GENETIC INFLUENCES ON PLATELET FUNCTION IN CORONARY ARTERY DISEASE AND MYOCARDIAL INFARCTION

For the Degree of Doctor of Medicine at Imperial College
University of London

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ABSTRACT

Coronary atherosclerosis with plaque disruption resulting in superimposed thrombosis is the main pathological mechanism in the acute coronary syndromes of unstable angina, myocardial infarction, and sudden death. In patients with significant obstructive coronary disease, high shear flow conditions are created in the stenosed vessel(s) and surface bound vWF is essential for platelet adhesion and circulating vWF for aggregation. The interaction between GP Ib-IX-V platelet surface complex and vWF is the primary event in the formation of an occlusive thrombus under conditions of high shear stress so initiating intracellular signalling resulting in activation of the integrin GP IIb/IIIa and subsequent platelet aggregation. There is strong evidence that predetermined genetic factors, in addition to traditional risk factors for atherosclerosis and arterial thrombosis, can influence haemostatic balance and consequently thrombotic risk. A large number of genetic polymorphisms have been implicated, mainly by case-control studies, to be associated with arterial thrombosis and coronary artery disease. The functional significance of some of the genotypes in the setting of coronary artery disease is yet unknown. However, my thesis hypothesises that two DNA polymorphisms in the GPIbα gene may be significant factors influencing the risk of myocardial infarction regardless of the degree of coronary atheroma.

Therefore, 256 patients, aged 33-80 were recruited at cardiac catheterisation for any diagnostic indication, and divided into two groups: group A, with confirmed previous myocardial infarction (98 patients) and group B, with no evidence of myocardial infarction (168 patients). All the patients were taking Aspirin 75 mg as their only antiplatelet medication. The platelet function was determined based on the closure time (CT) measured using PFA-100 with Col/Epi and Col/ADP cartridges in 84 consecutive

males from this study group. The plasma level of Von Willebrand factor (vWF) was also measured in all patients.

There was no significant difference in race, age, hypertension, smoking status or family history between the infarct and non-infarct groups. Genotype analysis showed an association between the GPIbα -5T/C Kozak homozygous TT genotype and the occurrence of myocardial infarction. The TC genotype may have a protective effect against MI. No significant association was found between myocardial infarction and the GPIbα VNTR or PL^{A1}/PL^{A2}, although analysis of the CC VNTR genotype against all other GPIbα VNTR genotypes showed a marginal association with myocardial infarction. There was no association between any of the genotypes polymorphism and the degree of vessel disease. The platelet reactivity was significantly enhanced in VNTR CC genotype using Col/Epi cartridge compared to the BC and CD genotype. In contrast the CD genotype had the longest closure time. The TT genotype of the –5T/C Kozak sequence polymorphism showed also a marginal increase in platelet reactivity compared to the TC genotype. The vWF plasma level was similar in all genotype groups.

I conclude that certain genotypes of GPIbα (-5T/C Kozak TT and VNTR CC) may independently increase the risk of non fatal myocardial infarction regardless of the extent of coronary disease. However, none of the polymorphisms studied for the purpose of this thesis increased the risk of coronary atheroma.

INTRODUCTION

Vascular injury and thrombus formation are key events in the pathogenesis of acute coronary thrombosis. The composition of thrombi found in arterial thrombosis is predominantly that of a platelet-rich core attached to an underlying atheromatous plaque in an epicardial segment of the corresponding coronary artery branch (Fuster V, *et al.* N Engl J Med 1992, Davies MJ, *et al.* Eur Heart J 1989). In the arterial circulation, platelets adhere to surface bound von Willebrand factor (vWF) via the platelet glycoprotein (GP) Ib/IX/V complex. This receptor is shear dependant and is "switched on" in the arterial microvasculature and environments such as those found in the coronary artery (Kroll MH, *et al.* Blood 1996, Roth GJ, *et al.*. Blood 1991, Berndt MC, *et al.* Aust NZ J Med 1995, Savage B, *et al.* Cell 1996). The platelet-collagen interaction then leads to activation and expression of functional GPIIb/IIIa receptors on the cell surface (Jang Y, *et al.* J Am Coll Cardiol 1994). Fibrinogen causes platelet aggregation via binding to GPIIb/IIIa receptors on adjacent activated platelets (Peerschke EI, J Lab Clin Med 1994). Activated platelets also provide a negatively charged phospholipid surface necessary for the activation of the coagulation system, with resultant generation of thrombin, leading to cleavage of fibrinogen to form fibrin and further activation of circulating platelets (Harker LA, *et al.* Clin Haematol. 1994). In addition the activated platelets release a number of factors leading to activation of factor XIII, which is essential for cross-linking of fibrin strands, leading to stabilization of the fibrin clot (Fitzgerald DJ, *et al.* Am J Cardiol 1991)

Compelling evidence from epidemiological studies suggests a genetic predisposition for atherosclerotic disease and arterial thrombosis. (Lane DA, *et al*, Blood 2000) Not surprisingly the platelet surface receptors have been the focus of such genetic researches.

Although the results of these published reports are controversial, the jury is still out as to whether there is a true association between these genetic polymorphisms and increased risk of coronary thrombosis.

AIM OF THE THESIS

In this thesis I intend to look at the direct association of some of the GPIbα and GPIIIa receptor polymorphisms and coronary thrombosis in subjects in whom the degree of coronary disease is known on angiography at the time of blood sampling. This study is unique and different from the traditional "case-control" studies. By comparing a myocardial infarction (MI) with a non-MI group of patients rather than incorporating a traditional "normal control group" we were able to compare two equivalent populations who were evenly matched with respect to other known risk factors (Table 1). Within any normal control group a degree of latent vessel disease would still be expected, therefore both the MI and non-MI cohorts were recruited from patients with known coronary angiographic data. Both study groups were well characterised with detailed clinical information. This enabled us to dissect out the influence of each of the two polymorphisms on the occurrence of MI and degree of vessel disease.

I looked at the frequency of these genotypes in the study subjects as well the functional effect of these genotypes on platelet reactivity. Firstly, the GPIbα –5T/C Kozak polymorphism which may potentially alter the level of expression of GPIb-IX-V, and secondly, the GPIbα variable number tandem repeat (VNTR) which alters the structure of this essential platelet adhesion receptor. Thirdly the effect of GP IIIa polymorphism on platelet aggregation and its association with coronary thrombosis was also evaluated.

MEANS OF MEASUREMENTS AND DESIGN OF THE THESIS

The author recognises that a number of polymorphisms have been described and their effects on coronary disease and arterial thrombosis have been reported. For the purpose of this study I have only concentrated on some of the most intensively investigated genetic polymorphisms of platelet surface receptors GPIbα and GPIIIa and their function. However this study was different from other large published data at the time the research was taking place because of the unique design and patient selection.

The utmost care was taken in the design of the study. The study group was selected from patients attending the day case coronary angiography. All our patients underwent coronary angiography and fasting blood sampling was done prior to the procedure. All patients were taking 75 mg Aspirin as their only antiplatelet therapy and statins in addition to other medications. None of the patients received Heparin prior to blood sampling.

Particular attention was given to DNA extraction and polymerase chain reaction (PCR) of the DNA to avoid contaminations. The Enzyme Linked Immuno Sorbent assay (ELISA) for von Willebrand factor antigen was done in triplicate and the final result reported as the mean of the three results which closely matched. Great care was taken with the blood sampling, particularly for the purpose of PFA-100, to avoid over stimulation of platelets so the *in vitro* measurements truly reflect the *in vivo*. The PFA-100^{TM} test was carried out in duplicate with both cartridges on 15 healthy volunteers to assure the reproducibility of the results hence to ensure the reliability of the method. With each new batch of cartridges the reproducibility test was carried with blood sample from a healthy volunteer. I myself carried out blood sample collection and all the molecular assays including DNA extraction, PCR, ELISA and PFA-100 assays.

The experimental work spanned four years and has been published in three peer-reviewed papers in 2004-2006 and partly in 1998. The main theme and aim of the research was set out at the beginning of my experimental work and the research was carried out at various stage of my training. I recognise that since my work was carried out other publications of similar work may have emerged but the originality of the thesis at the start of the research has been accepted as evidenced by my publications.

POTENTIAL CLINICAL RELEVANCE AND STATEMENTS OF ORIGINALITY OF THE THESIS

Identifying an association between platelet surface receptor gene polymorphisms and coronary thrombosis would help targeted therapy in high risk patients and reduce the risk of coronary thrombosis. Evaluation of the polymorphisms of the specific platelet surface receptors as an independent risk factor for myocardial infarction may lead to future screening in addition to blood glucose, lipids, etc. It will open the doors to possible gene therapy for coronary artery disease.

The entire work of this thesis is original. Most of the work has already been published in the journal Heart between 2004 and 2006. The author of this thesis is also the first author on all the papers indicating her major contribution to this study (copies of papers attached). The techniques of measurements are derived from the literature and adapted for the specific purpose

ACKNOWLEDGEMENT

My special thanks go to

-Prof Tuddenham, my supervisor for his constructive criticism and support throughout my work

-Dr Kate Michaelides, Medical Research Council, Hammersmith Hospital, whose help throughout the study is greatly appreciated. She spent a considerable amount of her precious time to teach me the DNA extraction and PCR and data analysis.

-Dr Graham Davies, my initial supervisor, who has been truly great help in the field of research as well as clinical duties

-Richard Manning, Haematology department, Hammersmith Hospital, For his significant help and teaching me ELISA methods for vWF assays and allowing me to use the lab for that purpose

CHAPTER 1: PLATELETS AND CORONARY THROMBOSIS

1.1 LITERATURE REVIEW

First discovered in 1842 by Donnè,(C R Acad Sci, Paris, 1842) platelets and their role in different physiological phenomenon and pathological conditions has been the interest of many clinicians and scientists over a century. Platelets not only participate in normal haemostasis but are also a central element in atherothrombosis, inflammatory and allergic reactions, tumour metastasis, etc. In 1882, Bizzozero (*Arch Path Anat Physiol.* 1882) first noted the term "white thrombus" and mechanism of adhesion and aggregation. The adhesion of platelets to collagen from an injured vessel with subsequent aggregation was first observed in the early 1960s. It was not until twenty years later that a number of platelet surface receptors for the initiation of adhesion, activation and aggregation were identified. In the last decade it is becoming clear that at high shear, the Glycoprotein (GP) Ib-IX-V complex is the primary receptor involved in platelet adherence to subendothelium, while at a low shear the GP IIb-IIIa serves the function.

1.1.1 PLATELETS AND ATHEROSCLEROSIS

Thirty years after Ross *et al* (N Engl J Med. 1976) proposed the involvement of platelets in atherosclerosis, recent evidences have provided insights into the important role of activated platelets in the development of atherosclerosis. Detection of activated platelets in peripheral blood of patients with unstable atherosclerosis disease was first reported by Fitzgerald *et al* (J Clin Invest. 1986). Circulating activated platelets were also detected in the blood of atherosclerotic rabbits (Tsa *et al* 1994) and patients with stable coronary disease (Furman *et al* 1 J Am Coll Cardiol, 1998). Platelet activation can be seen in

different phases of atherosclerosis. In the early phase of atherosclerosis, platelet activation as defined by P-selectin surface expression, may be attributed to (a) generation of hydroxyl radicals by risk factor; (b) reduction in production of nitric oxide and prostacyclin leading to loss of endothelial antithrombotic properties; (c) an increase in prothrombotic and pro-inflammatory mediators such as chemokines and tissue factor in the circulation. Upon activation, platelets release a wealth of adhesive and pro-inflammatory substances. Several groups of these substances such as platelet microparticles, platelet derived growth factor, PF-4, IL-1β, CD40 ligand are relevant to inflammation and atherosclerosis. Inhibition of thromboxane A2 (TX) production by aspirin (Cyrus *et al* Circulation, 2002) and indomethacin (Pratico *et al*, Proc Natl Acad Sci U S A, 2001) or TX receptor by antagonist (Cayatte *et al* Arterioscler Thromb Vasc Biol, 2000) is able to dramatically diminish the formation of the atherosclerotic lesion. Also, the effect of aspirin in atherosclerosis may well be explained not only by inhibition of TX production but also inhibition of cyclooxygenase-2 in macrophage and smooth muscle cells in the vessel wall. Interestingly most risk factors for atherosclerosis (diabetes, hypercholesterolemia and smoking) are able to increase the number of activated platelets in circulation. (Manduteanu *et al* J Submicrosc Cytol Pathol, 1992) (Broijersen *et al,* Thromb Haemost, 1998) (Nowak *et al*, Circulation, 1987).

1.1.2 PLATELETS AND THROMBOSIS

Following vessel wall injury as a result of atherosclerotic plaque rupture, platelets initially are tethered to the subendothelial extracellular matrix. Under condition of high shear rate, this interaction occurs through binding of glycoprotein Ib/V/IX to immobilized vWF. Stable platelet adhesion to the subendothelial extracellular matrix requires additional binding of glycoprotein Ia/IIa, as well as other interactions such as

glycoprotein IIb-IIIa-vWF and glycoprotein Ic/IIa-fibronectin. Platelet activation is initiated during tethering which results in platelet shape change, release of intracellular storage granule contents, expression of the activated form of glycoprotein IIb/IIIa and other activation markers (e.g. P-selectin) and generation of platelet membrane procoagulant activity. Binding of vWF and fibrinogen to activated platelets results in platelet aggregation stabilized by the interaction between activated glycoprotein IIb/IIIa and fibrinogen.

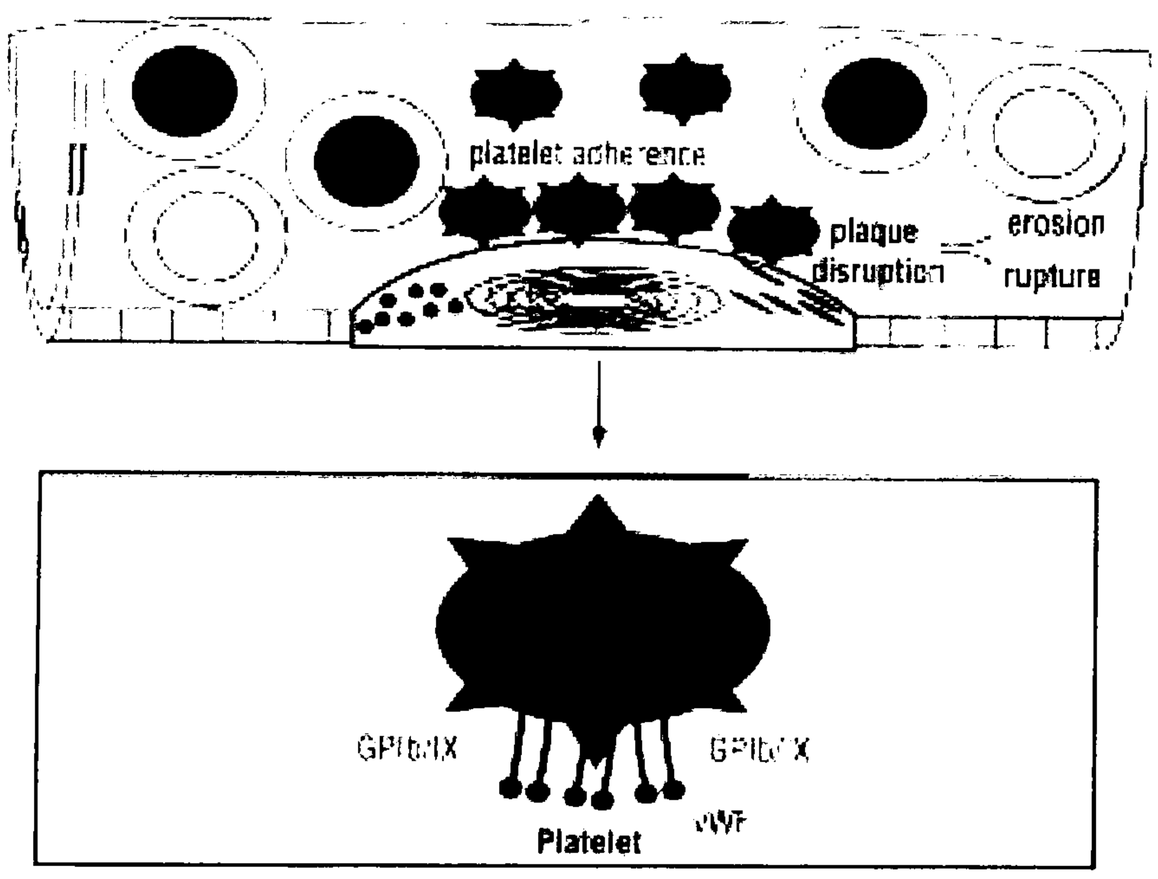


Figure 1. Plaque disruption (erosion or overt rupture) is followed by platelet adherence that is mediated by specific surface receptors and (GP Ib/IX) and adhesion molecules (von Willebrand factor [vWF]).

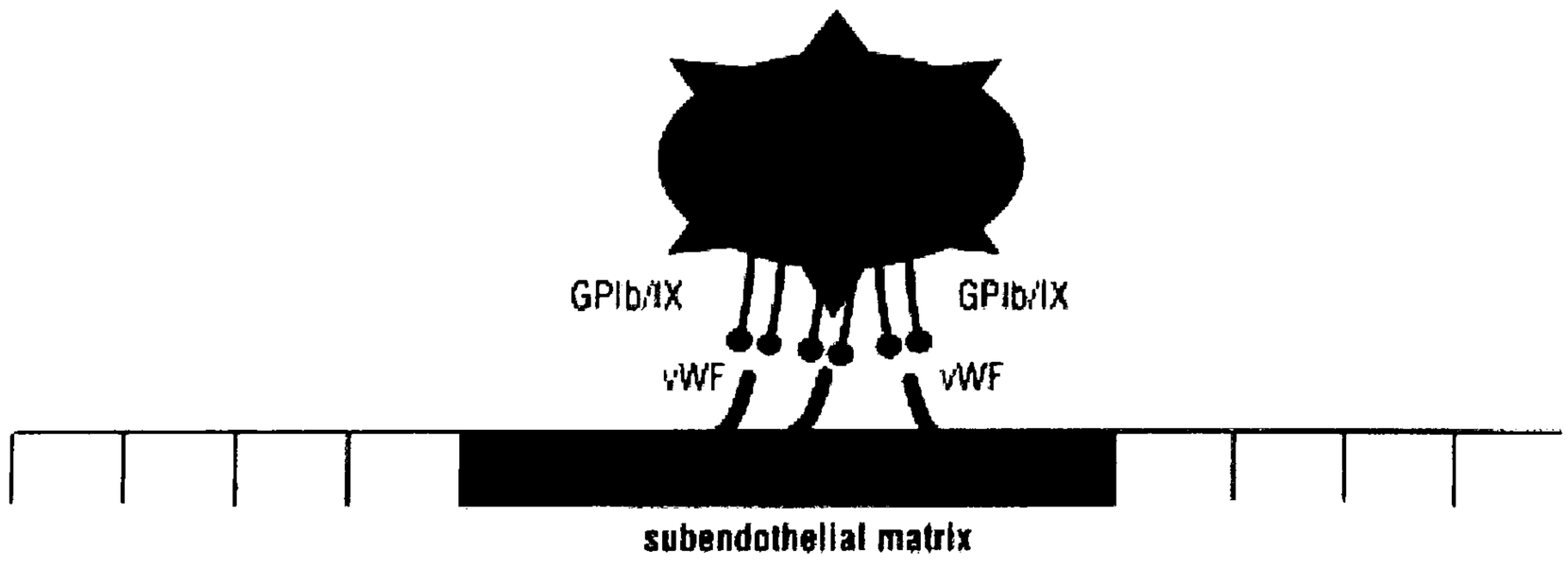


Figure 2. The platelet surface glycoprotein (GP) Ib/IX complex binds to von Willebrand factor (vWF) affecting adherence to the vessel's subendothelial matrix.

Figure 1 and 2 from : Becker RC, Am J Cardiolo 83(9, Symposium 1), pages 3-6

1.1.3 PLATELET HYPERREACTIVITY AND CORONARY ARTERY DISEASE

Enhanced platelet reactivity is well documented in acute coronary syndromes but also in patients with stable coronary disease as compared to a healthy population. Platelet activation occurs during episodes of chest pain in patients with coronary artery disease. It is accepted that such enhanced platelet reactivity has pathological importance and is associated with, if not directly responsible for, acute thrombotic events and poorer prognosis. Spontaneous platelet aggregation *in vitro* has been shown as a useful biological marker for the prediction of coronary events and mortality in survivors of myocardial infarction. It has also been suggested that *in vitro* analysis of platelet hyper-reactivity as measured by PFA-100 is a predictor of the extent of myocardial damage as measured by serum creatinine kinase and troponins. Furthermore, trials have demonstrated that various antiplatelet drugs including aspirin, clopidogrel and glycoprotein IIb/IIIa inhibitors provide substantial therapeutic benefit in patients with coronary disease and acute coronary thrombosis by reducing platelet activation, stabilising plaques, reducing incidence of death or MI and improving prognosis.

1.1.4 ASSESSMENT OF PLATELET FUNCTION USING PLATELET FUNCTION ANALYSER (PFA-100)

Kratzer and Born (Haemostasis, 1985) first described a system where primary haemostasis could be simulated *in vitro*. An anticoagulated blood sample passes through an aperture cut into a membrane coated with collagen and soaked with adenosine 5'-diphosphate (ADP). Platelets attach to the area surrounding the aperture and aggregate, forming a platelet plug that causes stoppage of blood flow through the aperture. The time

required for the formation of the platelet plug and the volume of the blood passed through the aperture correlated well with the standard clinical tests in patients with a variety of platelet dysfunction. The principle conceived by Kratzer *et al* ((Semin Thromb Hemost 1995,). was further developed as a prototyped system, the Thrombostat 4000 and subsequently to the PFA-100.

The PFA-100 is a high shear-inducing device that simulates primary haemostasis after injury to small vessels. The system consists of a microprocessor-controlled instrument and a disposable test cartridge. The test cartridge contains a reservoir for citrated whole blood and a capillary (200 μm internal diameter) surmounted by a cup containing a biologically active membrane with a central aperture (150 μm diameter). The membrane is coated with fibrillar type I equine tendon collagen. Additionally, either epinephrine (10 μg) or ADP (50 μg) is present on the membrane. These agents provide a controlled stimulation of platelets as the blood sample passes through the aperture. In response to stimulation by collagen and in conjunction with either epinephrine or ADP, as well as high shear rates (5000 to 6000 dynes/cm^2), platelets adhere and aggregate on the membrane surface at the area surrounding the aperture. Ultimately, a platelet plug forms which occludes the aperture and blood flow is stopped. The time required to obtain occlusion of the aperture is defined as the closure time (CT).

The closure time is measured in seconds. The PFA-100 test result is dependent on platelet number and function, plasma von Willebrand Factor (vWF) level, hematocrit (HCT≥28%, measured by our local lab at Hammersmith Hospital) and Aspirin therapy. In the haemostasis and thrombosis laboratory and as per manufacturer the normal values for closure time with collagen-epinephrine (CEPI) is <164 s and for the collagen-ADP (CADP) is <116 s. The ranges depend on the citrate anticoagulant concentration and the choice between glass and plastic collection tubes. Therefore it is recommended by the

manufacturer that each laboratory should determine its own normal range. A normal CEPI value excludes the presence of a significant platelet function defect. If CEPI is prolonged this may indicate "aspirin effect". The CEPI is very sensitive to aspirin in many patients and may be greater than 250 s. In contrast CADP is relatively insensitive to the aspirin-induced platelet defect. Some of the platelet storage pool and release defects can give the "aspirin pattern" on the PFA. The instrument performs a self-test on start up that tests the electronics and system vacuum.

The published coefficient of variation (CV) of the PFA-100^{TM} in normal subjects ranges from 3%-18% with average variability of 8.5%.(Kundu Sk, Semin Thromb Hemost.1995)

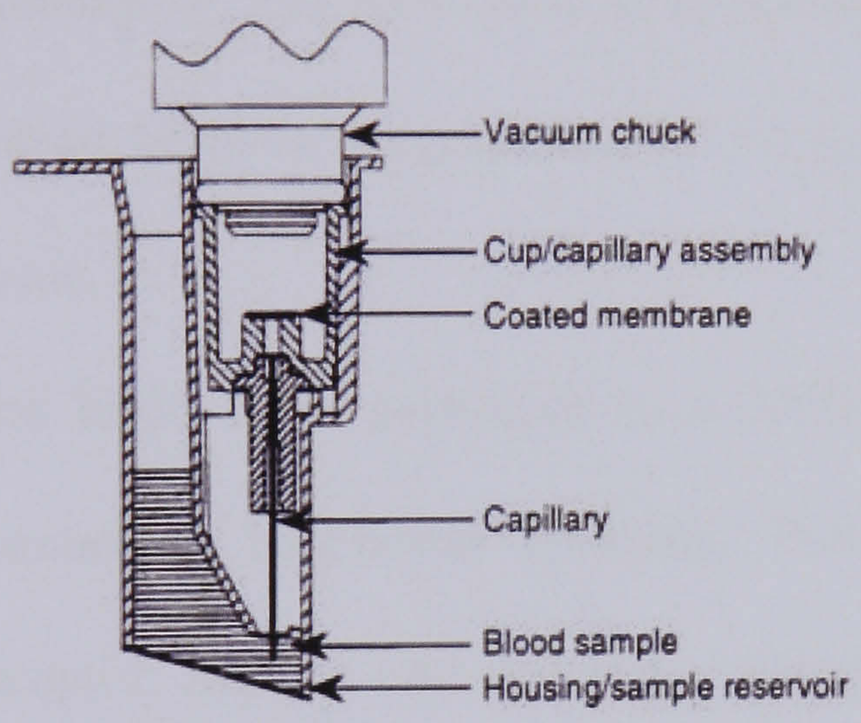


Fig 1: Schematic view of PFA-100^{TM}

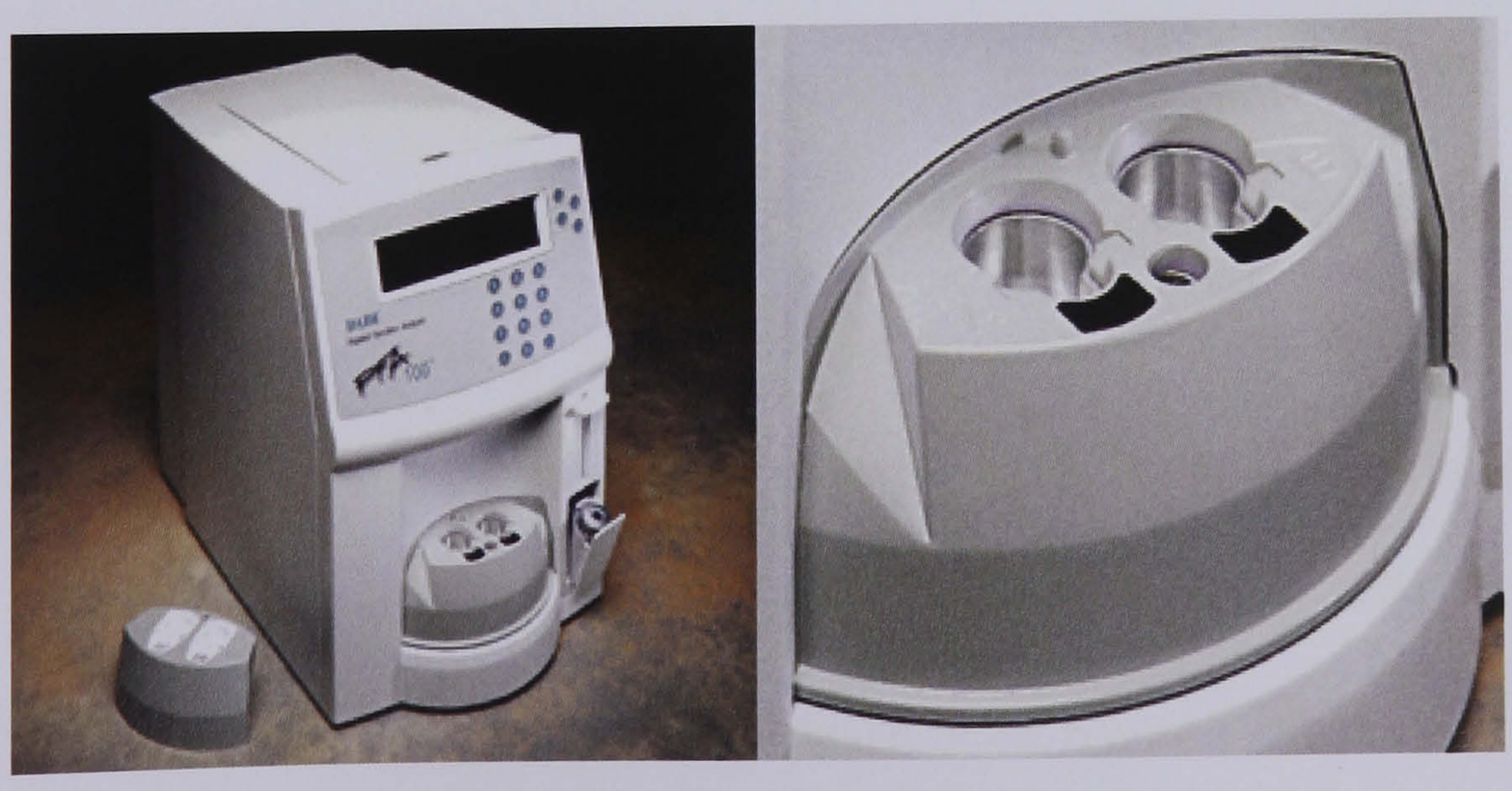


Fig 2: PFA-100^{TM} machine

CHAPTER 2: PLASMA VON WILLEBRAND FACTOR STRUCTURE AND FUNCTION

2.1 LITERATURE REVIEW

Von Willebrand factor (vWF) is named after Dr Erik von Willebrand (*Fin Läkaresällsk Handl*, 1926) who distinguished von Willebrand disease from other forms of bleeding diathesis in families from the Aland islands. It was first purified in 1970 and is a large multimeric glycoprotein (>20,000 kDa) present in blood plasma. It consists of over 80 subunits of 250 kDa each. It is produced constitutively in endothelium (in the Weibel-Palade bodies), megakaryocytes (α-granules of platelets) and subendothelial connective tissue.

The basic vWF monomer is a 2050 amino acid protein. Every monomer contains a number of functional domains. These include the binding sites for platelet GP 1b receptor, heparin (A1 domain) collagen (A3 and possibly A1 domain), IIb-IIIa complex of platelet surface receptor (C1 domain) and factor VIII (D'D3 domain). Their role in initiating the thrombosis by platelet-collagen and platelet-platelet interaction is pivotal. The vWF multimers are bound to the collagen surface of damaged vascular endothelium following an injury. Under conditions of high shear force, the VWF-A1 domain undergoes conformational transition from a globular state to an extended chain conformation exposing the individual domains and increasing the number of adhesive bonds for further platelet surface receptors and collagen causing a more stable and secure adhesion of platelets and collagen. This may explain why it is that only the large multimers are functional. (Siedlecki CA *et al*, Blood 1996)

There are two ways of detecting vWF in peripheral blood, quantitative and qualitative. The quantitative assays detect total vWF but give no accurate information on the functional capacity of vWF detected. Qualitative measurements detect only highly adhesive vWF and therefore a fraction of the total vWF hence less sensitive assay. The vWF:multimer analysis and the vWF :Collagen Binding Activity Assay are all functional assays compared to vWF:antigen assay which is the best quantitative assay. The vWF:Ristocetin Cofactor assay is both functional and quantitative but has significant variability and technical difficulties limit its usefulness. Generally, vWF qualitative assays are used in the diagnosis of variant Von Willebrand disease only since the ratio of vWF:Antigen to vWF qualitative activity is unity in virtually all patients without Von Willebrand disease.

2.2 VWF AND ARTERIAL THROMBOSIS

Elevated vWF level is a good index of endothelial cell damage/dysfunction (Blann AD, *et al* Br J Haem 1995)(Pearson JD *et al*, Br J Haematol 1993). The elevated levels of vWF have been reported in numerous cardiovascular conditions and predict poor outcome including myocardial infarction, first presentation of ischemic stroke and death in prospective studies. (Meade *et al*, Br J Haematol ,1994)(Kumari M *et al*, ATVB 2000)(Bongers T *et al*, Stroke 2006).

Factor VIII a pro-coagulant protein is bound to vWF while inactive in circulation and degrades rapidly when is released from vWF by the action of thrombin. The level of factor VIII coagulant activity correlates well with the vWF antigen level and high FVIII levels increase the risk of fatal ischemic heart disease. (Meade *et al*, Br J Haematol 1994)

CHAPTER 3: GPIb-IX-V and GPIIb-IIIa PLATELET SURFACE RECEPTOR POLYMORPHISMS

3.1 LITERATURE REVIEW: GLYCOPROTEIN Ib-IX-V COMPLEX

Each platelet contains 25000 copies of GPIb-IX-V complex. GPIb-IX-V is composed of four subunits: GPIbα, GPIbβ, GPIX and GPV, each being the product of a separate gene. GPIbα is disulfide linked to GPIbβ forming the heterodimer GPIb, which is non covalently associated with GPIX and GPV. (Ruggeri ZM *et al*, Prog Hemost Thromb 1991, Lopez JA. Blood Coagulation and Fibrinolysis 1994, Clemetson KJ *et al*, Seminars in Thrombosis and Haemostasis 1995). The binding site for von Willebrand factor (vWF) is located on the α subunit of GPIb.(Handa M *et al*, J Biol Chem 1989)

The major physiological role of the GPIb-IX-V complex is to mediate initial adhesion of circulating platelets to surface bound vWF on exposed subendothelial matrix under conditions of high shear such as those found in the arterial circulation and stenosed vessels. (Savage B *et al,* Cell 1996, Savage B *et al,* Curr Opin Hematol 2001) Other potential roles for the GPIb-IX-V complex are to serve as a high affinity α-thrombin binding site and as a receptor that participates in signal transduction to regulate the activation state of GPIIb-IIIa.(Yamamoto N *et al*, Throm Res 1985)

3.1.1 POLYMORPHISMS OF GPIbα

GPIbα is the largest glycoprotein of the complex. It contains binding sites for both vWF and α-thrombin . At least three polymorphisms may influence the function and surface expression of GPIbα. These polymorphisms are discussed below.

3.1.1.1 VARIABLE NUMBER TANDEM REPEAT (VNTR)

A variable number of tandem repeat of a 13-amino acid sequence within the mucin-like macroglycopeptide region of GPIbα and is due to the presence of either one, two, three or four copies (alleles D, C, B, A respectively). Each additional repeat of 13 amino-acids is predicted to add 32 Å to the distance that GPIbα extends from the platelet surface. (Lopez JA, Blood Coagul Fibrinolysis, 1994)

The four variants of the GPIbα VNTR range from 168 kDa to 153 kDa, designated A to D in order of decreasing molecular mass (D = 1, C = 2, B = 3 and A = 4 repeats)(Ishida F *et al*, Blood, 1995) This results in polypeptide lengths of 610, 623, 636 or 649 amino acids respectively (Lopez JA *et al*, J Biol Chem 1992). The C allele is the most prevalent among all human populations (ranging from 55% among Japanese to 85% among Caucasians). The smallest D variant is seen in 33% among Japanese, 8% of Caucasians and 4% of black people. The A isoform is found almost exclusively among Eastern Asian populations but is rarely present in white European populations.(Ishida F *et al*, Thromb Haemost 1996, and Blood 1995). In a Japanese study, the respective gene frequencies were reported to be: 0.35, 0.56, 0.01, 0.073 for D, C, B and A respectively.(Moroi M *et al*, Blood, 1984) It is therefore feasible that the length of GPIbα could affect the efficiency of platelet adhesion owing to differences in the distance between the GPIbα-vWF binding site and the vessel wall.

3.1.1.2 GPIbα -5T/C KOZAK SEQUENCE POLYMORPHISM

Kozak sequences are DNA motifs that surround ATG initiator codons. The sequence context of ATG codons is important, in particular the positions –3 to –9.(Kozak M, J Mol

Biol 1987) The GPIbα gene Kozak sequence polymorphism is the result of either a T (thymine) or a C (cytosine) at position –5 relative to the ATG start codon.(Kaski S et al, Immunogenetics 1996) Recently, this Kozak sequence has been reported to affect the expression of the GPIb-IX-V complex on the platelet surface. The less common allele C is closer to the Kozak consensus sequence, which is said to be more efficient at translation, and there is evidence from *in vitro* studies that it is associated with increased expression of the GPIb-IX-V complex on the surface of CHO cells. (Afshar-Kharghan V *et al*, Blood 1999)

The third polymorphisms of GPIbα is the Ko (HPA-2) polymorphism. It's association with platelet transfusion refractoriness was first described by Saji, *et al* and Murata *et al.* It is characterized by a C/T transition at nucleotide 1018, resulting in amino acid dimorphism (Thr/Met) at residue 145. (Saji H *et al,* Vox Sang 1989)(Murata M *et al*, Blood 1992) It is in nearly complete linkage disequilibrium with the VNTR. The A and B VNTR variants are associated with methionine 145(HPA-2b) and the C and D VNTR are linked to the Thr-isoform (HPA-2a allele). Most individuals with the Met allele have been reported to carry the B allele but 1-5 % of the B allele carriers are Thr homozygous. The allelic frequency in the white population has been reported as 0.89 for HPA-2a and 0.11 for HPA-2b. (Mazzucato M *et al*, Transfusion 1996) Thus any association of the Met allele with CAD may also be due to an effect of the VNTR polymorphism on platelet function.

3.2 LITERATURE REVIEW: GLYCOPROTEIN IIb/IIIa

GPIIb-IIIa is a platelet membrane glycoprotein complex important for platelet adhesion and aggregation. The complex is an integrin which recognises the arginine-glycine-

aspartic acid sequence present on several adhesive proteins such as fibrinogen and von Willebrand factor. GPIIb consists of two disulfide-linked subunits, GPIIb (MW=125,000) and GPII (MW=23,000) while GPIIIa has only one polypeptide chain (MW=108,000). The Fibrinogen binding site is located on GPIIIa. The role of GP IIb-IIIa in arterial thrombosis and the inhibition of this receptor is a major therapeutic advance in the management of coronary thrombosis. The use of anti glycoprotein IIb-IIIa receptor antibodies (known as Abciximab) has been shown to improve the outcome in patients with acute coronary syndrome and post-angioplasty.

3.2.1 POLYMORPHISMS OF GPIIb-IIIa

GPIIb-IIIa is highly polymorphic and carries PL^{A1}/PL^{A2}. (Kunicki TJ *et al*, Blood 1992) There is geographical variation in the prevalence of PL^{A2}. (Kim HO *et al*, Transfusion 1995)(Odawara M *et al*, Lancet 1996) The PL^{A1}/PL^{A2} polymorphism, also known as HPA-1a/HPA-1b, is due to a substitution of Leu-33 to proline in the β_3-subunit of GP IIb-IIIa. This results in an extracellularly positioned conformational change of the β_3-subunit. Therefore it has been considered biologically plausible to suggest a potential impact of Leu33Pro on platelet aggregation.

CHAPTER 4: FUNCTIONAL SIGNIFICANCE OF PLATELET SURFACE RECEPTOR POLYMORPHISMS

4.1 LITERATURE REVIEW:

4.1.1 GPIbα VNTR AND INCREASED RISK OF CORONARY ARTERY DISEASE AND ARTERIAL THROMBOSIS

This polymorphism may have important implications for the function of GP Ibα, as each added repeat would position the ligand-binding region further away from the platelet membrane surface, making it more accessible to ligand binding and more susceptible to shear forces. Several studies have assessed the relationship between the GP Ibα-VNTR and risk of thrombotic disease but the findings have been inconsistent. In a Japanese study of 91 patients with non-fatal MI or angina and 105 healthy volunteers, Murata *et al.* (Circulation 1997) reported an increase risk of coronary heart disease associated with the presence of the B or A allele. However this association was not seen in another study. (Ito T *et al*, Int J Hematol, 1999).

A case control study from Spain using risk factor-matched subjects without any vascular disease demonstrated a 2-fold increase in risk of both coronary heart disease and ischemic cerebrovascular disease associated with the presence of BC-VNTR genotype but no association was seen with deep venous thrombosis. (Gonzalez-Conejero R, Blood 1998). No association was found between VNTR genotype and risk of myocardial infarction in France (Mercier B *et al*, Thromb Haemost, 2000) or stroke in the UK (Carter AM *et al*, Arterioscler Thromb Vasc Biol, 1998).

4.1.2 GPIbα -5T/C KOZAK AND RISK OF CORONARY ARTERY DISEASE AND ARTERIAL THROMBOSIS

An effect of the glycoprotein Ibα Kozak sequence –5T to C substitution on translation efficiency is supported by the studies of Afshar-Khargan *et al* (Blood, 1999) who demonstrated increased surface levels of the GPIb-IX-V receptor on platelets carrying the –5C allele as well as CHO cells transfected with the –5C variant. In contrast, flow cytometric analysis of a larger number of individuals did not confirm the relationship

between –5 T/C genotype and platelet surface glycoprotein Ibα density among T/T and T/C individuals, although subjects carrying the C/C genotype were not examined (Corral J *et al*, Thrombosis Haemost, 2000). Cadroy Y *et al* (Blood 2001), using a parallel-plate perfusion chamber, reported a positive association between –5T/C polymorphism in the Kozak sequence and acute arterial thrombosis that is dependent on the local blood flow condition. The increase in platelet deposition was only seen *in vitro* at a physiological shear rate of 650/s and no difference was observed at the shear rate of 2600/s. Favaloro EJ *et al* 2002, using the PFA-100, found a shorter collagen-epinephrine-induced closure time in homozygotes with the (-5)T/T compared to (-5)C/T genotype.

This T/C polymorphism has also been identified to be associated with ischemic stroke. (Baker R *et al*, Blood 2001). A Japanese study reported an increased risk of ischemic stroke among patients carrying at least one copy of the -5C allele and the association was independent of the effect of other polymorphism (Sonoda A, Thrombosis Haemost, 2001). In contrast, in a population based study of North American young women with myocardial infarction, no association was seen between –5C allele and increased risk of MI or stroke, in fact, the C allele was less common among women with MI (Frank MB *et al*, Blood, 2001)

4.1.3 PL^{A1} AND PL^{A2} AND RISK OF ARTERIAL THROMBOSIS

Platelets from PL^{A2}- positive individuals show increased aggregability compared with platelets from PL^{A2}- negative individuals using a platelet aggregometer (Michelson AD *et al*, Thromb Haemost 2000)(Feng D *et al*, Arterioscler Thromb Vasc Biol 1999). The association of this polymorphism and ischemic coronary syndrome was also shown in other studies. (Weiss EJ *et al*, N Engl J Med 1996)(Pastinen T *et al*, Hum Mol Genet

1998). However, some investigators found no association between PL^{A2} genotypes and myocardial infarction. (Hermann SM *et al*, Thromb Haemost 1997) (Samani NJ *et al*, Cardiovasc Res 1997). Ridker PM and co-authors (Lancet 1997) found that the relative risk for any vascular event among men homozygous or heterozygous for PL^{A2} compared with men homozygous for PL^{A1} was 0.96 (95% CI 0.8-1.2). They also found no association between the PL^{A2} allele and myocardial infarction, stroke or venous thrombosis in subgroup analysis by traditional cardiac risk factor and aspirin intake. The meta-analysis of mainly case-control studies with conflicting results encompassing more than 17,000 individuals, aggregated results showed an odd ratio for ischemic cardiovascular disease in PL^{A1} /PL^{A2} heterozygotes combined with PL^{A2} /PL^{A2} homozygotes versus PL^{A1} /PL^{A1} noncarriers of 1.1 (95% confidence interval 1.03 to 1.18). The Copenhagen City Heart Study with 22 years follow up of the patients showed PL^{A2} /PL^{A2} homozygosity increased risk of ischemic cardiovascular and myocardial infarction in younger men.

CHAPTER 5: METHODS

5.1 PATIENTS AND CONTROLS

The study group consisted of 256 patients aged 33-80 years who were admitted for cardiac catheterisation for angina or reasons other than chest pain at Hammersmith hospital over a period of six months. This study was approved by the Research Ethics Committee. All patients gave informed consent. The clinical data was collected from a patient questionnaire (figure a) and from the patients' medical notes. Patients were divided into two matched groups according to their clinical history. Group A: 88 patients

with a definite previously diagnosed myocardial infarction (MI) and Group B: 168 patients with no previous myocardial infarction, with either normal or diseased coronary arteries (non-MI group) (Table 1 and 2). On presentation to the catheterization laboratory, the history of MI was established based on documented evidence in patients' hospital records or by ECG (Q wave) or ventriculogram (area of akinesia).

For the functional study PFA-100 was only carried out on 84 patients (Table 2). These patients were consecutive males selected from the 256 patients who had been genotyped for the VNTR and -5T/C Kozak sequence polymorphisms of the GPIbα receptor.

All the patients were fasting and taking 75mg aspirin as their only antiplatelet therapy. All patients with cholesterol above 5 mmol/litre were taking statins or other antilipid therapy. None of the patients received Heparin prior to blood sampling. All the patients were reminded to take their aspirin on a daily basis prior to blood sampling except on the day of the testing. They were also asked to abstain from smoking and alcohol for at least 12 hours. Patients who had suffered myocardial infarction within the previous 6 months were excluded from the study. The atherosclerosis risk factors were defined as: hypercholesterolemia (>5.2mmol per litre or on cholesterol lowering drugs), hypertension (blood pressure >140/90 mm Hg or on anti-hypertensive medication), smoking (at presentation), diabetes mellitus and a positive family history (1st degree relatives) of ischemic heart disease (IHD). The coronary arteriograms were interpreted by a single, independent observer. Each of the three main coronary vessels was considered to be diseased when the lumen was reduced to less than 50% of the normal diameter. We also recorded patients with minor atheromatous coronaries (irregularities on angiogram) without any significant stenosis and patients with normal coronaries in one sub group because of small sample group of patients with normal coronaries.

Separately, we also recruited 25 healthy volunteers (doctors and nurses, aged 25-50) without any significant cardiac risk factor or bleeding abnormality who were able to take aspirin 75mg for three days. The Ethics committee approved this late addition to the original study. The main purpose of recruiting a group of healthy volunteers was to determine our own laboratory normal range for vWF assays and PFA-100 prior to taking aspirin and after 3 days of taking aspirin.

5.2 BLOOD SAMPLING

Patients had routine blood tests (FBC, renal and liver function test) prior to their coronary angiography. Patients with anaemia and impaired renal or liver function were excluded from the study.

In addition to their routine blood sampling, 20 ml of blood was collected between 8am – 11am using a 21 gauge siliconised "butterfly" cannula (Abbott Co. Ltd. UK) without using a cuff. The blood was then transferred into two 4 ml of tubes containing 3.13% tri sodium citrate at a concentration of 1 part citrate to 9 parts blood. The first 4 ml was used for vWF:Ag assay; the second 4 ml citrated blood was used to measure platelet function with the PFA-100^{TM}. The remainder of the blood was stored into two 6 ml tubes that contained Ethylenediamine Tetracetic acid (EDTA) at –70^{o}C for subsequent DNA extraction.

5.3 VON WILLEBRAND FACTOR ANTIGEN ASSAYS

Since the aim of the study was to assess the functional effect of a rise in plasma vWF in patients with coronary disease it seemed reasonable to use a quantitative assay to quantify the rise in plasma vWF and not compromise on sensitivity. We selected the vWF:Ag assay as the best quantitative and highly sensitive assay. The enzyme linked

immunosorbent assay (ELISA) method was used to measure plasma vWF:Ag. The blood was collected into 3.2% citrate for vWF:Ag assay. The citrated blood was immediately centrifuged at 3000 rpm for 10 minutes, separated into Retic tubes and stored at –80°C for analysis as part of a batch. The frozen plasma was thawed only once at 37°C, 30 minutes before use. Anti-human vWF.Ag (DAKO A082) was diluted 1:500 in 0.05M carbonated buffer (40µl antibody in 20ml buffer). Each well of the microtitre plate was coated with 100 ml of diluted antibody and wrapped in parafilm and incubated for one hour at room temperature in a moist chamber. The test plasma ware then diluted in 1:10, 1:20, 1:40 with 0.01M Phosphate buffered saline (PBS), PH 7.2 added to the wells and incubated for one hour to allow the vWF.Ag to bind to the primary antibody. After removal of excess antigen by washing the plate a second antibody, anti-human vWF.Ag-peroxide conjugate, called "tag" antibody (DAKO P226) is added and incubated again for another hour, and this binds to the vWF.Ag already stuck to the plate. On addition of a specific substrate (colour reagent Ortho-phenylenediamine mixed with citric acid, $Na_2HPO_4.12H_2O$ and hydrogen peroxide) a colour change occurs. After the reaction has been stopped by 1.0M sulphuric acid, the optical density (OD) of each well can be measured using an electronic plate reader (DYNATECH MR 700) and the OD is directly proportional to the amount of vWF.Ag present in test plasma. The machine calculates the standard curve and gives an R^2 value. Results under 0.25U/ml or over 1.00U/ml should be disregarded. To calculate the results multiply the 1:20 result by 2 and the 1:40 result by 4. The final result is obtained by taking a mean of the three calculated results, which should be closely comparable. The normal range established locally, is 0.5-2.0 U/ml.

5.4 PFA-100 PLATELET FUNCTION ANALYSER

Platelet reactivity was assessed using PFA-100^{TM} system (Dade Behring, supplied by Sysmex UK Ltd.) as previously described.

Briefly, the citrated blood was allowed to stand for 30-60minutes only, 800 µl of whole blood was added to standardized, disposable cartridge and incubated at 38.5^{0}C. The blood was then aspirated at high shear rate (5000/s) through a ring coated with 2 µg equine type I collagen and 50 µg ADP. Platelets adhere to the ring and become activated forming an aggregate which occludes the opening. The time to occlusion (closure time, CT) was taken as a measure of platelet reactivity, shorter time indication greater activity. All the tests were performed by the same operator. A self-test and maintenance was carried out daily and with each new lot of cartridges a normal control sample was to assure proper instrument performance. The reproducibility of test variables was determined by comparing the results on both channels using the blood samples from the 25 healthy volunteers on two different occasions before and after taking aspirin 75 mg.

The coefficient of variation for closure time ranged from 5.2% and 11.2% before taking aspirin and 8.3% and 13.1% after taking aspirin. The PFA-100 sensitively detected the effect of aspirin. The average of coefficient of variation was 9.2% (less than 10% is acceptable). The closure time for Col/Epi in 95% of these selected individuals after taking aspirin was 100-200sec and for Col/ADP 80-120sec. Therefore, we accepted these values as the normal range for the purpose of our study.

5.5 DNA EXTRACTION

Genomic DNA was extracted from whole blood stored in EDTA tubes using BACC2 Nucleon kit (Nucleon Biosciences, Lanarkshire, Scotland).

The process of DNA extraction is described according to different phase of the procedure.

1. Cell preparation: Under aseptic technique 10ml of whole blood was diluted with Reagent A then rotary mixed for 4 minutes and centrifuged at 1300g (2400rpm) for 5 minutes. Without disturbing the pellet the supernatant was discarded.

2. Cell lysis: To the pellet Reagent B was added and incubated for 10minutes at 37°C.

3. Deproteinisation: 500µl of sodium perchlorate solution was added to the mix.

4. DNA extraction: 2ml of Choloroform was added and mixed by inverting at least 7 times. Then 300µl of Nucleon resin was added, centrifuged at 1300g (2400rpm) for 3 minute.

5. DNA precipitation: without disturbing the resin layer, the DNA containing upper phase was transferred to a new 1.5ml tube. 2 volumes of cold ethanol were added to the tubes and inverted several times until the DNA strands appeared. These were then spooled using out of the solution using a sterile innoculation loop. The DNA was re-dissolved in sterile DNase free water by mixing on a rotary mixer for 2 hours.

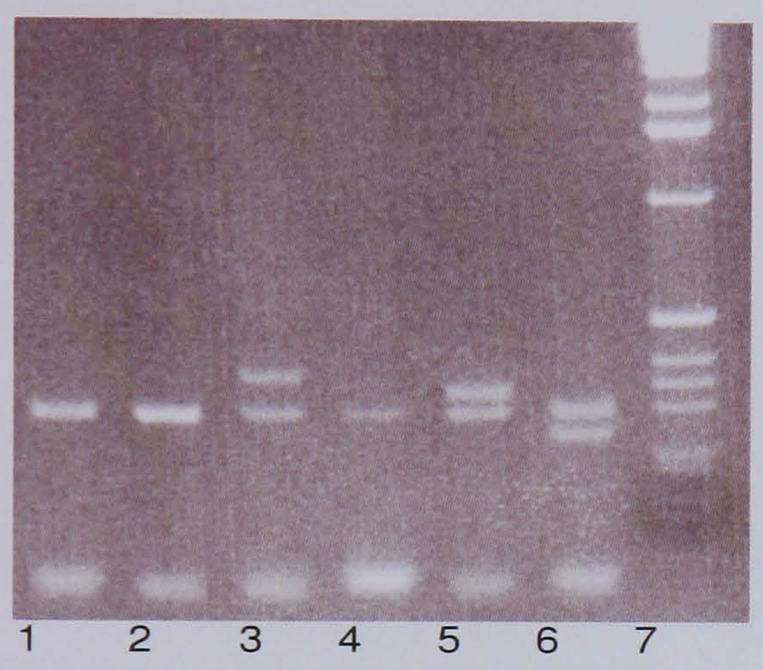
5.6 DNA GENOTYPING

VNTR GENOTYPE POLYMORPHISMS

The DNA sequence spanning the GPIbα VNTR polymorphism was amplified by polymerase chain reaction (PCR) of genomic DNA using Red Hot DNA polymerase (Advanced Biotechnologies, Surrey, UK) and the following primers: 5` ATCCACTACTGAACCAACCC3`nt 4199-4218 and 5' GAGTGATACGGGTTTTGTGG 3' nt 4451-4432 (GenBank Accession no. AC22403).

An initial DNA denaturation at 96°C for 5 minutes was followed by 32 cycles of 45 sec denaturation at 96°C, 1 minute annealing at 55°C, and 2 minutes extension at 72°C. A 10 minute final extension at 72°C completed the reaction.

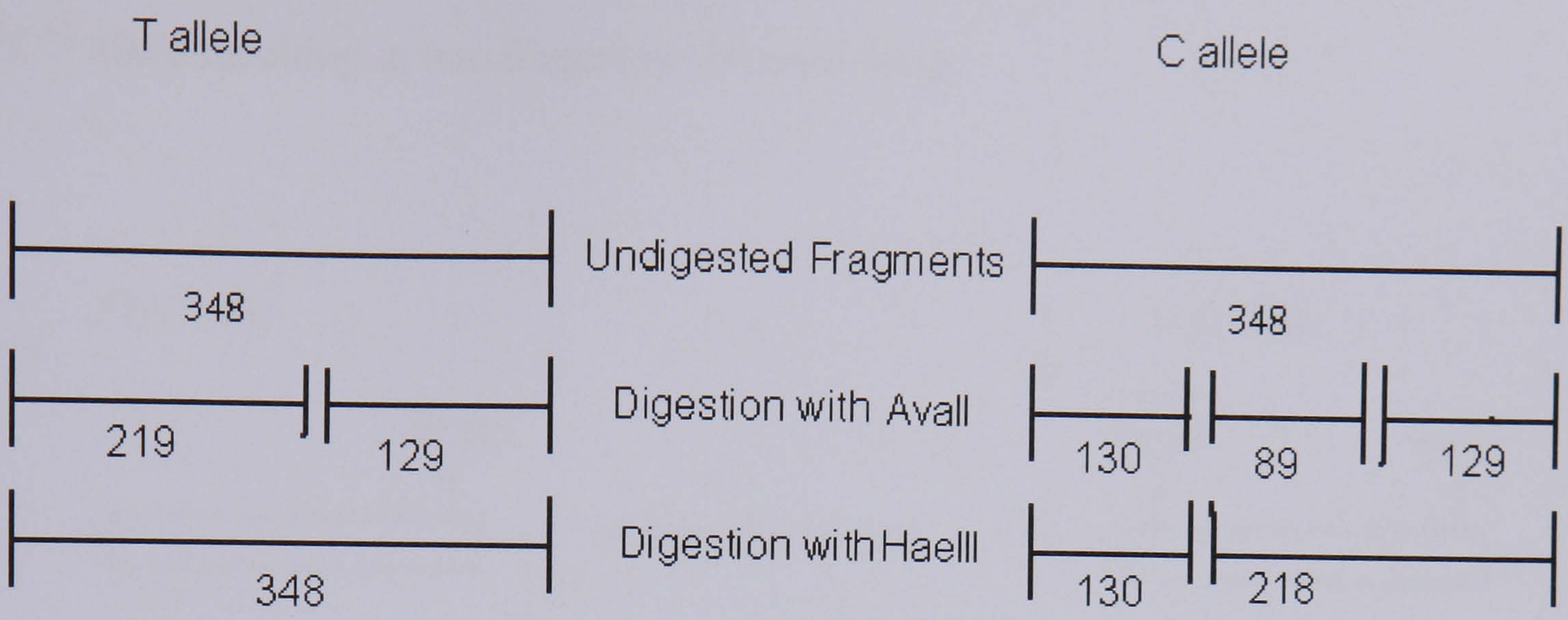
10µl of the PCR products and the restriction digests were analysed on 2.5% agarose gels (Seakem ME, FMC products, Maine, USA). The DNA fragments amplified were as follows: D allele 253bp, C allele 292bp, B allele 331bp and the A allele 370bp.



1. C/C
2. C/C
3. C/A
4. C/C
5. C/B
6. C/D
7. Marker Ladder

GPIbα -5T/C KOZAK SEQUENCE POLYMORPHISMS

The DNA sequence spanning the -5T/C Kozak polymorphism was amplified using the primers Kozak1 5` GGGAGTAGGGAGGACAGGAG 3` and Kozak2 5`AGTGTAAGGCATCAGGGTTG 3`. Amplification conditions were as above but with an annealing temperature of 59°C and using a hot start technique. The 348bp PCR products were subject to overnight digestion at 37°C with the restriction enzyme AvaII which recognises the T allele resulting in two fragments of 129 + 219bp. Any apparently homozygous CC patients were checked with HaeIII. In the presence of the C allele, HaeIII generates two fragments of 130 +218bp.





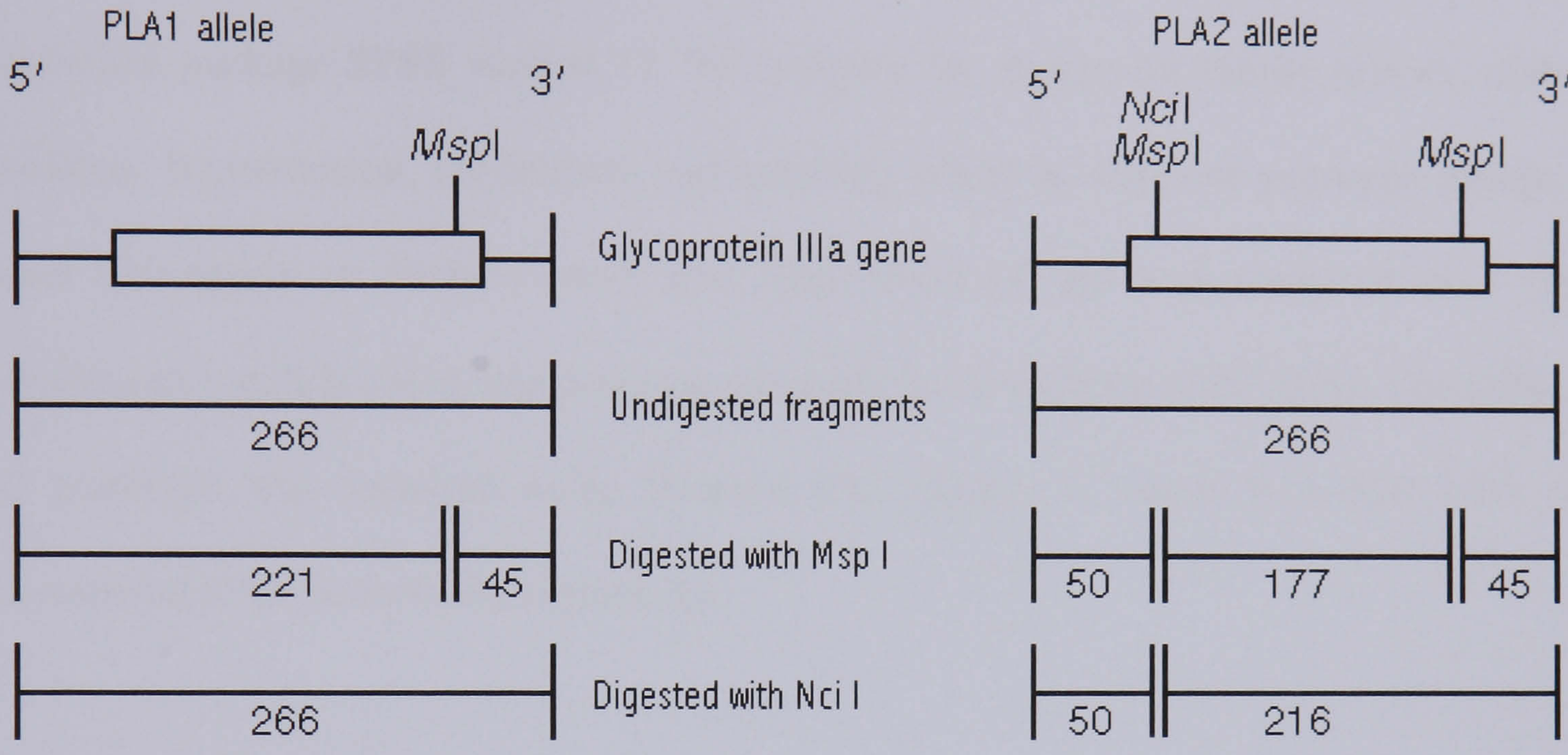
1 2 3 4 5 6 7 8 9 10 11 12 13 14

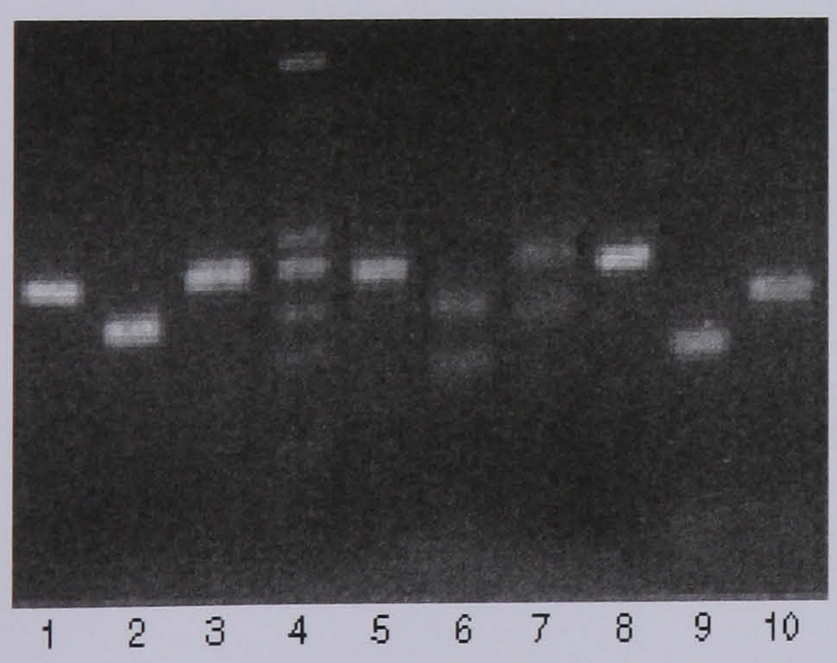
1. Marker Ladder
2. T/T
3. Uncut PCR
4 to 8. T/T
9. T/C
10 and 11. T/T
12. C/C
13 and 14 T/T

PL^{A1} AND PL^{A2} POLYMORPHISMS

The DNA sequence spanning the PL^{A1}/PL^{A2} polymorphism was amplified using the primers GP3Apla1 5`TTCTGATTGCTGGACTTCTCTT3` and GP3aPL^{A2} 5`TCTCTCCCCATGGCAAAGAGT 3`. Amplification conditions were as above but with denaturation at 94°C and annealing temperature of 53°C without using a hot start technique. The 266bp PCR product was then digested with the restriction endonuclease Msp I and run on 3% agarose gel and visualized using ethidium bromide. The homozygous PL^{A1} after digestion with Msp I results in two fragments 221+45. Any

apparently homozygous PL^{A2} patients were checked with Nci I, which recognizes the PL^{A2} allele resulting in two fragments 216 and 50bp.





1. PLA1/PLA1 uncut
2. PLA1/PLA1 cut using Msp I
3. PLA1/PLA1 cut using Nci I
4. marker ladder
5. PLA1/PLA2 uncut
6. PLA1/PLA2 cut using Msp I
7. PLA1/PLA2 cut using Nci I
8. PLA2/PLA2 uncut
9. PLA2/PLA2 cut using Msp
10. PLA2/PLA2 cut using Nci I

CHAPTER 6: STATISTICAL ANALYSIS

Genotype distribution in cases and controls were examined for significant deviation ($p<.05$) from Hardy Weinberg equilibrium. Association analysis was performed using the statistical package SPSS version 12. To compare the groups for family history, diabetes mellitus, hypertension, cholesterol and smoking effect in different genotype groups we used Chi-square or Fishers exact test. The effect of age was analyzed as a single continuous variable using linear regression analysis of variance (ANOVA). The influence of genotype was assessed using Pearson Chi square. P values less than 0.05 were considered to be statistically significant.

CHAPTER 7: RESULTS

7.1 CLINICAL CHARACTERISTICS OF STUDY GROUP

The study group consisted mainly of Caucasian (n=180) and males (n=180). Fifty patients were treated diabetic and 75 patients were hypertensive. Hypercholesterolemia was seen in 116 patients. Fifty-six patients were current smokers and 193 patients were ex-smoker or none smokers. One hundred and thirty four patients had family history of ischemic heart disease (first degree relatives). For the purpose of this thesis the total of patients were divided into two groups: patients with myocardial infarction (MI) and those without MI (no MI). The clinical characteristic of the total study group is shown in table 1.

Table 1.

Characteristics and prevalence of risk factors for Ischemic Heart Disease in the two subgroups with and without Myocardial Infarction

MI (n=88)	No MI (n=168)	p value † Odds Ratio (95% CI)
* [10] Age		
Range (years)	35-78	33-80 p= 0.117
Mean (SD)	59.06 (9.90)	56.96 (10.58)
Sex (%)		
Male	79 (89.8)	101 (60.1) p< 0.00001
Female	9 (10.2)	67 (39.9) OR 5.82 (2.61-13.38)
[1] Race		
Indian Asian	24 (27.3)	51 (30.5) p= 0.586
Caucasian	64 (72.7)	116 (69.5) OR 1.17 (0.64-2.17)
Risk Factors		
[12] Family history (%yes)	51 (60.7)	83 (51.9) p= 0.187
(% no)	33(39.3)	77 (48.1) OR 1.43 (0.81-2.54)
[3] Diabetes (%yes)	22 (25.3)	28 (16.9) p= 0.110
(% no)	65 (74.7)	138 (83.1) OR 1.67 (0.85-3.28)
[5] Hypertension (%yes)	30 (34.9)	45 (27.3) p= 0.211
(% no)	56 (65.1)	120 (72.7) OR 1.43 (0.78-2.60)
[1] Cholesterol (%yes)	46 (52.9)	70 (41.7) p= 0.088
(%no)	41 (47.1)	98 (58.3) OR 1.57 (0.90-2.74)
[7] Smoker (%yes)	20 (23.8)	36 (21.8) p= 0.72
(% no)	64 (76.2)	129 (78.2) OR 1.12 (0.57-2.18)
[16] Ex-smoker (% yes)	49 (60.5)	83 (52.2) p= 0.22
(% no)	32 (39.5)	76 (47.8) OR 1.40 (0.79-2.74)

* [n] Indicates the number of missing values

† p values are indicated together with the Odds Ratio and (95% confidence intervals)

I also looked at clinical characteristics of the 84 male patients selected for functional study. The comparison was done by dividing the patients into two groups: patients with

documented myocardial infarction (n=28) and those without MI (n=56). The characteristics and prevalence of risk factors for Ischemic Heart Disease in the two subgroups are shown in table 2.

Table 2:

	MI	No MI	*p* value
Age years, mean(SD)	63(6.1)	61(9.1)	0.223
Smoker, n(%)	9 (32.1)	12(21.4)	0.21
Diabetes, n(%)	7(25)	13(23.2)	0.53
Hypertension, n(%)	23(82.1)	41(73.2)	0.267
Hypercholesterolemia, n(%)	8(28.6)	15(26.8)	0.529
vWf Ag level, U/ml ,mean (SD)	1.15(0.79)	1.0(0.44)	0.21

7.2 RESULTS IN RELATION TO THE EXTENT OF CORONARY ATHEROMA

I also looked at the correlation between the severity of coronary disease and increased risk of myocardial infarction (table 3). The difference in the degree of vessel disease between the MI and non-MI groups was highly significant, $p<0.001$. Multivessel disease was more frequent in patients with previous MI than those without MI. The non-MI patients were mostly those with normal coronaries.

Table 3:

The distribution and severity of vessel disease detected by coronary angiography in the MI and non-MI patient groups

Vessel disease	MI	No MI
O*	2 (2.3%)	79 (47.0%)
X†	4 (4.6%)	14 (8.4%)
1‡	10 (11.5%)	17 (10.1%)
2‡	24 (27.3%)	23 (13.7%)
3‡	46 (52.3%)	35 (20.8%)

*O indicates normal coronaries

† X indicates irregularities on angiogram

‡1,2 and 3 indicate the number of diseased vessels.

§There were 2 subjects in the MI group whose degree of vessel disease was unavailable.

The sub analysis for the 84 patients is different because of small sample group. The extent of coronary disease were divided into those with normal coronaries or minor irregularies but without significant stenosis and those with one or more vessel disease .

Vessel disease	MI(%)	No MI (%)
No vessel disease(%)	12(42.8)	27(48.2)
> One vessel	16(57.2)	29(51.8)

ρ value 0.664

In this small group of patients the only significant atherosclerosis risk factor to influence the severity of coronary disease was hypercholesterolemia .

Table 4:

Cardiac risk factor (p value)	Normal coronaries Number (%)	More than one VD Number (%)
Smoking (p=0.08)	21 (38%)	35 (62%)
Hypercholesterolemia (p=0.0001)	15 (13%)	101 (87%)
Diabetes (p=0.45)	25 (50%)	25 (50%)
Hypertension (p=0.12)	31 (42%)	44 (58%)
Age (p=0.27)	61±9.2	63±7.4

7.3 VWF ANTIGEN LEVEL

Plasma von Willebrand factor antigen level was similar in MI and non-MI groups. The analysis of all genotypes also revealed similar plasma level in all genotype subgroups. (Figure 1) However, there was a difference in the plasma level of vWF in patients with coronary disease than those without (0.88±0.02 vs 1.06±0.59, p=0.003). However, the difference in the plasma vWF did not have any effect on the closure times measured by Col/Epi and Col/ADP cartridges in patients with normal coronaries and those with at least one or more diseased vessels. (Table 5)

In the 25 healthy controls group the vWF plasma level was 1.07±0.59.

7.4 PLATELET FUNCTION

The results of Platelet reactivity as measured by PFA-100 using collagen-epinephrine and collagen-ADP cartridges are shown here. The extent of coronary vessel disease appears to have no effect on platelet function (table 5).

Table 5: The platelet reactivity as measured by PFA-100 using Col/Epi and Col/ADP cartridges in relation to the degree of vessel disease

	Col/Epi closure time (sec)*	Col/ADP closure time (sec
Normal coronaries	252±82	93±28
One or more vessel disease	193±88	98±25

*p=0.23, † p=0.7

Results are Mean ± SD

No difference was seen in plasma vWF level and platelet reactivity in patients with previous myocardial infarction and those without MI. (table 6)

Table 6: The vWF plasma level and platelet reactivity as measured by PFA-100 using Col/Epi and Col/ADP cartridges in MI and non-MI patients

	vWF level (U/ml)*	Col/Epi closure time (sec) †	Col/ADP closure time (sec)**
MI	1.15±0.79	190±99	103±36
No MI	1.0±0.44	200±82	96±18

- p=0.2, † p=0.12, **p=0.07
- Results are Mean ± SD

Diabetes mellitus appears to be the only cardiac risk factor to marginally influence the platelet function observed by both Col/Epi and Col/ADP channels. (Table 7)

Table 7: Effect of Cardiovascular Risk Factors on Platelet Function

Cardiac risk factor		Col/Epi	Col/ADP	P value
Smoking	Yes	177.4±88.8	115.5±31.7	0.88
	No	173.6±82.1	103.9±25.8	
Hypercholesterolemia	Yes	169.8±83.9	99.5±19.7	0.6
	No	17.3±82.1	109.5±29.8	
Diabetes	Yes	159.6±67.9	106±26.6	0.05
	No	179.2±86	108±28.2	
Hypertension	Yes	181.5±85	106.4±25.2	0.2
	No	152.2±69.3	108±35.2	

- Results are Mean ± SD

Table 8: Platelet function in patients with or without history of myocardial infarction

Using Col/Epi cartridge

	closure time <100	**closure time =100-199 sec**	**closure time >200 sec**
MI	10(35.7)	10(35.7)	8(28.6)
Non-MI	8(14.3)	29(51.8)	19(33.9)
***p* value**		0.07	

Values are number (%)

Using Col/ADP cartridges

	closure time <80 sec	**closure time =80-119 sec**	**closure time >120 sec**
MI	3(10.7)	18(64.3)	7(25)
Non-MI	6(10.7)	34(60.7)	16(28.6)
***p* value**		0.939	

Values are number (%)

7.5 GPIbα POLYMORPHISMS

7.5.1 FREQUENCY OF GPIbα VNTR AND -5T/C KOZAK SEQUENCE POLYMORPHISMS

The frequency of the VNTR and –5T/C Kozak sequence polymorphisms in total population are shown in table 9 and 10. The frequency of these genotypes in our study group is similar to previously reported data. The frequency of theses genotypes were also analysed in sub-groups of patients with or without a history of myocardial infarction.

Table 9:

Genotype distribution and allele frequency of the GPIbα VNTR polymorphism in the Indian Asian and Caucasian patient subgroups

Genotype	AC	BC	BD	CC	CD	DD	Allele	A	B	C	D
% distribution of total study group	0.4	11.3	1.9	71.1	13.7	1.6		0.2	6.6	83.8	9.4
n=256	1	29	5	182	35	4	n=512	1	34	429	48
% distribution : Asian	1.3	11.8	1.3	56.6	25.0	4.0		0.7	6.6	75.6	17.1
n=76	1	9	1	43	19	3	n=152	1	10	115	26
% distribution : Caucasian	0	11.1	2.2	77.2	8.9	0.6		0	6.7	87.2	6.1
n=180	0	20	4	139	16	1	n=360	0	24	314	22

Table 10:

Genotype distribution and allele frequency of the GPIbα -5T/C Kozak polymorphism in the study group

Genotype	TT	TC	CC	Allele	T	C
% distribution	73.4	25.8	0.8		86.3	13.7
n=256	188	66	2	n=512	442	70

7.5.2 VNTR POLYMORPHISMS AND RISK OF MYOCARDIAL INFARCTION

Genotype distribution of the GPIbα VNTR polymorphisms between the MI and no-MI patient groups is shown in table 10. No significant association was found between the VNTR genotypes and MI, p=0.60.

Considering the CC genotype v all other VNTR genotypes, there was a marginal association, p=0.059, OR 1.8, 95%CI 0.94 - 3.48.

VNTR	AC	BC	BD	CC	CD	DD
MI n=88 (%)	0(0.0)	6(6.8)	3(3.4)	69(78.4)	8(9.1)	2(2.3)
No MI n=168 (%)	1(0.6)	23(13.6)	2(1.2)	113(67.3)	27(16.1)	2(1.2)

7.5.3 GPIbα -5T/C KOZAK POLYMORPHISMS AND RISK OF MYOCARDIAL INFARCTION

Genotype distribution of the GPIbα -5T/C Kozak sequence polymorphisms between the MI and no-MI patient groups are shown in this table.

-5T/C Kozak	TT	TC	CC
MI n=88 (%)	75 (85.2)	11 (12.5)	2 (2.3)
No MI n=168 (%)	113 (67.3)	55 (32.7)	0 (0.0)

A significant association was found between the –5T/C Kozak TT genotype and MI, p<0.001, OR 2.10, 95% CI 1.11 – 4.02

The TT v TC+CC Kozak genotype also shows a significant association with MI, p=0.002, OR 2.81, 95% CI 1.37 – 5.82.

7.5.4 EFFECT OF GPIbα POLYMORPHISMS ON PLATELET REACTIVITY

Comparison of GPIbα_VNTR and –5T/C Kozak sequence genotypes on closure time measured by PFA-100™ are shown. Platelet reactivity was significantly enhanced in the VNTR CC compared with BC and CD genotype (mean (SD), closure time: 117.66 (46.41) sec, closure time: 167.35 (73.3) sec, closure time: 247.96 (67.54) sec respectively, p=0.001) using Col/Epi cartridges. (Figure 1) A greater number of CC VNTR subjects had a closure time less than 100sec. (43%CC,17%BC,0%CD, p=0.0001). In contrast, the CD genotype had the longest closure time measured by Col/Epi (69%, CT>200, p=0.0001). The TT genotype of the –5T/C Kozak sequence polymorphism showed a marginal increase in platelet reactivity compared to the TC genotype (26% v 12.5% for closure time <100, 23% v 46% for closure time >200, p=0.05). No difference was seen in closure time measured by Col/ADP channel.

Table 11:

Using Col/ Epi cartridge

Genotype	closure time <100 sec	closure time =100-199 sec	closure time >200 sec
CC VNTR	13(43.3)	15(50)	2(6.7)
BC VNTR	5(17.9)	16(57.1)	7(25)
CD VNTR	0	8(30)	18(69.2)
p value	**0.0001**		
TT Kozak	14(26.9)	26(50)	12(23.1)
TC Kozak	4(12.5)	13(40)	15(46)
***p* value**	**0.05**		

Using Col/ADP cartridge

Genotype	closure time <80 sec	closure time = 80-119 sec	closure time >120 sec
CC VNTR	3(10)	20(66.7)	7(23.3)
BC VNTR	3(10.7)	17(60.7)	8(28.6)
CD VNTR	3(11.5)	15(57.7)	8(30.8)
p value	**0.971**		
TT Kozak	6(21.5)	31(59.6)	15(28.8)
TC Kozak	3(9.4)	21(65.6)	8(25)
***p* value**	**0.857**		

Values are number (%)

7.6 GPIIb-IIIa POLYMORPHISM

7.6.1 FREQUENCY OF THE PL^{A1}/PL^{A2} POLYMORPHISMS

PL^{A} genotype distribution in our study group was similar to previously reported data and did not differ from Hardy-Weinberg equilibrium.

Genotype distribution and allele frequency of the GPIIb-IIIa polymorphism in the study group

Genotype	A1/A1	A1/A2	A2/A2	Allele	A1	A2
% distribution	71.6	26.3	2.1		85	15
n=256	184	67	5	n=512	435	77

7.6.2 CORRELATION BETWEEN THE PL^{A1} AND PL^{A2} GENOTYPES AND MYOCARDIAL INFARCTION

The genotype frequency and risk of myocardial infarction

	$PL^{A1/A1}$	$PL^{A1/A2}$	$PL^{A2/A2}$
MI n=88 (%)	68(77.3)	18(20.5)	2 (2.2)
No MI n=168 (%)	116(69.3)	49(29)	3 (1.7)

P value=0.63

7.6.3 CORRELATION BETWEEN THE PL^{A1} AND PL^{A2} GENOTYPES AND SEVERITY OF CORONARY DISEASE

The PL^{A1} and PL^{A2} genotypes had no effect on the degree of coronary atheroma. Although not statistically significant, there is a suggestion that homozygosity for PL^{A2} is associated with more severe coronary disease.

Vessel disease		A1/A1	A1/A2	A2/A2
O*	(n=81)	60 (74%)	21 (26%)	0
X†	(n=18)	9 (50%)	9 (50%)	0
1‡	(n=27)	21 (77.7%)	5 (18.5)	1 (3.8%)
2‡	(n=47)	34 (72.3%)	11 (23.5%)	2 (4.2%)
3‡	(n=81)	60 (74%)	19 (23.6%)	2 (2.4%)

7.6.4 VWF LEVEL IN SUBGROUPS OF PL^{A1} AND PL^{A2} GENOTYPES

The von Willebrand factor level was similar in the three groups. Although there was a trend toward slightly raised plasma vWF in PL^{A2} allele.

7.6.5 PL^{A1} AND PL^{A2} GENOTYPES AND PLATELET REACTIVITY

PL^{A1} and PL^{A2} polymorphisms had no effect on the platelet function. The closure time using collagen-ADP cartridges was 95.4±24.3 sec for PL^{A1} heterozygotes, 90.7±24.2 sec for PL^{A1} homozygotes and 105±14.4 sec for PL^{A2} homozygotes. Also no effect was detected using Col/Epi cartridges. The closure time using Col/Epi are as follows: 200.4±82.7 secs for $PL^{A1/A1}$, 190.3±99.7 secs for $PL^{A1/A2}$, 193.2±88.9 secs for $PL^{A2/A2}$. (Figure 4).

7.7 EFFECT OF COMBINED GENOTYPES ON PLATELET FUNCTION

7.7.1 COMBINED GPIbα VNTR AND –5T/C KOZAK SEQUENCE POLYMORPHISMS ON PLATELET FUNCTION

The combined effect of VNTR polymorphisms and –5T/C Kozak sequence polymorphism on closure time in response to Col/Epi or Col/ADP is shown in table 12. Patients with combined CC VNTR/TT Kozak genotypes demonstrated the shortest closure time and patients with the CD VNTR/TC Kozak polymorphisms had the longest closure time (mean (SD), closure time: 104.05 (26.2) v 276.9 (50.1), p=0.002).

Table 12:

Genotypes	Col/Epi closure time	Col/ADP closure time
BC + TC* N=11	201.45(82.61)	103.72(19.56)
BC + TT* N=17	145.29(59.04)	108.35(29.64)
CC + TC* N=10	144.9(65.25)	105.2(36.32)
CC + TT* N=20	104.05(26.2)	106.95(26.79)
CD + TC* N=11	276.9(50.1)	113.72(34.29)
CD + TT* N=15	226.73(72.19)	103.26(23.48)
P value	0.002	0.839

*-5T/C Kozak sequence polymorphism
N number
Values are mean(SD)

GENERAL CONCLUSIONS

Coronary atherosclerosis with plaque disruption resulting in superimposed thrombosis is the main pathological mechanism in the acute coronary syndromes of unstable angina, myocardial infarction and sudden death with the amount and extent of associated thrombus formation affecting the final clinical outcome (Fuster V, *et al.* N Engl J Med 1992). The composition of thrombi found in arterial thrombosis is predominantly that of a platelet-rich core attached to an underlying atheromatous plaque in an epicardial segment of the corresponding coronary artery branch (Davies MJ, *et al.* Eur Heart J 1989). Blood passing through arteries that have been narrowed by atheroma will be subjected to higher local shear forces resulting in the possibility of increased risk of thrombotic occlusion via the shear dependant vWF and GPIb-IX-V interaction. It therefore appears that platelet adhesion to vascular sub-endothelium and subsequent platelet aggregation, are critical elements in the mechanism of coronary thrombosis (Fuster V, *et al.* Circulation 1990).

In the arterial circulation, platelets adhere to surface bound von Willebrand factor (vWF) via the platelet glycoprotein (GP) Ib-IX-V complex. This receptor is shear dependant and is "switched on" in the arterial microvasculature and environments such as those found in the coronary artery (Kroll MH, *et al* Blood 1996, Roth Gj, Blood 1991, Berndt MC, *et al,* NZ J Med 1995, Savage B, *et al,* Cell 1996).

There is an increasing body of evidence that predetermined genetic factors can influence haemostatic balance and consequently thrombotic risk (Lane DA, *et al,* Blood 2000). We hypothesized that two DNA polymorphisms in the GPIbα gene may be significant factors influencing the risk of myocardial infarction (MI). Firstly, the GPIbα –5T/C Kozak polymorphism which may potentially alter the level of expression of GPIb-IX-V, and

secondly, the GPIbα variable number tandem repeat (VNTR) which alters the structure of this essential platelet adhesion receptor.

We designed this study to explore the potential of the -5T/C Kozak and VNTR polymorphisms of GPIbα to act as independent risk factors for MI. By comparing a MI with a non-MI group of patients rather than incorporating a traditional "normal control group" we were able to compare two equivalent populations who were evenly matched with respect to other known risk factors (Table 1,2). Within any normal control group a degree of latent vessel disease would still be expected, therefore both the MI and non-MI cohorts were recruited from patients with known coronary angiographic data. Both study groups were well characterised with detailed clinical information. This enabled us to dissect out the influence of each of the two polymorphisms on the occurrence of MI and degree of vessel disease.

The pathogenesis of thrombosis hinges on an imbalance in the haemostatic system and polymorphisms of candidate coagulation genes have therefore been the targets of recent study. For example the PL^{A2} allele of GPIIIa has been associated with MI at a young age and also been linked with sudden death due to MI in a series of middle aged men (Ardissino D, *et al,* Blood 1999, Mikkelsson J, *et al,* Arterioscler Thromb Vasc Biol 1999).

Arterial thrombosis occurs predominantly in the older population and is affected by many environmental factors that may also act synergistically with pre-existing genetic influences. The GPIb-IX-V receptor functions within a high shear environment and altered binding to vWF due to changes in structure or levels of surface expression may therefore be associated with the development of coronary thrombosis and subsequent MI. The Kozak –5T/C and VNTR polymorphisms of the GPIbα gene may therefore hypothetically modulate the efficiency of platelet adhesion in the arterial

microvasculature. If this were the case, the genotype distribution may be expected to vary significantly between a group of patients with MI and a matched group without MI.

The results of our study show a significant difference in the genotype frequencies between the two study groups. In particular, the TT Kozak genotype was more frequent among those patients with MI and Kozak TC genotypes were more frequent in those patients without MI. Based on the *in vitro* work it might be expected that the -5C/C genotype would confer the greatest risk (Afshar-Kharghan V, *et al,* Blood 1999). Paradoxically, the results of our study suggest that the presence of the -5T/T genotype in the Kozak sequence is significantly associated with the occurrence of MI, $\chi^2 = 9.56$, p= 0.002, OR 2.81, 95%CI 1.37 to 5.82. Furthermore, it could be argued that the presence of the Kozak C allele may confer a degree of protection against a coronary event when present in the heterozygous state (TC 12.5% in MI, 32.7% in non-MI). This may give the impression that the TT genotype is associated with increased risk. Clearly the Kozak – 5T/C polymorphism has a potentially significant association with coronary thrombosis completed by MI and this will need a larger prospective study for clarification.

An elegant *in vitro* study in CHO cells investigated the effect of the T and C Kozak alleles on surface expression of GPIb-IX-V and suggested that the Kozak C allele results in increased expression of the complex that may correlate with increased platelet adhesion (Afshar-Kharghan V, *et al,* Blood 1999). However, studies in a static tissue culture system do not replicate the physiological environment that exists in the high shear forces of the arterial vasculature on which the adhesive function of this complex depends. It must also be considered that other regulatory sequences and transactivating factors may modulate *in vivo* gene expression (McBratney S, *et al,* Molecular and Cellular Biology 1996). It is therefore still credible that a C allele may confer protection, or alternatively a T allele act as a potential risk factor in the physiological situation.

Published data so far is inconsistent in its findings. In a series of CHD and CVA (cerebrovascular accident) patients, GPIb-IX-V expression was apparently not associated with the –5T/C Kozak dimorphism and showed no association with arterial thrombosis (Corral J, *et al,* Thrombosis and Haemostasis 2000).

A short report has found no association between the –5T/C Kozak sequence polymorphism and MI. (Croft SA, *et al,* Blood 2000) Their study populations however had significant differences in hypertension, cholesterol and particularly in smoking and diabetes between the normal and patient groups. Although the numbers tested were higher, this is not a truly matched control group and for neither patient nor control population were details of coronary vessel disease provided The data published from a series of non-fatal stroke and non-fatal MI in young women, suggested that the presence of a C allele apparently reduced the risk of MI in agreement with our results, but had no effect on CVA (Frank MB, *et al,* Blood 2001). Our study was based on MI survivors and this may mask the true effect of genotype on risk. Interestingly, in our study, the only 2 patients who were CC were in the MI group although this number is too small for statistical analysis. It could be argued, that if patients with sudden death due to acute coronary syndromes could be included, then a different picture of genotype v risk would emerge. The number of subjects in this study, although relatively small, still had adequate statistical power to provide valuable data. A much larger prospective study will address this issue.

A recent study of 1461 white participants genotyped for 85 genetic variants found no association between any of the genotypes and acute coronary syndrome. (Morgan TM *et al*, JAMA. 2007). The negative results in their study may be explained by varied effects of risk variants in different genetic backgrounds. Their results does not bear on the validity of positive associations reported in different populations and clinical

subgroups(eg, analyses sub stratified by age, sex or a clinical variable, such as hypertension, hyperlipidemia or smoking status).

Although our study population consisted of patients undergoing an invasive diagnostic procedure, some with suspected coronary artery disease, the overall distribution of alleles and genotypes observed was consistent with previous reports, based on general population screening. This study therefore provides some evidence that genetically determined differences in the structure of GPIbα may partly determine the risk of developing coronary thrombosis with consequent MI. It is interesting that no relationship was found between the severity of atheroma (stenosis) and the GPIbα VNTR or –5T/C Kozak genotypes, therefore the differences in risk associated with these polymorphisms appear to be more closely linked to thrombosis than to atheroma.

As the arterial lumen narrows in atheromatous coronary artery disease the turbulent flow increases the shear forces. The presence of atheroma appears to be a necessary, but not sufficient factor, for the occurrence of coronary thrombosis. This study was designed to elucidate the frequency of genotypes in coronary artery disease completed by thrombosis. It was therefore quite appropriate that the whole study population, including the controls, be recruited from a population of patients likely to have atheromatous coronary disease rather than a population of healthy volunteers. No significant difference was found between the two study groups in the prevalence of established risk factors for coronary disease (Table 1,2) and therefore no further stratification of the sample was necessary.

The different GPIbα VNTR polymorphic variants determine the distance between the vWF binding domain and the platelet surface. Some of these could create a minor spatial advantage for platelet adhesion to exposed vascular sub-endothelium. It has been implied that a longer GPIbα polypeptide may lower the threshold of shear force required for

platelet adhesion to immobilised vWF (Lopez JA, *et al,* The J Biol Chem 1992). A geometrically balanced arrangement of multiple complexes based on a hypothetical conformation of four copies of GPIb-IX-V and two copies of GPV has been proposed (López JA,*et al,* Advances in Vascular Biology, Volume Six, Platelets,Thrombosis and the Vessel Wall, 2000). The adhesion efficiency of the complex may therefore have subtle differences depending on whether it is assembled from GPIbα VNTR alleles of either equal or unequal length. In this study the CC VNTR genotype showed a marginal association with MI when compared against all other VNTR genotypes, χ^2 = 3.57, p= 0.059, OR 1.80, 95%CI 0.94 to 3.48 (Table 9). However, in a previous study of Mediterranean Caucasian patients the C/B VNTR genotype was reported as showing an association with CVA and CHD but not DVT in a study of patients in the age range 34-85(Gonzales-Conejero R, *et al,* Blood 1998). All patients had individually matched controls with no documented history of heart disease, although no information was available regarding the degree of vessel disease. In a cohort of Japanese patients, the A allele showed an association with CHD (Murata M, *et al*, Circulation 1997). As reports to date are inconsistent, a definitive answer will require a much larger prospective study to elucidate any potential association with CHD and MI.

The homozygous state also appears to be an important factor as the VNTR CC genotype was more frequent (78.4% v 67.3%) and the CD genotype less frequent (9.1% v 16.1%) among the patients with MI compared to those without MI respectively. Why expression of a shorter GPIbα receptor component may be associated with MI is open to speculation and further *in vitro* and *in vivo* studies are required.

As a result of our first study, we postulated that the difference in risk associated with these polymorphisms appear to be more closely linked to thrombosis than to atheroma. To further evaluate this hypothesis we designed this study to look at the effect of these

polymorphisms on platelet function of the same group of patients with or without angiographic evidence of coronary atherosclerosis using a method that mimics pathophysiological conditions *in vivo* involving platelet binding to vWF binding under condition of high shear stress. We therefore used the platelet function analyser, PFA-100^{TM} that measures the time taken for platelet adhesion/aggregation to occur in response to collagen under conditions of high shear following activation by epinephrine or ADP (Mammen EF, *et al,* Semin Thromb Haemost 1998)

Having shown that the GPIbα genotypes CC of the VNTR and Kozak –5TT increase risk of non fatal myocardial infarction, we then hypothesized that these genotypes increase the risk of platelet plug formation under the condition of high shear stress generated by stenosed vessels hence increasing the risk of myocardial infarction.

There have been variable reports of closure time values as measured by the PFA-100^{TM} in the literature. The influence of various factors, including blood collection, food and diurnal variability on platelet function as assessed by the PFA-100^{TM}, has been reported (Mani H, *et al,* Platelets 2004, Paglieroni TG, *et al, Vox Sang*. 2004, Dalby MC, *et al,* Platelets. 2000).

The effect of various cardiac risk factor and degree of vessel disease on the closure time as measured by the PFA-100^{TM} is reported in table 5-7. The effect of different genotypes on the platelet function is shown in table 11. These results are in some agreement with the recent study that showed the shortest closure time in individuals carrying the Kozak -5TT or VNTR CD genotypes. However, the individuals in this study were young healthy male and female not taking aspirin (Jilma-Stohlawetz P, *et al,* Br J Haematol. 2003). Our study group consists of older males who have been taking aspirin for a considerable period of time. We also showed that patients with combined VNTR CD and Kozak -5TC genotype have the longest closure time in response to aspirin thus lower risk. It is

possible that the VNTR B and D alleles and the C allele of the –5T/C Kozak polymorphism may have a protective effect.

Enhanced platelet reactivity is well documented in acute coronary syndromes, even in patients with stable coronary disease compared to a healthy population (Gawaz M, *et al.* Thromb Res. 1996, Furman MI, *et al.* J Am Coll Cardiol. 1998).

It is accepted that such enhanced platelet reactivity has pathological importance and is associated with, if not directly responsible for, acute thrombotic events and poorer prognosis (Frossard M, *et al.* Circulation. 2004, Furman MI, *et al.* J Am Coll Cardiol. 2001). Furthermore, treatments that reduce platelet activation are known to stabilise plaques, reduce the incidence of death or MI and improve prognosis (Fuster VV, *et al.* Curr Cardiol Rep. 1999, Peterson JG, *et al.* Am J Med. 2000, Helft G, *et al.* Arterioscler Thromb Vasc Biol. 2000). In our study we found a marginal increase in platelet reactivity in patients with a history of acute myocardial infarction (table 6). We demonstrated that more MI patients had the shortest closure time compared to non-MI patients, although, this did not reach statistical significance. This can be explained by the relatively small sample size particularly in the case of MI patients (28 v 56).

We found no correlation between the severity of coronary artery disease (CAD) and platelet function (table 5). This is consistent with the finding that, in patients with stable angina, the degree of luminal narrowing does not predict the likelihood of future myocardial infarction (Ambrose JA, *et* al. Heart 1998). However patients with raised cholesterol seem to have more severe CAD. This is consistent with previous studies showing that hypercholesterolemia is one of the most significant risk factors for the severity of CAD (Wang XL, *et al.* Circulation 1994).

In contrast to ADP and thrombin, epinephrine is a weak activator of human platelets. It interacts with α-adrenergic receptors on platelets and synergistically enhances the effects

of other aggregating agents (Lanza F, *et al.* Am J Physiol. 1988). It has also been suggested that the occurrence of stress related myocardial ischaemia and infarction may be related to haemodynamic and cardiovascular effects of sympathetic mediators and increasing plasma levels of epinephrine in the circulation (Dakak N, *et al.* Am J Cardiol. 1995). Generally, the closure time will be prolonged with the Col/Epi cartridge, but normal or near normal with the Col/ADP after the intake of aspirin (Mammen EF, *et al.* Semin Thromb Hemost 1995). In our study the effect of genotypes on the platelet function was only observed with Col/Epi not Col/ADP regardless of aspirin intake.

This thesis would indicate that GPIbα genotypes CC of the VNTR and Kozak –5TT polymorphisms are more closely associated with thrombosis than atheroma. We postulate that it is likely that genotypic variation in the GPIbα receptor may have functional significance perhaps resulting in increased binding to vWF. It has been suggested that in patients with longer GPIbα receptors (i.e. those with 3 or 4 tandem repeats), where the vWF-binding domain will be further away from the platelet surface membrane, surface bound vWF is more easily accessible to the binding site on that receptor (Lopez JA, *et al.* J Biol Chem 1992). However, in our study the VNTR CC genotype (2 tandem repeat) seems to be associated with increased platelet reactivity. We could hypothesize that a physically shorter receptor that brings the platelet into closer proximity with the sub-endothelial surface can result in more efficient binding and subsequently marginally increase the risk of coronary event. Additionally, a GPIb-IX-V receptor that has a heterozygous GPIbα genotype as in the CD VNTR, could be less favourable for efficient binding to surface bound vWF on damaged sub-endothelium under shear due to the unequal lengths of the GPIbα glycoprotein and this is supported by the increased closure times seen with the CD VNTR genotype in this study.

The effect of a substitution of proline for leucine on glycoprotein IIIa function is unclear. The presence of a proline in the peptide chain determines a different tertiary structure of the glycoprotein and this has been confirmed by previous reports of a different immunophenotype of the two polymorphic variants (Weiss EJ *et al*. Tissue Antigens 1995).

The PL^{A2} polymorphism of GPIIb-IIIa has been shown to be a strong independent risk factor for coronary thrombosis and the association with coronary thrombosis was found to be stronger in patients under the age of 60 years at the time of their acute myocardial infarction. (Weise *et al* N Engl J Med. 1996). This previously described association of myocardial infarction with the PL^{A2} polymorphism of the glycoprotein IIIa receptor gene is not evident in our study population. The prevalence of this polymorphism was similar in our group of patients with a previous myocardial infarction to that of our control group and is almost identical to that in the control population of the study reported by Weiss *et al*. This polymorphism was found in 23% of the patients with previous myocardial infarction and in 31% of the patients with no previous myocardial infarction ($p = 0.53$). There was also no significant association with the extent of coronary disease. There is no evidence, therefore, from this study of an association between the PL^{A1}/PL^{A2} polymorphisms and the occurrence of myocardial infarction.

The results of these two studies would not necessarily be contradictory if the presence of the PL^{A2} polymorphism was associated with an increased mortality rate after hospital discharge, as our patients were sampled an average of 60 months following the occurrence of myocardial infarction. The patients reported by Weiss *et al*. were sampled in the acute phase of myocardial infarction, during hospital admission. The results of our study are consistent with those reported by Marian *et al* (Curr Opin Cardiol. 1998). and Carter *et al*(Arterioscler Thromb Vasc Biol. 1998). who were also unable to document an

association between PL^{A2} and myocardial infarction. However, in these two reports the interval between myocardial infarction and the subsequent blood sampling for DNA extraction is not specified. The control group in our study is particularly appropriate to the study of coronary thrombosis and myocardial infarction as they had 53% had angiographic evidence of coronary artery disease. They are therefore very similar to our patient group, differing mainly by the absence of coronary thrombosis and myocardial infarction. The results of our study therefore do not exclude an association between coronary atherosclerosis and the PL^{A2} polymorphism.

The controls in the study by Weiss *et al.* were acutely ill patients with no evidence of ischemic heart disease, those of Marian *et al.* were healthy volunteers and the report by Carter *et al.* did not specify the nature of the control population. Our study population consisted of patients of both European (n = 180) and Asian (n = 75) origin. Only white patients were enrolled in the studies of Weiss *et al.* and Carter *et al.* The fact that 29.4% of our study population were of Indian Asian origin, the distribution of European and Asian patients between our two study groups was similar. Furthermore, the prevalence of the PL^{A2} polymorphism in our control group was similar to that of the control groups of European origin in the above mentioned studies and was also similar to that found by other authors in northern and central Europe (von dem Borne *et al* Transfusion 1990).

The main purpose of this study was to establish a correlation between the VNTR and –5T/C Kozak gene polymorphisms of the GPIbα platelet surface receptor and its function in males with documented coronary artery disease. We recognize the limitations of this pilot study. Our study population is small and did not include the less frequent VNTR A allele and homozygous CC –5T/C Kozak. A larger study may be required to fully assess the true implications of these genotypes in clinical practice and recognition of high-risk patients with CAD.

In summary, the preliminary findings of this study provide some evidence to suggest that genetically determined variations in the structure and expression of the platelet GP1bα receptor could potentially influence the risk of developing coronary thrombosis and myocardial infarction. We have demonstrated that men with known coronary artery disease and CC VNTR or TT –5T/C Kozak genotypes for the platelet GPIbα receptor have greatly enhanced platelet reactivity to collagen in the presence of epinephrine under high shear compared to those with the CD, BC VNTR and TC Kozak genotype. This suggests an association between platelet hyper-reactivity and GP Ibα receptor length polymorphism and the expression of the surface receptor. Taken in conjunction with our earlier demonstration of a link with risk of myocardial infarction we now propose a mechanistic connection between the genetically determined functional variants of this platelet surface receptor and its precise role as a risk factor for arterial thrombosis.

FIGURES

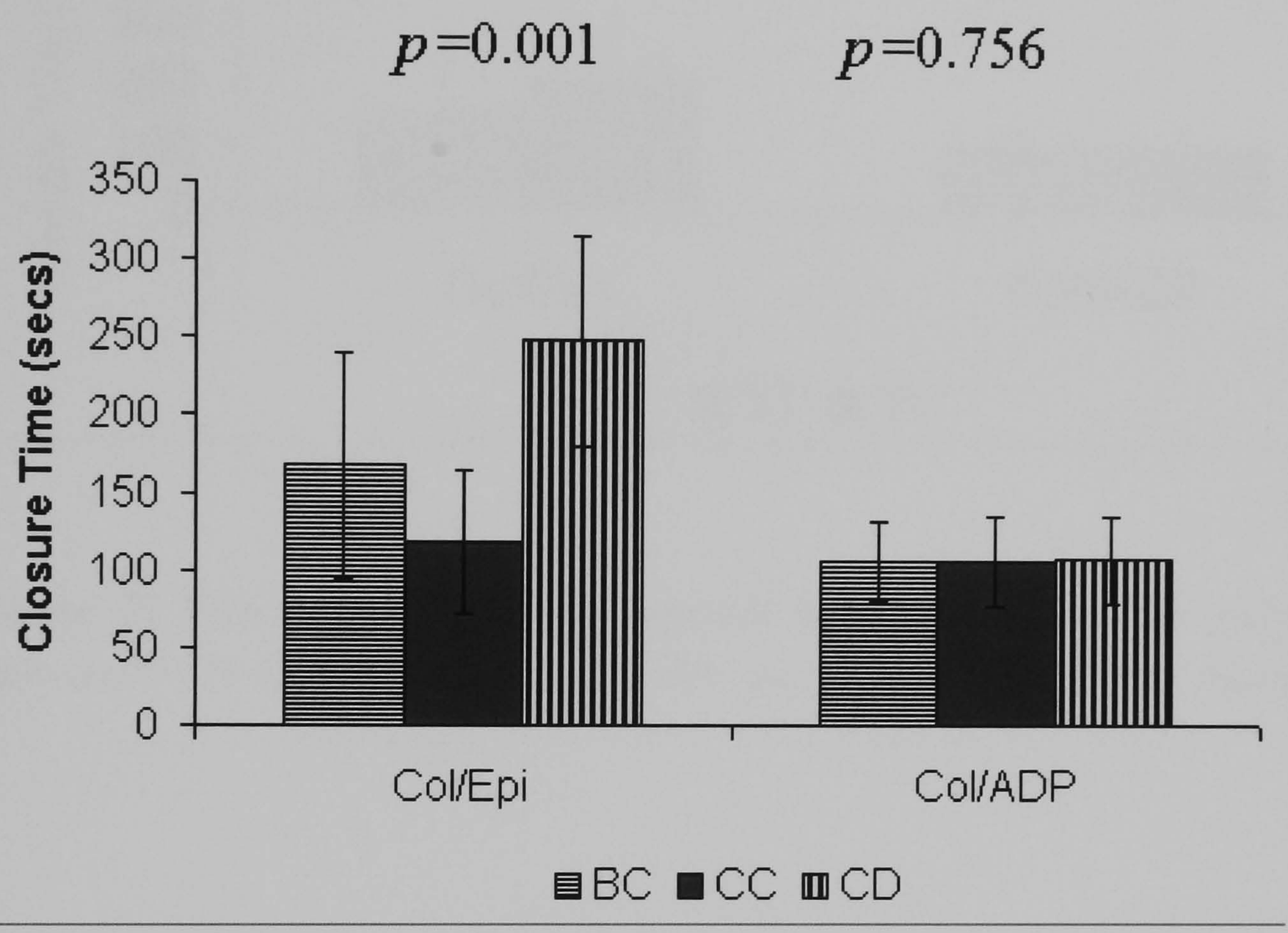


Figure 1: Effect of VNTR polymorphisms on the platelet function measured by PFA-100 using Collagen/ADP and Collagen/Epinephrine channels

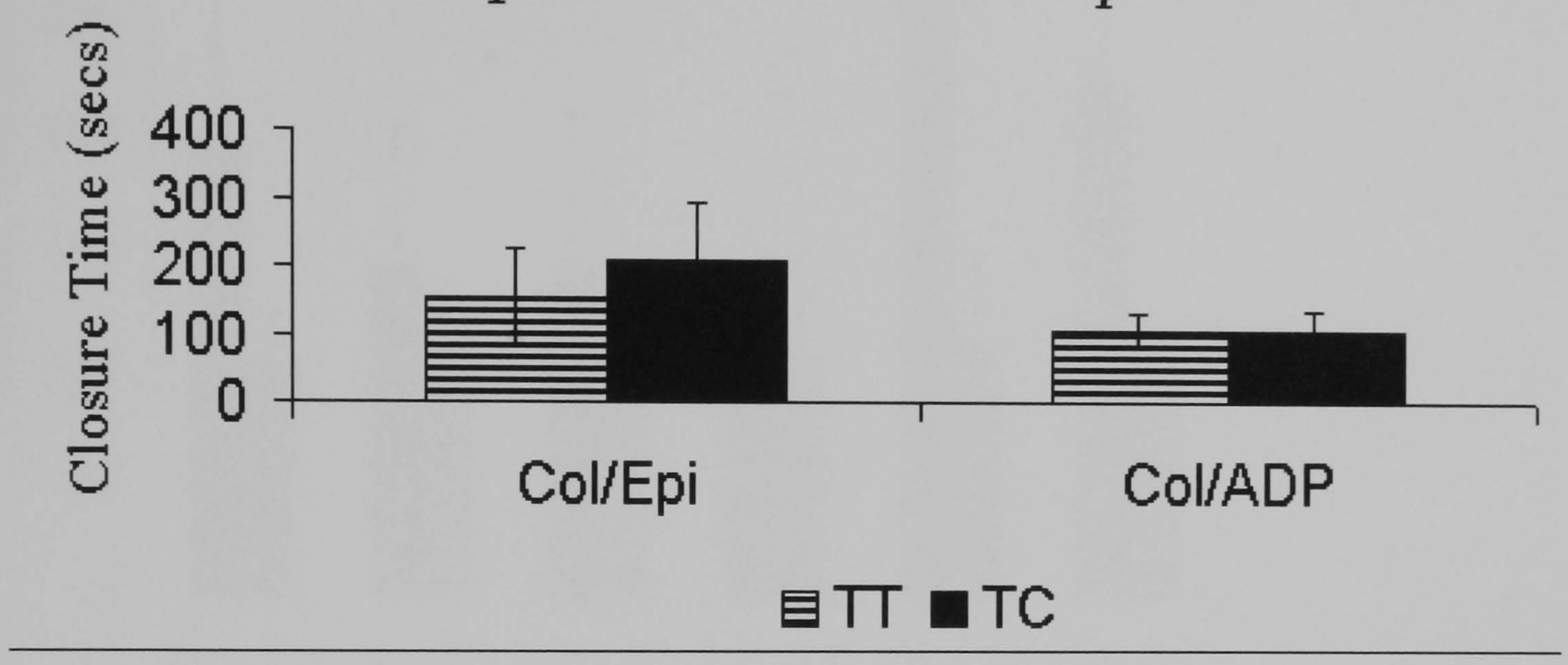


Figure 2: Effect of -5T/CKozak sequence polymorphism on the platelet function measured by PFA-100 using Collagen/ADP and Collagen/Epinephrine channels

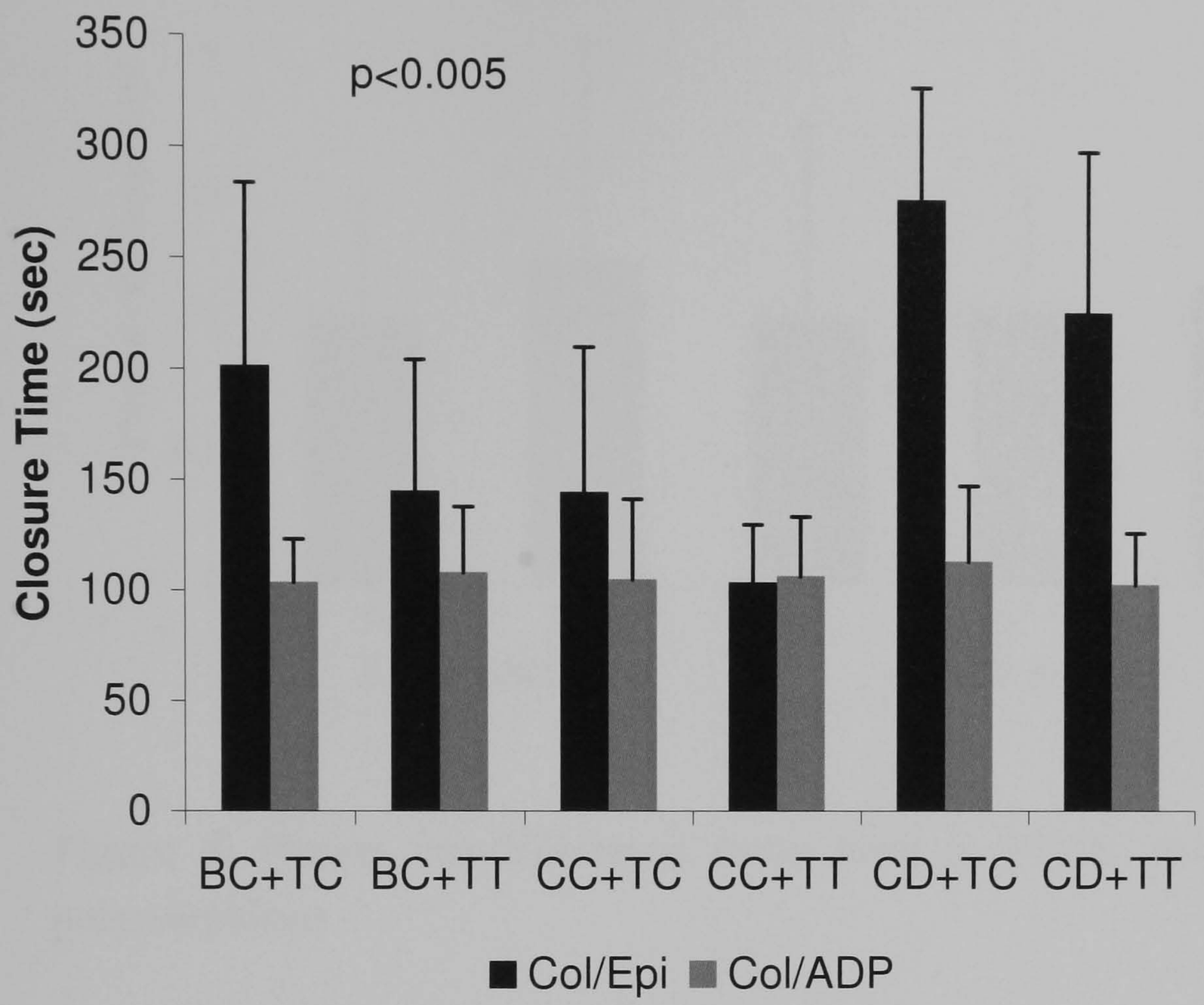


Figure 3: Effect of Combined VNTR and -5T/CKozak sequence polymorphisms on platelet function using PFA-100

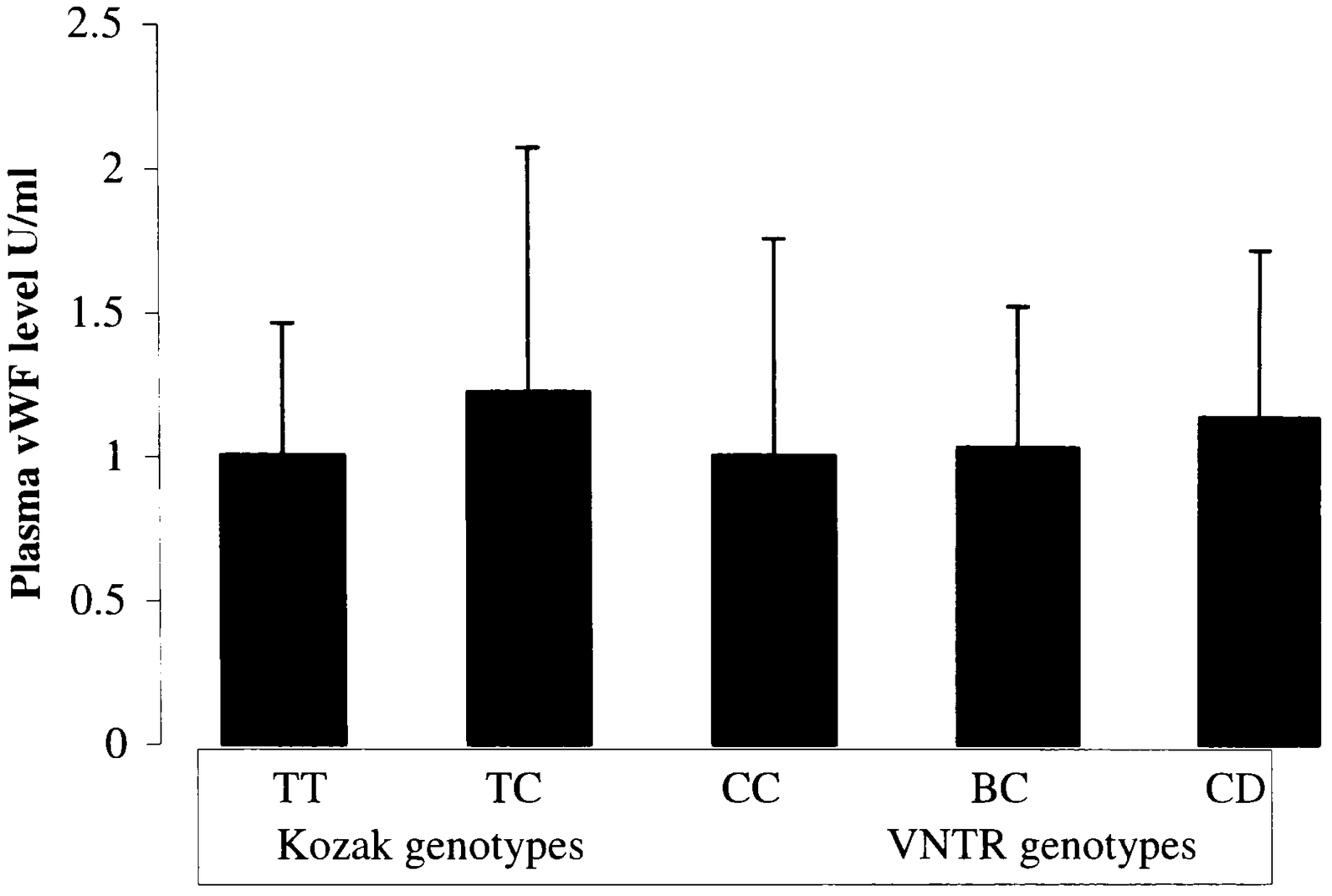


Figure 4: Plasma von-Willebrand factor level in VNTR and -5T/CKozak sequence polymorphisms

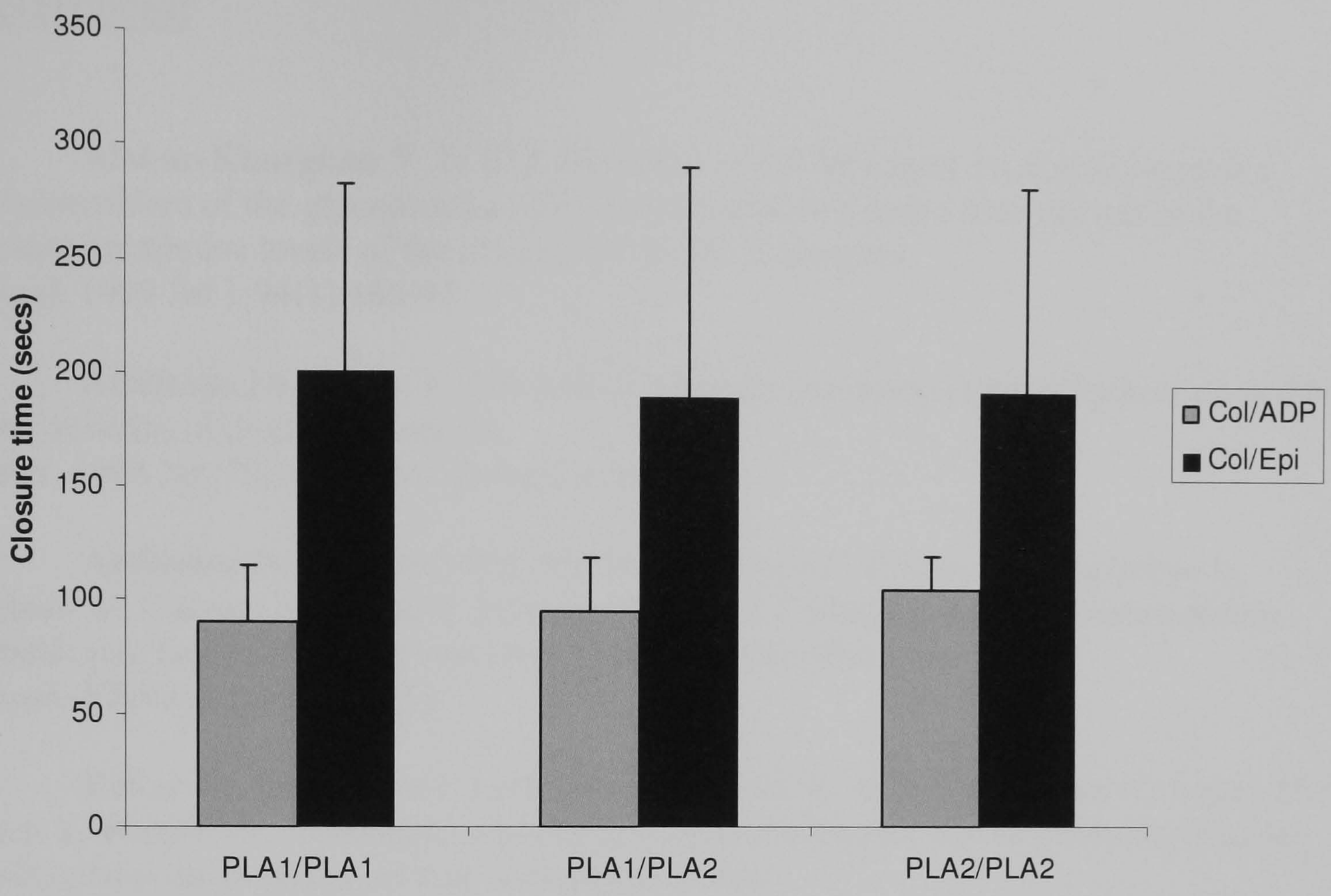


Figure 5: Effect of PLA$^{1/2}$ polymorphisms of GPIIb-IIIa on platelet function suing PFA-100

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