer, pH 2.2.
2) Solid phase. A 100x4.6mm Hichrom RBP (Hichrom, Reading UK) containing spherical 5μm silica particles.

The circuit also contained a precolumn (Hichrom, Reading, UK) to minimise degradation of the column in acidic conditions.

115µl were aspirated by the autosampler and injected into the mobile phase, which was passed over the column at a rate of 0.6ml/min, at a pressure of 70 bar generated by two pumps. After passing through the column the separated sample components were detected by fluorometry. Excitation with a 385nm light-source results in a 515nm signal from SBD-F derivatised thiols. This was processed with a microcomputer utilising 715 HPLC System Controller software (Gilson Medical Electronics, Villiers-Le-Bel, France), and a real-time chromatogram trace of fluorescence against time produced. Peaks corresponding to homocystine, cysteine and cysteinglycine were obtained *(figure 5.2.)*. The time a peak appears is known as the retention time (RT) and is dependent on the degree of interaction with the solid phase. This was usually between four and five minutes for homocysteine in the laboratory.

- **Calculation of total plasma homocysteine.** The mean area under the curve for each sample was obtained from the duplicate values, and this was compared with the value obtained from a standard concentration of 10µM to calculate the plasma

Hcy concentration. The area corresponding to a plasma concentration of 10μmol/l was calculated from the value obtained from a pooled normal plasma sample to which 10μM of L-Hcy were added ('spike sample') and subtracting the value obtained from the pooled normal plasma alone ('blank sample'). The value for each sample was therefore calculated thus:

$$\frac{\text{area from sample x 10}}{\text{'spike' minus 'blank'}}$$

Quality control considerations

Each assay batch contained a combination of acute, convalescent and control samples, and the operator was blinded to the identity of individual samples. Reproducibility of the assay was assessed by calculating intra- and inter- assay coefficients of variation (CV). The intra-assay CV was calculated by analysing a single sample in all tubes on a run, and yielded a value of 4.2%. The inter -assay CV was calculated from the value derived from the same plasma sample which was analysed in different runs. A value of 18.9% was obtained after the first tens runs by single sample analysis. This was reduced to 11.7% by using duplicates in subsequent runs, and samples initially analysed singly were therefore reassayed in duplicate. Samples with extreme values (<4μmol/l or >20μmol/l) were reanalysed to confirm reproducibility of those results. In each run the retention time was recorded and a value between four and five minutes considered acceptable. Prolongation of the RT indicated impaired homocystine elution and that batch was reanalysed.

5.3.2 Serum B12 and red cell folate assays.

These assays samples were prepared in the routine Haematology laboratory at Aberdeen Royal Infirmary, and analysed on a Technicon Immuno 1® autoanalyser (Bayer Technicon, Cedex, France). Both B12 and RCF concentrations were elucidated by a competitive magnetic separation assay (MSA). The principle of B12 estimation is summarised below:

1) Release of B12 from protein binding by incubation with pretreatment reagent.

2) Released B12 is mixed with Intrinsic Factor reagent (IF), which binds to it.

3) A B12-Alkaline Phosphatase (ALP) enzyme conjugate is added to the sample, which competes with endogenous B12 for IF binding sites.

4) Monoclonal immunomagnetic particle reagent (mIMP™) is incubated to separate the ALP enzyme bound to IF. Para-nitrophenyl phosphate (pNPP) substrate is added and the following reaction is catalysed by ALP:

pNPP+H_2O → p-Nitrophenoxide+phosphate.

This can be detected by spectrophotometry at 405nm. and is inversely proportional to the amount of serum B12 present, since only the activity of ALP bound to IF is detected. The B12 concentration is calculated by the production of a standard curve.

The red cell folate assay uses the same principle with modification;

1) Pretreatment to release folate from red cell proteins

2) Folate binds to added Folate binding protein (R1).

3) Folate-conjugated ALP is added which competes with endogenous folate for R1.

4) Enzyme activity detected by spectrophotometry is inversely proportional to endogenous folate concentration.

Quality assurance

The laboratory performing these assays is CPA accredited and performs satisfactorily in a national external quality assessment scheme (NEQAS). Internal quality control is monitored by the inclusion of three samples (Biorad, Hemel Hempstead, UK) of known B12 and red cell folate concentrations (high, normal and low). Results from a given assay batch are only accepted when the values for these controls are within the range provided by the manufacturers. Selected patient samples are repeatedly assayed to calculate intra-assay CVs (<5% in the laboratory).

5.3.3 Statistical analysis.

Calculations were performed using SPSS for Windows version 8.0 statistical software. Mean differences in normally distributed data between cases and controls were analysed by Student's *t*-test, and by the paired *t*-test for differences between acute and convalescent samples. Unpaired skewed continuous variables, including tHcy concentrations, were analysed by the Mann Whitney U test, and paired data by the Wilcoxon Ranked Sign test. A two-tailed p value of <0.05 was considered significant. Results obtained from non-parametric analyses were checked by parametric testing of log transformed data. Continuous variables influencing tHcy concentration were assessed by stepwise multiple regression analysis that included

age, red cell folate, serum B12 and serum creatinine concentrations in the model. Data were logarithmically transformed to a normal distribution if skewed, and multiple correlation coefficients (R) and partial Pearson correlation coefficients (r^2) calculated. The Beta weight, which expresses the change in the dependent variable in standard deviations that would result from a one SD change in the independent variable was calculated in order to predict the likely impact of any observed change in RCF, B12, or creatinine concentrations on tHcy concentrations. The distributions of clinical risk factors for arterial disease in cases and controls were compared by chi-squared analyses. Odds ratios and 95% confidence intervals were calculated using standard formulae.

5.4 Results

The outcome of patient recruitment and demography is described in chapter two. Data regarding homocysteine concentrations in the acute and convalescent periods are summarised in *table 5.1*. There was no statistically significant difference in the fasting median plasma homocystine concentrations in the acute phase compared to controls in females ($p=0.58$, Mann Whitney U test). In males there was a tendency to a higher median concentration in patients, but this was also not significant ($p=0.09$, Mann Whitney U test). In the 82 patients who underwent repeat testing the median fasting tHcy concentration increased significantly from 8.5µmol/l {range 4.8-19.2} to 10.1µmol/l {4.3-31.5} in the convalescent phase ($p<0.001$, Wilcoxon Ranked Signs test). This is illustrated in *figure 5.3*. Of these individuals, 56 had an increase, 25 a decrease, and one no change in tHcy between the acute and convalescent phases of their illness. In contrast to the acute phase, the tHcy concentrations in convalescent

patients were significantly greater than in matched controls (n=82). In both female and male patients median concentrations were raised significantly (p=0.048 and 0.035 respectively, Mann Whitney U-test).

Factors known to influence tHcy concentrations in subjects and controls were investigated by stepwise multiple regression analysis. The major determinants of $\log_{10}$ tHcy were creatinine concentration (partial $r^2=0.35$, $p<0.001$, Beta=0.42), red cell folate (partial $r^2=-0.32$, $p<0.001$, Beta=-0.27) and B12 (partial $r^2=-0.28$, $p<0.001$, Beta=-0.22). In 25 patients the alteration in concentrations of red cell folate and B12 between the acute and convalescent phases were assessed. The median red cell folate concentration in the acute phase (mean {SD} =223.2nmol/l {180.4}) did not differ significantly from that in the convalescent period (245.1nmol/l {172.3} p=0.061, paired *t*-test). Similarly there was no evidence of a significant reduction in B12 concentrations in the convalescent period to account for the rise in tHcy (acute mean 406μmol/l {243} versus convalescent mean 376μmol/l {128}, p=0.4, paired *t*-test. Alterations in renal function between the acute and convalescent periods were studied in a subgroup of 59 patients (37 men/22 women). There was a significant rise in mean serum creatinine when measured at least three months after acute stroke (acute mean{SD} 87.7μmol/l {19.5}, convalescent 94.1μmol/l {17.7}, p=0.006, paired samples *t*-test). It is calculated from the observed Beta weight of 0.42 that this predicts a rise in mean tHcy concentration of 0.3 μmol/l.

Other factors known to influence tHcy were examined. No patient began smoking, and there was a non-significant increase in the number of patients prescribed

medication associated with increased tHcy concentrations between acute CVA and follow-up (20/82 v 23/82 respectively, X^2= 0.28, p= 0.59).

5.5 Discussion

There are few reports of tHcy concentrations immediately following acute stroke, and these have recruited patients with a diversity of stroke aetiologies. This study was restricted to subjects with a single underlying pathophysiological process by recruitment of those with likely atherothrombotic stroke only. It was found that homocysteine concentrations were not significantly increased shortly after stroke, but that they rose significantly in the ensuing three months. There were no changes in factors commonly associated with mild hyperhomocysteinaemia to explain these findings.

Although red cell folate and serum B12 were significant inverse covariates, the rise in the convalescent phase tHcy was not associated with a corresponding reduction in their concentrations. Given that red cell folate and serum B12 reflect body stores, a longer follow up period may be required to detect such a change. Furthermore, this was assessed in a subset of the study cohort and it is therefore possible that a significant effect was undetected due to an inadequate sample size. The influence of the C677T polymorphism of MTHFR on tHcy is well documented, particularly when folate is also low (Selhub *et al*, 1993; Ma *et al*, 1996). It is clear that this is not a contributing factor in this study, since it is a constant in both periods. The number of patients taking medication associated with hyperhomocysteinaemia, such as

methotrexate, phenytoin, carbamazepine or oral contraceptives, or the number who smoked did not significantly increase following acute stroke.

The observed increases in serum creatinine concentrations following acute stroke may be responsible in part for the rise in homocysteine concentrations observed in the convalescent period, since serum creatinine was a significant covariate. However the predicted increase in tHcy concentrations arising from this (0.3μmol/l) is much smaller than the observed median increase of 1.6μmol/l. It is calculated that a mean rise of 73μmol/l would be required if creatinine were the sole determinant of tHcy concentration. An explanation for the observed rise in creatinine concentration is not apparent, but might reflect prescription of nephrotoxic drugs following stroke, or progressive decline in glomerular filtration in subjects with a diffusely diseased vasculature. The latter explanation is favoured since only 3/82 (3.6%) additional subjects were commenced on drugs associated with renal impairment (e.g. diuretics, angiotensin converting enzyme inhibitors or nonsteroidal anti-inflammatory drugs) following stroke. The noted deterioration in renal function may in fact reflect greater compliance with medication or tighter control of blood pressure in the convalescent phase.

In accordance with these observations, a previous smaller study reported that tHCy was not elevated in the acute phase of cerebral infarction, but noted that the median tHcy concentration was higher in 17 subjects resampled a median of 583 days later (Lindgren *et al*, 1995). However the median convalescent tHcy was not significantly

different from controls. Recruitment of a larger sample size in this study determined that this difference was statistically significant. This provides an explanation for data from case control studies which report an increased prevalence of hyperhomocysteinaemia in stroke survivors (Graham *et al*, 1997; Yoo *et al*, 1998). A study of 12 subjects reported a similar rise in convalescent phase tHcy following acute MI (Egerton *et al*, 1996).

A possible explanation for these findings is that tHcy is elevated in the period predating stroke or MI, and that concentrations temporarily fall in the acute phase by an as yet undetermined mechanism. It has been suggested that this may be related to the acute phase response, with dilution of tHcy by increased synthesis of plasma proteins (Egerton *et al*, 1996). Furthermore, fasting tHcy concentrations are inversely related to oral protein intake (Stolzenberg-Solomon *et al*, 1999), and concentrations may therefore decline due to reduced dietary intake around the acute event. Data regarding alterations in oral protein intake immediately after stroke and the subsequent effect on tHcy are lacking, and were not formally assessed in this study. However a significant period of reduced protein intake is considered unlikely in these patients, since they were admitted to an acute stroke unit where early nutritional supplementation was introduced if feeding difficulty was encountered. Furthermore the reduction in tHcy was not related to time of sampling in relation to stroke onset, which might be expected if the fall in concentration was related to this mechanism (those sampled at 24 hours, geometric mean{range} 9.3μmol/l{3.6-23.2};

at 36 hours 10.6 {5.0-32.3}; at 48 hours 8.8 {4.9-18.2}; 72 hours 8.4 {4.4-17.4}; at 96 hours 9.3 {6.9-18.2}, p=0.41, ANOVA).

The suggestion that tHcy may not be a causative risk factor for stroke at all, and that plasma levels merely rise secondarily to stroke development, possibly in response to tissue injury (Dudman, 1999) is more favourable. This hypothesis, unlike the former, provides an adequate explanation for the fact that prospective studies have found a much weaker association (Perry *et al*, 1995; Giles *et al*, 1998; Alfthan *et al*, 1994; Verhoef *et al*, 1994) than retrospective studies conducted in survivors of stroke (Graham *et al*, 1997; Yoo *et al*, 1998). Further evidence for this view comes from the presently unexplained observation that although homozygosity for the T allele of the C677T polymorphism of MTHFR is associated with mild hyperhomocysteinaemia (Jacques *et al*, 1996; Markus *et al*, 1997), studies have failed to demonstrate this genotype as a risk factor for MI and stroke (Deloughery *et al*, 1996; van Bockxmeer *et al*, 1997; Markus *et al*, 1997; Brattstrom *et al*, 1998; Reuner *et al*, 1998; Harmon *et al*, 1999). To explain this apparent contradiction it has been suggested the TT genotype may only enhance risk when folate is low, or when other risk factors are present (Markus *et al*, 1997; Gardemann *et al*, 1999b), but further studies are required. In addition, obligate heterozygotes for Cysathionine ß Synthase deficiency (i.e. parents of children with homocystinuria) do not exhibit evidence of carotid or femoral atherosclerosis despite elevated tHcy (de Valk *et al*, 1996). This lack of association between genetic abnormalities resulting in mild hyperhomocysteinaemia

and arterial disease further weakens the hypothesis that such elevations in tHcy directly promote atherosclerosis and thrombosis.

It should be acknowledged that homocysteine has been linked in numerous *in vitro* studies with a diversity of mechanisms that could potentiate atherothrombosis, including disrupted endothelial function, impaired protein C activation, increased thrombin generation and platelet aggregation (Harpel *et al*, 1996). However many of these studies were performed using supraphysiological concentrations of pure free single amino acid L-homocysteine and the data cannot be extrapolated to hyperhomocysteinaemia, where in addition to much lower concentrations, only 1% of tHcy is present as this reactive species. More recently transient moderate increases in tHCy following a methionine load have been associated with reversible disturbances in endothelium dependent arterial vasodilatation (Chambers *et al* 1999; Nappo *et al,* 1999), but data demonstrating that this promotes atherogenesis or thrombosis in humans in the longer term are lacking.

There is currently much interest in homocysteine as a risk factor for stroke, since it is possible to reduce plasma homocysteine concentrations in both healthy subjects (den Heijer *et al*, 1998) and patients with vascular disease (Malinow *et al*, 1998), with dietary folate, B12 and B6 supplementation. Several studies of homocysteine lowering agents in the secondary prevention of vascular disease are now underway (Hankey & Eikelboom, 1999). Many would argue that if these studies show a reduction in vascular events accompanied by a reduction in tHcy then a causative

association will be proved. However it should be noted that nutritional supplements may have a beneficial effect independently of an effect on homocyteine concentrations. For example folic acid enhances the synthesis of nitric oxide on endothelial cells (Verhaar *et al*, 1998), and may therefore be beneficial by promoting platelet inhibition and vasodilatation. In such circumstances correction of hyperhomocysteinaemia would merely be an epiphenomenon.

The temporal relationship described in this study between tHcy and stroke requires further characterisation. In particular data regarding the tHcy concentration predating stroke, the subsequent change immediately afterwards, and the time taken for concentrations to rise subsequently are lacking. In order to determine the benefit of folic acid and B vitamin supplementation in secondary stroke prevention, the timing of these events should be considered when designing such trials.

In conclusion, plasma homocysteine concentrations were not statistically significantly elevated immediately after atherothrombotic stroke, but then increased in the convalescent period. The mechanism for these observations was not identified, and it is possible that tHcy increases as a result of the disease process itself. Proposals for further study to test this hypothesis are discussed in chapter seven.

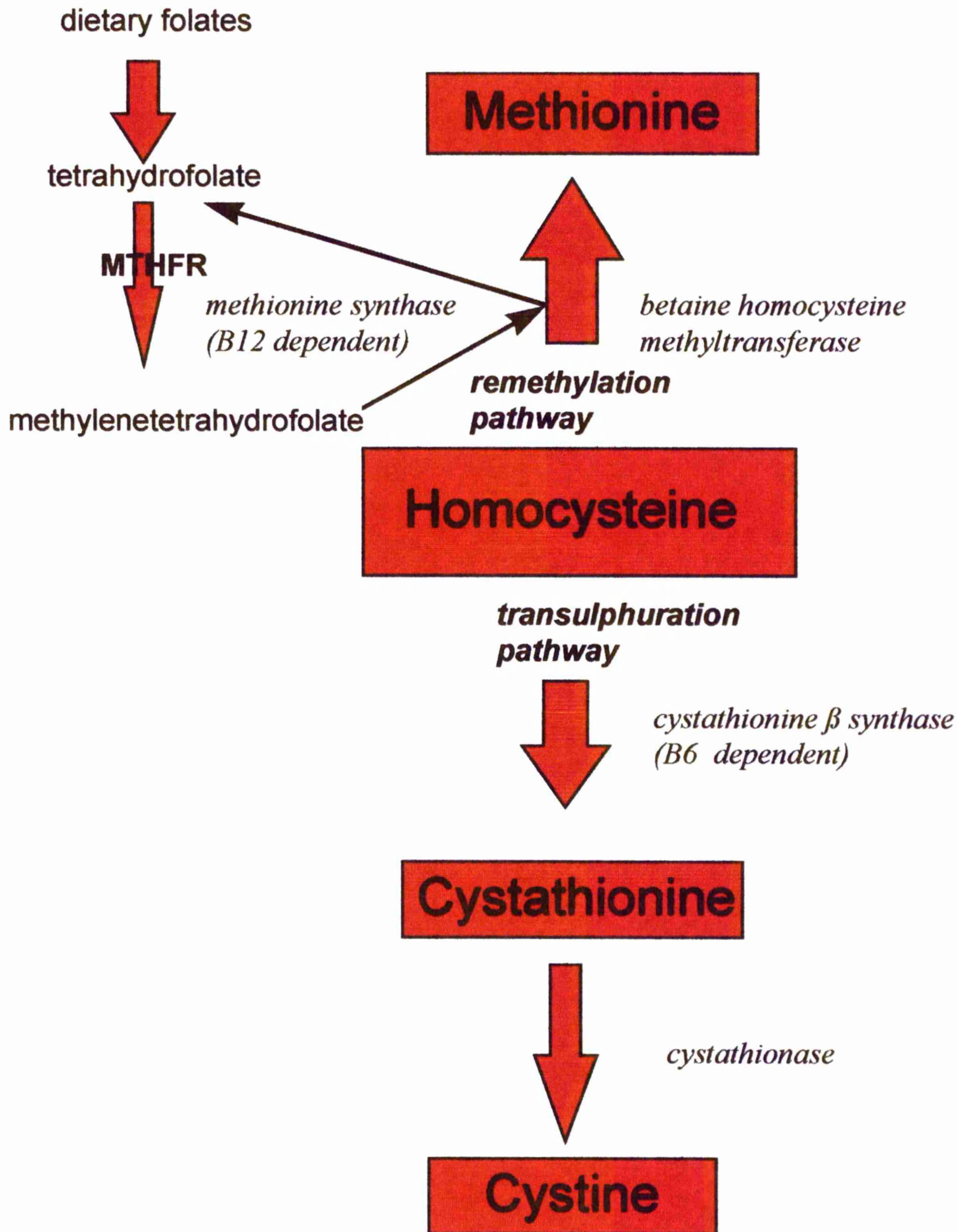


figure 5.1 Homocysteine metabolism.

MTHFR= methylene tetrahydrofolate reductase

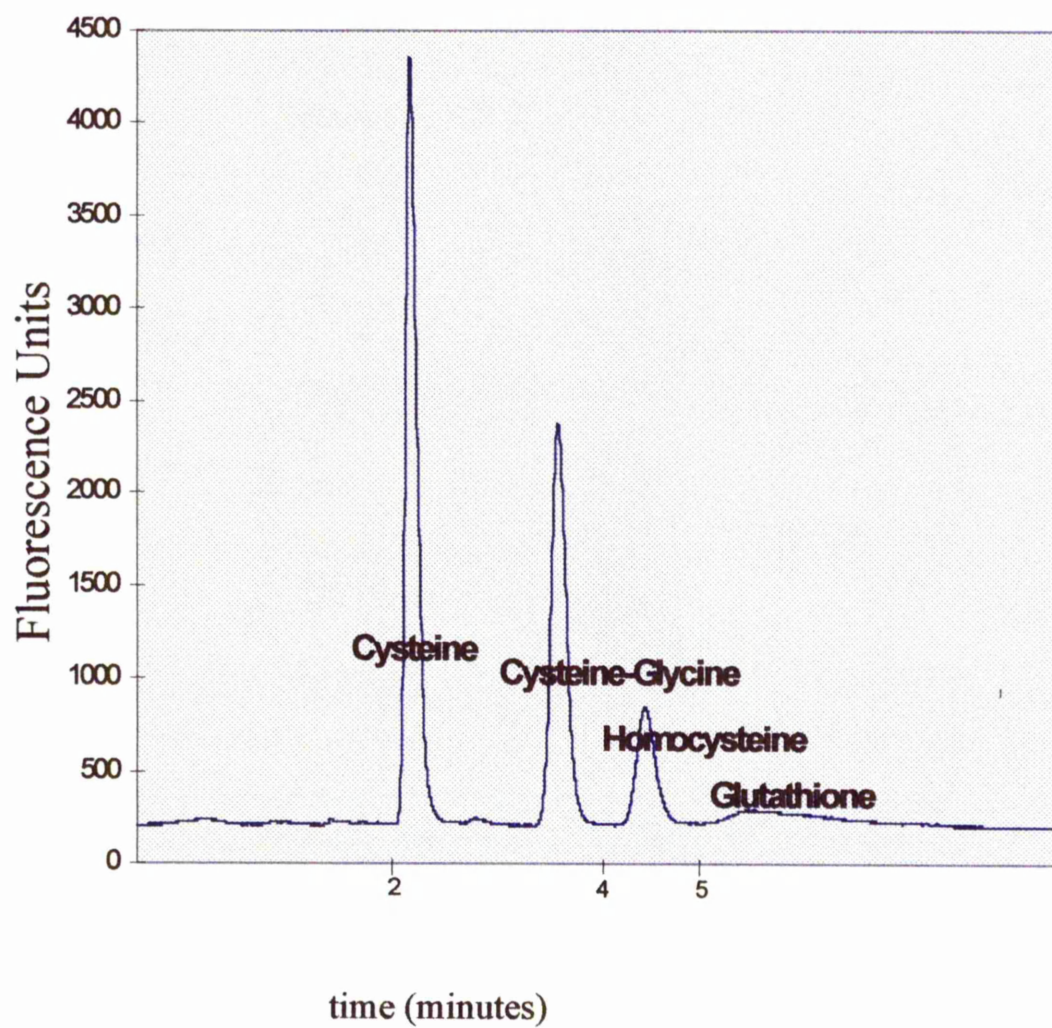
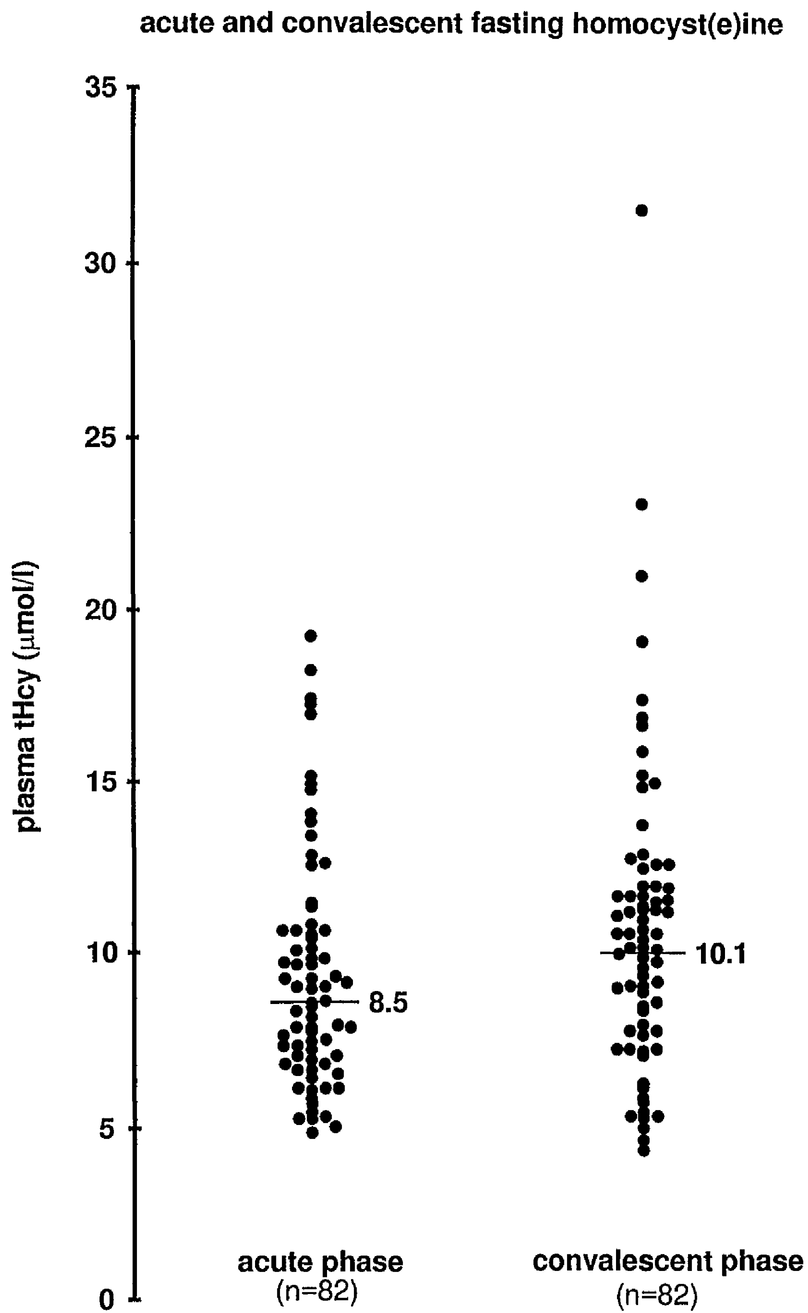


Figure 5.2. Real time HPLC chromatogram. Individual thiol amino acid peaks are indicated.

Figure 5.3



Group		median tHcy {μmol/l} (range)	p value
Female	acute (n=47) control (n=47)	8.1 (4.8-32.3) 7.6 (3.3-14.4)	0.58
	convalescent (n=32) control (n=32)	10.0 (4.9-23) 7.6 (3.3-14.4)	0.048
Male	acute (n=59) control (n=59)	9.2 (4.4-22.8) 8.7 (4.9-20)	0.09
	convalescent (n=50) control (n=50)	10.2 (4.3-31.5) 8.2 (4.9-20)	0.035

Table 5.1. Median fasting total plasma homocysteine concentrations in the acute and convalescent phases of atherothrombotic stroke. Differences were assessed by the Mann Whitney U test.

Chapter Six

The contribution of Factor VII (FVII) gene polymorphisms to longevity in nonagenarians.

6.1 Background

In the previous chapters polymorphisms that might increase the risk of stroke have been discussed. This chapter considers polymorphisms that have been suggested to protect against stroke and MI, and which might therefore influence lifespan. The determinants of longevity in an individual are complex, and include environmental and behavioural factors (Christensen & Vaupel, 1996). However, family studies have indicated that genetic factors are important (Herskind *et al*, 1996), for instance an association with specific HLA loci has been described (Ivanova *et al*, 1998). Stroke and MI are the major causes of mortality in the western world, with particularly high rates in Scotland (Chambless *et al*, 1997). Longevity is therefore likely to be partly dependent on avoiding or delaying the onset of these conditions, and so polymorphisms affecting their risk might exert an influence. Indeed, differences in the distributions of polymorphisms of the apolipoprotein E, angiotensin converting enzyme and plasminogen activator inhibitor type 1 (PAI -1) genes have been described in centenarians compared to younger individuals (Schachter *et al*, 1994; Mannucci *et al*, 1997).

The Northwick Park Heart Study identified elevated factor VII activity (VIIc) as an independent risk factor for fatal MI in middle aged males (Ruddock & Meade, 1994), although others reported that an association between VIIc and coronary or cerebrovascular disease was lacking in older individuals (Folsom *et al,* 1993; Tracy *et al*, 1999). While the determinants of FVII concentrations are complex, including obesity and triglyceride level (Mennen *et al,* 1997), a genetic influence has been established. At the time of this study, three polymorphisms of the factor VII gene had been described which are associated with different factor VII levels: (1) a missense mutation of exon 8 with substitution of arginine by glutamine at position 353, designated R353Q; (2) a decanucleotide insertion sequence in the promoter region at position -323, designated 5'F7 ; and (3) a variable number of tandem

repeats in the hypervariable region 4 of intron 7 designated IVS7. Possession of the Q allele of R353Q, the A2 insertion allele of 5'F7, and homozygosity for the H7 allele of IVS7 are associated with lower FVIIc (Green *et al*, 1991; Bernardi *et al*, 1997). A more recent study claimed that homozygosity (and to a lesser extent heterozygosity) for the Q allele of the R353Q polymorphism, and homozygosity for the H7 allele of the IVS7 polymorphism are protective against MI, possibly as a result of lower FVII concentrations (Iacoviello *et al*, 1998b). However, other studies have failed to confirm these observations (Lane *et al*, 1996; Heywood *et al*, 1997; Doggen *et al,* 1998). To assess their contribution to longevity in a population with high mortality rates due to MI and CVA, the R353Q and IVS7 polymorphisms were investigated, and evidence of enrichment of the genotypes previously associated with reduced MI risk was sought in a group of long-lived individuals living in the Grampian region of Scotland.

6.2 Methods

6.2.1. Subjects

113 consecutive subjects over the age of 90 who had a full blood count performed in the routine haematology laboratory at Aberdeen Royal Infirmary were identified by computer search between June and September 1998. The samples were retrieved after analysis and DNA extracted as described in chapter four. Samples from a sex matched control group were obtained from local blood donors under the age of 45 who were free of symptomatic vascular disease. The protocol was approved by the Grampian Regional Ethical Committee.

6.2.2 R353Q analysis.

Principle

Substitution of Adenine for Guanine at position 10976 in exon 8 of the FVII gene results in the loss an *msp 1* restriction endonuclease site, and in the substitution of arginine by glycine

at position 353 of the translated protein. Amplification of the following primers (Green *et al*, 1991) results in a 312 bp product:

sense 5'-GGGAGACTCCCCAAATATCAC-3'

antisense 5'-ACGCAGCCTTGGCTTTCTCTC-3'

The alleles can be discriminated electrophoretically, since digestion of the R allele results in 205, 67 and 40 bp fragments, whilst the Q allele results in 272 and 40 bp fragments.

PCR reaction conditions

Optimal conditions were determined by Mg titration. 4μl of 50ng/μl of genomic DNA were added to a thin walled PCR tube in a 25μl reaction. Each tube contained:

- 200ng genomic DNA.
- 10pmol each primer.
- 200μmol of dNTPs.
- 2 units of *Taq* DNA polymerase.
- $MgCl_2$, varying between 0.5-5mM.
- 20mM trisHCl.
- 25mM KCl.
- dH_2O to a final volume of 25μl.

The following conditions were applied using a Techne Progene thermal cycler (Cambridge UK): 1 cycle of denaturation at 93°C for 180s, annealing at 55°C for 60s and extension at 72°C for 120s; then 35 cycles of denaturation at 93°C for 60s, annealing at 55°C for 60s and extension at 72°C for 120s). 15 μl of product and 2 μl of loading dye were pipetted onto a 2% agarose gel, electrophoresed at 75V for two hours, and stained with ethidium bromide.

The brightest band was obtained using a Mg concentration of 1mM, and this was included in all subsequent reactions. 4μl of DNA were added to a mastermix consisting of the following (per tube):

- 2.5μl 10X Reaction Buffer.
- 0.5μl of dNTPs.
- 1μl sense primer (10pmol/μl).
- 1μl antisense primer (10pmol/μl).
- 1μl 25mM $MgCl_2$.
- 0.2μl *Taq* polymerase.
- 14.8μl dH_2O.

Restriction enzyme digest

- Conditions were optimised using subjects of known genotype. Initially 1 unit of *msp 1* was used per reaction, but this resulted in incomplete digestion of the QQ control sample, with a band at 205bp being obtained. The difficulty in discriminating between RQ and QQ genotypes was overcome on increasing the enzyme concentration tenfold. Thus the PCR product was incubated overnight at 37°C with 10 units of *msp 1*. 15μl of product were added to a mastermix consisting of (per reaction):
- 3μl Buffer Y.
- 1μl *msp 1* (10units/μl).
- 11μl dH_2O.

13μl of the digest product and 2μl of loading dye were electrophoresed on 2% agarose gel for 1 hour to discriminate the alleles.

Quality assurance

Each experiment included a blank (no DNA) to exclude reagent DNA contamination. Samples of known genotype were included in every experiment. Analyses were repeated at random to confirm the reproducibility of the method; two samples (one RR and one RQ) were genotyped three times each and the same result was obtained every time.

6.2.2 IVS7 polymorphism analysis.

These analyses were kindly performed by Mr Neil Youngson, undergraduate student in the Department of Genetics at the University of Aberdeen. 100ng genomic DNA were amplified using the primers (Marchetti *et al*, 1991):

sense 5'-AATGTGACTTCCACACCTCC-3'

antisense 5'-GATGTCTGTCTGTCTGTGGA-3'

A 25μl reaction was performed consisting of:

- 100ng genomic DNA.
- 7pmoles each primer.
- 200μM dNTPs.
- 2mM of $MgCl_2$ were determined optimum.
- 50mM KCl.
- 10mM Tris-HCl.

Initially the published cycling conditions (Marchetti *et al,* 1991), but no product was obtained. The cycling conditions described in chapter three for the HPA 1a/1b/1b genotyping by enzyme digest were therefore applied and successfully yielded a product. Three alleles were identified by electrophoresis on 2% agarose according to the number of monomeric

repeat sequences obtained: H5 (5 monomers, 406bp), H6 (6 monomers, 443bp) and H7 (7 monomers, 480bp). These are illustrated in *figure 6.1.* A blank and positive control DNA for the common IVS7 genotypes (H6H6, H6H7 and H7H7) were included in all experiments.

6.2.3 Statistical analysis.

Calculations were performed using the SPSS for Windows version 8.0 statistical software. The sex distributions, allele frequencies and distributions of the genotypes in the two groups were compared by chi squared analysis or Fisher's exact test. A p value <0.05 was considered significant. Since QQ homozygotes were very rare, RQ and QQ genotypes were considered together.

6.3 Results

Samples were obtained from 113 subjects (86 female/ 27 male) over 90 years of age (median age 96 years; range 90-103) and 100 (80 female/ 20 male) younger controls (median age 32 years, range 19-44). There was no significant difference in the sex distribution between the groups (p=0.49). Samples which failed to yield a PCR product were repurified and the reaction repeated. R353Q genotypes were successfully elucidated in 108 subjects and 94 controls, IVS7 genotype in 110 subjects and 100 controls, and both genotypes in 105 subjects and 94 controls.

The results are summarised in *tables 6.1 and 6.2.* Using Fisher's exact test, linkage disequilibrium was seen between the two loci (p<0.002), with the rarer H7 and Q alleles occurring together more frequently than expected.

The Q allele was enriched by 15.6% (0.111 v 0.096, p=0.61) and the H7 allele by 9.7% (0.395 v 0.360, p=0.45) in the nonagenarians compared to the control group, but these over-representations did not reach statistical significance (*table 6.1*). The distribution of the specific genotypes previously associated with a reduced risk of MI are shown in *table 6.2.* There is a non-significant tendency for both the RQ (0.222 v 0.17, p=0.36) and the H7H7 genotypes (0.182 v 0.13, p=0.3) to be 49% and 40% more frequent in the long-lived respectively.

6.4 Discussion

The frequencies of the R353Q and IVS7 polymorphisms of the FVII gene in this study are similar to those previously described in a Northern European population, with the Q and H7 alleles less common than in Southern Europe (Bernardi *et al*, 1997; Heywood *et al*, 1997). It has previously been reported that the FVII alleles associated with lower FVII concentrations, QQ, RQ and H7, are associated with a lower risk of MI (Iacoviello *et al*, 1998b). As atherosclerotic disease is the leading cause of death in Scotland, accounting for almost half of all deaths in the cohort under consideration (Scottish Health Statistics, 2000), overrepresentation of these alleles in nonagenarians was looked for. It was observed that the RQ and H7 alleles were overrepresented, but the difference did not reach statistical significance. There are two possible explanations for these findings. Firstly, there is no difference in frequencies between nonagenarians and younger subjects. If so, either the associations with MI previously reported are erroneous, or the effects of the polymorphisms are complex, perhaps only affecting events in the young or influencing non-fatal events. This viewpoint is supported by the observations that the role of VIIc in MI and CVA is itself subject to controversy. There does not appear to be a relationship between Factor VII and

atherosclerosis *per se*, since no association with the extent of coronary (Broadhurst *et al,* 1990) nor carotid disease (Wu *et al,* 1992; Folsom *et al,* 1993; Sosef *et al,* 1994) is apparent. However, given that an association with acute thrombotic events has been observed (Broadhurst *et al,* 1990), especially those that were fatal (Ruddock & Meade, 1994; Heinrich *et al,* 1994), it was reasoned that FVII concentrations might influence longevity. The second possibility is that there is an effect, but one too small for this study to identify at a statistically significant level. The number of subjects recruited was determined in order to achieve 80% statistical power to detect a twofold greater number of QQ homozygotes in the extremely elderly, based on the genotype frequencies previously described (Iacoviello *et al,* 1998b). This calculation was clearly an overestimate given the lower QQ geneotype frequency in Northern European populations. If the allele frequencies observed in this study reflect the true magnitude of any effect, it is calculated that between 750 (for the H7 allele) and 1600 (for the Q allele) subjects are required for these differences to reach significance with 80% power. Following the completion of this study a study of Danish centenarians (Bladbjerg *et al,* 1999) reported that there was no association with FVII gene polymorphisms and longevity, but the sample size was again modest (n=187). Thus larger study populations are required to confirm any relation between FVII polymorphisms and longevity. An apparent difference might also be demonstrated with a relatively modest sample size if young patients with premature vascular disease were recruited as controls, to amplify the differences in cardiovascular risk between groups.

These data are more suggestive of an association between the Q allele and longevity than the findings of Mannucci et al, who found no difference in R353Q allele frequencies between Italian Centenarians and younger controls (Mannucci *et al*, 1997). The current study investigated the influence of the polymorphism in a very different population. Since

cardiovascular mortality rates are three times those of Italy (Chambless *et al*, 1997), it was reasoned that any effect on longevity would be more apparent in this population. Indeed, the magnitude of effect of the Q allele, with 15.6% overrepresentation in nonagenerians, is comparable with the 25% underrepresentation observed in MI subjects (Iacoviello *et al*, 1998), as MI is not the sole cause of death in the cohort under consideration. The IVS7 polymorphism had not been investigated in the extremely elderly previously, although these data demonstrate linkage disequilibrium between the two loci.

Reaching the extremes of age may involve modification of genetically determined risk by environmental and other genetic factors. Indeed the Factor V Leiden polymorphism, which is associated with an increased risk venous thrombosis, is not associated with premature death and is not underrepresented in the extremely elderly (Mari *et al*, 1996; Rees *et al*, 1997). The current data suggest that polymorphisms of the FVII gene might exert a positive influence on longevity, but any such influence, if present, is weak. This is in accordance with the view that the predominant influence on cardiovascular mortality in a Western population is environmental, and that any genetic predisposition inherited by an individual reflect the interactions of many loci, each with a weak influence. Thus, any effect of FVII genotype on an individual's cardiovascular risk would be modified by other factors, both environmental and genetic (Mennen *et al*, 1997; Ridker & Stampfer, 1999a). This viewpoint is supported by reports which have been published since the completion of this study, as the effect of the R353Q polymorphism on risk of MI is modified by smoking (Iacoviello *et al* 1999). A further study from Italy demonstrated the alleles associated with lower FVII antigen concentrations were overrepresented in controls compared to young survivors of MI, suggesting a protective effect (Peyvandi *et al*, 2000). However a recent Dutch study did not identify an association (Lievers *et al,* 2000). Thus there appears to be an association between

FVII gene polymorphisms and cardiovascular disease in the Italian population (Iacoviello *et al,* 1998; Peyvandi *et al,* 2000) which is lacking in Northern European populations (Lane *et al,* 1996; Heywood *et al,* 1997; Doggen *et al,* 1998; Lievers *et al,* 2000; Feng *et al,* 2000). These differences might be related to the fact that these populations have lower allele frequencies than Italy, but it could also be speculated that different environmental factors modify the influence of genotype on FVII concentrations. Thus in the current study habitual differences in dietary fat intake between Scottish and Italian people might counteract any beneficial effect of the R353Q and IVS7 polymorphisms on the avoidance of stroke and MI.

In conclusion FVII gene polymorphisms claimed to be protective against MI were slightly overrepresented in nonagenarians, but a positive influence on longevity was not demonstrated since these differences were not statistically significant. A larger study is therefore required to investigate the effect of these polymorphisms on lifespan.

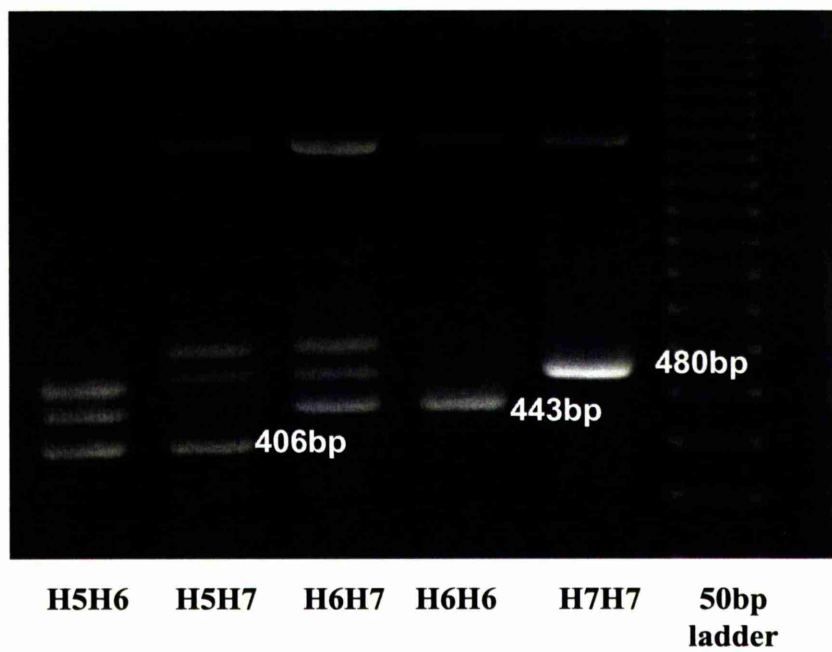


Figure 6.1. IVS7 gene polymorphisms. Additional bands seen at different sizes correspond to the formation of heteroduplexes.

Allele	**Cases** no.	(%)	**Controls** no.	(%)
R	192/216	(88.9)	170/188	(90.4)
Q	24/216	(11.1)	18/188	(9.6)
H5	3/220	(1.4)	4/200	(2)
H6	130/220	(59.1)	124/200	(62)
H7	87/220	(39.5)	72/200	(36)

Table 6.1. R535Q and IVS7 polymorphism allele frequencies.

Genotype	Cases no	(%)	Controls No	(%)
RR	84/108	(77.8)	78/94	(83)
RQ	24/108	(22.2)	14/94	(14.9)
QQ	0/108	(0)	2/94	(2.1)
H7H7	20/110	(18.2)	13/100	(13)
H6H7	45/110	(40.9)	42/100	(42)
H6H6	42/110	(38.2)	41/100	(41)
H5H6	1/110	(0.9)	0/100	(0)
H5H7	2/110	(1.8)	4/100	(4)
H5H5	0		0	

Table 6.2 Factor VII genotype frequencies.

Chapter seven

Conclusions

This thesis examined proposed risk factors for thrombotic stroke, and sought evidence of a causal relationship by examining both epidemiological and functional associations. Since it is impossible to accurately predict when an individual will have a stroke, and since the study of platelet function is labour intensive, prospective functional studies necessitate the study of a large number of subjects and considerable resource. While not truly prospective, it was considered that a study of platelet function and homocysteine concentrations both immediately after stroke and in the convalescent period would be informative.

In chapter four evidence of persisting platelet activation in patients with atherothrombotic stroke was demonstrated. Given that a substantial proportion of patients had significant pre-existing arterial disease (28% had a history of ischaemic heart disease, and 26% of previous stroke or TIA), and the observation that small absolute numbers of activated platelets were detected, it could be suggested that these results reflect ongoing plaque inflammation and thrombosis observed in diffuse arterial disease. However this suggestion is not supported by the lack of correlation between platelet activation markers and plasma fibrinogen concentration, an indicator of the acute phase reaction. In addition, analysis of the 83 patients with no history of a previous clinical arterial event reveals persisting platelet activation in these subjects (*table 7.1*). This therefore suggests these individuals these patients have a constitutional predisposition to atherothrombotic stroke. It was established that this tendency was not related to polymorphisms of GPIIIa or GPIb, but it is possible that other polymorphisms might mediate this effect. It is planned to investigate an

association between the C807T and G873A polymorphisms of the collagen receptor and platelet function in the cohort described in this thesis.

Platelet activation markers were not assessed in terms of clinical outcome in the study. A three-year mortality rate of 10% was recorded, but the cohort is too small and no statistical differences in platelet activation were observed between those who died and those who did not (data not shown). A retrospective assessment of the effect of platelet activation on disability score is therefore planned in order to establish whether a more subtle effect is present.

7.1 Polymorphism studies

Since this study was designed and completed there have been numerous studies of platelet glycoprotein polymorphisms and risk of vascular disease. These are discussed below, and the studies presented in this thesis are discussed in the context of these other reports.

7.1.1 Risk of MI.

Weiss *et al* were the first to observe an association with MI and the 1b allele in 1996, in a small study of 71 survivors of MI compared with healthy age and sex matched controls. This was followed by several conflicting reports (*table 7.2*). There are no obvious methodological differences between these studies, but in general those that refuted an association (Ridker *et al, 1997;* Samani *et al,* 1997; Gardemann *et al,* 1998; Mammotte *et al*, 1998; Bottiger *et al*, 2000) were larger than those in accord

with Weiss *et al* (Zotz *et al,* 1998; Ardissino *et al*, 1999; Mikkelson *et al*, 1999). It could be suggested that a genetic influence on a multifactorial disease like MI might be apparent only in younger subjects, and that an effect was obscured in these reports by the inclusion of older patients who had accrued additional lifestyle risk factors (Garcia-Ribes *et al,* 1998). However, restricting analysis to young cohorts does not support this hypothesis (Scaglione *et al,* 1998; Bottiger *et al*, 2000). It has been suggested (Di Castelnuovo *et al*, 2001) that the 1b allele is not a risk factor for coronary heart disease *per se*, but is associated with MI at a younger age and thrombosis in situations of heightened risk, such as following ruptured coronary plaque (Zotz *et al*, 1998), angioplasty (Abbate *et al*, 1998) or coronary stenting (Kastrati *et al*, 1999; Walter *et al*, 1997). However, negative associations in this context have also been reported (Laule *et al*, 1999).

GPIb polymorphisms have been studied since they might affect platelet function by an effect on vWF binding in the high shear stress conditions observed in stenosed arteries. Few studies of MI have been performed, and all have assessed small numbers of patients (*table 7.3*). Two did not observe an HPA 2a/2b genotype dependent influence overall in Japanese populations (Murata *et al,* 1997; Ito *et al,* 1999), but the risk increased threefold on subgroup analysis of subjects under 60 years who possessed the HPA 2b allele (Murata *et al*, 1997). These data require confirmation in larger studies, and in other ethnic groups.

The C807T polymorphism of GPIa has been investigated (*table 7.3*) as the density of platelet surface collagen binding sites is influenced by genotype, which might affect platelet adhesion following plaque rupture (Kunicki *et al, 1997*), and similar inconsistencies are identified (Moshfegh *et al*, 1999; Croft *et al,* 1999; Corral *et al*, 1999). A very large study of 1057 patients with MI calculated a more modest influence on risk of MI than the smaller studies which identified a positive outcome (Santoso *et al,* 1999).

7.1.2 Risk of stroke.

The studies of stroke are summarised in *table 7.4 and 7.5*, and comparisons made with the current study. The present study is similar in terms of the number and mean age of subjects studied. Most failed to identify an influence of HPA 1a/1b genotype on stroke risk. It could be suggested that this occurred because a diversity of pathogenic mechanisms underlies stroke development, making it unlikely that a single gene could influence aetiology. The study in this thesis, unlike others, attempted to restrict analysis to atherothrombotic stroke. Despite this no HPA 1a/1b genotype dependent effect was observed. It may be that risk is only apparent in certain subgroups of the population, or in younger patients. Wagner *et al* (1998) did not observe an influence in 45 young women with stroke, but reported a statistically significant effect in the 10 women of caucasian origin in whom a cause of stroke was identified. Carter *et al* (1998) also identified an effect in 37 patients under 50 years, and in 218 non-smokers. These effects were observed in a very small number of patients, and was not confirmed by analysis of the 79 subjects under 60 in this thesis.

These associations may therefore be spurious, and require confirmation in larger studies.

Apart from the study described in this thesis, there are four other reports of the influence of GPIb polymorphisms on stroke risk. They are comparable in terms of the number and ages of the patients recruited, and results are conflicting (*table7.4*). There has been a single case report of the C807T polymorphism of GPIa and stroke risk (Carlsson *et al,* 1999), but the association in requires confirmation in larger studies.

7.1.3 Conclusions

A recent meta-analysis of HPA 1a/1b (Di Castenuovo *et al,* 2001) combined the information from 34 studies, incorporating 9095 patients with IHD and 12508 controls. The risk of MI *per se* was modest, but statistically significant (OR 1.10{1.03-1.18}). This increased in patients under 60 years (1.21{1.05-1.38}) and in those undergoing revascularisation procedures (1.31{1.1-1.56}), but is still small compared to the positive studies listed in *table7.2.* The influence of HPA 1a/1b status is therefore unlikely to be of clinical relevance in individual patients, but remains of interest when considering risk factors for vascular disease on a population-wide basis. Most studies to date have therefore been inadequately statistically powered to detect such subtle genetic effects. It was recently calculated that recruitment of at least 4356 subjects would be necessary to demonstrate that HPA 1b is a risk for MI in a single population based study (Di Castelnuovo *et al,* 2001).

A similar conclusion is reached from consideration of the data discussed in chapter six. FVII gene polymorphisms have been suggested to be protective against the development of MI, and the hypothesis that they are associated with an increased lifespan was tested. The 'protective' genotypes were slightly overrepresented in nonagenarians in comparison to younger subjects, but it was calculated that a much larger sample size would be required to demonstrate a statistically significant effect. A study of a larger cohort is therefore necessary. In addition, the study described in chapter six did not measure FVIIc to establish the influence of genotype on plasma levels. Although such a relationship has been described in other populations (Green et al, 1991; Bernardi et al, 1997; Peyvandi et al, 2000) measurement of VIIc in this study would be informative, given the possibility that genetic determination of VIIc levels is modified by the diet of the Scottish population.

Given these findings, and the lack of association between genotypes and stroke, it is concluded that any relationships (if present) are likely to be small, and may be modified by environmental and/or other genetic factors. These effects may therefore only be apparent in small subpopulations of the study cohort. This study avoided such subgroup analyses, since the sample size has insufficient statistical power to make such comparisons. Indeed it is possible that spurious associations may be encountered by chance alone if numerous analyses are performed. Association studies should therefore restrict analysis to comparisons for which the sample size is adequate (Ridker and Stampfer, 1998a). Such an approach would avoid unnecessary

studies, exemplified by those of the angiotensin converting enzyme (ACE) I/D polymorphism and MI. Despite no evidence of a functional effect, numerous modestly sized case control studies generated conflicting data, and the controversy was only resolved when a large (n=5000) study reported no association of the D allele with MI (Keavney *et al*, 2000).

Although the study presented in this thesis is comparable in size to the others listed in *table 7.4*, it is clear with the benefit of hindsight that none of these are large enough to convincingly demonstrate an allele dependent risk. Although the power calculation to determine sample size was based on HPA 1b frequencies, this study was primarily designed to determine whether an effect of platelet genotype on function could be detected. From the outset the study was clearly underpowered to assess HPA1a/1b as an epidemiological risk factor, but the sample size is compares favourably with studies which have reported a functional consequence of the polymorphism (table 7.5).

An association of HPA 1b with stroke observed in non-smokers only (Carter *et al,* 1998) is in contradistinction to reports of Factor V Leiden (Rosendaal *et al,* 1997; Doggen *et al,* 1998) and the prothrombin G20210A mutation (Rosendaal *et al,* 1995; Doggen *et al,* 1999), which only observed an effect in smokers. The latter view is favourable since smoking produces a prothrombotic tendency, and it is plausible that this interacts with genes which also encourage thrombosis. Thus when considering

candidate genes or possible gene-environment interactions, there should be evidence of a plausible biological mechanism as well as epidemiological association.

7.2 Functional studies of platelet polymorphisms

Studies assessing the functional consequences of the HPA 1a/1b polymorphism are summarised in *table 7.5*, and comparisons with the studies in this thesis are drawn. As with the epidemiological studies the data are conflicting, and some (Feng *et al,* 1999; Michelson *et al,* 2000) reported greater responsiveness to agonists and increased activation in HPA 1b-containing platelets compared with 1a homozygotes. A 'gene-dose effect' was apparent as homozygous 1b platelets were more reactive than 1a/1b heterozygous platelets. Others found no allele dependent differences (Corral *et al,* 1997) or identified increased reactivity in 1a platelets (Lasne *et al,* 1997: Cooke *et al,* 1998). In chapter three no differences in basal fibrinogen activation or responsiveness to ADP were demonstrated in platelets from healthy donors. The present study was unable to assess homozygous 1b platelets since few subjects possessing this genotype were identified, but it was considered justifiable to search for functional effects in HPA 1a/1b heterozygotes since this genotype accounts for the majority of subjects claimed at increased risk in epidemiological studies.

Studies which reported an effect of genotype on platelet function have been performed using in platelets from normal subjects, and it is unclear whether any of these small physiological differences are sufficient to contribute to disease

pathogenesis. Goodall *et al* (1999) reported in 70 patients with stable coronary artery disease that 1b platelets from exhibited greater fibrinogen binding than 1a platelets following stimulation with a weak agonist. 22 patients had a history of previous MI, but were sampled at a timepoint distant from the acute thrombotic event, and it remains uncertain whether these differences could result in acute thrombosis. The authors have subsequently increased the number of subjects studied and an allele dependent difference is no longer apparent, suggesting that statistical error accounts for their initial observations (Goodall *et al,* 2001, personal communication). Thus the study described in chapter four was designed to determine whether allele dependent differences in platelet function were apparent around the time of an acute thrombotic event. This is currently the only study in the literature to consider this, and no allele determined influence was observed. Although it is possible that there are small physiological differences between 1a and 1b platelets, these were not observed at the time of stroke, and it is speculated that the cascade of events triggered by plaque rupture and tissue factor exposure swamp these relatively subtle effects.

7.3 Homocysteine as a causative risk factor.

It is apparent that demonstration of a plausible function effect of a genetic polymorphisms does not necessarily imply a causal relationship with stroke. This was discussed in chapter one, when in the case of fibrinogen ß-gene polymorphisms, no increased risk of vascular disease has been demonstrated despite an influence on plasma fibrinogen concentrations. As discussed in chapter four homozygosity for the T allele of MTHFR C677T polymorphism is associated with increased plasma tHcy

concentrations, but the genotype is not itself an independent risk factor for thrombosis (Brattstrom *et al,* 1998), and evidence of a plausible effect of tHcy on vascular risk is necessary. The relationship between plasma tHcy and acute thrombosis was therefore investigated in the stroke cohort. Median fasting tHcy concentrations did not statistically significantly differ between patients immediately after stroke and controls, but increased in the convalescent period so that homocysteine appears a significant risk factor when measured in the follow up period. These changes were not associated with alterations in factors known to influence tHcy, and the mechanisms of these changes require further study. There are two possible explanations for these observations:

- *Homocysteine is not a risk factor for stroke at all, but rises secondarily to the disease process.*
- *Homocysteine is a risk factor, and concentrations fall temporarily following stroke.*

The former is favoured since it provides an adequate explanation for the fact that prospective studies have found a much weaker association than retrospective studies. However the mechanism of homocysteine increase requires identification. It has been suggested the rise in tHcy is a secondary phenomenon mediated by an increase in methylation reactions in response to tissue damage, which generate S-adenosylhomocysteine, which is converted to Hcy (Dudman, 1999). If this is the case the magnitude of increase might be correlated with the extent of damage, and the

relationship between changes in homocysteine concentration and infarct size (assessed by CT scanning) is therefore planned. A similar study relating plasma concentrations with infarct size in MI is also being considered. Although disability does not necessarily correlate with infarct size, an assessment of functional outcome might also be informative.

A mechanism to support the hypothesis that tHcy merely falls transiently at the time of a stroke has not been elucidated, and the relationship between the change in tHcy and acute phase markers requires further study, as it has been suggested concentrations fall by dilution from increased plasma protein synthesis (Egerton *et al*, 1996). Furthermore a nutritional assessment would test the proposal that a reduction in dietary methionine intake following stroke might explain the observed changes.

7.4 Conclusions

In this thesis two main points are concluded. Firstly, the aetiology of atherothrombotic stroke is complex, and the influence of individual genetic risk factors may be small. Studies of genetic polymorphisms must be of appropriate statistical power to identify these subtle effects, and to investigate possible interactions. Furthermore plausible functional effects of polymorphisms should be sought to corroborate epidemiological associations. This could be studied in smaller cohorts, since one might reasonably expect to readily demonstrate an effect if it is clinically significant. Secondly, when considering the role of a proposed pathogenic

risk factor such as homocysteine, it is important to investigate the temporal nature of the association with the clinical event. Caution must be applied when considering the results of retrospective studies, since they do not necessarily apply causality and are likely to overestimate risk compared with prospective studies.

Group	Acute n=83	Convalescent n=47	Controls n=110	Significance
% fibrinogen binding. Geometric mean (range)	1.79 (0.2-15.1)	1.57 (0.2-19.0)	0.99 (0.2-10.3)	acute v control p<0.001[1] convalescent v control p=0.001[1] acute v convalescent p=0.70[2]
% CD62P expression. Geometric mean (range)	1.49 (0.1-3.72)	0.81 (0.2-7.76)	0.34 (0.1-1.56)	acute v control p<0.001[1] convalescent v control p<0.001[1] acute v convalescent p=0.005[2]

Table 7.1. Platelet activation markers in patients with stroke as the first arterial event.

[1]Student's *t*-test.

[2]Paired *t*-test.

Study	Number	Mean Age (range or SD)	?Association found	OR or P Value	Comments
Weiss et al, 1996	71	56.3 (12.8)	YES	2.8 (1.2-6.4)	
Ridker at, 1997	374 with MI	60.3	NO	P=0.4	Nested case control study of large prospective cohort .
Samani et al, 1997	242	65.8 (11.4)	NO	0.9 (0.6-1.4)	
Herrman et al, 1997	565 with MI	25-64	NO	P>0.05	ECTIM study
Zotz et al, 1998	298 undergoing coronary angiography	55.7 (9.6)	YES	2.3 (1.2-4.6)	In the 124 with MI
Durante-Mangoni et al, 1998	114 with IHD, 43 with MI	58.1 (10.1) 51 (10.9)	NO	P=0.6	
Gardemann et al 1998	2252 undergoing angiography	62.7 (9.3)	NO	P>0.05	No association found in subgroups with other risk factors.
Mamotte et al, 1998	589 with IHD or MI	<50	NO	P>0.1	
Scaglione et al 1998	98 with MI	<45	NO	P=0.41	
Anderson et al, 1999	791 undergoing angiography, 221 with MI	64 (17-89)	NO	1.4 (0.95-2.0)	
Mikkelson et al, 1999	300 men who died suddenly	(33-69)	YES	6.6 (2.1-22.8)	HPA 2b associated with ruptured plaque morphology, but not with the extent of stable coronary artery narrowing at autopsy.
Ardissino et al, 1999	200 with MI	<45	YES	1.84 (1.0-3.0)	
Bottiger et al, 2000	998 with IHD, 793 with MI	64.1 (10.2) 62.6 (11.6)	NO	P>0.05 P>0.05	No interactions with other risk factors observed.

Table7.2. The risk of myocardial infarction with HPA-1b allele.

Polymorphism	Study	Number	Mean age (range or SD)	?Association found	OR (95%CI) or P value	Comments
HPA 2a/2b and VNTR of GPIb	Murata et al, 1997	91 with MI or IHD	60.7 (41-74)	YES-on subgroup analysis	2.5 (1.1-5.8) in the 44 under 60 years.	OR for all patients 1.5 (0.7-3.2)
	Gonzalez-Conejero et al, 1998	101 with MI or unstable angina	62.9 (32-86)	YES	OR 2.1 (0.98-4.5) for 2a/2b OR 2.8 (1.3-6.4) for VNTR	
	Ito et al, 1999	158 with IHD	59.3 (SD 10.0)	NO	2.0 (0.9-4.3)	No relationship between HPA 2a/2b or VNTR and extent of coronary disease on angiography.
S/R of GPIb	Corral et al, 2000	101 with MI or unstable angina	62.9 (34-85)	NO	P=0.5	
C807T of GPIa	Moshfegh et al, 1999	177 with MI	57 (32-72)	YES	3.3 (1.2-8.8)	
	Santoso et al, 1999	1057 with MI	62.2 (1.1-2.1)	YES	1.6 (1.1-2.1)	Not associated with stable coronary disease
	Croft et al, 1999	546 with MI	62.1 (SD 9.1)	NO	0.9 (0.7-1.05)	No associations in subgroups with other risk factors.
	Corral et al, 1999	101 with MI or unstable angina	62.9 (34-85)	NO	P=0.3	

Table7.3. GPIb and Ia polymorphisms and MI.

Polymorphism	Study	Numbers	Mean age (range or SD)	?Association found	OR (95%CI) or P Value	Comments
HPA 1a/1b of GPIIIa	Ridker et al, 1997	209	60.3	NO	P=0.5	All subtypes
	Corral et al, 1997	103	62.9 (24-88)	NO	P=0.8	
	Wagner et al, 1998	65	<45	YES- in subgroup analysis only	1.1 (0.6-2.3)	Risk in subgroup of white women with identifiable cause (n=10) OR 12.8 (1.2-135.0)
	Carter et al, 1998	505	70 (61-77) male 75 (68-82) female	NO overall	P>0.05	All subtypes of cerebral infarction. Association in 218 non-smokers, and in the 37 who were <50 years
	Kekomaki et al, 1999	234	50 (SD 7.2)	NO	P>0.05	Cases of cerebral infarction
	This Study	**150**	**58.3 (25-70)**	**NO**	**0.8 (0.5-1.3)**	**Atherothrombotic stroke**
Polymorphisms of GPIb	Gonzales-Conejero et al, 1998	104	65.8 (24-88)	YES	2.4 (1.0-5.6)	For HPA 2b. OR 2.8 (1.2-7.1) for C/B alleles of VNTR polymorphism.
	Carter et al, 1998	505	70 (61-77) male (68-82) female	NO	P>0.05	No associations withVNTR polymorphism on subgroup analysis.
	Sonoda et al, 2000	200	58.2 (SD 7.7)	YES	OR 2.2 P<0.001	HPA 2a/2b polymorphism. OR increases in younger Association with all subtypes
	Corral et al, 2000	104	65.8 (24-88)	NO	P=0.29	No association with S/R polymorphism.
	This Study	**150**	**58.3 (25-70)**	**NO**	**1.4 (0.8-2.4)**	
Polymorphisms of GP IaIIa	Carlsson et al, 1999	227	62.2(SD 14.3)	YES-in subgroup only	3.0 (1.2-7.6)	In the 45 who were <50 years

Table 7.4. Platelet polymorphisms and the risk of stroke.

Study	Subjects	Numbers	Technique	Conclusion
Corral *et al*, 1997b	9 healthy subjects	3 with 1a/1a 3 with 1a/1b 3 with 1b/1b	Aggregometry and FACS of PRP	No effect on response to agonists. No effect on vWF or fibrinogen binding. No effect on P-Selectin expression.
Lasne *et al*, 1997	102 healthy subjects	with 1a/1a with 1a/1b with 1b/1b	Aggregometry of ???	HPA 1b platelets less responsive to TRAP-induced aggregation.
Cooke *et al*, 1998	26 healthy subjects	15 with 1a/1a 11 with 1a/1b 0 with 1b/1b	Aggregometry of PRP	Response to ADP and adrenaline identical. HPA 1b platelets more sensitive to inhibition of aggregation by aspirin.
Goodall *et al*, 1999	70 patients with stable angina	50 with 1a/1a 19 with 1a/1b 1 with 1b/1b	Whole blood FACS	Increased ADP induced fibrinogen binding in 1b platelets. No difference in basal fibrinogen binding.
Feng *et al*, 1999	1422 healthy subjects	1017 with 1a/1a 369 with 1a/1b 36 with 1b/1b	Aggregometry of PRP	Greater aggregability of HPA 1b platelets with adrenaline, but not ADP. Dose effect, as 1b/1b platelets more responsive than 1a/1b.
Michelson *et al, 2000*	56 healthy subjects	20 with 1a/1a 20 with 1a/1b 16 with 1b/1b	Aggregometry of PRP Whole blood FACS	1b/1b>1a/1b>1a/1a CD62P expression, fibrinogen binding, and ADP-induced GPIIbIIIa activation.
This study, chapter 3	70 healthy volunteers	35 with 1a/1a 34 with 1a/1b 1 with 1b/1b	Whole blood FACS	No difference in basal fibrinogen binding or CD62P expression. No difference in ADP-induced fibrinogen binding.
This study, chapter 4	148 patient with acute stroke	108 with 1a/1a 29 with 1a/1b 1 with 1b/1b	Whole blood FACS	No difference in basal fibrinogen binding or CD62P expression. No difference in ADP-induced fibrinogen binding.
	75 patients in convalescent period	62 with 1a/1a 12 with 1a/1b 1 with 1b/1b	Whole blood FACS	No difference in basal fibrinogen binding or CD62P expression. No difference in ADP-induced fibrinogen binding.

Table 7.5. Functional studies of HPA 1a/1b polymorphism.

References

Abbate, R., Marcucci, R., Camacho-Vanegas, O., Pepe, G., Gori, A.M., Capanni, M., Simonetti, I., Prisco, D. & Gensini, G.F. (1998) Role of platelet glycoprotein PL(A1/A2) polymorphism in restenosis after percutaneous transluminal coronary angioplasty. *American Journal of Cardiology*, **82**, 524-525.

Alfthan, G., Pekkanen, J., Jauhiainen, M., Pitkaniemi, J., Karvonen, M., Tuomilehto, J., Salonen, J.T. & Ehnholm, C. (1994) Relation of serum homocysteine and lipoprotein(a) concentrations to atherosclerotic disease in a prospective Finnish population based study. *Atherosclerosis*, **106**, 9-19.

Antiplatelet Trialists' Collaboration. (1994) Collaborative overview of randomised trials of antiplatelet therapy--I: Prevention of death, myocardial infarction, and stroke by prolonged antiplatelet therapy in various categories of patients. *British Medical Journal*, **308**, 81-106.

Arnesen, E., Refsum, H., Bonaa, K.H., Ueland, P.M., Forde, O.H. & Nordrehaug, J.E. (1995) Serum total homocysteine and coronary heart disease. *International Journal of Epidemiology*, **24**, 704-709.

Ardissino, D., Mannucci, P.M., Merlini, P.A., Duca, F., Fetiveau, R., Tagliabue, L., Tubaro, M., Galvani, M., Ottani, F., Ferrario, M., Corral, J. & Margaglione, M. (1999) Prothrombotic genetic risk factors in young survivors of myocardial infarction. *Blood*, **94**, 46-51.

Arruda, V.R., Siquiera, L.H., Chiaparini, L.C., Coelho, O.R., Mansur, A.P., Ramires, A. & Annichino-Bizzacchi, J.M. (1998) Prevalence of the

prothrombin gene variant 20210 G --> A among patients with myocardial infarction. *Cardiovascular Research*, **37**, 42-45.

Basha, B.J. & Sowers, J.R. (1996) Atherosclerosis: an update. *American Heart Journal,* **131**, 1192-1202.

Behague I., Poirier O., Nicaud V., Evans A., Arveiler D., Luc G., Cambou J.P., Scarabin P.Y., Bara L., Green F. and Cambien F. (1996) Beta fibrinogen gene polymorphisms are associated with plasma fibrinogen and coronary artery disease in patients with myocardial infarction. *Circulation,* **93,** 440-449.

Berliner, J., Leitinger, N., Watson, A., Huber, J., Fogelman, A. & Navab, M. (1997) Oxidized lipids in atherogenesis: formation, destruction and action. *Thrombosis & Haemostasis*, **78**, 195-199.

Bernardi, F., Arcieri, P., Bertina, R.M., Chiarotti, F., Corral, J., Pinotti, M., Prydz, H., Samama, M., Sandset, P.M., Strom, R., Garcia, V.V. & Mariani, G. (1997) Contribution of factor VII genotype to activated FVII levels. Differences in genotype frequencies between northern and southern European populations. *Arteriosclerosis, Thrombosis & Vascular Biology*, **17**, 2548-2553.

Bertina, R.M., Koeleman, B.P., Koster, T., Rosendaal, F.R., Dirven, R.J., de Ronde, H., van der Velden, P.A. & Reitsma, P.H. (1994) Mutation in blood coagulation factor V associated with resistance to activated protein C. *Nature*, **369**, 64-67.

Bird, I.M. (1989) High performance liquid chromatography: principles and clinical applications. *British Medical Journal*, **299**, 783-787.

Bladbjerg, E.M., Andersen-Ranberg, K., de Maat, M.P., Kristensen, S.R., Jeune, B., Gram, J. & Jespersen, J. (1999) Longevity is independent of common variations in genes associated with cardiovascular risk. *Thrombosis & Haemostasis*, **82**, 1100-1105.

Boers, G.H., Smals, A.G., Trijbels, F.J., Fowler, B., Bakkeren, J.A., Schoonderwaldt, H.C., Kleijer, W.J. & Kloppenborg, P.W. (1985) Heterozygosity for homocystinuria in premature peripheral and cerebral occlusive arterial disease. *New England Journal of Medicine*, **313**, 709-715.

Bottema, C.D. & Sommer, S.S. (1993) PCR amplification of specific alleles: rapid detection of known mutations and polymorphisms. *Mutation Research*, **288**, 93-102.

Bottiger C., Kastrati A., Koch W., Mehilli J., Seidl H., Schomig K., von Beckerath N. and Schomig A. (2000) HPA-1 and HPA-3 polymorphisms of the platelet fibrinogen receptor and coronary artery disease and myocardial infarction. *Thrombosis & Haemostasis*, **83,** 559-562.

Brass, L.M., Isaacsohn, J.L., Merikangas, K.R. & Robinette, C.D. (1992) A study of twins and stroke. *Stroke*, **23**, 221-223.

Brass, L.F., Ahuja, M., Belmonte, E., Blanchard, N., Pizarro, S., Tarver, A. & Hoxie, J.A. (1994) Thrombin receptors: turning them off after turning them on. *Seminars in Hematology*, **31**, 251-260.

Brattstrom, L., Wilcken, D.E., Ohrvik, J. & Brudin, L. (1998) Common methylenetetrahydrofolate reductase gene mutation leads to hyperhomocysteinemia but not to vascular disease: the result of a meta-analysis. *Circulation*, **98**, 2520-2526.

Bray, P.F. (1999) Integrin polymorphisms as risk factors for thrombosis. *Thrombosis & Haemostasis*, **82**, 337-344.

Broadhurst, P., Kelleher, C., Hughes, L., Imeson, J.D. & Raftery, E.B. (1990) Fibrinogen, factor VII clotting activity and coronary artery disease severity. *Atherosclerosis*, **85**, 169-173.

Burke, A.P., Farb, A., Malcom, G.T., Liang, Y.H., Smialek, J. & Virmani, R. (1997) Coronary risk factors and plaque morphology in men with coronary disease who died suddenly. *New England Journal of Medicine*, **336**, 1276-1282.

Butterworth R.J., Marshall W.J. & Bath P.M.W. (1997) Changes in serum lipid measurements following acute ischaemic stroke. *Cerebrovascular Diseases*, **7**, 10-13.

Carlsson, L.E., Santoso, S., Spitzer, C., Kessler, C. & Greinacher, A. (1999) The alpha2 gene coding sequence T807/A873 of the platelet collagen receptor integrin alpha2beta1 might be a genetic risk factor for the development of stroke in younger patients. *Blood*, **93**, 3583-3586.

Carter A.M., Mansfield M.W., Stickland M.H. and Grant P.J. (1996) Beta-fibrinogen -455 G/A polymorphism and fibrinogen levels. Risk factors for coronary artery disease in subjects with NIDDM. *Diabetes Care,* **19,** 1265-1268.

Carter, A.M., Catto, A.J., Bamford, J.M. & Grant, P.J. (1997a) Gender-specific associations of the fibrinogen B beta 448 polymorphism, fibrinogen levels, and acute cerebrovascular disease. *Arteriosclerosis, Thrombosis & Vascular Biology*, **17**, 589-594.

Carter, A.M., Ossei-Gerning, N., Wilson, I.J. & Grant, P.J. (1997b) Association of the platelet Pl(A) polymorphism of glycoprotein IIb/IIIa and the fibrinogen Bbeta 448 polymorphism with myocardial infarction and extent of coronary artery disease. *Circulation*, **96** , 1424-1431.

Carter, A.M., Catto, A.J., Bamford, J.M. & Grant, P.J. (1998) Platelet GP IIIa PlA and GP Ib variable number tandem repeat polymorphisms and markers of platelet activation in acute stroke. *Arteriosclerosis, Thrombosis & Vascular Biology*, **18**, 1124-1131.

Carter A.M., Catto A.J. and Grant P.J. (1999) Association of the alpha-fibrinogen Thr312Ala polymorphism with poststroke mortality in subjects with atrial fibrillation. *Circulation,* **99,** 2423-2426.

Catto, A., Carter, A., Ireland, H., Bayston, T.A., Philippou, H., Barrett, J., Lane, D.A. & Grant, P.J. (1995) Factor V Leiden gene mutation and thrombin generation in relation to the development of acute stroke. *Arteriosclerosis, Thrombosis & Vascular Biology*, **15**, 783-785.

Catto A.J., Carter A.M., Stickland M., Bamford J.M, Davies J.A. and Grant P.J. (1997) Plasminogen activator inhibitor-1 (PAI-1) 4G/5G promoter polymorphism and levels in subjects with cerebrovascular disease. *Thrombosis & Haemostasis*, **77**, 730-734.

Catto A.J., Kohler H.P., Bannan S., Stickland M., Carter A. and Grant P.J. (1998) Factor XIII Val34Leu: a novel association with primary intracerebral hemorrhage. *Stroke,* **29,** 813-816.

Cavanagh, G., Dunn, A.N., Chapman, C.E. & Metcalfe, P. (1997) HPA genotyping by PCR sequence-specific priming (PCR-SSP): a streamlined method for rapid routine investigations. *Transfusion Medicine*, **7**, 41-45.

Chambers, J.C., McGregor, A., Jean-Marie, J., Obeid, O.A. & Kooner, J.S. (1999) Demonstration of rapid onset vascular endothelial dysfunction after hyperhomocysteinemia: an effect reversible with vitamin C therapy. *Circulation*, **99**, 1156-1160.

Chambless, L., Keil, U., Dobson, A., Mahonen, M., Kuulasmaa, K., Rajakangas, A.M., Lowel, H. & Tunstall-Pedoe, H. (1997) Population versus clinical view of case fatality from acute coronary heart disease: results from the WHO MONICA Project 1985-1990. Multinational MONItoring of Trends and Determinants in CArdiovascular Disease. *Circulation*, **96**, 3849-3859.

Chasan-Taber, L., Selhub, J., Rosenberg, I.H., Malinow, M.R., Terry, P., Tishler, PV, Willett, W., Hennekens, C.H. & Stampfer, M.J. (1996) A prospective study of folate and vitamin B6 and risk of myocardial infarction in US physicians. *Journal of the American College of Nutrition*, **15**, 136-143.

Christensen, K. & Vaupel, J.W. (1996) Determinants of longevity: genetic, environmental and medical factors. *Journal of Internal Medicine*, **240**, 333-341.

Cooke, G.E., Bray, P.F., Hamlington, J.D., Pham, D.M. & Goldschmidt-Clermont, P.J. (1998) PlA2 polymorphism and efficacy of aspirin. *Lancet*, **351**, 1253

Corral, J., Gonzalez-Conejero, R., Lozano, M.L., Rivera, J., Heras, I. & Vicente (1997a) The venous thrombosis risk factor 20210 A allele of the prothrombin gene is not a major risk factor for arterial thrombotic disease. *British Journal of Haematology*, **99**, 304-307.

Corral, J., Gonzalez-Conejero, R., Rivera J., Iniesta J.A., Lozano M.L. and Vicente V. (1997b) HPA-1 genotype in arterial thrombosis—role of HPA-1b polymorphism in platelet function. *Blood Coagulation and Fibrinolysis,* **8,** 284-290.

Corral, J., Gonzalez-Conejero, R., Rivera J., Ortuno F., Aparacio P. and Vicente V. (1999) Role of the 807 T/C polymorphism of the alpha2 gene in platelet GPIa collagen receptor expression and function—effect in thromboembolic diseases. *Thrombosis & Haemostasis,* **81,** 951-956.

Corral, J., Lozano, M.L., Gonzalez-Conejero, R., Martinéz C, Iniesta, J.A., Rivera, J. & Vicente, V. (2000) A common polymorphism flanking the ATG initiator codon of GP1ba does not affect expression and is not a major risk factor for arterial thrombosis. *Thrombosis & Haemostasis*, **83**, 23-28.

Couch, J.R. & Hassanein, R.S. (1976) Platelet aggregation, stroke, and transient ischemic attack in middle- aged and elderly patients. *Neurology*, **26**, 888-895.

Croft, S.A., Hampton, K.K., Sorrell, J.A., Steeds, R.P., Channer, K.S., Samani, N.J. & Daly, M.E. (1999) The GPIa C807T dimorphism associated with platelet collagen receptor density is not a risk factor for myocardial infarction. *British Journal of Haematology*, **106**, 771-776.

Cushman M., Rosendaal F.R, Psaty B.M., Cook E.F., Valliere J., Kuller L.H. and Tracy R.P. (1998) Factor V Leiden is not a risk factor for arterial vascular disease in the elderly: results from the Cardiovascular Health Study. *Thrombosis and Haemostasis*, **79**, 912-915.

Dawson S., Hamsten A., Wiman B., Henney A. and Humphries S. (1991) Genetic variation at the plasminogen activator inhibitor-1 locus is associated with altered levels of plasma plasminogen activator inhibitor-1 activity. *Arteriosclerosis & Thrombosis*, **11,** 183-190.

De Maat M.P., Kastelein J.J., Jukema J.W., Zwinderman A.H., Jansen H., Groenemeier B., Bruschke A.V. and Kluft C. (1998) -455 G/A polymorphism of the beta-fibrinogen gene is associated with the progression of coronary atherosclerosis in symptomatic men: proposed role for an acute-phase reaction pattern of fibrinogen. *Arteriosclerosis, Thrombosis & Vascular Biology,* **18,** 265-271.

den Heijer, M., Koster, T., Blom, H.J., Bos, G.M., Briet, E., Reitsma, P.H., Vandenbroucke, J.P. & Rosendaal, F.R. (1996) Hyperhomocysteinemia as

a risk factor for deep-vein thrombosis. *New England Journal of Medicine*, **334**, 759-762.

den Heijer, M., Brouwer, I.A., Bos, G.M., Blom, H.J., van der Put, N.M., Spaans, A.P., Rosendaal, F.R., Thomas, C.M., Haak, H.L., Wijermans, P.W. & Gerrits, W.B. (1998) Vitamin supplementation reduces blood homocysteine levels: a controlled trial in patients with venous thrombosis and healthy volunteers. *Arteriosclerosis, Thrombosis & Vascular Biology*, **18**, 356-361.

De Stefano, V., Chiusolo, P., Paciaroni, K., Casorelli, I., Rossi, E., Molinari, Servidei, S., Tonali, P.A. & Leone, G. (1998) Prothrombin G20210A mutant genotype is a risk factor for cerebrovascular ischemic disease in young patients. *Blood*, **91**, 3562-3565.

de Valk, H.W., van Eeden, M.K., Banga, J.D., van der Griend, R., de Groot, E., Haas, F.J., Meuwissen, O.J., Duran, M., Smeitink, J.A., Poll-The, B.T., de, K., JB, Wittebol-Post, D. & Rolland, M.O. (1996) Evaluation of the presence of premature atherosclerosis in adults with heterozygosity for cystathionine-beta-synthase deficiency. *Stroke*, **27**, 1134-1136.

del Zoppo, G.J. (1998) The role of platelets in ischemic stroke. *Neurology*, **51**, S9-14.

Di Castelnuovo, A., de Gaetano G., Donati, M.B. & Iacoviello, L. (2001) Platelet glycoprotein receptor IIIa polymorphism $Pl^{A1/A2}$ and coronary risk: a meta-analysis. *Thrombosis & Haemostasis*, **85**, 626-633.

Diez-Tejedor E. & Fuentes B. (2001) Acute care in stroke: do stroke units make the difference? *Cerebrovascular Disease*, **11Suppl 1,** 31-9.

Deloughery, T.G., Evans, A., Sadeghi, A., McWilliams, J., Henner, W.D., Taylor, L.M., Jr & Press, R.D. (1996) Common mutation in methylenetetrahydrofolate reductase. Correlation with homocysteine metabolism and late-onset vascular disease. *Circulation*, **94**, 3074-3078.

Doggen C.J., Cats V.M., Bertina R.M. and Rosendaal F.R. (1998a) Interaction of coagulation defects and cardiovascular risk factors: increased risk of myocardial infarction associated with factor V Leiden or prothrombin 20210A. *Thrombosis and Haemostasis,* **97,** 1037-1041.

Doggen, C.J., Manger, C.V., Bertina, R.M., Reitsma, P.H., Vandenbroucke, J.P. & Rosendaal, F.R. (1998b) A genetic propensity to high factor VII is not associated with the risk of myocardial infarction in men. *Thrombosis & Haemostasis*, **80**, 281-285.

Doggen C.J., Bertina R.M., Cats V.M., Reitsma P.H. & Rosendaal F.R. (1999) The 4G/5G polymorphism in the plasminogen activator inhibitor-1 gene is not associated with myocardial infarction. *Thrombosis & Haemostasis,* **82,** 115-120.

Dougherty, J.H.J., Levy, D.E. & Weksler, B.B. (1977) Platelet activation in acute cerebral ischaemia. Serial measurements of platelet function in cerebrovascular disease. *Lancet*, **1**, 821-824.

Dougherty, J.H.J., Levy, D.E. & Weksler, B.B. (1979) Experimental cerebral ischemia produces platelet aggregates. *Neurology*, **29**, 1460-1465.

Dudman, N.P. (1999) An alternative view of homocysteine. *Lancet*, **354**, 2072-2074.

Durante-Mangoni, E., Davies, G.J., Ahmed, N., Ruggiero, G. & Tuddenham, E.G. (1998) Coronary thrombosis and the platelet glycoprotein IIIA gene PLA2 polymorphism. *Thrombosis & Haemostasis*, **80**, 218-219.

Dyker A.G., Weir C.J. and Lees K.R. (1997) Influence of cholesterol on survival after stroke: retrospective study. *British Medical Journal*, **314,**1584-1588.

Egerton, W., Silberberg, J., Crooks, R., Ray, C., Xie, L. & Dudman, N. (1996) Serial measures of plasma homocyst(e)ine after acute myocardial infarction. *American Journal of Cardiology*, **77**, 759-761.

Eikelboom, J.W., Baker, R.I., Parsons, R., Taylor, R.R. & van Bockxmeer, F.M. (1998) No association between the 20210 G/A prothrombin gene mutation and premature coronary artery disease. *Thrombosis & Haemostasis*, **80**, 878-880.

Eriksson, P., Kallin, B., van 't, H., Bavenholm, P. & Hamsten, A. (1995) Allele-specific increase in basal transcription of the plasminogen-activator inhibitor 1 gene is associated with myocardial infarction. *Proceedings of the National Academy of Science of U.S.A.*, **92**, 1851-1855.

Evans, R.W., Shaten, B.J., Hempel, J.D., Cutler, J.A. & Kuller, L.H. (1997) Homocyst(e)ine and risk of cardiovascular disease in the Multiple Risk Factor Intervention Trial. *Arteriosclerosis, Thrombosis & Vascular Biology*, **17**, 1947-1953.

Feng, D., Lindpaintner, K., Larson, M.G., Rao, V.S., O'Donnell, C.J., Lipinska, I., Schmitz, C., Sutherland, P.A., Silbershatz, H., D'Agostino, R.B., Muller, J.E., Myers, R.H., Levy, D. & Tofler, G.H. (1999) Increased platelet aggregability associated with platelet GPIIIa PlA2 polymorphism: the Framingham Offspring Study. *Arteriosclerosis Thrombosis & Vascular Biology*, **19**, 1142-1147.

Feng, D., Tofler, G.H., Larson, M.G., O'Donnell, C.J., Lipinska, I., Schmitz, C., Sutherland, P.A., Johnstone, M.T., Muller, J.E., D'Agostino, R.B., Levy, D. & Lindpaintner, K.(2000) Factor VII gene polymorphism, factor VII levels, and prevalent cardiovascular disease: the Framingham Heart Study. *Arteriosclerosis Thrombosis & Vascular Biology*, **20**, 593-600.

Ferraresi P., Marchetti G., Legnani C., Cavallari E., Castoldi E., Mascoli F., Ardissino D., Palareti G. and Bernardi F. (1997) The heterozygous 20210 G/A prothrombin genotype is associated with early venous thrombosis in inherited thrombophilias and is not increased in frequency in artery disease. *Arteriosclerosis Thrombosis & Vascular Biology,* **17,** 2418-2422.

Folsom, A.R., Wu, K.K., Shahar, E. & Davis, C.E. (1993) Association of hemostatic variables with prevalent cardiovascular disease and asymptomatic carotid artery atherosclerosis. The Atherosclerosis Risk in Communities (ARIC) Study Investigators. *Arteriosclerosis & Thrombosis*, **13**, 1829-1836.

Folsom, A.R., Nieto, F.J., McGovern, P.G., Tsai, M.Y., Malinow, M.R., Eckfeldt, J.H., Hess, D.L. & Davis, C.E. (1998) Prospective study of coronary heart disease incidence in relation to fasting total homocysteine, related genetic

polymorphisms, and B vitamins: the Atherosclerosis Risk in Communities (ARIC) study. *Circulation*, **98**, 204-210.

Franco, R.F., Trip, M.D., ten Cate, H., van den Ende, A., Prins, M.H., Kastelein, J.J. & Reitsma, P.H. (1999) The 20210 G-->A mutation in the 3'-untranslated region of the prothrombin gene and the risk for arterial thrombotic disease. *British Journal of Haematology*, **104**, 50-54.

Frosst, P., Blom, H.J., Milos, R., Goyette, P., Sheppard, C.A., Matthews, R.G., Boers, G.J., den Heijer, M., Kluijtmans, L.A. & van den Heuvel, L.P. (1995) A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. *Nature Genetics*, **10**, 111-113.

Garcia-Ribes, M., Gonzalez-Lamuno, D., Hernandez-Estefania, R., Colman, T., Pocovi, M., Delgado-Rodriguez, M., Garcia-Fuentes, M. & Revuelta, J.M. (1998) Polymorphism of the platelet glycoprotein IIIa gene in patients with coronary stenosis. *Thrombosis & Haemostasis*, **79**, 1126-1129.

Gardemann A., Schwartz O., Haberbosch W., Katz N., Weiss T., Tillmanns H., Hehrlein F.W., Waas W. and Eberbach B. (1997) Positive association of the beta fibrinogen H1/H2 gene variation to basal fibrinogen levels and to the increase in fibrinogen concentration during acute phase reaction but not to coronary artery disease and myocardial infarction. *Thrombosis & Haemostasis,* **77,** 1120-1126.

Gardemann, A., Humme, J., Stricker, J., Nguyen, Q.D., Katz, N., Philipp, M., Tillmanns, H., Hehrlein, F.W., Rau, M. & Haberbosch, W. (1998)

Association of the platelet glycoprotein IIIa PlA1/A2 gene polymorphism to coronary artery disease but not to nonfatal myocardial infarction in low risk patients. *Thrombosis & Haemostasis*, **80**, 214-217.

Gardemann, A., Arsic, T., Katz, N., Tillmanns, H., Hehrlein, F.W. & Haberbosch, W. (1999a) The factor II G20210A and factor V G1691A gene transitions and coronary heart disease. *Thrombosis & Haemostasis*, **81**, 208-213.

Gardemann A., Lohre J., Katz N., Tillmanns H., Hehrlein F.W. and Haberbosch W. (1999b) The 4G4G genotype of the plasminogen activator inhibitor 4G/5G gene polymorphism is associated with coronary atherosclerosis in patients at high risk for this disease. *Thrombosis & Haemostasis,* **82,** 1121-1126.

Gardemann, A., Weidemann, H., Philipp, M., Katz, N., Tillmanns, H., Hehrlein, FW & Haberbosch, W. (1999c) The TT genotype of the methylenetetrahydrofolate reductase C677T gene polymorphism is associated with the extent of coronary atherosclerosis in patients at high risk for coronary artery disease. *European Heart Journal*, **20**, 584-592.

Giles, W.H., Croft, J.B., Greenlund, K.J., Ford, E.S. & Kittner, S.J. (1998) Total homocyst(e)ine concentration and the likelihood of nonfatal stroke: results from the third national health and nutrition examination survey, 1988-1994. *Stroke*, **29**, 2473-2477.

Gonzalez-Conejero, R., Lozano, M.L., Rivera, J., Corral, J., Iniesta, J.A., Moraleda, J.M. & Vicente, V. (1998) Polymorphisms of platelet

membrane glycoprotein Ib associated with arterial thrombotic disease. *Blood*, **92**, 2771-2776.

Goodall, A.H., Curzen, N., Panesar, M., Hurd, C., Knight, C.J., Ouwehand, W.H. & Fox, K.M. (1999) Increased binding of fibrinogen to glycoprotein IIIa-proline33 (HPA-1b, PlA2, Zwb) positive platelets in patients with cardiovascular disease. *European Heart Journal*, **20**, 742-747.

Gowda M.S., Zucker M.L., Vacek J.L., Carriger W.L., Van Laeys D.L., Rachel J.M. and Strope B.D. (2000) Incidence of factor V Leiden in patients with acute myocardial infarction. *Journal of Thrombosis and Thrombolysis,* **9,** 43-45.

Graham, I.M., Daly, L.E., Refsum, H.M., Robinson, K., Brattstrom, L.E., Ueland, P.M., Palma-Reis, R.J., Boers, G.H., Sheahan, R.G., Israelsson, B., Uiterwaal, C.S., Meleady, R., McMaster, D., Verhoef, P., Witteman, J., Rubba, P., Bellet, H., Wautrecht, J.C., de Valk, H.W., Sales, L.A., Parrot-Rouland, F.M., Tan, K.S., Higgins, I., Garcon, D. & Andria, G. (1997) Plasma homocysteine as a risk factor for vascular disease. The European Concerted Action Project. *JAMA*, **277**, 1775-1781.

Green, F., Kelleher, C., Wilkes, H., Temple, A., Meade, T. & Humphries, S. (1991) A common genetic polymorphism associated with lower coagulation factor VII levels in healthy individuals. *Arteriosclerosis & Thrombosis*, **11**, 540-546.

Hankey, G.J. & Eikelboom, J.W. (1999) Homocysteine and vascular disease. *Lancet*, **354**, 407-413.

Harmon, D.L., Doyle, R.M., Meleady, R., Doyle, M., Shields, D.C., Barry, R., Coakley, D., Graham, I.M. & Whitehead, A.S. (1999) Genetic analysis of the thermolabile variant of 5, 10-methylenetetrahydrofolate reductase as a risk factor for ischemic stroke. *Arteriosclerosis, Thrombosis & Vascular Biology*, **19**, 208-211.

Harpel, P.C., Zhang, X. & Borth, W. (1996) Homocysteine and hemostasis: pathogenic mechanisms predisposing to thrombosis. *Journal of Nutrition*, **126**, 1285S-1289S.

Heinrich, J., Balleisen, L., Schulte, H., Assmann, G. & van de Loo, J. (1994) Fibrinogen and factor VII in the prediction of coronary risk. Results from the PROCAM study in healthy men. *Arteriosclerosis & Thrombosis*, **14**, 54-59.

Herrmann, S.M., Poirier, O., Marques-Vidal, P., Evans, A., Arveiler, D., Luc, G., Emmerich, J. & Cambien, F. (1997) The Leu33/Pro polymorphism (PlA1/PlA2) of the glycoprotein IIIa (GPIIIa) receptor is not related to myocardial infarction in the ECTIM Study. Etude Cas-Temoins de l'Infarctus du Myocarde. *Thrombosis & Haemostasis*, **77**, 1179-1181.

Herskind, A.M., McGue, M., Holm, N.V., Sorensen, T.I., Harvald, B. & Vaupel, J.W. (1996) The heritability of human longevity: a population-based study of 2872 Danish twin pairs born 1870-1900. *Human Genetics*, **97**, 319-323.

Heywood, D.M., Carter, A.M., Catto, A.J., Bamford, J.M. & Grant, P.J. (1997) Polymorphisms of the factor VII gene and circulating FVII:C levels in

relation to acute cerebrovascular disease and poststroke mortality. *Stroke*, **28**, 816-821.

Hoogendijk, E.M., Jenkins, C.S., van Wijk, E.M., Vos, J. & ten Cate, J.W. (1979) Spontaneous platelet aggregation in cerebrovascular disease II. Further characterisation of the platelet defect. *Thromb.Haemost.*, **41**, 512-522.

Iacoviello, L., Burzotta, F., Di Castelnuovo, A., Zito, F., Marchioli, R. & Donati, M.B. (1998a) The 4G/5G polymorphism of PAI-1 promoter gene and the risk of myocardial infarction: a meta-analysis. *Thrombosis & Haemostasis*, **80**, 1029-1030.

Iacoviello, L., Di Castelnuovo, A., de Knijff, P., D'Orazio, A., Amore, C., Arboretti, R., Kluft, C. & Benedetta, D.M. (1998b) Polymorphisms in the coagulation factor VII gene and the risk of myocardial infarction. *New England Journal of Medicine*, **338**, 79-85.

Iacoviello, L., Di Castelnuovo, A., D'Orazio, A. & Donati, B. (1999) Cigarette smoking doubles the risk of myocardial infarction in carriers of a protective polymorphism in the blood coagulation factor VII gene [letter]. *Thrombosis & Haemostasis*, **81**, 658.

Ito T., Ishida F., Shimodaira S. and Kitano K. (1999) Polymorphisms of platelet membrane glycoprotein Ib alpha and plasma von Willebrand factor antigen in coronary artery disease. *International Journal of Hematology,* **70,** 47-51.

Ivanova, R., Henon, N., Lepage, V., Charron, D., Vicaut, E. & Schachter, F. (1998) HLA-DR alleles display sex-dependent effects on survival and

discriminate between individual and familial longevity. *Human Molecular Genetics*, **7**, 187-194.

Iwai N., Shimoike H., Nakamura Y., Tamaki S. and Kinoshita M. (1998) The 4G/5G polymorphism of the plasminogen activator inhibitor gene is associated with the time course of progression to acute coronary syndromes. *Atherosclerosis,* **136,** 109-114.

Jacques, P.F., Bostom, A.G., Williams, R.R., Ellison, R.C., Eckfeldt, J.H., Rosenberg, I.H., Selhub, J. & Rozen, R. (1996) Relation between folate status, a common mutation in methylenetetrahydrofolate reductase, and plasma homocysteine concentrations. *Circulation*, **93**, 7-9.

Janes, S.L., Wilson, D.J., Chronos, N. & Goodall, A.H. (1993) Evaluation of whole blood flow cytometric detection of platelet bound fibrinogen on normal subjects and patients with activated platelets. *Thrombosis & Haemostasis*, **70**, 659-666.

Jin, Y., Dietz, H.C., Nurden, A. & Bray, P.F. (1993) Single-strand conformation polymorphism analysis is a rapid and effective method for the identification of mutations and polymorphisms in the gene for glycoprotein IIIa. *Blood*, **82**, 2281-2288.

Kastrati, A., Schomig, A., Seyfarth, M., Koch, W., Elezi, S., Bottiger, C., Mehilli, J., Schomig, K. & von Beckerath, N. (1999) PlA polymorphism of platelet glycoprotein IIIa and risk of restenosis after coronary stent placement. *Circulation*, **99**, 1005-1010.

Katagiri, Y., Hayashi, Y., Yamamoto, K., Tanoue, K., Kosaki, G. & Yamazaki, H. (1990) Localization of von Willebrand factor and thrombin-interactive domains on human platelet glycoprotein Ib. *Thrombosis & Haemostasis*, **63**, 122-126.

Keavney, B., Mckenzie, C., Parish, S., Palmer, A., Clark, S., Youngman, L., Delépine, M., Lathrop, M., Peto, R., Collins, R. & for the International Studies of Infarct Survival (ISIS) collaborators (2000) Large-scale test of hypothesised associations between the angiotensin-converting-enzyme insertion/deletion polymorphism and myocardial infarction in about 5000 cases and 6000 controls. *Lancet*, **355**, 434-442.

Kekomaki S., Hamaleinen L., Kappunen-Makelin R., Palomaki H., Kaste M. and Kontula K. (1999) Genetic polymorphism of platelet glycoprotein IIIa in patients with acute myocardial infarction and acute ischaemic stroke. *Journal of Cardiovascular Risk*, **6**, 13-17.

Kiely, D.K., Wolf, P.A., Cupples, L.A., Beiser, A.S. & Myers, R.H. (1993) Familial aggregation of stroke. The Framingham Study. *Stroke*, **24**, 1366-1371.

Kohler H.P., Stickland M.H., Ossei-Gerning N., Carter A., Mikkola H. and Grant P.J. (1998) Association of a common polymorphism in the factor XIII gene with myocardial infarction. *Thrombosis & Haemostasis*, **79**, 8-13.

Konstantopoulos, K., Grotta, J.C., Sills, C., Wu, K.K. & Hellums, J.D. (1995) Shear-induced platelet aggregation in normal subjects and stroke patients. *Thrombosis & Haemostasis*, **74**, 1329-1334.

Kuller, L.H. & Evans, R.W. (1998) Homocysteine, vitamins, and cardiovascular disease. *Circulation*, **98**, 196-199.

Kunicki, T.J., Orchekowski, R., Annis, D. & Honda, Y. (1993) Variability of integrin alpha 2 beta 1 activity on human platelets. *Blood*, **82**, 2693-2703.

Kunicki, T.J.,Kritzik M., Annis, D. & Nugent D.J. (1997) Hereditary variation in platelet integrin $alpha_2beta_1$ density is associated with two silent polymorphisms in the $alpha_2$ gene coding sequence. *Blood*, **89,** 1939-43.

Lane, A., Green, F., Scarabin, P.Y., Nicaud, V., Bara, L., Humphries, S., Evans, Luc, G., Cambou, J.P., Arveiler, D. & Cambien, F. (1996) Factor VII Arg/Gln353 polymorphism determines factor VII coagulant activity in patients with myocardial infarction (MI) and control subjects in Belfast and in France but is not a strong indicator of MI risk in the ECTIM study. *Atherosclerosis*, **119**, 119-127.

Lasne, D., Krenn, M., Pingault, V., Arnaud, E., Fiessinger, J.N., Aiach, M. & Rendu, F. (1997) Interdonor variability of platelet response to thrombin receptor activation: influence of PlA2 polymorphism. *British Journal of Haematology*, **99**, 801-807.

Laule, M., Cascorbi, I., Stangl, V., Bielecke, C., Wernecke, K.D., Mrozikiewicz, PM, Felix, S.B., Roots, I., Baumann, G. & Stangl, K. (1999) A1/A2 polymorphism of glycoprotein IIIa and association with excess procedural risk for coronary catheter interventions: a case-controlled study. *Lancet*, **353**, 708-712.

Lievers, K.J., Mennen, L.I., Rattink, A.P., Zwinderman, A.H., Jukema, J.W., Schouten, E.G. & de Maat, M.P. (2000) The -323Ins10 polymorphism for factor VII is not associated with coronary atherosclerosis in symptomatic men. The REGRESS study group. *Thrombosis Research*, **97**, 275-280.

Lindgren, A., Brattstrom, L., Norrving, B., Hultberg, B., Andersson, A. & Johansson, B.B. (1995) Plasma homocysteine in the acute and convalescent phases after stroke. *Stroke*, **26**, 795-800.

Longstreth, W.T.J., Rosendaal, F.R., Siscovick, D.S., Vos, H.L., Schwartz, S.M., Psaty, B.M., Raghunathan, T.E., Koepsell, T.D. & Reitsma, P.H. (1998) Risk of stroke in young women and two prothrombotic mutations: factor V Leiden and prothrombin gene variant (G20210A). *Stroke*, **29**, 577-580.

Ma, J., Stampfer, M.J., Hennekens, C.H., Frosst, P., Selhub, J., Horsford, J., Malinow, M.R., Willett, W.C. & Rozen, R. (1996) Methylenetetrahydrofolate reductase polymorphism, plasma folate, homocysteine, and risk of myocardial infarction in US physicians. *Circulation*, **94**, 2410-2416.

Malinow, M.R., Nieto, F.J., Szklo, M., Chambless, L.E. & Bond, G. (1993) Carotid artery intimal-medial wall thickening and plasma homocyst(e)ine in asymptomatic adults. The Atherosclerosis Risk in Communities Study. *Circulation*, **87**, 1107-1113.

Malinow, M.R., Nieto, F.J., Kruger, W.D., Duell, P.B., Hess, D.L., Gluckman, R.A., Block, P.C., Holzgang, C.R., Anderson, P.H., Seltzer, D., Upson, B. & Lin, Q.R. (1997) The effects of folic acid supplementation on plasma

total homocysteine are modulated by multivitamin use and methylenetetrahydrofolate reductase genotypes. *Arteriosclerosis, Thrombosis & Vascular Biology*, **17**, 1157-1162.

Malinow, M.R., Duell, P.B., Hess, D.L., Anderson, P.H., Kruger, W.D., Phillipson, B.E., Gluckman, R.A., Block, P.C. & Upson, B.M. (1998) Reduction of plasma homocyst(e)ine levels by breakfast cereal fortified with folic acid in patients with coronary heart disease. *New England Journal of Medicine*, **338**, 1009-1015.

Mamotte C.D., van Bockxmeer F.M. and Taylor R.R. (1998) Pla1/a2 polymorphism of glycoprotein IIIa and risk of coronary artery disease and restenosis following coronary angioplasty. *American Journal of Cardiology,* **82,** 13-16.

Mannucci, P.M., Mari, D., Merati, G., Peyvandi, F., Tagliabue, L., Sacchi, E., Taioli, E., Sansoni, P., Bertolini, S. & Franceschi, C. (1997) Gene polymorphisms predicting high plasma levels of coagulation and fibrinolysis proteins. A study in centenarians. *Arteriosclerosis, Thrombosis & Vascular Biology*, **17**, 755-759.

Mansfield M.W., Stickland M.H. and Grant P.J. (1995) Plasminogen activator inhibitor-1 (PAI-1) promoter polymorphism and coronary artery disease in non-insulin-dependent diabetes. *Thrombosis & Haemostasis,* **74,** 1032-1034.

Mari, D., Mannucci, P.M., Duca, F., Bertolini, S. & Franceschi, C. (1996) Mutant factor V (Arg506Gln) in healthy centenarians. *Lancet*, **347**, 1044

Marian, A.J., Brugada, R. & Kleiman, N.S. (1996) Platelet glycoprotein IIIa PlA polymorphism and myocardial infarction. *New England Journal of Medicine*, **335**, 1071-1072.

Marchetti, G., Gemmati, D., Patracchini, P., Pinotti, M. & Bernardi, F. (1991) PCR detection of a repeat polymorphism within the F7 gene. *Nucleic Acids Research*, **19**, 4570

Markus, H.S., Ali, N., Swaminathan, R., Sankaralingam, A., Molloy, J. & Powell, J. (1997) A common polymorphism in the methylenetetrahydrofolate reductase gene, homocysteine, and ischemic cerebrovascular disease. *Stroke*, **28**, 1739-1743.

Martin, J.F., Bath, P.M. & Burr, M.L. (1991) Influence of platelet size on outcome after myocardial infarction. *Lancet*, **338**, 1409-1411.

Mennen, L.I., de Maat, M.P., Schouten, E.G., Kluft, C., de Jong, P.T., Hofman, A. & Grobbee, D.E. (1997) Coagulation factor VII, serum-triglycerides and the R/Q353 polymorphism: differences between older men and women. *Thrombosis & Haemostasis.*, **78**, 984-986.

Michelson, A.D. (1996) Flow cytometry: a clinical test of platelet function. *Blood*, **87**, 4925-4936.

Michelson, A.D., Furman, M.I., Goldschmidt-Clermont, P., Mascelli, M.A., Hendrix, C., Coleman, L., Hamlington, J., Barnard, M.R., Kickler, T., Christie, D.J., Kundu, S. & Bray, P.F. (2000) Platelet GP IIIa Pl(A) polymorphisms display different sensitivities to agonists. *Circulation,* **101**, 1013-1018.

Mikkelsson, J., Perola, M., Laippala, P., Savolainen, V., Pajarinen, J., Lalu, K., Penttila, A. & Karhunen, P.J. (1999) Glycoprotein IIIa Pl(A) polymorphism associates with progression of coronary artery disease and with myocardial infarction in an autopsy series of middle-aged men who died suddenly. *Arteriosclerosis, Thrombosis & Vascular Biology*, **19**, 2573-2578.

Moshfegh, K., Wuillemin, W.A., Redondo, M., Lammle, B., Beer, J.H., Liechti-Gallati, S. & Meyer, B.J. (1999) Association of two silent polymorphisms of platelet glycoprotein Ia/IIa receptor with risk of myocardial infarction: a case-control study. *Lancet*, **353**, 351-354.

Mudd, S.H., Skovby, F., Levy, H.L., Pettigrew, K.D., Wilcken, B., Pyeritz, R.E., Andria, G., Boers, G.H., Bromberg, I.L. & Cerone, R. (1985) The natural history of homocystinuria due to cystathionine beta-synthase deficiency. *American Journal of Human Genetics*, **37**, 1-31.

Murata, M., Matsubara, Y., Kawano, K., Zama, T., Aoki, N., Yoshino, H., Watanabe, Ishikawa, K. & Ikeda, Y. (1997) Coronary artery disease and polymorphisms in a receptor mediating shear stress-dependent platelet activation. *Circulation*, **96**, 3281-3286.

Nappo, F., De Rosa, N., Marfella, R., De Lucia, D., Ingrosso, D., Perna, A.F., Farzati, B. & Giugliano, D. (1999) Impairment of endothelial functions by acute hyperhomocysteinemia and reversal by antioxidant vitamins. *JAMA*, **281**, 2113-2118.

Newman, P.J., Derbes, R.S. & Aster, R.H. (1989) The human platelet alloantigens, PlA1 and PlA2, are associated with a leucine33/proline33 amino acid polymorphism in membrane glycoprotein IIIa, and are distinguishable by DNA typing. *Journal of Clinical Investigation*, **83**, 1778-1781.

Nishiuma S., Kario K., Yakuijin K., Maeda M., Murai R., Matsuo T., Ikeda U., Shimada K. and Matsuo M. (1998) Genetic variation in the promoter region of the beta-fibrinogen gene is associated with ischemic stroke in a Japanese population. *Blood Coagulation and Fibrinolysis,* **9,** 373-379.

Nurden, A.T. (1995) Polymorphisms of human platelet membrane glycoproteins: structure and clinical significance. *Thrombosis & Haemostasis*, **74**, 345-351.

Nurden, A.T. (1997) Platelet glycoprotein IIIa polymorphism and coronary thrombosis. *Lancet* , **350**, 1189-1191.

Nygard, O., Vollset, S.E., Refsum, H., Stensvold, I., Tverdal, A., Nordrehaug, JE, Ueland, M. & Kvale, G. (1995) Total plasma homocysteine and cardiovascular risk profile. The Hordaland Homocysteine Study. *JAMA*, **274**, 1526-1533.

Osborn, S.V., Hampton, K.K., Smillie, D., Channer, K.S. & Daly, M.E. (1996) Platelet glycoprotein IIIa gene polymorphism and myocardial infarction. *Lancet*, **348**, 1309-1310.

Perry, I.J., Refsum, H., Morris, R.W., Ebrahim, S.B., Ueland, P.M. & Shaper, A.G. (1995) Prospective study of serum total homocysteine concentration and risk of stroke in middle-aged British men. *Lancet*, **346**, 1395-1398.

Peyvandi, F., Mannucci, P.M., Bucciarelli, P., Zeinali, S., Akhavan, S., Sacchi, E., Merlini, P.A. & Perry, D.J. (2000) A novel polymorphism in intron 1a of the human factor VII gene (G73A): study of a healthy Italian population and of 190 young survivors of myocardial infarction. *British Journal of Haematolo*gy, **108**, 247-253.

Poort S.R., Rosendaal F.R., Reitsma P.H. and Bertina R.M. (1996) A common genetic variation in the 3'-untranslated region of the prothrombin gene is associated with elevated plasma prothrombin levels and an increase in venous thrombosis. *Blood,* **88,** 3698-3703.

Powell, J.T. (1998) Vascular damage from smoking: disease mechanisms at the arterial wall. *Vascular Medicine*, **3**, 21-28.

Pullicino, P., Greenberg, S. & Trevisan, M. (1997) Genetic stroke risk factors. *Current Opinion in Neurology*, **10**, 58-63.

Rees, D.C., Liu, Y.T., Cox, M.J., Elliott, P. & Wainscoat, J.S. (1997) Factor V Leiden and thermolabile methylenetetrahydrofolate reductase in extreme old age. *Thrombosis & Haemostasis*, **78**, 1357-1359.

Reuner, K.H., Ruf, A., Kaps, M., Druschky, K.F. & Patscheke, H. (1998) The mutation C677-->T in the methylene tetrahydrofolate reductase gene and stroke. *Thrombosis & Haemostasis*, **79**, 450-451.

Ridker P.M., Hennekens C.H., Lindpainter K., Stampfer M.J., Eisenberg P.R. and Miletich J.P. (1995) Mutation in the gene coding for coagulation factor V and the risk of myocardial infarction, stroke and venous thrombosis in apparently healthy men. *New England Journal of Medicine,* **332,** 912-917.

Ridker, P.M., Cushman, M., Stampfer, M.J., Tracy, R.P. & Hennekens, C.H. (1997a) Inflammation, aspirin, and the risk of cardiovascular disease in apparently healthy men. *New England Journal of Medicine*, **336**, 973-979.

Ridker, P.M., Hennekens, C.H., Lindpaintner, K., Stampfer, M.J. & Miletich, J.P. (1997b) Arterial and venous thrombosis is not associated with the 4G/5G polymorphism in the promoter of the plasminogen activator inhibitor gene in a large cohort of US men. *Circulation*, **95**, 59-62.

Ridker, P.M., Hennekens, C.H., Schmitz, C., Stampfer, M.J. & Lindpaintner, K. (1997c) PIA1/A2 polymorphism of platelet glycoprotein IIIa and risks of myocardial infarction, stroke, and venous thrombosis. *Lancet*, **349**, 385-388.

Ridker, P.M. & Stampfer, M.J. (1999a) Assessment of genetic markers for coronary thrombosis: promise and precaution. *Lancet*, **353**, 687-688.

Ridker, P.M., Hennekens, C.H. & Miletich, J.P. (1999b) G20210A mutation in prothrombin gene and risk of myocardial infarction, stroke, and venous thrombosis in a large cohort of US men. *Circulation*, **99**, 999-1004.

Robertson, R.M., Robertson, D., Friesinger, G.C., Timmons, S. & Hawiger, J. (1980) Platelet aggregates in peripheral and coronary-sinus blood in patients with spontaneous coronary-artery spasm. *Lancet*, **2**, 829-831.

Rosendaal, F.R. (1997a) Thrombosis in the young: epidemiology and risk factors. A focus on venous thrombosis. *Thrombosis & Haemostasis*, **78**, 1-6.

Rosendaal, F.R., Siscovick, D.S., Schwartz, S.M., Beverly, R.K., Psaty, B.M., Longstreth, W.T.J., Raghunathan, T.E., Koepsell, T.D. & Reitsma, P.H. (1997b) Factor V Leiden (resistance to activated protein C) increases the risk of myocardial infarction in young women. *Blood*, **89**, 2817-2821.

Rosendaal, F.R., Siscovick, D.S., Schwartz, S.M., Psaty, B.M., Raghunathan, T.E. & Vos, H.L. (1997c) A common prothrombin variant (20210 G to A) increases the risk of myocardial infarction in young women. *Blood*, **90**, 1747-1750.

Ross, R. (1999) Atherosclerosis--an inflammatory disease. *N.ew England Journal of Medicine*, **340**, 115-126.

Ruddock, V. & Meade, T.W. (1994) Factor-VII activity and ischaemic heart disease: fatal and non-fatal events. *QJM*, **87**, 403-406.

Samani, N.J. & Lodwick, D. (1997) Glycoprotein IIIa polymorphism and risk of myocardial infarction. *Cardiovascular Research*, **33**, 693-697.

Santoso S., Kunicki T.J., Kroll H., Haberbosch W. and Gardemann A. (1999) Association of the platelet glycoprotein Ia C807T polymorphism with nonfatal myocardial infarction in younger patients. *Blood,* **93,** 2449-2453.

Scaglione L., Bergerone S., Gaschino G., Imazio M., Maccagnani A., Gambino R., Cassader M., DiLeo M., Macchia G., Brusca A., Pagnano G. and Cavallo-Perin P. (1998) Lack of relationship between the PlA1/PlA2

polymorphism of platelet glycoprotein IIIa and premature myocardial infarction. *European Journal of Clinical Investigation,* **28,** 385-388.

Schachter, F., Faure-Delanef, L., Guenot, F., Rouger, H., Froguel, P., Lesueur-Ginot, L. & Cohen, D. (1994) Genetic associations with human longevity at the APOE and ACE loci. *Nature Genetics*, **6**, 29-32.

Schmidt, H., Schmidt, R., Niederkorn, K., Horner, S., Becsagh, P., Reinhart, B., Schumacher, M., Weinrauch, V. & Kostner, G.M. (1998) Beta-fibrinogen gene polymorphism (C148-->T) is associated with carotid atherosclerosis: results of the Austrian Stroke Prevention Study. *Arteriosclerosis, Thrombosis & Vascular Biology*, **18**, 487-492.

Scottish Health Statistics. (2000) Information and Statistics Division. National Health Service in Scotland Website.

Selhub, J., Jacques, P.F., Wilson, P.W., Rush, D. & Rosenberg, I.H. (1993) Vitamin status and intake as primary determinants of homocysteinemia in an elderly population. *JAMA*, **270**, 2693-2698.

Selhub, J., Jacques, P.F., Bostom, A.G., D'Agostino, R.B., Wilson, P.W., Belanger, AJ, O'Leary, D.H., Wolf, P.A., Schaefer, E.J. & Rosenberg, I.H. (1995) Association between plasma homocysteine concentrations and extracranial carotid-artery stenosis. *New England Journal of Medicine*, **332**, 286-291.

Selhub, J., Jacques, P.F., Bostom, A.G., D'Agostino, R.B., Wilson, P.W., Belanger, AJ, O'Leary, D.H., Wolf, P.A., Rush, D., Schaefer, E.J. & Rosenberg, I.H. (1996) Relationship between plasma homocysteine,

vitamin status and extracranial carotid-artery stenosis in the Framingham Study population. *Journal of Nutrition*, **126**, 1258S-1265S.

Shah, A.B., Beamer, N. & Coull, B.M. (1985) Enhanced in vivo platelet activation in subtypes of ischemic stroke. *Stroke*, **16**, 643-647.

Shattil, S.J. (1999) Signaling through platelet integrin alpha IIb beta 3: inside-out, outside-in, and sideways. *Thrombosis & Haemostasis*, **82**, 318-325.

Sonoda, A., Murata, M., Ito, D., Tanahashi, N., Ohta, A., Tada, Y., Takeshita, E., Yoshida, T., Saito, I., Yamamoto, M., Ikeda, Y., Fukuuchi, Y. & Watanabe, K. (2000) Association between platelet glycoprotein Iba genotype and ischemic cerebrovascular disease. *Stroke*, **31**, 493-497.

Sosef, M.N., Bosch, J.G., van Oostayen, J., Visser, T., Reiber, J.H. & Rosendaal, F.R. (1994) Relation of plasma coagulation factor VII and fibrinogen to carotid artery intima-media thickness. *Thrombosis & Haemostasis*, **72**, 250-254.

Stampfer, M.J., Malinow, M.R., Willett, W.C., Newcomer, L.M., Upson, B., Ullmann, D., Tishler, P.V. & Hennekens, C.H. (1992) A prospective study of plasma homocyst(e)ine and risk of myocardial infarction in US physicians. *JAMA*, **268**, 877-881.

Stolzenberg-Solomon, R.Z., Miller, E.R., Maguire, M.G., Selhub, J. & Appel, L.J. (1999) Association of dietary protein intake and coffee consumption with serum homocysteine concentrations in an older population. *American Journal of Clinical Nutrition*, **69**, 467-475.

Taubman, M.B., Fallon, J.T., Schecter, A.D., Giesen, P., Mendlowitz, M., Fyfe, B.S., Marmur, J.D. & Nemerson, Y. (1997) Tissue factor in the pathogenesis of atherosclerosis. *Thrombosis & Haemostasis*, **78**, 200-204.

Tracy, R.P., Arnold, A.M., Ettinger, W., Fried, L., Meilahn, E. & Savage, P. (1999) The relationship of fibrinogen and factors VII and VIII to incident cardiovascular disease and death in the elderly: results from the cardiovascular health study. *Arteriosclerosis Thrombosis & Vascular Biology*, **19**, 1776-1783.

Trip, M.D., Cats, V.M., van Capelle, F.J. & Vreeken, J. (1990) Platelet hyperreactivity and prognosis in survivors of myocardial infarction. *New England Journal of Medicine*, **322**, 1549-1554.

Trowbridge, E.A., Reardon, D.M., Hutchinson, D. & Pickering, C. (1985) The routine measurement of platelet volume: a comparison of light-scattering and aperture-impedance technologies. *Clinical Physics & Physiological Measurement*, **6**, 221-238.

Tyjbaerg-Hansen A., Agerholm-Larsen B., Humphries S.E, Abildgaard S., Schnohr P. and Nordestgaard B.G. (1997) A common mutation (G-455A) in the beta-fibrinogen promoter is an independent predictor of plasma fibrinogen, but not of ischaemic heart disease. A study of 9,127 individuals based on the Copenhagen City Heart Study. *Journal of Clinical Investigation,* **99,** 3.34-3039.

Ubbink, J.B., Hayward, V.W. & Bissbort, S. (1991) Rapid high-performance liquid chromatographic assay for total homocysteine levels in human serum. *Journal of Chromatography*, **565**, 441-446.

Ueland, P.M., Refsum, H., Stabler, S.P., Malinow, M.R., Andersson, A. & Allen, R.H. (1993) Total homocysteine in plasma or serum: methods and clinical applications. *Clinical Chemistry*, **39**, 1764-1779.

van Bockxmeer, F.M., Mamotte, C.D., Vasikaran, S.D. & Taylor, R.R. (1997) Methylenetetrahydrofolate reductase gene and coronary artery disease. *Circulation*, **95**, 21-23.

van Kooten, F., Ciabattoni, G., Koudstaal, P.J., Dippel, D.W. & Patrono, C. (1999) Increased platelet activation in the chronic phase after cerebral ischemia and intracerebral hemorrhage. *Stroke*, **30**, 546-549.

van Kooten, F., Ciabattoni, G., Patrono, C., Dippel, D.W. & Koudstaal, P.J. (1997) Platelet activation and lipid peroxidation in patients with acute ischemic stroke. *Stroke*, **28**, 1557-1563.

Verhaar, M.C., Wever, R.M., Kastelein, J.J., van Dam, T., Koomans, H.A., Rabelink & TJ (1998) 5-methyltetrahydrofolate, the active form of folic acid, restores endothelial function in familial hypercholesterolemia. *Circulation*, **97**, 237-241.

Verhoef, P., Hennekens, C.H., Malinow, M.R., Kok, F.J., Willett, W.C. & Stampfer, M.J. (1994) A prospective study of plasma homocyst(e)ine and risk of ischemic stroke. *Stroke*, **25**, 1924-1930.

Wagner, K.R., Giles, W.H., Johnson, C.J., Ou, C.Y., Bray, P.F., Goldschmidt, C., PJ, Croft, J.B., Brown, V.K., Stern, B.J., Feeser, B.R., Buchholz, D.W., Earley, C.J., Macko, R.F., McCarter, R.J., Sloan, M.A., Stolley, P.D., Wityk, R.J., Wozniak, M.A., Price, T.R. & Kittner, S.J. (1998) Platelet glycoprotein receptor IIIa polymorphism P1A2 and ischemic stroke risk: the Stroke Prevention in Young Women Study. *Stroke*, **29** , 581-585.

Wald, N.J., Watt, H.C., Law, M.R., Weir, D.G., McPartlin, J. & Scott, J.M. (1998) Homocysteine and ischemic heart disease: results of a prospective study with implications regarding prevention. *Archives of Internal Medicine*, **158**, 862-867.

Walter, D.H., Schachinger, V., Elsner, M., Dimmeler, S. & Zeiher, A.M. (1997) Platelet glycoprotein IIIa polymorphisms and risk of coronary stent thrombosis. *Lancet*, **350**, 1217-1219.

Wang X.L., Wang J., McCredie R.M. and Wilcken D.E. (1997) Polymorphisms of factor V, factor VII, and fibrinogen genes. Relevance to severity of coronary artery disease. *Arteriosclerosis Thrombosis & Vascular Biology,* **17,** 246-251.

Warkentin, T.E., Powling, M.J. & Hardisty, R.M. (1990) Measurement of fibrinogen binding to platelets in whole blood by flow cytometry: a micromethod for the detection of platelet activation. *British Journal of Haematology*, **76**, 387-394.

Wartiovaara U., Perola M., Mikkola H., Totterman K., Savolainen V., Pentilla A., Grant P.J., Tikkanen M.J., Vartiainen E., Karhunen P.J., Peltonen L. and

Palotie A. (1999) Association of FXIII Val34Leu with decreased risk of myocardial infarction in Finnish males. *Atherosclerosis,* **142,** 295-300.

Warlow, C.P. (1996) *Oxford Textbook of Medicine* (ed. by D.J. Weatherall, J.G.G. Ledingham and D.A. Warrell), p.3947. Oxford University Press.

Weiss, E.J., Bray, P.F., Tayback, M., Schulman, S.P., Kickler, T.S., Becker, L.C., Weiss, J.L., Gerstenblith, G. & Goldschmidt-Clermont, P.J. (1996) A polymorphism of a platelet glycoprotein receptor as an inherited risk factor for coronary thrombosis. *New England Journal of Medicine*, **334**, 1090-1094.

Wu, K.K., Folsom, A.R., Heiss, G., Davis, C.E., Conlan, M.G. & Barnes, R. (1992) Association of coagulation factors and inhibitors with carotid artery atherosclerosis. Early results of the Atherosclerosis Risk in Communities (ARIC) Study. *Annals of Epidemiology*, **2**, 471-480.

Wu, K.K. (1996) Platelet activation mechanisms and markers in arterial thrombosis. *Journal of Internal Medicine*, **239**, 17-34.

Ye, S., Green, F.R., Scarabin, P.Y., Nicaud, V., Bara, L., Dawson, S.J., Humphries, S.E., Evans, A., Luc, G. & Cambou, J.P. (1995) The 4G/5G genetic polymorphism in the promoter of the plasminogen activator inhibitor-1 (PAI-1) gene is associated with differences in plasma PAI-1 activity but not with risk of myocardial infarction in the ECTIM study. *Thrombosis & Haemostasis*, **74**, 837-841.

Yoo, J.H., Chung, C.S. & Kang, S.S. (1998) Relation of plasma homocyst(e)ine to cerebral infarction and cerebral atherosclerosis. *Stroke*, **29**, 2478-2483.

Yu, Q., Safavi, F., Roberts, R. & Marian, A.J. (1996) A variant of beta fibrinogen is a genetic risk factor for coronary artery disease and myocardial infarction. *Journal of Investigative Medicine*, **44** , 154-159.

Zeller, J.A., Tschoepe, D. & Kessler, C. (1999) Circulating platelets show increased activation in patients with acute cerebral ischemia. *Thrombosis & Haemostasis*, **81**, 373-377.

Zito F., Di Castelnuovo A., D'Orazio A., Negrini R., De Lucia D., Donati M.B. and Iacoviello L. (1999) Helicobacter pylori infection and the risk of myocardial infarction: role of fibrinogen and its genetic control. *Thrombosis & Haemostasis,* **82,** 14-18.

Zotz, R.B., Winkelmann, B.R., Nauck, M., Giers, G., Maruhn-Debowski, B., Marz, W. & Scharf, R.E. (1998) Polymorphism of platelet membrane glycoprotein IIIa: human platelet antigen 1b (HPA-1b/PlA2) is an inherited risk factor for premature myocardial infarction in coronary artery disease. *Thrombosis & Haemostasis*, **79**, 731-735.

APPENDICES

Appendix I. Reagents

i) Extraction of DNA from leucocytes

- Nucleon BACC 2 DNA extraction kit (Nucleon Biosciences, Coatbridge, UK). Contains:
    - ♦ Reagent A (red cell lysis solution) 420ml; 10mM Tris-HCL, 320mM Sucrose, 5mM $MgCl_2.6H_2O$, 1% Triton X-100.
    - ♦ b) Reagent B 110ml.
    - ♦ c) Sodium Perchlorate 26ml.
    - ♦ d) Nucleon Resin 16ml.

- 100% Chloroform (BDH Laboratory Supplies, Poole, UK).

- 100% Ethanol (BDH Laboratory Supplies, Poole, UK). Diluted to 70% by adding 70ml to 30ml H_2O.

- 1M Tris. 121.1g Tris (2-Amino-2-(hydroxymethyl)-1,3-propanediol, Boehringer, Mannheim, Germany) added to 1L H_2O, adjusted to pH 8.0 and stored at 4 °C.

- 10mM Tris/EDTA (TE). 10 ml of 1M Tris added to 20ml of 0.5M EDTA (18.612g ethylene diaminetetrahydroacetic acid, BDH Laboratory Supplies, Poole, UK in 100ml dH_2O). Adjusted to pH8.0 and stored at 4 °C.

ii) HPA 1a/1b and 2a/2b genotyping by SSP-PCR.

- *Thermus aquaticus* (*Taq*) DNA polymerase 5 units /µl: (Appligene Oncor, a division of Perkin Elmer, Foster City, California, USA).

- dNTPs: 2.5mM each of dATP, dTTP, dGTP and dCTP (Applied Biosystems, Warrington, UK).

- NW3 PCR reaction Buffer consisted of: 160mM$(NH_4)_2SO_4$; 1.5mM $MgCl_2$; 670 mM Tris-HCl pH 8.8/0.1% w/v Tween 20 (purchased from University of Bristol, Department of Transplantation, Bristol, UK).

- Primer mix: contained 1µl each of allele specific primer (3.5-5.0mM) and control primer (0.4-2.0mM). Kindly supplied by G. Cavanagh, National Blood Service, Newcastle.

- Cresol Red dissolved in dH_20, 10 mg/ml (Sigma Chemical Co., St Louis, USA).

- 1.5% Agarose gel: 1.5g Agarose (Appligene Oncor, Foster City, California, USA) dissolved in 100 ml of TBE.

- TBE. A 10x stock solution was made as follows: 108g Tris ((Amresco, Solon, Ohio, USA), 55g Boric acid (Amresco, Solon, Ohio, USA) and 40 ml of 0.5M EDTA (BDH Laboratory Supplies, Poole, UK), pH 8.0 were dissolved in

200ml of dH_2O and made up to a final volume of 1000ml. Working dilution prepared by the addition of 500ml to 4500ml dH_2O.

- Gene ruler 100bp DNA Ladder (MBI Fermentas, Vilnius, Lithuania).

iii) HPA 1a/1b genotyping by PCR and restriction enzyme digest, and Factor VII R353Q and IVS7 genotyping.

- Oligonucleotides, diluted to 10pmol/µl. Synthesised by Oswel Ltd, Southampton, UK.

- dNTP's. 2x500µl containing 12.5mM each of dATP, dCTP, dGTP and dTTP (Bioline, London, UK).

- 10x Reaction Buffer. Contains 1ml of 100mM Tris-HCl (pH 8.8 at 25 °C), 500mM KCl and 0.8% Nonidet P40 (MBI Fermentas, Vilnius, Lithuania).

- $MgCl_2$. Contains 1ml of 25mM $MgCl_2$ (MBI Fermentas, Vilnius, Lithuania).
- *Thermus aquaticus* (*Taq)* DNA polymerase. 5 units/µl (MBI Fermentas, Vilnius, Lithuania).

- *MSP 1 (HPA II)* restriction endonuclease, 10 units/µl (MBI Fermentas, Vilnius, Lithuania).

- Buffer Y^+/Tango restriction enzyme buffer (MBI Fermentas, Vilnius, Lithuania). Contains 1ml of 33mM Tris-acetate, 10mM magnesium acetate, 66mM potassium acetate and 0.1mg/ml bovine serum albumin (pH7.9 at 37°C).

- Agarose and TBE as described above.

- 6x Loading dye solution (MBI Fermentas, Vilnius, Lithuania). Contains bromophenol blue and xylene cyanol, diluted 1:6 in dH_2O prior to use.

- Gene ruler™ 100 base pair DNA Ladder (MBI Fermentas, Vilnius, Lithuania).

iv) Platelet activation markers by whole blood flow cytometry.

- Na_3Citrate 3.2%. 3.2g (Trisodium citrate, BDH Laboratory supplies, Poole, UK) were dissolved in 100ml dH_2O. One ml aliquots stored at 4°C.

- HEPES-Mg buffer. The following are dissolved in H_2O to a final volume of 100ml and adjusted to pH 7.4:

  -NaCl 0.847g (Sodium chloride, BDH Laboratory Supplies).

  -KCl 0.037g (Potassium chloride, BDH Laboratory Supplies).

  -$MgSO_4.7H_2O$ 0.025g (Magnesium sulphate, BDH Laboratory Supplies).

  -HEPES 0.260g (N-2-hydroxyethyl piperazine-N-2-ethane sulphonic acid, monosodium salt, BDH Laboratory Supplies).

- The solution was filtered and stored in eppendorfs containing 450μl at 4°C.

- Phosphate buffered saline (PBS). The following were dissolved in dH_2O to final volume of 100ml and adjusted to pH 7.4:

  -$NaH_2PO_4.2H_2O$ 0.035g (Sodium dihydrogen orthophosphate, Fisher Scientific Ltd, Loughborough, UK).

  -Na_2HPO_4anhydrous 0.107g (diSodium hydrogen orthophosphate anhydrous, BDH Laboratory Supplies, Poole, UK).

  -NaCl 0.850g (Sodium Chloride, BDH Laboratory Supplies, Poole, UK).

- The solution was filtered and stored at 4°C. The solution was replaced weekly.

- ADP. (Adenosine diphosphate, Sigma, St. Louis, USA). 1mM solution was diluted 1:10 by adding 5µl to 45µl of PBS.
- PMA (phorbol 12-myristate 13-acetate, Sigma, St Louis, USA). Dissolved 1mg/ml in DMSO(dimethyl sulfoxide, Sigma, St Louis, USA).One ml aliquots stored at -20°C prior to use. This was diluted 1:100 by adding 5µl to 495µl PBS.
- FITC (fluorescein isothyocyanate) conjugated murine anti-Keyhole limper hemocyanin (KLH) IgG_1 isotype control (Becton Dickinson, California, USA). 50µg / ml. IgG. 1:25 dilution to yield 2ng/µl final concentration.

- PE (phycoerythrin) conjugated mouse anti-KLH IgG_1 isotype control (Becton Dickinson , California, USA). 50µg/ml IgG. 1:25 dilution to yield 2ng/µl final concentration.

- FITC conjugated monoclonal mouse antiCD61 (GPIIIa) antibody (Dako, Glostrup, Denmark). 100µg/ml. 1:50 dilution to yield 2ng/µl final concentration.

- PE conjugated monoclonal mouse anti-CD62P (P-Selectin) antibody (Immonotech, Marseilles, France). 40µg/ml. 1:20 dilution to yield 2ng/µl final concentration.

- FITC conjugated polyclonal rabbit antifibrinogen antibody (Dako, Glostrup, Denmark). 100µg/ml. 1:50 dilution to yield 2ng/µl final concentration.

- Flowcheck™ fluorospheres (Coulter, Luton, UK). Fluorescent microspheres for assessment of optical alignment of fluidics. HPCV(half peak coefficient of variation) of <3% is considered acceptable for assay of cell surface markers.

- Immunobrite beads. EPICS Immuno-brite Standards Kit (Coulter, Luton, UK). Five vials of fluorospheres of known intensity:

  I) Blank

  II) Medium-low

  III) Medium-reference

  IV) Medium-high

V) Brite

Run each day to determine voltage settings required to attain identical fluorescence. In practice this did not require adjustment.

v) Total plasma Homocyst(e)ine by reverse phase HPLC and fluorescent detection.

- QC plasma. Pooled normal plasma obtained from healthy volunteers and stored at -20 °C in 200μl aliquots for single use.

- L-Homocystine standard (Sigma, St Louis, USA). 67.6mg dissolved in 100ml dH_2O to a concentration of 50μM. 20μl in 100μl yields a final concentration of 10μM.

- 10% TBP. Tri-n-butylphosphine 0.81g/ml (Sigma, St Louis, USA) 0.1ml added to 0.9ml dimethylformamide 0.94g/ml (Sigma, St Louis, USA).

- 10% TCA. 10g trichloroacetic acid (BDH Laboratory Supplies, Poole, UK) dissolved in 100ml dH_2O.

- SBD-F (ammonium-7-fluoro-3,1,3-benzoxadiazole sulphonate, Fluka, New-Ulm, Switzerland). 0.5mg added to 1ml of borate buffer, thoroughly mixed and stored in the dark, as light sensitive. Prepared daily.

- 0.1M Borate buffer. One litre made by dissolving 38.14g disodium tetrahydroborate (BDH Laboratory Supplies, Poole, UK) and 0.74g ethylene diaminetetra-acetic acid (BDH Laboratory Supplies, Poole, UK) in 1000ml of dH_2O.

- 3M NaOH. 6g sodium hydroxide (BDH Laboratory Supplies, Poole, UK) in 50ml dH_2O.

- Orthophosphoric acid 85% solution, 'HiPerSolv for HPLC™ (BDH Laboratory Supplies, Poole, UK).

- Mobile phase: 7% ACN, acetonitrile (Rathburn Chemicals Ltd, Walkerburn, Scotland, UK)70g in 1000ml 25mM Potassium dihydrogen phosphate buffer (BDH Laboratory Supplies, Poole UK). To make up 2L of mobile phase: 6.8g buffer in 1840ml dH_2O, plus 140ml ACN. Adjusted to pH 2.2 by the addition of 20ml neat orthophosphoric acid.

Appendix II

PATIENT INFORMATION SHEET

Homocysteine is a chemical in the blood (an amino acid) which, when raised, is associated with narrowing of the arteries (atherosclerosis). This can cause such illnesses as 'heart attacks' (myocardial infarctions) and 'strokes' (cerebrovascular accidents).

Platelets are the cells in the blood which help it to clot when an injury occurs. It is thought that abnormal platelets may be involved in inappropriate clotting, resulting in heart attacks or strokes.

The purpose of this study is to investigate the role of these two mechanisms in the causation of strokes.

This will involve taking a sample of your blood to measure homocysteine levels and to look at whether the platelets are working normally. We will also investigate why homocysteine is raised. This may be due to your diet or to an inherited predisposition. A sample of your DNA will be extracted from the blood to look at this.

The samples will all be taken together after you have fasted from midnight the night before. They will be taken at 9am so that you do not have to wait long for breakfast.

In order for the results to make sense we also need to do the same tests on a control who has not had a stroke. It would be very helpful if you could nominate a friend or relative of the same sex and roughly the same age who would be willing to have this done. It would involve them attending hospital in the morning after fasting from midnight to have some blood taken.

It will also be necessary to repeat the tests after 3 months and you will be sent an appointment to attend the outpatient clinic for this.

All the tests are done in an anonymous fashion and are completely confidential. If you refuse to take part it will in no way influence your treatment in hospital.

Dr D Meiklejohn. June 1997

VOLUNTEER CONTROL INFORMATION SHEET

Thank you for considering giving a blood sample as a control sample for the study investigating the causes of stroke.

We are investigating the role a chemical in the blood called homocysteine has in the cause of strokes, since it has been suggested that raised homocysteine levels can cause narrowing of arteries (atherosclerosis). In addition we are interested in looking at platelets. These are the cells in the blood that help it to clot after an injury. It is thought that they may be overactive in patients with a stroke causing inappropriate blood clotting.

We are interested in assessing if these features have a role in patients in Aberdeen, and are taking blood from newly diagnosed stroke victims at A.R.I.. In order to assess the results properly it is necessary to test the blood of someone who has not suffered a stroke.

If you agree to this a sample of approximately 30 ml of blood will be taken from you in the morning at the clinic, after you have fasted from midnight and before you have your breakfast. This will be as soon as possible after you arrive so that you don't have to wait too long before eating. This will be used to extract DNA and to check vitamin and homocysteine levels.

The blood tests are done anonymously and the results are kept strictly confidential. You will also be asked to fill in a questionnaire about your eating habits.

Dr D Meiklejohn June 1997.

CONSENT BY PATIENT/VOLUNTEER TO PARTICIPATE IN: "STUDY OF PLATELET GENOTYPE, GENETIC DETERMINANTS OF HYPERHOMOCYSTEINAEMIA AND PLATELET AND COAGULATION ACTIVATION IN THROMBOTIC STROKE."

Name of patient/volunteer:..........................

Principal Investigator: Dr D Meiklejohn

I have read the patient/volunteer information sheet and have had the opportunity to discuss the details with......................... and ask questions. The doctor has explained to me the nature and purpose of the tests to be undertaken. I understand fully what is proposed to be done.

I have agreed to take part in the study as it has been outlined to me, but I understand that am free to withdraw from the study or any part of the study at any time I wish and that this will not affect my continuing medical treatment in any way.

I understand that these trials are part of a research project designed to promote medical knowledge, which has been approved by a Joint Ethical Committee, and may be of no benefit to me personally.

I agree to a sample of my DNA being kept so that further investigations may be performed at a later time, as new
developments in medical science arise.

I understand tat the tests are done on an anonymous basis, so that it will not be possible to disclose my individual results.

I also understand that where appropriate, my General Practitioner will be informed that I have taken part in the study.

I hereby fully and freely consent to participate in the study which has been fully explained to me.

Signature of patient/volunteer:........................
date:.......................

I confirm that I have explained to the patient/volunteer named above, the nature and purpose of the tests to be undertaken.

Signature of investigator:......................
date:......................

NOTE: FILE ONE COPY IN SECTION A OF PATIENT NOTES AND RETAIN A SECOND COPY

INITIAL ASSESSMENT CHECK LIST

1) Name and unit no:..

Affix addressograph here

2) Age/dob...................

3) CT confirmation of completed infarct rather than TIA or haemorrhage. Yes/No (delete as appropriate).

4) Excluded AF/ valvular heart disease. Yes/No.

5) Past Medical History (HBP, DM, Hyperlipidaemia)

....
....
....
....
....
....

6) Drug History (Aspirin/NSAIDs or B12/Folate/B6)

....
....
....
....
....
....

7) Dietary assessment ????

8) Family History of atherosclerosis. Yes/No

9) Details of friend/relative who will be control.

....

9) Sample checklist (tick):

i) 2x 4ml EDTA; FBC and rcf to haematology

; frozen sample for homocysteine.

ii) 1x 10ml Na Cit; for platelet genotype and

coagulation markers.

iii) 1x 4ml EDTA; for DNA extraction.

iv) 2x 5ml clotted ; 1 to haem for B12/folate.

; 1 stored for B6 estimation.

v) Ensure lipids checked on routine biochemistry.

vi) Coag screen if not routinely done.

Comments

....

9) Consent form signed Yes/No.

10) Date for repeat testing given Yes/No.
If yes, when.......................

D Meiklejohn. June 1997.

DONOR PLATELET STUDY

Study number..............

1)Donor Number..................

2) Date of sample.............

3) Previously Tested Genotype................

4) Sample Taken for DNA extraction Yes/No

5) Repeat Genotyping performed? Yes/No Date........

6) Result of repeat genotyping..............

7) Consent form signed Yes/No

8) Results

GPIIIa %	GPIIIa +CD62P H2, FL2/FL1	GPIIIa +CD62p +PMA H2, FL2/FL1	FBG %	FBG + ADP %
	/	/		

D Meiklejohn March 1998

Appendix III. Publications arising from the work in this thesis.

Abstracts

Meiklejohn DJ, Urbaniak S.J. and Greaves M. 'Platelet Glycoprotein IIIa polymorphism HPA 1b and fibrinogen binding.' Oral communication at the annual meeting of the British Society for Haemostasis and Thrombosis, St Thomas's Hospital, London 17/09/98. Abstract in *Blood Coagulation and Fibrinolysis* 1998;**9:** 11

Meiklejohn DJ, Urbaniak S.J and Greaves M. 'Platelet Glycoprotein IIIa polymorphism HPA 1b and fibrinogen binding.' Poster presentation at 40th annual meeting of ASH, Miami, USA on 06/12/98. Abstract in *Blood* 1998;**92:** 190a.

Meiklejohn DJ, Riches Z, Youngson N and Vickers M. 'The contribution of Factor VII polymorphisms to longevity.' Poster presentation at the 39th Annual Scientific Meeting of the British Society for Haematology, Brighton 12/04/99. Abstract *British Journal of Haematology* 1999;**105S**:38.

Meiklejohn DJ, Riches Z, Youngson N and Vickers MA. 'The contribution of Factor VII (FVII) gene polymorphisms to longevity.' Poster presentation at the XVIIth congress of the International Society for Thrombosis and Haemostasis, Washington DC, USA, 17/08/99. Abstract published in *Thrombosis and Haemostasis* 1999;supplement: 187S.

Meiklejohn DJ, Morrison ER, Urbaniak SJ, Vickers MA and Greaves M. 'Circulating activated platelets in the acute and convalescent phases of thrombotic stroke.' Oral presentation at the annual scientific meeting of the British Society for Haemostasis and Thrombosis, Cambridge, UK, 24/09/99. Abtract published in *Blood Coagulation and Fibrinolysis* 1999;Supplement.

Meiklejohn DJ, Morrison ER, Urbaniak SJ, Vickers MA and Greaves M. 'Circulating activated platelets in the acute and convalescent phases of thrombotic stroke.' Oral presentation at the annual meeting of the Scottish Haematology Group, 02/10/99, Dunkeld House Hotel. Awarded prize for best training grade presentation.

Meiklejohn DJ, Vickers MA, and Greaves M. 'Plasma homocyst(e)ine levels in the acute and convalescent phases of atherothrombotic stroke.' Poster presentation at annual scientific meeting of the British Society for Haematology, 28/03/00, Bournmouth. Abstract published in *British Journal of Haematology* 2000:108(S1);56.

Meiklejohn DJ, Vickers MA, and Greaves M. 'Circulating activated platelets in the acute and convalescent phases of atherothrombotic stroke: no relationship with HPA 1a/1b Genotype.' Oral presentation at the 5th Annual meeting of the European Haematology Association, 27/06/00, Birmingham. Abstract published in *The Haematology Journal* 2000:1(S);60.

Meiklejohn DJ, Vickers MA, and Greaves M. 'Plasma homocyst(e)ine levels in the acute and convalescent phases of atherothrombotic stroke.' Poster presentation at the 5th Annual meeting of the European Haematology Association, 27/06/00, Birmingham. Abstract published in *The Haematology Journal* 2000:1(S);126.

Meiklejohn DJ and Greaves M. 'Circulating activated platelets in the acute and convalescent phases of atherothrombotic stroke: no relationship with HPA 1a/1b Genotype.' Oral presentation at the Scottish Society of Physicians annual meeting, 07/10/00, Royal College of Physicians and Surgeons, Glasgow.

Papers

Meiklejohn DJ, Urbaniak S.J and Greaves M. 'Platelet Glycoprotein IIIa polymorphism HPA 1b (PLA2): No Association With Platelet Fibrinogen Binding.' *British Journal of Haematology* 1999;**105:**664-666.

Meiklejohn DJ, Riches Z, Youngson N and Vickers MA. 'The contribution of Factor VII gene polymorphisms to longevity in Scottish Nonagenarians.' *Thrombosis and Haemostasis* 2000;**83**:519.

Meiklejohn DJ, Vickers MA, Dijkhuisen R and Greaves M. 'Plasma homocysteine concentrations in the acute and convalescent periods of atherothrombotic stroke.' *Stroke* 2001;**32**:57-62.

Meiklejohn DJ, Vickers MA, Morrison ER, Dijkhuisen R, Moore I, Urbaniak SJ and Greaves M. 'In vivo platelet activation in atherothrombotic stroke is not determined by polymorphisms of human platelet glycoprotein (GP) IIIa or GPIb.' *British Journal of Haematology*, 2001;**112**:621-631.