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Adaptation of existing methods of genotyping platelet polymorphisms associated with cerebrovascular disease for use within the routine laboratory setting and determining the relative frequency in a cohort of stroke patients

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2009-11-09T13:06:00Z
http://hdl.handle.net/10539/7426

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CHAPTER ONE

1. INTRODUCTION

1.1 Stroke

Stroke is a multifactorial, debilitating disease that afflicts numerous people in both the developed and developing countries of the world (Murray *et al.*, 1997). It is a major public health problem and is one of the leading causes of death world-wide (Zagaria, 2004). Hankey *et al.*, (1999) suggested that the risk of a recurrent stroke is high amongst individuals surviving a stroke episode, with at least one in six suffering a second stroke within 5 years.

Stroke is characterized by the sudden loss of blood circulation to the brain with concomitant loss of neurologic function (Meschia, 2002). The pathophysiology of stroke is due to either rupture or occlusion of blood vessels in the brain. These events deprive the brain of essential nutrients and oxygen, leading to brain necrosis with loss of function and irreparable damage (Larrue *et al*., 1997).

Stroke may be broadly classified into either ischaemic stroke or haemorrhagic stroke. Ischaemic stroke is caused by a blockage of blood vessels in the brain, which severely reduces blood flow. Ischaemic strokes can be either thrombotic or embolic. Thrombotic stroke occurs when a thrombus in one of the arteries blocks the blood supply to the brain while an embolic stroke occurs when part of a thrombus formed at a distal part of the vasculature dislodges and is then swept through the bloodstream

and subsequently lodge in narrower blood vessels that are commonly found in the brain (Zoppo, 1998).

Ischaemic strokes may also be caused by a rupture of an atherosclerotic plaque, leading to activation of both platelets and the coagulation cascade, triggering thrombosis. The latter could lead to complete occlusion and tissue ischaemia. Furthermore, there is also growing evidence suggesting that inflammation plays an important role in the pathophysiology of ischaemic stroke (Taman *et al.,* 2005).

Haemorrhagic stroke occurs when a blood vessel in the brain bursts or ruptures from an aneurysm. The haemorrhage may be intracerebral haemorrhage or occur in the subarachnoid space. Intracerebral haemorrhage is often associated with hypertension, causing small vessels in the brain to rupture. The ruptured vessels allow blood to infiltrate and disperse into the surrounding brain tissue damaging the cells (de Jong *et al.,* 2002).

Subarachnoid haemorrhage occurs when an artery supplying blood to the brain ruptures usually at the site of an aneurysm, causing blood to be trapped between the surface of the brain and the skull. Stroke can be potentially fatal, with those surviving having varying degrees of disability (Wilkinson *et al.,* 1997).

Carma *et al.,* (2001) reported that in the United States of America, ischaemic stroke is the most common type of stroke accounting for about 70-80% of all strokes. In a report that was submitted to the South African Medical Research Council as “Initial burden of disease estimates for South Africa 2000”, Bradshaw *et al.,* (2000) identified stroke as one of the most common causes of mortality in South Africa. Furthermore, the mortality profile for the year 2003 showed that men over 65 years had a higher incidence of ischaemic heart disease followed by stroke while women of the same age group showed higher incidence of stroke (Bradshaw *et al.,* 2005). Since stroke is associated with long-term disability and hospitalisation, it places an immense burden on an already cash strapped national health system.

Stroke is a multifactorial disorder with various risk factors playing a vital role in the aetiology of the disease (Hillen *et al*., 2003). Risk factors such as stasis, hypercoagulability and intimal changes (Virchow’s triad) may independently increase the risk of stroke (Thorvaldsen *et al.,*1995).

Effective prevention remains the best treatment for reducing the risk of stroke as reported by Adams *et al.*, (2005). Primary prevention is particularly important because more than 70% of of all strokes are first time events (Feinberg *et al*., 1996). There are various established risk factors, that may be divided into either modifiable or non-modifiable risk factors.

Modifiable risk factors for ischaemic stroke, include hypertension, smoking, hypercholestrolaemia, obesity and diabetes mellitus. Hypertension is a major risk factor for both cerebral infarction and intracerebral haemorrhage (Fields *et al*., 2004). The higher the blood pressure, the greater the risk of stroke (Lewington *et al*., 2002).

Smoking has been shown to cause major damage to endothelial cells lining the vessel walls thereby creating potential sites for artherosclerosis and thrombosis. Shinton *et al.,* (1989) concluded that smoking increases the risk of ischaemic stroke by nearly two fold. In addition, Broderick *et al.,* (2003) and Kurth *et al.,* (2003), showed that smoking was associated with a 2 to 4 fold increase in the risk for haemorrhagic stroke.

Elevated blood cholesterol levels have been known to increase the risk of stroke. In three prospective population-based studies, men who had elevated levels of total serum cholesterol, as well as low levels of HDL cholesterol were shown to have higher rates of ischaemic strokes (Leppala *et al.,* 1999; Wannamethee *et al.,* 2000 and Soyama *et al.,* 2003). Furthermore, women with low levels of HDL have been shown to be at a higher risk of ischaemic strokes than men with similar HDL levels, as reported by Goldstein *et al*., (2006). Thus, it appears that low levels of HDL may well be associated with a risk for ischaemic stroke in both men and women.

Several studies have shown a strong association of diabetes mellitus with a number of atherogenic risk factors such as obesity, abnormal blood lipid

profiles and hypertension (Angiolillo *et al.,* 2005). Burchfiel *et al*., (1994) reported that individuals with diabetes had twice the risk of thromboembolic stroke than persons without diabetes concluding that diabetes is an independent risk factor for stroke.

In a study investigating the prevalence of stroke in the rural South African black population (Southern African Stroke Prevention Initiative – SASPI study, 2004), the most common risk factor identified, was hypertension (71%), followed by diabetes mellitus (12 %) and cigarette smoking (9 %) as reported by Connor *et al.,* (2004).

Non-Modifiable risk factors such as age, gender and hereditary factors have also been shown to play a significant role in the development of stroke. In 1992, Wolf *et al.,* showed that age was one of the single most important risk factor predisposing to stroke. It has been shown that although children and young adults are at risk of stroke, the risk increases with age, and almost doubles for each successive decade post 55 years of age (Brown *et al.,* 1996). Although there is no clear evidence of any difference between the different genders, Brown *et al.,* (1996) also showed that stroke was more common in men than in women. However, studies have shown that pregnant women have a higher risk of developing stroke, possibly because they are already in a prothrombotic state (Kittner *et al*., 1996). It has also been suggested that the risk of stroke is increased if a family member such as a parent, grandparent, or a sibling has had a stroke. Both paternal and maternal history of stroke has been associated

with an increased stroke risk indicating possible herediatory factors (Kiely *et al.,* 1993).

It is clearly evident that the development and progession of stroke is multifactorial, in which blood supply and blood flow to various tissues and organs appears to play a critical role. One of the key physiological components in this process is the balance of the haemostatic system.

Haemostasis may be defined as a finely tuned system of sequential activation of pro-enzymes in the coagulation system and the fibrinolytic system and simultaneous platelet activation whereby excessive blood loss is prevented after vessel wall injury (Hansen, 1986; Bick and Munrano, 1994). Under normal physiological conditions, haemostasis is regulated by a number of checks and balances involving positive and negative feedback mechanisms (Davie *et al.,* 1991). However, when the balance of this system is compromised in any way, either through inherited or acquired conditions, inappropriate responses predisposing to either thrombosis or haemorrhage are effected (Gonias and Pizzo, 1986; Davie *et al.,* 1991).

Increasing evidence has indicated that the formation of the haemostatic plug is a multifactorial process, in which platelets play key roles in a large number of complex interactions between blood and the constituents of the vessel wall (Aviram, 1992; Bick and Munrano, 1994).

Platelets are small anucleate cells that are produced from megakaryocytes by cytoplasmic budding (Heyns, 1994) and have a life span of 7-10 days

(Sims *et al.,* 1988). The platelet plasma membrane is composed of a bilayer of phospholipids containing cholesterol, glycolipids, and glycoproteins (GP's) (White *et al.,* 1993). Under normal physiological conditions platelets circulate in a quiescent state but upon injury to the sub endothelium, they form a haemostatic plug at the site of injury. In achieving and preserving haemostasis, platelets function in several different, though interrelated ways (Dörmann *et al.,* 2000).

Upon activation platelets undergo a process of shape change from a smooth discoid shape to spiny spheres with extending "pseudopodias" that may be several time the length of the platelet. The shape change facilitates the process of adhesion of platelets to the exposed subendothelium. Platelet adhesion to the exposed subendothelium is facilitated by various GP's found on the platelet surface and ligands such as fibrinogen, von Willebrand Factor (vWF) and fibronectin. Once activated, the platelets release or secrete their granular contents which facilitates the recruitment and activation of surrounding platelets. This is the process of platelet aggregation thus forming a positive feedback mechanism.

In the 1990's a number of studies characterized platelet glycoproteins, their mobilization and translocation involved in platelet activation (Suzuki *et al.,* 1992 and Nurden *et al.,* 1994). Michelson *et al.,* (1992) showed that activation of platelets resulted in the conformational changes and

movement of glycoprotein Ib-IX (GPIb-IX) receptors on the surface of platelet membrane. This was confirmed by Suzuki *et al.,* 1992 and Nurden *et al.,*1994, who demonstrated the mobilisation and translocation of GP's from the platelet internal membrane and the surface connecting canalicular system to the outer surface during platelet activation.

Platelet GPIa/IIa (intergrin α_2 $ß_1$) found on the platelet surface and granule membrane have been shown to play an important role in platelet interaction with subendothelial collagen (Nurden *et al.,* 1994). Platelet GPIIb/IIIa (intergrins α_2 $ß_3$) has been identified as the receptor that expresses the ligand binding sites for fibrinogen, vWF and fibronectin (Lefkovits,1995). In a latent unstimulated state GPIIb and IIIa are randomly distributed sub units, however, upon platelet stimulation and activation, the sub units undergo a conformational change, and come into close proximity to form a GPIIb/IIIa complex. During this process the reactive sites for the various adhesive ligands previously mentioned become exposed (Woods *et al.,* 1986).

Platelets have been implicated to play a vital role in a number of pathological disorders. As result there has been an active and continuing interest in evaluating the effects of pharmacological interventions to suppress platelet function. It is important to note that since most drugs have different pharmacological actions the demonstration of the

effectiveness of an individual anti-platelet agent cannot be generalized to others.

Anti-platelet agents may be classified into three broad categories, 1) non-steroidal anti-inflammatory drugs such as aspirin (acetylsalicylic acid), a drug that has potent antiplatelet effects, 2) drugs such as dipyridimole that inhibits cyclic AMP levels which influence platelet phosphodiesterase and 3) drugs such as clopidogrel that antagonises the platelet ADP receptor.

Aspirin is one of the most studied antiplatelet agents. It was first shown in 1971 to inhibit prostaglandin synthesis in several tissues (Vane, 1971) and platelets (Smith and Willis, 1971). Aspirin irreversibly acetylates the active site of the enzyme cyclooxygenase (cox) thereby impeding the formation of prostaglandin endoperoxides from which thromboxane A_2, a potent vasocontrictor and platelet agonist, is synthesized (Patrono *et al.,* 2004).

Recent studies have shown a diminished anti-platelet effect of aspirin in some subjects, commonly refered to as non responders or individuals with aspirin resistance. However, given the multifactorial nature of thrombosis, a thrombotic episode in an aspirin treated patient may not necessarily suggest aspirin resistance (Macchi *et al.,* 2003).

The exact nature of aspirin resistance has not yet been clearly elucidated. Cattaneo (2007), suggested that aspirin resistance as determined by

specific laboratory tests, is a rare occurrence and may only account for 1% to 2% of non-responders. Other explanations for aspirin resistance may be poor aspirin absorption, poor compliance, increased cyclooxygenase synthesis and activity, as well as platelet polymorphisms which indirectly influence the effectiveness of aspirin (Martin *et al.,* 2005). There are a few laboratory tests available that test for aspirin resistance. One of the most common techniques used to determine platelet function is platelet aggregrometry.

1.2. Platelet Function

The role of platelet in thrombogenesis and specifically the role of platelet GP's in platelet activation and function as been the subject of interest for many years. A number of methods for preparing platelet suspensions (platelet rich plasma) have been developed. The simplest and most widely used method is the one developed by Born and Cross (1963).

The extent of platelet function or aggregation may be determined by the change in light transmission (optical density) through the platelet suspension. Platelet aggregation may be induced by specific platelet agonists such as collagen, ADP, adrenaline and arachidonic acid. The amount of platelet aggregation is related to the amount of light that passes through the suspension, and the results are reported in units of percentage of light transmission on a scale of 0-100%. When there is no light transmission (no aggregation) then the response would be

considered as “flat” and as such the resultant interpretation would be that the patient is responding to anti-platelet therapy. If platelet aggregation was present, the patient would be considered to not be responding to therapy. However, platelet aggregation is dictated by a number of limitations such as the time in which platelet aggregation must be completed, which is usually within two hours of collection as the platelets begin to deteriorate and therefore only a limited number of patients can be tested per day (Michelson *et al.,* 2005). In addition there are no commercial controls available for aggregation studies. The amount of blood required is substantial, in excess of 30 mililiters, and it is not always possible to obtain adequate quantity of platelets for analysis. Furthermore, there is no international standardization and the method lacks universal reference values. The limiting factor that has the most impact, is that the assay is cumbersome, requiring a specialized laboratory setting. Thus, it may be only available in academic centres and to which patients need to be referred. Recent advances in medical technology, specifically molecular techniques, have been shown to be complementary to platelet aggregation studies in determining platelet function.

Although, there is ample data to support the efficacy of aspirin therapy for prevention of strokes or for the prevention of recurrent strokes, there is limited data on platelet polymorphisms and their relationship to the effectiveness of aspirin therapy. It has since been established that platelet membrane glycoprotein polymorphisms may contribute significantly to

aspirin resistance (Benze *et al.,* 2002). Studies have shown that a number of platelet GP's are polymorphic and may influence the sensitivity of the receptors influencing platelet function (Carlsson *et al.,* 1997). Platelets have a number of receptors, such as GPIb/IX/V, GPIIb/IIIa and GPIa/IIa, each playing a major role in platelet function. The GPIb/IX/V complex has been shown to initiate the adhesion of platelets to vWF. There are three polymorphisms that have been described in the platelet GPIbα region, the Kozak –5 T/C polymorphism, the Human Platelet Antigen 2 (HPA-2) polymorphism and the Variable Number of Tandem Repeats (VNTR) polymorphism, each of which, may enhance vWF and platelet interaction (Ishida *et al.,* 1995). Polymorphisms in the GPIIIa gene such as PI^A polymorphism may influence the platelet activation process and could potentially modify the thrombogenicity of platelets (Benze *et al.,* 2002).

1.3. PLATELET POLYMORPHISMS

Studies by Szczeklik *et al.,* (2000), suggested that it was possible that variations in the GP receptors may be responsible for resistance to antithrombotic effects of aspirin. Baker *et al.,* (2001) identified that the Kozak GPIbα polymorphism was an independent risk factor implicated in patients for first time ischaemic stroke.

1.3.1. Glycoprotein Ib/IX/V

GPIb/IX/V is composed of four subunits: GPIbα, GPIbβ, GPIX, and GPV, each being the product of a separate gene. Glycoprotein GPIbα, GPIbβ

are two polypeptide chains, which are linked together by disulfide bonds. GPIb/IX/V mediates adhesion of platelets to the extracellular matrix under high sheer rates via the binding of vWF to GPIbα. GPIb play a crucial role as a receptor mediating platelet-platelet, platelet-endothelium and platelet leukocyte interactions (Stoll *et al.,* 2008).The GPIb/IX/V complex is important in platelet adhesion and therefore any change in the GPIb structure may alter the way in which platelets function and thus could potentially lead to thrombosis.

Three polymorphisms in GPIbα region have been described. Two of the polymorphisms affect the structure while the third has been shown to alter the gene expression of the subunit (Baker *et al.,* 2001). The first of the two polymorphisms that affect the structure is known as the Human Platelet Antigen–2 (HPA-2). In this polymorphism there is a cytosine (C) to thymidine (T) substitution, which results in a threonine to methionine amino acid change at position 145. The second polymorphism is the variable number of tandem repeats (VNTR). This polymorphism, results in the duplication of 13 amino acid sequences. The resulting fragment may be a single copy or repeated up to four times with each varying by 39bp.
The third polymorphism known as the Kozak polymorphism is a T to C substitution at position 5 from the ATG initiator codon. This polymorphism may influence the translational efficiency of GPIbα. Furthermore, it was shown to increase the receptor density on the platelet surface (Clemetson

et al., 1995). Studies have shown that all three polymorphisms significantly influence platelet function (Baker *et al.,* 2001)

1.3.1.1. Kozak –5 T/C polymorphism

GPIb is composed of GPIbα (610 amino acids) disulphide bonded to GPIbβ (122 amino acid). GPIbα is a component of the GPIb-IX-V complex, which mediates platelet adhesion to the subendothelium at sites of injury. An important polymorphism the Kozak –5T/C defined as either the presence of thymine (T) or cytosine (C) at position –5 from the initiator ATG start codon has been identified in this region. This polymorphism has been shown to influence messenger RNA translation and the expression of GP Ibα on the platelet surface. Afshar-Kharghan *et al.,* (1999) reported that the presence of the C allele leads to increased expression of GPIbα on the platelet surface. Baker *et al.,* (2001) concluded that the C allele of the Kozak polymorphism plays a role in the pathogenesis of ischaemic stroke. Furthermore, Hsieh *et al.,* (2004) using data from the Vienna Stroke Registry found that patients who were homozygous for the Kozak CC genotype had a 3.5 fold increased risk for ischaemic cerebrovascular events compared with the TT or TC genotype carriers. The over expression of platelet GPIbα on the surface of the platelets renders the platelets much more susceptible to increased binding, and hence an independent risk factor for first ischaemic stroke. Maguire *et al.,* (2008) reported that the Kozak polymorphism may be close to another causative locus that was associated with ischaemic stroke.

1.3.1.2. Variable Number of Tandem Repeats (VNTR) polymorphism

The second platelet polymorphism described within the Kozak GPIbα region was the VNTR polymorphism. This polymorphism causes a change in the polypeptide length by the variation in the number of 13 amino acid tandem repeats. The variation occurs in the mucin like macroglycopeptide region that alters the molecular weight of the protein (Lopez *et al.,* 1992). This is a size polymorphism with four possible repeats, with the length, which varies by multiples of either 1 (D allele), 2 (C allele), 3 (B allele) or 4 (A allele) differing by multiples of 39 base pairs, which results in a polypeptide length of 610, 623, 636 and 649 amino acids, respectively (Ishida *et al.,* 1995). The VNTR polymorphism interferes with the functioning of GPIbα since each repeat would position the ligand binding region further away from platelet membrane surface making it more accessible to binding but more susceptible to sheer forces. Zhang *et al.,* (2007) concluded that the D allele of the VNTR polymorphism was associated with atherothrombotic stroke. It was also reported by Cervera et al., (2007) that the BC genotype of the VNTR polymorphism was a contributing factor associated with aspirin treatment failure.

1.3.1.3. Human Platelet Antigen- 2 (HPA-2) Polymorphism

The third platelet polymorphism that exists within the Kozak GPIbα region is known as the HPA-2 polymorphism. This polymorphism exists

within the vWF and thrombin binding leucine-rich repeat region of GPIbα and is determined by the presence of threonine (Thr) or methionine (Met) at position 145, which affects the structure of the protein. The HPA-2 polymorphism is a C to T nucleotide substitution which results in a Thr to Met transition which in turn is responsible for the altered ligand binding of GP Ib (Shen *et al.,* 2000). This polymorphism forms the basis of many platelet alloantigen systems, HPA-2a/2b, which is involved in the development of platelet transfusion refractoriness (Murata *et al.,* 1992). This C to T substitution results in Met^{145} (HPA-2b) being in linkage disequilibrum with the VNTR allele A and B. (Sonoda *et al.,* 2000), while Thr ^{145} (HPA-2a) is in linkage disequilibrum with the C and D allele (Baker *et al.,* 2001). Kaski *et al.,* (1996) and Corral *et al.,* (1998) also reported on similar findings that the HPA-2 polymorphism is in nearly complete linkage disequilibrium with a size polymorphism in the same gene. It has been shown that individuals carrying a combination of the B allele for the VNTR polymorphism as well as the HPA-2 Met^{145} polymorphism had increased platelet activity, despite aspirin therapy (Sonoda *et al.,* 2000). All individuals with the Met^{145} polymorphism have been reported to carry the B allele, but 1% to 5% of B allele carriers are Thr^{145} homozygotes. Murata *et al.,* (1997) reported that the presence of the Met^{145} allele in GPIbα is a risk factor for the prevalence and severity of coronary artery disease (CAD). Mikkelsson *et.al,* (2001) suggesed that the HPA-2 Met^{145}/VNTR B haplotype of the platelet vWF and thrombin receptor protein GP Ib-V-IX may be considered to be a major risk factor of coronary thrombosis.

Sonoda *et al.,* (2000) as well as Reiner *et al.,* (2000) showed that the HPA-2 polymorphism was found to be associated with ischaemic cerebrovascular disease and ischaemic stroke. Futhermore, Magurie *et al.,* (2008) suggested that the Thr/Met polymorphism was associated with ischaemic stroke in a dominant genetic model.

1.3.2. GP IIIa PI^A polymorphism

GP IIb/IIIa is a platelet membrane receptor for fibrinogen and vWF factor (Slowik *et al.,* 2004) and represents approximately 15 % of platelet surface protein. The genes encoding GP IIb and IIIa are located on chromosome 17q21 (Bray *et al.,* 1987). The GPIIIa gene exhibits two allelic forms, PI^{A1} and PI^{A2} (Michalak *et al.,* 1998). The PI^A polymorphism of the GPIIIa gene, is produced by a single point mutation, which results in a substitution of a cytosine for a thymine at position 1565 in exon 3 of the GPIIIa gene. Wagner *et al.,* (1998) showed, the more common allele PI^{A1} encodes a leucine and the less common allele PI^{A2} encodes a proline at residue 33 of the protein amino acid sequence and this leads to consequent changes in the protein conformation and spatial orientation of the fibrinogen binding region. Data suggests that the PI^{A2} polymorphism of GPIIIa may be associated with an increased risk for cardiovascular disease (Feng *et al.,* 1999). It has also been suggested to play an important role in thrombosis. The hyperaggregability conferred by the PI^{A2} homozygote was attributed to increased surface expression of GP IIb/IIIa receptors and an increased affinity for fibrinogen.

As early as 1989, Newman *et al.,* and Stakos *et al*., (2002) showed that a platelet polymorphism which was described in the GP IIb/IIIa receptor , the PI^{A2} polymorphism was much more reactive in both the homozygote and heterozygotes state, with enhanced fibrinogen binding capacity and therefore showed variable response to antiplatelet effects of aspirin.

Studies by Naran *et al.,* (2008) showed that the frequency of the unfavourable allele PI^{A2} was higher in subjects with CAD than in subjects without CAD. Furthermore, they showed that platelets carrying the PI^{A2} allele were hypersensitive to platelet aggregating agents and had produced higher amounts of thromboxane A_2 than platelet having the PI^{A1} genotype.

In a recent study, the Copenhagen City Heart Study (Bojesen *et al.*, 2003) supports the finding of both Newman *et al.,* (1989) and Stakos *et al.,* (2002) that men less than 50 years of age with the homozygote form of the PI^{A2} polymorphism showed a four fold increase in ischaemic cardiac events. However, the findings in premenopausal women suggested that the effect of oestrogen on the PI^{A2} polymorphism may have conferred some protection.

There is substantial evidence pointing towards platelet polymorphisms as being associated with thrombotic events and this could be a new avenue of pursuit with regards to laboratory testing. With the advent of polymerase

chain reaction (PCR), molecular testing is now at the forefront for diagnosing genetic disorders.

1.4 Rationale for this study

Molecular techniques have been used to screen for a number of genetic disorders from sickle cell disease to factor V Leiden gene mutation. Since the incidence of stroke is high, the current methods for assessing stroke that are based on clinical findings as well as neurological scores such as doppler, cat scans and magnetic resonance imagining (MRI) may be limiting. Although laboratory based tests such as platelet aggregation are well established diagnostic tool for determining platelet function, perhaps a complementary technique is required to test for platelet polymorphisms that have been implicated in stroke and aspirin resistance.

Thus the focus of this study is on platelet polymorphisms, to detect such polymorphisms and the adaptation of conventional PCR to a Real- Time platform using the Roche LightCycler™.

CHAPTER TWO

2. Study Design

2.1 The Evolution of Molecular Techniques

The advances in molecular techniques used to characterise genetic material have revolutionised the field of molecular biology. It has improved the quality, precision and accuracy of detecting specific target sequences. The advancement in the field of molecular biology has been intimately dependent on and restricted by advances in technology. Therefore, it is only due to the advances in the detection methodology or techniques that have lead to the progress in the field of molecular biology. Early methods although antiquated and laborious, played a significant role in the evolution of the methodology. Techniques such as protein sequencing, protein electrophoresis, DNA hybridisation and restriction fragment analysis paved the way for more advanced methods to be developed such as polymerase chain reaction (PCR). The early techniques such as DNA hybridisation were not direct measures but rather a measure of indirect differences in nucleic acid based on inferred similarity of sequences (Mable, 2001). When first developed, DNA sequencing depended on cloning, which was achieved by inserting the portion of DNA to be studied into a bacterial plasmid, which then replicated the DNA fragments. The replicated DNA was removed and this concentrated DNA was sequenced, to reveal nucleotides that constitute its code (Paterson 1977).

Sanger *et al.,* (1977) and Maxam and Gilbert (1980) proposed the direct DNA sequencing technique, but it was not until the discovery of

thermostable DNA polymerase together with PCR as described by Mullis and Faloona, (1987) that large scale automation was feasible. Thus, the discovery of PCR revolutionized molecular biology by allowing DNA sequencing to become tools for studying genetic disorders in diagnostic laboratories.

Restriction Fragment Length Polymorphism (RFLP) was used as a method to examine genetic changes in specific locations within the DNA. It required large amounts ribosomal and mitochondial DNA. The RFLP method relied heavily on the use of restriction enzymes in order to visualise the final product. Restriction enzymes are DNA cutting enzymes found in bacteria and isolated for use. The enzyme is capable of cutting within the DNA sequence and hence they are often called restriction endonucleases. Restriction enzymes recognise and cut DNA only at specific sites and only at a particular sequence of nucleotides. The enzyme recognition site is usually 4 to 6 base pairs in length. Depending on the number of restriction sites, the DNA segment will be cut into a number of fragments which are then electrophoresed together with a known molecular marker or ladder and visualised using ethidium bromide (a dye that binds to DNA). Restriction enzymes are named by using the first letter of the genus, the first two letters of the species, and in order of discovery (National Health Museum website). This method provided an indirect comparison of sequence variations. RFLP's were used for rapid screening of large number of samples (van Ebden *et al.,* 1993).

2.2. MOLECULAR TECHNIQUES OF CONVENTIONAL PCR

2.2.1 DNA amplification

The ability to identify a target region of a particular DNA sequence and then produce multiple copies of the target DNA is known as DNA amplification. A number of methods have been used to amplify DNA, such as transcription-mediated amplification, strand displacement amplification and the PCR (Mullis *et al.,* 1987). Of all the methods used in research, PCR, is the most successful and widely used technique to obtain amplified product (Ross, 1996). This technique makes it possible to identify genetic disorders and also allows for identification of gene polymorphisms. PCR reaction takes place in a thermocycler, an instrument that was designed to rapidly heat and cool DNA samples in a controlled environment allowing for amplification of DNA product.

2.2.2. Polymerase chain reaction (PCR)

Kary Mullis described the PCR technique in 1985. In the same year Saiki and co workers made it possible to target segments of DNA using specific primers to amplify only those segments. The technique was used to detect rare sequences due to its high sensitivity. The method allowed only the target sequence to be amplified and thus many copies of the product were produced for further analysis. The entire process was based on three temperature cycles that were repeated from between 25-40 times in a thermocycler in order to yield specific DNA products. The temperature cycle consist of 1) denaturation, 2) annealing and 3) extension.

1) Denaturation

Denaturation is a process whereby the double stranded DNA is heated to 94-95°C, to allow the bonds between the strands to be separated to yield two single stranded DNA. The complete denaturation of the DNA template is crucial since incomplete denaturation results in the under utilisation of the template and thus leading to a poor yield of PCR product. Hence the time and temperature is of extreme importance. Usually denaturation between 0.5 – 2 minutes at 94-95°C is adequate. The product formed during the first cycle at this temperature and duration is shorter than the template DNA and under these conditions it is completely denatured. If the DNA that needs to be amplified has a high GC content the time of the cycle should be increased to 3-4 minutes (Fermentas).

2) Annealing

The binding of primers to the complementary ends of the target DNA is known as annealing. Specific annealing leads to amplification of the required PCR product. Annealing temperature has to be optimised empirically. The choice of primer annealing temperature is probably the most critical factor in designing high specificity PCR. Usually the optimal annealing temperature is 5°C lower than the melting temperature of the primer-template DNA duplex .Too high temperatures result in no annealing taking place, and too low temperatures leads to the formation of primer-dimers which results from non specific annealing. This could occur due to primers having complementary bases so that base pairing between the 3'

end of both the primers can occur (Roche Biochemicals, Germany). Annealing temperature is important in detecting polymorphisms. Slight mismatches, even 1 base-pair mutations in any one of sequences bound by the two primers used to amplify a DNA locus, can be detected by slight variations in annealing temperature.

3) Extension

Primer extension is carried out at 72°C, at this temperature *Taq* DNA polymerase can add approximately 60 bases per second. A 45-60 seconds extension is sufficient for fragments up to 1kb. The one minute cycle extension allows the enzyme more time to work optimally, as the PCR progresses, there is more template to amplify and less enzyme to complete the extension, this may be due to denaturation of *Taq* polymerase during the prolonged high PCR temperatures (Roche Biochemicals, Germany).

An additional extension for 10 minutes may be initiated at the end of the cycle, to allow for the completion of partial extension products and annealing of single stranded complementary products (Roche, Biochemicals, Germany).

4) Detection Procedure

An agarose gel (1-2%) containing ethidium bromide is frequently used to separate out the PCR products by electrophoresis. The products were

identified by exposing the gel to a UV light source (transilluminator) that allowed for visualisation of the various fragments. A known molecular weight marker is run in parallel with the samples for the identification of the various fragment sizes that could be matched to the known marker.

Although conventional PCR remains the “Gold Standard” in genetic testing, the advent of automation such as real-time PCR and improvements in automated sequencing has allowed for entire genomes to be sequenced (Mable,2001).

2.3. REAL-TIME PCR

2.3.1. Principle of real-time PCR using the Roche LightCycler ™

The LightCycler™ is essentially a rapid version of the thermocycler, it combines the process of both DNA amplification and product detection (Roche Biochemicals, Germany). This method is ideal for routine nucleic acid analysis involving rapid thermal cycling and real-time online detection of PCR reaction kinetics (Roche, Biochemicals, Germany). The rapid thermocycling is achieved by alternating heated and ambient air around the sample. Since air has a low heat capacity, it can absorb and dissipate heat quickly and evenly. The high ratio of surface to volume of the glass capillaries allows for this to occur (Roche Biochemicals, Germany). The process of heating and cooling is what allows each step of the PCR amplification program to take place in less than 30 seconds as compared to a minute or more for the conventional thermocycler. (Roche

Biochemicals, Germany). Smaller reactions are now made possible by using glass capillaries and it is also essential for fluorescent monitoring. A light emitting diode (LED) is housed in the LightCycler's fluorimeter. This LED produces a blue light which excites the fluorescent molecules. The fluorescent signals emitted are detected by the fluorimeter in either the green spectrum (wavelength of 530nm), or the red spectrum (wavelength of 640 or 710nm). By using fluorescent labelled oligonucleotide probes and sophisticated software it is now possible to monitor the PCR reaction in real-time while it is in progress (LightCycler™ online resource site). An increase in fluorescence is proportional to the increase in DNA concentration. There are many types of detection formats used for monitoring the various fluorescent dyes (Roche-applied science).

2.3.2. Detection Formats

There are various formats that can be used to detect a target DNA sequence, each with its own advantages and disadvantages. It is therefore essential to have good understanding of the various formats when designing the experiment to detect the intended platelet polymorphism. There are four types of detection formats that can be used on the lightCycler™ each with a specific function.

1) Monitor PCR with SYBR Green I Dye

During the annealing phase, PCR primers hybridize to the target and form small regions of double stranded DNA where SYBR Green I intercalates

thereby releasing a fluorescent signal. As the reaction proceeds in the elongation phase, more double stranded DNA is formed resulting in SYBR Green I dye to intercalate, thus a higher fluorescent signal is emitted. Furthermore, at the end of the elongation phase where all DNA has become double-stranded a maximum amount of SYBR Green I is intercalated and a maximum fluorescence signal is emitted. The fluorescence is measured (530 nm) at the end of each elongation phase.

2) Monitor PCR with Hydrolysis probes

The probe carries two fluorescent dyes (quencher and reporter) in close proximity, with the quencher dye suppressing the reporter fluorescence signal. During denaturation the target double-stranded DNA is separated. In the annealing phase of PCR, primers and probes specifically anneal to the target sequence. Since the 3' end of the hydrolysis probe is phosphorylated, so it cannot be extended during PCR. As the DNA polymerase extends the primer, it encounters the probe. The polymerase then cleaves the probe with its inherent 5´ nuclease activity, displaces the probe fragments from the target and continues to polymerize the new amplicon. In the cleaved probe, the reporter dye is no longer quenched and therefore can emit fluorescent light that can be measured by one channel of the LightCycler® optical unit. Thus, the increase in fluorescence from the reporter dye directly correlates to the accumulation of PCR products.

3) Genotyping and Mutation Detection with SimpleProbe Probes

SimpleProbe probe is a special type of hybridisation probe which differs from the HybProbe assays in that each assay requires only a single probe. The probe will only hybridise to the target sequence that contains the SNP of interest. The fluorescence signal of the hybridised probe is much higher than when not hybridised to its target. Thus, the change in the intensity of the signal depends on the hybridisation status of the probe. SimpleProbe probes are extremely useful in SNP genotyping and mutation detection because they can readily identify the wild type, heterozygous and mutant samples. In the intact probe, both the quencher and reporter dyes are close to each other such that the quencher dye supressess the reporter fluorescent signal. During PCR, the 5' nuclease activity of the polymerase cleaves the hydrolysis probe, separating the reporter and quencher. Thus in the cleaved probe, the reporter is now free and no longer quenched and emits a fluorescence signal.

4) Monitor PCR with the LightCycler® HybProbe Format

For the purpose of this study the HybProbe format was chosen. This unique format is based on fluorescence resonance energy transfer (FRET). Two sequence specific oligonucleotide probes are labelled with different dyes (acceptor and donor), and are added with the primers to the reaction mix. During annealing the Hybprobe probes hybridise to the target sequence of the amplified DNA fragment in a head-to-tail arrangement, thereby bringing the two dyes close to each other. The fluorescein (donor

dye) is excited by the blue LED. The two dyes must lie close to each other (within 15 nucleotides) in order for the energy emitted by the donor to excite the acceptor dye of the second HybProbe probe. When this occurs fluorescent light of a different wavelength is emitted. This fluorescence is directly proportional to the amount of target DNA generated during PCR. During the elongation and denaturation steps the Hybprobe probe is displaced. (Roche Diagnostics Corporation).

Melting curve analysis follows PCR amplification. The melting profile is the specific melting temperature of an oligonucleotide. It allows for the detection of a specific single nucleotide mutation (de Silva *et al.,* 1998). A melting curve is generated in three steps. Firstly, the amplified DNA is denatured, secondly, the DNA is cooled to below its annealing temperature and finally it is heated slowly at 0.1–0.2°C/second. The fluorescence is monitored continuously during the final step. There is a characteristic drop in fluorescence at the melting temperature of the hybridisation probe. Both the detector and anchor probes are complementary to the specific region on the amplified DNA to which they hybridise. When the amplified DNA contains a single nucleotide mutation within the specific region, there is a mismatch between the probe and the target DNA (Roche). This mismatch significantly reduces the melting temperature of the hybridisation probes. Due to this mismatch, genotyping a single nucleotide mutation was made possible by analysing the melting curve profile. The use of melting curve analysis for the detection of

specific nucleotide mutations has proven to be reliable and rapid (de Silva *et al.,* 1998).

2.4. Experimental Design

Thrombosis plays an integral part in acute ischaemic stroke. The genes selected for this study have been shown to play a role in thrombosis as well as aspirin resistance in previously published studies (Newman *et al.,* 1989; Gonzalez-Conejero *et al.,* 1998; Carlsson *et al.,* 1999; Sonoda *et al.,* 2000 and Stakos *et al.,* 2002). The study was designed to compare conventional PCR with real-time LightCycler™ PCR by genotyping the GPIIIa PI^A, HPA-2, VNTR and Kozak-5T/C platelet polymorphisms. The main focus of this study was to adapt of conventional methods to real-time PCR. The essence was to develop an assay for routine diagnostic use in a clinical setting by economising on time while striving to reduce costs as well as maintaining high levels of quality control. The study also aims to provide a surrogate marker to compliment platelet aggregometry. The advantages and disadvantages two PCR methods were compared. Candidate genes were selected to study the effect of polymorphisms on structure, expression and function of gene products.

CHAPTER THREE

3. MATERIALS AND METHODS

3.1.1. Patients and Controls

A total of 60 caucasian patients were recruited for the study, 32 males and 28 females with the mean age of 66.5 years. The patients were classified as having stroke by virtue of a magnetic resonance imaging (MRI) scan as determined by clinicians at the Johannesburg Stroke Clinic. The inclusion and exclusion criteria for acceptance are shown in appendix one.

A total of 38 age and gender matched healthy caucasian subjects, 20 females and 18 males, who had no history of stroke or any chronic disorder, were recruited from the National Health Laboratory Service (NHLS) and the University of Witwatersrand as controls.

The subjects that were enrolled for this study were part of a larger study the “Stroke and Aspirin Resistance” (STAR) Study. The study was explained to all the subjects and written informed consent obtained. The subjects were free to discontinue their participation at any time without predjudice. Ethical clearance for this study was also obtained from the University of the Witwatersrand ethics committee (appendix).

3.1.2. Samples

A 5 ml venous blood sample was collected into a EDTA anticoagulated specimen tube from each individual for DNA extraction.

3.1.3. Genomic DNA Extraction

3.1.3.1. High Pure PCR Template Preparation Kit (Roche)- Method

To reduce contamination and improve the quality of the extracted DNA, a commercially available kit -based DNA extraction method from Roche was used. The system utilized a filter with glass fibres to which the DNA selectively binds. The process was initiated by pipetting 200µl of anticoagulated (EDTA) whole blood into a 1.5ml Eppendorf tube. To this, 200µl of Binding Buffer as well as 40µl of Proteinase K solution was added. The sample was then incubated at 72°C for 10 minutes before adding 100µl isopropanol and mixing well. The sample was then transferred into the upper reservoir of a High Pure filter tube which was then placed in a collection tube assembly and microcentrifuged for 1 minute at 8000rpm. The flow-through solution in the collection tube was discarded, the filter tube was then transferred to second collection tube assembly and 500µl of Inibitor Removal Buffer was added. The centrifugation process was repeated once more for 1 minute at 8000rpm. The flow-through was discarded and the filter placed on a new assembly. Wash Buffer was then added to the upper reservoir of the filter collection tube and it was centrifuged at 1 minute at 8000rpm, this process was repeated twice with a final centifugation at 14000 rpm for 10 seconds to remove residual wash buffer from the filter. The upper reservoir was then inserted into a clean 1.5ml eppendorf tube and 200µl of pre-warmed (70°C) Elution Buffer was added to the upper reservoir and the sample was microcentrifuged for 1 minute at 8000rpm. The eluted solution

contained the extracted DNA. The DNA was stored at -20°C until required. (Roche Biochemicals, Germany)

3.2. DNA Quantification

The quantity and quality of the extracted DNA was determined by photometric analysis using ultraviolet light (UV) by analysing the absorbance at 260nm and 280nm respectively. A 1 in 50 dilution of the sample was used for analysis (20µl of DNA in 980µl of distilled water). The quantity of DNA was determined by the optical density (OD) reading at 260nm. An OD reading of 1 corresponds to approximately 50µg/ml of double stranded DNA. The OD reading at 280nm indicates that amount of protein present in the sample. Therefore, the purity of the DNA in the sample can be assessed by dividing OD260 by OD280. Pure DNA has a ratio of 1.8 – 2.0. The following equation was used to calculate the DNA concentration in the original sample.

$$\text{DNA concentration (µg/µl)} = \frac{OD_{260} \times 50\ (\mu g/ml) \times 50\ (\text{dilution factor})}{1000}$$

3.3. CONVENTIONAL PCR METHODOLOGY

3.3.1. BUFFERS AND SOLUTIONS

3.3.1.1. Tris-Acetic acid-EDTA (TAE), (50x concentrated stock buffer)

Stock TAE buffer consists of 242g of Tris-base, 57.1ml Glacial acetic acid and a 100ml of 0.5 M EDTA (pH 8.0), made up to a 1000ml with distilled water.

3.3.1.2. Tank Buffer (electrophoresis)

Working solution for electrophoresis tank buffer: dilute 20ml 50X TAE buffer in 980ml distilled water and add 50ul ethidium bromide working solution to make up a working solution of tank buffer.

3.3.1.3. Gel Buffer

Working solution for the agarose gel buffer: dilute 20ml 50X stock TAE in 980ml of distilled water.

3.3.1.4 Ethidium Bromide (10mg/ml Stock Solution)

0.1 gram of ethidium bromide was added into a 100 mililitres of distilled water, and swirled (stock solution). To prepare a working solution, an equal volume of ethidium bromide was diluted with distilled water. When not in use the solution must be stored in the dark at 4°C.

3.3.1.5. 2% Agarose gel preparation

2g of (D1 LE) agarose was weighed and added to a conical flask to which a 100ml of TAE buffer (working solution for gels) was added. The flask was placed in a microwave and the solution was heated, it was stirred occasionally to ensure that all the agarose was completely dissolved. The flask was removed from the microwave and 5-6ul of ethidium bromide was added to the agarose and mixed. This solution was then poured into the casting tray and allowed to cool for 30 minutes at room temperature prior to use.

3.4.1 Reagents and Materials - Conventional PCR

Primers are one of the components that are essential for PCR analysis. Primers are used to detect specific alleles sequences that exist for a given platelet polymorphism. Primer sequences for the Kozak, VNTR, GPIIIa PI^A and HPA-2 polymorphisms are shown in table 1. The various reagents that were used in the preparation of the master mix solutions for the different platelet polymorphisms (Kozak, VNTR, HPA-2 and GPIIIa PI^A) are shown in tables, 2, 3 and 4

Table 1: Primer sequences for each platelet polymorphism used in conventional PCR analysis.

Kozak forward primer-20mer GPIb-V-IX (von Willebrand receptor)	5'-GAGAGAAGGACGGAGTCGAG-3' Baker *et al.*,(2001)
Kozak reverse primer-19mer GPIb-V-IX (von Willebrand receptor)	5'-GGTTGTGTCTTTCGGCAGG-3' Baker *et al.*,(2001)
VNTR forward primer-20mer GPIb-V-IX (von Willebrand receptor)	5'-TCCACTGCTTCTCTAGACAG-3' Ishida *et al.,* (1995)
VNTR reverse primer-20mer GPIb-V-IX (von Willebrand receptor)	5'-GGCTGATCAAGTTCAGGGAT-3' Ishida *et al.,* (1995)
GPIIIa PI^{A} forward primer-22mer GPIIbIIIa (Fibrinogen receptor)	5'-TTCTGATTGCTGGACTTCTCTT-3' Weiss *et al.,* (1996)
GPIIIa PI^{A} reverse primer-21mer GPIIbIIIa (Fibrinogen receptor)	5'-TCTCTCCCCATGGCAAAGAGT-3' Weiss *et al.,* (1996)
HPA-2 common -17mer forward primer GPIb-V-IX (von Willebrand receptor)	5'-GATGGGACGCTGCCAGT-3' Baker *et al*.,(2001)
HPA-2reverse primer for Thr^{45} -23mer GPIb-V-IX (von Willebrand receptor)	5'-CTTCTCCAGCTTGGGTGTGGGAG-3' Baker *et al.*,(2001)
HPA-2 reverse primer for Met^{45} –23mer GPIb-V-IX (von Willebrand receptor)	5'-CTTCTCCAGCTTGGGTGTGGGAA-3' Baker *et al.*,(2001)

Table 2: Reagents, volumes and concentrations that were used to prepare a master mix solution for both the Kozak and VNTR polymorphism

Reagents	**Kozak**	**VNTR**	**Manufacturer**
DNA (100-200ng)	5µl	5µl	-
Master Mix (1.5mM $MgCl_2$, 200µM of each dNTP and *Taq* 1.0 U/reaction)	25µl	25µl	Promega
Primer-Forward (10 ρmol)	1µl	1µl	Fermentas, supplied by Inqaba biologics SA
Primer-Reverse (10 ρmol)	1µl	1µl	Fermentas, supplied by Inqaba biologics SA
Water	18µl	18µl	Sabax
Total	50µl	50µl	

Table 3: Reagents, volumes and concentrations used to prepare a master mix solution for the HPA-2 polymorphism.

Reagents **HPA-2**	**Reaction x1** **Tube 1**	**Reaction x1** **Tube 2**	**Manufacturer**
DNA (100-200ng)	5µl	5µl	-
Master Mix (1.5mM $MgCl_2$, 200µM of each dNTP and *Taq* 1.0 U/reaction)	25µl	25µl	Promega
Primer-forward (common) (10 ρmol)	1µl	1µl	Fermentas, supplied by Inqaba biologics SA
Primer reverse no 1 (10 ρmol)	1µl	-	Fermentas, supplied by Inqaba biotech SA
Primer reverse no 2 (10 ρmol)	-	1µl	Fermentas, supplied by Inqaba biotech SA
Water	18µl	18µl	Sabax
Total	50µl	50µl	

In this reaction one common forward primer and two reverse primers were used to perform the test and the reactions were set up in two separate tubes.

Table 4: Reagents, volumes and concentrations used to prepare a master mix solution for the GPIIIa PI^A polymorphism.

Reagent	**GPIIIa PI^A**	**Manufacturer**
DNA (100-200ng)	5µl	-
PCR buffer containing $MgCl_2$ (1.5mM)	5µl	Roche Molecular Diagnostics
dNTP's (3.2mM)	0.75µl	Roche Molecular Diagnostics
Forward primer -10pmol	1µl	Fermentas, supplied by Inqaba biotech SA
Reverse primer-10pmol	1µl	Fermentas, supplied by Inqaba biotech SA
Taq polymerase (1unit)	0.2µl	Roche Molecular Diagnostics
Water	37.05µl	Sabax
Total	50µl	

3.5. PCR Cycling Conditions for Conventional PCR

The DNA used in the conventional PCR experiments was amplified in a Perkin Elmer 2400 thermal cycler and table 5 below decribes the cycling conditions used for the various platelet polymorphisms.

Table 5: The cycling conditions demonstrated in the table are for GPIIIa PI^A, Kozak, VNTR and HPA-2 polymorphism detection.

	GPIIIa PI^A	**Kozak**	**VNTR**	**HPA-2**
Hotstart	94°C for 1 minute-single cycle	94°C for 1 minute-single cycle	94°C for 1 minute-single cycle	94°C for 1 minute-single cycle
Denaturation 35 cycles	94°C for 1 minute	94°C for 1 minute	94°C for 1 minute	94°C for 1 minute
Annealing 35 cycles	55°C for 1 minute	55°C for 1 minute	55°C for 1 minute	63°C for 1 minute
Extension 35 cycles	72°C for 1 minute	72°C for 1 minute	72°C for 1 minute	72°C for 1 minute
Final Extension	72°C for 10 minutes-single cycle	72°C for 10 minutes-single cycle	72°C for 10 minutes-single cycle	72°C for 10 minutes-single cycle
Hold	4°C -indefinite	4°C – indefinite	4°C – indefinite	4°C -indefinite

All the thermal cycles (denaturation, annealing and extension) and transitions rates were identical with one exception, that of HPA-2 where the annealing temperature was 63°C for 1 minute instead of 55°C for 1 minute.

3.6. Restriction Fragment Length polymorphism (RFLP) Analysis

RFLP analysis was performed on GPIIIa PI^A and Kozak polymorphisms using *Msp1* and *Ppu* also known as *(Psp)* respectively. 15µl of the post PCR DNA product was then incubated with a specific restriction enzyme to produce fragments that could be identified electrophoresis. Table 6 below describes the various enzymes and method used.

Table 6: Restriction enzymes and the volumes used for each individual reaction in both the PI^A and Kozak polymorphism.

Reagents	**GPIIIa PI^A**	**Kozak**	**Manufacturer**
Enzyme *Msp1* (5u/µl)	0.5µl	-	Roche Molecular Diagnostic
Enzyme *Ppu (Psp)* (10u/µl)	-	0.25µl	Fermentas
Cut Buffer 10x concentration	2.0µl	2.0µl	Roche/Fermentas
Water	2.5µl	2.75µl	Sabax
PCR product used	15µl	15µl	-
Total volume	20µl	20µl	-
Incubation Time	2 hours	2 hours	-
Temperature	37°C	37°C	-

3.7. Representations of the Kozak, HPA-2, VNTR and GPIIIa PI^A Polymorphisms using conventional PCR.

3.7.1. Kozak – 5T/C polymorphism determination – Coventional PCR

The Kozak –5T/C polymorphism was detected by electrophoresis on 2% agarose gel (described previously) using a UV transilluminator. The digestion of the amplified products produced 3 bands for T/T genotype (125 base pair (bp), 157bp, and 175bp), for the T/C genotype 4 bands (125bp, 157bp, 175bp and 332 bp) and for the C/C genotype 2 bands (125 and 332bp) Baker *et al.,* (1998). Each subject was classified into one of three possible genotypes: C/C, T/T, T/C as shown in figure 1.

3.7.2. Human Platelet Antigen (HPA-2) polymorphism – Conventional PCR

The HPA-2 polymorphism was detected by performing two separate PCR using allele specific primers. The primers that were used in this PCR reaction were described by Baker *et al.,* (1998). Met^{145} (A genotype) and Thr^{145} (B genotype) were visualised using Gel docking station that uses the same principle as the transilluminator. Since the method, as described by Baker *et al.,* (1998) does not include an internal PCR control, homozygous samples Met^{145} and Thr^{145} was confirmed by repeating the PCR. Each subject was classified into one of three possible genotypes: A, B or AB. These are shown in figures 2a and 2b.

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

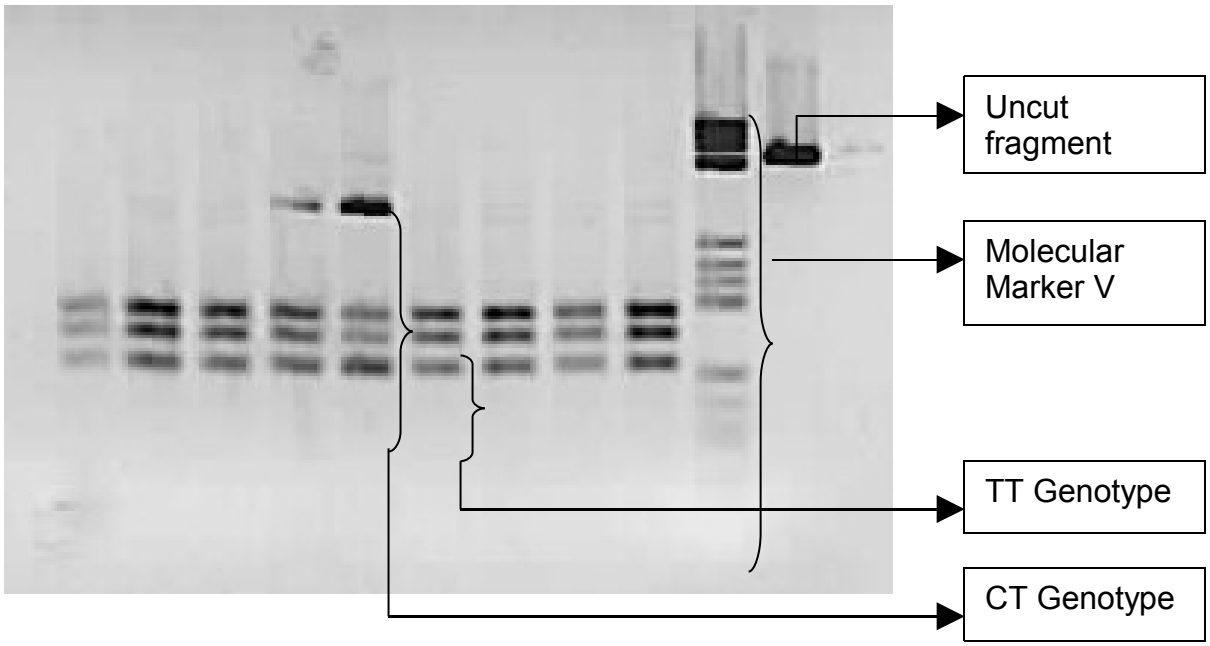
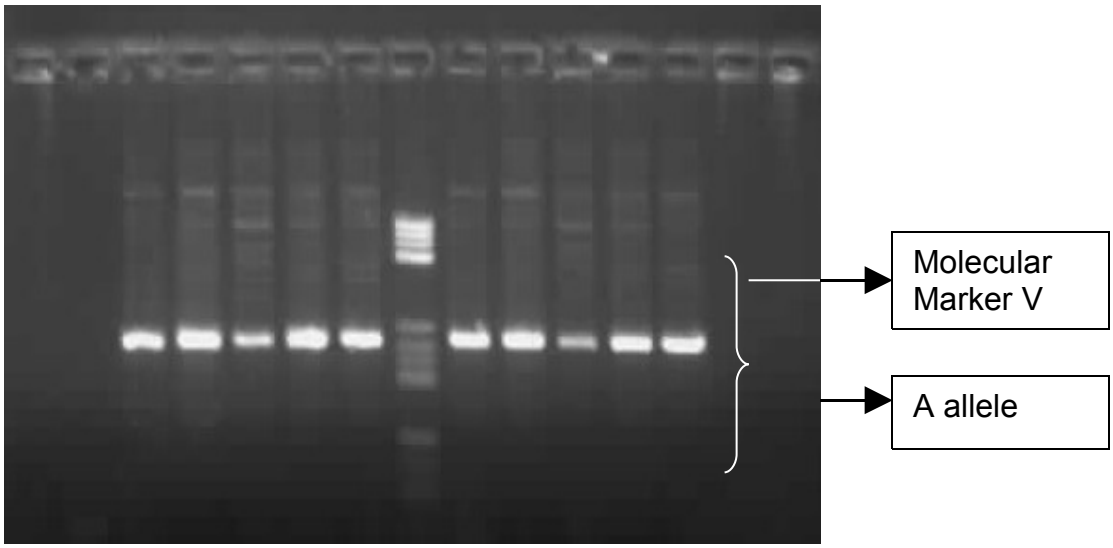
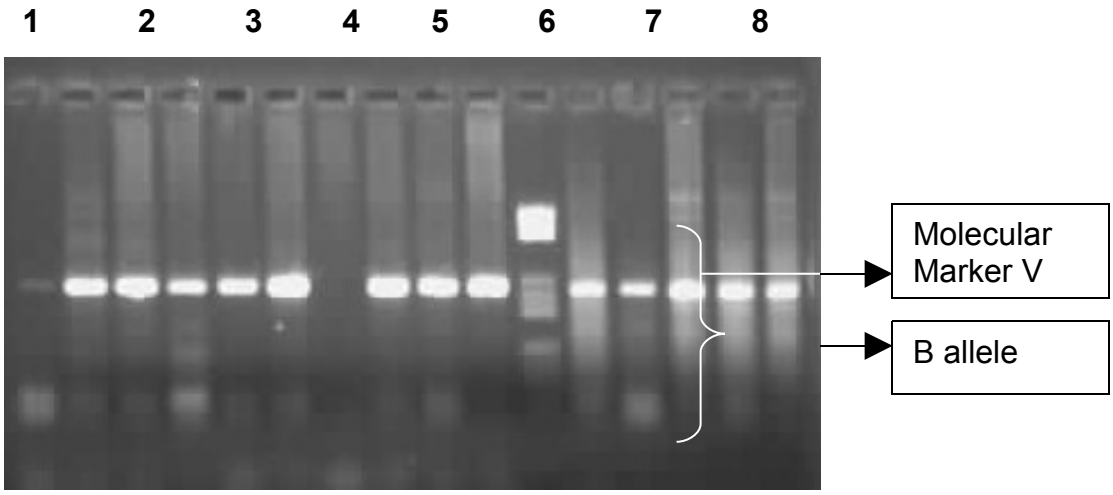


Figure 1: A representation of the Kozak genotypes that was separated on a 2% agarose gel electrophoresis. Lane 1 represents the blank control, while lane 2,3,4,7,8,9 and 10 represents the TT homozygote genotype. Lanes 5 and 6 represents the CT heterozygote genotype. Lane 11 is molecular marker V (8 – 587bp), while lane 12 is an uncut fragment of DNA. The CC homozygote genotype was not detected in both the study subjects and controls. Since the frequency of the CC genotype is low and the sample study size being small, a homogeneous CC genotype was therefore not present in this study (Baker *et al.,* 2001).

1 2 3 4 5 6 7 8



Figures 2a)



Figures 2b)

Figures 2a and 2b. A representation off HPA-2 Thr^{145} 234bp (fig 2a) and Met^{145}234bp (fig 2b) genotypes are shown. The genotypes were separated on 2% agarose gel containing ethidium bromide. Lane 1 represents Met^{145}234bp homozygous (B allele). Lane 3 represents the Thr^{145} 234bp homozygous (A allele). Lanes 2, 4, 5, 6 and 8 represents the heterozygous (AB) genotype while lane 7 was marker V.

When no results were observed on the gel, the sample was repeated to confirm the finding, this was done to exclude sample failure and to ensure that the result obtained was a true negative.

3.7.3 VNTR polymorphism determination – Conventional PCR

The primers sequence for the VNTR polymorphism was adapted from the method described by Baker *et al.,* (1998). The following bands, 692bp (D/D), 596bp (C/C) and 489(B/B), were visualised. Each subject was classified into one of six possible genotypes: C/C, C/B, C/D, B/D, D/D and B/B, as shown in figures 3a, 3b and 3c,Ishida *et al.,* (1995)

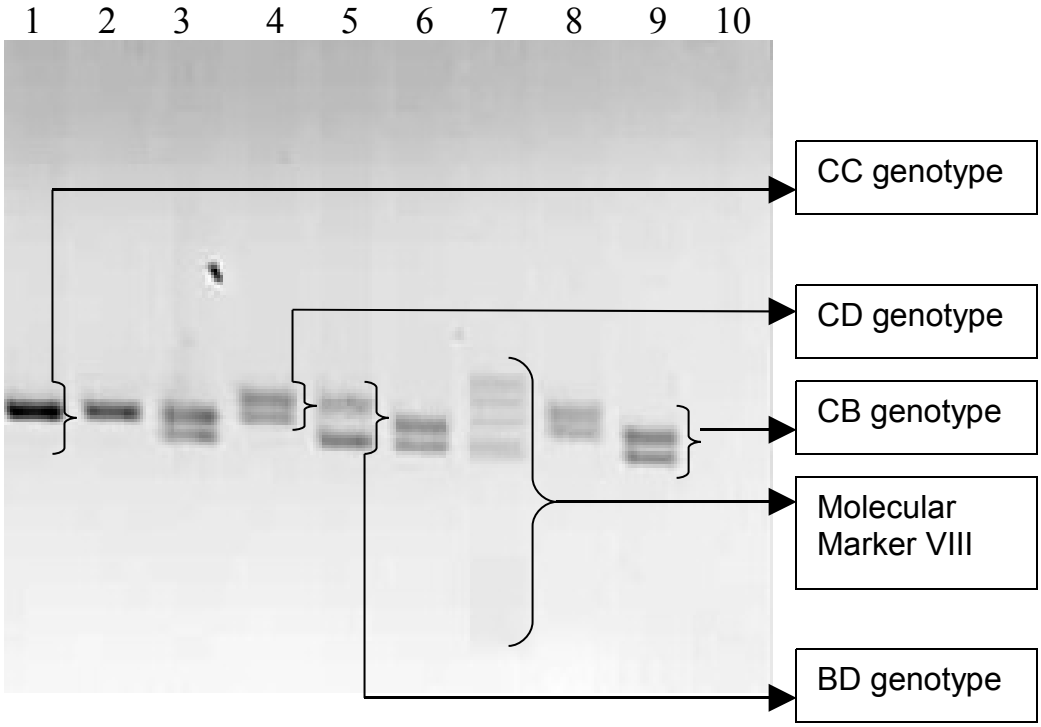


Figure 3a: Representation of the VNTR genotypes that were separated on a 2% agarose gel electrophoresis. Lanes 1 and 2 represents the CC homozygote genotype. Lanes 3, 6 and 9 represents the CB heterozygote genotype, lanes 4 and 8 represents the CD heterozygote genotype while lane 5 represents the BD heterozygote genotype. Lane 7 represents molecular marker VIIII (19 -1114 bp), while lane 10 was the blank.

1 2 3 4 5 6

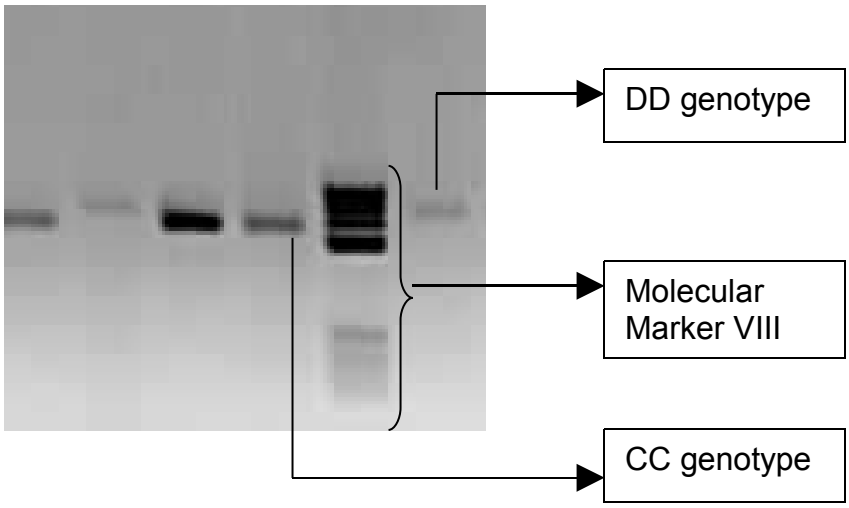


Figure 3b: Representation of the VNTR genotypes that were separated on a 2% agarose gel electrophoresis. Lanes 1, 3 and 4 represents the CC homozygote genotype. Lanes 2 and 6 represents the DD homozygote genotype. Lane 5 represents molecular marker VIIII (19 -1114 bp)

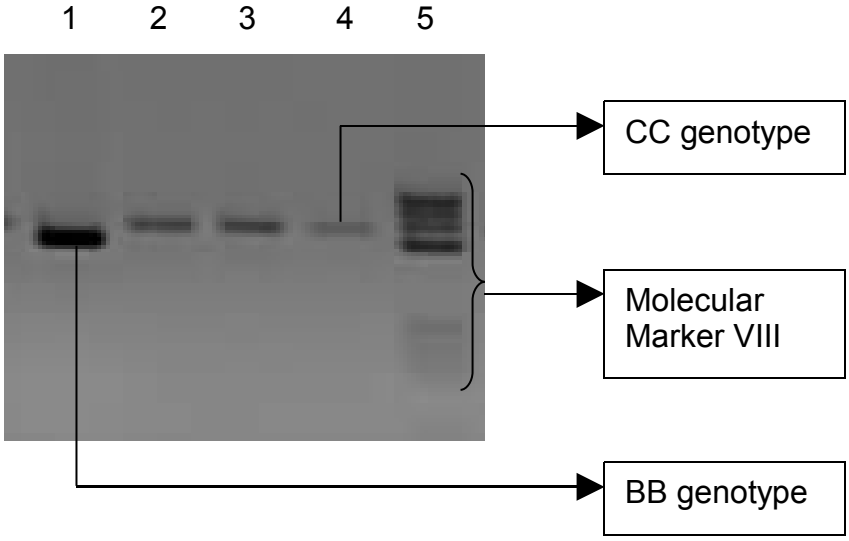
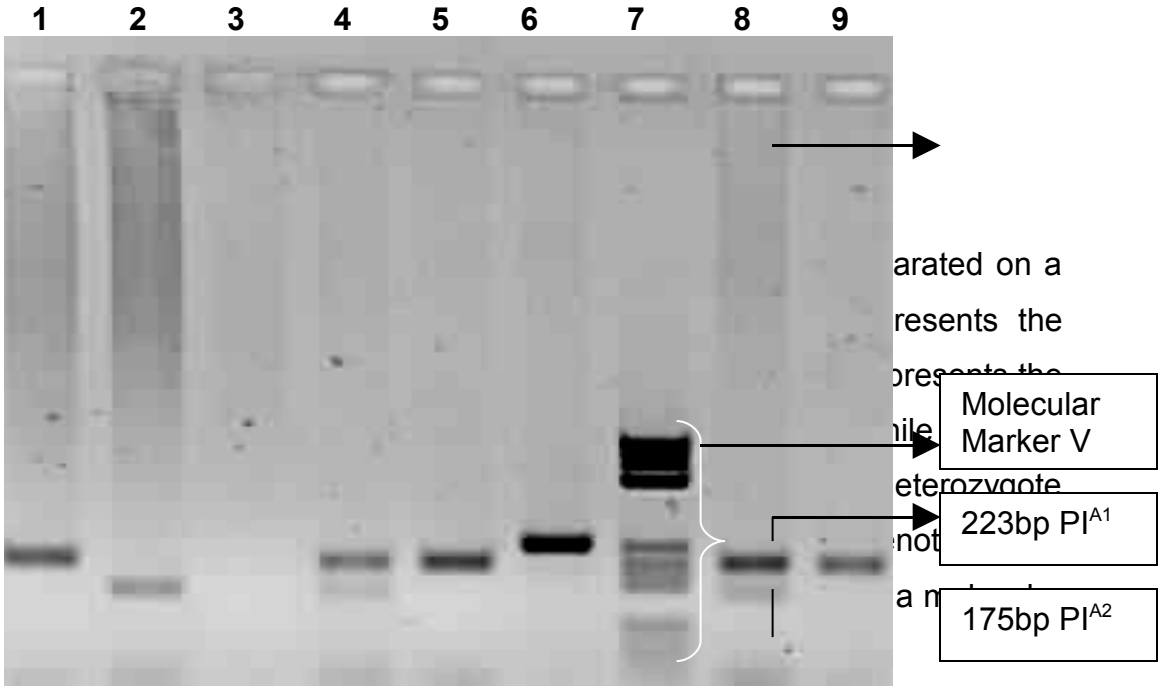


Figure 3c: Representation of the VNTR genotypes that were separated on a 2% agarose gel electrophoresis. Lanes 1 represents the BB homozygote genotype. Lanes 2, 3 and 4 represents the CC homozygote genotype, while lane 5 represents molecular marker VIIII (19 -1114 bp).

3.7.4. GPIIIa $PI^{A1/A2}$ polymorphism determination – Conventional PCR

The primers that were used in GPIIIa $PI^{A1/A2}$ polymorphism determination assay was described by Durante-Mangoni *et al.,* (1998). Two bands, 223bp (PI^{A1}) and 173 (PI^{A2}) were observed. Each subject was classified into one of three possible genotypes: $PI^{A1/A1}$, $PI^{A1/A2}$ or $PI^{A2/A2}$ as shown in figure 4.



3.8.1 Real-Time PCR using LightCycler ™

Compared to the conventional PCR methodology, the Real-Time PCR is well suited for a routine laboratory setting. The Real-Time PCR assay makes use of a commercial master mix kit that was specifically designed to be optimised on the LightCycler™. For this study the FastStart DNA Master^{PLUS}HybProbe was chosen.

3.8.1. LightCycler™ FastStart DNA Master^{PLUS}HybProbe

This was a ready to use hot start reaction mix for the PCR using the LightCycler™ system (Roche Biochemicals, Germany)

3.8.2. Kit Content

The kit consisted of a ready to use hot start PCR reaction mix. It also contained Fast Start 1.0U *Taq* DNA polymerase, reaction buffer, magnesium chloride (5mM $MgCl_2$, is optimised for most primers) and 3.2mmol dNTP mix (with dUTP instead of dTTP).

3.8.3. Primers and Probes used to detect the Target Polymorphisms using Real-Time PCR.

3.8.3a Kozak – 5T/C polymorphism determination – Real Time PCR

The Kozak polymorphism was amplified using a set of forward and reverse primers together with labelled hybridisation probes which were adapted from Baker *et al.,*(2001). The primers and probes used to detect the Kozak polymorphism are shown in table 8.

Table 7: Primer and Hybridization probe sequence used for genotyping the Kozak polymorphism.

Primer/Probe	Sequence
Forward Primer	5'- GCAGGGGGATCCACTCAA -3'
Reverse Primer	5'- AGGATGGTTGTGTCTTTCGGC -3'
Detection Probe	5'- CCACAGGCCCTCATGCCTC -Fluo-3'*
Anchor Probe	5'-LCRed-640- CCTCCTCTTGCTGCTCCTGCTGCC -Pho-3'**

* Fluorescein

** Phosphorylated

2.8.3b Human Platelet Antigen (HPA-2) polymorphism – Real Time PCR

The HPA-2 polymorphism (Kozak region) was amplified using a set of forward and reverse primers together with labelled hybridisation probes which were adapted from Baker *et al.,*(2001). The primers and probes used to detect the HPA-2 polymorphism are shown in table 9.

Table 8: Primer and Hybridization probe sequence used for genotyping the HPA-2 platelet polymorphism.

Primer/Probe	Sequence
Forward Primer	5'- CGC TCT GTG CCT TCG GA-3'

Reverse Primer	5'- AGC TCA GTC AAG TTG TTG TTA G-3'
Detection Probe	5'- AGC TCT ACC TGA AAG GCA ATG AGC TGA AGA CCC TG-Fluo-3'*
Anchor Probe	5'-LCRed-640-CCC AGG GCT CCT GAC GCC CAC A-Pho-3'**

* Fluorescein

** Phosphorylated

3.8.3c GPIIIa $PI^{A1/A2}$ polymorphism determination – Real Time PCR

The GPIIIa PI^{A} polymorphism was amplified using a set of forward and reverse primers together with labelled hybridisation probes which were adapted from Weiss *et al.,* (1996). The various primers and probes used to detect the GPIIIa PI^{A} polymorphism are shown in table 7.

Table 9: Primer and Hybridization probe sequence for genotyping GPIIIa PI^{A} polymorphism.

Primer/Probe	**Sequence**
Forward Primer	5'-GGACAGGCACCTCAGCG -3'
Reverse Primer	5'- CTCTATGCCCACCTGCTTCA-3'
Detection Probe	5'- GCAATCCTCTGGGGACTGACTTGAGTGACCT-Fluo-3'*

Anchor Probe	5'-LCRed-640-GGAGCTGTCTCCAGAGCCCTTGT - Pho-3'**

* Fluorescein

** Phosphorylated

3.8.4. Preparation of the PCR Mix for Real-time PCR

The preparation of a master mix for real-time PCR is shown in table 10

Table 10: A generic protocol for the preparation of a 20µl standard master mix reaction for the GPIIIa PI^A, Kozak and HPA-2 polymorphism using their respective probes and primers for real-time PCR.

Reagent	**Reaction x1**	**Manufacturer**
DNA	5µl	-
Master-Mix–1x concentration(1.5mM $MgCl_2$, 1.0U *Taq* and 3.2mmol dNTP))	4µl	Roche,Biochemicals Germany
Forward primer 10pmol	0.5µl	Roche,Biochemicals Germany
Reverse primer 10ρmol	0.5µl	Roche,Biochemicals Germany
Anchor probe 10ρmol	0.1µl	Roche,Biochemicals Germany
Detector probe 10ρmol	0.1µl	Roche,Biochemicals

		Germany
Water	9.8µl	Sabax
Total	20µl	-

The cycling conditions for the platelet polymorphisms GPIIIa PI^A, Kozak and HPA-2 using real–time LightCycler™ PCR, are shown in table 11.

Table 11: The PCR cycling conditions for the GPIIIa PI^A, Kozak and HPA-2 polymorphism on the Real-time Lightcycler™

	GPIIIa PI^A	**Kozak**	**HPA-2**
Hotsart	95°C for 10 minute-single cycle	95°C for 10 minute-single cycle	95°C for 10 minute-single cycle
Denaturation for 45 cycles	95°C for 10 seconds	95°C for 10 seconds	95°C for 10 seconds
Annealing, and flouresence acquisition for 45 cycles	56°C for 10 seconds	58°C for 10 seconds	60°C for 10 seconds
Extension for 45 cycles	72°C for 10 seconds	72°C for 10 seconds	72°C for 10 seconds
Melting curve analysis heating cycle	95°C for 20 seconds, single cycle	95°C for 30 seconds, single cycle	95°C for 30 seconds, single cycle
Cooling cycle	40°C for 20 seconds	38°C for 30 seconds	38°C for 30 seconds
Ramping	From 40°C at 0.1ºC/s until	From 38°C at 0.1ºC/s until	From 45°C at 0.1ºC/s until

	85°C is reached	95°C is reached	95°C is reached
Cooling –once cycle is complete	From 85°C to 40°C at 0.1ºC/s	From 95°C to 38°C at 0.1ºC/s	From 95°C to 45°C at 0.1ºC/s
Flourescence Measurements	At 0.1ºC/s intervals	At 0.1ºC/s intervals	At 0.1ºC/s intervals

3.8.5. HPA-2, Kozak and PI^{A} Cycling Program for Real-Time PCR

All temperature transition rates were programmed at 20ºC/s. Each experiment included a heterozygote control together with a blank control (distilled water) and the test samples.

Melting curves were converted into melting peaks by plotting the negative derivative of the fluorescence with respect to temperature (-dF/dT) against temperature (T).

3.9. Representation of the Melting Curve Analysis of the Platelet Polymorphisms for HPA-2, Kozak and GP IIIa PI^A using Real-Time PCR

3.9.1. HPA-2, Kozak and GPIIIa PI^A polymorphisms

All the samples which were used to detect the platelet HPA-2, PI^A and Kozak polymorphisms using conventional PCR were repeated using Real-Time PCR on the lightCyler™. For all the polymorphisms the amplification was monitored in the LightCycler F2 channel. Representation of typical results for genotyping with this method are shown in figures 5, 6 and 7(LightCycler peaks) for HPA-2, GPIIIa PI^A and Kozak polymorphism. The melting peaks for the HPA-2 showed the (Met^{145}) wildtype homozygous genotype was at 73.9°C and the mutant homozygote (Thr^{145}) was at 67.5°C. The heterozygote mutant produced two characteristic melting peaks which was observed at 67.5°C and 73.9°C (Fig 5). The melting peak or the PI^A polymorphism showed that the melting temperature for the homozygote (PI^{A2}) mutant allele was at 65.7°C, while for the normal wild type (PI^{A1}) allele it was observed at 69.6°C. The heterozygote genotype

had both (65.7°C and 69.6°C) melting temperatures (Fig 6). The melting peak for the Kozak polymorphism revealed that the (T/T) wildtype genotype was observed at 47.6°C. The heterozygote mutant produced two characteristic melting peaks which was observed at 47.6°C and 55.7°C (Fig 7). There was no homozygote C/C genotype recorded in this study.

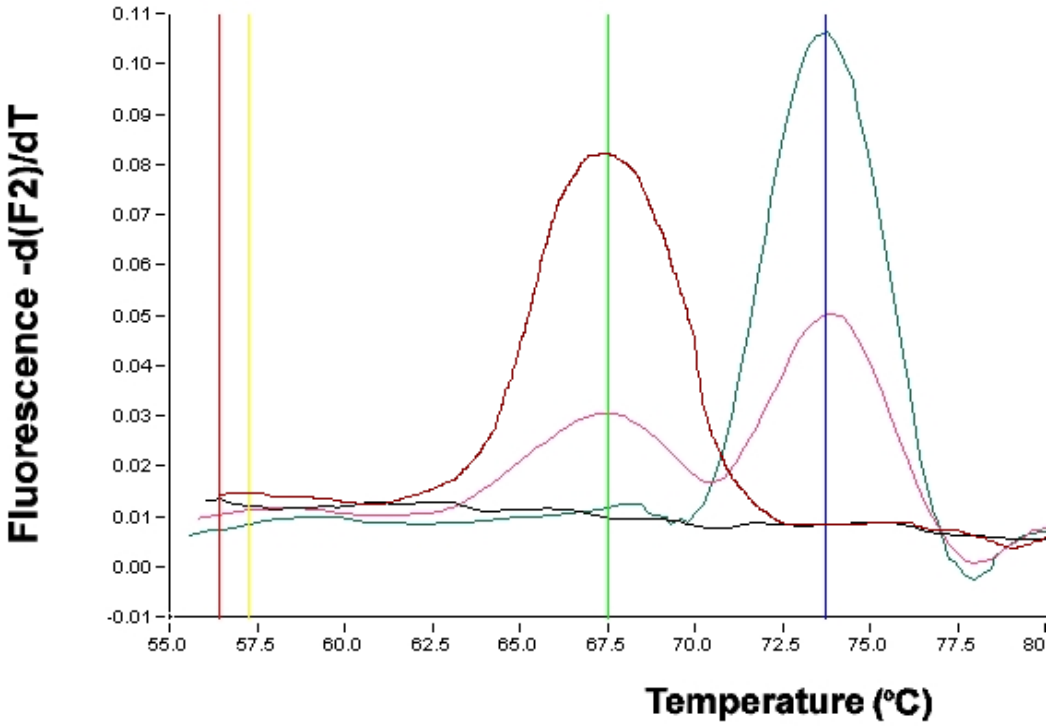


Figure 5: Melting curve analysis for HPA-2 polymorphism. The Green curve is homozygous for the wild type (Met^{145} AA genotype), while the brown curve is homozygous for the mutant (Thr^{145} BB genotype), the magenta curve indicates the heterozygous form (AB genotype) with the black line indicating the blank. The melting temperature for the mutant genotype was observed at 67.5°C, while for the wild type the melting temperature was observed at 73.6°C. The heterozygote has two peaks containing both the mutant and wild type hence mimicking the temperatures of each.

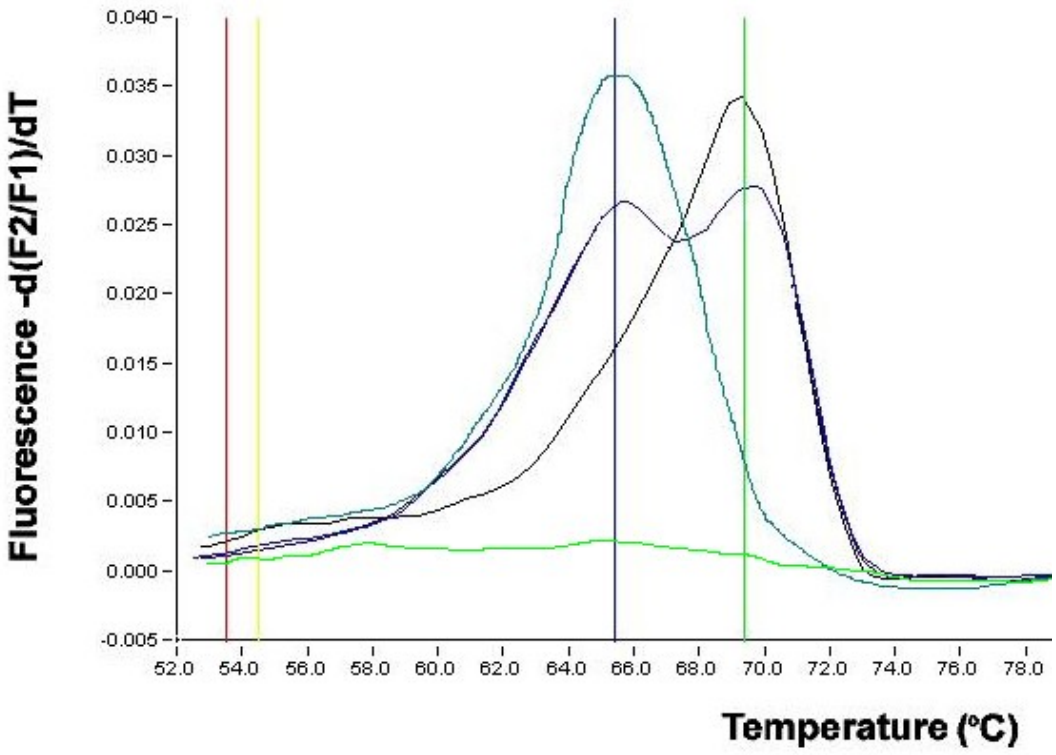


Figure 6: Melting curve analysis for PI^{A} polymorphism. The black curve is homozygous for the wild type genotype ($PI^{A1/A1}$), while the green curve is homozygous for the mutant genotype ($PI^{A2/A2}$), the blue curve indicates the heterozygous form ($PI^{A1/A2}$) with the light green line indicating the blank. Melting temperature was observed at 65.7°C for the mutant genotype and 69.6°C for the wild type genotype. The heterozygote presented with two melting peaks, with the melting temperatures indentical to the wild type and mutant genotype.

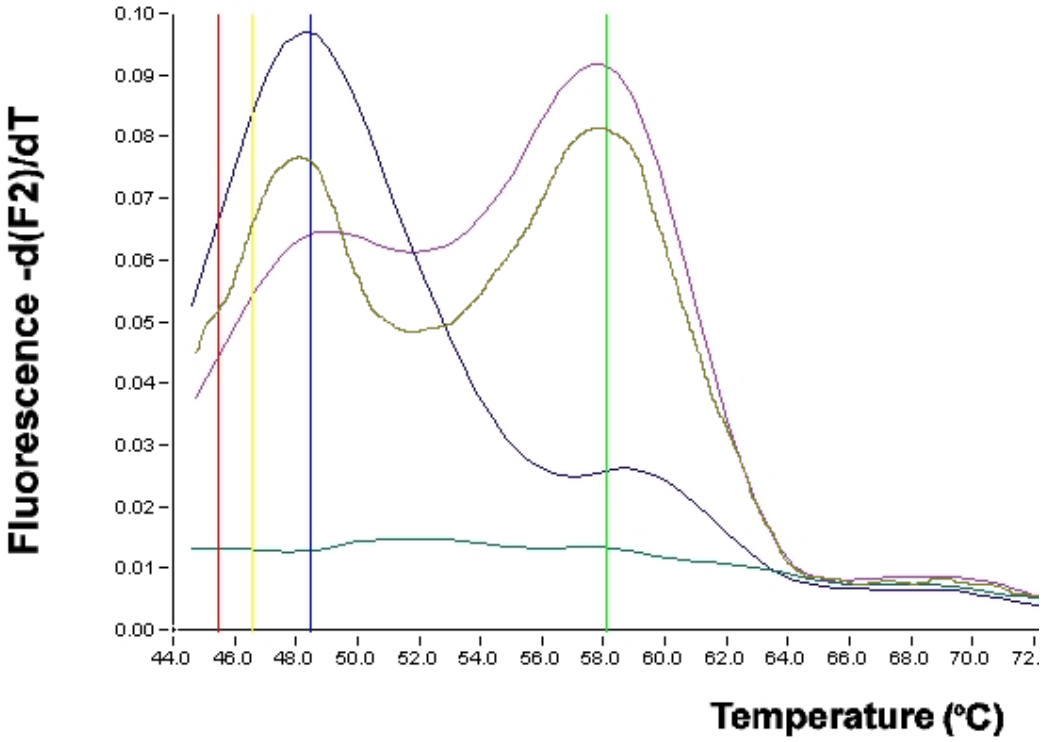


Figure 7: Melting curve analysis for Kozak –5T/C polymorphism. The black curve indicates the homozygous or wild type genotype (TT), while the megenta and gold curves are heterozygous (CT) and the green line indicating the blank. Melting temperature was observed at 48.5°C and 57.8°C for the for the heterozygote genotype while for wild type a single peak was observed at 48.5°C. There was no mutant (CC) genotype in either the patient or normal population studied.

3.9.2. The Application of Real-Time PCR to the VNTR Polymorphism.

VNTR Primers and Probes

Numerous attempts were made to design a set of primers and probes to detect the VNTR polymorphism on the LightCycler™ but were unsuccessful. This was due to the fact that the VNTR consisted of four repeats of 39bp each, and it was not possible to create standard probes to detect the full VNTR polymorphism sequence. Primary models using melting curve analysis indicated that there would be no distinction between the B and D repeats and therefore no interpretation would be possible. Since the melting curve analysis relies on the GC content of the product, the A and T both melt at 2°C and C and G both melt at 4°C, it does not matter what combination of (A,T,C and G) content was presented the melting temperature could always be calculated. It is for this reason that the VNTR B and D alleles could not be distinguished on the LightCycler™ by using melting curve analysis. Both the B and D alleles contain the same GC content in sequences. However, visualisation of these products using conventional PCR is not a problem when using hybridising probes since the fragments would be observed at different locations on the gel electrophoresis. The B and D allele occupy different sites on the gel and therefore can be accurately genotyped without ambiguity. While on the real-time LightCycler™ both the B and D alleles melt at the same temperature and therefore cannot be distinguished.

CHAPTER FOUR

4. RESULTS AND STATISTICAL ANALYSIS OF ALL PCR RESULTS

For the purpose of this study, the Hardy-Weinburg equilibrum, Fisher exact and the Kappa statistical models were used to analyse the results.

The Hardy Weinberg Equilibrium was used to calculate allele and genotype frequencies.

4.1. Calulating gene frequencies as per formula:

The formula as follows:

$$\frac{2 \text{ X (homozygous genotype)} + 1 \text{ X (Heterozygous genotype)}}{2 \text{ X (Total genotype)}}$$

Fisher's Exact Test is based on exact probabilities from a specific distribution (the hypergeometric distribution). It is used in place of the chi-square test in small 2-by-2 tables. A p value of ≤ 0.05 was considered significant.

Kappa statistics was used to compare the two methods, the conventional PCR and the real-time PCR. Kappa measures the percentage of data values in the main diagonal of the table and then adjusts these values for the amount of agreement that could be expected due to chance alone.

Kappa is always less than or equal to 1. A value of 1 implies perfect agreement and values less than 1 implies less than perfect agreement. Therefore, a poor agreement is idicated by a value of <0.2, while a fair agreement is between 0.20 and 0.40, moderate agreement is between 0.41 and 0.60, good agreement between 0.61 and 0.80 and very good agreement between 0.81 and 0.99 (Altman DG, 1991)

4.2 Hardy-Weinberg Equilibrium and Fisher's Exact Test

While the Hardy-Weinberg Equilibrium was used to calculate allele frequencies from the population studied shown in tables (12, 13 ,14, 14b and 15), the Fisher's Exact Test was used to obtain the p value for each polymorphism. A p value of ≤ 0.05 was considered significant.

Table 12. The percentage distribution of GPIIIa (PI^A) polymorphism and PI^{A2} Allele frequency in stroke vs controls

Genotype PI^A	Patients n=60	**% Patients**	Controls n=38	**% Control**	**Total**
$PI^{A1/A1}$	41	68.3	26	68.4	67
$PI^{A1/A2}$	16	26.7	11	28.9	27
$PI^{A2/A2}$	3	5	1	2.7	4
PI^{A2} Allele Frequency		**0.18**		**0.17**	

(Fisher exact) p = 0.997 (no significance)

No significant difference was observed in the percentage distribution of the GPIIIa PI^A genotypes between the patient and control subjects.

Table 13. The percentage distribution of Kozak (-5T/C) polymorphism and T Allele frequency in stroke vs controls

Genotype **Kozak**	Patients: n=60	**% Patients**	Controls n=38	**% Control**	**Total**
TT	46	76.7	28	73.7	74
TC	14	23.3	10	26.3	24
CC	0	0	0	0	0
T Allele Frequency		**0.88**		**0.86**	

(Fisher exact) p = 0.811 (no significance)

No significant difference was observed in the percentage distribution of the Kozak -5T/C genotypes between the patient and control subjects.

Table 14a. The percentage distribution of VNTR polymorphism and C Allele frequency in stroke vs controls

Genotype **VNTR**	Patients: n=60	**% Patients**	Controls n=38	**% Control**	**Total**
CC genotype	49	81.7	28	73.6	78
CB genotype	2	3.3	5	13.15	7
CD genotype	5	8.3	1	2.63	6
BD genotype	0	0	2	5.2	2
BB genotype	1	1.7	0	0.0	2
DD genotype	3	5.0	2	5.2	5
C Genotype Frequency		**0.83**		**0.84**	

(Fisher exact) p = 0.731(no significance)

No significant difference was observed in the percentage distribution of the VNTR genotypes between the patient and control subjects.

Table 14b. Breakdown of the calculations for the C allele frequency for VNTR polymorphism, as it appears in three different genotypes.

Genotype	**Patient**	**Control**
CC	0.87	0.80
CB	0.07	0.09
CD	0.06	0.11
Total	1	1

Table 15. The percentage distribution of HPA-2 polymorphism and BB genotype frequency in stroke vs controls

Genotype **HPA-2**	Patients: n=60	**% Patients**	Controls n=38	**% Control**	**Total**
AA genotype	50	83.3	30	78.9	80
AB genotype	9	15.0	7	18.4	16
BB genotype	1	1.7	1	2.7	2
BB Genotype Frequency		**0.09**		**0.11**	

(Fisher exact) p = 0.512 (no significance)

No significant difference was observed in the percentage distribution of the HPA-2(A-B) genotypes between the patient and control subjects.

4.3 Kappa statistics

A perfect correlation of two methods, that of the conventional PCR and real-time PCR is shown in table 16, 17, and 18 using kappa statistics. Below, the horizontal axis reflects the real-time PCR method while the vertical axis reflects the conventional PCR method. The number next to each polymorphism reflects samples that were positive for that specific polymorphism and the respective methods. The numbers were the same

for each method therefore, there was perfect correlation between methods.

Table 16: Correlation between the Real-Time PCR and the Conventional PCR method for PI^A polymorphism

	Real-Time	**PCR**		
Conventional PCR	**PI^A**	$PI^{A1/A1}$ n=67	$PI^{A1/A2}$ n=27	$PI^{A2/A2}$ n=4
	$PI^{A1/A1}$ n=67	67 (**1**)		
	$PI^{A1/A2}$ n=27		27 (**1**)	
	$PI^{A2/A2}$ n=4			4 (**1**)

A perfect correlation (Kappa=1) was obtained between the conventional and real-time PCR for GPIIIa PI^A as shown in table 16.

Table 17: Correlation between the Real-time PCR and the Conventional PCR method for Kozak polymorphism

	Real-Time	**PCR**		
Conventional PCR	**Kozak**	TT n=74	CT n=24	CC n=0
	TT n=74	74(**1**)		
	CT n=24		24(**1**)	
	CC n=0			0(**1**)

A perfect correlation (Kappa=1) was obtained between the conventional and real-time PCR for Kozak (T/C) polymorphism as shown in table 17.

Table 18: Correlation between the Real-time PCR and the Conventional PCR method for HPA-2 polymorphism

	Real-Time	**PCR**		
Conventional **PCR**	 **HPA-2**	AA n=78	AB n=18	BB n=2
	AA n=78	78 (**1**)		
	AB n=18		18 (**1**)	
	BB n=2			2(**1**)

A perfect correlation (Kappa=1) was obtained between the conventional and real-time PCR for HPA-2 as shown in table 18.

CHAPTER FIVE

5. Discussion

Platelet polymorphisms are, without doubt, making an impact on the molecular diagnosis of thrombotic diseases. DNA is the basis for genetic studies, and platelet polymorphisms are no exception. The use of molecular tools is of great value, especially in the understanding of the dynamics and complexity of thrombosis and stroke. Recently there have been advances in antiplatelet therapy in the treatment of thrombosis in which aspirin still remains the cornerstone for treatment of stroke and together with other antiplatelet drugs such as clopidogrel, has managed to reduce the incidence of secondary strokes (Dierner *et al.,* 2004). In order to monitor the efficacy of aspirin *in-vivo*, one requires *in-vitro* laboratory tests on platelet function. Platelet aggregrometery remains one of the few laboratory tests available for monitoring the effects of aspirin on platelets. This technique is reliable to assist clinicians in making decisions on

treatment. However, it is only accessible in tertiary health care centres and impractical for most routine laboratories.

It is therefore crucial that an alternative laboratory test be made available which may assist clinicians to make meaningful therapeutic decisions. Assessment of platelet polymorphisms may be a potential solution. Studies have shown that platelet polymorphisms play a major role in stroke and could also be implicated in aspirin non responsiveness (Martin *et al.,* 2005). Genotyping is an exact science. It cannot change over time and therefore it is definitive. It is highly sensitive and specific. It is also amenable to multiplexing and thus high-throughput could be achieved by analysing two genotypes simultaneously. PCR methodologies are well defined and are routinely used in most diagnostic laboratories all over the world to detect various genotypes for a multitude of diseases. The specimen requirement for performing platelet polymorphism testing is a 5ml EDTA sample that does not require any special collection conditions. Provided that the sample reaches the reference laboratory within a few days, the test could be done almost immediately. The only requirement for this test is DNA. Samples can therefore be batched and tested at a prescribed time thus reducing the cost per test. The simplicity and convenience of genetic testing is most attractive. The patients do not have to present themselves at the tertiary centres. Blood samples can be transported from afar and individuals could be genotyped within one week.

Although, the conventional PCR still remains a well established method for genotyping existing polymorphisms, real-time PCR is extremely fast when compared to conventional PCR. However, the initial costs for the real-time PCR is high, due to the fact that materials and reagents may not be routinely available. Most of material and reagents need to be custom designed and would therefore require additional scientific resources. The cost, however, is transitional and will depend on the demand for the test, which could lead to kits being produced for routine use thus reducing the cost of the tests significantly, (see appendix 2 for cost comparison). The added advantage of real-time PCR is the fact that there are fewer manual steps thus rendering the test less prone to contamination. Furthermore, there is no longer a need to use mutagenic substances, such as ethidium bromide.

Since platelet phenotypic expressions are prone to change over time and with treatment, the presentation of the platelet genotypic expression may complement the platelet phenotypic expression thus, allowing the clinicians to offer the best possible treatment specific to the individual (Helgason *et al.,* 1993).

In the present study there was a spectrum of genotypes for each of the polymorphisms studied. Previous studies done by Newman *et al.,* (1989) and Stakos *et al*., (2002) showed that the GPIIIa PI^{A2} in both homozygote as well as the heterozygote state were implicated in thrombosis and

stroke. Their findings showed that men less than 50 years of age with the homozygote form of the GPIIIa PI^{A2} polymorphism experienced a higher incidence of stroke while women of the same age group did not since oestrogen seems to confer some form of protection. Studies have also shown that GPIIIa PI^{A} and Kozak polymorphisms were implicated in stroke and coronary thrombosis. Hsieh *et al.,* (2004) using data from the Vienna Stroke Registry found that patients who were homozygous for the Kozak CC genotype had a 3.5 fold increased risk for ischaemic cerebrovascular events compared with the TT or TC genotype carriers. Baker *et al.,* (2001) concluded that the Kozak -5T/C polymorphism was an independent risk factor for first-ever ischaemic stroke. This T/C polymorphism was associated with an increase in platelet GP Ibα surface expression. In this study there was no age or gender related differences with regards to the various genotypes studied.

Mikkelsson *et al.,* (2001) suggested that the HPA-2b/VNTR B haplotype of the platelet von Willebrand factor and thrombin receptor protein GP Ib-V-IX may be considered to be a major risk factor of coronary thrombosis, fatal myocardial Infarction and sudden cardiac death in early middle age. The CB genotype of the VNTR and HPA-2b polymorphism were found to be associated with an increased risk of stroke (Carlsson *et al.,* 1999; Gonzalez-Conejero *et al.,* 1998; Sonoda *et al.,* 2000). However, some studies suggest that the HPA-2a and VNTR B polymorphism confer some sort of protection from thrombotic events (Carter *et al.,* 1998 and Carlsson

et al., 1997). Magurie *et al.,* (2008) suggested that the Thr/Met polymorphism was associated with ischaemic stroke in a dominant genetic model, while studies conducted on a cohort of ethnic Chinese by Zhang *et al.,* (2007) concluded that the D allele of the VNTR polymorphism was associated with atherothrombotic stroke. Furthermore, recent studies by Cervera *et al.,* (2007) showed that the BC genotype of the VNTR polymorphism was a contributing factor associated with aspirin treatment failure and it was therefore concluded that this genotype was an independent predictor of recurrent events in stroke patients on aspirin.

In the present study there was no difference between patients and controls in respect to statistics performed on the various polymorphisms. There was also no significant difference between the control subjects and the patient group with regards to all the polymorphisms studied. Infact there was almost perfect correlation of data. One of the possible explaination for this is that the sample size was small and further studies using a larger sample size is required. The VNTR genotype distributions in patients and controls were in Hardy-Weinberg equilibrium. There was no difference in the genotype distribution of VNTR in patients and control, with one exception that of the VNTR polymorphism in the CB genotype was found in 13.2% in the control population and only 3.3% of the patient population. Thus, the data of this study, for the CB genotype in particular supports the findings of both Carter *et al.,* 1998 and Carlsson *et al.,* 1997 that the HPA-2a and VNTR B polymorphism could confer some sort of protection from thrombotic events.

In the VNTR polymorphism the A allele was absent in this study. This allele as been shown to occur in the Japanese population (Ishida *et al.,* 1995) and therefore this study was in keeping with the findings by Ishida *et al.,* (1995). For the Kozak polymorphism the absence of the C/C genotype was not surprising since literature shows that it is not a common allele (Baker *et al.,* 2001). The frequency of the C/C genotype is reported to be less than three percent in the population that was studied (Douglas *et al.,* 2002). However, subsequent studies conducted by Douglas *et al.,* (2006) proposed that the glycoprotein Ibα genotypes C/C of the VNTR and the -5T/T of the Kozak polymorphism increased the risk of platelet plug formation under higher sheer rates that was generated when vessels are stenosed, thus causing an increase in the risk of myocardial infarction. A second important observation was that the Kozak -5T/C genotype was reported to confer some protective effect against thrombosis.

Data obtained from platelet function studies of both the control and patients (with permission from Dr Penelope Bernstein, unpublished data) was compared to data for the platelet genotyping to test for association. Platelet aggregation was performed on all the individuals enrolled for this study. The arachidonic acid was chosen as an agonist to confirm platelet resistance or sensitivity to aspirin. If the platelets did not aggregate in the presence of arachidonic acid it was considered sensitive and if it did it was considered resistant to aspirin.

Of the 60 caucasian patients tested only four were identified as possible aspirin non responders (6.7%), and when their genotypes were compared to the platelet function, no trends where detected. One of the possible explainations for this is that, aspirin resistance or aspirin non responsiveness may be due to, poor aspirin absorption, poor compliance, increased cyclooxygenase synthesis and activity, as well as platelet polymorphisms which indirectly influence the effectiveness of aspirin (Martin *et al.,* 2005).

Nevertheless, the aim of this study was to compare the conventional PCR to the real-time PCR. Although the real-time technique is relatively simple and time saving, it does require initial hands on troubleshooting to optimise the assay. The initial set up and analysis of the polymorphism was accomplished by trial and error. There were a number of remelts that had to be performed to fine tune the assay. Indeed, molecular techniques have progressed and for the purpose of this study the conventional PCR method that was used was not complex. The quoted primers worked well for the conventional PCR. Although with real-time PCR problems were encountered, examples of such problem arose during the analysis of PI^A, in which two sets of primers were designed according to the original primers sequence (Durante-Mangoni, 1998) and in both instances the primers failed to amplify the heterozygote state and thus need to be redesigned. Similarly, the Kozak polymorphism real-time PCR required trouble shooting, initially to determine the correct combination of sense

and antisense primers and secondly to determine the correct melting temperatures together with a slower ramping rate (46°C to 65°C at 0.1°C/s). After numerous attempts the correct combination was found and the method was adapted for the real-time PCR. Furthermore, with the VNTR polymorphism it was not possible to automate this method using real-time PCR. This was due to the fact that the VNTR consisted of four repeats of 39bp each, and it was not possible to create standard probes to detect the full VNTR polymorphism sequence. Primary models using melting curve analysis indicated that there would be no distinction between the B and D repeats and therefore no interpretation would be possible. Since the melting curve analysis relies on the GC content of the product, the A and T both melt at 2°C and C and G both melt at 4°C, it does not matter what combination of (A,T,C and G) content was presented the melting temperature could always be calculated. It is for this reason that the VNTR B and D alleles could not be distinguished on the LightCycler™ by using melting curve analysis. Both the B and D alleles contain the same GC content in sequences. However, visualisation of these products using conventional PCR is not a problem when using hybridising probes since the fragments would be observed at different locations on the gel electrophoresis. The B and D allele occupy different sites on the gel and therefore can be accurately genotyped without ambiguity. While on the real-time LightCycler™ both the B and D alleles melt at the same temperature and therefore cannot be distinguished.

One of the advantages of real-time PCR technology is the possibility of multiplexing tests. Multiplexing allows for two or even three different polymorphisms to be simultaneously detected in the same reaction tube. This will substantially reduce cost, time and the amount of technical skill required to perform the test. Therefore, the ability to adapt the conventional PCR to a real-time PCR platform is the most logical progression in the evolution of methodology. This study clearly showed that it was possible to adapt the technique successfully.

CHAPTER SIX

6. Conclusion

In conclusion, this study clearly shows that the adaptation of the conventional PCR method to Real-time PCR for platelet GPIIIa PI^A, HPA-2 and Kozak polymorphisms is possible. This adapatation makes it possible to screen for platelet polymorphisms that have been implicated in platelet resistance not only in subjects that are at risk of developing strokes but in subjects at risk of developing premature coronary artery disease. However, a larger population based study on subjects at risk of ischaemic attacks needs to be performed to confirm the viability of platelet polymorphisms as a surrogate marker for platelet function.

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Promega: http://www.promega.com

National Health Museum:

http://www.accessexcellence.org/AE/AEC/CC/restriction.html

Appendix

Appendix 1

Sampling Technique

For the purpose of this study patients were screened by a neurologist using a set of criteria available at the department of neurology, Johannesburg hospital to include or exclude patients in this trial.

All the participants in this study were classified has having a stroke as confirmed by Magnetic resonance imaging (MRI) at the Stroke Clinic, located at the Johannesburg hospital - Parktown

Patient group:

The doctor attending the patient were responsible for filling out a Patient Data form. This form included relevant demographic data (including name and hospital number) as well as relevant medical history. The doctor

assessed whether the patient met the inclusion/exclusion criteria stated on the form.

Inclusion Criteria (Patient Group)

The following patients were eligible for the study:

a) White male or female
b) Older than 20 years and younger than 80 years
c) Has had a previous stroke or transient ischaemic attack.

   Stroke: Must have documented infarct on CT scan/MRI

   Transient Ischaemic Attack (TIA): Must be diagnosed by a qualified neurologist according to most recent diagnostic criteria

d) In the case of stroke – at least one month must have elapsed after the stroke.This is not a necessary criterion in the case of TIA
e) Patients must be taking daily aspirin 75-150mg daily. This may be any preparation eg. enteric coated

Exclusion Criteria (Patient Group)

The following patients were excluded from the study:

a) Patients of race groups other than White
b) Patients younger than 20 years or older than 80 years
c) Pregnant women
d) Patients whose daily dose of aspirin is less than 75mg or more than 150mg

e) Patients on other antiplatelet agents such as dipyridamole (Persantin, Asasantin), clopidogrel (Plavix), ticlopidine (Ticlid), abciximab (Reopro), eptifibatide, tirofiban.
f) Patients who have used nonsteroidal anti-inflammatory drugs (NSAIDs) within 7 days of testing.
g) Patients whose stroke was of cardiac embolic origin
h) Patients who have eaten onions or garlic within 3 days of testing

The following were NOT exclusion criteria:

a) concomitant ischaemic heart disease, peripheral vascular disease, diabetes, connective tissue disease
b) chronic NSAID use, provided the person stops all NSAIDs for at least a week prior to having blood tests
c) Patients who have been diagnosed with a thrombophilic state eg Protein C or S deficiency, activated protein C resistance, factor V Leiden, prothrombin gene 20210A mutation,
d) hyperhomocysteinaemia, antithrombin deficiency
e) Patients with hypercholesterolaemia, hereditary or acquired
f) Patients with mild, moderate or severe hypertension, provided that their cerebrovascular event is known to be ischaemic and not haemorrhagic

Control group:

A Control Data Form was filled out for each participant in the control group. This included relevant demographics (including name and contact details of a doctor) and medical history. This form may be filled out by any doctor or nursing sister appointed to the task. This person was assessed to meet the inclusion/exclusion criteria stated on the form.

Inclusion Criteria (Control group)

a) White male or female
b) Older than 20 years and younger than 80 years

Exclusion Criteria (Contol group)

a) Subjects of race groups other than White
b) Subjects younger than 20 years or older than 80 years
c) Pregnant women
d) Subjects taking chronic aspirin therapy
e) Subjects on antiplatelet agents such as dipyridamole (Persantin, Asasantin), clopidogrel (Plavix), ticlopidine (Ticlid), abciximab (Reopro), eptifibatide, tirofiban.
f) Subjects who have used nonsteroidal anti-inflammatory drugs (NSAIDs) or aspirin within 1 week of testing.
g) Subjects who have had a stroke (ischaemic or haemorrhagic) or transient ischaemic attack
h) Subjects with ischaemic heart disease, peripheral vascular disease or connective tissue disease, familial hypercholesterolaemia

i) Subjects with any previously diagnosed bleeding or clotting disorders for example (but not limited to) haemophilia, von Willebrands disease, Protein C or S deficiency, activated protein C resistance, factor V Leiden, prothrombin gene 20210A mutation, hyperhomocysteinaemia, antithrombin deficiency

j) Subjects with a past or current malignant disease.

k) Subjects who have eaten onions or garlic within 3 days of testing

Results:

A copy of each patient's results was made available to the referring neurologist. The genotype of each study participant was handed to the principle investigator of the bigger study to be communicated to the referring neurologist.

Appendix 2

Comparison of Costing for Real-Time PCR vs Conventional PCR

Table 19 Real Time PCR - Initial Cost

Consumables	**Manufacturer/ Catalogue No**	**Cost per test**
DNA Extraction Kit	Roche-11796828001	R18
Primers	Roche- manufactured as per customer request	R1.00
Probes	Roche- manufactured as per customer request	R12.00
Probe Set-Anchor	Roche- manufactured as per customer request	R12.00
Primer Set-Detector	Roche- manufactured as per customer request	R1.00
DNA grade water	Sabex	R1.00
Capillaries Lightcycler	Roche-11909339001	R6.30
DNA Master Mix	Roche-030003248001	R23.42
Other Consumables (Tips,gloves, tubes etc)	-	R29.50
Total Cost of Test		**R104.22**
Turn around Time for	1 hour	-

Preparation		
Turn around time for analysis	45 minutes	-

Table 20 Conventional PCR – Initial Cost

Consumables	**Manufacturer/ Catalogue No**	**Cost per test /Rands**
DNA Extraction Kit	Roche-11796828001	R18
Primers- Foward	Roche- manufactured as per customer request	R5.05
Primers- Reverse	Roche- manufactured as per customer request	R5.05
DNA grade water	Sabex	R0.25
Promega Master Mix Master Mix (1.5mM $MgCl_2$, 200μM of each dNTP and *Taq* 1.0 U/reaction)	Whitehead Scientific/M7502	R5.00
Gel –D1LE	Whitehead Scientific/ D1LE-H091086	R2.00
Restriction enzymes	Fermentas/	R6.00
Other Consumables (Tips,gloves, tubes etc)	-	R26.00
Total Cost		**R 67.35**
Turn around time for preparation	10 hours	
Turn around time for analysis	1 hour	

Ethics approval certificate

Ethics approval has been granted from the Ethics Committee of the University of the Witwatersrand. Ethics Protocol No: 03-09-53. Ethics clearance (Number: M080991 - R14/49 Moodly).