COMPARISON OF PLATELET RECEPTORS P2Y12, GPIIb/IIIa, GPVI, AND GPIbα BETWEEN THE CAPE CHACMA BABOON AND THE HUMAN

By

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List of abbreviations

ACS	Acute coronary syndrome
ADP	Adenosine diphosphate
AIDS	Acquired immunodeficiency syndrome
APS	Antiphospholipid syndrome
ATP	Adenosine triphosphate
bp	Basepairs
C-terminus	Carboxyl terminus
cAMP	Cyclic adenosine monophosphate
CAD	Coronary artery disease
CBD	Collagen-binding domain
CCCP	Carbonyl cyanide m-chlorophenyl-hydrazone
CD	Cluster of differentiation
cDNA	Complementary DNA
CHD	Coronary heart disease
CRP	Collagen-related peptide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
Epi	Epinephrine
EU	European Union
F(ab)	Antigen-binding fragment
F(c)	Crystallisable fragment
FXa	Activated factor X
GP	Glycoprotein
GT	Glanzmann thrombasthaenia
HIV	Human immunodeficiency virus
HEL	Human erythroleukaemia
HUVEC	Human umbilical vein endothelial cells
HPA	Human Platelet Antigen
IgG	Immunoglobulin gamma
ITAM	Immunoreceptor tyrosine-based activation motif
LRC	Leukocyte receptor cluster

MI	Myocardial infarction
MPV	Mean platelet volume
mRNA	Messenger ribonucleic acid
N-terminal	Amino-terminal
NAIT	Neonatal alloimmune thrombocytopenia
NETs	Neutrophil extracellular traps
NHLS	National Health Laboratory Service
NSPCA	National Council of SPCA's
OCS	Open canicular system
PAD	Peripheral arterial disease
PCI	Percutaneous coronary intervention
PCR	Polymerase chain reaction
PFA-100	Platelet Function Analyser 100
PI	Phosphoinositide
PKC	Protein kinase C
PLCβ	Phospholipase Cβ
PPP	Platelet-poor plasma
PRP	Platelet-rich plasma
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SLE	Systemic lupus erythematosus
SNP	Single-nucleotide polymorphism
STEMI	ST-elevation myocardial infarction
TACE	Tumor Necrosis Factor-α-Converting Enzyme
TXA_2	Thromboxane A_2
USA	United States of America
USDA	United States Department of Agriculture
UV	Ultraviolet
VNTR	Variable number of tandem repeats
VWF	Von Willebrand factor
WHO	World Health Organisation

List of amino acids

Amino Acid	3-letter code	Single-letter code
Arginine	Arg	R
Alanine	Ala	A
Asparagine	Asn	N
Aspartic Acid	Asp	D
Cysteine	Cys	C
Glutamic Acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine (start)	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

List of DNA nucleotide bases

DNA Nucleotide	Single-letter Abbreviation
Guanine	G
Cytosine	C
Thymine	T
Adenine	A

Chapter 1 – Introduction

Platelets are small, discoid, anuclear megakaryocytic fragments that play a pivotal role in haemostasis and thrombosis. When the vessel wall is damaged the resting platelets are rapidly activated and form a plug to occlude the site of damage. Decreased platelet numbers and defective platelet function can, therefore, lead to excessive blood loss. On the other hand, damage to the vessel wall (usually associated with vascular disorders like atherosclerosis) or a significant increase in platelet numbers or reactivity, can cause intravascular thrombosis and vessel occlusion, as seen in acute coronary syndrome (Watson & Harrison, 2005).

Acute coronary syndrome (ACS) is a collection of conditions caused by occlusion of the coronary arteries around the heart. These diseases include unstable angina, ST-elevation myocardial infarction (STEMI) and non-STEMI (Achar *et al*., 2005). Occlusion of the vessels is mainly due to atherosclerotic plaque rupture and the resulting platelet deposition on the lesion (Khan, 2006). In response to vessel wall damage or plaque rupture, platelets undergo conformational changes from a smooth discoid to a more spherical shape with extending pseudopodia (Carlsson *et al*., 1979). Activation of platelets is brought about via agonists such as thrombin, thromboxane A_2 (TXA_2), adenosine diphosphate (ADP), collagen and von Willebrand factor (VWF), that are released or exposed with vessel damage (Italiano, 2008). After vessel wall damage platelets adhere to the site of damage via the platelet glycoprotein (GP) receptors (Watson & Harrison, 2005).

The platelet membrane receptors that play a role in platelet adhesion, together with the ligands they bind to, are (figure 1): GPIa/IIa ($\alpha_2\beta_1$) and GPVI bind collagen; GPIc/IIa ($\alpha_5\beta_1$) binds fibronectin; GPIb of the GPIb/IX/V complex binds to VWF; $\alpha_V\beta_3$ binds vitronectin, fibrinogen, VWF and thrombospondin; and GPIIb/IIIa ($\alpha_{IIb}\beta_3$) binds VWF, fibrinogen, fibronectin and vitronectin. The fact that a single ligand can bind more than one receptor

enhances the platelet interaction with the microenvironment (Israels & Israels, 2002).

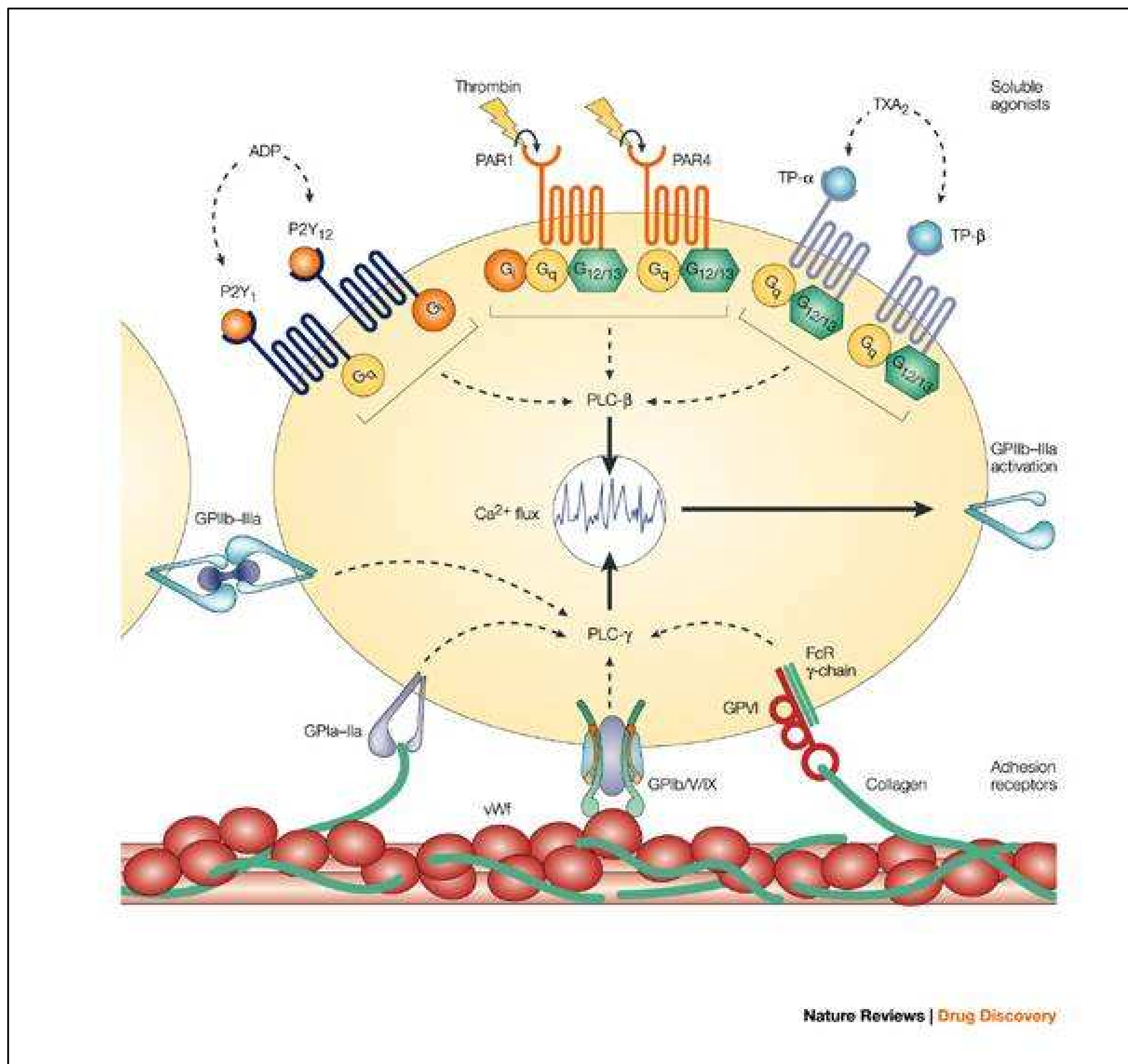


Figure 1. Platelet membrane receptors. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Drug Discovery (Jackson SP & Schoenwaelder SM. Antiplatelet therapy: in search of the 'magic bullet.' *Nature Reviews Drug Discovery* 2:775-789), copyright 2003.

The rate of shear within the damaged vessel determines the adhesion process. Under high- and intermediate-shear rates, adhesion relies on VWF to form a bridge between the exposed subendothelial matrix proteins such as collagen and fibrinogen and the platelet GPIb/IX/V receptor complex. Under

low-shear rate conditions, adhesion of GPIb/IX/V can take place without VWF, but VWF can also support adhesion under these conditions (Watson & Harrison, 2005). During these conditions, platelets can also adhere through the interactions of GPIc/IIa with fibronectin, GPIa/IIa and GPVI with collagen, and GPIIb/IIIa with immobilised fibrinogen (Israels & Israels, 2002).

After platelets form a single layer on the exposed subendothelium, they attach to each other in a process called aggregation. This process is facilitated by fibrinogen, fibronectin, GPIb/IX and GPIIb/IIIa. Aggregation is further promoted by agonists such as collagen, ADP, thrombin, adrenaline (epinephrine), serotonin and some arachidonic acid metabolites, for example, TXA_2 (Laffan & Manning, 2006).

The initial treatment and management of patients presenting with ACS is very similar (Achar *et al*., 2005). The recommended treatment regimens for patients presenting with symptoms of ACS include intravenous nitroglycerin (relieves chest pain), morphine (analgesic), a beta-blocker (counters adverse effects of norepinephrine like increased heart rate), chewable aspirin (inhibits platelet aggregation), calcium antagonist (if beta-blocker is contraindicated), subcutaneous heparin/low molecular weight heparin (inhibits coagulation), a statin (lowers LDL-cholesterol), and clopidogrel plus a GPIIb/IIIa inhibitor (inhibit arterial thrombosis in high-risk patients undergoing angiography and/or angioplasty) (Khan, 2006). The American College of Cardiology Foundation and American Heart Association recommend multi-drug treatment for ACS management, since thrombosis is such a multi-factorial and complex process. Essential to the successful management of ACS is an effective and safe anti-platelet drug, such as aspirin, clopidogrel or a GPIIb/IIIa inhibitor (Cannon *et al*., 2013).

Morbidity and mortality are very high in patients presenting with ACS (Grech & Ramsdale, 2003). Each year coronary heart disease (CHD), including ACS, causes more than half of the deaths resulting from cardiovascular disease (AHA and ASA, 2006). It equates to one in every six deaths in the United States of America (USA). ACS also produced approximately 1 141 000

hospital admissions in 2010 in the USA alone (Go *et al.*, 2014). According to statistics released by the World Health Organisation (WHO) in 2013, ischaemic heart disease caused approximately seven million deaths globally in 2011; this equates to 11.2% of all deaths recorded (WHO Factsheet No. 310, 2013). The WHO approximates that by 2030, nearly 23.6 million people will die each year of cardiovascular disease. Therefore, ACS remains a main cause of mortality in the world (WHO Factsheet No. 317, 2011). In the developing world, the human immunodeficiency virus (HIV) and acquired immunodeficiency syndrome (AIDS) associated thrombotic complications are on the increase, with the prevalence of HIV in acute thrombotic cases being as high as 84% in some settings (Louw *et al.*, 2008).

The high mortality rate of ACS gives rise to the need to develop antithrombotic drugs with better efficacy but with fewer side-effects. With the development of new antithrombotic drugs, there is an on-going need to develop safe and more effective animal models to evaluate these drugs. Novel antithrombotic drugs are usually initially tested in lower mammals (e.g. rats, mice, rabbits etc.). More discriminating animal models are needed to evaluate promising drugs before it can be clinically evaluated in humans. The type of animal is selected by comparing its blood coagulation, platelet adhesion and aggregation and fibrinolytic systems with that of humans. Non-human primates are considered the best-suited animals taking these requirements into consideration (Mason *et al.*, 1976).

Many of the new anti-thrombotic drugs are molecules that specifically target different platelet receptors. Platelet receptors play a pivotal role in the adhesion and aggregation of platelets at the site of vascular injury (Harker & Mann, 1992). Therefore, by targeting these receptors, platelet adhesion and aggregation can be inhibited, thus preventing thrombosis and the resultant occlusion of vessels that lead to ACS. Some of the most common targets are GPVI, GPIb/IX/V, GPIIb/IIIa, P2Y1 and P2Y12. Commercial drugs include abciximab (GPIIb/IIIa inhibitor), tirofiban (GPIIb/IIIa inhibitor) and clopidogrel (P2Y12 inhibitor) (Mousa *et al.*, 2001, Shanmugam, 2005 and

http://products.sanofi-aventis.us/plavix/plavix.html, 2008). Currently, there are no commercial GPVI, GPIb/IX/V or P2Y1 inhibitors available.

Due to advances in molecular pharmacology, more drugs are specifically designed to interact with certain features of a molecule to alter, increase or inhibit the particular function of the molecule. An example of this is the anti-platelet drug tirofiban, where a non-peptide molecule was designed and produced to mimic the Arg-Gly-Asp (RGD) recognition sequence on fibrinogen. The RGD recognition sequence plays a pivotal role in platelet aggregation, as this is the binding site of platelets to fibrinogen via the glycoprotein IIb/IIIa receptor on platelets. Tirofiban inhibits platelet aggregation by inhibiting the binding of fibrinogen to platelets (Mousa *et al*., 2001 and Shanmugam, 2005). Therefore, it has become paramount to not only select a test animal on its physical characteristics, but also on its genetic make-up.

Through the ages, animals have been used in research in an attempt to limit the harm caused to humans by certain products and procedures. Animals have been used extensively to explore areas where it would be ethically impossible to do the research on humans. This field has, however, been widely criticized by animal rights groups, and calls have been made to ban animal testing on a global scale (www.bwcsa.co.za; www.animalrightsafrica.org). Since 1997 ten countries around the world passed laws and policies that either banned or strictly limited the use of great apes in experiments (www.releasechimps.org/mission/end-chimpanzee-research/country-bans/).

According to statistics published by the United States Department of Agriculture (USDA) 1,027,450 animals were used in monitored US research laboratories during 2007. Of these, only 69,990 (6.81%) were non-human primates (Animal Care Annual Report of Activities, 2008). In the European Union (EU), the number of non-human primates used in research average roughly 10,000 animals per year. The EU states that non-human primates should only be used if no alternative is available for the purpose of the

research. Furthermore, the EU legislation on the marketing authorization of pharmaceuticals entails the use of non-rodent species, as closely related to humans as possible, to study the safety aspects of pharmaceuticals that have highly human-specific effects. Therefore, around 67% of all non-human primates used in the EU are for the safety and efficacy testing of pharmaceutical products (European Commission, 2009). According to statistics released by the National Council of SPCA's (NSPCA) in South Africa, only 0.1% of animals used in research in South Africa are non-human primates (baboons and monkeys). They further state that non-human primates are exclusively used when there is no alternative available (NSPCA Annual Report 2012-2013).

According to the Declaration of Helsinki, "the wellbeing of the human subject should take precedence over the interests of science and society." Therefore, the risk assessment of pharmaceuticals in animal models with the intention to safeguard humans in clinical trials takes precedent over other ethical concerns (European Commission, 2009). The high-level similitude between humans and non-human primates makes the non-human primate a highly dependable model to evaluate pharmaceuticals. Taking it into account, research-minded entrepreneurs have changed the image of non-human primates from pests to valuable assets, and in some instances saved non-human primates from eradication. Research on non-human primates can, therefore, be seen potentially beneficial not only for the research community, but also for the survival of the species (Hau & Schapiro, 2006).

Cook *et al.* (2012) recommended that larger animals, and if possible non-human primates, be used as translational proof of concept prior to commencing clinical trials in humans. The use of non-human primates in scientific research is well documented (Hill, 1977). Their similarity to humans makes them treasured as an animal model for biochemical research. Due to the genetic similarities between humans and non-human primates, the latter are considered good models to study the genetic blueprint of common human diseases (Rogers & Hixon, 1997). Therefore, they will also make suitable models for studying drugs to treat these human diseases. The non-human

primate of choice at the Department of Haematology and Cell Biology, UFS, is the Cape chacma baboon (*Papio ursinus*).

Much work has been done to map the similarities and differences in the genetic make-up of humans and baboons (Caccone & Powell, 1989, Graves *et al*., 1995, Perelygin *et al*., 1996 and Haudek *et al*., 1998). It is, however, important to note that significant genetic variety exists between subspecies of baboons and that it is important genetically to type each of the subspecies, as different subspecies cannot be interchangeably used (Williams-Blangero *et al*., 1989). There is also a growing need to sequence more non-human primate species genomes and to conduct further studies to determine the impact of genetic variance on specific experimental outcomes (Haus *et al*., 2014).

The Baboon Genome Project of the Baylor College of Medicine Human Genome Sequencing Center (BCM-HGSC) has successfully produced a draft sequence for the Olive baboon (*Papio anubis*) reference genome. This project also involves whole genome sequencing of the Cape chacma baboon (Baboon Genome Project, 2015). However, at the time of writing (May 2015) no substantial data has been made available for the Cape chacma baboon. Taking the pressing nature of our research question into account, it was decided to continue with sequencing of these specific genes, as a definite date for the availability of whole genome data could not be established.

Papio, better known as the baboon, is a thriving non-human primate found in sub-Saharan Africa. Baboons can be very flexible and adaptive in their diet; this contributes to their overall success and ultimate prosperity as species (Van Doorn *et al*., 2010). *Papio ursinus* is native to Angola, Botswana, Lesotho, Mozambique, Namibia, South Africa, Swaziland, Zambia and Zimbabwe. They are on the "least concern"-list of the IUCN Red List of threatened species as there are no major threats to them. There is, however, the matter of problem animals being shot as vermin or hunted for traditional medicine (www.iucnredlist.org, 2010). Limited impact is made on the environment by using a non-endangered species.

The Cape chacma baboon or *Papio ursinus* is classified as an Old World monkey and falls in the Cercopithecidae family. It is further sub-classified in the sub-family Cercopithecinae, which includes the guenons, macaques and mangabeys. These non-human primates are usually large, omnivorous, terrestrial animals. Baboons, *Papio spp.*, are commonly used in research. They are relatively large and on average range from 11 kg to 15 kg for adult females and 22 kg to 30 kg for adult males (Fortman *et al.*, 2002). Because baboons are large animals venepuncture on them are relatively easy, and multiple samples can also be drawn without compromising the haemodynamic system (Leadley *et al.*, 2000). However, the large size of the animals also offers disadvantages, such as the need for larger quantities of trial agents and reagents. The physical size of the baboon also poses a potential physical risk, as these animals are more dangerous to handle than smaller animals.

Currently in South Africa there is a debate on whether wild-caught baboons should be replaced by rhesus monkeys (*Macaca mulatta*) in biochemical research given the large number of captivity bred rhesus monkeys available in the country. However, the blood volume of baboons has been determined at 50-70 ml/kg and for rhesus monkeys at 50-96 ml/kg. Given that rhesus monkeys range from 4 kg for small females to 11 kg for large males, more blood is available from the larger baboons than from the much smaller rhesus. Therefore, the baboon is a more suitable animal to work on when large amounts of blood need to be analysed, as with thrombosis studies (Fortman *et al.*, 2002).

Taking the three R's of animal research, namely reduction, replacement and refinement, into consideration, it is imperative to make a thorough comparative study of primate platelet receptor genetics. The three R's are addressed as follows:

Reduction: Fewer animals will be used, by only using drugs that can interact with receptor sequences similar to those in humans. Thus, animals that are genetically incompatible with human targeted drugs will not be wasted.

Replacement: Knowing the specific DNA sequences will lead to the better selection of animal- and non-animal models that may better suit a new drug.

Refinement: Optimising drugs to limit the invasiveness to the animals (HPCSA Booklet 6, 2008).

The main reason behind this study comes from prior experience. In a previous contract research study done at the Pre-clinical Drug Testing Unit of the Department of Haematology and Cell Biology, it was found that a certain anti-platelet drug did not interact with baboon platelets. This drug was specifically designed to bind to and block a particular platelet receptor on human platelets. It was subsequently found that this particular platelet receptor has a different amino-acid sequence in baboons. It explains why the baboon could not be used as a model to test this drug. Therefore, the Rhesus macaque model, with more similar amino-acid sequence to humans at this region, had to be used. Due to contractual obligations further information on the drug cannot be divulged. It was also found in previous studies that a higher concentration of agonist is needed to stimulate baboon platelet aggregation in platelet aggregation tests than with human platelets (Cauwenberghs *et al.,* 2000 and Fontayne *et al.*, 2008). Currently, there is no explanation for this phenomenon, but it is hypothesized that it may be due to differences in receptor quantity between humans and baboons.

A study done in canines demonstrated the variance in platelet function even within species between breeds (Nielsen *et al.*, 2007). It is, therefore, important to determine the effect of different platelet aggregation agonists on baboon platelet function in comparison to human platelet function, considering that results from pre-clinical tests are used to predict the possible effect of anti-platelet drugs on human platelet aggregation.

The aim of the study was to comprehensively characterize four common platelet receptors in Cape chacma baboons (*Papio ursinus*), by sequencing the genomic deoxyribonucleic acid (DNA) of the species' receptors, quantifying the receptors and doing platelet aggregometry. It was followed by a comparison of the sequencing results with reference sequences available in the Ensembl or GenBank DNA reference databases of the same receptors on human platelets (www.ensembl.org or www.ncbi.nlm.nih.gov). Flow cytometry

was done on both human and baboon platelets to determine the difference in the percentage of platelet receptors on the platelet surface between the two species. Results found with the baboon platelet aggregometry were compared with human control results done previously at the National Health Laboratory Service (NHLS) Laboratory at Universitas Hospital in Bloemfontein, South Africa.

Chapter 2 – Literature review

The following chapter contains numerous historical references. This was done in light of the following quote taken from Nurden (2014): “It is quite remarkable that so many of the early studies still stand up in 2014. Their significance grew in importance when it was shown that the mechanisms responsible for platelet function in hemostasis were also pathologically involved in ischemic syndromes.”

Platelet receptors:

Nomenclature:

In the literature, several systems have been used as nomenclature to name the same platelet membrane receptor proteins. The first method used was based on the mobility of these proteins on gel electrophoresis. This system gave rise to the names GPI, GPII, GPIII etc. GPI has the highest molecular weight and is followed numerically according to fragment size from largest to smallest (Phillips & Agin, 1977a and Phillips, 1979). With gel separation and protein identification becoming more refined, several proteins with similar mobility were identified and resulted in more discerning names (Plow, 2003). Two dimentional SDS-PAGE procedures allowed for greater separation of glycoproteins, leading to expansion of the numbers of glycoproteins. The expansion brought names such as GPIa, GPIb, GPIc, GPIIa, GPIIb and so forth. Reduction of the intermolecular disulphide bonds leads to a reduction of molecular weight and the subsequent acceleration of migration. Migration of other glycoproteins (such as GPIIIa) was slowed due to the loss of intramolecular disulphide bonds. Some of the glycoproteins also consist of two subunits, namely the α- and β-chains (such as GPIbα). The light β-chains are separated from the heavier α-chains by reducing the disulphide bonds (Nurden, 2014).

Other nomenclature systems are based on the physical characteristics of the protein, and in which molecular family the protein falls, as is the case with GPIIb/IIIa. GPIIb/IIIa is designated the alternative name integrin $\alpha_{IIb}\beta_3$, due to its physical properties that classify it as part of the integrin family (Phillips *et al.*, 1988). However, the system based on electrophoretic mobility remains a commonly used system (Plow, 2003). Currently, there is no consensus or guidelines on the nomenclature for these receptors.

It is also important to note, that anti-platelet drugs such as abciximab, eptifibatide and tirofiban are commonly referred to as GPIIb/IIIa inhibitors, and not $\alpha_{IIb}\beta_3$ inhibitors (EPIC Investigators, 1994, EPILOG investigators, 1997, and Coller, 1997). Therefore, to limit confusion, the GP*n*-nomenclature system was used, when available.

The name of the ADP-receptor P2Y12 was used as is, as according to the nomenclature guidelines of the International Union of Basic and Clinical Pharmacology (Burnstock *et al.*, 2010).

P2Y12:

Physiology:

The role of ADP in platelet aggregation was first described by Ollgard in 1961, as well as in a later article by Born in 1962. Born (1985), through numerous animal models, subsequently came to the conclusion that ADP is one of the principal mediators of platelet aggregation, and, therefore, also a major role-player in thrombogenesis. ADP is stored in the dense granules of platelets and is released upon platelet activation. ADP is an agonist that supports platelet aggregation through G-protein-coupled, transmembrane receptors (Watson & Harrison, 2005).

Jin & Kunapuli (1998) determined that there are two G-protein-coupled proteins, namely P2Y1 and $P2T_{AC}$ (later identified as P2Y12), that play essential roles in ADP-induced platelet aggregation, and that these receptors do not work independently from each other. These are the two primary ADP

receptors involved in platelet activation and aggregation. P2Y12 is coupled to the G_i family of G-proteins. The G_i proteins inhibit adenyl cyclase and activate phosphoinositide (PI) 3-kinase. P2Y1 is coupled to the G_q and $G_{12/13}$ families which regulate phospholipase Cβ (PLCβ) and Rho kinase. P2Y12 needs to be in synergy with G_q-coupled and tyrosine kinase-linked receptors to bring on platelet activation; without this synergy, P2Y12 has very minimal effect on platelet activity (Watson & Harrison, 2005).

The P2Y1 receptor plays a role in platelet shape change and aggregation, TXA_2 production, procoagulant activity, adhesion of platelets to fibrinogen and platelet thrombus formation under high-shear stress. The P2Y12 receptor has very similar functions to the P2Y1 receptor. Other than the functions of P2Y1, P2Y12 also potentiates platelet activation caused by collagen, VWF and TXA_2 (Murugappan & Kunapuli, 2006).

Hechler *et al.* (1998a) found that P2Y1 is required for ADP-induced platelet aggregation, but subsequently found that P2Y1 alone was not sufficient to sustain complete ADP-induced platelet aggregation, and was also not the target of the ADP-receptor antagonist clopidogrel (Hechler *et al.*, 1998b). Hollopeter *et al.* (2001) subsequently identified the P2Y12 receptor as the ADP receptor that is the target of anti-platelet agents such as clopidogrel, ticlopidine and AR-66096. Foster *et al.* (2001) also identified P2Y12 (SP1999) as the target of the thienopyridine anti-platelet drugs.

P2Y12 was identified to play a role in dense granule secretion, fibrinogen-receptor activation and thrombus formation. The aggregation caused by this receptor is irreversible and can be activated not only by ADP but also by thromboxane A_2 and the PAR1 selective peptide agonist SFLLRN. P2Y12 activation causes platelet aggregation, either through G_i signalling in the presence of high concentrations of ADP or with concurrent stimulation of either the G_q or $G_{12/13}$ pathways. P2Y12 is also critical in ADP-mediated thromboxane A_2 generation. TXA_2 is also an important platelet activator. P2Y12 activation causes secretion of platelet α-granules that subsequently

leads to the expression of P-selectin on the surface of activated platelets (Dorsam & Kunapuli, 2004).

Kauffenstein *et al*. (2001) found that P2Y12 preferentially associates with a G_{i2}-protein, which subsequently triggers the inside-out activation of GPIIb/IIIa, which leads to platelet aggregation. They also established that this mechanism is independent of P2Y1 and PKC activity, but is reliant on the action of PI 3-kinase. Interestingly, epinephrine can mimic all the functions of the P2Y12 receptor (Dorsam & Kunapuli, 2004), but Kauffenstein *et al*. (2001) determined that ADP and epinephrine uses different, but synergistic, signalling pathways to activate GPIIb/IIIa.

The thienopyridines, ticlopidine (Ticlid®), clopidogrel bisulphate (Plavix®), and prasugrel are anti-platelet drugs that inhibit ADP-induced platelet aggregation. Di Minno *et al*. (1985) found that ticlopidine causes a complete inhibition of ATP secretion from platelets in response to ADP or epinephrine. However, it did not influence the intraplatelet cyclic adenosine monophosphate (cAMP) levels and ristocetin-induced platelet agglutination, nor did it impede the platelets' shape change in response to ADP, collagen, thrombin or arachidonic acid. Prostaglandin E_1 could also bind uninhibited to resting platelets in the presence of ticlopidine. They further determined that ticlopidine does not have an effect on the quantity of GPIIb/IIIa in the platelet, but creates a functionally thrombasthaenic state, by preventing adenosine triphosphate (ATP) secretion and subsequent GPIIb/IIIa surface expression.

In 1989, Hass *et al.* reported that ticlopidine is somewhat more effective than aspirin in the prevention of stroke, but carried higher risks of side-effects. However, clopidogrel bisulphate was shown to be a much more potent anti-platelet drug and was better tolerated than ticlopidine (Herbert *et al., 1993).* Clopidogrel bisulphate does not directly cause platelet inhibition. The initial drug needs to be metabolised before being active, the active metabolites of clopidogrel then covalently bind to the P2Y12 receptor for the entire lifespan of the platelet, causing irreversible inhibition of the receptor (Wallentin, 2009).

Moussa *et al.* (1999) determined that the combination of clopidogrel and aspirin was as effective as the combination of ticlopidine and aspirin in preventing thrombosis in stents after coronary implantation. However, the clopidogrel and aspirin combination had a much lower probability of causing side-effects such as neutropenia, diarrhoea and rash as the ticlopidine and aspirin combination. Helft *et al.* (2000) suggested a front-loaded regimen of a combination of clopidogrel and aspirin to attain significant antithrombotic effects in patients with atherosclerotic disease that is already on chronic aspirin therapy. In 2002, the American College of Cardiology made the recommendation that the combination of clopidogrel and aspirin should replace the combination of ticlopidine and aspirin as the standard anti-platelet treatment after coronary stent placement (Bhatt *et al.*, 2002). Dual anti-platelet therapy with clopidogrel and aspirin is approved for the treatment of atherothrombotic events in ACS and is still currently the gold standard in treating patients with ACS to prevent ischaemic complications (Han *et al.*, 2009).

Ticlopidine and clopidogrel have some disadvantages, such as delayed activity (due to the need to be metabolised), prolonged effects (due to irreversible binding to platelets), and inter-individual variability (due to individual differences in metabolism). Both drugs also elicit severely toxic side-effects such as bone marrow aplasia and thrombotic thrombocytopenic purpura (Cattaneo, 2006).

Prasugrel (CS-747) is the third member of the thienopyridine class of oral anti-platelet drugs. This drug has shown to be roughly ten times more potent than clopidogrel and 100 times more potent than ticlopidine when evaluated in rats (Niitsu *et al.*, 2005). Prasugrel overcomes some of the disadvantages of the previous thienopyridine drugs, by having a more rapid and high-grade anti-platelet effect, even in poor responders to the other drugs (Cattaneo, 2006). It was, however, reported in the TRILOGY ACS clinical trial that prasugrel did not have a significant advantage over clopidogrel when taking reduction of the primary endpoint and the decrease in bleeding risk in ACS into account (TRILOGY ACS Investigators, 2012).

Novel P2Y12 inhibitors have been developed to overcome some of the disadvantages of the thienopyridines. Ticagrelor (AZD6140) is a P2Y12 receptor antagonist which can bind reversibly to P2Y12 and reveals a rapid onset and offset of its effect, closely following drug exposure. When evaluated in mouse models, ticagrelor showed greater separation between the anti-platelet effects of the drug and the bleeding caused than the thienopyridines. One major advantage of this drug is that it does not need to be metabolized to be active. However, this drug still has some adverse effects associated with its' use, such as in increased bleeding-risk (Husted & Van Giezen, 2009). Therefore, the importance and need of developing novel P2Y12 antagonist agents, in suitable animal models, becomes even more evident. Fontayne *et al*. (2008) found that clopidogrel was successful in inhibiting arterial thrombosis in *Papio ursinus* baboons. Therefore, this study will contribute to the translational value of their results.

Molecular biology:

The P2Y12 gene is situated on chromosome 3q21-q25 (Cattaneo, 2011). The P2Y12 receptor gene (P2RY12-001, transcript ID: ENST00000302632) consists of three exons, containing 1766 bases. Interestingly, out of all three exons, only 1022 bases of exon 3 are ultimately translated into the P2Y12 protein (P2Y12, www.ensemble.org, 2011). P2Y12 receptor (figure 2 and figure 3) consists of a 26 amino acid extracellular N-terminal domain, seven transmembrane regions, three extracellular loops, three intracellular loops and a 38 amino acid cytoplasmic C-terminal domain (P2Y12_Human, www.uniprot.org, 2015).

P2Y12 is a 342-amino acid receptor (Fontana *et al*., 2003a), which include four extracellular Cys residues (position 17, 97, 175 and 270). Cys97 and Cys175 have been shown as the binding site for the active metabolites of prasugrel (Algaier *et al*., 2008). They also play vital roles in receptor expression and are linked by disulphide bonds (Cattaneo, 2011). Ding *et al*. (2009) also proposed that Cys97 and/or Cys175 are the binding sites of the active metabolites of prasugrel and clopidogrel. In two recent studies Cys97 has been implicated as the most likely target for anti-P2Y12 agents (Zhang *et*

al., 2014a and Zhang *et al.*, 2014b). Ignatovica *et al.* (2012) identified Glu181, Arg256, Arg265 and Lys280 as amino acids that are important for the functional integrity of P2Y12. Furthermore, they revealed that Lys280 is the vital amino acid in the ligand-binding pocket of the receptor.

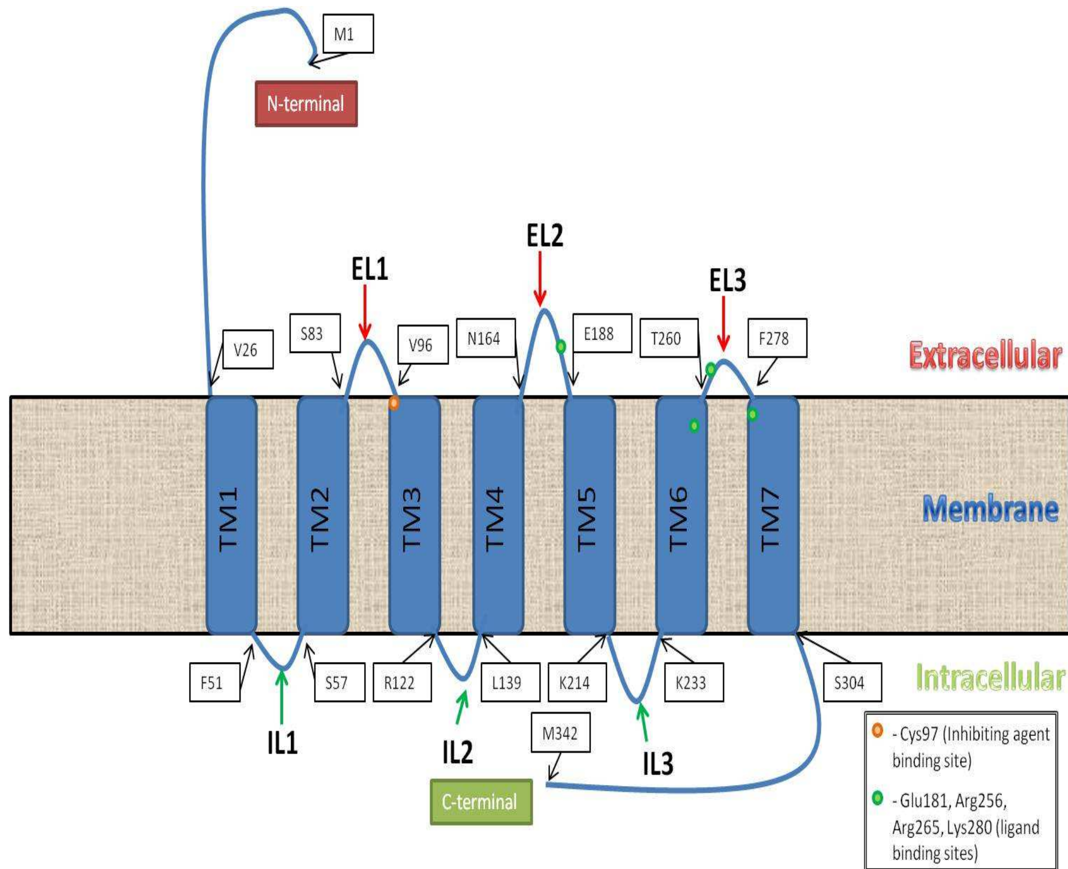


Figure 2: Diagram of P2Y12 structure. TM1 to TM7 are the seven transmembrane regions. EL1 to EL3 are the three extracellular loops and IL1 to IL3 are the three intracellular loops. The putative P2Y12-inhibiting agent binding amino acid, Cys97, is indicated with an orange circle. The ligand binding amino acids are indicated with green circles. The first and last amino acids of each extra-membrane region are indicated. The diagram was drawn from the regional information available from www.uniprot.org (P2Y12_Human, www.uniprot.org, 2015) together with the amino acid sequence available from www.ensembl.org (P2Y12, www.ensemble.org, 2011).

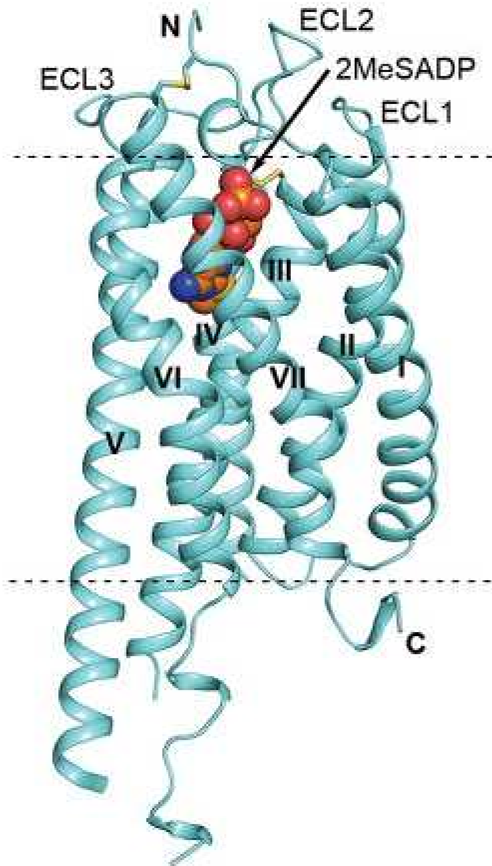


Figure 3: Ribbon structure of P2Y12 in complex with its agonist, ADP. The transmembrane regions are indicated with Roman numerals I to VII. The extracellular loops are designated ECL1 to ECL3. The N- and C-terminals are indicated with an N and C, respectively. The dashed lines indicate the membrane boundries. Reprinted by permission from Nature Publishing Group: Nature (Zhang J, Zhang K, Gao ZG, Paoletta S, Zhang D, Han GW *et al*. Agonist bound structure of the human P2Y12 receptor. *Nature* 509:119-122), copyright 2014.

Fontana *et al*. (2003a) discovered two haplotypes for the P2Y12 gene. Based on the work of Hollopeter *et al*. (2001), Fontana *et al*. (2003a) worked on the assumption that the P2Y12 gene consists of two exons and an intron. They found a C139T (i-C139T) and a T744C (i-T744C) single-nucleotide polymorphism (SNP), as well as a single-nucleotide insertion of an A at position 801 (i-ins810A) in the intron, and a C34T and a G52T SNP in exon 2. They regarded the H1 haplotype as the major haplotype containing the i-139C, i-744T and 52G, without i-ins810A. H2 is the minor haplotype containing the i-139T, i-744C, 52T, with the i-ins810A. They further showed

an association between the H2 haplotype and a higher maximum ADP-induced aggregation. They postulated that this relationship may lead to an increased risk of atherothrombosis in people with the H2 haplotype, as well as possible decreased P2Y12 antagonist effectiveness. Fontana *et al*. (2003b) subsequently confirmed this postulation, by proving the relationship between the H2 haplotype and the occurrence of peripheral arterial disease (PAD) in a case-controled study in 184 patients with PAD and 330 healthy controls. Cavallari *et al*. (2007) also determined that the H2 haplotype is significantly associated with the occurrence of coronary artery disease, particularly in non-smoking individuals.

GPVI:

Physiology:

GPVI was given its designation by using the technique of Phillips (1979), visualising its fragment with nonreduced-reduced two-dimensional gel electrophoresis. GPVI is a member of the immunoglobulin superfamily and contains two immunoglobulin C2-domain loops. GPVI traverses the membrane once, with the N-terminus at the exterior (Clemetson *et al*., 1999). GPVI is a 58-kDa (62-kDa in reduced form) platelet membrane receptor that plays a vital role in platelet activation and aggregation via collagen (Watson & Gibbins, 1998, Jandrot-Perrus *et al*., 2000 and Nieswandt *et al*., 2001a). Intracellular signals from GPVI are vital for firm adhesion of platelets to collagen under high-shear conditions (Nieswandt & Watson, 2003). There are numerous types of collagen, and GPVI has been shown to react only to types that can form large filaments, which are types I to III (Moroi & Jung, 2004). Lecut *et al*. (2005) showed that the interaction of GPVI with fibrillar type I collagen enhances platelet-dependent thrombin generation. Therefore, GPVI does not only have a role in platelet adhesion and aggregation, but also in coagulation.

Morton *et al*. (1995) proved that collagen-like peptides that contain a Gly-Pro-Hyp-rich repeat sequence are potent platelet activators. It was, therefore, postulated that collagen reacts with GPVI via a similar sequence. Knight *et al*.

(1999) confirmed that the Gly-Pro-Hyp sequence is specific for GPVI and is necessary for collagen-mediated platelet activation. However, a similar Gly-Pro-Hyp-rich area could not be identified within the collagen sequence; thus, it was further hypothesised that collagen become Gly-Pro-Hyp-enriched following polymerization, after which GPVI will bind to these areas (Moroi & Jung, 2004).

The collagen-binding domain (CBD) of GPVI is located inside the two immunoglobulin C2-domains (D1 and D2). The collagen-binding sites have been identified as two parallel grooves on the GPVI surface. An 11-residue deletion in the GPVI sequence in relation to other leukocyte receptor cluster (LRC) receptors is responsible for the formation of a shallow groove on the surface of D1. In the crystal form, the CBD forms a back-to-back dimer; this creates two virtually parallel putative collagen-binding grooves separated by a 5.5-nm gap. An intact collagen fibre has the exact dimensions to match these grooves. Therefore, it is accepted that these grooves are the collagen-binding region on GPVI (Horii *et al.*, 2006). Two distinct collagen-binding sites have been discovered on human GPVI by two independent studies. Smethurst *et al.* (2004) determined the first collagen-binding site at lysine 59 (Lys59) by using the blocking phage antibody 10B12. Lecut *et al.* (2004) subsequently identified the second site at valine 34 (Val34) and leucine 36 (Leu36), using the monoclonal antibody, 9O12.

Platelet membrane GPVI is associated with the common gamma chain of a low-affinity Fc receptor for immunoglobulin gamma (IgG) (FcRγ), which is an immunoreceptor tyrosine-based activation motif (ITAM)-bearing receptor. The binding of collagen to GPVI induces tyrosine phosphorylation of FcRγ, which in turn recruits and activates Syk, a protein-tyrosine kinase, through the Src homology 2 (SH2) domain (Tsuji *et al.*, 1997). Platelet activation is brought about through phosphorylation of several cellular proteins, and Syk was identified as playing a central role in these phosphorylation events (Clark *et al.*, 1994). Phospholipase Cγ2 (PLCγ2) is also phosphorylated by binding of GPVI to collagen (Suzuki-Inoue *et al.*, 2004). Phosphorylation of PLCγ2 hydrolyse phosphatidylinositol 4,5-biphosphate (PIP_2) to inositol 1,4,5-

triphosphate (IP_3) and 1,2-diacylglycerol (DAG). DAG subsequently serves as a receptor for protein kinase C, which leads to platelet granule secretion and GPIIb/IIIa up-regulation. IP_3 binds to its receptor (PI_3R) located on the membrane of the dense tubular system (which is a selective calcium channel) and subsequently increases calcium release from the dense tubular system (Israels and Israels, 2002, Broos *el al.*, 2011).

Berlanga *et al.* (2002) discovered that GPVI possesses a transmembrane arginine (Arg252), which, together with part of the cytoplasmic tail of GPVI, is responsible for the association of GPVI with FcRγ. Furthermore, they determined that FcRγ is essential and adequate to mediate signalling through activated GPVI. GPVI contains a proline-rich (RPLPPLPPLP) domain within the cytoplasmic tail (Clemetson *et al.*, 1999). Rickles *et al.* (1994) determined that the ligands for the Src family kinases Fyn is XXXRPLP(I/L)PXX, and for Lyn is RXXRPLPPLPXP respectively. Subsequently, Suzuki-Inoue *et al.* (2002) determined that Fyn and Lyn, activate Syk through the phosphorylation of the ITAM in FcRγ by binding to the proline-rich motif within the cytoplasmic tail of GPVI. FcRγ is only expressed in association with GPVI in human platelets. Therefore, platelets with a GPVI-deficiency will also lack FcRγ (Tsuji *et al.*, 1997). The co-expression and co-function of GPVI and FcRγ have been demonstrated in GPVI-deficient human platelets that lack collagen-stimulated Syk activation (Ichinohe *et al.*, 1997).

It was suggested that GPVI is the central platelet receptor for collagen and that the integrin α2β1 (GPIa/IIa) plays a supportive role to GPVI-mediated collagen-induced platelet aggregation (Nieswandt & Watson, 2003 and Nieswandt *et al.*, 2001a). It was, however, found that both GPVI and GPIa/IIa play important roles in collagen-induced platelet adhesion and aggregation under flow conditions, independent of each other (Sarratt *et al.*, 2005). Subsequently, due to the conflicting reports, Pugh *et al.* (2010) defined the roles of GPVI and GPIa/IIa in platelet-collagen interaction. They concluded that GPVI plays a pivotal part in signalling but that it is unlikely that it has an adhesive role. On the other hand, they proved that GPIa/IIa and the VWF-GPIb/V/IX axis are both primarily responsible for platelet adhesion to collagen.

In addition to the physiological agonist collagen, laminin is a naturally occurring protein in the basement membrane of the vessel wall that can stimulate GPVI activation through binding to integrin α6β1 (Inoue *et al.*, 2006). Non-physiological proteins that act as agonists for GPVI are the C-type lectin, convulxin, obtained from the venom of the tropical rattlesnake (*Crotalus durissus terrificus*) and alborhagin, venom functionally similar to convulxin, but related to venom metalloproteinases, obtained from the white-lipped tree viper (*Trimeresurus albolabris*) (Polgár *et al.*, 1997 and Andrews *et al.*, 2001). The triple-helical structure of collagen is also mimicked by the synthetic peptide collagen-related peptide (CRP). CRP selectively binds to GPVI after stabilization of the peptide by cross-linkage (Morton *et al.*, 1995; Polanowska-Grabowska *et al.*, 2003). CRP has been found to independently mediate tyrosine phosphorylation of Syk and PLCγ2, without co-stimulation of α2β1 (Asselin *et al.*, 1997). All these non-physiological agonists may also be used in the study of GPVI function.

The first publication on the possible interaction of GPVI with collagen was in 1987, when Sugiyama *et al.* (1987) found a platelet aggregating antibody in a patient with defective collagen-induced platelet aggregation and immune thrombocytopenia (ITP). This antibody mainly precipitated a 62-kDa (reduced state) protein, indicating GPVI as the most likely candidate. They further found that this protein (the putative collagen-receptor) was absent on the patient's platelets, giving a possible reason for the defective collagen-induced platelet aggregation.

Subsequently, Moroi *et al.* (1989) reported on a patient whose platelets had a GPVI deficiency and lacked collagen-induced aggregation and adhesion, indicating the importance of GPVI in the platelet–collagen interaction. The parents of this patient had roughly half the amount of GPVI than normal, but did not show any signs of bleeding, signifying that 50% of the normal amount of GPVI is still enough to maintain normal haemostasis. Furihata *et al.* (2001) indicated a five-fold range in GPVI receptor number among 23 healthy individuals. However, Best *et al.* (2003) found that the number of GPVI is highly regulated and does not vary by more than 1.5-fold in healthy

individuals. It was also found that human platelets with only 50% of the normal value are not affected during thrombus formation under high-shear stress in the Platelet Function Analyser 100 (PFA-100). It can, therefore, be derived that GPVI is still efficient at low levels. Arai *et al.* (1995) described a patient with only 10% of the usual amount of GPVI on her platelets' surface and an impaired collagen response that consequently resulted in a mild bleeding tendency. Therefore, it can be postulated that GPVI can still be effective somewhere between 10% and 50% receptor density.

GPVI deficiency is mostly associated with either an autoantibody and/or another haematological disorder such as systemic lupus erythematosus (SLE) or gray platelet syndrome. Usually, no genetic abnormality is detected in these patients (Sugiyama *et al.*, 1987; Takahashi & Moroi, 2001; Nurden *et al.*, 2004; Boylan *et al.*, 2004). Kojima *et al.* (2006) described a patient with a GPVI deficiency, but with normal expression of GPVI messenger ribonucleic acid (mRNA), a normal sequence of the total GPVI coding region, and degraded GPVI present in her plasma. They could also not detect any autoantibody in her blood. However, they did postulate that this deficiency can still be due to an autoantibody, either as a result of short-term exposure to a GPVI-autoantibody which was cleared from the circulation but which caused a persistent GPVI-deficiency, or prolonged exposure to low-titre GPVI-autoantibody not detectable by conventional methods.

The first patient described with a genetically proven GPVI deficiency defect was a 31-year-old woman with a history of ecchymosis, epistaxis, menorrhagia and posttraumatic and post-surgery bleeding. She had no detectable coagulation or VWF abnormalities, and her full blood count was normal. There was also no family history. Blood was collected from both the parents and the patient for further testing. Genetic analysis showed that she had a paternal inherited heterozygous out-of-frame 16-basepair deletion and a maternally inherited heterozygous p.S175N missense mutation. It was found that the deletion caused a premature stop codon, producing a protein of only 56 amino acids compared to the 339 amino acids of a typical GPVI protein.

The patient's platelets also showed an absent response in reaction to HORM® collagen with platelet aggregation tests (Hermans *et al.*, 2009).

In recent years GPVI has become a very promising target for anti-platelet drugs, as patients with GPVI deficiency only has mild bleeding tendencies (Sugiyama *et al.*, 1987; Moroi *et al.*, 1989; Takahashi & Moroi, 2001; Nurden *et al.*, 2004; Boylan *et al.*, 2004; Kojima *et al.*, 2006; Hermans *et al.*, 2009). JAQ1 was the first anti-GPVI monoclonal antibody that was tested *in vivo* as anti-platelet drug. It was directed against mouse GPVI and proved effective in preventing thrombosis in a mouse pulmonary thromboembolism model. The platelets from mice treated with JAQ1 had no response to collagen and collagen-related peptide (CRP) for at least 14 days. JAQ1 treated mice also had significantly shorter bleeding times than mice treated with GPIIb/IIIa antagonists. It was also the first study to describe the loss of GPVI from platelets after treatment with an anti-GPVI antibody. It was, however, found that this phenomenon only took place *in vivo* and not *in vitro*. Results of this study suggested that the JAQ1-GPVI complex was internalised by the platelet and degraded within two days, giving a possible explanation for the depletion of GPVI from platelets after treatment. The divalent form of the antibody, $F(ab')_2$, was able to cause GPVI depletion. However, injection of only the antigen-binding fragment, F(ab), of JAQ1 also induced GPVI depletion. Antibody-mediated GPVI depletion is, therefore, not only independent of the crystallisable fragment, F(c), of the antibody, but also independent of crosslinking for internalization to take place (Nieswandt *et al.*, 2001b).

Schulte *et al.* (2003) tested two more mouse anti-GPVI antibodies JAQ2 and JAQ3. These antibodies are similar to JAQ1 but bind to different epitopes on GPVI. They found that JAQ2 and JAQ3 both also induced GPVI depletion *in vivo*, and similar to JAQ1, not *in vitro*. Therefore, they made the assumption that GPVI depletion is not dependent on exact epitope binding. They also confirmed that GPVI depletion is not dependent on the F(c) portion of the antibody, as injection of only the F(ab) portion also caused GPVI depletion.
Bergmeier *et al.* (2004a) established a link between GPVI depletion and a metalloproteinase. They found that GPVI was completely cleaved from the

platelet surface of murine platelets after inducing mitochondrial injury through carbonyl cyanide m-chlorophenyl-hydrazone (CCCP) treatment. The GPVI depletion was due to proteolytic cleavage of the surface GPVI, as confirmed by the presence of the 55 kDa extracellular GPVI domain in the supernatant. This phenomenon is known as GPVI shedding. CCCP-treated platelets showed impaired responses to collagen and collagen-related peptide (CRP). After the metalloproteinase inhibitor GM6001 was added, GPVI depletion was inhibited, and CRP responses restored, establishing the pivotal role of a metalloproteinase in the proteolytic cleavage of surface GPVI.

Andrews *et al*. (2002) showed that calmodulin binds to the cytoplasmic domain of GPVI, and that this interaction diminishes subsequent to platelet activation. Calmodulin is an acronym for **Cal**cium **Modul**ated Prote**in** and is used to describe a protein that is regulated by changes in calcium ion concentrations (Chin & Means, 2000). Gardiner *et al.* (2004) found that agonist binding to GPVI disrupts calmodulin binding, which subsequently results in metalloproteinase-mediated GPVI depletion from the platelet surface of human platelets. They were, therefore, the first group to show that binding of specific GPVI agonists to platelets did not only cause platelet activation, but proteolytic shedding of the receptor as well.

The study by Stephens *et al.* (2005) confirmed that platelet activation by both anti-GPVI antibody and specific GPVI agonists (collagen and convulxin) results in metalloproteinase-dependent GPVI shedding. This event was presented as a possible mechanism to regulate platelet-collagen interaction. The GPVI sheddase (GPVI shedding enzyme) was identified as the metalloproteinase ADAM10, with the cleavage site identified at Arg242/Gln243 within the GPVI peptide (Gardiner *et al*., 2007). Subsequently, Bender *et al.* (2010) determined that ADAM17 is also able to cleave GPVI under certain experimental conditions. However, they did find that agonist activation of ADAM17 alone is not adequate to induce GPVI shedding. They also found that platelets possess a third GPVI sheddase, insensitive to GM6001 inhibition, but the exact metalloproteinase is still unknown. Al-Tamimi *et al.* (2011) concluded that the activation of the coagulation cascade induces

GPVI shedding. They identified activated factor X (FXa) as a primary mediator in this process, suggesting that FXa directly activates ADAM10. Their results further suggest that thrombin only plays a minor role in GPVI shedding. Figure 4 presents the interactions of GPVI with FcRγ and the subsequent activation of the second messanger systems, as well as where the sheddase is active on GPVI.

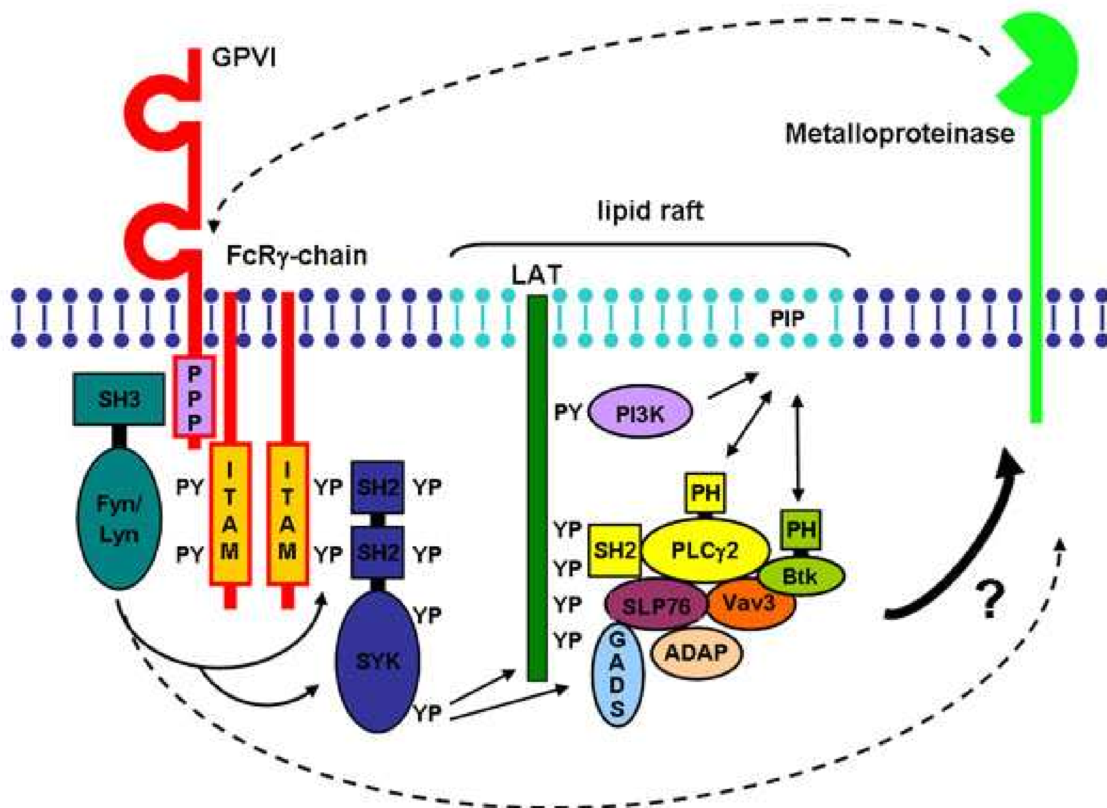


Figure 4: Diagram presenting the interaction of GPVI with FcRγ, activation of the second messanger systems, as well as where the metalloproteinase cleaves the receptor during receptor shedding. Reprinted with personal permission from Prof. Bernhard Nieswandt, Chair of Experimental Biomedicine – Vascular Medicine, University Clinic of Wuerzburg & Rudolf Virchow Center, Germany. Available from http://www.virchow.uni-wuerzburg.de/lab_pages/nieswandt/index.php/research-projects/96-studies-on-the-cellular-regulation-of-platelet-surface-receptors.html.

Due to its role in thrombosis, and the fact that patients with GPVI deficiency only have a mild bleeding diathesis, GPVI has become a very promising

target for anti-platelet therapy. Miura *et al*. (2002) determined that a dimeric form of GPVI, linked with human immunoglobulin F(c) domain (GPVI-Fc) can inhibit collagen-induced platelet aggregation. Subsequently, Massberg *et al*. (2004) showed that GPVI-Fc was not only able to inhibit platelet adhesion to collagen under different shear conditions *in vitro*, but also inhibited platelet adhesion and aggregation at the site of vascular injury in a mouse arterial thrombosis model *in vivo*. Grüner *et al*. (2005), however, found that even though complete anti-GPVI antibodies were effective in preventing thrombosis in two different mouse arterial thrombosis models, GPVI-Fc did not achieve any significant protection against thrombosis in the same models.

Lecut *et al*. (2003) demonstrated that the monoclonal anti-GPVI antibody, 9O12.2, can inhibit the binding of soluble GPVI to collagen and convulxin. Furthermore, they found that binding of cells expressing recombinant GPVI to collagen and CRP was also inhibited by 9O12.2. The F(ab) fragments of 9O12.2 were shown to inhibit the adhesion of platelets to collagen under static conditions, and to prevent thrombus formation under arterial flow conditions *in vitro*. The *ex vivo effect* of 9O12.2 F(ab) was evaluated in cynomolgus monkeys (*Macaca fascicularis*), and found to not only inhibit collagen-induced platelet aggregation, but also thrombus formation on collagen under flowing conditions and collagen-induced or tissue factor-induced thrombin generation, without GPVI depletion (Ohlmann *et al*., 2008).

The anti-human anti-GPVI monoclonal antibody, OM2, was also evaluated in Cynomolgus monkeys. It was found that the F(ab) fragment of OM2 was successful in inhibiting collagen-induced platelet aggregation *ex vivo*, and only caused a slight prolongation of the skin bleeding time, without thrombocytopenia and GPVI depletion. OM2 was also found to cause less bleeding than abciximab in the same model (Matsumoto *et al*., 2006). Takayama *et al*. (2008) evaluated two human-specific anti-GPVI mouse antibodies (mF1201 and mF1232) in cynomolgus monkeys. They determined that mF1201 bound at Lys59, the same epitope as 10B12. Furthermore, mF1232 was found to bind to a novel epitope on loop 9 (Thr116-Gln122) of the D2 domain of human GPVI. mF1201 caused severe thrombocytopenia

and was, therefore, an unsuitable anti-thrombotic candidate for clinical use. Subcutaneous injection of a chimeric form of mF1232 (cF1232) produced long-term inhibition of collagen-induced platelet aggregation due to GPVI depletion, without significant thrombocytopenia. It was demonstrated that GPVI depletion was due to internalization and endocytosis of the receptor via a particular antibody-induced mechanism.

It is important to note that the other components of the subendothelial matrix, excluding collagen, can induce a thrombogenic response in GPVI deficient mice (Konstantinides *et al.*, 2006). Moreover, the haemostatic and thrombotic defect caused by GPVI deficiency can also be overcome by thrombin (Mangin *et al.*, 2006). Therefore, it is postulated that GPVI inhibition alone is not efficient to elicit a full antithrombotic effect *in vivo* and that concomitant anticoagulation is needed for successful *in vivo* thrombosis treatment.

Molecular biology:

The complementary DNA (cDNA) of GPVI consists of 1017 basepairs (bp) which encodes for 339 amino-acids (Clemetson *et al.*, 1999, Jandrot-Perrus *et al.*, 2000 and Ezumi *et al.*, 2000). A 20 amino acid signal sequence makes up the first part of the GVPI sequence; therefore, the mature human GPVI protein consists of only 319 amino acids (Miura *et al.*, 2000). The mature GPVI protein encloses a 19 amino acid transmembrane domain (situated between residues 247 and 265 of the mature protein), and a cytoplasmic tail consisting of 51 amino acids (Clemetson *et al.*, 1999). GPVI contains an extracellular region consisting of two immunoglobulin-like domains and a mucin-like Ser/Thr-rich region. Carbohydrate residues, conjugated to the polypeptide chain consisting of amino acids, constitute roughly 45% of the total molecular weight of GPVI (Moroi & Jung, 2004). The GPVI-gene is located on chromosome 19q13.4 of the human genome (Ezumi *et al.*, 2000).

Human GPVI (transcript ID: ENST00000417454) is not designated a cluster of differentiation (CD) number. GPVI is encoded by eight exons (GPVI, www.ensemble.org, 2011). Smethurst *et al.* (2004) found that human GPVI has a much higher affinity for CRP than mouse GPVI, indicating a difference

in the platelet membrane organization between the two species. In a study where mouse and human GPVI sequences were compared, the mouse GPVI sequence showed only roughly 64% homology to the human GPVI sequence (Jandrot-Perrus *et al.*, 2000). Amino acid sequence alignment of Cynomolgus monkey and mouse with human GPVI revealed 90% and 63% homology, respectively (Takayama *et al.*, 2008).

Ohlmann *et al.* (2008) found that 9O12.2 F(ab) does not react with the platelets of mice, rats, rabbits, or pigs. They did, however, determine that it can cross-react with platelets from Cynomolgus monkeys. It can, therefore, be derived that a non-human primate is the best model to evaluate drugs that interact with GPVI when humans are the ultimate target of the drug. However, Mangin *et al.* (2011) developed a successful humanized mouse model to evaluate human targeted anti-GPVI agents in. In this model they genetically modified a mouse strain to express human GPVI by using a knockin strategy. They then evaluated 9O12.2 in this model, and it showed interaction with the humanized mouse platelets. This strategy may present a viable and sustainable alternative to non-human primate use. Nevertheless, this strategy and technique is not that widely available, and may not be accessible in poorly resourced countries, such as South Africa.

Joutsi-Korhonen *et al.* (2003) discovered that two common alleles ("a" and "b") of GPVI differ by five amino acid substitutions. Substitutions Ser199Pro, Lys217Glu and Thr229Ala, were identified in the stem region of GPVI, and Gln297Leu and His302Asn were identified in the cytoplasmic domain. Allele "a" is a high-frequency polymorphism which encodes the amino acids Ser, Lys, Thr, Gln and His (SKTQH), and allele "b" is a low-frequency polymorphism which encodes the amino acids Pro, Glu, Ala, Leu and Asn (PEALN). It was also determined that the "b" allele has a reduction in glycosylation, which leads to reduced functionality and expression of GPVI. Based on these results, Joutsi-Korhonen *et al.* (2003) postulated that patients with the "bb" genotype are more likely to bleed during a haemostatic challenge.

GPVI defects are, however, not only associated with bleeding, but some GPVI defects can also result in thrombosis. Croft *et al*. (2001) identified five dimorphisms in the GPVI locus that result in an amino acid substitution, namely T13254C, A19871G, A21908G, A22630T and C22644A. Patients with the T13254C dimorphism, more specifically homozygous patients with the 13254CC genotype (results in a substitution of serine 219 by proline, Ser219Pro, S219P) have an increased risk for myocardial infarction (MI). These results were confirmed by Ollikainen *et al*. (2004) where they showed the relationship between coronary thrombosis and the presence of the C-allele of the T13254C dimorphism. Croft *et al*. (2001) did, however, determine that heterozygosity for 13254C did not increase the risk for MI. Therefore, it can be derived that the presence of the 13254T allele eliminates the prothrombotic effect of the 13254C allele. Cole *et al*. (2003) subsequently evaluated the association of A22630T (results in a substitution of glutamine 317 by leucine, Gln317Leu, Q317L) with the risk of ischaemic stroke and found no relationship between any of the genotypes and the risk for ischaemic stroke. They, however, found a significant interaction between the 22630AA genotype and hypertension, but the clinical importance of this finding is still unknown. Bigalke *et al*. (2006) also determined that an increase in GPVI surface expression is associated with ACS.

GPIIb/IIIa complex:

Physiology:

The GPIIb/IIIa complex is part of the integrin family of cell-surface receptors. Hynes (1987) described the integrin family as a group of cell-surface receptors that contain an α- and β-subunit. GPIIb/IIIa is also called integrin $\alpha_{IIb}\beta_3$ and is considered the prototype of integrin receptors. GPIIb is the α-subunit, and GPIIIa is the β-subunit within the integrin receptor (Phillips *et al*., 1988).

McEver *et al.* (1980) determined that GPIIb and GPIIIa are in complex with each other by the use of a monoclonal antibody directed against the complex.

Jennings & Phillips (1982), however, found that this heterodimer complex is dependent on the presence of calcium and that in the absence of calcium, GPIIb and GPIIIa exist in their monomeric forms. GPIIb and GPIIIa are arranged in a 1:1 configuration (Phillips *et al*., 1988).

Activation of GPIIb/IIIa is the final step of platelet aggregation. GPIIb/IIIa undergoes a conformational change from a low-affinity to a high-affinity state, leading to irreversible binding of its primary ligand, fibrinogen, and subsequent cross-linking of platelets in a stable platelet plug (Huber *et al*., 1995). In addition to conformational changes, increased ligand binding capacity is related to clustering of receptors on the surface of the platelet and/or an increase in surface receptor expression (Hato *et al*., 1998).

Activational changes (figure 5) seen in GPIIb/IIIa take place in an “inside-out” and “outside-in” fashion. “Inside-out” activation is as result of signalling process initiated by agonist binding to the receptor that result in the conformational changes seen in GPIIb/IIIa, which leads to an increased affinity of GPIIb/IIIa for fibrinogen binding. “Outside-in” activation is when fibrinogen binds to GPIIb/IIIa, subsequently causing further conformational changes, and finally produces acceleration in aggregation (Fullard, 2004).

In resting platelets the GPIIb/IIIa has a low affinity for fibrinogen with a closed bent conformation. In this state GPIIb has contact with a seven-bladed β-propeller structure at its N-terminus and a β3-I-like domain. Activation of the receptor causes the cytoplasmic tails to separate and the extracellular domains, containing the ligand binding site, to be extended, subsequently revealing the ligand binding site (Luo & Springer, 2006). Talins and kindlins are proteins that have an essential role the conformational change in integrin receptors, such as GPIIb/IIIa. Talins actively competes with the cytoplasmic tail of GPIIb for binding to the GPIIIa tail. Thus, talins are necessary for the cytoplasmic tails to separate, with the subsequent extension of the extracellular domains and exposure of the ligand binding regions. Kindlin binds to the tail region of the β-subunit in integrins, and has a cytoskeletal linker molecule function in “outside-in” signalling processes (Moser *et al*.,

2009). Both talin-1 and kindlin-3 are necessary for total receptor activation, as illustrated in cases of defective platelet aggregation with either talin-1 (Nieswandt *et al.*, 2007) or kindlin-3 absence (Moser *et al.*, 2008).

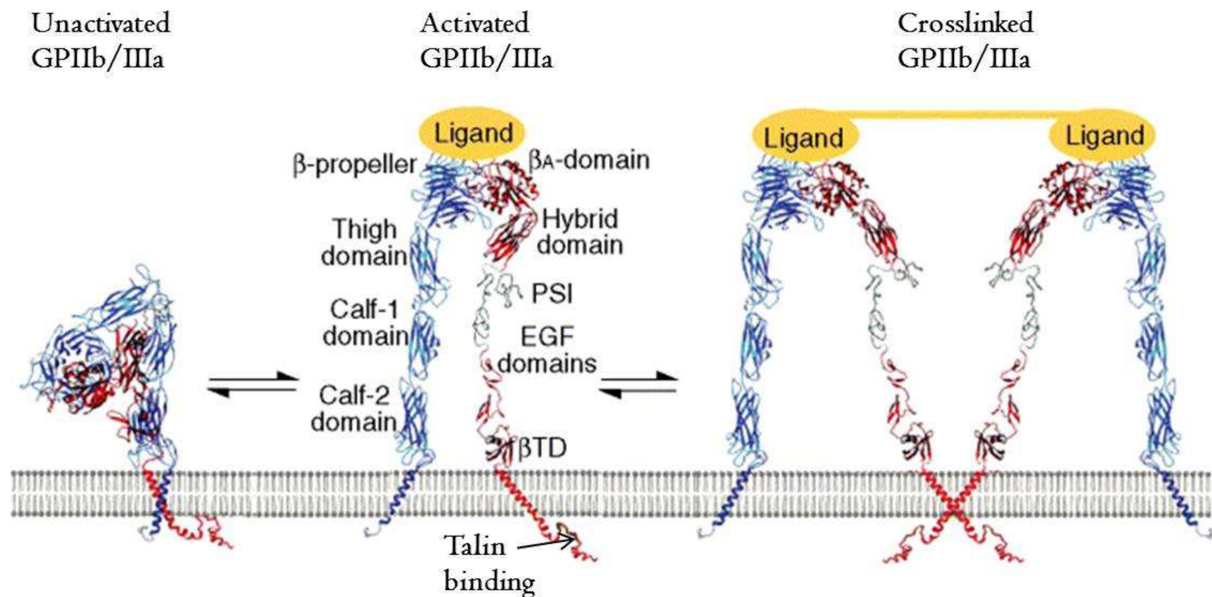


Figure 5: Crystal structure of GPIIb/IIIa indicating a resting (bent configuration), activated (extended configuration) and crosslinked state. The blue structure is GPIIb and the red structure GPIIIa. Image was adapted from Ma *et al.* (2007). Reprinted by permission from John Wiley and Sons: Journal of Thrombosis and Haemostasis (Ma YQ, Qin J and Plow EF. Platelet integrin $\alpha_{IIb}\beta_3$: activation mechanisms. *Journal of Thrombosis and Haemostasis*; 5:1345-1352), copyright 2007.

GPIIb/IIIa is reportedly the most abundant receptor on the platelet surface, with roughly 80,000 receptors per platelet (Wagner *et al.*, 1996). This number is nonetheless debatable as other studies with different monoclonal antibodies found noteworthy different receptor numbers. To illustrate the difficulty in establishing a definite receptor number, the next section contains results from different studies starting from 1980.

McEver *et al.* (1980) used the monoclonal antibody *Tab*, to determine the number of GPIIb/IIIa receptors at 39 000 ± 4600. Pidard *et al.* (1983) found the number to be 57 400 ± 9 700 using the murine monoclonal antibody, AP-2. Newman *et al.* (1985) used an anti-GPIIIa murine antibody, AP-3, to

estimate the number at roughly 40 000 per platelet. Niiya *et al.* (1987) used three different monoclonal antibodies (LJ-CP3, LJ-CP8 and LJ-P9) directed against the GPIIb/IIIa complex to determine receptor numbers ranging from 27 500 to 81 100. Furthermore, Tsao *et al.* (1995) determined the number at approximately 53 800, using flow cytometry with a fluoresceinated cyclic RGD peptide.

Wagner *et al.* (1996) used complete antibody, F(ab) fragments, as well as $F(ab')_2$ fragments, to explain the diverse results from the previous studies. The F(ab) fragment of the c7E3 monoclonal antibody determined the number of GPIIb/IIIa receptors at ± 80 000 per platelet using a radiolabelled direct binding method. However, in the same study the number of GPIIb/IIIa were estimated at ± 40 000 per platelet using the full 7E3 antibody. They attributed this finding to the full antibody being able to bind at two receptor binding sites at the same time while the F(ab) fragment could only bind to one receptor at a time. However, this did not provide a suitable explanation regarding studies where the results were not close to 40 000 or 80 000 receptors per platelet. Quinn *et al.* (1999) used two different antibodies to determine the number of complete GPIIb/IIIa receptors at 53 300 ± 5423 (Mab1 antibody) and 50 120 ± 5066 (Mab2 antibody). Taking into consideration all the studies done on this specific topic, the amount of GPIIb/IIIa can be estimated at between 40 000 and 80 000 receptors per platelet.

Phillips & Agin (1977a) first estimated the molecular weight of GPIIb with nonreduced-reduced two-dimensional gel electrophoresis at approximately 142-kDa (nonreduced form), and that of GPIII at roughly 99-kDa (nonreduced form). GPIIb consists of two subunits, a large 125-kDa chain, and a smaller 25-kDa chain, linked together by disulfide bonds (Phillips *et al.*, 1988). GPIIb is only present on the platelet-megakaryocyte membrane (Bray *et al.*, 1987). GPIIIa is a 105-kDa single-polypeptide chain. GPIIb/IIIa on activated platelets can bind to fibrinogen, fibronectin, VWF and vitronectin, but only fibrinogen binding to the GPIIb/IIIa receptor is a requirement for platelet aggregation to take place (Phillips *et al.*, 1988). Piétu *et al.* (1984) found that fibrinogen inhibits the interaction between VWF and GPIIb/IIIa after ADP and thrombin

stimulation. Subsequently, Gralnick *et al.* (1984) determined that both ADP and thrombin induce binding of VWF to GPIIb/IIIa but that fibrinogen actively competes with VWF for GPIIb/IIIa binding. Thus, there is limited VWF-binding to platelet GPIIb/IIIa in the presence of normal plasma fibrinogen levels. Schullek *et al.* (1984) showed that VWF does not interact with GPIIb/IIIa under normal physiological conditions in the plasma, further emphasising the important role of fibrinogen during ADP- and thrombin-induced platelet aggregation.

Cross (1964) was the first to demonstrate that fibrinogen is needed for efficient ADP-induced platelet aggregation. Mustard *et al.* (1978) showed that fibrinogen binds to platelets, but only after being activated by the addition of ADP. Therefore, they postulated that fibrinogen plays a role in the cross-linking of platelets. Bennett & Vilaire (1979) demonstrated that fibrinogen receptors are exposed on the platelet membrane after stimulation by ADP and epinephrine. Marguerie *et al.* (1979) also determined that platelets possess a specific receptor for fibrinogen, which is unmasked via ADP-induced activation. Niewiarowski *et al.* (1977) found that only intact fibrinogen was able to support ADP-induced platelet aggregation and that fragments of fibrinogen had a reduced/absent ability to sustain ADP-induced platelet aggregation. They also discovered that the amount of aggregation increased with the increase in concentration of fibrinogen.

Plow & Ginsberg (1981) found that fibronectin specifically binds to platelets stimulated with thrombin. Ginsberg *et al.* (1983) determined that platelets from patients with Glanzmann's thrombasthaenia had decreased/absent fibronectin binding after thrombin stimulation. However, they found that these platelets still showed thrombin-induced stimulation by secreting serotonin, but lacked subsequent aggregation.

Pierschbacher & Ruoslahti (1984) recognized that the amino acid sequence of arginine (Arg), glycine (Gly), aspartic acid (Asp), and serine (Ser) is the cell-binding sequence within fibronectin. They did, however, also establish Arg, Gly and Asp as the essential residues within this sequence. Therefore, some

studies only looked at the tripeptide sequence (RGD) and not the tetrapeptide (Arg-Gly-Asp-Ser). Doolittle *et al*. (1979) showed that fibrinogen also contain this sequence. Ginsberg *et al*. (1985) determined that platelets bind to fibronectin via the Arg-Gly-Asp-Ser sequence. Therefore, the question was raised whether platelets bound to fibrinogen via the same sequence.

Nachman & Leung (1982) demonstrated that, under the correct physiological conditions, GPIIb/IIIa is the binding site for fibrinogen. They found that fibrinogen binding to GPIIb/IIIa is calcium dependent, fibrinogen specific, saturable and can also be inhibited by specific amino sugars and -acids. They also determined that this binding is required to enable normal platelet aggregation to occur. Gogstad *et al*. (1982) also showed that fibrinogen binds to GPIIb/IIIa in a very precise manner. However, they also showed that neither fibronectin nor thrombin bind to GPIIb/IIIa.

Marguerie *et al*. (1984) determined that the α-chain of fibrinogen and GPIIb/III are in very close proximity when fibrinogen is bound to the platelet surface, which further strengthened the hypothesis that GPIIb/III was possibly the receptor for fibrinogen on platelets. Gartner & Bennett (1985) subsequently found that by inhibiting the Arg-Gly-Asp-Ser site on fibrinogen via competitive inhibition (with an Arg-Gly-Asp-Ser analogue), one could selectively inhibit platelet aggregation and fibrinogen binding to activated platelets. Pytela *et al.* (1986) later established that GPIIb/IIIa and the Arg-Gly-Asp containing fibrinogen receptor on platelets are, in fact, identical. Therefore, currently it is accepted that fibrinogen binds to the GPIIb/IIIa receptor on platelets via the RGD recognition sequence (Mousa *et al*., 2001). A Lys-Gly-Asp (KGD) recognition sequence is present in some snake venom that also has specificity for the GPIIb/IIIa receptor (Madan *et al*., 1998).

Woods *et al.* (1986) identified a large internal pool of GPIIb/IIIa, which is translocated to the platelet surface after thrombin stimulation. Subsequently, Wencel-Drake *et al.* (1986) determined that this internal pool of GPIIb/IIIa is located on the membrane surface of the α-granules, which is transported to the platelet surface in reaction to thrombin-induced activation. Later, Wencel-

Drake (1990) found that GPIIb/IIIa is actively cycled between the plasma membrane and internal vacuoles. These GPIIb/IIIa containing vacuoles were shown to be a separate entity to the α-granules, but that these newly internalised GPIIb/IIIa molecules were also transported to the cell-surface after thrombin-induced activation. This could provide a possible explanation to why these particular receptor numbers vary to such a great extent.

Cramer *et al*. (1990) found that in normal, non-activated platelets GPIIb/IIIa is present on the intracellular side of the plasma membrane, the membranes of the platelet open canicular system (OCS) and on the internal surface of the α-granule membrane. They further found that platelets from a patient with Glanzmann's thrombasthaenia did not express any GPIIb/IIIa. They also showed that in platelets from patients with gray platelet syndrome GPIIb/IIIa was highly expressed on both the plasma membrane and OCS, as well as on the small abnormal α-granules and the inside surface of large intracellular vacuoles. GPIIb/IIIa was also shown to be expressed on the plasma membrane, the demarcation membrane system, and α-granule membrane of megakaryocytes. Youssefian *et al*. (1997) observed that GPIIb/IIIa can also be found in the dense granules of platelets.

Shattil *et al*. (1986) found that the expression of the fibrinogen receptor on platelets is increased, and fibrinogen-mediated aggregation augmented after the binding of epinephrine (Epi) to the α_2-adrenergic receptor and subsequent activation of the platelet but only in the presence of extracellular free calcium. They also established that this reaction decreases in potency after prolonged exposure to Epi, thus indicating desensitization to Epi activation. Brass & Shattil (1984) demonstrated that GPIIb/IIIa is the primary Ca^{2+}-binding receptor on non-activated platelets and that Ca^{2+} binding to this complex is necessary for maximal ADP- and Epi-induced platelet aggregation.

Coller (1985) found that an anti-GPIIb/IIIa antibody (7E3) bound much faster to activated platelets than to native platelets, giving evidence that activation of platelets resulted in a change in the conformation and/or microenvironment of GPIIb/IIIa. Isenberg *et al*. (1989) determined that binding of fibrinogen to the

GPIIb/IIIa on the platelet surface caused the receptor molecules to cluster on the platelet surface. Sims *et al.* (1991) showed that the activation of a platelet causes a change in the spatial arrangement of the exoplasmic domains of GPIIb/IIIa, which allows GPIIb/IIIa to become a functional adhesion receptor. However, Savage & Ruggeri (1991) showed that GPIIb/IIIa on unstimulated platelets is capable of specific interaction with intact fibrinogen, which subsequently causes spreading and irreversible adhesion without the need of an exogenous agonist. This observation provides a mechanism by which platelets can interact with a forming fibrin clot, which is regulated by the fibrinolytic system.

Müller *et al.* (1993) determined that binding of fibrinogen to GPIIb/IIIa takes place in two steps. Fibrinogen initially binds with a quick, weak bond, followed by a slow, strong bond. Huber *et al.* (1995) identified a fast, reversible, low-affinity interaction between fibrinogen and GPIIb/IIIa, followed by a slower, more stable, high-affinity interaction between fibrinogen and GPIIb/IIIa. They found that this strong complex could only be dissociated by a strong competitive inhibitor, such as an RGDV containing molecule. This multi-step interaction between fibrinogen and GPIIb/IIIa were later also confirmed by Litvinov *et al.* (2005) using force spectroscopy.

The importance of GPIIb/IIIa is seen in the clinical manifestation of Glanzmann thrombasthaenia (GT). GT was first discovered in 1918 by Glanzmann, a Swiss paediatrician. The children Glanzmann studied all came from the same village where marriages between close relatives were frequent (Lupien *et al.*, 2001). Caen *et al.* (1966) found that patients with this disorder have absent platelet aggregation, but regular release of aggregating agents after activation. Therefore, they hypothesised that this disorder was due to an inherent membrane defect. Nurden & Caen (1974) also found that platelets from patients with GT had an abnormal membrane glycoprotein composition compared to platelets from healthy volunteers. Phillips & Agin (1977b) subsequently determined that GT is characterised by a decrease in GPIIb and GPIII.

McEver *et al*. (1980) also identified the deficient glycoproteins as GPIIb and GPIIIa. However, they further determined that the glycoproteins are in complex with each other, and, therefore, the defect was not attributed to a deficiency of one or the other, but to the complex as a whole. Bennett & Vilaire (1979) also found that fibrinogen did not bind to platelets from patients with GT. Nurden *et al*. (1987) subsequently found a woman that showed the typical signs and symptoms of GT, but who's platelets still had normal expression of GPIIb/IIIa, and concluded that her condition was due to an inherent defect within the GPIIb/IIIa. Therefore, GT is characterised by either quantitative or qualitative GPIIb/IIIa abnormalities. Patients present with easy and spontaneous bruising, mucosal membrane bleeding, subcutaneous haemorrhages, and petechiae (Margaglione, 2005).

GT is classified into three categories according to the severity, which depends on the magnitude of the GPIIb/IIIa deficiency. Type I (severe) is where a patient has less than 5% of the normal level of GPIIb/IIIa. Type II (less severe) is where a patient has between 5% and 20% of the normal level of GPIIb/IIIa. Type III (least severe / variant) is where a patient has more than 50% of the normal level of GPIIb/IIIa, but with significant aggregatory abnormalities (Lupien *et al*., 2001).

The pivotal role that GPIIb/IIIa plays in platelet aggregation makes it a favourite target for anti-thrombotic agents. It is recommended that ACS patients with on-going ischaemic symptoms or haemodynamic or rhythm flux undergo percutaneous coronary intervention (PCI) to open the occluded vessel and restore blood flow (Anderson *et al*., 2007). The use of GPIIb/IIIa inhibitors for the prevention of ischaemic thrombotic events during PCI has been shown to provide some protection against ischaemic events (EPIC Investigators, 1994 and EPILOG investigators, 1997).

The first GPIIb/IIIa antagonist that was approved in 1994 for human use was the F(ab) fragment monoclonal antibody c7E3 F(ab) (abciximab) (Coller, 1997). The 7E3 monoclonal antibody was first described by Coller (1985), who found that this antibody can block the interaction between platelets and

fibrinogen. This drug is very effective in reducing the risk for ischaemic complications after percutaneous coronary intervention by inhibiting binding of fibrinogen, VWF, fibronectin and vitronectin to activated platelets (Mousa *et al.*, 2001). C7E3 F(ab) was the first rationally designed anti-platelet agent, and, therefore, serves as a prototype for other anti-receptor agents (Coller, 1997). Other agents that contain an RGD or KGD sequence can also potently inhibit platelet aggregation by blocking GPIIb/IIIa-fibrinogen interaction (Reiss *et al.*, 2006). Tirofiban is a synthetic agent that mimics the RGD recognition sequence, therefore, inhibiting fibrinogen-GPIIb/IIIa interaction (Shanmugam, 2005). Eptifibatide is a synthetic cyclic heptapeptide that contain a KGD motif, and it also inhibits the fibrinogen-GPIIb/IIIa interaction (Madan *et al.*, 1998).

Molecular biology:

Both the GPIIb- and GPIIIa-genes are located on chromosome 17q2-22 of the human genome (Bray *et al.*, 1988). Sosnoski *et al.* (1988) proposed that the concurrent expression of these two genes may be due to the close physical proximity of the genes on chromosome 17. Human platelet antigens (HPA) are found in this receptor. HPA-3 is found on GPIIb and HPA-1 and HPA-4 is found on GPIIIa (Deckmyn *et al.*, 2004).

GPIIb (transcript ID: ENST00000262407) is encoded by the *ITGA2B* gene and is also called CD41b; it is encoded by 30 exons and consists of 1039 amino-acids (GPIIb, www.ensemble.org, 2011). Poncz *et al.* (1987) first determined the cDNA and amino acid sequence of GPIIb, and subsequently found that out of the 1039 amino acids, 137 represent the smaller subunit, 871 the larger subunit and 30 an NH_2-terminal signal peptide. They also identified that GPIIb has a 41% DNA homology, and 74% and 63% amino acid homology with the α-subunits of vitronectin and fibronectin, respectively. In the same paper they identified a putative transmembrane domain consisting of a 26-residue hydrophobic sequence near the COOH terminus of the smaller subunit of GPIIb, as well as a region within the larger subunit, consisting of four areas of 12 amino acids, similar to the calcium binding sites of calmodulin and troponin C.

According to www.ensemble.org the first 31 amino acids of GPIIb constitute the signal peptide, followed by 451 amino acids constituting the β-propeller region. The β-propeller region is followed by a thigh region and two calf regions (calf-1 and calf-2). The β-propeller region is the main contact point between GPIIb and GPIIIa, with minor contacts present in the two calf regions (Losonczy *et al.*, 2007). The transmembrane region spans from amino acid 997 to 1019. Kamata *et al.* (2001) found that 40 amino acid residues within the β-propeller region of GPIIb were vital for ligand binding to the receptor. Podolnikova *et al.* (2014) subsequently confirmed these findings by determining that the binding of fibrin to the GPIIb/IIIa complex occurs at multiple sites within the β-propeller region of GPIIb. A salt bridge between Arg995 of GPIIb and Asp723 of GPIIIa, together with the hydrophobic residues KVGFF994 of GPIIb and KLLITIH722 of GPIIIa, stabilises the interaction between the cytoplasmic portions of GPIIb and GPIIIa. This stabilisation keeps the receptor in the low affinity state (Vinogradova *et al*, 2002). Binding of talin to the cytoplasmic tail of GPIIIa, dislocates the bond between GPIIb and GPIIIa leading to the "inside-out" activation of the receptor (Anthis *et al.*, 2009). Binding of talin prompts a conformational change, which is conducted through the transmembrane domains, and ultimately leads to the extension of the extracellular domains and unmasking of the ligand binding site (Shattil *et al.*, 2010). The GVLGG976 amino acid sequence in the transmembrane domain of GPIIb has been shown to play a role in the conformational change needed for unmasking of the ligand binding sites (Bennet, 2005).

GPIIIa (transcript ID: ENST00000559488) is encoded by the *ITGB3* gene and is also called CD61, is encoded by 15 exons, and consists of 788 amino-acid residues (GPIIIa, www.ensemble.org, 2011). Fitzgerald *et al.* (1987) were the first to publish the cDNA sequence for GPIIIa. The sequence they published was derived from human umbilical vein endothelial cells (HUVEC) and was found to consist of 788 amino acids. They determined that the mature GPIIIa polypeptide contained 762 amino acid residues, together with a 26 amino acid signal peptide at the 5'-end of the amino terminus. They also found that there is a 29 amino acid hydrophobic transmembrane domain within the mature

polypeptide, as well as four tandem repeat cysteine-rich areas consisting of roughly 40 amino acids each and a 41 amino acid intracellular C-terminal segment. In addition, they showed that GPIIIa and "integrin" (a chicken fibronectin receptor) has 47% sequence homology.

Subsequently, Zimrin *et al.* (1988) determined that the sequence of GPIIIa on human erythroleukaemia (HEL) cells, platelets and HUVEC are identical. Furthermore, they showed that endothelial cells do not express GPIIb, therefore proving that the integrins on platelets and endothelial cells share a common β-subunit, but not a α-subunit. Zimrin *et al.* (1990) later published the full genomic DNA sequence of platelet GPIIIa, but this sequence only contained 14 exons. Wilhide *et al.* (1997) subsequently found that the GPIIIa gene contains 15 exons, with the first and second exon being linked. They designated the first upstream exon as exon 0, to avoid confusion with the numbering system used by Zimrin *et al.* (1990). In a study by Lin *et al.* (1997) it was determined that amino acid residues at positions 1 to 334 (within the β-propeller region) of GPIIb, and residues 164 to 202 of GPIIIa were the minimum integrin domains required for specific RGD motive containing ligand binding to the GPIIb/IIIa receptor complex.

Currently, there are 186 records of mutations for GPIIb listed on Glanzmann Thrombasthaenia database of the Samuel Bronfman Department of Medicine at the Icahn School of Medicine at Mount Sinai, as well as 123 records listed for GPIIIa. All these mutations were detected in patients diagnosed with Glanzmann's thrombasthaenia. This database contains all mutations identified within the GPIIb and GPIIIa genes up to date. Mutations have been identified within all 30 exons of the GPIIb gene, as well as within all 15 exons of GPIIIa (http://sinaicentral.mssm.edu/intranet/research/glanzmann). Some of the mutations lead to more severe phenotypes, with the symptoms ranging from severe bruising and heavy bleeding to silent mutations with no apparent symptoms (Nurden *et al.*, 2011). To illustrate some of the clinical implications of mutations in these genes, a couple of published examples of mutations with clinical manifestations are described in the following section.

Vannier *et al*. (2010) described a homozygous mutation in a newborn boy with type I GT, who presented with petechiae. They discovered a single G deletion at position 175 in exon 1 of the *ITGA2B* gene, which causes a reading frameshift and a premature stop codon. This mutated gene code for a protein only 109 amino acids long, in contrast with the 1039 amino acids of a normal GPIIb protein. This mutant variant of GPIIb was undetectable with immunoblotting analysis, but it also caused a marked decrease in GPIIIa surface expression.

The first variant mutation in the *ITGB3* gene was described by Loftus *et al*. (1990) in two homozygous siblings. They found that a single G to a T mutation caused a substitution of aspartic acid at position 119 (Asp119) by a tyrosine (Tyr). This mutation caused an abolishment of fibrinogen binding in these patients' platelets. Subsequently, Bajt & Loftus (1994) determined that substituting Asp119, serine121 (Ser121) and serine123 (Ser123) with alanine (Ala) in the β_3 domain also caused an elimination of ligand binding to GPIIb/IIIa. Bajt *et al*. (1992) found that a G to an A mutation in the β_3 domain of a thrombasthaenic patient caused a substitution of arginine at position 214 (Arg214) to glutamine (Gln), which caused a 50-fold reduction in GPIIb/IIIa's affinity for its ligand.

An interesting case was reported by Jallu *et al*. (2002) involving a newborn boy who presented with severe thrombocytopenia at birth. They found that the boy inherited a heterozygous single-base substitution C to a T at position 517 in the *ITGB3* gene from his father, which causes an amino acid change from threonine (Thr) to isoleucine (Ile) at position 140 within the RGD binding region of GPIIIa. Even though this polymorphism did not cause a functional defect within the GPIIb/IIIa complex, it did encode for the Duv^{a+} epitope on the infant's platelets, which elicited an immune response from the mother, which in turn resulted in neonatal alloimmune thrombocytopenia (NAIT).

An extensive study in India involving 45 patients with GT identified 31 different mutations in 36 of the patients. Nine of the patients did not have any identifiable genetic abnormality. Out of the 31 mutations, 17 were present in

the *ITGA2B* gene and 14 in the *ITGB3* gene. They further determined that only an Ala313Thr missense mutation in the *ITGA2B* gene and four missense mutations, Asp139His, Leu318Ser, Tyr344Cys and Cys547Trp, in the *ITGB3* gene, could have an adverse effect on the functional domains of the GPIIb/IIIa receptor (Kannan *et al.*, 2009).

Wilcox *et al.* (1994) found that a Gly418Asp mutation in the *ITGA2B* gene did not interfere with the production on the GPIIb/IIIa complex, but caused a failure of the complex to reach the surface of the platelet membrane, therefore, creating the physical abnormalities seen in GT. Subsequently, Nelson *et al.* (2005) found three more mutations, namely Gly128Ser, Ser287Leu and Gly357Ser, in the *ITGA2B* gene, which caused normal production of GPIIb, had no effect on the ability of GPIIb to bind with GPIIIa or on the stability of the complex. However, it caused a failure of the complex to be efficiently transported from the endoplasmic reticulum to the Golgi, therefore, preventing normal exposure of the complex on the platelet membrane.

González-Manchón *et al.* (2003) found a Gly188Asp mutation in the *ITGA2B* gene which leads to reduced mRNA levels, and subsequently causes a type II GT. They postulated that this decrease in mRNA is caused by either inefficient RNA splicing or the formation of an in-frame stop codon, or both. Mansour *et al.* (2011) found an Asn2Asp mutation in the *ITGA2B* gene in four Israeli Arab siblings, all affected with GT. GPIIb/IIIa is formed normally, but due to disruption of a hydrogen bond between Asn2 and Leu366, which is a calcium-binding domain of GPIIb, calcium binding that is vital for intracellular GPIIb/IIIa trafficking is impaired. This form of GT also emphasises the importance of calcium in the intracellular trafficking of GPIIb/IIIa.

The differences between populations regarding GT have been clearly demonstrated by Newman *et al.* (1991). They found an 11-base deletion (starting at base 2051) in exon 12 of the *ITGB3* gene in six out of six Iraqi-Jewish families. This deletion caused the translation of the GPIIIa protein to be prematurely terminated, leading to the protein lacking a transmembrane

region. In the same study they found another deletion, this time in the Arab community in Israel. In this population, GT was caused by a 13-base deletion in exon 4 of the *ITGA2B* gene. Subsequently, this deletion causes an 18-base deletion (nucleotide 410-427) within the mRNA of GPIIb, resulting in an amino acid deletion from 106-111. These amino acids include a cysteine residue, which possibly plays an important role in the normal folding and intracellular processing of GPIIb/IIIa. Tadokoro *et al.* (2002) also found that a His280Pro mutation in the *ITGB3* gene, which is prevalent in the Japanese population, leads to GT by causing decreased expression of GPIIb/IIIa. Interestingly, this mutation, however, did not affect the expression of the other β_3-integrin, $\alpha_v\beta_3$.

Mutations in GPIIb/IIIa are, however, not only associated with bleeding. It was found that the presence of the HPA-1b and HPA-3b alleles pose a higher risk for patients to develop thrombosis associated coronary artery disease (CAD) (Abboud *et al.*, 2010). HPA-1b/b is also associated with a higher risk for ischemic stroke (Saidi *et al.*, 2008). HPA-1b is also associated with hypertension, and subsequent end-stage renal disease (Chiras *et al.*, 2009).

It has been found that rodent platelets are less responsive to RGD/KGD motif-containing peptides, like tirofiban and eptifibatide, than human platelets, due to small sequence differences between species (Basani *et al.*, 2009). It is, therefore, vital to evaluate anti-GPIIb/IIIa drugs in animal models with high sequence homology to humans to ensure that results found in the animal model can be extrapolated to humans. Kotzé *et al.* (1995) showed that a human targeted anti-GPIIb/IIIa monoclonal antibody, MA-16N7C2, successfully bound to *P. ursinus* platelets, with up to 65% receptor occupancy. This illustrated the usefulness of this specific spesie in human targeted anti-GPIIb/IIIa studies. However, in a later study performed at the Department of Haematology and Cell Biology, UFS, on tirofiban in an arterial thrombosis model in baboons, questions were raised by some reviewers on the suitability of the Cape chacma baboon as a model for human targeted anti-GPIIb/IIIa agents (Janse van Rensburg *et al.*, 2012b). Therefore, it is important to adequately characterize the GPIIb/IIIa receptor in the Cape chacma baboon, to establish its suitability as a model.

GPIb/IX/V complex:

Physiology:

The GPIb/IX/V complex consists of four distinctive gene products namely two GPIb subunits, GPIbα (135-kDa) and GPIbβ (25-kDa), GPIX (22-kDa) and GPV (82-kDa). The complex is arranged in a ratio for GPIbα:GPIbβ:GPIX:GPV of 2:2:2:1. All these receptors are part of the leucine-rich protein family with 24-residue leucine-rich sequences in their extracellular domains (Andrews *et al.*, 1999). Roughly 25 000 copies of GPIb/IX and 12 000 copies of GPV are expressed on the surface of non-activated platelets (Bergmeier *et al.*, 2000). It is, however, important to note that GPIbα, GPIbβ and GPIX all need to be present for the GPIb/GPIX complex to be expressed. On the other hand, GPV is not essential for the expression of the GPIb/GPIX complex, though the GPIb/GPIX complex is necessary for GPV expression (López *et al.*, 1992a). Murata *et al.* (1991) also confirmed that the functional VWF-binding site is not dependent on the whole complex, but rather GPIbα alone. In addition to being expressed on the platelet membrane surface, the GPIb/IX/V complex is also found on the membranes of the platelet α-granules (Berger *et al.*, 1996) and in the platelet dense granules (Youssefian *et al.*, 1997).

Due to the pivotal role that GPIbα play in platelet function, and taking into consideration that GPIX and GPV only have minor roles in platelet function, it was decided to focus solely on the GPIbα in the GPIb/IX/V complex for this study. Numerous studies have also been done to examine the expression of GPIb in other human cells (Sprandio *et al.*, 1988; Asch *et al.*, 1988; Konkle *et al.*, 1990; Rajagopalan *et al.*, 1992, Perrault *et al.*, 1997), but this study is solely focussing on its role in platelet function.

Phillips & Agin (1977a) first identified GPIb with nonreduced-reduced two-dimensional gel electrophoresis. They estimated the molecular weight of GPIb at approximately 170-kDa (nonreduced form). They further identified the two subunits, GPIbα and GPIbβ, with apparent molecular weights of 143-kDa and 22-kDa respectively. The main function of the GPIb/IX/V complex is to help

the initial adhesion of platelets to the subendothelium of damaged blood vessels (Wu *et al*., 2002). The GPIb/IX/V complex binds to subendothelial bound VWF at high-shear stress. Within the Leu480-Gly718 dispase fragment of VWF, which include the A1 domain, lays the recognition site for the GPIb/IX/V complex. The GPIb/IX/V complex does not spontaneously recognize VWF, but needs VWF to be either bound to the subendothelium or that both the platelets and VWF be exposed to high-shear stress. Once VWF and GPIb/IX/V bind to each other, GPIb/IX/V is activated, which leads to cytoskeletal changes that induce shape change, spreading, secretion, aggregation and contraction of the platelet (Andrews *et al*., 1999).

The GPIbα chain is the most essential component of the GPIb/IX/V complex. It is not only the largest subunit in the complex, but also has the functional binding sites for the A1-domain of VWF, and thrombin (Uff *et al*., 2002). It also anchors the complex to the cytoskeleton of the platelet (Huizinga *et al*., 2002). Chow *et al.* (1992) determined that binding of GPIb together with extracellular Ca^{2+} is essential for VWF-mediated Ca^{2+} influx and subsequent platelet aggregation associated with high-shear stress. Cosemans *et al*. (2011) determined that the GPIb/V/IX complex not only plays a pivotal role in high-shear stress related platelet function, but also plays a role, together with VWF, in low-shear platelet-mediated fibrin formation.

Handa *et al*. (1986) determined that the VWF-binding domain of GPIb is situated within the amino terminal (N-terminal) domain of its α-chain, and identified GPIbα as the receptor for VWF. GPIbα binds to the A1-domain of VWF via at least three regions in the GPIbα N-terminal domain. Binding of GPIbα to VWF causes an increase in intracellular Ca^{2+} and protein kinase C (PKC) activation, which triggers platelet activation and inside-out activation of GPIIb/IIIa (Israels & Israels, 2002). Okumura & Jamieson (1976a) established that the glycoprotein, glycocalicin, is a platelet receptor responsible for thrombin-induced platelet aggregation and ristocetin-induced platelet agglutination. The same authors subsequently found that glycocalicin and GPIb have the same electrophoretic motility but that GPIb stays membrane-bound whereas glycocalicin is released in a soluble state following platelet

homogenization (Okumura & Jamieson, 1976b). Clemetson *et al*. (1981) found evidence that supported the concept that glycocalicin is derived from membrane-bound GPIb.

Okumura *et al*. (1978) demonstrated that glycocalicin is a competitive inhibitor of thrombin-binding to platelets, and derived that glycocalicin is a platelet surface receptor for thrombin; however, Harmon & Jamieson (1986) found that, in fact, the glycocalicin portion of GPIb contains receptor sites for thrombin. Parker & Gralnick (1987) subsequently found that binding of platelet-derived VWF to the glycocalicin portion of GPIb is greatly enhanced on thrombin-stimulated platelets, but only when fibrin monomers also bind to the glycocalicin portion of GPIb. Wicki & Clemetson (1985) determined that a 45-kDa portion of the glycocalicin portion of GPIb is situated on the outside of GPIb and functions as a receptor for VWF. They also found that inhibition of this receptor leads to decreased thrombin-response.

Fox *et al*. (1988) determined that the GPIb/IX complex is an asymmetrical flexible rod, consisting of a large globular domain with a membrane-bound hydrophobic region and an extracellular smaller globular domain. The rod has an elongated portion attributed to the glycocalicin portion of GPIbα, which positions the VWF-binding amino-terminal end of the GPIbα molecule to interact with VWF.

Huizinga *et al*. (2002) determined the crystal structure of GPIbα bound to VWF. They found that GPIbα has an elongated and curved shape, with a central region consisting of eight leucine-rich repeats. A 14 amino acid β-hairpin is found at the N-terminal end, defined by a disulphide bond between Cys4 and Cys17. A disordered protrusion from the top of the β-hairpin is called the β-finger. The C-terminal end holds a nine amino acid α helix and four secondary structures called 3_{10} helices. Futhermore, the C-terminal flanking region contain disordered β-switch loop (amino acids 227-241) that aligns with the β3 strand of VWF A1. The contact sites of GPIbα for VWF are the leucine-rich repeats 5 to 8 and the C-terminal flanking region, as well as the β-finger and the first leucine-rich repeat (figure 6).

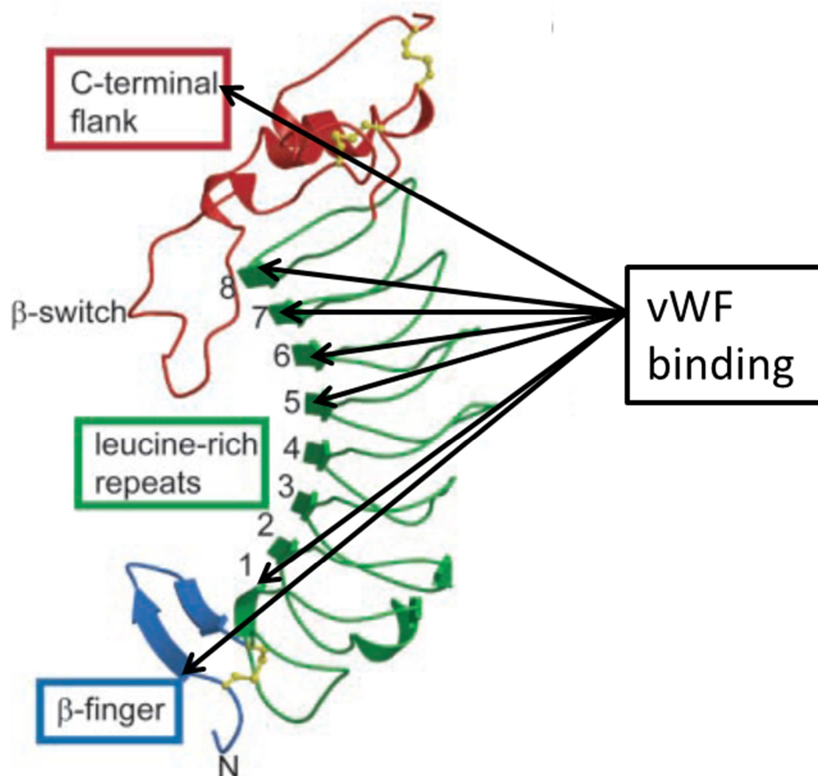


Figure 6: Structure of GPIα VWF binding domain. The VWF binding regions are indicated with arrows. Image adapted from Huizinga *et al.* (2002). Reprinted by permission from The American Association for the Advancement of Science: Science (Huizinga EG, Tsuji S, Romijn RAP, Schiphorst ME, De Groot PG, Sixma JJ, Gros P. Structures of Glycoprotein Ibα and Its Complex with von Willebrand Factor A1 Domain. *Science*; 297:1176-1179), copyright 2002.

Gitz *et al.* (2013) found that GPIbα receptors cluster at high-shear conditions. During clustering the GPIbα is translocated to lipid rafts within the platelet membrane, a process dependent on the arachidonic acid-mediated binding of 14-3-3ζ to the cytoplasmic tail of GPIbα. This clustering improves the VWF-GPIbα platelet interaction. However, high-shear clustering is reversible and does not cause platelet granule release or GPIIb/IIIa activation.

Similar to GPVI, GPIbα is depleted from the surface of activated platelets through the process of shedding (Fox, 1994). This phenomenon was first observed by Okumura *et al.* (1978), with an increase in glycocalicin in the supernatant after platelet activation. Bergmeier *et al.* (2004b) determined that

the metalloprotease Tumor Necrosis Factor-α-Converting Enzyme (TACE), also called ADAM17, is the sheddase responsible for the shedding of the extracellular portion of GPIbα. An interesting finding was that the surface expression of GPIX was not affected by TACE-treatment, further accentuating that GPIbα is the main role-player within the GPIb/V/IX complex during platelet activation.

The importance of GPIbα is shown in the clinical manifestation of a GPIbα deficiency, known as Bernard-Soulier Syndrome. The disease was discovered by Jean Bernard and Jean-Pierre Soulier, French haematologists, in 1948. They reported their findings of a boy that suffered from abnormal bleeding, who had a sibling who died of a haemorrhage. They reported abnormally large platelets and defective adherence of platelets to the vessel wall, leading to a prolongation in bleeding (Bernard & Soulier, 1948).

Gröttum & Solum (1969) determined that platelets from patients with congenital macrothrombocytic thrombocytopenia had reduced electrophoretic mobility as well as abnormally low sialic acid content, suggesting that the abnormality is caused by a platelet membrane defect. Howard & Firkin (1971) observed that the addition of the antibiotic, ristocetin, to normal platelets, caused the platelets to agglutinate. Subsequently, Howard *et al.* (1973a) determined that the VWF is necessary for ristocetin-induced platelet agglutination. Later that same year Howard *et al.* (1973b) discovered that platelets from patients with Bernard-Soulier syndrome failed to have ristocetin-induced agglutination. However, unlike platelets from patients with vWD, the defect failed to correct after addition of VWF containing normal plasma. It indicated that platelets from patients with Bernard-Soulier syndrome lacked a component necessary to interact with VWF for ristocetin-induced agglutination to continue.

Weiss *et al.* (1974) also concluded that platelets from patients with Bernard-Soulier syndrome not only failed to agglutinate with ristocetin, but also showed decreased adhesion to the subendothelium, and hypothesised that this may be due to a lack of a VWF receptor. Coller *et al.* (1983) subsequently

found supporting evidence that GPIb is the receptor for VWF. They blocked the VWF-binding site on GPIb with monoclonal antibodies and consequently inhibited ristocetin-induced platelet agglutination, as well as VWF antigen binding to platelets.

Jenkins *et al*. (1976) compared the membrane surface of normal platelets to the platelets of patients with Bernard-Soulier syndrome and found that GPI concentration was considerably reduced on platelets of patients with Bernard-Soulier syndrome. Nurden *et al*. (1981) used improved single and two-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis procedures to determine the glycoprotein and protein content of Bernard-Soulier platelets. They determined that these platelets were absent of GPIb, but evidently contained GPIa, GPIIb, GPIIIa and GPIIIb.

Berndt *et al*. (1983) determined that Bernard-Soulier syndrome is a genetically linked disorder, by studying two siblings that had the disorder, as well as their parents. They were also first to describe that GPIb is complexed with GPIX, and also found a link between the GPIb/GPIX complex and GPV. It was later determined that the stoichiometry of the GPIb/GPIX complex is 1:1 (Berndt *et al*., 1985). Du *et al*. (1987) found that GPIb and GPXI are not individually expressed in the intact platelet membrane, but are fully complexed, as the complex is immunoprecipitated as a whole and not as individual components. Modderman *et al*. (1992) confirmed the earlier findings of Berndt *et al*. (1983) by determining that the GPIb/GPIX complex and GPV are noncovalently linked to form a complex in the platelet membrane.

Cadroy *et al*. (1994) studied an anti-GPIb monoclonal antibody, AP1, in *Papio anubis* baboons, but found that it caused irreversible thrombocytopenia in these animals. Studies with the anti-human anti-GPIbα monoclonal antibody F(ab) fragment, 6B4, showed similar *in vitro* effects in non-human primate (*Papio ursinus*) platelets and in human platelets, but did not react to dog, hamster, pig, or guinea pig platelets (Cauwenberghs *et al*., 2000). 6B4 was effective in inhibiting arterial thrombosis in non-human primate models *in vivo* (Cauwenberghs *et al*., 2000 and Fontayne *et al*., 2008). Fontayne *et al*. (2007)

determined that 6B4 binds to residues Asp235 and Lys237 within the flexible loop (β-switch region; amino acids 230 to 242) of GPIbα, and, therefore, can inhibit binding of the GPIbα to VWF. Thus, it is postulated that the success of 6B4 in non-human primates is due to a high degree of homology in the VWF-binding sequence, at that particular area, between the human and baboon GPIbα amino acid sequence. Other smaller promising molecules have also emerged that target GPIbα, such as the GPIbα blocking peptide OS1 (Benard *et al*., 2008) and small modulating molecule G6 (Broos *et al*., 2012), which also have to be tested in animal models before clinical trials can be done.

Ulrichts *et al.* (2011) also showed that the anti-VWF antibody, ALX-0081, is highly effective in preventing arterial thrombosis in baboons, by inhibiting the binding of GPIb to VWF. They determined that ALX-0081 was superior to current marketed anti-platelet drugs regarding preclinical safety and efficacy. This further highlights, not only the importance of the VWF-GPIb axis as anti-platelet target, but also the importance of having a suitable animal model, such as the baboon, to evaluate these compounds.

Molecular biology:

The GPIbα-gene is located on chromosome 17p12-ter (Wenger *et al*., 1989), the GPIbβ-gene on chromosome 22q11.2 (Kelly *et al*., 1994 and Yagi *et al*., 1994), the GPIX-gene on chromosome 3q21 and the GPV-gene on chromosome 3q29 (Yagi *et al*., 1995). HPA-2 is associated with GPIbα (Deckmyn *et al*., 2004).

Lopez *et al.* (1987) were the first to successfully clone and sequence GPIbα. They determined that GPIbα is an integral membrane protein unrelated to the integrin- and cytoadhesin adhesive protein families, such as GPIIb/IIIa, vitronectin and fibronectin. They found that the cDNA was 2420 nucleotides in length, with a 42 nucleotide non-coding sequence at the 5’-end, followed by a putative signal peptide of 16 amino acids. They further suggested that GPIbα contain an extracytoplasmic domain of roughly 485 amino acids, a transmembrane segment of roughly 29 amino acids, and an intracellular (cytoplasmic) domain of roughly 100 amino acids. The amino-terminal

fragment of the GPIbα sequence contains seven tandem repeats of 24 amino acids which correspond to 13 tandem repeats in the leucine-rich α_2-glycoprotein in plasma, making the GPIbα receptor part of the leucine-rich protein family. Currently the domains of GPIbα are defined as follows: the signal peptide (amino acids 1-16), the extracellular domain (amino acids 17-531), the transmembrane domain (amino acids 532-552) and the cytoplasmic domain (amino acids553-652) (GP1BA_Human, www.uniprot.org, 2014). The GPIbα-receptor (transcript ID: ENST00000329125) is also known as CD42b, consists of 652 amino acid residues and is encoded by two exons (GPIbα, www.ensemble.org, 2011).

Dong *et al*. (1997) found that the cytoplasmic carboxyl terminus (C-terminus) of GPIbα anchored the GPIb/IX complex on the platelet membrane, which is essential for the VWF-binding ability of the complex. They determined that the mobility of the GPIb/IX complex within the plasma membrane increased after systematically removing between 6 and 92 amino acids from the carboxyl end of GPIbα (t604 to t518; 604 to 518 amino acid residues for the mutants versus 610 residues for the wild type).

Practically the entire capacity of the GPIb/IX/V complex to bind its ligands is located within the N-Terminal globular region of GPIbα, consisting of amino acids 1 to 282 of the GPIbα-protein (Vanhoorelbeke *et al*., 2007). Katagiri *et al*. (1990) determined the VWF-binding domain as being between the amino acids aspartic acid at position 235 (Asp235) and lysine at position 262 (Lys262) of the GPIbα sequence. Furthermore, they found that the thrombin-binding domain is located between phenylalanine at position 216 (Phe216) and alanine at position 274 (Ala274), with the region between Phe216 and threonine at position 240 (Thr240) possibly being the centre of thrombin interaction in the GPIbα sequence. Murata *et al*. (1991) proposed amino acids 252 to 287 as important for normal GPIbα-VWF interaction. However, they found that mutation of amino acids 280 to 302 did not affect the epitopes for anti-GPIbα monoclonal antibodies. Therefore, amino acids 252-279 are considered the most important when developing inhibiting agents directed against the GPIbα-VWF axis. Andrews *et al.* (2003) located the contact

residues between the VWF-A1 domain and GPIbα at serine 11 (Ser11), histidine 12 (His12), glutamine 14 (Gln14), asparagine 16 (Asn16), histidine 37 (His37), glutamic acid 128 (Glu128), lysine 152 (Lys152), aspartic acid 175 (Asp 175), threonine 176 (Thr176), phenylalanine 199 (Phe199), glutamic acid 225 (Glu225), asparagine 226 (Asn226), tyrosine 228 (Tyr228) and serine 241 (Ser241).

Du *et al*. (1994) showed that the GPIb/IX complex is physically linked to the 29-kDa intracellular messenger phospholipase A_2, also known as the ζ-form of the 14-3-3 protein. Subsequently, it was found that 14-3-3ζ binds to the C-terminus of the cytoplasmic domain within GPIbα. The binding region for 14-3-3ζ was determined to be between aspartic acid at position 596 (Asp596) and leucine at position 610 (Leu610). This region is serine-rich, with a serine present at every 3-4 residues. Five amino acid residues, namely serine at position 606 (Ser606), glycine at 607 (Gly607), histidine at 608 (His608), serine at 609 (Ser609) and Leu610, were found to be critical within this binding domain (Du *et al*., 1996). Gu *et al*. (1999) determined that the binding of 14-3-3ζ to GPIbα is necessary for the signalling that ultimately leads to the activation of integrin GPIIb/IIIa. Munday *et al*. (2000) showed that the GPIb/IX/V complex and 14-3-3ζ forms a complex with the signalling protein PI 3-kinase.

After observing functional differences between mini pig and human platelets, Shi *et al*. (2012) published a comparison of the DNA sequences of GPIbα between the mini pig and human. They recognized the following regions inside the mini pig amino acid sequence: the signal peptide (amino acids -16 to 0), the VWF-binding region (amino acids 1 to 280), the proline-, glutamate-, serine-, threonine-rich (PEST) region (amino acids 281 to 477), the transmembrane domain (amino acids 496 to 523), and the intracytoplasmic region (amino acids 524 to 611). They subsequently determined that the PEST region only has a 35.3% homology between the species. The PEST region is believed to play a part in the acceleration of protein degradation. However, the function of this region is yet to be defined.

Moroi *et al*. (1984) determined that GPIb is a genetically controlled, heterogeneous protein. They analysed platelets from 131 healthy donors, and identified four different gene alleles that could be differentiated by slight differences in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) mobilities. The different gene alleles were designated A, B, C, and D, with frequencies of 0.073, 0.011, 0.561 and 0.355, and molecular weights of 168 kDa, 162 kDa, 159 kDa and 153 kDa, respectively. The molecular weights of the α-chains of GPIb were determined as 141 kDa, 136 kDa, 132 kDa and 128 kDa for the different alleles, respectively. It was suggested that these differences can be attributed to molecular variance on the extracellular portion of GPIb. They did, however, find that there was no distinct difference in the biological activities of the different phenotypes and that all phenotypes showed similar aggregatory activities and retention.

Subsequently, Meyer & Schellenberg (1990) found that out of 128 German volunteers, only the B, C and D alleles were present but that all alleles were present in a smaller Vietnamese cohort. They further determined that the polymorphic region of GPIb is located in the C-terminal carbohydrate-rich glycocalicin portion (also known as the macroglycopeptide) of GPIb. López *et al*. (1992b) recognised three different length variants when evaluating GPIb mobility on denaturing polyacrylamide gels. DNA sequence analysis showed that all four alleles were represented by the three length variants and that two alleles only differed by one base pair, which did not result in an amino acid change. Tandem repeats of a 39 base pair sequence, resulting in a 13 amino acid sequence duplication, were identified as the origin of the length variants. The different isoforms contained one, two, or three of these 13 amino acid sequences, leading to the different length variants and the average incremental difference of 6 kDa between the isoforms. This occurrence is called "variable number of tandem repeats" (VNTR). The C/B genotype has been associated with an increased risk of coronary heart disease and cerebral vascular disease, but not venous thrombosis (Gonzalez-Conejero *et al*., 1998).

Kuijpers *et al*. (1992a) localised the alloantigen, Human Platelet Antigen 2 (HPA-2) on the N-terminal globular section of GPIbα. Autoantibodies against HPA-2 lead to decreased ristocetin-induced agglutination, causing a functional Bernard-Soulier syndrome. The same group identified a single base C to T polymorphism at position 434 of the mature GPIbα gene. This polymorphism leads to a substitution of threonine (Thr) to methionine (Met) at position 145 of the amino acid sequence. This polymorphism was subsequently found to be associated with HPA-2 alloantigens (Kuijpers *et al*., 1992b). Murata *et al*. (1997) found a correlation between the presence of the Met145 allele and an increased risk for coronary artery disease. However, Carlsson *et al*. (1997) found no correlation between HPA-2 polymorphisms and an increased risk for stroke. Both these findings were confirmed by Gonzalez-Conejero *et al*. (1998) who found that HPA-2b, which contains the Met145 allele, is associated with an increased risk of coronary heart disease and cerebral vascular disease, but not venous thrombosis.

Li *et al*. (1995) described a point mutation in codon 129 of the GPIbα gene, which resulted in leucine being substituted by a proline (CTC: leucine to CCC: proline). The mutation is found within the fifth leucine-rich repeat area of GPIbα. This mutation leads to reduced surface expression of GPIbα but does not affect transcription. The mutation causes a conformational change within the GPIb/IX/V receptor, which consequently results in a functional Bernard-Soulier phenotype. Kenny *et al*. (1997) identified a homozygous dinucleotide deletion in the tyrosine 508 codon that causes a frameshift within the transmembrane coding region. This frameshift alters the hydrophobic properties of the region, which negatively affects the anchoring of GPIbα in platelets. The ultimate result of this deletion is a functional Bernard-Soulier syndrome, but with soluble GPIbα detectable in the plasma. Several mutations have been identified that do not lead to amino acid changes, and are thus considered silent mutations (Kaski *et al*., 1996; Suzuki *et al*., 1996).

Marilyn Kozak (1987) found a sequence flanking the AUG start codon, which influences the ribosome recognition and plays a pivotal role in translation. Afshar-Kharghan *et al*. (1999) found a C/T polymorphism at position -5 (taken

from the initiator codon) in the Kozak sequence of the GPIbα gene. The presence of the -5C allele leads to better translation of the mRNA, and, thus, increased surface expression of the receptor. In contrast, Corral *et al.* (2000) could not establish a correlation between either the C/T polymorphism and surface expression of the GPIbα receptor or the C/T polymorphism and an increased risk for arterial thrombosis. Similar to Corral *et al.* (2000), Jilma-Stohlawetz *et al.* (2003) could not establish a link between GPIb genotypes and surface expression of the GPIb receptor, as all genotypes showed comparable surface expression. Subsequently, Baker *et al.* (2001) determined that the presence of this polymorphism in the Kozak sequence of the GPIbα gene can be linked to an increased risk of first-ever ischaemic stroke. Meisel *et al.* (2001) also concluded that the presence of the -5C allele is associated with an increased risk for ACS, as well as for complications after PCI.

Douglas *et al.* (2002) paradoxically found evidence in a study that involved 256 patients that the -5T/T genotype is also associated with an increased risk for MI and coronary thrombosis and that the -5C/T genotype grants protection against MI and coronary thrombosis. Conversely, Kenny *et al.* (2002) found evidence in a much larger study (n=1014), which indicated that the risk for MI increased with the number of -5C alleles. The occurrence of MI in this study was 2.3% for -5T/T, 5% for -5C/T, and 16% for -5C/C, clearly indicating the higher associated risk with the presence of the -5C allele.

Jilma-Stohlawetz *et al.* (2003) established that blood from patients with the -5T/T allele and the VNTR C/D alleles have increased platelet plug formation when evaluated under high-shear conditions. Subsequently, Ozelo *et al.* (2004) confirmed that the VNTR CD genotype has an increased risk for MI and that the presence of a D allele leads to more severe occlusion of blood vessels. They could, however, not ascertain a precise link between the Kozak polymorphisms and an increased risk for MI. Afshar-Kharghan *et al.* (2004) established that the presence of the VNTR CC genotype confers some protection against CHD in the African-American population, but could not find an association between any of the other genotypes and an increased risk for CHD.

Lugli *et al*. (2011) determined that the presence of the -5C allele is a compounding risk factor for cardiovascular disease in smokers with high-grade carotid stenosis. Esen *et al*. (2012) also suggested that the presence of the -5C allele might be a risk factor for ischaemic stroke in patients with undetermined aetiology. Yonal *et al*. (2012) established that the -5T/C genotype is a risk factor for arterial thrombosis in patients with antiphospholipid syndrome (APS). They also found that the VNTR D-allele confers some protection against APS. It can, therefore, be assumed that the presence of the -5C allele in the Kozak region, and the longer VNTR alleles may present bigger risk factors for the development of cardiovascular disease and stroke development.

Ware *et al*. (1997) found that mouse GPIbα lacks the ability to effectively bind to human VWF, due to differences in the linear sequence in the human and mouse GPIbα genes. Mice are, therefore, not suitable models to use when evaluating drugs that interact with GPIbα at molecular level. Shi *et al*. (2012) found that the ristocetin-VWF-GPIbα interaction differs between the mini-pig and human. Consequently, the mini-pig as an animal model in human targeted anti-GPIbα studies was found to be unsuitable. It further highlights the importance of determining the suitability of *Papio ursinus* for these studies.

Rationale of the study

In this experiment the DNA sequence of specific platelet receptors of Cape chacma baboons (*Papio ursinus*) were compared with the corresponding receptors on humans. These receptors were also quantified, and comparative platelet aggregometry was done. By comparing receptor numbers on baboon platelets with that on human platelets, it was hypothesised that it would be possible to better predict optimal dosages for anti-platelet treatment in humans, by making the necessary ratio adjustments. Results of this study may also have a substantial impact on the use of this species of non-human primate in the preclinical evaluation of anti-platelet drugs. Optimal agonist concentrations were also determined for baboon platelet aggregometry, which

will lead to better *ex vivo* monitoring of anti-platelet effects of drugs on baboon platelets.

This study will greatly contribute to the knowledge about the common Cape chacma baboon (*Papio ursinus*), a species that, even though being a common species, has been neglected in molecular science compared to species such as the macaque and chimpanzee.

Currently, no work, outside of this study, has been published regarding platelet receptors of *Papio ursinus*. Therefore, this was the first formal study to characterise the platelet receptors in this species.

Research question

Formerly queries have been raised about the translatability of *Papio ursinus* in human targeted anti-platelet studies. Therefore, the research question was as follows: “Is the platelet receptors of the Cape chacma baboon (*Papio ursinus*) similar enough to human platelet receptors for the continued use of this species in human targeted anti-platelet studies?”

Aim

The aim of this study was to characterise four common platelet receptors in Cape chacma baboons (*Papio ursinus*) and to compare the data with information of normal humans.

Chapter 3 – Methodology

Ethics

Humans:

Ethics approval was obtained from the Ethics committee of the Faculty of Health Sciences at the University of the Free State (ECUFS NR 133/2011). Volunteers were recruited at the Department of Haematology and Cell Biology, as well as at the NHLS service laboratory at Universitas Tertiary Hospital.

Blood was collected by either a registered medical practitioner or phlebotomist. Participation was wholly voluntary, and volunteers signed an informed consent form prior to blood collection. Blood were only used to determine the percentage of platelet receptors on the platelet surface with flow cytometry, and was discarded after use. Volunteers were assigned a specific number to ensure anonymity and results were not disclosed to anyone other than the principle investigator. Volunteers did not receive any remuneration for their participation.

Animals:

Ethics approval was granted by the Control Committee for Animal Experimentation at the Faculty of Health Sciences, UFS (Animal experiment NR 18/2011).

Study design:

This study was an observational and cross-sectional study.

Test subjects:

For the receptor quantification, blood from ten healthy adult human volunteers was collected. Volunteers, aged between 19 and 49, were recruited at the Department of Haematology and Cell Biology, as well as at the NHLS Laboratories at Universitas Tertiary Hospital. Volunteers were non-smokers and were not on any medication. Volunteers were in general good health, with no underlying infection or medical condition that could influence the results.

Experimental animals required:

Species	Phylum	Sex	Age/mass	Number
Papio ursinus	**Primata**	**Male and female**	**12 to 15 kg**	**10**

Drugs:

Drug/compound	Route Dosage Frequency
Ketamine (Anaket V®) –*anaesthetic*	0.1 ml/kg Intramuscular every 30 minutes or as needed
Euthanaze®	1 mg/kg Intravenous if needed

Methods

Anaesthetic (Animals):

Ketamine [Ketamine-2-(2-chlorophenyl)-2-methylaminocyclohexanone hydrochloride] was the anaesthetic of choice. Ketamine is preserved with benzethonium chloride and is lipophilic. Ketamine is more often than not administered intravenously, but can also be introduced via the intramuscular or rectal routes. The onset of anaesthesia is rapid and can also be used to maintain anaesthesia. Ketamine is not only an anaesthetic but also an analgesic (pain-suppressing) agent even at sub-anaesthetic doses; this is not true for other induction agents. Ketamine maintains muscle tone, preserves spontaneous respiration and has a profound analgesic and amnesia effect; therefore, it is especially useful for animal experiments. Ketamine leads to an increase in heart rate, blood pressure, cardiac output and myocardial consumption; all symptoms that may have an adverse effect on the animal. Ketamine reduces central sensitivity after tissue injury and secondary hyperalgesia and is thus a suitable agent to control pain during the procedure (Davies & Cashman, 2006).

Adverse effects (Animals):

No serious adverse effects were expected, as only routine venipuncture was performed. An adverse effect that could have been expected was nausea and vomiting due to the anaesthetic or superficial bruising at the site of venipuncture. No excessive bleeding or discomfort due to the venipuncture was expected.

Post-procedure care (Animals):

Animals were housed in holding cages at the primate facility on the main campus of the UFS. Animals were monitored at least three times a day by staff working at the primate facility. An animal welfare sheet was filled in once a day to observe the animals for seven days. The time just after anaesthesia was the most important time to watch the animal for any adverse effects caused by the anaesthetic, but out of the numerous studies done at the Primate Research Facility, this was very seldom observed.

Sample acquisition and preparation:

Human volunteers

One 5 ml tube of venous blood was collected in tubes containing ethylenediaminetetraacetic acid (EDTA) (BD Vacutainer, BD South Africa). Venipuncture was done by a registered physician in the Paternity Clinic of the Department of Haematology and Cell Biology. Whole blood was used for flow cytometry.

Animals

Animals were anaesthetized by injection of 0.1 ml/kg Ketamine intramuscularly. Twenty millilitres (4 x 5 ml tubes) of venous blood were collected in tubes containing 3.2% sodium-citrate (1 part sodium citrate to 9 parts blood; BD Vacutainer, BD South Africa) and 5 ml of blood were collected in a tube containing EDTA (BD Vacutainer, BD South Africa). Citrated blood was used for platelet aggregometry, and the EDTA blood was used for flow cytometry. 500 µl of the EDTA whole blood was blotted onto FTA™ paper (Whatman, Buckinghamshire, UK) for storage. FTA™ paper contains chemicals that lyse cells and binds nucleic acids for preservation. FTA™ paper also contains anti-bacterial and anti-viral agents to preserve sample integrity. According to the manufacturer, DNA can be preserved and stored on FTA™ paper at room temperature for up to seventeen years.

DNA extraction:

DNA was extracted from the FTA™ paper using a methanol extraction method as described by Labea & Pretorius (2008).

Methanol DNA extraction method (Labea & Pretorius, 2008):

i. Punch 2 mm discs out of FTA™ paper
ii. Place discs into separate 0.2 ml Eppendorf tubes
iii. Add 50 µl methanol to each tube
iv. Incubate for 10 min at room temperature
v. Remove the methanol with pipette
vi. Dry the discs for 15 min at 28˚C on a PCR block
vii. Add 60 µl TE^{-1}
viii. Incubate for 15 min at 95˚C
ix. Transfer the solution to a 0.2 ml Eppendorf tube.
x. Add another 60 µl TE^{-1} to the initial tube containing the discs
xi. Incubate for 15 min at 95˚C
xii. Add the solution to that of the first incubation step
xiii. Vortex the combined solution
xiv. Centrifuge for 5 min at 13 400 rpm
xv. Transfer the solution to a 0.6 ml Eppendorf tube, without disturbing the pellet.
xvi. Store the DNA solution at 4˚C.

Polymerase chain reaction:

The extracted DNA was amplified with the polymerase chain reaction (PCR). The Phusion® Blood Direct PCR Kit (Thermo Scientific, available from Inqaba Biotec, Pretoria, South Africa) was used to set up a PCR reaction. Specific primers (Appendix F) were designed by using the Primer3plus online primer designing tool (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/). The published human reference sequences were used as a template for the primer design. The PCR conditions were

optimised by doing a few trial runs. Initial primers weren't always successful; therefore, subsequent primers were designed.

The standard PCR reagent cocktail consisted of the following:

Component	20 µl reaction	60 µl master mix	Final concentration
2x Phusion Blood PCR Buffer	10 µl	30 µl	1x
Primer F	1 µl	3 µl	0.5 µM
Primer R	1 µl	3 µl	0.5 µM
DNA	1 µl	3 µl	3 to 12.5 ng/µl
Phusion Blood II DNA Polymerase	0.4 µl	1.2 µl	
H_2O	Add to 20 µl	Add to 60 µl	
Total	**20 µl**	**60 µl**	

In some cases, primer dimer was found; therefore, subsequent PCRs were done with less primer to limit the amount of primer dimer formation. MgCl and DMSO were also added to some reactions to optimise PCR reactions.

Gel electrophoresis:

The PCR product was run on a 2% agarose gel at 150 Volts for roughly 40 minutes to an hour, to determine if a DNA fragment was produced and if it was of the right fragment size. A Lonza DNA Marker 50-2500 bp (Lonza Rockland Inc, Rockland, USA) was used as molecular weight marker to determine fragment size. This marker can determine fragment sizes from 50 to 2500 bp.

The agarose gel was prepared as follows:

i. Add 1 gr of agarose powder (Seakem Agarose LE, WhiteSci, South Africa) to an Erlenmeyer flask
ii. Add 50 ml 1 x TAE buffer

iii. Boil in a microwave for roughly two minutes until the solution is clear
iv. Add 3 µl of ethidium bromide
v. Mix well by slowly swirling the flask
vi. Pour the solution in casting tray
vii. Insert well spacer
viii. Leave to set
ix. Store at 4℃ until needed

PCR product purification:

If successful in producing a DNA fragment, but with more than one band present, the fragment was purified by removing the particular band from the agarose gel after electrophoresis. The removed product was then purified with a Zymoclean™ Gel DNA Recovery Kit (Zymo Research, available from Inqaba Biotec, Pretoria, South Africa). This product was used for further processing for sequencing. In the case where a single band was present on the gel, the PCR product was purified with the Illustra™ ExoStar™ 1-Step reagent (GE Healthcare, Buckinghamshire, UK). The Illustra™ ExoStar™ 1-Step reagent enzymatically removes excess primers and nucleotides from the PCR product, to prevent interference of these components with the subsequent sequencing reaction. Four µl of the Illustra™ ExoStar™ 1-Step reagent was added to 10 µl of PCR product. The mixture was then incubated at 37℃ (optimal enzyme activity) for 15 minutes, followed by another 15 minutes at 80℃ (enzyme deactivation). The purified PCR product from this reaction was used as is for further processing for sequencing.

DNA concentration:

The DNA concentration was estimated by measuring the optical density of the purified sample. Absorbance was measured at 260 nm (A_{260}) on a spectrophotometer equipped with an ultraviolet (UV) lamp and UV-transparent

cuvettes. The A_{260} of 1.0 was equal to 50 µg/ml pure DNA. The A_{260} measurement had to be adjusted for turbidity that was measured by absorbance at 320 nm (A_{320}). The formula used to calculate the DNA concentration is as follows:

(A_{260} reading – A_{320} reading) x Dilution factor x 50 µg/ml = Concentration (µg/ml)

Sequencing reaction:

The purified PCR product was sequenced on an ABI Prism 3130 sequencer of the Department of Haematology and Cell Biology. The BigDye® Terminator v1.1 Cycle Sequencing Kit (Product #: 4337450) available from Applied Biosystems (Johannesburg, South Africa) were used. The same primers used for PCR was used for the sequencing. For a standard quarter reaction the following reagents were used:

Reagent	**Amount**
Purified PCR product	5 µl (60 to 100 ng)
Big Dye Terminator Mix (dNTP and ddNTP)	2 µl
Primer	2 µl (0.8 pmol)
Sequencing buffer (5x)	1 µl
Total	10 µl

Sequence Cycle conditions:

Time	**Temperature**	**Number of cycles**
1 minute	96℃	1
10 seconds	96℃	25
5 seconds	58℃	
4 minutes	60℃	
Hold	25℃	Hold

Purification of sequencing product:

After the sequencing reaction, the sequence reaction products were purified using an ethanol/sodium acetate precipitation method in micro-centrifuge tubes. It was performed according to the BigDye® Terminator v1.1 Cycle Sequencing Kit protocol.

The procedure was as follows:

i. Add 3 µl 3M sodium acetate (pH 5.0, 4℃) to a 0. 6 ml Eppendorf tube
ii. Add 62.5 µl 90% ethanol (-20℃)
iii. Add 14.5 µl sterile distilled water
iv. Add 20 µl sequence product
v. Mix with pipette
vi. Vortex for 30 seconds
vii. Centrifuge in a micro-centrifuge for 10 seconds
viii. Incubate at room temperature in the dark for 30 minutes
ix. Centrifuge at 13 500 x g for 30 minutes (carefully orientate tubes, as precipitate is not visible)
x. While waiting for step ix to finish, heat a water bath to 90℃
xi. Carefully aspirate the supernatants (use separate tips for each sample)
xii. Add 250 µl 70% ethanol (-20℃) to the product pellet
xiii. Vortex for two minutes
xiv. Centrifuge at 13 500 x g for 10 minutes (note tube orientation)
xv. Aspirate the supernatants
xvi. Place open tubes in 90℃ water bath for one mi nute to dry

Resuspending samples for sequencing:

Purified samples were resuspended for sequencing with POP-7. The following procedure is standard practice in the laboratory to resuspend samples:

i. Add 25 µl Hi-di formamide
ii. Vortex samples for one minute
iii. Centrifuge at 12700 x g for 30 seconds

iv. Denature samples at 95℃ in a water bath for two minutes
v. Chill on ice for five minutes
vi. Vortex samples for 10 seconds
vii. Centrifuge at 12700 x g for 30 seconds
viii. Place product in the dark at 4℃ until loaded on sequencer

Capillary electrophoresis:

Resuspended samples were run on an ABI Prism 3130 sequencer (Applied Biosystems®, available from Life Technologies™, Johannesburg, South Africa) of the Department of Haematology and Cell Biology. Separation took place in a coated capillary (internal diameter of 36 cm x 50 µm) filled with a separation polymer, Performance Optimized Polymer 7 (POP-7).

Outsourcing of sequencing:

Problems with the sequencing of some of the exons of GPIIb/IIIa and GPIbα were encountered. Therefore, some of the sequencing was outsourced to a company doing commercial sequencing for diagnostic purposes, GSTS Pathology, London, England. GSTS Pathology was not able to get amplification of some of the exons due to non-binding of the human-specific primers (possibly due to sequence differences). In these cases, new primers were designed by the candidate around those regions to try and get amplification for subsequent sequencing. Problems were also encountered with the ABI Prism 3130 sequencer of the Department of Haematology and Cell Biology, such as the instrument not being able to handle fragment sizes of larger than 1000 bases. However, Inqaba Biotec (Pretoria, South Africa) was able to do the sequencing using purified PCR product as well as the PCR primers from the initial PCR reactions. The only difference between sequencing at the Department of Haematology and Cell Biology and Inqaba Biotec was that Inqaba Biotec used an ABI 3130 XL sequencer (Applied

Biosystems®, available from Life Technologies™, Johannesburg, South Africa).

Sequence data analysis:

Sequencing results were analysed using Sequencing Analyses software version 5.3.1. The settings for analysis were the SeqPop7Vs3 protocol. The instrument protocol was LongRapidSeqPop7 using the Z: bigDyeV3 Dye Set. All data were checked using Chromas lite (available online at http://chromas-lite.software.informer.com/2.0) and compared with the known human sequence data from the Ensembl online database (www.ensembl.com). The Chromas lite data were converted to text using the Chromas lite program. The text sequence data were then aligned and compared to the human sequence using the Lalign sequence alignment program (www.ch.embnet.org/software/LALIGN_form.html, June 2011). The Lalign program calculated the percentage similarity between the two sequences using the algorithm from Huang & Miller (1991). The nucleotide sequences were translated to amino acid sequences by the ExPasy translation tool (http://web.expasy.org/translate). The amino acid sequences were also analyzed with Lalign.

Platelet function tests:

The first-line tests for platelet function are the platelet count, skin bleeding time or the PFA-100 (*in vitro* platelet function test). These tests are, however, not sensitive for some platelet function disorders (Quiroga *et al.,* 2004). Therefore, more in depth platelet function tests are done to determine platelet function. In the Department of Haematology and Cell Biology, UFS, platelet function is determined by doing platelet aggregation tests by adding different platelet agonists.

The principle of the test used is that light absorbance of platelet-rich plasma (PRP) decreases with platelet aggregation. The temperature and mixing speed of the sample are controlled. Therefore, the amount and rate of decrease is directly proportional to platelet reactivity to the added agonist. The absorbance is monitored on a chart recorder for further analysis. The agonists used are ADP, collagen, ristocetin, arachidonic acid and adrenaline (epinephrine) (Laffan & Manning, 2006).

In the past, the platelet count was adjusted by adding platelet-poor plasma (PPP) to standardise the method to a particular platelet count. It was, however, found that the addition of PPP to PRP may inhibit platelet aggregation, thus rendering erroneous results (Cattaneo *et al.*, 2007). Another study showed that adjusting the platelet count with PPP does not offer any advantage over using unadjusted PRP, therefore, platelet count adjustment is deemed unnecessary (Linnemann *et al.*, 2008).

All aggregations were done at an accredited NHLS Service laboratory, according to a strict approved standard operating procedure used for human platelet function testing. All reagents were freshly prepared, and all batches of reagents were tested with normal human samples to ensure reactivity. Human sample testing was only performed once, prior to performing the baboon aggregations. All baboon platelets were isolated from citrated blood. This was done in the light of evaluating baboon platelets with current standardized human methods in an accredited laboratory and comparing the results to human results using the same method.

Platelet function agonists:

ADP:

ADP binds to the G-protein coupled P2Y1 and P2Y12 receptors on the platelet surface. Early binding of ADP to these receptors leads to the release of calcium from the platelet from intracellular stores into the cytosol of the platelet (Israels and Israels, 2002). This release of intracellular calcium

confers a shape change to the platelet, which is seen as primary aggregation. In the secondary wave of platelet aggregation ADP is released from the platelet's storage α-granules (http://www.practical-haemostasis.com, 2011).

ADP binding to P2Y1 activates phospholipase C, which leads to the formation of IP3 and Ca^{2+} release from intracellular stores. When ADP binds to P2Y12 platelet adenylyl cyclase is inhibited, leading to a reduction in cAMP. These receptors need to be stimulated simultaneously by ADP to induce platelet aggregation (Israels & Israels, 2002).

When using low-dose ADP (1, 2.5 or 5 µM) only primary, reversible aggregation is induced. High dose ADP (10 µM) induces both primary and secondary aggregation. ADP is considered as a mild platelet agonist. Activation of the P2Y1 receptor only initiates the primary wave of aggregation. The P2Y12 receptor inhibits adenyl cyclase and initiates full platelet aggregation. The P2Y12 receptor is considered the principal ADP receptor and is also a target for clopidogrel, an anti-platelet drug. Aspirin inhibits the secondary wave of aggregation (http://www.practical-haemostasis.com, 2011).

Collagen:

Collagen is a very thrombogenic subendothelial matrix protein (Israels & Israels, 2002). Binding of collagen to the GPVI and GPIa/IIa receptors induces platelet granule release, TXA_2 production and subsequently, sustained GPIIb/IIIa activation. The GPIa/IIa receptor functions in platelet adhesion to subendothelium and the GPVI receptor plays a vital role in platelet signalling and TXA_2 production (http://www.practical-haemostasis.com, 2011).

Usually, a lag phase between 10 and 60 seconds is seen after the addition of collagen to the PRP. Collagen is used in platelet function testing at concentrations of 1 and 4 µg/ml. Doses of more than 2 µg/ml can cause a sudden increase in intracellular calcium, leading to granule release, but not

TXA_2 production (Laffan & Manning, 2006). Therefore, it is important always to use the low and high doses of collagen. From previous experience it was also found that baboon platelets do not aggregate with all types of collagen as is the case for human platelets but that HORM® collagen (equine tendon type 1 collagen fibrils) is needed to induce aggregation.

Ristocetin:

Ristocetin is an antibiotic that has shown to have an agglutinating effect on PRP of healthy patients (Howard & Firkin, 1971). Ristocetin causes platelets to clump together by reacting with VWF and the GPIb-IX-V receptor. This process differs from aggregation and is named agglutination (http://www.practical-haemostasis.com, 2011). Ristocetin dimers promote the formation of a bridge between VWF and GPIb, independent of the VWF A1 loop binding site of GPIb. Under low-shear conditions, in the absence of ristocetin, VWF-GPIb interaction is minor (Hoylaerts *et al.*, 1995), which illustrate the usefulness of ristocetin in VWF-GPIb interaction evaluation. The platelet response is initially studied at a concentration of 1.2 mg/ml ristocetin. Ristocetin concentrations of more than 1.4 mg/ml may cause non-specific platelet agglutination due to a ristocetin-fibrinogen interaction and protein precipitation (Laffan & Manning, 2006).

Arachidonic acid:

Arachidonic acid-induced aggregation detects abnormalities in the later stages of platelet activation. Arachidonic acid is normally metabolised to substances such as TXA_2. TXA_2 production and granule release continues regardless whether there is a defect in agonist binding to the platelet membrane or decreased phospholipase-induced endogenous arachidonate release. Arachidonic acid aggregation will be abnormal in cases where cyclooxygenase is absent or impaired, such as with the aspirin effect (Laffan & Manning, 2006).

Epinephrine:

EPI binds to the alpha-2 adrenergic receptor on the platelet membrane. This results in inhibition of adenyl cyclase and the release of calcium ions. EPI causes a similar pattern of aggregation to that of ADP with an initial primary wave of aggregation, the release of endogenous ADP from the platelet granules, followed by a second wave of sustained aggregation. Aspirin also inhibits this second wave of aggregation, as with ADP. EPI is a weak agonist. Abnormalities in the alpha-2 receptor signalling pathway have been associated with a bleeding disorder (http://www.practical-haemostasis.com, 2011). It is important to note that EPI-induced aggregation is not included in the table of differential diagnosis of platelet function disorders (Laffan & Manning, 2006). Thus, it is not seen as a crucial agonist to determine platelet function.

Platelet aggregometry:

Platelet aggregometry was done on ten baboons, and the results compared to historical human control values used for routine platelet function tests at the NHLS service laboratory at Universitas Tertiary Hospital. Aggregometry was done on a Chrono-log platelet aggregometer at 37℃. Tests were done on citrated PRP prepared by centrifugation of the citrated tubes at 200 x g for ten minutes. PRP was aspirated and aliquoted for further preparation. For each test, 225 µl of the PRP was used together with 25 µl of each agonist.

To determine how baboon platelets react with the standard concentration of agonist used for human platelet function tests, the reactions were set up using the highest agonist concentration used for humans, as well as using agonist at 1.5-times the highest human concentration.

The following agonist concentrations were initially used (the first concentration is the highest human concentration as per standard operating procedure for human platelet function tests at the NHLS Service Laboratory at Universitas

Tertiary Hospital, and the second concentration is 1.5-times the highest concentration):

Initial agonist concentration	
Agonist	**Concentrations (1x and 1.5x)**
ADP (Helena Biosciences, United Kingdom)	10 and 15 µM
*HORM® Collagen (Nycomed, Austria)	4 and 6 µg/ml
Ristocetin (Helena Biosciences, United Kingdom)	1.25 and 1.875 mg/ml
Arachidonic Acid (Helena Biosciences, United Kingdom)	500 and 750 µg/ml
Epinephrine (Helena Biosciences, United Kingdom)	10 and 15 µM

**Used HORM® collagen, as it was previously found that not all types of collagen used for human platelet aggregations always reacted with the platelets of other species.*

After the first couple of tests were done, additional concentrations of agonist were added, in order to evaluate the effect of stronger agonists on aggregation, as some of the weaker concentrations did not have an effect, or had a feeble effect on aggregation. None of the baboons' platelets reacted against EPI. Therefore, it was decided to discontinue the use of EPI as agonist for this study. It was done in the light of EPI known to be a weak agonist, it is not always being used in the differential diagnosis, and that in some clinically healthy people a severely reduced EPI response is seen (Laffan & Manning, 2006).

Additional agonist concentrations	
Agonist	**Concentrations**
ADP (Helena Biosciences, United Kingdom)	20 and 40 µM

Horm Collagen (Nycomed, Austria)	8 µg/ml
Ristocetin (Helena Biosciences, United Kingdom)	2.5 mg/ml
Arachidonic Acid (Helena Biosciences, United Kingdom)	1 mg/ml

Differential diagnosis of platelet function disorders:

Table 1. Differential diagnosis of platelet function disorders as described by Laffan & Manning (2006).

Condition	Agonist induced aggregation			
	ADP	**Collagen**	**Ristocetin**	**Arachidonic acid**
Thrombasthaenia	Absent	Absent	Primary wave only	Absent
Bernard-Soulier syndrome	Normal	Normal	Absent	Normal
Storage pool defect	Primary wave only	Reduced	Primary wave only	Primary wave only / absent
Cyclo-oxygenase deficiency	Primary wave only / Normal	Reduced	Normal	Reduced
Thromboxane synthase deficiency	Primary wave only / Normal	Reduced	Normal	Reduced / absent
Aspirin ingestion	Primary wave only	Reduced	Normal	Reduced / absent
Ehlers-Danlos syndrome	Normal	Normal	Normal	Normal
Von Willebrand disease	Normal	Normal	Absent / reduced	Normal

Flow Cytometric Analysis:

Receptor quantification was done on ten healthy human volunteers and ten baboons utilising flow cytometry. Based on published studies it was considered that ten healthy human volunteers would be adequate to set a human laboratory reference range (Stricker *et al.*, 2006 and Patel *et al.*, 2000). Taking ethical considerations into account and considering reduction as part of the three R's of animal research, and in view of previously published work where less than ten animals were used to come to rational conclusions (Cauwenberghs *et al.*, 2000 and Fontayne *et al.*, 2008), ten animals were regarded as adequate to come to logical conclusions. Flow cytometry was done on the BD FACSCalibur® flow cytometer at the Department of Haematology and Cell Biology. The Platelet Calibrator kit (Biocytex, Marseille, France) were used to quantify the expression. This kit can be used to measure platelet GP expression level or any other platelet surface molecule, given that there is a monoclonal antibody available against the molecule. A no wash indirect immunofluorescence technique is utilised to stain platelets with specific monoclonal antibodies. Calibration beads, coated with increasing and accurately known numbers of mouse IgG, are used to determine the expression level of a specific antigen. Each kit contains the specific number of determinants of each bead population. Analysis was performed on a calculation template made available from Biocytex (Appendix G). The kit contains 10x concentrated diluent, three different negative isotypic controls (IgG1, IgG2a and IgG2b), calibration beads, and staining reagent. All monoclonal antibodies were used at a concentration of 10 µg/ml. 20 mM of EDTA was added to all the buffers used to try and limit activation of platelets. It was the first time this kit was utilised in the Department of Haematology and Cell Biology, UFS.

The reactions were set up as follows:

i. Label tubes T1-T8
ii. Add 20 µl Anti-CD42b to tube T1
iii. Add 20 µl Anti-CD41a to tube T2

iv. Add 20 µl Anti-CD61 to tube T3
v. Add 20 µl Anti-GPVI to tube T4
vi. Add 20 µl of corresponding negative isotypic control (IgG_1, IgG_{2a} or IgG_{2b}) in Tube T(5-7) (The number of tubes depends on the number of different isotypes in the panel)
vii. Add 40 µl calibration beads to tube T8
viii. Label a sample dilution tube S1
ix. Add 50 µl of whole blood to tube S1
x. Add 150 µl of 1x diluent to tube S1
xi. Vortex for 1-2 seconds
xii. Add 20 µl of S1 to tubes T1-7
xiii. Vortex for 1-2 seconds
xiv. Incubate at room temperature for ten minutes
xv. Add 20 µl staining reagent to tubes T1-8
xvi. Vortex for 1-2 seconds
xvii. Incubate at room temperature for ten minutes
xviii. Add 2 mL 1x diluent to tubes T1-8
xix. Vortex for 1-2 seconds
xx. Store at 2-8℃ for up to two hours until analys ed on FACSCalibur

The following monoclonal antibodies were initially used:

Monoclonal Antibody	**Receptor**
Anti-CD42b (BD Cat #: 555471, Mouse IgG_1)	GPIbα
Anti-CD41a (BD Cat #: 555465, Mouse IgG_1)	GPIIb
Anti-CD61 (BD Cat #: 555752, Mouse IgG_1)	GPIIIa
Anti-GPVI (Monoclonal antibody 3J24.2, Mouse IgG_1, available from Dr. Martine Jandrot-Perrus, Hôspital Bichat, Paris, France)	GPVI

After limited binding was found with the commercial anti-CD42b antibody, an additional anti-GPIbα antibody, known to react with the Cape chacma baboon, 6B4, was courteously provided by Prof. Karen Vanhoorelbeke (Laboratory for Thrombosis Research, KU Leuven, KULAK, Kortrijk, Belgium). This antibody

did react with the baboon platelets. The 3J24.2 antibody also did not react with the baboon platelets. Prof. Rob Andrew (Monash University, Melbourne, Australia) graciously provided five different anti-GPVI antibodies (IA12, 4B8, 12A5, 12C9, 12H1), but, unfortunately, none of them reacted with the baboon platelets either.

Please note:

Currently, there is no method to test for the presence of the P2Y12 receptor and no monoclonal anti-P2Y12 antibody available; therefore, the P2Y12 receptor was not quantified in this study. However, it is planned to develop a technique for P2Y12 quantification after completion of this study, either as a masters or post-doctoral study.

Statistical analysis

Because clear cut results were expected from this study no expert statistical analysis of the results by a biostatistician was needed. Sequence results were given as the percentage equivalence of the baboon sequence to the human sequence, taking the human sequence as 100%. The Student T-test was used to determine if there is a significant difference between human and baboon platelet aggregation studies at the different agonist concentrations, with $p<0.05$ regarded as significant. Flow cytometry results were compared by determining the receptor number difference between the human and baboon samples. The Student T-test was used to determine if there is a significant difference between human and baboon receptor numbers, with $p<0.05$ regarded as significant.

Experiment facility

All animal work was performed at the animal holding facility on the West-Campus of the University of the Free States' Main Campus. Laboratory preparation and analysis was done at the laboratories of the Department of Haematology and Cell Biology, in the Faculty of Health Sciences, UFS, as well as at the laboratories of the NHLS at Universitas Tertiary Hospital.

Chapter 4 – Results and discussion

Platelet aggregation results:

Figures 7 to 11 contain examples of tracing acquired for the different agonists, respectively. The amount of aggregation was determined using the maximum amplitude of the graph. Tables 2 to 5 contain the results for platelet aggregation studies using light transmission aggregometry, with the various agonists at different concentrations. Aggregation of less than 10% is highlighted in yellow, and the maximum mean aggregation is highlighted in green. Results for aggregations using epinephrine as agonist have been omitted, because none of the initial baboons showed aggregation with this agonist at any concentration. Each table also contains the mean aggregation results of 38 healthy human volunteers, used as controls from 2008 to 2012 at the NHLS Service laboratory at Universitas Tertiary Hospital, for platelet aggregation tests. The baboon and human results were compared, to determine any significant differences in platelet aggregation.

Table 2 contains the results for ADP-induced platelet aggregation. The concentration of ADP was increased to four times the maximum human concentration (40 µM) because minimal aggregation was found at the lower concentrations. However, the amount of aggregation reached a plateau after addition of twice the human maximum concentration (20 µM). At all concentrations of ADP, the baboon results differed significantly ($p<0.05$) from the human results. When taking the maximum mean amount of baboon platelet aggregation into account (54% of normal human aggregation), one can deduce that ADP was only able to cause the primary wave of aggregation in baboon platelets, and not the complete, irreversible secondary wave as seen in humans.

The difference of ADP-induced aggregation between the baboon and human platelets are still unexplained. One can hypothesise that it may be due to small sequence differences between the baboon and human ADP receptors

(especially P2Y12), which results in ADP on its own not being a strong enough agonist to induce complete platelet aggregation in the baboon. It is possible that it does not bind as effectively to baboon ADP receptors than to human ADP receptors. This phenomenon may also be caused by a difference in the quantity of ADP receptors on the baboon platelets. Unfortunately, this theory could not be tested as there is no method available to quantify the number of ADP receptors on platelets. On the other hand, considering the close relationship between ADP-induced platelet activation and subsequent GPIIb/IIIa mediated aggregation, an increase in GPIIb/IIIa receptor number may also have an effect on the ADP-induced platelet aggregation results.

Interestingly, only the primary wave of aggregation as observed in the baboons is seen in patients with a storage pool defect or who ingested aspirin. It may also be seen in patients with a cyclo-oxygenase- or thromboxane synthetase deficiency, but in these cases aggregation can occasionally be normal (Laffan & Manning, 2006). These findings will later be discussed as part of the differential diagnosis of platelet function disorders.

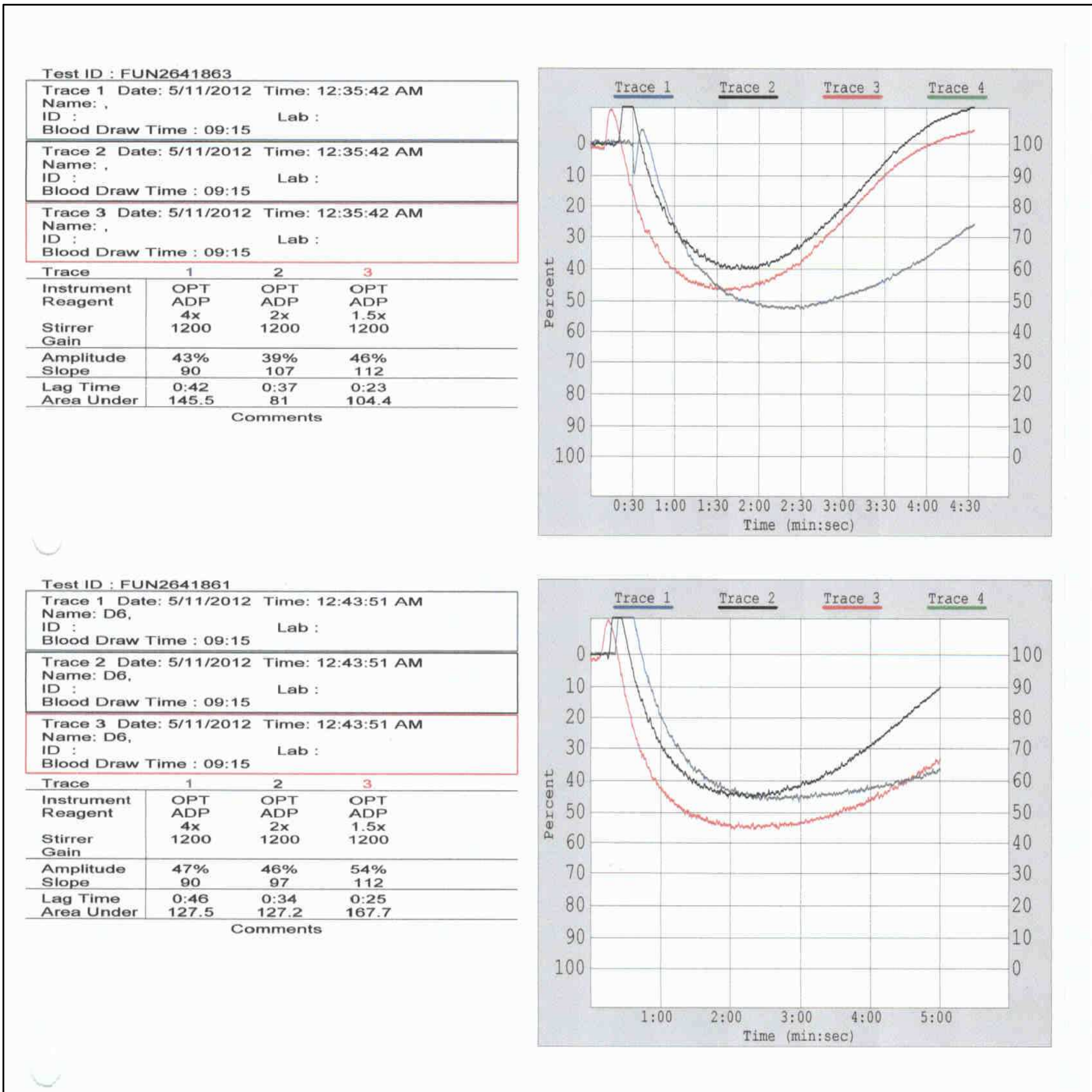


Figure 7: Example of ADP-induced aggregation tracings. Each graph contains information for three different ADP concentrations. It can be noted that the highest ADP concentration did not produce the highest amount of aggregation.

Table 2. ADP-induced platelet aggregation expressed as a percentage. The checkered block indicates the maximum percentage aggregation.

ADP					
Baboon	**10 µM**	**15 µM**	**20 µM**	**40 µM**	**Human 10 µM**
1	31	36	44	43	n=38
2	32	28	42	49	
3	23	33	51	37	
4	16	25	31	18	
5	28	24	35	27	
6	36	35	44	46	
7	11	12	21	30	
8	22	23	30	39	
9	42	46	39	43	
10	41	54	46	47	
Mean	28.2	31.6	38.3	37.9	71
SD	10.26	12.08	9.02	10.02	10
T-Test	0.00000001	0.00000043	0.00000004	0.00000019	
	Significant	Significant	Significant	Significant	

Table 3 contains the results for collagen-induced platelet aggregation. As with the ADP tests, the concentration of collagen used as agonist had to be increased. Double the maximum human concentration of 8 µg/ml of collagen reagent (HORM®) was used. At the maximum human concentration of 4 µg/ml, 50% (5/10) of the samples did not reach 10% aggregation. At 1.5 times the maximum human concentration (6 µg/ml) 40% (4/10) of the samples did not reach 10% aggregation. All samples showed substantial aggregation at 8 µg/ml.

Statistically, there was a significant difference between baboon aggregation at 8 µg/ml and maximum human aggregation results (baboon: 56.7%±5.46% vs. human: 66%±16%). However, this difference may not be of clinical importance as all the baboon samples did aggregate at this concentration, with a minimum aggregation of 48%, only 2% less than the lower limit of the human reference range. Therefore, the aggregation of baboon platelets at double the maximum human collagen concentration is comparable to human platelet aggregation at the maximum concentration.

As with ADP-induced aggregation, it can be hypothesised that this finding may be due to sequence differences between the baboon and human collagen receptor (GPVI), resulting in more collagen being needed to induce sufficient platelet aggregation in the baboon. These sequence differences can also explain why none of the other collagen agonists (type II to type IV) reacts with baboon platelets, as only HORM® collagen induces aggregation in baboon platelets.

It may also be explained by the manufacturing process of the HORM® collagen. Non-proteolytic digestion of equine tendon is used to produce HORM® collagen. This process may cause platelet reactive substances, such as decorin, to remain in the reagent (Farndale & Siljander, 2003). Therefore, the aggregation obtained using HORM® collagen cannot be solely attributed to the collagen within the reagent.

As with the ADP-induced aggregation the findings may also be explained by receptor number differences between baboon and human platelets. However, if the postulation derived from Arai *et al*. (1995) and Best *et al*. (2003) is correct, that platelets still aggregate at GPVI levels between 10% and 50% receptor density, the former hypothesis of the sequence differences being the causative factor for the aggregation differences is more probable. Unfortunately, the speculation regarding receptor numbers could not be confirmed as there is currently no monoclonal antibody available to quantify the number of GPVI receptors on baboon platelets.

For differential diagnosis purposes, it is important to note that reduced collagen-induced aggregation is associated with patients with a storage pool defect, patients who ingested aspirin, or patients with a cyclo-oxygenase- or thromboxane synthetase deficiency (Laffan & Manning, 2006).

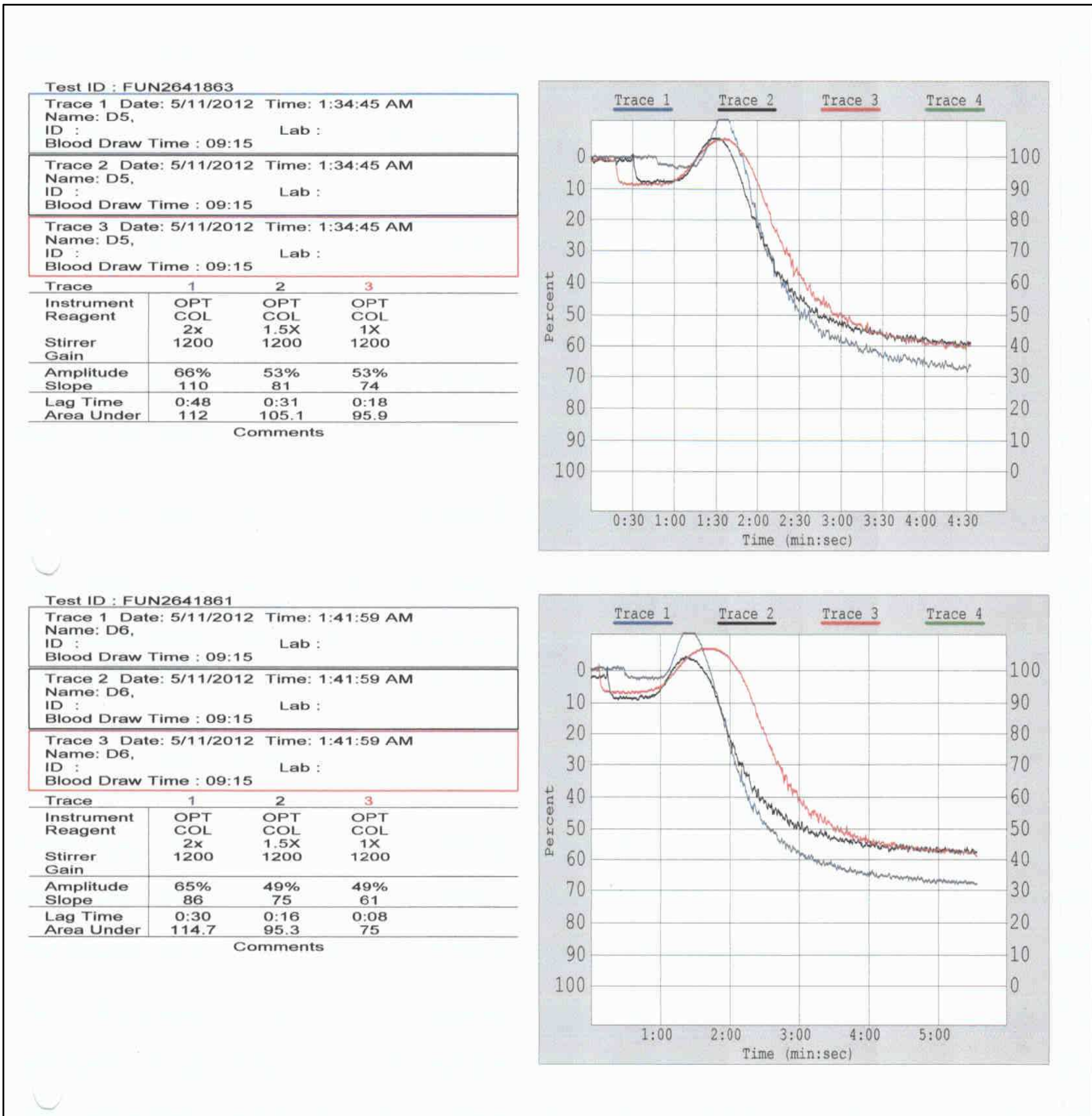


Figure 8: Example of Collagen-induced aggregation tracings. Each graph contains information for the three different collagen concentrations. An increase in aggregation associated with an increased agonist concentration is clearly observable.

Table 3. Collagen-induced platelet aggregation expressed as a percentage. The checkered block indicates the maximum percentage aggregation, and the shaded blocks indicate samples that had less than 5% aggregation.

Collagen				
Baboon	**4 µg/ml**	**6 µg/ml**	**8 µg/ml**	**Human 4 µg/ml**
1	20	50	53	n=38
2	1	1	56	
3	2	50	59	
4	30	49	53	
5	8	53	55	
6	1	3	56	
7	2	2	48	
8	1	3	56	
9	53	53	66	
10	49	49	65	
Mean	16.7	31.3	56.7	66
SD	20.54	25.05	5.46	16
T-Test	0.000014	0.0017	0.0065	
	Significant	Significant	Significant	

Table 4 contains the results for ristocetin-induced platelet agglutination. In these tests a higher concentration of twice the maximum human concentration of ristocetin (2.5 mg/ml) was added to induce a strong reaction in the baboon platelets. With the maximum human concentration, 50% (5/10) of the baboon samples did not reach 10% agglutination, and with 1.5 times the maximum human concentration (1.875 mg/ml) one baboon sample did not react to the addition of agonist. At both these concentrations, the baboon and human results had a significant difference ($p<0.05$). However, the higher concentration of 2.5 mg/ml ristocetin was the most successful of all the agonists. It was the only agonist that produced results that did not significantly ($p=0.32$) differ from the human results.

One can hypothesize that sequence differences may cause the need for higher ristocetin concentrations in baboons. However, Shi *et al*. (2012) found that porcine platelets did not respond adequately to ristocetin, even at very high concentrations (3.6 mg/ml). They speculated that this was due to species-specific differences between the human and porcine VWF and

GPIbα. Hence, because a proper platelet response was elicited from baboon platelets with ristocetin, it is improbable that sequence differences caused the need for higher ristocetin concentrations in baboons.

Thus, the requirement for a higher concentration of ristocetin to induce agglutination in the baboons can rather be attributed to the fact that baboons have significantly more GPIbα receptors on the surface of their platelets than humans; therefore, more ristocetin is needed to elicit a platelet response in baboons than in humans. It is, nevertheless, only a hypothesis, as an increase in receptor numbers could rather be expected to lead to a stronger response. However, the quantification of the receptors will be discussed in the following section.

For the intention of differential diagnosis, it is essential to note that in patients with thrombasthaenia and a storage pool defect, ristocetin only induces the primary wave of agglutination. In patients with Bernard-Soulier syndrome, ristocetin does not illicit any response from the platelets, and with von Willebrand disease (vWD) ristocetin-induced agglutination is either absent or reduced depending on the type of vWD. However, normal ristocetin-induced agglutination is seen in the other known platelet function disorders, and an increased reaction is seen in vWD type 2B and platelet-type vWD (Laffan & Manning, 2006).

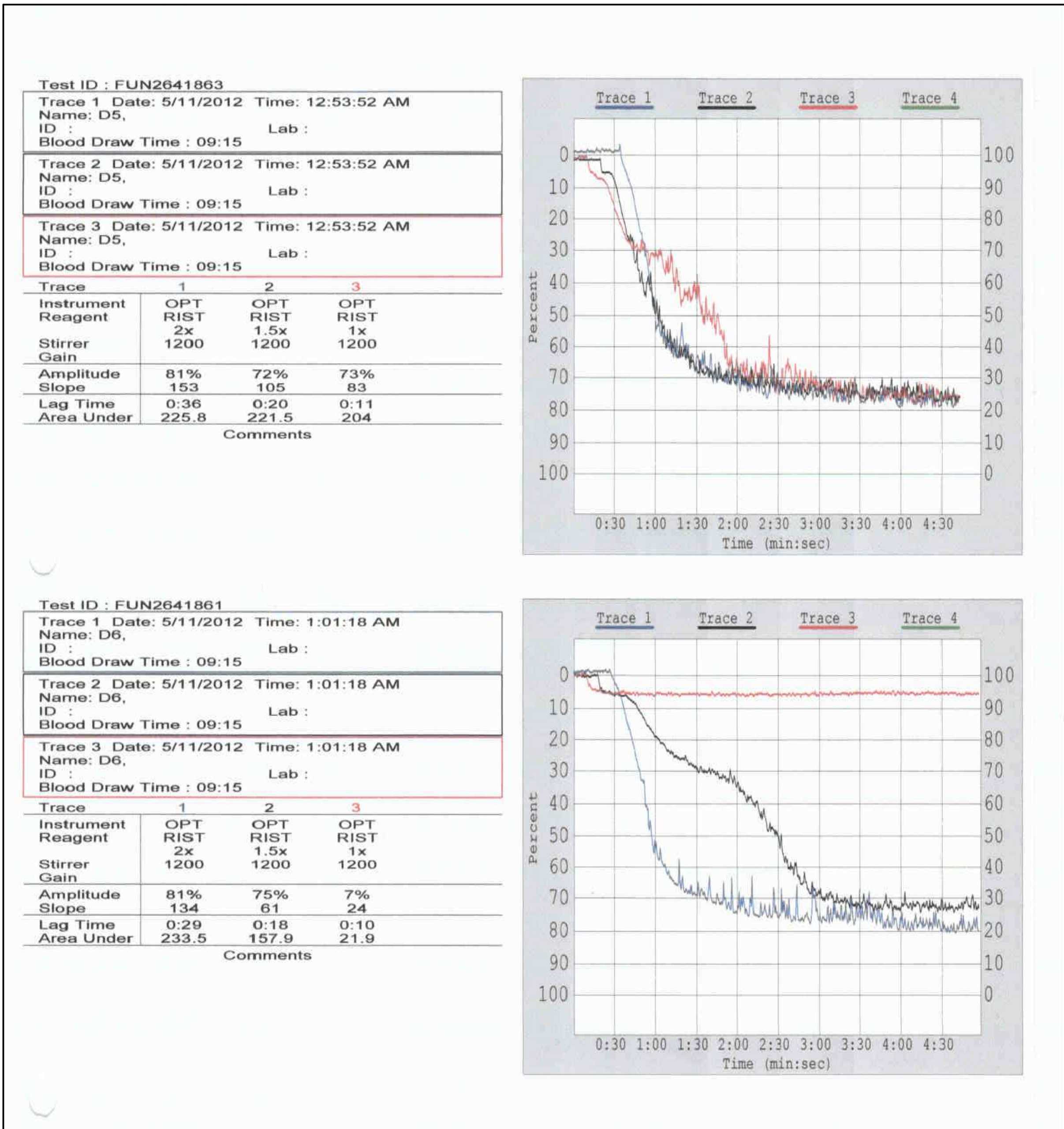


Figure 9: Example of ristocetin-induced agglutination tracings. Each graph contains information for three different ristocetin concentrations. The second graph is a good example of how different concentrations of ristocetin show different agglutination reactions, with the lowest concentration not causing a reaction, the middle concentration causing a slow response, and the highest concentration causing a fast and powerful respons.

Table 4. Ristocetin-induced platelet agglutination expressed as a percentage. The checkered block indicates the maximum percentage aggregation, and the shaded blocks indicate samples that had less than 10% aggregation.

Ristocetin				
Baboon	**1.25 mg/ml**	**1.875 mg/ml**	**2.5 mg/ml**	**Human 1.25 mg/ml**
1	61	55	71	n=38
2	3	56	76	
3	1	2	61	
4	56	65	69	
5	1	64	82	
6	1	51	87	
7	76	65	68	
8	63	74	70	
9	73	72	81	
10	7	75	81	
Mean	34.2	57.9	74.6	78
SD	33.82	21.27	8.07	13
T-Test	0.0026	0.016	0.32	
	Significant	Significant	Not Significant	

Table 5 contains the results for the arachidonic acid-induced platelet aggregation. As with all the previous agonists an additional higher concentration of arachidonic acid had to be used because some of the baboon samples did not react to the lower concentrations. With the maximum human concentration, 30% (3/10) of the baboon samples did not reach 10% aggregation, and with 1.5 times the maximum human concentration (0.75 mg/ml) 20% (2/10) of the baboon samples did not react to the addition of agonist.

It was evident from the results that baboon platelets did not follow a predictable pattern of aggregation in response to arachidonic acid, as some animals had maximum aggregation at the lowest concentration; some had maximum aggregation at the middle concentration, and others had maximum aggregation at the highest concentration. All concentrations produced significantly ($p<0.05$) different results when compared with the human results. When taking the maximum mean amount of baboon platelet aggregation into account (58% of normal human aggregation), it can be derived that

arachidonic acid was also only able to cause the primary wave of aggregation in baboon platelets. It did not cause the total permanent secondary wave of aggregation as seen in humans, such as was the case with ADP-induced aggregation in the baboon.

Taking the unpredictability as well as the significant differences from the human results into account, arachidonic acid-induced platelet aggregation can be regarded as not a good test to evaluate baboon platelet aggregation because it is impossible to determine an optimal arachidonic acid concentration to use routinely to evaluate baboon platelet aggregation. Because arachidonic acid-induced aggregation detects abnormalities in the later stages of platelet activation, any of the numerous small differences between baboon and human platelets could be responsible for defective arachidonic acid-induced aggregation.

According to Laffan & Manning (2006) arachidonic acid aggregation will be reduced or absent in cases where cyclooxygenase is absent or impaired, such as with the aspirin effect. Arachidonic acid aggregation is also absent in patients with thrombasthaenia, has only the primary wave or is absent in patients with a storage pool defect, is reduced or absent in patients with a thromboxane synthetase deficiency, and reduced in patients with a cyclo-oxygenase deficiency.

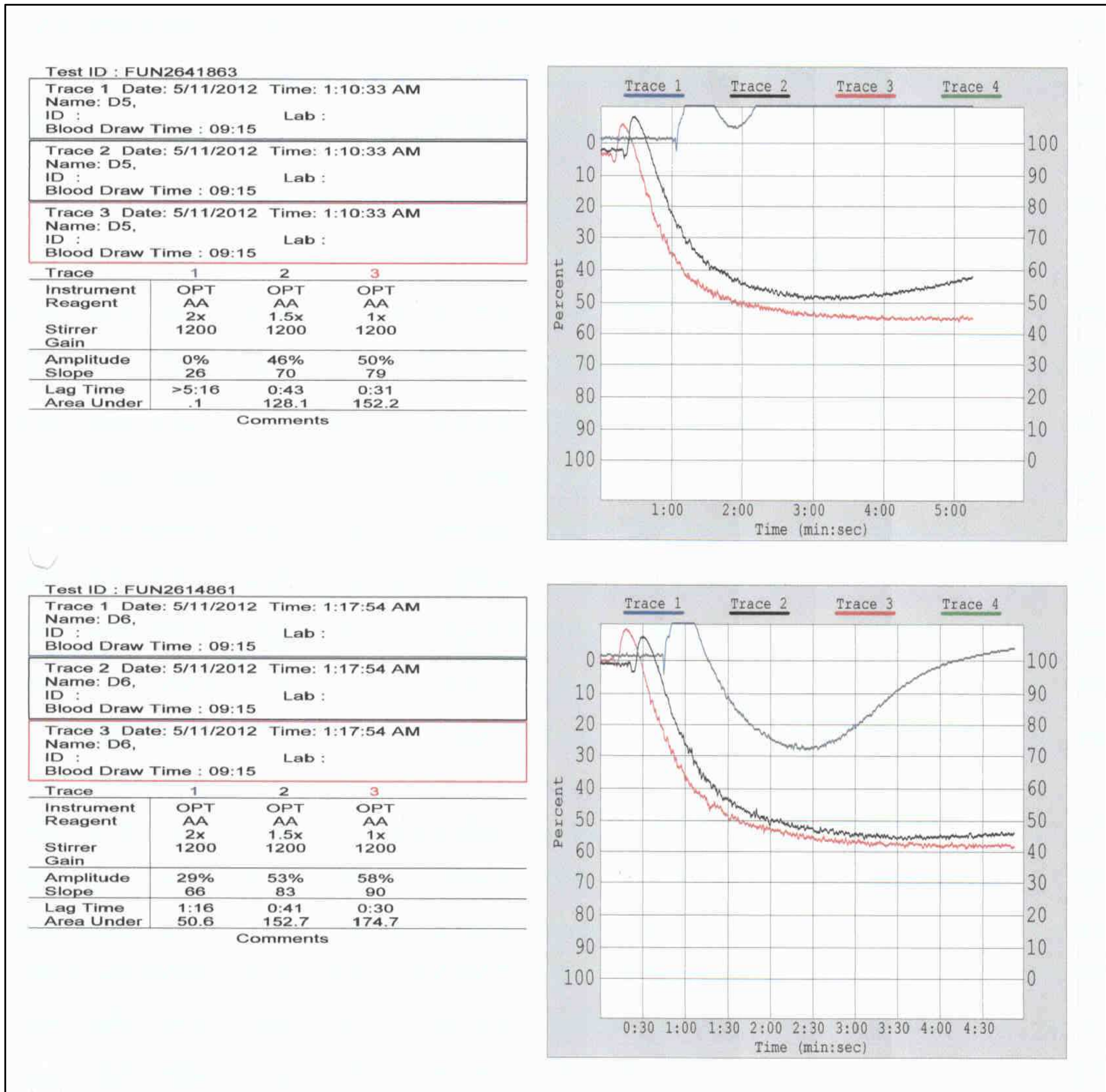


Figure 10: Example of arachidonic acid-induced aggregation tracings. Each graph contains information for three different arachidonic acid concentrations. In the first graph the highest concentration did not cause aggregation, this reaction was repeated in the following figure.

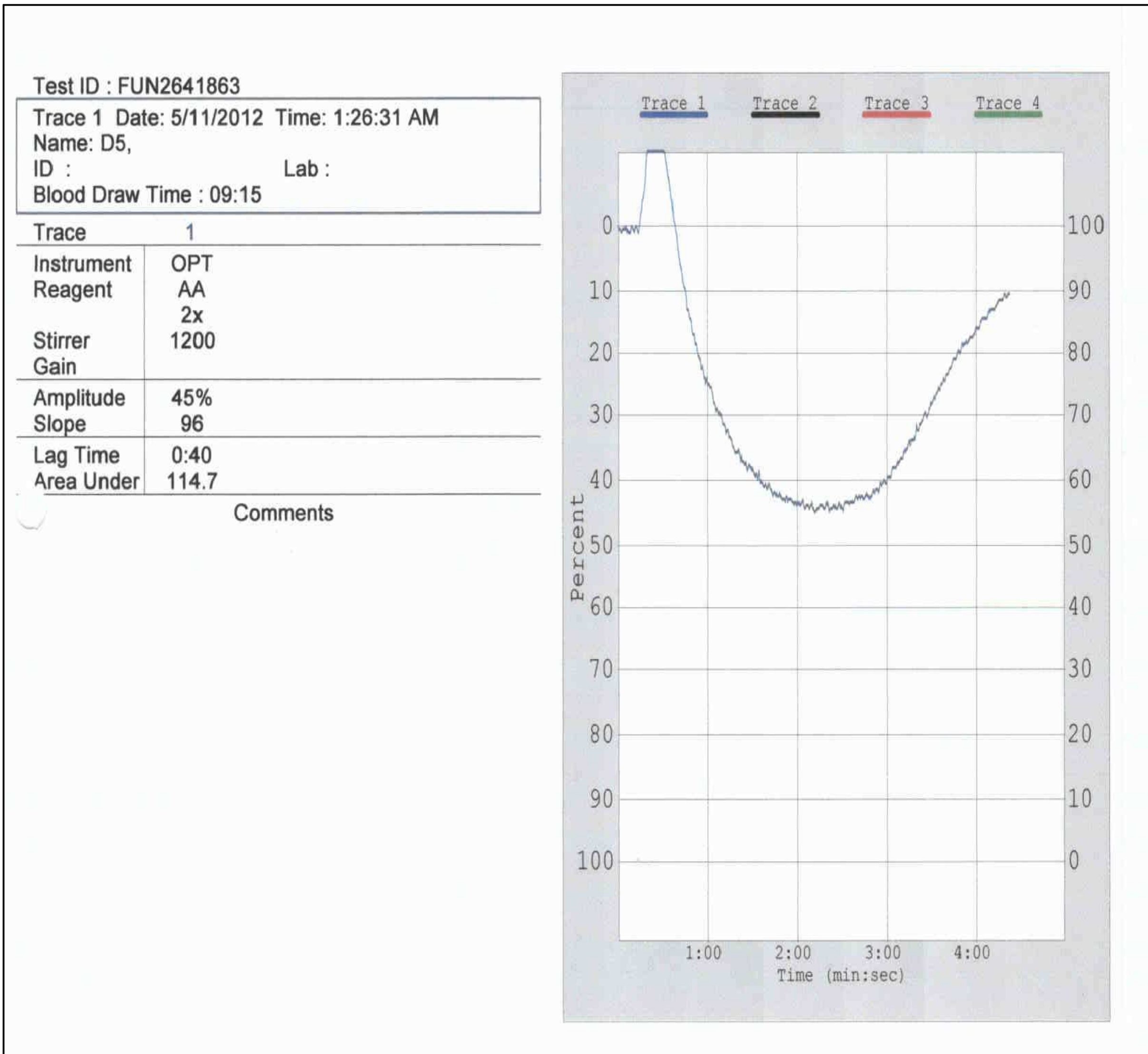


Figure 11: Example of arachidonic acid-induced aggregation tracing using the highest concentration. This reaction was a repeat as the reaction was unsuccessful previously, as shown in the previous figure.

Table 5. Arachidonic acid-induced platelet aggregation expressed as a percentage. The checkered block indicates the maximum percentage aggregation, and the shaded blocks indicate samples that had less than 5% aggregation.

Arachidonic Acid				
Baboon	**0.5 mg/ml**	**0.75 mg/ml**	**1 mg/ml**	**Human 0.5 mg/ml**
1	38	37	36	n=38
2	0	0	38	
3	50	55	49	
4	45	47	24	
5	1	49	39	
6	64	55	47	
7	0	1	37	
8	44	38	43	
9	50	46	45	
10	58	53	29	
Mean	35	38.1	38.7	67
SD	24.98	20.77	7.85	8
T-Test	0.0026	0.0015	0.00000005	
	Significant	Significant	Significant	

Considering the differential diagnosis of platelet function disorders (table 1), as set out by Laffan & Manning (2006), and taking all the maximum aggregation results of each agonist into account, there are only three conditions that fit the baboons' aggregation profile. These conditions are a cyclo-oxygenase deficiency, a thromboxane synthetase deficiency, or aspirin ingestion. It was ensured that no medication was given to the animals prior to taking the blood, thus it can be eliminated that aspirin ingestion as a possible explanation for the baboons' aggregation profile. It leaves only the possibility of a cyclo-oxygenase, thromboxane synthetase or TXA_2-receptor deficiency. It may also be possible that baboon platelets do not produce, or produces less TXA_2, after activation, resulting in a reduced reaction to agonists.

It was a very interesting finding, since, in a previous unpublished study done in the Department of Haematology and Cell Biology by Roodt and colleagues, it was found that in an arterial thrombosis model in the baboon, aspirin was not able to inhibit platelet aggregation. It is in contrast to what is seen in humans, where aspirin is very effective in preventing thrombosis associated cardiovascular incidences, such as myocardial infarction (Antithrombotic

Trialists' Collaboration, 2002). The reason for this finding may be because of a difference in baboon and human cyclo-oxygenase- or thromboxane synthetase activity, or a discrepancy in TXA_2 secretion. Further studies on the cyclo-oxygenase and thromboxane synthetase activities and TXA_2 production in baboons have to be done to determine whether this hypothesis is correct. Detection of the TXA_2-receptor may also be done to determine if it is indeed expressed on baboon platelets.

In a poster presented at the 58th Scientific and Standardization Committee meeting of the International Society on Thrombosis and Haemostasis, Liverpool, United Kingdom (2012), it was demonstrated that when using whole blood impedance platelet aggregometry (Multiplate®), baboon platelet aggregation was closely related to the anticoagulant used in sample collection. In that study the manufacturer of the Multiplate® instrument's recommended agonists were used: ADP (6.5 µM and 9.75 µM), ristocetin (0.77 mg/ml and 1.155 mg/ml), type I collagen (3.2 µg/ml and 4.8 µg/ml), arachidonic acid (0.5 mM and 0.75 mM) and thrombin receptor activator peptide 6 (TRAP-6, 32 µM and 48 µM). The lower concentration was the one recommended, and the higher concentration was 1.5 times higher (Janse van Rensburg *et al.*, 2012a).

In that study baboon platelet aggregation was evaluated using sodium citrate, heparin and hirudin as anticoagulants. It was found that with citrated baboon whole blood all agonists, at both concentrations, with the exception of collagen, had significantly ($p<0.05$) different results to those obtained for humans. In heparinized blood, collagen at the higher concentration showed a significant difference from humans. TRAP-6 at the lower concentration was the only other agonist that showed a significant difference using heparin as an anticoagulant. Hirudin was the most successful anticoagulant in the study, with only TRAP-6 at the lower concentration showing a significant difference from normal human results (Janse van Rensburg *et al.*, 2012a). The baboon impedance platelet aggregation gave comparable results to normal humans using ADP, collagen and arachidonic acid. It is in contrast to what was found in the current study using light transmission aggregometry, where reduced

baboon platelet aggregation was observed even at high concentrations of these three agonists. Unfortunately, those experiments were performed as part of another study, and as such the results could not be further included in this thesis.

A possible explanation is related to the type of sample used in the different methods. Light transmission aggregometry uses platelet-rich plasma as sample. These samples contain platelets and plasma, with the cellular components mostly removed. On the other hand, impedance aggregometry uses whole blood, which still contains all the other cellular components. Caudrillier *et al.* (2012) described that activated platelets have the potential to induce the formation of neutrophil extracellular traps (NETs). NETs are extracellular fibers consisting mostly of DNA that is released by activated neutrophils (Brinkmann *et al.*, 2004). Fuchs *et al.* (2010) explained that NETs promote platelet thrombus formation. Therefore, it is hypothesised that the presence of neutrophils in the impedance aggregometry sample may contribute to platelet aggregation, causing the platelets to have stronger aggregation than when evaluated in isolation, such as in the case with light transmission aggregometry.

A follow-up study can be done to determine if other anticoagulants, such as heparin and hirudin, produces better results for baboon LTA, than sodium citrate. High levels of sodium citrate have shown to produce a reduction in platelet aggregation and lower plasma calcium levels (Kingston *et al.*, 2001). The lack of aggregation could be attrubited to the interaction between the stromal interaction molecule 1 (STIM1) and the calcium channel moiety Orai1 and subsequent further platelet activation. STIM1 and Orai1 interaction causes an increase in cytosolic calcium, which further contributes to platelet activation and aggregation (Broos *et al.*, 2011). Therefore, the presence of a calcium chelator, such as sodium citrate, may inhibit calcium to such an extent as to inhibit strong platelet aggregation. However, heparin is also not an optimal anticoagulant, as it has shown to cause platelet clumping, it is thus not so suitable for platelet aggregation testing (Kingston *et al.*, 2001). As was the case with the whole blood impedance study, it seems as if hirudin would

be the preferred anticoagulant to use in LTA studies. However, this theory must still be tested. One potential drawback of hirudin is the availability of the tubes. Currently, hirudin containing tubes are not standard stock in South Africa, and it has to be specially ordered. This will increase the cost and time associated with these tests.

In the next section the platelet receptor number quantification and how it relates to the light transmission aggregation results as set out above will be discussed.

Platelet receptor quantification:

Table 6 contains the platelet receptor quantification results. The number of GPIIb, GPIIIa and GPIbα receptors differed significantly ($p<0.05$) between baboon and human platelets. It was found that the mean platelet volume (MPV) of baboon platelets is 7.23 ± 0.62 fl. This finding shows that baboon platelets are smaller than human platelets (normal range: 8.8 – 12.5 fl) when measured with the same instrument (Sysmex XE-2100, Roche, South Africa) (Botma *et al.*, 2012), which is important when evaluating surface receptor numbers.

A mean of 55 801 ± 16 317 GPIIb receptors per platelet was found in the humans. Interestingly, the average number of GPIIb receptors on baboon platelets (76 677 ± 7 747) correlated better with the published human GPIIb average as determined by Wagner *et al*. (1996) of roughly 80 000 per platelet, than the mean results with the healthy human volunteers (55 801 ± 16 317) in this study. This finding was also seen with GPIIIa receptor numbers, where the baboon GPIIIa numbers (65 048 ± 13 921) were closer to the Wagner *et al.* (1996) human GPIIIa numbers (roughly 80 000 per platelet), than in the healthy human volunteers (51 142 ± 10 318). On the other hand, the human volunteer results correlated very well with the GPIIb/IIIa complex numbers estimated by Pidard *et al*. (1983), Tsao *et al*. (1995) and Quinn *et al*. (1999) of

57 400 ± 9 700, approximately 53 800, and 53 300 ± 5423 (Mab1 antibody) and 50 120 ± 5066 (Mab2 antibody), respectively. The mean human volunteer GPIbα numbers (33 266 ± 7 013) closely resemble the published human numbers of roughly 25 000 (Bergmeier *et al.*, 2000). Unfortunately, the article published by Kotzé *et al.* (1995) only included receptor occupancy and not quantity, therefore, this is the first published record of receptor quantity on the baboon platelets.

When comparing the baboon results with the healthy human volunteer results, it was determined that the baboon platelet has 37% more GPIIb receptors, 27% more GPIIIa receptors, and 25.5% more GPIbα receptors on the platelet surface than humans. It was also found that GPIIb/IIIa is arranged closer to a 9:7 configuration than to the 1:1 configuration seen in humans (Phillips *et al.*, 1988). The increased receptor numbers become even more noteworthy, taken the fact that the baboon platelets are roughly 30% smaller than the average human platelet.

The need of ADP-induced platelet activation for fibrinogen to cause irreversible platelet aggregation has been comprehensively described (Cross, 1964; Mustard *et al.,* 1978; Bennett & Vilaire, 1979; Marguerie *et al.*, 1979; Niewiarowski *et al.*, 1977). The fact that GPIIb/IIIa is the receptor for fibrinogen on the platelet surface after platelet activation has also been well described (Nachman & Leung, 1982; Gogstad *et al.*, 1982; Marguerie *et al.*, 1984). Landolfi *et al.* (1991) found a correlation between the velocity of ADP-induced platelet aggregation and the saturation of the fibrinogen receptor, GPIIb/IIIa. They determined that the velocity of ADP-induced aggregation is directly proportional to the amount of GPIIb/IIIa receptor saturation, up to a certain saturation point, after which a plateau is reached. They based their finding on experiments using different fibrinogen concentrations and not GPIIb/IIIa receptor numbers.

However, Hainsey *et al.* (1993) established that the baboon and normal clinical human fibrinogen levels are comparable. It can, therefore, be postulated that the increase in GPIIb/IIIa receptor number in the baboon

required a higher ADP agonist concentration to elicit a full cross-linked secondary wave of aggregation in the baboon platelets.

However, small sequence differences have been found between the human and baboon P2Y12 ADP receptor DNA sequences (see sequencing results). This finding together with the increased GPIIb/IIIa receptor number, could explain why ADP-induced platelet aggregation only produced the primary wave of aggregation in the baboon.

The mean baboon GPIbα receptor number was 25.5% more than the mean normal human volunteer GPIbα receptor number. However, 100% higher concentration of ristocetin (2.5 mg/ml vs. 1.25 mg/ml) was consistently required to induce baboon platelet agglutination similar to normal human platelets. Ristocetin causes platelet agglutination reacting with VWF and the GPIb-IX-V receptor (http://www.practical-haemostasis.com, 2011).

The positive antithrombotic properties shown in studies using the humanized anti-GPIbα antibody, 6B4 (Cauwenberghs *et al*., 2000 and Fontayne *et al*., 2008), and the humanized anti-VWF antibody, ALX-0081 (Ulrichts *et al*., 2011) have revealed that baboon and human GPIbα and VWF have very similar properties. It can consequently be theorised that the need for a higher ristocetin concentration to elicit a similar platelet response in baboon and human platelets is caused by an increase in GPIbα receptor number in the baboon. Thus, more ristocetin is needed to reach a certain threshold of receptor saturation, at which baboon platelets are activated.

It is, however, important to note that when a commercial anti-GPIbα antibody (Anti-CD42b, BD Cat #: 555471) was used to quantify the baboon and human receptors, no binding of the antibody to the baboon platelets were found. It was in contrast to what was seen with the healthy human volunteer platelets, where decent binding was observed. Therefore, it can be derived that the higher amount of ristocetin is not only needed because of the increase in GPIbα receptor number, but may also be due to small sequence differences

between the baboon and human GPIbα receptors DNA sequence (see sequencing results).

As previously described, none of the six different monoclonal antibodies directed against GPVI were able to bind to the baboon platelets in all ten animals. The GPVI quantification was, therefore, excluded from further discussion.

Table 6. Platelet receptor quantification results expressed as numbers.

Platelet receptor quantification								
Baboon	**GPIIb**	**GPIIIa**	**GPIbα**		**Human**	**GPIIb**	**GPIIIa**	**GPIbα**
1	74023	58104	45466		**1**	59162	50392	35869
2	73818	52864	38423		**2**	46474	45611	27598
3	93945	84464	43359		**3**	47761	53318	28065
4	77669	64461	35223		**4**	46643	39761	29368
5	77906	57571	42665		**5**	51238	48657	27268
6	75852	52914	42426		**6**	48903	45581	32960
7	67061	73354	42450		**7**	38085	41789	28798
8	79714	80815	40705		**8**	50016	47906	31601
9	80468	80862	48226		**9**	80134	67609	45830
10	66309	45066	38668		**10**	89590	70800	45303
Mean	76677	65048	41761		**Mean**	55801	51142	33266
SD	7747	13921	3718		**SD**	16317	10318	7013
T-Test	0.0018	0.021	0.0033					
	Significant	Significant	Significant					

Sequencing Results

Figure 12 is an example of a gel with amplified fragments. This example taken from GPVI was typical for this study. The two different lanes for each sample represent different annealing temperatures used during the PCR reactions. The two end lanes contain negative controls, for exon 4 two fragments were present, for exons 5, 6 and 7 single fragments were obtained, and for exon 8 (fragment 1) multiple fragments were obtained. Both fragments of exon 4 were removed and sequenced, with only the bottom fragment successfully sequenced. At exon 5, only one temperature produced a proper fragment, which was successfully sequenced. None of the fragments of exon 8 gave a successful sequence, thus, the primers were redesigned and the experiment repeated. All unsuccessful reactions were first repeated under different cycling conditions and with different reagent concentrations, before it was decided to design new primers.

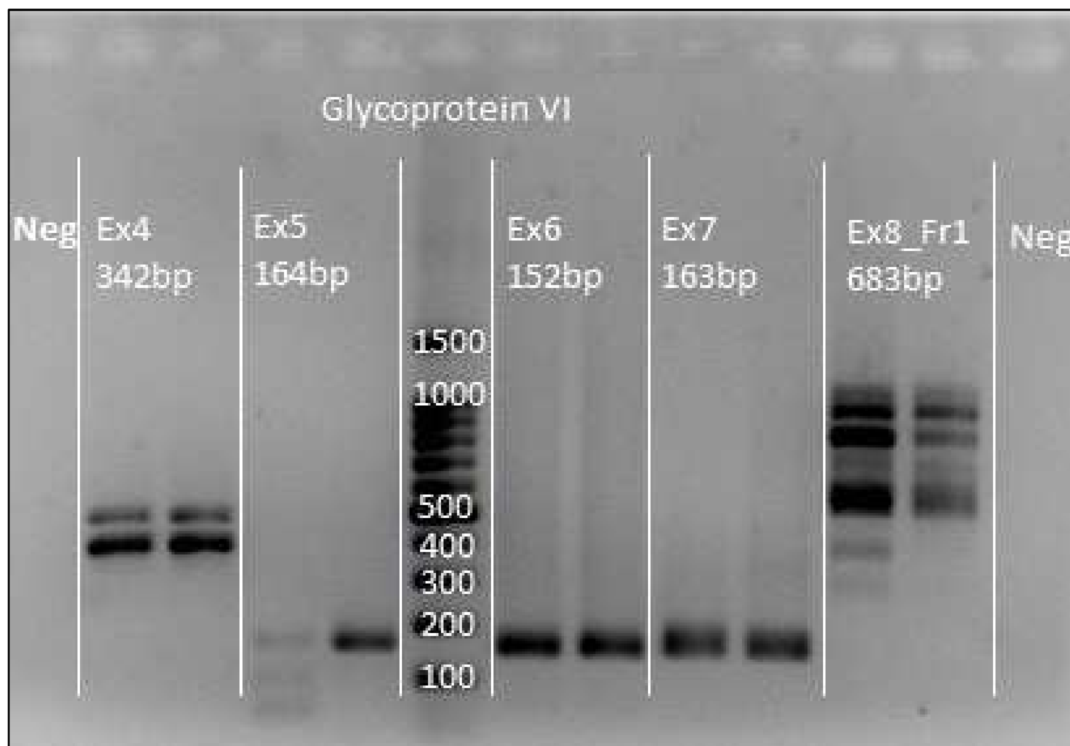


Figure 12: Gel picture for amplification of GPVI showing two negative controls, two fragments for exon 4, one fragment each for exons 5, 6 and 7, and multiple fragments for exon 8 (fragment 1).

P2Y12 sequence:

Table 7 contains the detected SNPs in the baboon P2Y12 sequence. Figure 13 shows a comparison between the protein translating region nucleotide sequence between the P2Y12 gene of the baboon and human reference sequence as found on www.ensembl.com (P2RY12-001, transcript ID: ENST00000302632). In figure 13, an SNP that does not cause a change in amino acid is shown in green, and one that does is indicated in red. A total of 17 SNPs were detected in the baboon P2Y12 sequence when compared with the human P2Y12 reference sequence. Of the 17, 12 SNPs were silent mutations, which did not cause an amino acid change. There were, however, five SNPs that caused a change in four amino acids. When evaluating the nucleotide sequence, the baboon P2Y12 has a 98.3% similarity to the human.

Table 7. SNPs detected in baboon P2Y12 sequence.

SNP	Position	Amino Acid Change
G to A	10	Yes
C to G	24	No
T to G	36	No
T to C	120	No
G to A	130	Yes
C to T	158	No
T to C	207	No
T to C	549	No
T to C	615	No
T to C	627	No
C to T	660	No
G to A	669	No
C to A	777	No
A to G	811	Yes
T to C	813	Yes
C to T	846	No
T to A	965	Yes

```
Baboon Translating Region of P2Y12 1029 bp        1029 nt vs.
Human Translating Region of P2Y12 1029 bp         1029 nt
 using matrix file: DNA, gap open/ext: -14/-4
  98.3% identity in 1029 nt overlap;        Global score: 4992

               10        20        30        40        50        60
Baboon ATGCAAGCCATCGACAACCTCACGTCTGCGCCTGGGAACACCAGTCTGTGCACCAGAGAC
       ::::::::: ::::::::::::: ::::::::::: ::::::::::::::::::::::::
Human  ATGCAAGCCGTCGACAACCTCACCTCTGCGCCTGGTAACACCAGTCTGTGCACCAGAGAC
               10        20        30        40        50        60

               70        80        90       100       110       120
Baboon TACAAAATCACCCAGGTCCTCTTCCCACTGCTCTACACTGTCCTGTTTTTGTTGGACTC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::: 
Human  TACAAAATCACCCAGGTCCTCTTCCCACTGCTCTACACTGTCCTGTTTTTGTTGGACTT
               70        80        90       100       110       120

              130       140       150       160       170       180
Baboon ATCACAAATAGCCTGGCGATGAGGATTTCTTTCAAATTCGGAGTAAATCAAACTTTATT
       ::::::::: ::::::::::::::::::::::::::: ::::::::::::::::::::::
Human  ATCACAAATGGCCTGGCGATGAGGATTTCTTTCAAATCCGGAGTAAATCAAACTTTATT
              130       140       150       160       170       180

              190       200       210       220       230       240
Baboon ATTTTTCTTAAGAACACAGTCATTTCCGATCTTCTCATGATTCTGACTTTTCCATTCAAA
       :::::::::::::::::::::::::: :::::::::::::::::::::::::::::::::
Human  ATTTTTCTTAAGAACACAGTCATTTCTGATCTTCTCATGATTCTGACTTTTCCATTCAAA
              190       200       210       220       230       240

              250       260       270       280       290       300
Baboon ATTCTTAGTGATGCCAAACTGGGAACAGGACCACTGAGAACTTTTGTGTGTCAAGTTACC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Human  ATTCTTAGTGATGCCAAACTGGGAACAGGACCACTGAGAACTTTTGTGTGTCAAGTTACC
              250       260       270       280       290       300

              310       320       330       340       350       360
Baboon TCCGTCATATTTTATTTCACAATGTATATCAGTATTTCATTCCTGGGACTGATAACTATC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Human  TCCGTCATATTTTATTTCACAATGTATATCAGTATTTCATTCCTGGGACTGATAACTATC
              310       320       330       340       350       360

              370       380       390       400       410       420
Baboon GATCGCTACCAGAAGACCACCAGGCCATTTAAAACATCCAACCCCAAAAATCTCTTGGGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Human  GATCGCTACCAGAAGACCACCAGGCCATTTAAAACATCCAACCCCAAAAATCTCTTGGGG
              370       380       390       400       410       420

              430       440       450       460       470       480
Baboon GCTAAGATTCTCTCTGTTGTCATCTGGGCATTCATGTTCTTACTCTCTTTGCCTAACATG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Human  GCTAAGATTCTCTCTGTTGTCATCTGGGCATTCATGTTCTTACTCTCTTTGCCTAACATG
              430       440       450       460       470       480

              490       500       510       520       530       540
Baboon ATTCTGACCAACAGGCAGCCGAGAGACAAGAATGTGAAGAAATGCTCTTTCCTTAAATCA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Human  ATTCTGACCAACAGGCAGCCGAGAGACAAGAATGTGAAGAAATGCTCTTTCCTTAAATCA
              490       500       510       520       530       540

              550       560       570       580       590       600
Baboon GAGTTCGGCCTAGTCTGGCATGAAATAGTAAATTACATCTGTCAAGTCATTTTCTGGATT
       :::::::: :::::::::::::::::::::::::::::::::::::::::::::::::::
Human  GAGTTCGGTCTAGTCTGGCATGAAATAGTAAATTACATCTGTCAAGTCATTTTCTGGATT
              550       560       570       580       590       600

              610       620       630       640       650       660
Baboon AATTTCTTAATTGTCATTGTATGTTACACACTCATTACAAAAGAACTGTACCGGTCATAT
       :::::::::::::: ::::::::::: :::::::::::::::::::::::::::::::: 
Human  AATTTCTTAATTGTTATTGTATGTTATACACTCATTACAAAAGAACTGTACCGGTCATAC
              610       620       630       640       650       660

              670       680       690       700       710       720
Baboon GTAAGAACAAGGGGTGTAGGTAAAGTCCCCAGGAAAAAGGTGAACGTCAAAGTTTTCATT
       :::::::: :::::::::::::::::::::::::::::::::::::::::::::::::::
Human  GTAAGAACGAGGGGTGTAGGTAAAGTCCCCAGGAAAAAGGTGAACGTCAAAGTTTTCATT
              670       680       690       700       710       720

              730       740       750       760       770       780
Baboon ATCATTGCTGTATTCTTTATTTGTTTTGTTCCTTTCCATTTTGCCCGAATTCCTTATACC
       :::::::::::::::::::::::::::::::::::::::::::::::::::::::: :::
Human  ATCATTGCTGTATTCTTTATTTGTTTTGTTCCTTTCCATTTTGCCCGAATTCCTTACACC
              730       740       750       760       770       780

              790       800       810       820       830       840
Baboon CTGAGCCAAACCCGGGATGTCTTTGACTGCGCCGCTGAAAATACTCTGTTCTATGTGAAA
       :::::::::::::::::::::::::::::: : :::::::::::::::::::::::::::
Human  CTGAGCCAAACCCGGGATGTCTTTGACTGCACTGCTGAAAATACTCTGTTCTATGTGAAA
              790       800       810       820       830       840

              850       860       870       880       890       900
Baboon GAGAGTACTCTGTGGTTAACTTCCTTAAATGCATGCCTGGATCCGTTCATCTATTTTTTC
       ::::: ::::::::::::::::::::::::::::::::::::::::::::::::::::::
Human  GAGAGCACTCTGTGGTTAACTTCCTTAAATGCATGCCTGGATCCGTTCATCTATTTTTTC
              850       860       870       880       890       900

              910       920       930       940       950       960
Baboon CTTTGCAAGTCCTTCAGAAATTCCTTGATAAGTATGCTGAAGTGCCCCAATTCTGCAACA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Human  CTTTGCAAGTCCTTCAGAAATTCCTTGATAAGTATGCTGAAGTGCCCCAATTCTGCAACA
              910       920       930       940       950       960

              970       980       990      1000      1010      1020
Baboon TCTCAGTCCCAGGACAATAGGAAAAAAGAACAGGATGGTGGTGACCCAAATGAAGAGACT
       :::: :::::::::::::::::::::::::::::::::::::::::::::::::::::::
Human  TCTCTGTCCCAGGACAATAGGAAAAAAGAACAGGATGGTGGTGACCCAAATGAAGAGACT
              970       980       990      1000      1010      1020

Baboon CCAATGTAA
       :::::::::
Human  CCAATGTAA
```

Figure 13. Comparison of the protein translating region nucleotide sequence between the P2Y12 gene of the baboon and human reference sequence. SNPs that cause amino acid changes are shown in red and silent mutations in green.

Figure 14 shows a comparison between the amino acid sequences of the P2Y12 protein of the baboon and human. As mentioned previously, there was a four amino acid difference between the baboon and human P2Y12 proteins. It equates to 98.8% similarity between the protein sequences. The amino acid changes are Val4Ile, Gly44Ser, Thr271Ala and Leu322Gln.

The algorithm of Huang & Miller (1991) used by the sequence alignment tool (Lalign) can also predict if an amino acid change is a conservative or a radical

(non-conservative) substitution. A conservative substitution is defined by Li & Graur (1991a) as “the substitution of an amino acid by another with similar chemical properties,” which, therefore, has minimal effect on the protein structure. In contrast, Li & Graur (1991b) define a radical substitution as “the substitution of an amino acid by another with markedly different chemical properties,” which may then potential influence the protein structure. According to amino acid alignment, only Leu322Gln is a radical substitution, with the other three substitutions seen as conservative substitutions. Leu and Gln differ in that Leu is a neutral nonpolar amino acid, and Gln is a neutral polar amino acid (McKee & McKee, 2003). It can be postulated that this single radical amino acid substitution may be a contributing factor to the reduction in ADP-induced platelet aggregation found with the baboon platelets.

All four extracellular Cys residues (position 17, 97, 175 and 270) as described in humans were present in the baboon. The P2Y12 receptor expression was, therefore, not altered in the baboon as both the Cys 97 and the Cys 175 which play vital roles in receptor expression were present in the baboon sequence (Cattaneo, 2011). Cys97 and Cys175 have also been presented as the binding sites for the active metabolites of prasugrel and clopidogrel in humans (Algaier *et al*., 2008 and Ding *et al*., 2009), with Cys97 implicated as the most likely target for P2Y12 inhibiting agents (Zhang *et al*., 2014a and Zhang *et al*., 2014b). In the papers of Zhang (2014a and 2014b) the crystal structure of P2Y12 (figure 16) was determined in combination with its agonist. Therefore, these anti-P2Y12 agents should bind with the same affinity to baboon platelets as they do to human platelets; thus having the same inhibitory effect in baboons as in humans. All four amino acids identified by Ignatovica *et al*. (2012) as important for the functional integrity of human P2Y12, namely Glu181, Arg256, Arg265 and Lys280 (vital for ligand-binding), were also intact in baboon platelets. Hence, it can be postulated that baboon platelets will have similar P2Y12 mediated properties to human platelets.

Figure 15 shows the predicted secondary structures (Jones, 1999), as well as predicted disorders (Ward *et al*., 2004) of the baboon and human P2Y12 proteins. The secondary structure prediction shows two minor changes within

the baboon protein (indicated with arrows). However, no disorder was predicted in the baboon protein. Nevertheless, it cannot be ruled out that these small variations in protein are the cause of the reduction of ADP-induced platelet aggregation as found in the baboon.

A comparison was done between *P. ursinus* and *M. fascicularis* (accession number: Q95KC3). The amino acid sequences were 99.1% similar. Three conservative amino acid changes, Thr89Ala, Val147Leu and Gln166Arg, were detected. All the changes the baboon had in relation with the human were also present in *M. fascicularis*, resulting in a 97.95% similarity between *M. fascicularis* and the human. Therefore, *P. ursinus* is more similar to humans than *M. fascicularis*. Unfortunately, no data for P2Y12 regarding other baboon subspecies were available on the NCBI database.

These findings are crucial for future anti-P2Y12 drug development, as they highlight the areas where the baboon and human proteins differ. Therefore, better predictive value is given to the baboon as research model when evaluating novel, molecular-based anti-P2Y12 agents. However, receptor number quantification may become essential for future anti-P2Y12 studies as P2Y12 protein expression has been linked with residual platelet reactivity after P2Y12 inhibiting treatment (Braun *et al*., 2007).

It is also important to note that not only amino acid changes in the protein may cause a difference in P2Y12 function, as is demonstrated by the intronic P2Y12 SNP, T742C. Patients with this SNP have increased ADP-induced aggregation, even though the physical protein is not affected (Lee *et al*., 2011). Therefore, a future study exploring the intronic regions of this gene may yield important information regarding P2Y12 function.

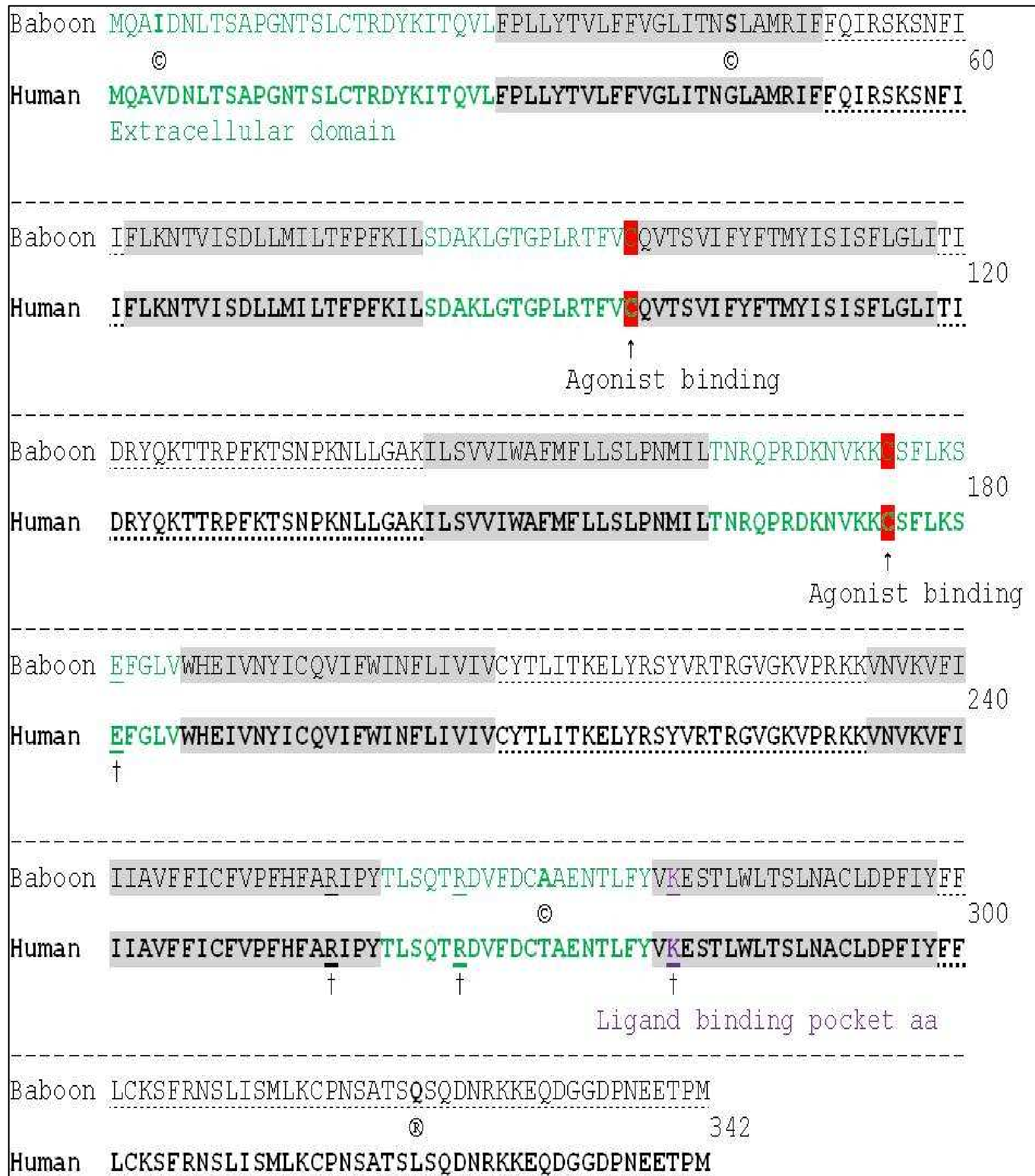


Figure 14. Comparison of the baboon and human P2Y12 amino acid sequences. A conservative amino acid change is indicated with ©. A radical amino acid change is indicated with ®. The agonist-binding sites are indicated with arrows and highlighted in red. The amino acids vital for functional integrity of the receptor are indicated with a cross (†). The vital amino acid for ligand binding is in purple lettering. Green lettering indicates an extracellular domain. The seven transmembrane regions are shaded with grey. The cytoplasmic regions are underlined with a dotted line.

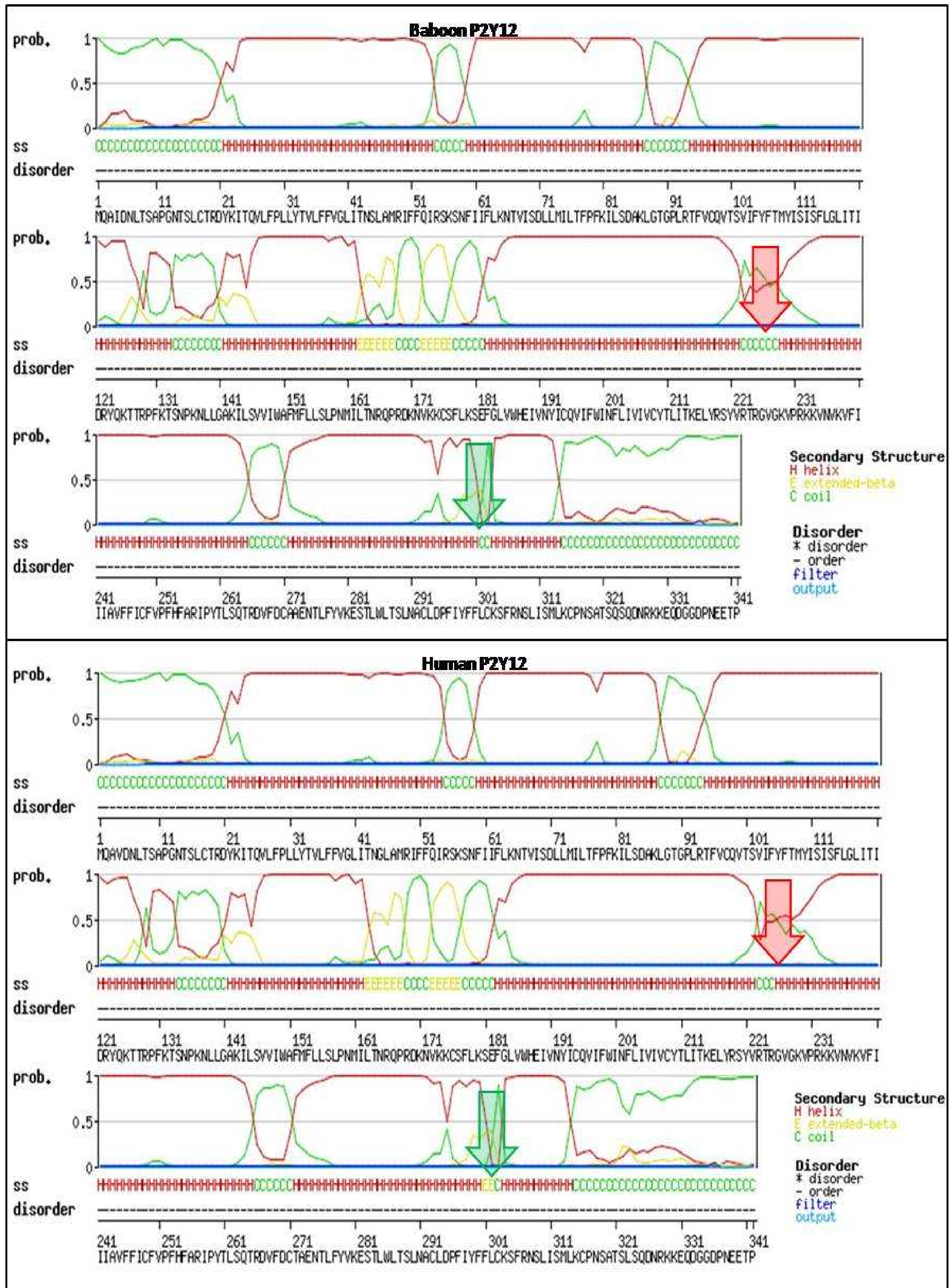


Figure 15. Prediction of the secondary protein structure of the baboon and human P2Y12 proteins. The red arrow indicates the differences at positions 226 to 228, and the green arrow indicates the differences at positions 300 and 301.

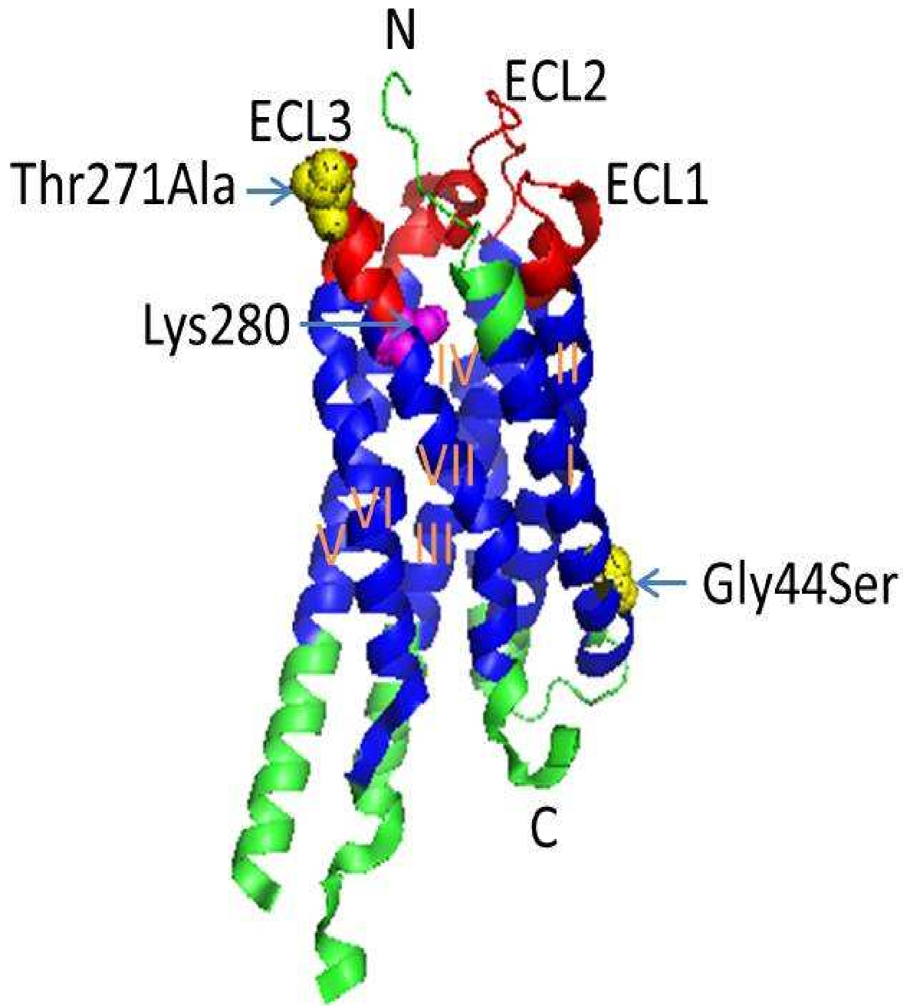


Figure 16. Crystal structure of P2Y12 as determined by Zhang *et al*. (2014a), depicting amino acid changes found in the *P. ursinus* sequence (shown in yellow dots). The amino acid Lys280, vital for ligand binding, is also indicated (shown in magenta dots). Cartoon drawn with PyMol. Crystal structure available from RCSB Protein Data Bank (accession # 4PXZ). The crystal structure did not contain the full C-terminal cytoplasmic region, therefore, Leu322Gln could not be indicated in this figure.

GPVI sequence:

Table 8 contains the detected SNPs in the baboon GPVI sequence. Figure 17 shows a comparison of the protein translating region nucleotide sequence between the GPVI gene of the baboon and human reference sequence as found on www.ensembl.com (GP6-001, transcript ID: ENST00000417454). In figure 17 an SNP that does not cause a change in amino acid is shown in green and one that does is indicated in red. A total of 36 SNPs was detected in the baboon GPVI sequence when compared with the human GPVI reference sequence. Of the 36, 23 SNPs were silent mutations, which did not cause an amino acid change. There were, however, 14 SNPs that caused a change in 14 amino acids. A 9 bp deletion was also found from position 942 to 950 (after position 166 in exon 8). When evaluating the nucleotide sequence, the baboon GPVI has a 95.6% similarity to the human.

Table 8. SNPs detected in baboon GPVI sequence.

SNP	Position	Amino Acid Change
A to C	69	No
G toA	72	No
C to G	75	No
C to T	78	No
G to T	198	Yes
A to G	213	No
C to G	216	No
G to A	239	Yes
A to C	241	Yes
T to C	249	No
T to C	293	Yes
G to C	324	No
A to G	339	No
C to G	342	No
G to C	375	No

T to C	408	No
A to C	484	No
T to C	495	No
C to T	516	No
A to G	537	No
A to G	576	No
C to T	579	No
T to C	600	No
G to A	601	Yes
C to T	655	Yes
T to A	670	Yes
T to C	700	Yes
T to C	780	No
T to C	790	Yes
A to C	844	Yes
G to A	852	No
G to T	905	Yes
T to C	927	No
9-bp deletion	942-950	Yes
A to C	964	Yes
G to A	971	Yes
A to C	989	Yes

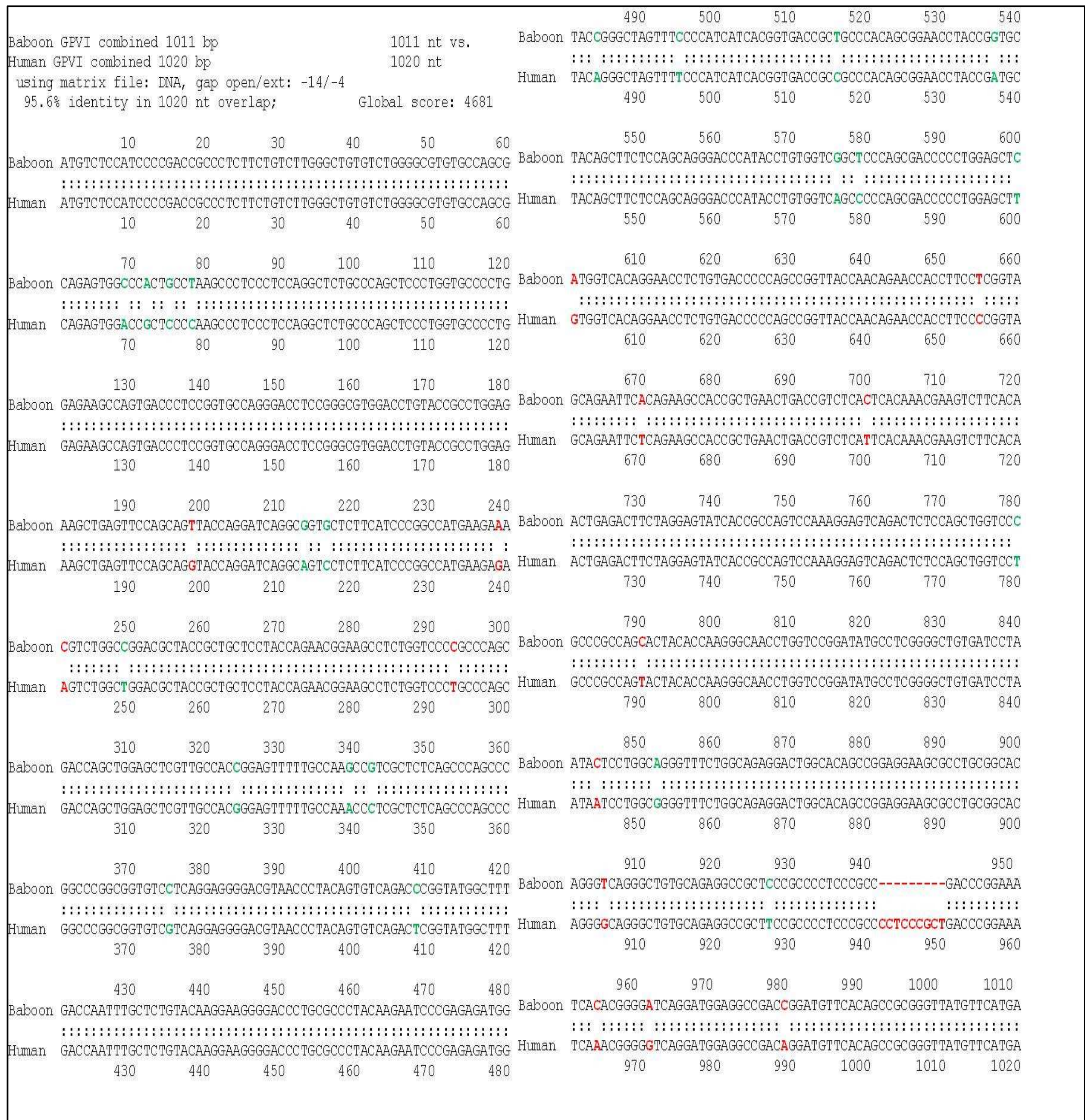


Figure 17. Comparison of the protein translating region nucleotide sequence between the GPVI gene of the baboon and human reference sequence. SNPs that cause amino acid changes are shown in red and silent mutations are indicated in green.

Figure 18 shows a comparison between the amino acid sequences of the GPVI protein of the baboon and human. There is 95% similarity between the protein sequences. The amino acid changes are Arg46Ser, Arg60Lys, Ser61Arg, Leu78Pro, Pro199Ser, Val201Met, Ser204Thr, Phe214Leu, Tyr244His, Ile262Leu, Gly282Val, Asn302His, Gly304Asp and Gln310Pro.

Leu295, Pro296 and Leu297 are deleted from the baboon sequence. The *Papio ursinus* sequence is a mix between the “a” and “b” alleles as determined by Joutsi-Korhonen *et al*. (2003). *Papio ursinus* gave a SEA-H sequence, in contrast with the SKTQH (“a” allele) and PEALN (“b” allele) sequences.

The collagen binding regions on GPVI as determined by Smethurst *et al*. (2004) and Lecut *et al*. (2004) are Lys59, Val34 and Leu36. Most monoclonal antibodies bind to Lys59 (Smethurst *et al*., 2004 and Takayama *et al*., 2008). The mF1232 monoclonal antibody that binds to the Thr116-Gln122 region does not directly inhibit the binding of collagen to GPVI, but rather depletes the surface expression of GPVI (Takayama *et al*., 2008). It can, therefore, be postulated that for a monoclonal antibody to directly inhibit collagen binding to GPVI, it needs to bind to Lys59, Val34 or Leu36. The monoclonal antibodies used for the flow cytometry receptor quantification should bind to these epitopes as these antibodies should only bind to GPVI without causing depletion of the receptor.

It was found that Lys59, Val34 and Leu36 are intact in the baboon sequence, however, the two amino acids directly following Lys59 are different between the baboon and human (Arg60Lys – conservative, and Ser61Arg - radical). Radical amino acid substitutions Arg46Ser and Leu78Pro are also present in the vicinity of the binding epitopes. Stave & Lindpainter (2013) determined that the average number of linear amino acids needed as contact residues for proper antibody-antigen interaction is 50-79 amino acids.

It can, therefore, be hypothesized that the presence of the radical amino acid changes within the nearby vicinity (within 50-79 amino acids) of the ligand binding regions of GPVI could interfere with the binding of monoclonal antibodies to GPVI to such an extent that limited or no antibody binding takes place. Thus, the reason baboon GPVI could not be quantified using human targeted anti-GPVI antibodies may lay in the amino acid sequence differences between the two species.

The reason only HORM® collagen elicited aggregation in baboon platelets may be due to the size of the collagen fibrils, together with the amino acid changes described above. The longer fibrils in HORM® collagen probably being able to bind more of the different collagen binding amino acids, and, therefore, being more efficient, than shorter fibrils found in other types of collagen at binding to GPVI.

The fact that more collagen is required for induction of collagen-induced platelet aggregation in baboons can also be explained in part by the sequencing results. A mutation in Arg60 of GPVI has previously been implicated in reduced collagen affinity (O'Connor *et al.*, 2006). Therefore, the Arg60Lys mutation within the baboon sequence could be the cause of the reduced collagen-induced platelet aggregation seen in baboons. As described earlier GPVI contains a Proline-rich (RPLPPLPPLP) domain within the cytoplasmic tail from Arg287 to Pro296 (Clemetson *et al.*, 1999), and that Fyn and Lyn activate Syk through the phosphorylation of the ITAM in FcRγ by binding to this Proline-rich motif within GPVI (Suzuki-Inoue *et al.*, 2002). The baboon GPVI does not contain the last part (Leu295 and Pro296) of this domain due to a 9-bp deletion within the baboon GPVI gene. It can be postulated that these missing amino acids could cause the signal to be weaker in the baboons than in humans. Thus, more collagen is required to elicit the same amount or strength of aggregation in the baboon platelets as in the human platelets.

Figure 19 shows the predicted secondary structures (Jones, 1999), as well as predicted disorders (Ward *et al.*, 2004) of the baboon and human GPVI proteins. The secondary structure prediction shows six minor changes within the baboon protein (indicated with arrows). However, a major change in the secondary structure takes place between position 260 and 320, due to the six amino acid changes between the baboon and human (indicated with a red frame). These changes can have a noteworthy role in the ability of the baboon GPVI to signal for aggregation to take place, and can further explain the lack of collagen-induced platelet aggregation when using standard human collagen concentrations.

According to the crystal structure of GPVI (figure 20) as determined by Horii *et al.* (2006) Ser61 and Arg46 form part of the periphery of the putative collagen binding groove within GPVI, and the Arg60 is directly adjacent to collagen-related peptide (CRP) when bound to GPVI. The Arg46Ser, Arg60Lys and Ser61Arg mutations were found within these regions. These mutations may contribute to the fact that collagen does not bind as effectively to baboon platelets as to human platelets. The radical amino acid changes found in this region is indicated in the crystal structure in figure 20.

The baboon sequence was subsequently compared with the mouse, chimpanzee, cynomolgus monkey, and olive baboon sequences. The mouse and baboon sequences have a 62.9% similarity, and the chimpanzee sequence shows an 85% similarity. Comparison with the cynomolgus monkey sequence (*Macaca fascicularis*; Accession number: XP_005590417) was very interesting. Even though the cynomolgus monkey GPVI has 18 amino acids less than the baboon, it showed a 91.1% similarity. The cynomolgus monkey has the same amino acids as the human at Arg46 and Arg60, but contains a Ser61His mutation where the baboon has a Ser61Arg mutation at the same place. The cynomolgus monkey also has the Leu78Pro, Val181Met, Phe214Leu, Tyr244His, Ile262Leu, Gly282Val, Asn302His, Gly304Asp and Gln310Pro mutations, as well as the deletions of Leu295, Pro296 and Leu297, as seen in the baboon. The cynomolgus monkey has been successfully used to study human-targeted anti-GPVI antibodies in the past (Matsumoto *et al.*, 2006; Ohlmann *et al.*, 2008; Takayama *et al.*, 2008). Therefore, it is hypothesized that the Arg46Ser and Arg60Lys mutations are the major changes responsible for the functional differences seen between human and baboon GPVI studies.

The olive baboons' GPVI (*Papio anubis*; Accession number: XP_009193594) has 29 less amino acids than the *P. ursinus* baboon, and is 89.9% similar in sequence. Five amino acid changes were detected between the species, other than the amino acids missing (amino acids Gln1 to Gly3, and Gly183 to Ala201). Out of the five, only one change Thr204Pro, were at the same position as a change found between *P. ursinus* and the human, Ser204Thr.

P. anubis also had the changes Arg46Ser, Arg60Lys, Ser61Arg, Leu78Pro, Ser204Thr, Phe214Leu, Tyr244His, Ile262Leu, Gly282Val, Asn302His, Gly304Asp and Gln310Pro, as well as the deletions of Leu295, Pro296 and Leu297, as seen in the *P. ursinus* baboon. It would be interesting to evaluate anti-GPVI agents in the olive baboon, to confirm if it is in deed the Arg46Ser and Arg60Lys mutations causing the discrepancy in GPVI functional assessments in *P. ursinus*.

The *P. ursinus* baboon GPVI does contain the metalloproteinase ADAM10 cleavage site at Arg242/Gln243 (Gardiner *et al.*, 2007). Therefore, care should be taken to prevent shedding of GPVI from the platelet surface, whenever baboon GPVI is studied in future.

When taking all the GPVI results into consideration, the *P. ursinus* baboon is not considered to be a suitable model to evaluate human targeted anti-GPVI agents, therefore, more suited animals, with a better-matched genetic make-up should be considered for future anti-GPVI studies.

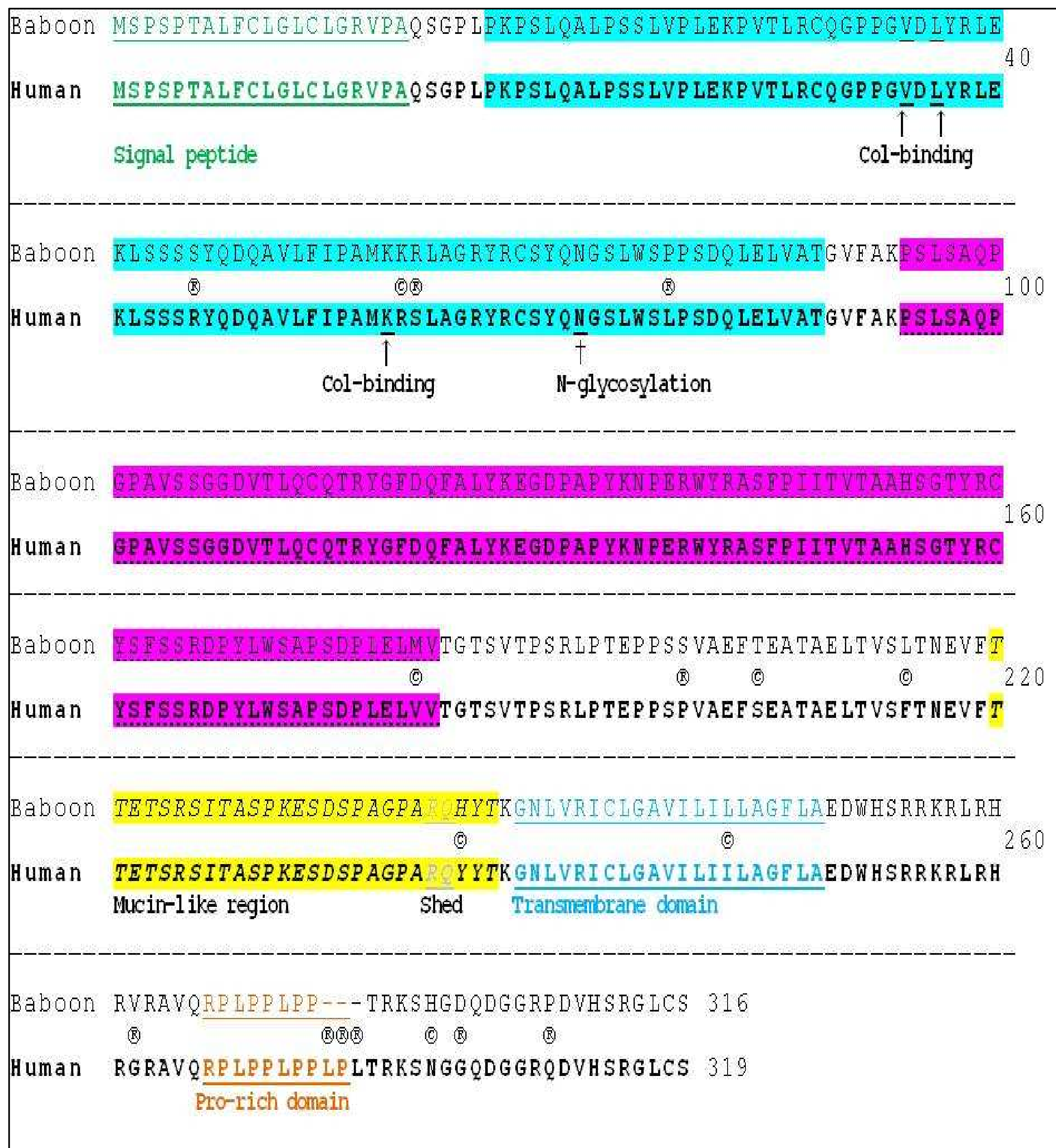


Figure 18. Comparison of the baboon and human GPVI amino acid sequences. A conservative amino acid change is indicated with ©. A radical amino acid change is indicated with ®. The signal peptide (green lettering), sheddase cleaving site (grey lettering), transmembrane domain (blue lettering) and proline-rich domain (orange lettering) is underlined. The ligand-binding sites are indicated with arrows. The N-glycosylation site is indicated with a dagger (†). The D1 immunoglobulin-like domain is highlighted with turquoise, and the D2 immunoglobulin-like domain is highlighted with purple and underlined with a dotted line. The mucin-like domain is in italics and highlighted with yellow.

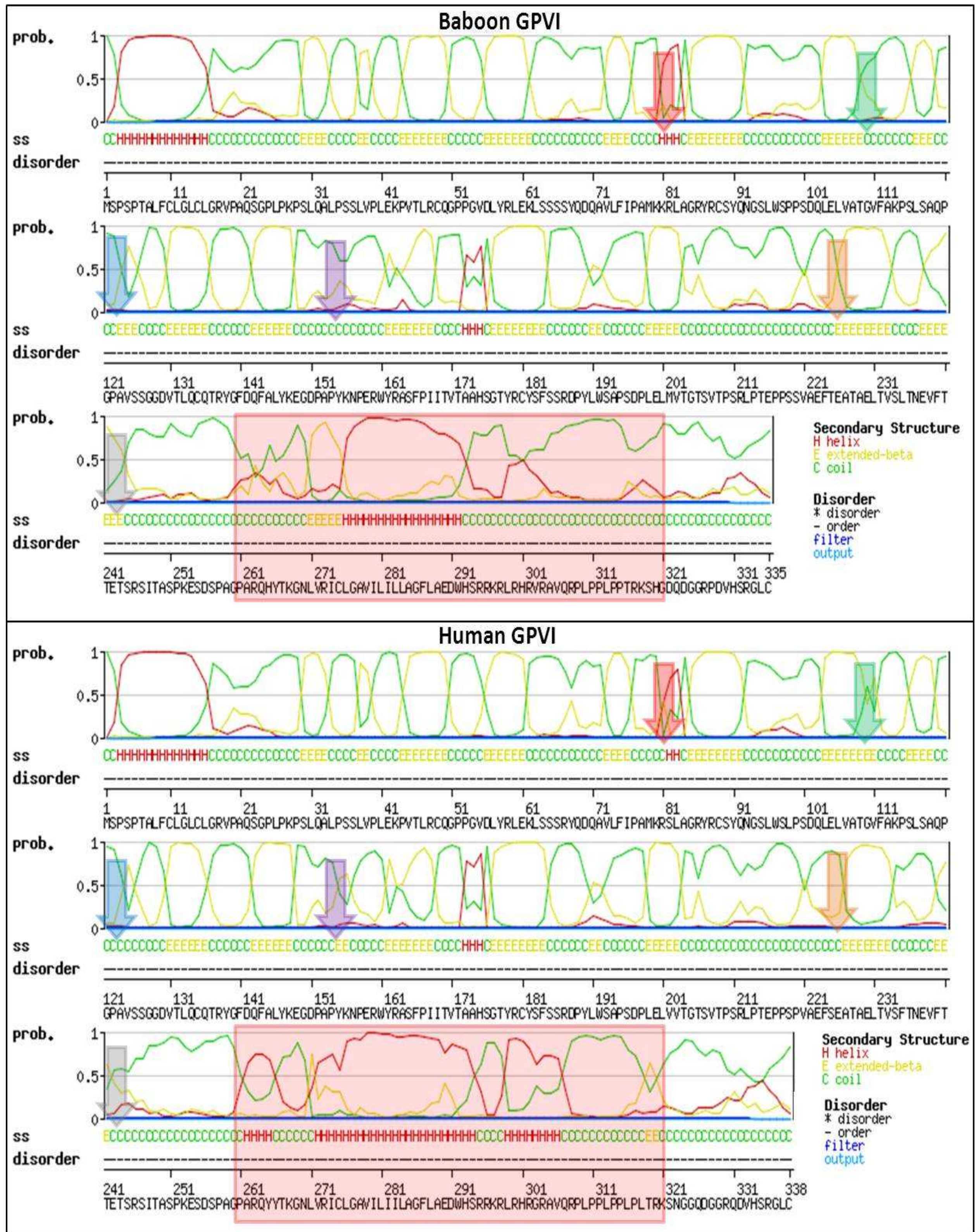


Figure 19. Prediction of the secondary protein structure of the baboon and human GPVI proteins. The arrows indicate all the differences between the baboon and human proteins. The red frame indicates the major differences between the baboon and human cytoplasmic regions.

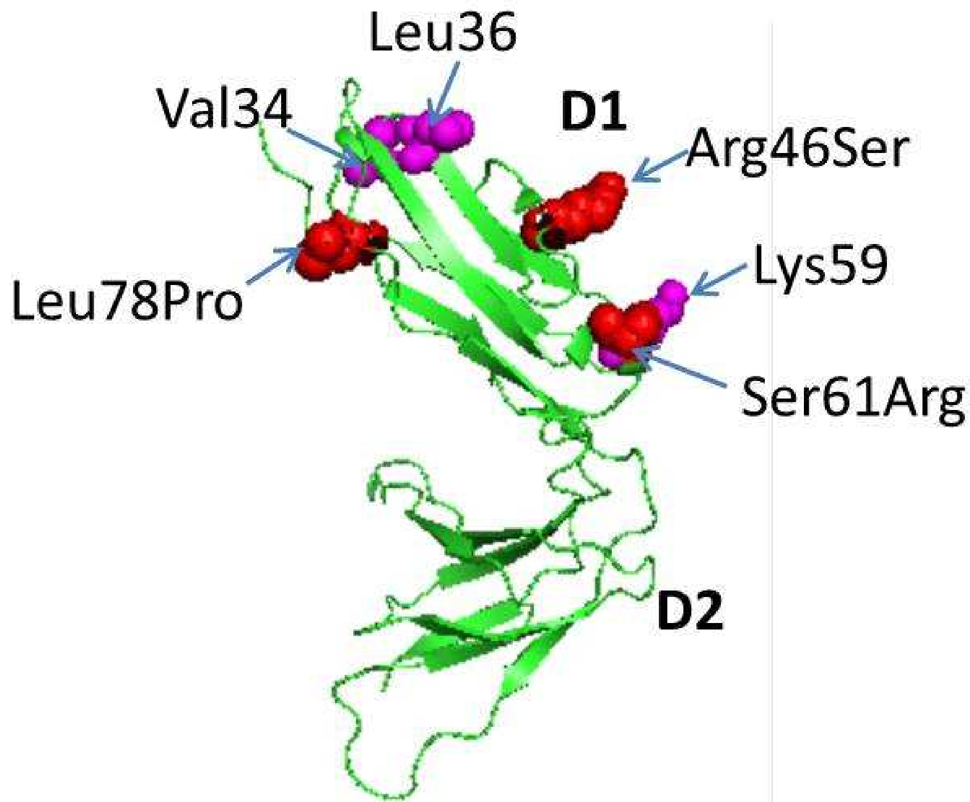


Figure 20. Crystal structure of the GPVI collagen binding region as determined by Horii *et al*. (2006), depicting amino acid changes found in the *P. ursinus* sequence (shown in red dots). The collagen binding amino acids are also indicated (shown in magenta dots). Cartoon created with PyMol. Crystal structure available from RCSB Protein Data Bank (accession # 2GI7). Unfortunately, the crystal structure did not contain the full GPVI sequence, thus, only some of the amino acid changes could be indicated.

GPIIb sequence:

Because of the size of the gene, GPIIb is divided into the individual exons for illustration purposes. Table 9 contains the detected SNPs in the baboon GPIIb sequence. Figures 21 to 49 shows a comparison of the protein translating region nucleotide sequence (exon 1 to 30) between the GPIIb gene of the baboon and human reference sequence as found on www.ensembl.com (ITGA2B-001, transcript ID: ENST00000262407). Unfortunately, after numerous attempts, exon 28 could not be sequenced. Three different primer pairs (Appendix F: 28SIIBF and 28ASIIBR; GPIIb Ex28-29F and GPIIb Ex28-29R; GPIIb Ex28F and GPIIb Ex28R) were used at different cycling conditions, using different concentrations of sample, primer, MgCl and DMSO. The commercial company also failed to sequence the exon. Unfortunately, due to lack of local expertise and budgetary constraints, other techniques to sequence this exon were not explored as part of this study. This finding could indicate major sequence differences between the human and baboon GPIIb sequences in the area flanking exon 28. It was decided to leave exon 28 out of further discussion as this exon does not contain any amino acid essential for ligand binding or signalling.

In figures 21 to 49 an SNP that does not cause a change in amino acid is indicated in green, and one that does in red. A total of 62 SNPs was detected in the baboon GPIIb sequence when compared with the human GPIIb reference sequence. Of the 62, 42 SNPs were silent mutations, which did not cause an amino acid change. There were 20 SNPs that caused changes in 18 amino acids. The nucleotide sequences are 97.9% similar between baboon and human GPIIb for all the exons sequenced.

Table 9. SNPs detected in baboon GPIIb sequence.

Exon	SNP	Position	Amino Acid Change
1	G to T	114	Yes
	G to C	147	Yes
	A to T	187	No

2	G to A	25	No
	C to G	31	No
	C to T	37	No
	C to T	91	No
	G to A	109	No
3	G to A	62	No
4	T to C	42	No
	T to C	69	No
	C to T	135	No
	A to C	157	Yes
	G to A	160	Yes
6	A to G	6	No
	C to A	30	No
7	G to A	21	Yes
	C to T	84	Yes
	T to C	92	No
9	A to G	2	No
	C to G	17	No
	C to G	35	No
10	A to G	14	Yes
	T to C	15	Yes
	A to T	23	Yes
	T to C	35	No
	G to A	42	No
	G to A	44	Yes
	C to T	45	Yes
11	G to T	6	No
12	C to T	10	No
	G to A	110	Yes
	G to A	198	Yes
13	G to T	83	Yes
	C to T	155	No

14	C to T	8	No
15	G to A	40	No
	T to C	82	No
20	G to A	4	No
	G to A	56	Yes
	G to A	76	No
	C to G	115	No
	T to C	142	No
	C to T	145	No
23	C to T	43	No
	G to A	49	No
24	A to T	31	No
	G to C	46	Yes
25	T to C	54	No
	A to G	60	No
	C to A	71	Yes
	C to T	123	No
	T to C	147	No
26	T to C	51	No
	C to A	94	Yes
	G to C	102	No
27	C to T	87	No
	T to C	109	Yes
29	T to C	4	Yes
	C to T	18	No
	A to T	45	No
	T to C	75	No

```
Baboon GPIIb Ex1 188 bp                                  188 nt vs.
Human GPIIb Ex1 188 bp                                   188 nt
 using matrix file: DNA, gap open/ext: -14/-4
  97.9% identity in 188 nt overlap;           Global score: 904

                10        20        30        40        50        60
Baboon ATGGCCAGAGCTTTGTGTCCACTGCAAGCCCTCTGGCTTCTGGAGTGGGTGCTGCTGCTC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Human  ATGGCCAGAGCTTTGTGTCCACTGCAAGCCCTCTGGCTTCTGGAGTGGGTGCTGCTGCTC
                10        20        30        40        50        60

                70        80        90       100       110       120
Baboon TTGGGACCTTGTGCTGCCCCTCCAGCCTGGGCCTTGAACCTGGACCCAGTGCATCTCACC
       :::::::::::::::::::::::::::::::::::::::::::::::::::: ::::::
Human  TTGGGACCTTGTGCTGCCCCTCCAGCCTGGGCCTTGAACCTGGACCCAGTGCAGCTCACC
                70        80        90       100       110       120

               130       140       150       160       170       180
Baboon TTCTATGCAGGCCCCAATGGCAGCCACTTTGGGTTTTCACTGGACTTCCACAAGGACAGC
       :::::::::::::::::::::::::: ::::: ::::::::::::::::::::::::::
Human  TTCTATGCAGGCCCCAATGGCAGCCAGTTTGGATTTTCACTGGACTTCCACAAGGACAGC
               130       140       150       160       170       180


Baboon CATGGGTG
       :::::: :
Human  CATGGGAG
```

Figure 21. Comparison of the protein translating region nucleotide sequence between the GPIIb exon 1 of the baboon and human reference sequence. SNPs that cause amino acid changes are shown in red and silent mutations in green.

```
Baboon GPIIb Ex 2 122 bp                                122 nt vs.
Human GPIIb Ex 2 122 bp                                 122 nt
 using matrix file: DNA, gap open/ext: -14/-4
  95.9% identity in 122 nt overlap;           Global score: 565

                10        20        30        40        50        60
Baboon AGTGGCCATCGTGGTGGGCGCCCCACGGACGCTGGGTCCCAGCCAGGAGGAGACGGGCGG
       :::::::::::::::::::::::: ::::: ::::: :::::::::::::::::::::::
Human  AGTGGCCATCGTGGTGGGCGCCCCGCGGACCCTGGGCCCCAGCCAGGAGGAGACGGGCGG
                10        20        30        40        50        60

                70        80        90       100       110       120
Baboon CGTGTTCCTGTGCCCCTGGAGGGCCGAGGGTGGCCAGTGCCCCTCGCTACTCTTTGACCT
       :::::::::::::::::::::::::::::: ::::::::::::::::: :::::::::::
Human  CGTGTTCCTGTGCCCCTGGAGGGCCGAGGGCGGCCAGTGCCCCTCGCTGCTCTTTGACCT
                70        80        90       100       110       120


Baboon CC
       ::
Human  CC
```

Figure 22. Comparison of the protein translating region nucleotide sequence between the GPIIb exon 2 of the baboon and human reference sequence. SNPs that are silent mutations are indicated in green.

```
Baboon GPIIb Ex 3 98 bp                                  98 nt vs.
Human GPIIb Ex 3 98 bp                                   98 nt
 using matrix file: DNA, gap open/ext: -14/-4
  99.0% identity in 98 nt overlap;            Global score: 481

                10        20        30        40        50        60
Baboon GTGATGAGACCCGAAATGTAGGCTCCCAAACTTTACAAACCTTCAAGGCCCGCCAAGGAC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Human  GTGATGAGACCCGAAATGTAGGCTCCCAAACTTTACAAACCTTCAAGGCCCGCCAAGGAC
                10        20        30        40        50        60

                70        80        90
Baboon TAGGGGCGTCGGTCGTCAGCTGGAGCGACGTCATTGTG
       : ::::::::::::::::::::::::::::::::::::
Human  TGGGGGCGTCGGTCGTCAGCTGGAGCGACGTCATTGTG
                70        80        90
```

Figure 23. Comparison of the protein translating region nucleotide sequence between the GPIIb exon 3 of the baboon and human reference sequence. SNPs that are silent mutations are indicated in green.

```
Baboon GPIIb Ex4 166 bp                                166 nt vs.
Human GPIIb Ex4 166 bp                                 166 nt
 using matrix file: DNA, gap open/ext: -14/-4
  97.0% identity in 166 nt overlap;               Global score: 785

               10        20        30        40        50        60
Baboon GCCTGCGCCCCCTGGCAGCACTGGAACGTCCTAGAAAAGACCGAGGAGGCTGAGAAGACG
       ::::::::::::::::::::::::::::::::::::::::: ::::::::::::::::::
Human  GCCTGCGCCCCCTGGCAGCACTGGAACGTCCTAGAAAAGACTGAGGAGGCTGAGAAGACG
               10        20        30        40        50        60

               70        80        90       100       110       120
Baboon CCCGTAGGCAGCTGCTTTTTGGCTCAGCCAGAGAGCGGCCGCCGCGCCGAGTACTCCCCC
       :::::::: :::::::::::::::::::::::::::::::::::::::::::::::::::
Human  CCCGTAGGTAGCTGCTTTTTGGCTCAGCCAGAGAGCGGCCGCCGCGCCGAGTACTCCCCC
               70        80        90       100       110       120

              130       140       150       160
Baboon TGTCGCGGGAACACTCTGAGCCGCATTTACGTGGAACATAATTTTA
       :::::::::::::: :::::::::::::::::::::: :: ::::::
Human  TGTCGCGGGAACACCCTGAGCCGCATTTACGTGGAAAATGATTTTA
              130       140       150       160
```

Figure 24. Comparison of the protein translating region nucleotide sequence between the GPIIb exon 4 of the baboon and human reference sequence. SNPs that cause amino acid changes are indicated in red and silent mutations are indicated in green.

```
Baboon GPIIb Ex5 50 bp                                  50 nt vs.
Human GPIIb Ex5 50 bp                                   50 nt
 using matrix file: DNA, gap open/ext: -14/-4
 100.0% identity in 50 nt overlap;                Global score: 250

               10        20        30        40        50
Baboon GCTGGGACAAGCGTTACTGTGAAGCGGGCTTCAGCTCCGTGGTCACTCAG
       ::::::::::::::::::::::::::::::::::::::::::::::::::
Human  GCTGGGACAAGCGTTACTGTGAAGCGGGCTTCAGCTCCGTGGTCACTCAG
               10        20        30        40        50
```

Figure 25. Comparison of the protein translating region nucleotide sequence between the GPIIb exon 5 of the baboon and human reference sequence.

```
Baboon GPIIb Ex6 46 bp                                     46 nt vs.
Human GPIIb Ex6 46 bp                                      46 nt
 using matrix file: DNA, gap open/ext: -14/-4
  95.7% identity in 46 nt overlap;                   Global score: 212

               10        20        30        40
Baboon GCCGGGGAGCTGGTGCTTGGGGCTCCTGGAGGCTATTATTTCTTAG
       ::::: ::::::::::::::::::::::: :::::::::::::::
Human  GCCGGAGAGCTGGTGCTTGGGGCTCCTGGCGGCTATTATTTCTTAG
               10        20        30        40
```

Figure 26. Comparison of the protein translating region nucleotide sequence between the GPIIb exon 6 of the baboon and human reference sequence. SNPs that are silent mutations are indicated in green.

```
Baboon GPIIb Ex7 129 bp                                   129 nt vs.
Human GPIIb Ex7 129 bp                                    129 nt
 using matrix file: DNA, gap open/ext: -14/-4
  97.7% identity in 129 nt overlap;                  Global score: 618

               10        20        30        40        50        60
Baboon GTCTCCTGGCCCAGGCTCCAATTGCGGATATTTTCTCGAGTTACCGCCCAGGCATCCTTT
       :::::::::::::::::::: :::::::::::::::::::::::::::::::::::::::
Human  GTCTCCTGGCCCAGGCTCCAGTTGCGGATATTTTCTCGAGTTACCGCCCAGGCATCCTTT
               10        20        30        40        50        60

               70        80        90       100       110       120
Baboon TGTGGCACGTGTCCTCCCAGAGCTTCTCCTTCGACTCCAGCAACCCAGAGTACTTCGACG
       ::::::::::::::::::::::: ::::::: ::::::::::::::::::::::::::::
Human  TGTGGCACGTGTCCTCCCAGAGCCTCTCCTTTGACTCCAGCAACCCAGAGTACTTCGACG
               70        80        90       100       110       120


Baboon GCTACTGGG
       :::::::::
Human  GCTACTGGG
```

Figure 27. Comparison of the protein translating region nucleotide sequence between the GPIIb exon 7 of the baboon and human reference sequence. SNPs that cause amino acid changes are indicated in red and silent mutations in green.

```
Baboon GPIIb Ex8 48 bp                                    48 nt vs.
Human GPIIb Ex8 48 bp                                     48 nt
 using matrix file: DNA, gap open/ext: -14/-4
 100.0% identity in 48 nt overlap;                Global score: 240

               10        20        30        40
Baboon GGTACTCGGTGGCCGTGGGCGAGTTCGACGGGGATCTCAACACTACAG
       ::::::::::::::::::::::::::::::::::::::::::::::::
Human  GGTACTCGGTGGCCGTGGGCGAGTTCGACGGGGATCTCAACACTACAG
               10        20        30        40
```

Figure 28. Comparison of the protein translating region nucleotide sequence between the GPIIb exon 8 of the baboon and human reference sequence.

```
Baboon GPIIb Ex9 44 bp                                    44 nt vs.
Human GPIIb Ex9 44 bp                                     44 nt
 using matrix file: DNA, gap open/ext: -14/-4
  93.2% identity in 44 nt overlap;                Global score: 193

               10        20        30        40
Baboon AGTATGTCGTCGGTGCGCCCACTTGGAGCTGGACGCTGGGAGCG
       : :::::::::::::: ::::::::::::::::: :::::::::
Human  AATATGTCGTCGGTGCCCCCACTTGGAGCTGGACCCTGGGAGCG
               10        20        30        40
```

Figure 29. Comparison of the protein translating region nucleotide sequence between the GPIIb exon 9 of the baboon and human reference sequence. SNPs that are silent mutations are indicated in green.

```
Baboon GPIIb Ex10 54 bp                                   54 nt vs.
Human GPIIb Ex10 54 bp                                    54 nt
 using matrix file: DNA, gap open/ext: -14/-4
  87.0% identity in 54 nt overlap;                Global score: 207

               10        20        30        40        50
Baboon GTGGAAATTTTGGGCTCCTACTTTCCAGAGGCTGCACCGGCTACATGGAGAGCAG
       :::::::::::::  ::::::: :::::::::::: ::::: :  :::::::::
Human  GTGGAAATTTTGGATTCCTACTACCAGAGGCTGCATCGGCTGCGCGGAGAGCAG
               10        20        30        40        50
```

Figure 30. Comparison of the protein translating region nucleotide sequence between the GPIIb exon 10 of the baboon and human reference sequence. SNPs that cause amino acid changes are indicated in red and silent mutations in green.

```
Baboon GPIIb Ex11 53 bp                                 53 nt vs.
Human GPIIb Ex11 53 bp                                  53 nt
 using matrix file: DNA, gap open/ext: -14/-4
  98.1% identity in 53 nt overlap;            Global score: 256

               10        20        30        40        50
Baboon ATGGCTTCGTATTTTGGGCATTCAGTGGCTGTCACTGACGTCAACGGGGATGG
       ::::: :::::::::::::::::::::::::::::::::::::::::::::::
Human  ATGGCGTCGTATTTTGGGCATTCAGTGGCTGTCACTGACGTCAACGGGGATGG
               10        20        30        40        50
```

Figure 31. Comparison of the protein translating region nucleotide sequence between the GPIIb exon 11 of the baboon and human reference sequence. SNPs that are silent mutations are indicated in green.

```
Baboon GPIIb Ex12 212 bp                              - 212 nt
Human GPIIb Ex12 212 bp                               - 212 nt
 using matrix file: DNA (5/-4), gap-open/ext: -14/-4 E(limit)   0.05
  98.6% identity in 212 nt overlap (1-212:1-212); score: 1033 E(10000):
4.2e-79

               10        20        30        40        50        60
Baboon GAGGCATGACCTGCTGGTGGGCGCTCCACTGTATATGGAGAGCCGGGCAGACCGAAAACT
       ::::::::: ::::::::::::::::::::::::::::::::::::::::::::::::::
Human  GAGGCATGATCTGCTGGTGGGCGCTCCACTGTATATGGAGAGCCGGGCAGACCGAAAACT
               10        20        30        40        50        60

               70        80        90       100       110       120
Baboon GGCCGAAGTGGGGCGTGTGTATTTGTTCCTGCAGCCGCGAGGCCCCCACACGCTGGGTGC
       ::::::::::::::::::::::::::::::::::::::::::::::::: ::::::::::
Human  GGCCGAAGTGGGGCGTGTGTATTTGTTCCTGCAGCCGCGAGGCCCCCACGCGCTGGGTGC
               70        80        90       100       110       120

              130       140       150       160       170       180
Baboon CCCCAGCCTCCTGCTGACTGGCACACAGCTCTATGGGCGATTCGGCTCTGCCATCGCACC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Human  CCCCAGCCTCCTGCTGACTGGCACACAGCTCTATGGGCGATTCGGCTCTGCCATCGCACC
              130       140       150       160       170       180

              190       200       210
Baboon CCTGGGCGACCTCGACCAGGATGGCTACAATG
       ::::::::::::::::: ::::::::::::::
Human  CCTGGGCGACCTCGACCGGGATGGCTACAATG
              190       200       210
```

Figure 32. Comparison of the protein translating region nucleotide sequence between the GPIIb exon 12 of the baboon and human reference sequence. SNPs that cause amino acid changes are indicated in red and silent mutations in green.

```
Baboon GPIIb Ex13 183 bp                                   183 nt vs.
Human GPIIb Ex13 183 bp                                    183 nt
 using matrix file: DNA, gap open/ext: -14/-4
  98.9% identity in 183 nt overlap;                   Global score: 897

                10         20         30         40         50         60
Baboon ACATTGCAGTGGCTGCCCCCTACGGGGGTCCCAGTGGCCGGGGCCAAGTGCTGGTGTTCC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Human  ACATTGCAGTGGCTGCCCCCTACGGGGGTCCCAGTGGCCGGGGCCAAGTGCTGGTGTTCC
                10         20         30         40         50         60

                70         80         90        100        110        120
Baboon TGGGTCAGAGTGAGGGGCTGAGTTCACGTCCCTCCCAGGTCCTGGACAGCCCCTTCCCCA
       :::::::::::::::::::::: :::::::::::::::::::::::::::::::::::::
Human  TGGGTCAGAGTGAGGGGCTGAGGTCACGTCCCTCCCAGGTCCTGGACAGCCCCTTCCCCA
                70         80         90        100        110        120

               130        140        150        160        170        180
Baboon CAGGCTCTGCCTTTGGCTTCTCCCTTCGAGGTGCTGTAGACATCGATGACAACGGATACC
       :::::::::::::::::::::::::::::::::: :::::::::::::::::::::::::
Human  CAGGCTCTGCCTTTGGCTTCTCCCTTCGAGGTGCCGTAGACATCGATGACAACGGATACC
               130        140        150        160        170        180


Baboon CAG
       :::
Human  CAG
```

Figure 33. Comparison of the protein translating region nucleotide sequence between the GPIIb exon 13 of the baboon and human reference sequence. SNPs that cause amino acid changes are indicated in red and silent mutations in green.

```
Baboon GPIIb Ex 14 46 bp                                    46 nt vs.
Human GPIIb Ex 14 46 bp                                     46 nt
 using matrix file: DNA, gap open/ext: -14/-4
  97.8% identity in 46 nt overlap;                    Global score: 221

                10         20         30         40
Baboon ACCTGATTGTGGGAGCTTACGGGGCCAACCAGGTGGCTGTGTACAG
       ::::::: ::::::::::::::::::::::::::::::::::::::
Human  ACCTGATCGTGGGAGCTTACGGGGCCAACCAGGTGGCTGTGTACAG
                10         20         30         40
```

Figure 34. Comparison of the protein translating region nucleotide sequence between the GPIIb exon 14 of the baboon and human reference sequence. The SNP that is a silent mutation is indicated in green.

```
Baboon GPIIb Ex 15 105 bp                                   105 nt vs.
Human GPIIb Ex 15 105 bp                                    105 nt
 using matrix file: DNA, gap open/ext: -14/-4
  98.1% identity in 105 nt overlap;           Global score: 507

               10        20        30        40        50        60
Baboon AGCTCAGCCAGTGGTGAAGGCCTCTGTCCAGCTACTGGTACAAGATTCACTGAATCCTGC
       :::::::::::::::::::::::::::::::::::::::: ::::::::::::::::::::
Human  AGCTCAGCCAGTGGTGAAGGCCTCTGTCCAGCTACTGGTGCAAGATTCACTGAATCCTGC
               10        20        30        40        50        60

               70        80        90       100
Baboon TGTGAAGAGCTGTGTCCTACCCCAGACCAAGACACCCGTGAGCTG
       ::::::::::::::::::::: :::::::::::::::::::::::
Human  TGTGAAGAGCTGTGTCCTACCTCAGACCAAGACACCCGTGAGCTG
               70        80        90       100
```

Figure 35. Comparison of the protein translating region nucleotide sequence between the GPIIb exon 15 of the baboon and human reference sequence. The SNPs that are silent mutations are indicated in green.

```
Baboon GPIIb Ex16 56 bp                                     56 nt vs.
Human GPIIb Ex16 56 bp                                      56 nt
 using matrix file: DNA, gap open/ext: -14/-4
 100.0% identity in 56 nt overlap;            Global score: 280

               10        20        30        40        50
Baboon CTTCAACATCCAGATGTGTGTTGGAGCCACTGGGCACAACATTCCTCAGAAGCTAT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Human  CTTCAACATCCAGATGTGTGTTGGAGCCACTGGGCACAACATTCCTCAGAAGCTAT
               10        20        30        40        50
```

Figure 36. Comparison of the protein translating region nucleotide sequence between the GPIIb exon 16 of the baboon and human reference sequence.

```
Baboon GPIIb Ex17 152 bp                                  152 nt vs.
Human GPIIb Ex17 152 bp                                   152 nt
 using matrix file: DNA, gap open/ext: -14/-4
 100.0% identity in 152 nt overlap;             Global score: 760

                10        20        30        40        50        60
Baboon CCCTAAATGCCGAGCTGCAGCTGGACCGGCAGAAGCCCCGCCAGGGCCGGCGGGTGCTGC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Human  CCCTAAATGCCGAGCTGCAGCTGGACCGGCAGAAGCCCCGCCAGGGCCGGCGGGTGCTGC
                10        20        30        40        50        60

                70        80        90       100       110       120
Baboon TGCTGGGCTCTCAACAGGCAGGCACCACCCTGAACCTGGATCTGGGCGGAAAGCACAGCC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Human  TGCTGGGCTCTCAACAGGCAGGCACCACCCTGAACCTGGATCTGGGCGGAAAGCACAGCC
                70        80        90       100       110       120

               130       140       150
Baboon CCATCTGCCACACCACCATGGCCTTCCTTCGA
       ::::::::::::::::::::::::::::::::
Human  CCATCTGCCACACCACCATGGCCTTCCTTCGA
               130       140       150
```

Figure 37. Comparison of the protein translating region nucleotide sequence between the GPIIb exon 17 of the baboon and human reference sequence.

```
Baboon GPIIb Ex18 126 bp                                  126 nt vs.
Human GPIIb Ex18 126 bp                                   126 nt
 using matrix file: DNA, gap open/ext: -14/-4
 100.0% identity in 126 nt overlap;             Global score: 630

                10        20        30        40        50        60
Baboon GATGAGGCAGACTTCCGGGACAAGCTGAGCCCCATTGTGCTCAGCCTCAATGTGTCCCTA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Human  GATGAGGCAGACTTCCGGGACAAGCTGAGCCCCATTGTGCTCAGCCTCAATGTGTCCCTA
                10        20        30        40        50        60

                70        80        90       100       110       120
Baboon CCGCCCACGGAGGCTGGAATGGCCCCTGCTGTCGTGCTGCATGGAGACACCCATGTGCAG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Human  CCGCCCACGGAGGCTGGAATGGCCCCTGCTGTCGTGCTGCATGGAGACACCCATGTGCAG
                70        80        90       100       110       120


Baboon GAGCAG
       ::::::
Human  GAGCAG
```

Figure 38. Comparison of the protein translating region nucleotide sequence between the GPIIb exon 18 of the baboon and human reference sequence.

```
Baboon GPIIb Ex 19 68 bp                          68 nt vs.
Human GPIIb Ex 19 68 bp                           68 nt
 using matrix file: DNA, gap open/ext: -14/-4
 100.0% identity in 68 nt overlap;          Global score: 340

               10        20        30        40        50        60
Baboon ACACGAATCGTCCTGGACTGTGGGGAAGATGACGTATGTGTGCCCCAGCTTCAGCTCACT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Human  ACACGAATCGTCCTGGACTGTGGGGAAGATGACGTATGTGTGCCCCAGCTTCAGCTCACT
               10        20        30        40        50        60


Baboon GCCAGCGT
       ::::::::
Human  GCCAGCGT
```

Figure 39. Comparison of the protein translating region nucleotide sequence between the GPIIb exon 19 of the baboon and human reference sequence.

```
Baboon GPIIb Ex20 148 bp                          148 nt vs.
Human GPIIb Ex20 148 bp                           148 nt
 using matrix file: DNA, gap open/ext: -14/-4
  95.9% identity in 148 nt overlap;          Global score: 686

               10        20        30        40        50        60
Baboon GACAGGCTCCCCGCTCCTAGTTGGGGCAGATAATGTCCTGGAGCTGCAGATGGACACAGC
       ::: ::::::::::::::::::::::::::::::::::::::::::::::::::: ::::
Human  GACGGGCTCCCCGCTCCTAGTTGGGGCAGATAATGTCCTGGAGCTGCAGATGGACGCAGC
               10        20        30        40        50        60

               70        80        90       100       110       120
Baboon CAACGAGGGCGAGGGAGCCTATGAAGCAGAGCTGGCCGTGCACCTGCCCCAGGGGGCCCA
       ::::::::::::::: :::::::::::::::::::::::::::::::::::::: :::::
Human  CAACGAGGGCGAGGGGGCCTATGAAGCAGAGCTGGCCGTGCACCTGCCCCAGGGCGCCCA
               70        80        90       100       110       120

              130       140
Baboon CTACATGCGGGCCCTAAGCAACGTTGAG
       ::::::::::::::::::::: :: :::
Human  CTACATGCGGGCCCTAAGCAATGTCGAG
              130       140
```

Figure 40. Comparison of the protein translating region nucleotide sequence between the GPIIb exon 20 of the baboon and human reference sequence. SNPs that cause amino acid changes are shown in red and silent mutations in green.

```
Baboon GPIIb Ex21 93 bp                         93 nt vs.
Human GPIIb Ex21 93 bp                            93 nt
 using matrix file: DNA, gap open/ext: -14/-4
 100.0% identity in 93 nt overlap;           Global score: 465

               10        20        30        40        50        60
Baboon GGCTTTGAGAGACTCATCTGTAATCAGAAGAAGGAGAATGAGACCAGGGTGGTGCTGTGT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Human  GGCTTTGAGAGACTCATCTGTAATCAGAAGAAGGAGAATGAGACCAGGGTGGTGCTGTGT
               10        20        30        40        50        60

               70        80        90
Baboon GAGCTGGGCAACCCCATGAAGAAGAACGCCCAG
       :::::::::::::::::::::::::::::::::
Human  GAGCTGGGCAACCCCATGAAGAAGAACGCCCAG
               70        80        90
```

Figure 41. Comparison of the protein translating region nucleotide sequence between the GPIIb exon 21 of the baboon and human reference sequence.

```
Baboon GPIIb Ex22 80 bp                            80 nt vs.
Human GPIIb Ex22 80 bp                             80 nt
 using matrix file: DNA, gap open/ext: -14/-4
 100.0% identity in 80 nt overlap;           Global score: 400

               10        20        30        40        50        60
Baboon ATAGGAATCGCGATGTTGGTGAGCGTGGGGAATCTGGAAGAGGCTGGGGAGTCTGTGTCC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Human  ATAGGAATCGCGATGTTGGTGAGCGTGGGGAATCTGGAAGAGGCTGGGGAGTCTGTGTCC
               10        20        30        40        50        60

               70        80
Baboon TTCCAGCTGCAGATACGGAG
       ::::::::::::::::::::
Human  TTCCAGCTGCAGATACGGAG
               70        80
```

Figure 42. Comparison of the protein translating region nucleotide sequence between the GPIIb exon 22 of the baboon and human reference sequence.

```
Baboon GPIIb Ex23 81 bp                                  81 nt vs.
Human GPIIb Ex23 81 bp                                   81 nt
 using matrix file: DNA, gap open/ext: -14/-4
  97.5% identity in 81 nt overlap;            Global score: 387

               10        20        30        40        50        60
Baboon CAAGAACAGCCAGAATCCAAACAGCAAGATTGTGCTGCTGGATGTGCCAGTCCGGGCAGA
       :::::::::::::::::::::::::::::::::::::::::: ::::: :::::::::::
Human  CAAGAACAGCCAGAATCCAAACAGCAAGATTGTGCTGCTGGACGTGCCGGTCCGGGCAGA
               10        20        30        40        50        60

               70        80
Baboon GGCCCAAGTGGAGCTGCGAGG
       :::::::::::::::::::::
Human  GGCCCAAGTGGAGCTGCGAGG
               70        80
```

Figure 43. Comparison of the protein translating region nucleotide sequence between the GPIIb exon 23 of the baboon and human reference sequence. SNPs that are silent mutations are indicated in green.

```
Baboon GPIIb Ex24 100 bp                                100 nt vs.
Human GPIIb Ex24 100 bp                                 100 nt
 using matrix file: DNA, gap open/ext: -14/-4
  98.0% identity in 100 nt overlap;           Global score: 482

               10        20        30        40        50        60
Baboon GAACTCCTTTCCAGCCTCCCTGGTGGTGGCTGCAGAAGAAGGTGACAGGGAGCAGAACAG
       :::::::::::::::::::::::::::::: ::::::::::::::: ::::::::::::::
Human  GAACTCCTTTCCAGCCTCCCTGGTGGTGGCAGCAGAAGAAGGTGAGAGGGAGCAGAACAG
               10        20        30        40        50        60

               70        80        90       100
Baboon CTTGGACAGCTGGGGACCCAAAGTGGAGCACACCTATGAG
       ::::::::::::::::::::::::::::::::::::::::
Human  CTTGGACAGCTGGGGACCCAAAGTGGAGCACACCTATGAG
               70        80        90       100
```

Figure 44. Comparison of the protein translating region nucleotide sequence between the GPIIb exon 24 of the baboon and human reference sequence. SNPs that cause amino acid changes are indicated in red and silent mutations are indicated in green.

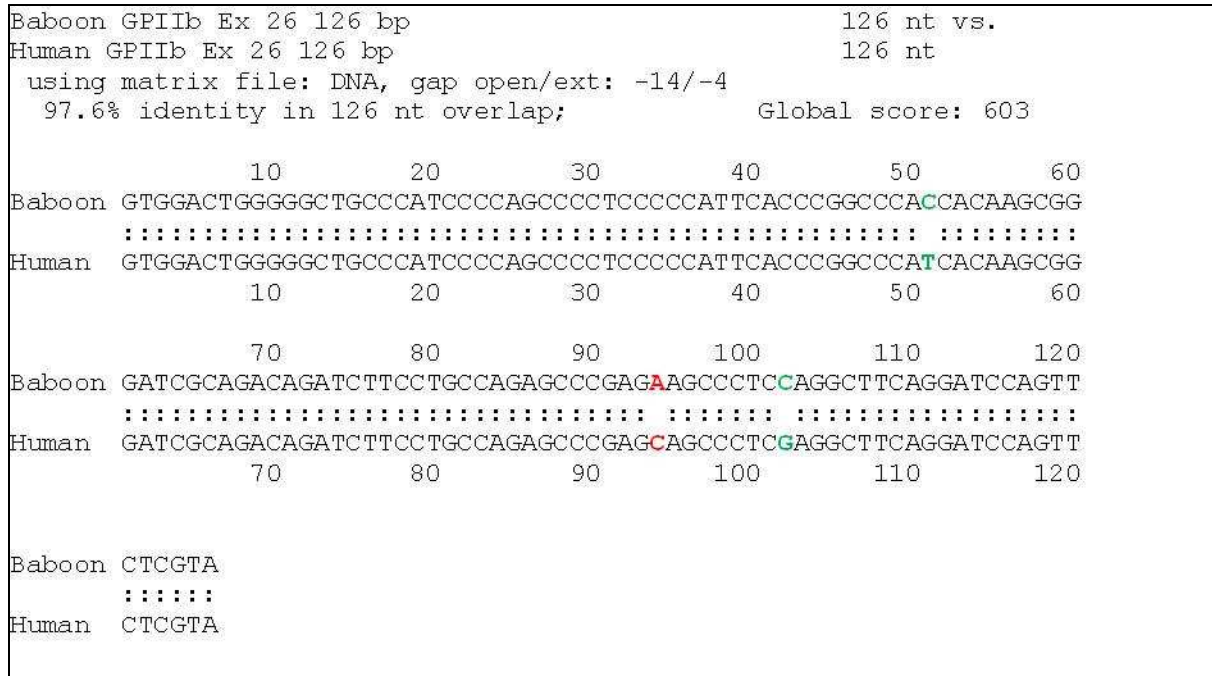
```
Baboon GPIIb Ex 25 153 bp                              153 nt vs.
Human GPIIb Ex 25 153 bp                               153 nt
 using matrix file: DNA, gap open/ext: -14/-4
  96.7% identity in 153 nt overlap;            Global score: 720

                10        20        30        40        50        60
Baboon CTCCACAACAATGGCCCTGGGACTGTGAATGGTCTTCACCTCAGCATCCACCTCCCGGGG
       :::::::::::::::::::::::::::::::::::::::::::::::::::::: :::::
Human  CTCCACAACAATGGCCCTGGGACTGTGAATGGTCTTCACCTCAGCATCCACCTTCCGGGA
                10        20        30        40        50        60

                70        80        90       100       110       120
Baboon CAGTCCCAGCACTCCGACCTGCTCTACATCCTGGATATACAGCCCCAGGGGGGCCTTCAG
       :::::::::: :::::::::::::::::::::::::::::::::::::::::::::::::
Human  CAGTCCCAGCCCTCCGACCTGCTCTACATCCTGGATATACAGCCCCAGGGGGGCCTTCAG
                70        80        90       100       110       120

               130       140       150
Baboon TGTTTCCCACAGCCTCCTGTCAACCCCCTCAAG
       :: :::::::::::::::::::::: ::::::
Human  TGCTTCCCACAGCCTCCTGTCAACCCTCTCAAG
               130       140       150
```

Figure 45. Comparison of the protein translating region nucleotide sequence between the GPIIb exon 25 of the baboon and human reference sequence. SNPs that cause amino acid changes are indicated in red and silent mutations in green.



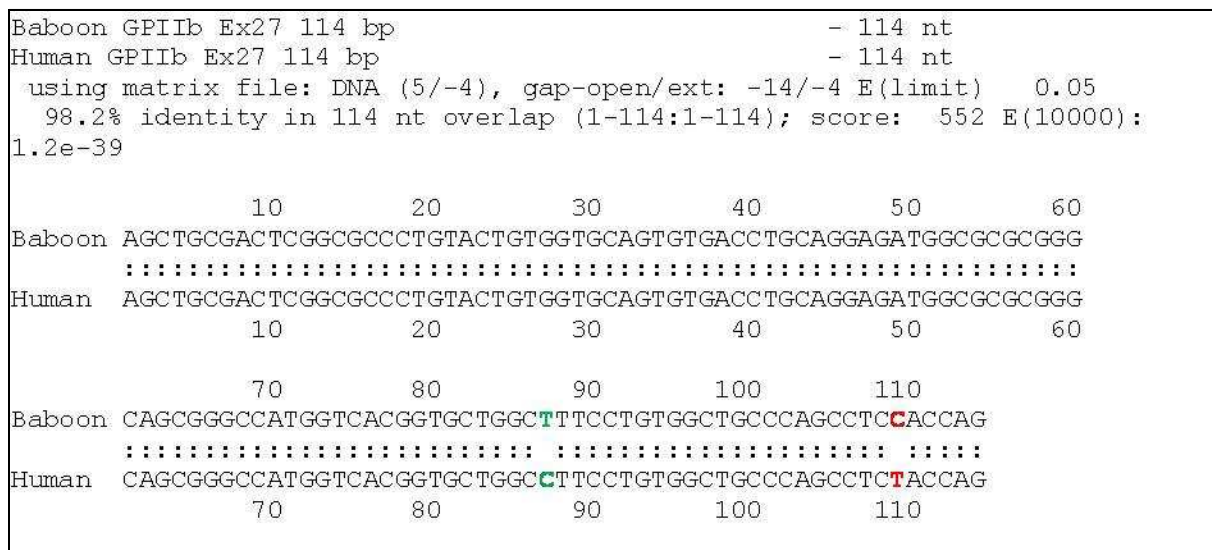
```
Baboon GPIIb Ex 26 126 bp                                126 nt vs.
Human GPIIb Ex 26 126 bp                                 126 nt
 using matrix file: DNA, gap open/ext: -14/-4
  97.6% identity in 126 nt overlap;          Global score: 603

               10        20        30        40        50        60
Baboon GTGGACTGGGGGCTGCCCATCCCCAGCCCCTCCCCCATTCACCCGGCCCACCCACAAGCGG
       :::::::::::::::::::::::::::::::::::::::::::::::::::: :::::::::
Human  GTGGACTGGGGGCTGCCCATCCCCAGCCCCTCCCCCATTCACCCGGCCCATCACAAGCGG
               10        20        30        40        50        60

               70        80        90       100       110       120
Baboon GATCGCAGACAGATCTTCCTGCCAGAGCCCGAGAAGCCCTCCAGGCTTCAGGATCCAGTT
       ::::::::::::::::::::::::::::::::: ::::::: :::::::::::::::::::
Human  GATCGCAGACAGATCTTCCTGCCAGAGCCCGAGCAGCCCTCGAGGCTTCAGGATCCAGTT
               70        80        90       100       110       120


Baboon CTCGTA
       ::::::
Human  CTCGTA
```

Figure 46. Comparison of the protein translating region nucleotide sequence between the GPIIb exon 26 of the baboon and human reference sequence. SNPs that cause amino acid changes are indicated in red and silent mutations in green.



```
Baboon GPIIb Ex27 114 bp                              - 114 nt
Human GPIIb Ex27 114 bp                               - 114 nt
 using matrix file: DNA (5/-4), gap-open/ext: -14/-4 E(limit)   0.05
  98.2% identity in 114 nt overlap (1-114:1-114); score:  552 E(10000):
1.2e-39

               10        20        30        40        50        60
Baboon AGCTGCGACTCGGCGCCCTGTACTGTGGTGCAGTGTGACCTGCAGGAGATGGCGCGCGGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Human  AGCTGCGACTCGGCGCCCTGTACTGTGGTGCAGTGTGACCTGCAGGAGATGGCGCGCGGG
               10        20        30        40        50        60

               70        80        90       100       110
Baboon CAGCGGGCCATGGTCACGGTGCTGGCTTTCCTGTGGCTGCCCAGCCTCCACCAG
       :::::::::::::::::::::::::: ::::::::::::::::::::: :::::
Human  CAGCGGGCCATGGTCACGGTGCTGGCCTTCCTGTGGCTGCCCAGCCTCTACCAG
               70        80        90       100       110
```

Figure 47. Comparison of the protein translating region nucleotide sequence between the GPIIb exon 27 of the baboon and human reference sequence. SNPs that cause amino acid changes are indicated in red and silent mutations in green.

```
Baboon GPIIb Ex29 117 bp                                117 nt vs.
Human GPIIb Ex29 117 bp                                 117 nt
 using matrix file: DNA, gap open/ext: -14/-4
  95.7% identity in 117 nt overlap;            Global score: 540

              10        20        30        40        50        60
Baboon GTGCGGACACAGCTGCTTCGAGCCTTGGAGGAGAGGGCCATTCCTATCTGGTGGGTGCTG
       ::: ::::::::::::: :: :::::::::::::::::::::: ::::::::::::::
Human  GTGTGGACACAGCTGCTCCGGGCCTTGGAGGAGAGGGCCATTCCAATCTGGTGGGTGCTG
              10        20        30        40        50        60

              70        80        90       100       110
Baboon GTGGGTGTGCTGGGCGGCCTGCTGCTGCTCACCATCCTGGTCCTGGCCATGTGGAAG
       :::::::::::::: ::::::::::::::::::::::::::::::::::::::::::
Human  GTGGGTGTGCTGGGTGGCCTGCTGCTGCTCACCATCCTGGTCCTGGCCATGTGGAAG
              70        80        90       100       110
```

Figure 48. Comparison of the protein translating region nucleotide sequence between the GPIIb exon 29 of the baboon and human reference sequence. SNPs that cause amino acid changes are indicated in red and silent mutations in green.

```
Baboon GPIIb Ex30 60 bp                                  60 nt vs.
Human GPIIb Ex30 60 bp                                   60 nt
 using matrix file: DNA, gap open/ext: -14/-4
 100.0% identity in 60 nt overlap;             Global score: 300

              10        20        30        40        50        60
Baboon GTCGGCTTCTTCAAGCGGAACCGGCCACCCCTGGAAGAAGATGATGAAGAGGGGGAGTGA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Human  GTCGGCTTCTTCAAGCGGAACCGGCCACCCCTGGAAGAAGATGATGAAGAGGGGGAGTGA
              10        20        30        40        50        60
```

Figure 49. Comparison of the protein translating region nucleotide sequence between the GPIIb exon 30 of the baboon and human reference sequence.

Figure 50 shows a comparison between the amino acid sequences of the GPIIb protein of the baboon and human. There is 98.2% similarity between the protein sequences of exons 1 to 27 and 98.3% similarity between the protein sequences of exons 29 to 30. The amino acid changes are Gln7His, Gln18His, Asn158His, Asp159Asn, Val200Ile, Leu221Phe, Asp271Gly, Tyr274Phe, Arg281His, Ala339Thr, Arg368Gln, Arg400Ser, Ala637Thr,

Glu767Asp, Pro809His, Gln868Lys and Tyr915His for exons 1 to 27, and Trp952Arg for exons 29 to 30. Out of the 18 amino acid changes, only four amino acid changes, Arg281His, Arg400Ser, Pro809His and Trp952Arg, are radical amino acid changes. Only Arg281His is located within the RGD binding region of GPIIb (amino acids 1 to 334) as determined by Lin *et al.* (1997). Arg400Ser was situated in the β-propeller region of GPIIb, but in a section not described as functionally important. Pro809His and Trp952Arg were located in the calf-regions of the protein. Mutations in the calf-regions have been reported to negatively influence the transport of the receptor from the ER to ultimately the plasma membrane (Rosenberg *et al.*, 2003). Therefore, taken the fact that GPIIb/IIIa was still expressed on the babbon platelet surface, the influence of these changes is seen as minor. However, if a mutation in this area is able to cause a decrease in surface expression, it may be possible that the converse is also true. Thus, the increase in GPIIb/IIIa surface expression may be related to the radical changes in the calf-region of GPIIb.

From the 40 amino acids Kamata *et al.* (2001) identified as ligand binding regions, only one amino acid, Val200Ile, differed between the baboon and human. However, this is a conservative amino acid substitution and is unlikely to adversely affect ligand binding to GPIIb. Arg995 and KVGFF994 of the cytoplasmic domain, which is essential for GPIIb interaction with GPIIIa, and GVLGG976 of the transmembrane domain, which is important for the conformational change seen during receptor activation, were all unchanged in the *P. ursinus* sequence. None of the mutations described in patients with GT was detected in the baboon GPIIb sequence (Wilcox *et al.*, 1994; González-Manchón *et al.*, 2003; Nelson *et al.*, 2005; Kannan *et al.*, 2009; Vannier *et al.*, 2010; Mansour *et al.*, 2011).

A noteworthy conservative substitution was Arg368Gln. It is located within a calcium-binding area of GPIIb. Thus, the subtle change within this region may be the reason why baboon platelets are possibly more sensitive to sodium citrate, and could perhaps explain why baboon platelets are less reactive than human platelets in the presence of sodium citrate.

The GPIIb amino acid sequences between *P. ursinus* and *P. anubis* (Accession number: XM_003913168) were compared. Collectively the similarity between the two baboon species is 99.4%, when excluding exon 28. Six amino acid changes were found. Asp557Glu, Ala697Thr, Ile849Val and Lys868Gln were conservative changes, and Leu663Arg and Ile843Thr were radical changes. Only Gln868 is actually also present in the human, thus this is a change that is specific to *P. ursinus*. All the other changes were specific for *P. anubis*, and did not differ between *P. ursinus* and the human. When comparing *P. anubis* to the human a 97.88% similarity was found. This included 18 conservative substitutions and four radical changes. The radical changes were Arg400Ser, Leu663Arg, Ile843Thr and Trp952Arg. Leu663Arg and Ile843Thr are conserved in the *P. anubis*, and was not detected in the *P. ursinus* sequence.

Figure 51 shows the predicted secondary structures (Jones, 1999), as well as predicted disorders (Ward *et al*., 2004) of the baboon and human GPIIb proteins. The secondary structure prediction shows eight minor changes within the baboon protein (indicated with arrows), and one region with major changes (indicated in a red frame). These variations in protein may be the cause of the reduction of ADP, collagen and arachidonic acid-induced platelet aggregation as found in the baboon.

```
Baboon MARALCPLQALWLLEWVLLLLGPCAAPPAWALNLDPVHLTFYAGPNGSHFGFSLDFHKDS
                                          ©         ©          29
Human  MARALCPLQALWLLEWVLLLLGPCAAPPAWALNLDPVQLTFYAGPNGSQFGFSLDFHKDS
       Signal peptide
------------------------------------------------------------
Baboon HGRVAIVVGAPRTLGPSQEETGGVFLCPWRAEGGQCPSLLFDLRDETRNVGSQTLQTFKA
                                                               89
Human  HGRVAIVVGAPRTLGPSQEETGGVFLCPWRAEGGQCPSLLFDLRDETRNVGSQTLQTFKA
                                              ↑        ↑  ↑
------------------------------------------------------------
Baboon RQGLGASVVSWSDVIVACAPWQHWNVLEKTEEAEKTPVGSCFLAQPESGRRAEYSPCRGN
                                                               149
Human  RQGLGASVVSWSDVIVACAPWQHWNVLEKTEEAEKTPVGSCFLAQPESGRRAEYSPCRGN
                           ↑↑↑↑↑  ↑      ↑↑                 ↑
------------------------------------------------------------
Baboon TLSRIYVEHNFSWDKRYCEAGFSSVVTQAGELVLGAPGGYYFLGLLAQAPIADIFSSYRP
                ©©                                   ©         209
Human  TLSRIYVENDFSWDKRYCEAGFSSVVTQAGELVLGAPGGYYFLGLLAQAPVADIFSSYRP
            ↑    ↑  ↑↑↑↑                         ↑↑↑ ↑      ↑  ↑↑  ↑
------------------------------------------------------------
Baboon GILLWHVSSQSFSFDSSNPEYFDGYWGYSVAVGEFDGDLNTTEYVVGAPTWSWTLGAVEI
                 ©                                             269
Human  GILLWHVSSQSLSFDSSNPEYFDGYWGYSVAVGEFDGDLNTTEYVVGAPTWSWTLGAVEI
         ↑↑↑↑      ↑ ↑ ↑    ↑↑ ↑       Ca+ binding      ↑   ↑
------------------------------------------------------------
Baboon LGSYFQRLHRLHGEQMASYFGHSVAVTDVNGDGRHDLLVGAPLYMESRADRKLAEVGRVY
        ®  ©     ©                                              329
Human  LDSYYQRLHRLRGEQMASYFGHSVAVTDVNGDGRHDLLVGAPLYMESRADRKLAEVGRVY
                     ↑↑           Ca+ binding
------------------------------------------------------------
Baboon LFLQPRGPHTLGAPSLLLTGTQLYGRFGSAIAPLGDLDQDGYNDIAVAAPYGGPSGRGQV
                ©                         ©                    389
Human  LFLQPRGPHALGAPSLLLTGTQLYGRFGSAIAPLGDLDRDGYNDIAVAAPYGGPSGRGQV
                                      Ca+ binding
------------------------------------------------------------
Baboon LVFLGQSEGLSSRPSQVLDSPFPTGSAFGFSLRGAVDIDDNGYPDLIVGAYGANQVAVYR
                 ®                                             449
Human  LVFLGQSEGLRSRPSQVLDSPFPTGSAFGFSLRGAVDIDDNGYPDLIVGAYGANQVAVYR
                                       Ca+ binding
------------------------------------------------------------
Baboon AQPVVKASVQLLVQDSLNPAVKSCVLPQTKTPVSCFNIQMCVGATGHNIPQKLSLNAELQ
                                                               509
Human  AQPVVKASVQLLVQDSLNPAVKSCVLPQTKTPVSCFNIQMCVGATGHNIPQKLSLNAELQ
------------------------------------------------------------

Baboon LDRQKPRQGRRVLLLGSQQAGTTLNLDLGGKHSPICHTTMAFLRDEADFRDKLSPIVLSL
                                                               569
Human  LDRQKPRQGRRVLLLGSQQAGTTLNLDLGGKHSPICHTTMAFLRDEADFRDKLSPIVLSL
------------------------------------------------------------
Baboon NVSLPPTEAGMAPAVVLHGDTHVQEQTRIVLDCGEDDVCVPQLQLTASVTGSPLLVGADN
                                                               629
Human  NVSLPPTEAGMAPAVVLHGDTHVQEQTRIVLDCGEDDVCVPQLQLTASVTGSPLLVGADN
------------------------------------------------------------
Baboon VLELQMDTANEGEGAYEAELAVHLPQGAHYMRALSNVEGFERLICNQKKENETRVVLCEL
              ©                                                689
Human  VLELQMDAANEGEGAYEAELAVHLPQGAHYMRALSNVEGFERLICNQKKENETRVVLCEL
------------------------------------------------------------
Baboon GNPMKKNAQIGIAMLVSVGNLEEAGESVSFQLQIRSKNSQNPNSKIVLLDVPVRAEAQVE
                                                               749
Human  GNPMKKNAQIGIAMLVSVGNLEEAGESVSFQLQIRSKNSQNPNSKIVLLDVPVRAEAQVE
------------------------------------------------------------
Baboon LRGNSFPASLVVAAEEGDREQNSLDSWGPKVEHTYELHNNGPGTVNGLHLSIHLPGQSQH
                        ©                                     ® 809
Human  LRGNSFPASLVVAAEEGEREQNSLDSWGPKVEHTYELHNNGPGTVNGLHLSIHLPGQSQP
------------------------------------------------------------
Baboon SDLLYILDIQPQGGLQCFPQPPVNPLKVDWGLPIPSPSPIHPAHHKRDRRQIFLPEPEKP
                                                              © 869
Human  SDLLYILDIQPQGGLQCFPQPPVNPLKVDWGLPIPSPSPIHPAHHKRDRRQIFLPEPEQP
------------------------------------------------------------
Baboon SRLQDPVLVSCDSAPCTVVQCDLQEMARGQRAMVTVLAFLWLPSLHQ------------ 916
                                                 ©
Human  SRLQDPVLVSCDSAPCTVVQCDLQEMARGQRAMVTVLAFLWLPSLYQRPLDQFVLQSHAW 929
                                                        Exon 28
------------------------------------------------------------
Baboon -------------------VRTQLLRALEERAIPIWWVLVGVLGGLLLLTILVLAMWK 955
                          ®
Human  FNVSSLPYAVPPLSLPRGEAQVWTQLLRALEERAIPIWWVLVGVLGGLLLLTILVLAMWK 989
                Exon 28            Transmembrane domain
------------------------------------------------------------
Baboon VGFFKRNRPPLEEDDEEGE 974

Human  VGFFKRNRPPLEEDDEEGE 1008
           GPIIIa interaction
       Cytoplasmic domain
```

Figure 50: Comparison of the amino acid sequences of GPIIb between the baboon and human. The signal peptide is indicated in green lettering. The β-propeller region is shaded in grey. The missing exon 28 is indicated in red lettering. The transmembrane domain is indicated in blue lettering. The cytoplasmic domain is indicated in orange lettering. Conservative amino acid changes are indicated with an ©. Radical amino acid changes are indicated with an ®. All ligand binding amino acids are indicated with an arrow (↑) and in red lettering. The amino acid vital for interaction with GPIIIa (Arg995) is in purple lettering. The hydrophobic region N-terminal to Arg995, also implicated in GPIIIa interaction is underlined with a dotted line. The GVLGG sequence that plays a role in the conformational change and subsequent GPIIb/IIIa activation is underlined with a double line. The calcium binding amino acids are in italics and underlined.

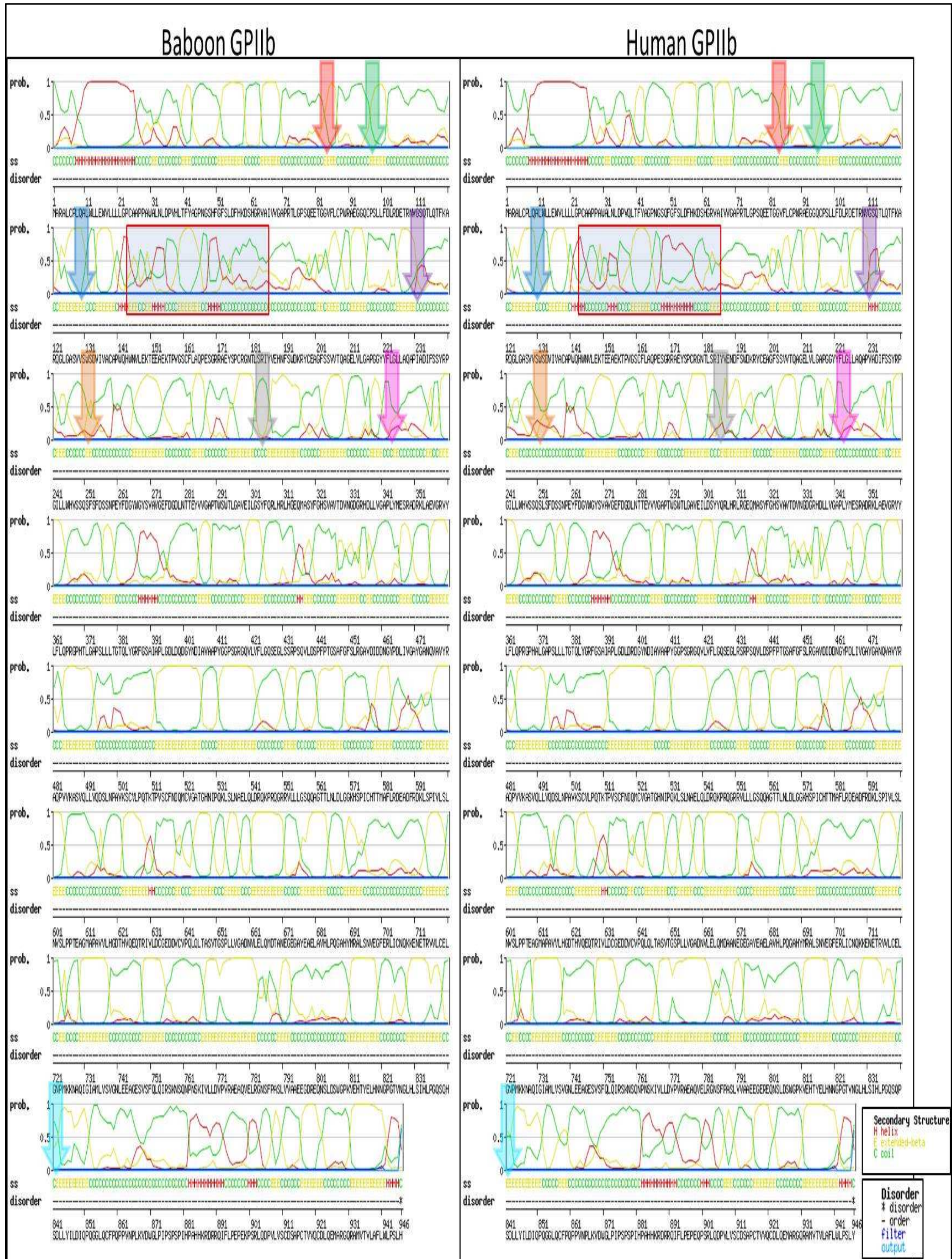


Figure 51. Prediction of the secondary protein structure of the baboon and human GPIIb proteins for exons 1-27. The arrows and frame indicate all the differences between the baboon and human proteins.

GPIIIa sequence:

Because of the size of the gene, GPIIIa is divided into the individual exons for illustration purposes. Table 10 contains the detected SNPs in the baboon GPIIIa sequence. Figures 52 to 66 shows a comparison of the protein translating region nucleotide sequence (exon 1 to 15) between the GPIIIa gene of the baboon and human reference sequence as found on www.ensembl.com (ITGB3-001, transcript ID: ENST00000559488). Table 10 contains the detected SNPs in the baboon GPIIIa sequence. In figures 50 to 64 an SNP that does not cause a change in amino acid is indicated in green and one that does is indicated in red. A total of 31 SNPs was detected in the baboon GPIIIa sequence when compared with the human GPIIIa reference sequence. Of the 31, 28 SNPs were silent mutations, which did not cause an amino acid change. There were only 3 SNPs that caused changes in amino acids. The nucleotide sequences are 98.7% similar between baboon and human GPIIIa.

Table 10. SNPs detected in baboon GPIIIa sequence.

Exon	SNP	Position	Amino Acid Change
3	G to A	129	No
4	G to A	53	No
	T to C	95	No
5	A to T	47	Yes
6	C to G	30	No
	T to C	73	No
	T to C	105	No
	C to T	111	No
7	A to C	36	No
9	T to C	9	No
	A to C	18	No
	G to A	48	No
	T to C	66	No
10	A to C	66	No

	A to G	155	Yes
	G to A	158	Yes
	A to G	195	No
	A to G	273	No
	C to T	321	No
	C to T	390	No
11	T to C	35	No
	T to C	68	No
	G to A	74	No
	C to T	194	No
12	T to C	7	No
	G to A	10	No
	C to T	46	No
	T to C	73	No
	C to T	76	No
13	T to C	80	No
15	G to C	51	No

```
Baboon GPIIIa Ex1 79 bp                                  79 nt vs.
Human GPIIIa Ex1 79 bp                                   79 nt
 using matrix file: DNA, gap open/ext: -14/-4
 100.0% identity in 79 nt overlap;          Global score: 395

               10        20        30        40        50        60
Baboon ATGCGAGCGCGGCCGCGGCCCCGGCCGCTCTGGGCGACTGTGCTGGCGCTGGGGGCGCTG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Human  ATGCGAGCGCGGCCGCGGCCCCGGCCGCTCTGGGCGACTGTGCTGGCGCTGGGGGCGCTG
               10        20        30        40        50        60

               70
Baboon GCGGGCGTTGGCGTAGGAG
       :::::::::::::::::::
Human  GCGGGCGTTGGCGTAGGAG
               70
```

Figure 52. Comparison of the protein translating region nucleotide sequence between the GPIIIa exon 1 of the baboon and human reference sequence.

```
Baboon GPIIIa Ex2 86 bp                                  86 nt vs.
Human GPIIIa Ex2 86 bp                                   86 nt
 using matrix file: DNA, gap open/ext: -14/-4
 100.0% identity in 86 nt overlap;          Global score: 430

               10        20        30        40        50        60
Baboon GGCCCAACATCTGTACCACGCGAGGTGTGAGCTCCTGCCAGCAGTGCCTGGCTGTGAGCC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Human  GGCCCAACATCTGTACCACGCGAGGTGTGAGCTCCTGCCAGCAGTGCCTGGCTGTGAGCC
               10        20        30        40        50        60

               70        80
Baboon CCATGTGTGCCTGGTGCTCTGATGAG
       ::::::::::::::::::::::::::
Human  CCATGTGTGCCTGGTGCTCTGATGAG
               70        80
```

Figure 53. Comparison of the protein translating region nucleotide sequence between the GPIIIa exon 2 of the baboon and human reference sequence.

```
Baboon GPIIIa Ex3 196 bp                                196 nt vs.
Human GPIIIa Ex3 196 bp                                 196 nt
 using matrix file: DNA, gap open/ext: -14/-4
  99.5% identity in 196 nt overlap;           Global score: 971

               10        20        30        40        50        60
Baboon GCCCTGCCTCTGGGCTCACCTCGCTGTGACCTGAAGGAGAATCTGCTGAAGGATAACTGT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Human  GCCCTGCCTCTGGGCTCACCTCGCTGTGACCTGAAGGAGAATCTGCTGAAGGATAACTGT
               10        20        30        40        50        60

               70        80        90       100       110       120
Baboon GCCCCAGAATCCATCGAGTTCCCAGTGAGTGAGGCCCGAGTACTAGAGGACAGGCCCCTC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Human  GCCCCAGAATCCATCGAGTTCCCAGTGAGTGAGGCCCGAGTACTAGAGGACAGGCCCCTC
               70        80        90       100       110       120

              130       140       150       160       170       180
Baboon AGCGACAAAGGCTCTGGAGACAGCTCCCAGGTCACTCAAGTCAGTCCCCAGAGGATTGCA
       :::::::: :::::::::::::::::::::::::::::::::::::::::::::::::::
Human  AGCGACAAGGGCTCTGGAGACAGCTCCCAGGTCACTCAAGTCAGTCCCCAGAGGATTGCA
              130       140       150       160       170       180

              190
Baboon CTCCGGCTCCGGCCAG
       ::::::::::::::::
Human  CTCCGGCTCCGGCCAG
              190
```

Figure 54. Comparison of the protein translating region nucleotide sequence between the GPIIIa exon 3 of the baboon and human reference sequence. The SNP that is a silent mutation is indicated in green.

```
Baboon GPIIIa Ex4 253 bp                                253 nt vs.
Human GPIIIa Ex4 253 bp                                 253 nt
 using matrix file: DNA, gap open/ext: -14/-4
  99.2% identity in 253 nt overlap;           Global score: 1247

               10        20        30        40        50        60
Baboon ATGATTCGAAGAATTTCTCCATCCAAGTGCGGCAGGTGGAGGATTACCCTGTAGACATCT
       :::::::::::::::::::::::::::::::::::::::::::::::::::: :::::::
Human  ATGATTCGAAGAATTTCTCCATCCAAGTGCGGCAGGTGGAGGATTACCCTGTGGACATCT
               10        20        30        40        50        60

               70        80        90       100       110       120
Baboon ACTACTTGATGGACCTGTCTTACTCCATGAAGGACGATCTGTGGAGCATCCAGAACCTGG
       :::::::::::::::::::::::::::::::::: :::::::::::::::::::::::::
Human  ACTACTTGATGGACCTGTCTTACTCCATGAAGGATGATCTGTGGAGCATCCAGAACCTGG
               70        80        90       100       110       120

              130       140       150       160       170       180
Baboon GTACCAAGCTGGCCACCCAGATGCGAAAGCTCACCAGTAACCTGCGGATTGGCTTCGGGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Human  GTACCAAGCTGGCCACCCAGATGCGAAAGCTCACCAGTAACCTGCGGATTGGCTTCGGGG
              130       140       150       160       170       180

              190       200       210       220       230       240
Baboon CATTTGTGGACAAGCCTGTGTCACCATACATGTATATCTCCCCACCAGAGGCCCTCGAAA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Human  CATTTGTGGACAAGCCTGTGTCACCATACATGTATATCTCCCCACCAGAGGCCCTCGAAA
              190       200       210       220       230       240

              250
Baboon ACCCCTGCTATGA
       :::::::::::::
Human  ACCCCTGCTATGA
              250
```

Figure 55. Comparison of the protein translating region nucleotide sequence between the GPIIIa exon 4 of the baboon and human reference sequence. SNPs that are silent mutations are indicated in green.

```
Baboon GPIIIa Ex5 163 bp                                  163 nt vs.
Human GPIIIa Ex5 163 bp                                   163 nt
 using matrix file: DNA, gap open/ext: -14/-4
  99.4% identity in 163 nt overlap;            Global score: 806

               10        20        30        40        50        60
Baboon TATGAAGACCACCTGCTTGCCCATGTTTGGCTACAAACACGTGCTGTCGCTAACTGACCA
       :::::::::::::::::::::::::::::::::::::::::::::: :::::::::::::
Human  TATGAAGACCACCTGCTTGCCCATGTTTGGCTACAAACACGTGCTGACGCTAACTGACCA
               10        20        30        40        50        60

               70        80        90       100       110       120
Baboon GGTGACCCGCTTCAATGAGGAAGTGAAGAAGCAGAGTGTGTCACGGAACCGAGATGCCCC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Human  GGTGACCCGCTTCAATGAGGAAGTGAAGAAGCAGAGTGTGTCACGGAACCGAGATGCCCC
               70        80        90       100       110       120

              130       140       150       160
Baboon AGAGGGTGGCTTTGATGCCATCATGCAGGCTACAGTCTGTGAT
       :::::::::::::::::::::::::::::::::::::::::::
Human  AGAGGGTGGCTTTGATGCCATCATGCAGGCTACAGTCTGTGAT
              130       140       150       160
```

Figure 56. Comparison of the protein translating region nucleotide sequence between the GPIIIa exon 5 of the baboon and human reference sequence. The SNP that caused an amino acid change is indicated in red.

```
Baboon GPIIIa Ex6 162 bp                                  162 nt vs.
Human GPIIIa Ex6 162 bp                                   162 nt
 using matrix file: DNA, gap open/ext: -14/-4
  97.5% identity in 162 nt overlap;            Global score: 774

               10        20        30        40        50        60
Baboon GAAAAGATTGGCTGGAGGAATGATGCATCGCACTTGCTGGTGTTTACCACTGATGCCAAG
       ::::::::::::::::::::::::::::: ::::::::::::::::::::::::::::::
Human  GAAAAGATTGGCTGGAGGAATGATGCATCCCACTTGCTGGTGTTTACCACTGATGCCAAG
               10        20        30        40        50        60

               70        80        90       100       110       120
Baboon ACTCATATAGCACTGGACGGAAGGCTGGCAGGCATTGTCCAGCCCAATGATGGGCAGTGT
       :::::::::::: ::::::::::::::::::::::::::::::: ::::: :::::::::
Human  ACTCATATAGCATTGGACGGAAGGCTGGCAGGCATTGTCCAGCCTAATGACGGGCAGTGT
               70        80        90       100       110       120

              130       140       150       160
Baboon CATGTTGGTAGTGACAATCATTACTCTGCCTCCACTACCATG
       ::::::::::::::::::::::::::::::::::::::::::
Human  CATGTTGGTAGTGACAATCATTACTCTGCCTCCACTACCATG
              130       140       150       160
```

Figure 57. Comparison of the protein translating region nucleotide sequence between the GPIIIa exon 6 of the baboon and human reference sequence. SNPs that are silent mutations are indicated in green.

```
Baboon GPIIIa Ex7 96 bp                                   96 nt vs.
Human GPIIIa Ex7 96 bp                                    96 nt
 using matrix file: DNA, gap open/ext: -14/-4
  99.0% identity in 96 nt overlap;            Global score: 471

              10        20        30        40        50        60
Baboon GATTATCCCTCTTTGGGGCTGATGACTGAGAAGCTCTCCCAGAAAAACATCAATTTGATC
       ::::::::::::::::::::::::::::::::::: ::::::::::::::::::::::::
Human  GATTATCCCTCTTTGGGGCTGATGACTGAGAAGCTATCCCAGAAAAACATCAATTTGATC
              10        20        30        40        50        60

              70        80        90
Baboon TTTGCAGTGACTGAAAATGTAGTCAATCTCTATCAG
       ::::::::::::::::::::::::::::::::::::
Human  TTTGCAGTGACTGAAAATGTAGTCAATCTCTATCAG
              70        80        90
```

Figure 58. Comparison of the protein translating region nucleotide sequence between the GPIIIa exon 7 of the baboon and human reference sequence. The SNP that is a silent mutation is indicated in green.

```
Baboon GPIIIa Ex8 90 bp                                   90 nt vs.
Human GPIIIa Ex8 90 bp                                    90 nt
 using matrix file: DNA, gap open/ext: -14/-4
 100.0% identity in 90 nt overlap;            Global score: 450

              10        20        30        40        50        60
Baboon AACTATAGTGAGCTCATCCCAGGGACCACAGTTGGGGTTCTGTCCATGGATTCCAGCAAT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Human  AACTATAGTGAGCTCATCCCAGGGACCACAGTTGGGGTTCTGTCCATGGATTCCAGCAAT
              10        20        30        40        50        60

              70        80        90
Baboon GTCCTCCAGCTCATTGTTGATGCTTATGGG
       ::::::::::::::::::::::::::::::
Human  GTCCTCCAGCTCATTGTTGATGCTTATGGG
              70        80        90
```

Figure 59. Comparison of the protein translating region nucleotide sequence between the GPIIIa exon 8 of the baboon and human reference sequence.

```
Baboon GPIIIa Ex9 135 bp                                135 nt vs.
Human GPIIIa Ex9 135 bp                                 135 nt
 using matrix file: DNA, gap open/ext: -14/-4
  97.0% identity in 135 nt overlap;           Global score: 639

                10        20        30        40        50        60
Baboon AAAATCCGCTCTAAAGTCGAGCTGGAAGTGCGTGACCTCCCTGAAGAATTGTCTCTATCC
       :::::::: :::::::: ::::::::::::::::::::::::::::: :::::::::::
Human  AAAATCCGTTCTAAAGTAGAGCTGGAAGTGCGTGACCTCCCTGAAGAGTTGTCTCTATCC
                10        20        30        40        50        60

                70        80        90       100       110       120
Baboon TTCAACGCCACCTGCCTCAACAATGAGGTCATCCCTGGCCTCAAGTCTTGTATGGGACTC
       ::::: ::::::::::::::::::::::::::::::::::::::::::::::::::::::
Human  TTCAATGCCACCTGCCTCAACAATGAGGTCATCCCTGGCCTCAAGTCTTGTATGGGACTC
                70        80        90       100       110       120

               130
Baboon AAGATTGGAGACACG
       :::::::::::::::
Human  AAGATTGGAGACACG
               130
```

Figure 60. Comparison of the protein translating region nucleotide sequence between the GPIIIa exon 9 of the baboon and human reference sequence. SNPs that are silent mutations are indicated in green.

```
Baboon GPIIIa Ex10 430 bp                              430 nt vs.
Human GPIIIa Ex10 430 bp                               430 nt
 using matrix file: DNA, gap open/ext: -14/-4
  98.4% identity in 430 nt overlap;             Global score: 2087

               10        20        30        40        50        60
Baboon GTGAGCTTCAGCATTGAGGCCAAGGTGCGAGGCTGTCCCCAGGAGAAGGAGAAGTCCTTT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Human  GTGAGCTTCAGCATTGAGGCCAAGGTGCGAGGCTGTCCCCAGGAGAAGGAGAAGTCCTTT
               10        20        30        40        50        60

               70        80        90       100       110       120
Baboon ACCATCAAGCCCGTGGGCTTCAAGGACAGCCTGATCGTCCAGGTCACCTTTGATTGTGAC
       ::::: ::::::::::::::::::::::::::::::::::::::::::::::::::::::
Human  ACCATAAAGCCCGTGGGCTTCAAGGACAGCCTGATCGTCCAGGTCACCTTTGATTGTGAC
               70        80        90       100       110       120

              130       140       150       160       170       180
Baboon TGTGCCTGCCAGGCCCAAGCTGAACCTAATAGCCGTCACTGCAACAATGGCAATGGGACC
       :::::::::::::::::::::::::::::::::: :: ::::::::::::::::::::::
Human  TGTGCCTGCCAGGCCCAAGCTGAACCTAATAGCCATCGCTGCAACAATGGCAATGGGACC
              130       140       150       160       170       180

              190       200       210       220       230       240
Baboon TTTGAGTGTGGGGTGTGCCGTTGTGGGCCTGGCTGGCTGGGATCCCAGTGTGAGTGCTCA
       :::::::::::::: :::::::::::::::::::::::::::::::::::::::::::::
Human  TTTGAGTGTGGGGTATGCCGTTGTGGGCCTGGCTGGCTGGGATCCCAGTGTGAGTGCTCA
              190       200       210       220       230       240

              250       260       270       280       290       300
Baboon GAGGAGGACTATCGCCCTTCCCAGCAGGACGAGTGCAGCCCCCGGGAGGGTCAGCCCGTC
       ::::::::::::::::::::::::::::::::: ::::::::::::::::::::::::::
Human  GAGGAGGACTATCGCCCTTCCCAGCAGGACGAATGCAGCCCCCGGGAGGGTCAGCCCGTC
              250       260       270       280       290       300

              310       320       330       340       350       360
Baboon TGCAGCCAGCGGGGCGAGTGTCTCTGTGGTCAATGTGTCTGCCACAGCAGTGACTTTGGC
       ::::::::::::::::::::: ::::::::::::::::::::::::::::::::::::::
Human  TGCAGCCAGCGGGGCGAGTGCCTCTGTGGTCAATGTGTCTGCCACAGCAGTGACTTTGGC
              310       320       330       340       350       360

              370       380       390       400       410       420
Baboon AAGATCACGGGCAAGTACTGCGAGTGTGATGACTTCTCCTGTGTCCGCTACAAGGGGGAG
       :::::::::::::::::::::::::::::: :::::::::::::::::::::::::::::
Human  AAGATCACGGGCAAGTACTGCGAGTGTGACGACTTCTCCTGTGTCCGCTACAAGGGGGAG
              370       380       390       400       410       420

              430
Baboon ATGTGCTCAG
       ::::::::::
Human  ATGTGCTCAG
              430
```

Figure 61. Comparison of the protein translating region nucleotide sequence between the GPIIIa exon 10 of the baboon and human reference sequence. SNPs that cause amino acid changes are indicated in red and silent mutations are in green.

```
Baboon GPIIIa Ex11 223 bp                          223 nt vs.
Human GPIIIa Ex11 223 bp                           223 nt
 using matrix file: DNA, gap open/ext: -14/-4
  98.2% identity in 223 nt overlap;          Global score: 1079

               10        20        30        40        50        60
Baboon GCCATGGCCAGTGCAGCTGTGGGGACTGCCTGTGCGACTCCGACTGGACCGGCTACTACT
       :::::::::::::::::::::::::::::::::: :::::::::::::::::::::::::
Human  GCCATGGCCAGTGCAGCTGTGGGGACTGCCTGTGTGACTCCGACTGGACCGGCTACTACT
               10        20        30        40        50        60

               70        80        90       100       110       120
Baboon GCAACTGCACCACACGTACTGACACCTGCATGTCCAGCAATGGGCTGCTGTGCAGCGGCC
       ::::::: ::::: ::::::::::::::::::::::::::::::::::::::::::::::
Human  GCAACTGTACCACGCGTACTGACACCTGCATGTCCAGCAATGGGCTGCTGTGCAGCGGCC
               70        80        90       100       110       120

              130       140       150       160       170       180
Baboon GCGGCAAGTGTGAATGTGGCAGCTGTGTCTGTATCCAGCCGGGCTCCTATGGGGACACCT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Human  GCGGCAAGTGTGAATGTGGCAGCTGTGTCTGTATCCAGCCGGGCTCCTATGGGGACACCT
              130       140       150       160       170       180

              190       200       210       220
Baboon GTGAGAAGTGCCCTACCTGCCCAGATGCCTGCACCTTTAAGAA
       ::::::::::::: :::::::::::::::::::::::::::::
Human  GTGAGAAGTGCCCCACCTGCCCAGATGCCTGCACCTTTAAGAA
              190       200       210       220
```

Figure 62. Comparison of the protein translating region nucleotide sequence between the GPIIIa exon 11 of the baboon and human reference sequence. SNPs that are silent mutations are indicated in green.

```
Baboon GPIIIa Ex12 101 bp                                101 nt vs.
Human GPIIIa Ex12 101 bp                                 101 nt
 using matrix file: DNA, gap open/ext: -14/-4
  95.0% identity in 101 nt overlap;            Global score: 460

                10        20        30        40        50        60
Baboon AGAATGCGTAGAGTGTAAGAAGTTTGACCGGGGAGCCCTACATGATGAAAATACCTGCAA
       :::::: :: :::::::::::::::::::::::::::::::::: ::::::::::::::
Human  AGAATGTGTGGAGTGTAAGAAGTTTGACCGGGGAGCCCTACATGACGAAAATACCTGCAA
                10        20        30        40        50        60

                70        80        90       100
Baboon CCGTTACTGCCGCGATGAGATTGAGTCAGTGAAAGAGCTTA
       :::::::::::: :: :::::::::::::::::::::::::
Human  CCGTTACTGCCGTGACGAGATTGAGTCAGTGAAAGAGCTTA
                70        80        90       100
```

Figure 63. Comparison of the protein translating region nucleotide sequence between the GPIIIa exon 12 of the baboon and human reference sequence. SNPs that are silent mutations are indicated in green.

```
Baboon GPIIIa Ex13 120 bp                                120 nt vs.
Human GPIIIa Ex13 120 bp                                 120 nt
 using matrix file: DNA, gap open/ext: -14/-4
  99.2% identity in 120 nt overlap;            Global score: 591

                10        20        30        40        50        60
Baboon AGGACACTGGCAAGGATGCAGTGAATTGTACCTATAAGAATGAGGATGACTGTGTCGTCA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Human  AGGACACTGGCAAGGATGCAGTGAATTGTACCTATAAGAATGAGGATGACTGTGTCGTCA
                10        20        30        40        50        60

                70        80        90       100       110       120
Baboon GATTCCAGTACTATGAAGACTCTAGTGGAAAGTCCATCCTGTATGTGGTAGAAGAGCCAG
       ::::::::::::::::::: ::::::::::::::::::::::::::::::::::::::::
Human  GATTCCAGTACTATGAAGATTCTAGTGGAAAGTCCATCCTGTATGTGGTAGAAGAGCCAG
                70        80        90       100       110       120
```

Figure 64. Comparison of the protein translating region nucleotide sequence between the GPIIIa exon 13 of the baboon and human reference sequence. The SNP that is a silent mutation is indicated in green.

```
Baboon GPIIIa Ex14 167 bp                              167 nt vs.
Human GPIIIa Ex14 167 bp                               167 nt
 using matrix file: DNA, gap open/ext: -14/-4
 100.0% identity in 167 nt overlap;          Global score: 835

               10        20        30        40        50        60
Baboon AGTGTCCCAAGGGCCCTGACATCCTGGTGGTCCTGCTCTCAGTGATGGGGGCCATTCTGC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Human  AGTGTCCCAAGGGCCCTGACATCCTGGTGGTCCTGCTCTCAGTGATGGGGGCCATTCTGC
               10        20        30        40        50        60

               70        80        90       100       110       120
Baboon TCATTGGCCTTGCCGCCCTGCTCATCTGGAAACTCCTCATCACCATCCACGACCGAAAAG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Human  TCATTGGCCTTGCCGCCCTGCTCATCTGGAAACTCCTCATCACCATCCACGACCGAAAAG
               70        80        90       100       110       120

              130       140       150       160
Baboon AATTCGCTAAATTTGAGGAAGAACGCGCCAGAGCAAAATGGGACACA
       :::::::::::::::::::::::::::::::::::::::::::::::
Human  AATTCGCTAAATTTGAGGAAGAACGCGCCAGAGCAAAATGGGACACA
              130       140       150       160
```

Figure 65. Comparison of the protein translating region nucleotide sequence between the GPIIIa exon 14 of the baboon and the human reference sequence.

```
Baboon GPIIIa Ex15 65 bp                                65 nt vs.
Human GPIIIa Ex15 65 bp                                 65 nt
 using matrix file: DNA, gap open/ext: -14/-4
  98.5% identity in 65 nt overlap;           Global score: 316

               10        20        30        40        50        60
Baboon GCCAACAACCCACTGTATAAAGAGGCCACGTCTACCTTCACCAATATCACCTACCGGGGC
       ::::::::::::::::::::::::::::::::::::::::::::::::: :::::::::
Human  GCCAACAACCCACTGTATAAAGAGGCCACGTCTACCTTCACCAATATCACGTACCGGGGC
               10        20        30        40        50        60


Baboon ACTTA
       :::::
Human  ACTTA
```

Figure 66. Comparison of the protein translating region nucleotide sequence between the GPIIIa exon 15 of the baboon and human reference sequence. The SNP that is a silent mutation is indicated in green.

Figure 67 shows a comparison between the amino acid sequences of the GPIIIa protein of the baboon and human. There is 99.6% similarity between

the protein sequences. The amino acid changes are Thr195Ser, His446Arg and Arg447His. All three the substitutions are conservative substitutions. Therefore, they have limited impact on the folding of the protein. Lin *et al.* (1997) determined that amino acid residues 164 to 202 of GPIIIa were the minimum integrin domains required for specific RGD motive containing ligand binding to the GPIIb/IIIa receptor complex. Only the Thr195Ser SNP is present in this region. Therefore, as this amino acid change is only a conservative substitution, this mutation is unlikely to have an effect on ligand binding. Asp723 and KLLITIH722 are intact in the baboon sequence; therefore the interaction of GPIIIa with GPIIb and talin is not likely to be influenced. Thus, taken together with the fact that the GPIIIb interacting- and the conformation change enhancing amino acids in GPIIb are also intact, the conformational change seen in GPIIb/IIIa after talin binding is unlikely to differ between the baboon and human. Not one of the mutations previously described in patients with GT were detected in the baboon GPIIIa sequence (Loftus *et al.*, 1990; Bajt *et al.*, 1992; Bajt & Loftus, 1994; Jallu *et al.*, 2002; Tadokoro *et al.*, 2002; Kannan *et al.*, 2009).

Figure 68 shows the predicted secondary structures (Jones, 1999), as well as predicted disorders (Ward *et al.*, 2004) of the baboon and human GPIIIa proteins. The secondary structure prediction shows eight minor changes within the baboon protein (indicated with arrows). However, no disorder was predicted in the baboon protein. However, it cannot be ruled out that these small variations in protein are the cause of the reduction of ADP-, collagen-, and arachidonic acid-induced platelet aggregation as found in the baboon.

Comparison between *P. ursinus* and *P. anubis* (Accession number: XP_003913295) found a 99.6% amino acid similarity between the two species. Three amino acid changes were detected. Arg4Gln and Ala737Thr were conservative changes, and Arg6Gly was a radical change. All three these changes were not present when comparing *P. ursinus* with human GPIIIa. Three further conservative changes were found between the human and *P. anubis*, namely Thr195Ser, His446Arg and Arg447His. These changes were also detected between *P. ursinus* and the human. *P. anubis* GPIIIa is

99.2% similar to human, thus, *P. ursinus* is more similar to humans than *P. anubis*. Concidering that *P. ursinus* also has less variation in GPIIb than *P. anubis*, it may be derived that *P. ursinus* is a slightly more suitable model for human targeted GPIIb/IIIa studies.

```
Baboon MRARPRPRPLWATVLALGALAGVGVGGPNICTTRGVSSCQQCLAVSPMCAWCSDEALPLG
                                                                       34
Human  MRARPRPRPLWATVLALGALAGVGVGGPNICTTRGVSSCQQCLAVSPMCAWCSDEALPLG
       Signal peptide
--------------------------------------------------------------------
Baboon SPRCDLKENLLKDNCAPESIEFPVSEARVLEDRPLSDKGSGDSSQVTQVSPQRIALRLRP
                                                                       94
Human  SPRCDLKENLLKDNCAPESIEFPVSEARVLEDRPLSDKGSGDSSQVTQVSPQRIALRLRP

--------------------------------------------------------------------
Baboon DDSKNFSIQVRQVEDYPVDIYYLMDLSYSMKDDLWSIQNLGTKLATQMRKLTSNLRIGFG
                                                                       154
Human  DDSKNFSIQVRQVEDYPVDIYYLMDLSYSMKDDLWSIQNLGTKLATQMRKLTSNLRIGFG

--------------------------------------------------------------------
Baboon AFVDKPVSPYMYISPPEALENPCYDMKTTCLPMFGYKHVLSLTDQVTRFNEEVKKQSVSR
                                              ©                        214
Human  AFVDKPVSPYMYISPPEALENPCYDMKTTCLPMFGYKHVLTLTDQVTRFNEEVKKQSVSR
                      Ligand binding
--------------------------------------------------------------------
Baboon NRDAPEGGFDAIMQATVCDEKIGWRNDASHLLVFTTDAKTHIALDGRLAGIVQPNDGQCH
                                                                       274
Human  NRDAPEGGFDAIMQATVCDEKIGWRNDASHLLVFTTDAKTHIALDGRLAGIVQPNDGQCH

--------------------------------------------------------------------
Baboon VGSDNHYSASTTMDYPSLGLMTEKLSQKNINLIFAVTENVVNLYQNYSELIPGTTVGVLS
                                                                       334
Human  VGSDNHYSASTTMDYPSLGLMTEKLSQKNINLIFAVTENVVNLYQNYSELIPGTTVGVLS

--------------------------------------------------------------------
Baboon MDSSNVLQLIVDAYGKIRSKVELEVRDLPEELSLSFNATCLNNEVIPGLKSCMGLKIGDT
                                                                       394
Human  MDSSNVLQLIVDAYGKIRSKVELEVRDLPEELSLSFNATCLNNEVIPGLKSCMGLKIGDT

--------------------------------------------------------------------
Baboon VSFSIEAKVRGCPQEKEKSFTIKPVGFKDSLIVQVTFDCDCACQAQAEPNSRHCNNGNGT
                                                    ©©                 454
Human  VSFSIEAKVRGCPQEKEKSFTIKPVGFKDSLIVQVTFDCDCACQAQAEPNSHRCNNGNGT

--------------------------------------------------------------------
Baboon FECGVCRCGPGWLGSQCECSEEDYRPSQQDECSPREGQPVCSQRGECLCGQCVCHSSDFG
                                                                       514
Human  FECGVCRCGPGWLGSQCECSEEDYRPSQQDECSPREGQPVCSQRGECLCGQCVCHSSDFG

--------------------------------------------------------------------
Baboon KITGKYCECDDFSCVRYKGEMCSGHGQCSCGDCLCDSDWTGYYCNCTTRTDTCMSSNGLL
                                                                       574
Human  KITGKYCECDDFSCVRYKGEMCSGHGQCSCGDCLCDSDWTGYYCNCTTRTDTCMSSNGLL

--------------------------------------------------------------------
Baboon CSGRGKCECGSCVCIQPGSYGDTCEKCPTCPDACTFKKECVECKKFDRGALHDENTCNRY
                                                                       634
Human  CSGRGKCECGSCVCIQPGSYGDTCEKCPTCPDACTFKKECVECKKFDRGALHDENTCNRY

--------------------------------------------------------------------
Baboon CRDEIESVKELKDTGKDAVNCTYKNEDDCVVRFQYYEDSSGKSILYVVEEPECPKGPDIL
                                                                       694
Human  CRDEIESVKELKDTGKDAVNCTYKNEDDCVVRFQYYEDSSGKSILYVVEEPECPKGPDIL

--------------------------------------------------------------------
Baboon VVLLSVMGAILLIGLAALLIWKLLITIHDRKEFAKFEEERARAKWDTANNPLYKEATSTF
                                                                       754
Human  VVLLSVMGAILLIGLAALLIWKLLITIHDRKEFAKFEEERARAKWDTANNPLYKEATSTF
                                    GPIIb and talin interaction
       Transmembrane domain  Cytoplasmic domain
--------------------------------------------------------------------
Baboon TNITYRGT
                 762
Human  TNITYRGT
```

Figure 67. Comparison of the amino acid sequences of GPIIIa between the *P. ursinus* baboon and human. The signal peptide is indicated in green lettering. The ligand binding region is highlighted in purple. The cysteine-rich tandem repeat region is shaded in grey. The transmembrane domain is indicated in blue lettering. The cytoplasmic domain is indicated in orange lettering. Conservative amino acid changes are indicated with an ©. Radical amino acid changes are indicated with an ®. The amino acid vital for interaction with GPIIb and talin is in purple lettering (Asp723). The hydrophobic region N-terminal to Asp723, also implicated in GPIIIa interaction is underlined with a dotted line.

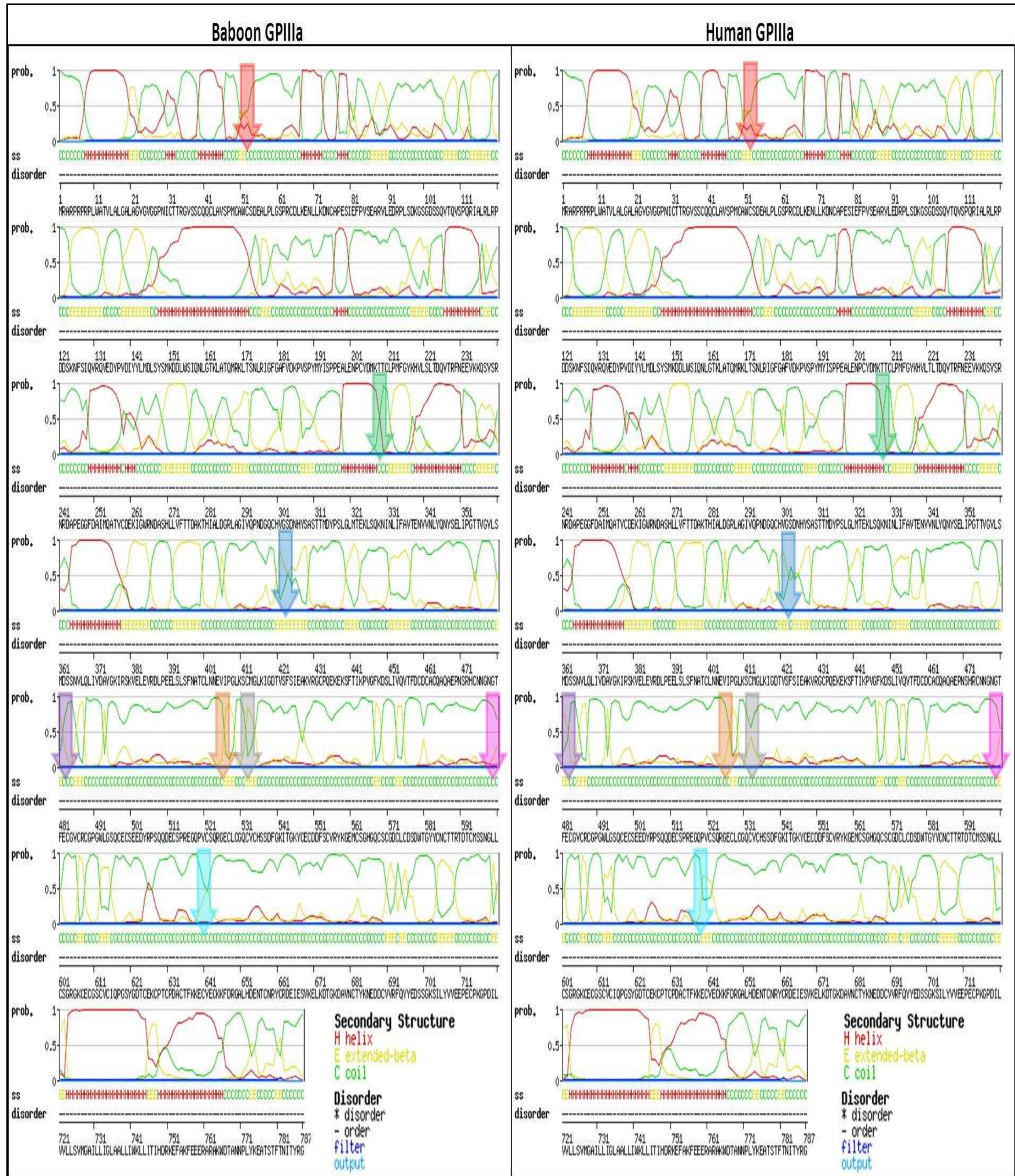


Figure 68. Prediction of the secondary protein structure of the baboon and human GPIIIa proteins. The arrows indicate all the differences between the baboon and human proteins.

Considering both the GPIIb and GPIIIa sequencing results it is believed that due to the minimal changes observed (figure 69) between the human and baboon, the Cape chacma baboon (*Papio ursinus*) is a suitable model to

evaluate human-targeted anti-GPIIb/IIIa agents in. However, the radical amino acid changes, as found in this study, should be taken into consideration when evaluating molecular-based anti-GPIIb/IIIa agents in the future.

Reflecting on the ADP-, collagen-, and arachidonic acid-induced aggregation, quantification and sequencing results, it is judged that the higher concentration of ADP, collagen and arachidonic acid needed to induce aggregation similar to humans, may be mostly attributed to the greater number of GPIIb/IIIa receptors present on the baboon platelet surface. The minor amino acid changes within the baboon sequence probably have only a limited effect on the aggregation results. However, because sodium citrate is used as anticoagulant in platelet function tests, it may also be possible that the baboon platelets are more reliant on the STIM-1/Orai1 interaction for strong aggregation. Thus, the presence of of a calcium chelator, such as sodium citrate, may negatively influence baboon platelet aggregation. The presence of the Arg368Gln substitution in a calcium binding region of GPIIb may further contribute to this outcome. This possibility must be further explored in future studies.

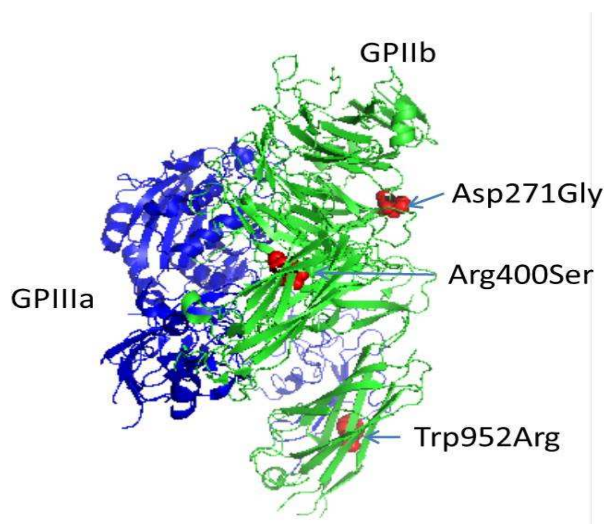


Figure 69. Crystal structure of GPIIb/IIIa in its resting conformation, depicting radical amino acid changes found in the *P. ursinus* sequence (shown in red dots). GPIIb is in green and GPIIIa is in blue. Cartoon drawn with PyMol. Crystal structure available from RCSB Protein Data Bank (accession # 4CAK). None of the radical changes is found at a contact point between GPIIb and GPIIIa, and thus, is unlikely to influence the interaction between the two proteins.

In a previous study performed in our setting, it was found that the GPIIb/IIIa inhibitor, tirofiban, is only effective in preventing arterial thrombosis in baboons at nine times the prescribed dose for humans (Janse van Rensburg *et al.*, 2012b). However, questions were raised on whether the baboon data could be extrapolated to humans. Considering that the baboon only has 37% more GPIIb receptors and merely 27% more GPIIIa receptors on its platelet surface, it can be assumed that even though nine times may be too much, an increase in dose between three and nine times may be a reasonable option in humans. In that same study, abciximab was able to inhibit platelet aggregation at the presecribed human dose, thus highlighting that the tirofiban results obtained with the baboon model was rather drug specific and not model specific. The high similarity between the RGD binding regions, the GPIIb/IIIa contact regions, and regions involved in conformational changes of the human and baboon GPIIb/IIIa receptors also contributes to the fact that results from the baboon model can be extrapolated to human with only limited interpretation and calculations needed.

The example above illustrates how the current study will contribute to making relevant and more accurate conclusions and predictions from baboon model studies in the future.

GPIbα sequence:

Table 11 contains the detected SNPs in the baboon GPIbα sequence. Due to big differences detected in the protein translating region it was decided to evaluate each domain within the baboon GPIbα sequence separately. These domains included the signal peptide, the VWF-binding domain, the PEST / macroglycoprotein domain, transmembrane domain, and cytoplasmic domain.

GPIbα proved to be the most challenging of all the receptors to sequence. After five months of failed reactions and numerous different primer pairs and all new reagents, 249 bases (equating to 83 amino acids) could not be

sequenced. The non-sequenced region starts at base 1186 and ends at base 1435 of the GPIbα translation region. The final 528 bases of the sequence (base 1436 to 1959) were successfully sequenced. The non-sequenced region is located in the extracellular portion of the protein, between the VWF-binding domain and the transmembrane domain. This region is known to be very polymorphic, with the presence of the VNTR in humans. The region was named the macroglycoprotein (Meyer & Schellenberg, 1990) and also contained the PEST domain (Shi *et al.*, 2012). The three different size variations of GPIbα in humans as described by López *et al.* (1992b) have a sequence difference of 78 bases (26 amino acids) between the largest (B allele) and smallest (D allele) variants. These differences are also located within the macroglycoprotein region. The VNTR is located in the middle of the non-sequenced region as depicted in figure 70.

380APNMTTLEPTPSPTTPEPTSEPAPSPTTPEPTSEPAPSPTTPEPTSEPAPSPTTPEPTPIPTIATSPTILVSATSLITPKST462

Variable number of tandem repeats

Figure 70: The non-sequenced region of GPIbα, depicting the location of the VNTR. The figure contains three 13 amino acid (SEPAPSPTTPEPT) repeats (underlined in different styles) as seen in allele B.

The following primers were used to try and sequence this region, without success: GPIba 2X.1F and GPIba 2X.1R, Iba Final F and Iba Final R, GPIba 2X.2 F and GPIba 2X.2 R, GPIba Ex2CF and GPIba 2.3R, GPIba 2.3F and GPIba Ex2_3R, GPIba 1_F and GPIba 2.3R, GPIa 2.3F and GPIba 2_R, GPIba 1_F and GPIba 2_R, GPIba 1_F and GPIba 1_R, GPIba 2_F and GPIba 2_R, GPIba 2.1F and GPIba 2.1R, as well as GPIba 2.2F and GPIba 2.2R. Gradient PCR cycling conditions, as well as different reagent concentrations, were used for all primer pairs. These PCR reactions either produced no fragments or multiple fragments (both smaller and larger than expected). The multiple fragments were all removed from the gel, and subsequently sequenced. However, none of the sequencing reactions gave results that could be interpreted. These results may indicate that this part of the sequence may indeed be lacking, thus the *P. ursinus* GPIbα receptor may

be shorter than the human receptor. But, as is evident from inter-specie comparison of this region, a shorter sequence does not necessarily influence functionality of the receptor. As was the case with GPIIb exon 28, alternative avenues for sequencing could not be explored due to the lack of local know-how and a limited budget. However, a follow-up study is planned to answer this question. Due to the differences detected in this region it was decided to evaluate each domain within the baboon GPIbα sequence separately. Because the whole translated sequence of GPIbα is situated within exon 2 it was decided to deviate from the analysis method used previously where exons were evaluated separately, and rather focus on different domains within the sequence.

Table 12 contains the percentage similarity of the macroglycoprotein region between the human B- (largest) and D-alleles (smallest), and different animals. The mini-pig (*Sus scrofa* – Accession # FJ228700) and mouse (*Mus musculus* – Accession # NM_01326) were selected as they are also popular animal models used in platelet research. The chimpanzee (*Pan troglodytes* – Accession # XM_523557) was selected due to its close evolutionary link to humans. The results illustrate the massive variability of this region and could give a possible explanation why it is difficult to sequence this region. However, other than the chimpanzee, *Papio ursinus* remains the closest to the human sequence at this region, despite the non-sequenced region. *Papio ursinus* was also the only species that had an increased similarity with human the D-allele.

Figures 68 to 72 shows a comparison of the protein translating region nucleotide sequence between the different domains of the GPIbα gene of the baboon and the human reference sequence as found on www.ensembl.com (GP1BA-001, transcript ID: ENST00000329380). In figures 68 to 72 an SNP that does not cause a change in amino acid is indicated in green, and one that does is in red.

A total of 89 SNPs was detected in the baboon GPIbα sequence when compared with the human GPIbα reference sequence. Of the 89, 29 SNPs

were silent mutations, which did not cause an amino acid change. There were, however, 60 SNPs that caused a change in 54 amino acids. The baboon signal peptide nucleotide sequence showed a 95.8% similarity to humans. The baboon VWF-binding domain was 94.7% similar to humans on the nucleotide level. The nucleotide sequences of the PEST / macroglycoprotein region were found to be 60.8% similar between the two species. The nucleotide sequences of the transmembrane domain showed a 96.8% similarity. Finally, the cytoplasmic domain had a 96.0% nucleotide sequence homology. The Kozak region was also evaluated (nucleotide data not shown, as it is not part of the translated region). The baboon sequence showed to have the -5C/C genotype, associated with increased mRNA and surface receptor expression (Afshar-Khargan *et al.*, 1999).

Table 11. SNPs detected in baboon GPVI sequence.

Domain	SNP	Position	Amino Acid Change
Signal Peptide	A to G	44	Yes
	C to T	45	Yes
VWF-binding Domain	G to C	108	Yes
	A to G	115	Yes
	G to A	138	No
	C to A	150	No
	C to G	158	Yes
	C to T	160	No
	A to G	163	Yes
	T to G	165	Yes
	G to A	166	Yes
	A to T	196	Yes
	A to G	202	Yes
	A to T	209	Yes
	A to T	229	Yes
	C to T	232	No
	G to A	239	Yes
	G to A	273	No

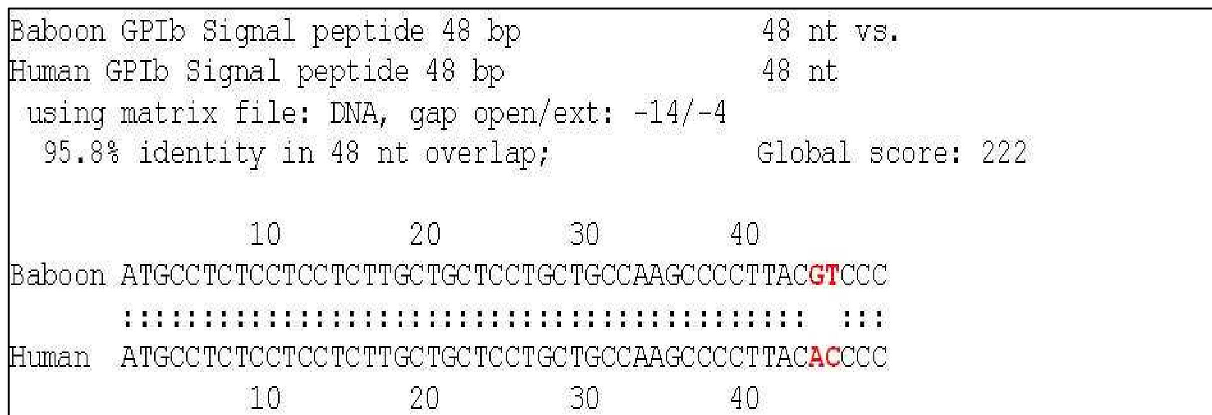
	C to A	309	Yes
	G to A	358	Yes
	G to A	380	Yes
	G to A	410	Yes
	G to A	421	Yes
	A to C	523	Yes
	T to C	531	No
	G to C	532	Yes
	T to C	543	No
	A to G	553	Yes
	A to G	563	Yes
	A to G	606	No
	A to C	632	Yes
	C to T	657	No
	T to C	720	No
	A to T	723	Yes
	G to A	778	Yes
	T to A	808	Yes
	T to C	809	Yes
	C to T	813	No
	T to C	817	Yes
	A to G	828	No
	G to A	837	No
	G to A	860	Yes
	A to C	865	Yes
	A to G	869	Yes
	T to G	880	Yes
	C to A	886	Yes
	A to C	891	Yes
PEST / macroglycoprotein Domain	C to T	906	No
	A to G	910	Yes
	T to A	932	Yes

	A to G	960	No
	C to T	963	No
	A to G	975	No
	T to C	979	Yes
	C to T	990	No
	A to G	991	Yes
	A to G	1006	Yes
	C to T	1008	Yes
	T to C	1013	Yes
	T to C	1071	No
	A to G	1084	Yes
	T to C	1100	Yes
	A to G	1103	Yes
	C to T	1107	No
	A to G	1108	Yes
	T to C	1109	Yes
	C to T	1145	Yes
	A to G	1146	Yes
	G to A	1179	No
	A to G	1437	No
	C to A	1481	Yes
	C to T	1492	Yes
	C to A	1502	Yes
	G to C	1536	Yes
	C to A	1554	Yes
Transmembrane Domain	T to C	1615	Yes
	C to A	1651	Yes
Cytoplasmic Domain	G to T	1658	Yes
	T to C	1665	No
	G to A	1666	Yes
	G to C	1708	Yes
	A to G	1714	Yes

	C to T	1737	No
	A to G	1767	No
	G to A	1803	No
	T to C	1806	No
	G to A	1833	No
	T to C	1854	No
	A to G	1921	Yes

Table 12. Percentage similarity of the PEST / macroglycoprotein region between different species and the human B- and D alleles of GPIbα.

Species	Human B allele	Human D allele
Papio ursinus (Cape chacma baboon)	57.9%	65.2%
Sus scrofa (mini pig)	39.7%	38.0%
Pan troglodytes (chimpanzee)	93.1%	82.5%
Mus musculus (mouse)	34.3%	31.5%



```
Baboon GPIb Signal peptide 48 bp              48 nt vs.
Human GPIb Signal peptide 48 bp               48 nt
 using matrix file: DNA, gap open/ext: -14/-4
  95.8% identity in 48 nt overlap;            Global score: 222

               10        20        30        40
Baboon ATGCCTCTCCTCCTCTTGCTGCTCCTGCTGCCAAGCCCCTTACGTCCC
       ::::::::::::::::::::::::::::::::::::::::::  :::
Human  ATGCCTCTCCTCCTCTTGCTGCTCCTGCTGCCAAGCCCCTTACACCCC
               10        20        30        40
```

Figure 71. Comparison of the protein translating region nucleotide sequence between the signal peptide of the GPIbα gene of the baboon and human reference sequence. SNPs that cause amino acid changes are indicated in red and silent mutations in green.

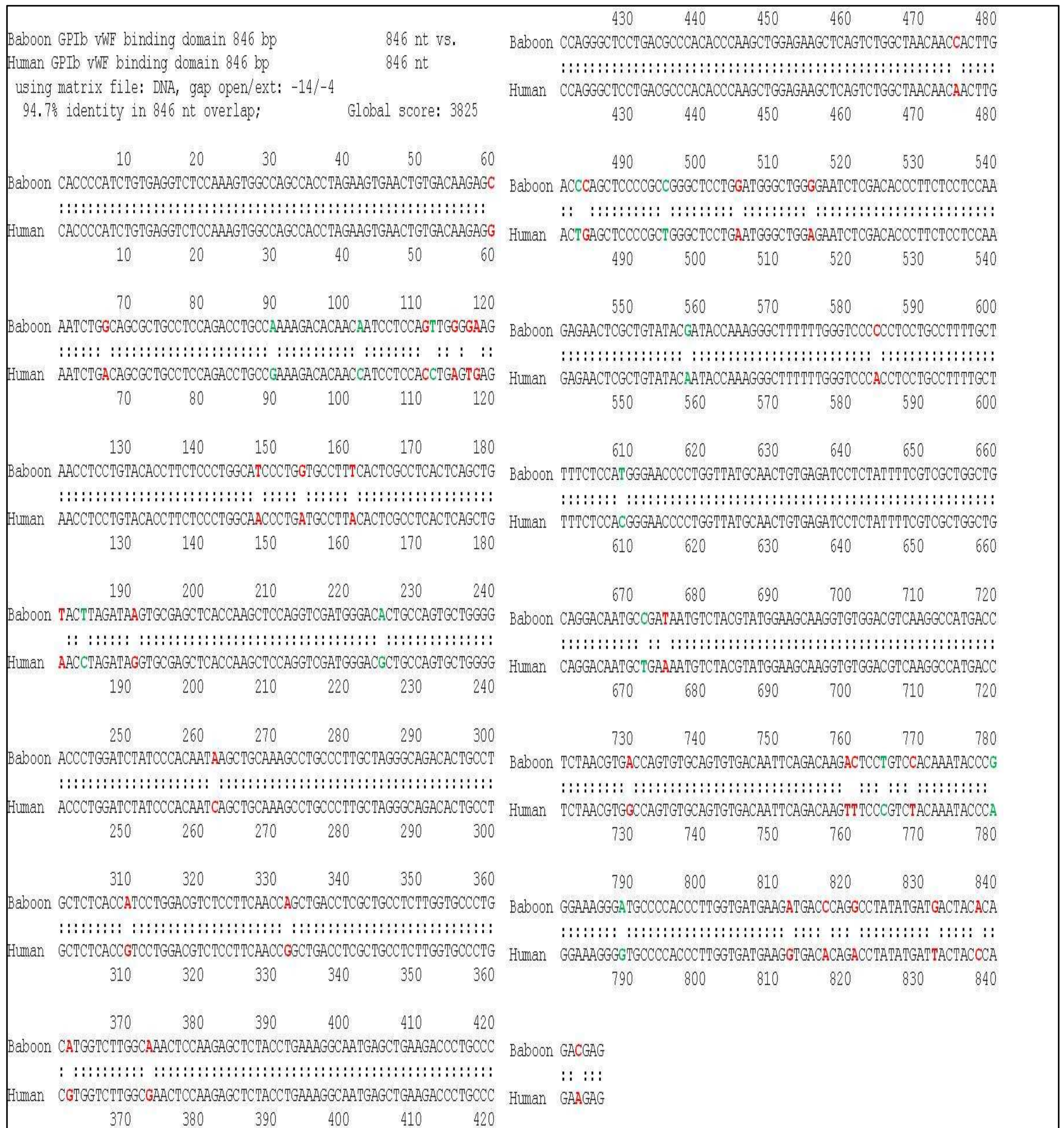


Figure 72. Comparison of the protein translating region nucleotide sequence between the VWF-binding domain of the GPIbα gene of the baboon and human reference sequence. SNPs that cause amino acid changes are indicated in red and silent mutations in green.

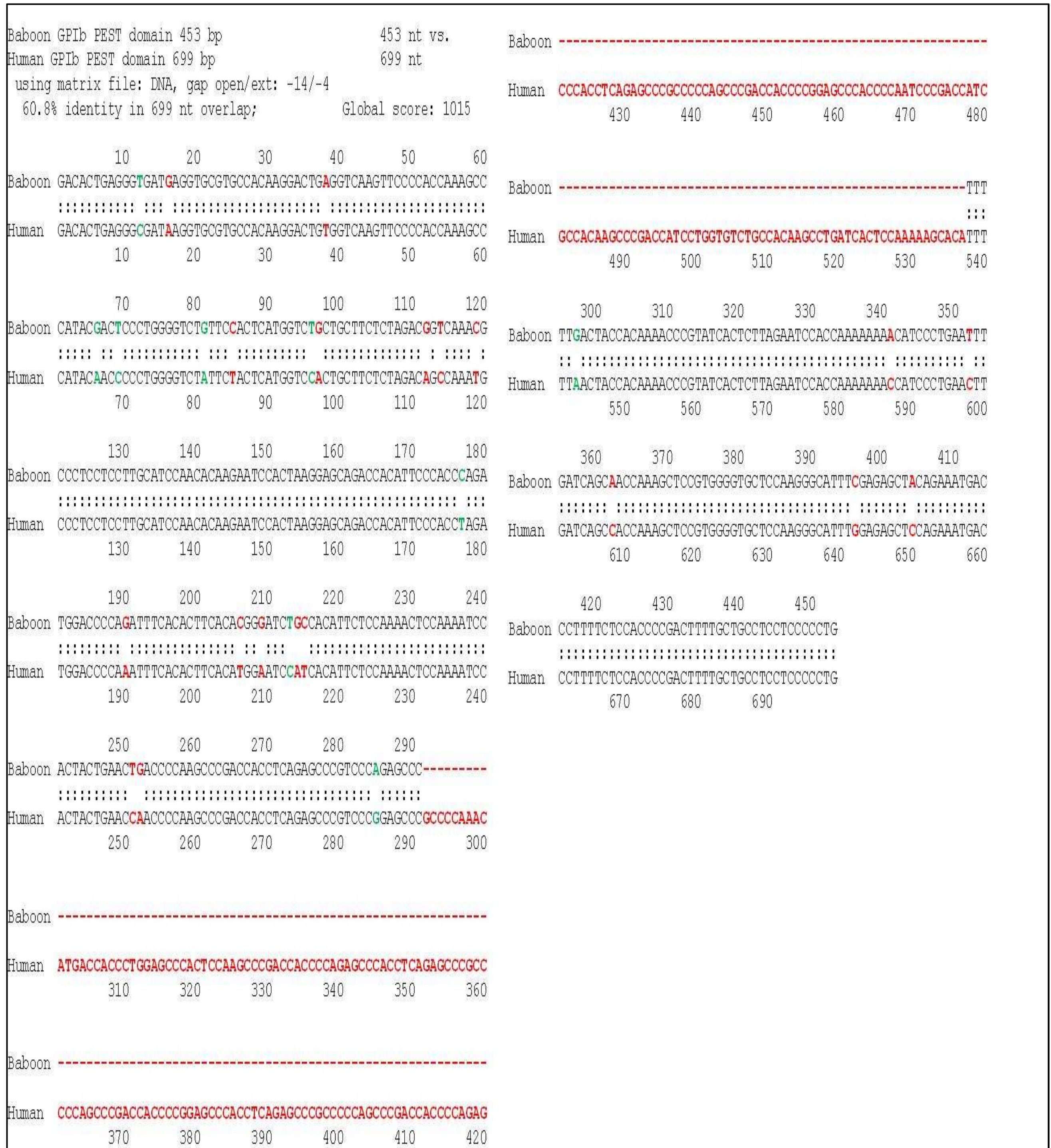


Figure 73. Comparison of the protein translating region nucleotide sequence between the PEST / macroglycoprotein region of the GPIbα gene of the baboon and human reference sequence. SNPs that cause amino acid changes are indicated in red and silent mutations in green.

```
Baboon GPIb Transmembrane domain 63 bp              63 nt vs.
Human GPIb Transmembrane domain 63 bp               63 nt
 using matrix file: DNA, gap open/ext: -14/-4
  96.8% identity in 63 nt overlap;            Global score: 297

               10        20        30        40        50        60
Baboon GGCTTCTATGTCTTGGGTCTCCTCTGGCTGCTCTTTGCCTCTGTGGTCCTCATCCTGATG
       ::::::::::::::::::::: :::::::::::::::::::::::::::::::::::: ::
Human  GGCTTCTATGTCTTGGGTCTCTTCTGGCTGCTCTTTGCCTCTGTGGTCCTCATCCTGCTG
               10        20        30        40        50        60


Baboon CTG
       :::
Human  CTG
```

Figure 74. Comparison of the protein translating region nucleotide sequence between the transmembrane domain of the GPIbα gene of the baboon and human reference sequence. SNPs that cause amino acid changes are indicated in red.

```
Baboon GPIb Cytoplasmic domain 303 bp          303 nt vs.
Human GPIb Cytoplasmic domain 303 bp           303 nt
 using matrix file: DNA, gap open/ext: -14/-4
  96.0% identity in 303 nt overlap;          Global score: 1407

                10        20        30        40        50        60
Baboon ATCTGGGTCAGGCATGTGAAACCACAGGCCCTGGACTCTGGCCAAGGTGCTCCTCTGGCC
       : ::::::  ::::::::::::::::::::::::::::::::::::::::: ::::: ::
Human  AGCTGGGTTGGGCATGTGAAACCACAGGCCCTGGACTCTGGCCAAGGTGCTGCTCTGACC
                10        20        30        40        50        60

                70        80        90       100       110       120
Baboon ACAGCCACACAAACCACACATCTGGAGCTGCAGAGGGGACGGCAAGTGACGGTGCCCCGG
       :::::::::::::::::::: :::::::::::::::::::::::::::::: :::::::::
Human  ACAGCCACACAAACCACACACCTGGAGCTGCAGAGGGGACGGCAAGTGACAGTGCCCCGG
                70        80        90       100       110       120

               130       140       150       160       170       180
Baboon GCCTGGCTGCTCTTCCTTCGAGGTTCACTCCCCACTTTCCGCTCCAGCCTCTTCCTATGG
       ::::::::::::::::::::::::::: :: ::::::::::::::::::::::::: :::
Human  GCCTGGCTGCTCTTCCTTCGAGGTTCGCTTCCCACTTTCCGCTCCAGCCTCTTCCTGTGG
               130       140       150       160       170       180

               190       200       210       220       230       240
Baboon GTACGGCCTAATGGCCGCGTGGGGCCTCTAGTGGCAGGAAGGAGGCCCTCAGCTCTGAGT
       ::::::::::::::::: ::::::::::::::::::::::::::::::::::::::::::
Human  GTACGGCCTAATGGCCGTGTGGGGCCTCTAGTGGCAGGAAGGAGGCCCTCAGCTCTGAGT
               190       200       210       220       230       240

               250       260       270       280       290       300
Baboon CAGGGTCGTGGTCAGGACCTGCTGGGCACAGTGAGCATTAGGTACTCTGGCCACAGCCTC
       :::::::::::::::::::::::: :::::::::::::::::::::::::::::::::::
Human  CAGGGTCGTGGTCAGGACCTGCTGAGCACAGTGAGCATTAGGTACTCTGGCCACAGCCTC
               250       260       270       280       290       300


Baboon TGA
       :::
Human  TGA
```

Figure 75. Comparison of the protein translating region nucleotide sequence between the cytoplasmic domain of the GPIbα gene of the baboon and human reference sequence. SNPs that cause amino acid changes are indicated in red and silent mutations in green.

Table 13 contains all the amino acid changes detected in the baboon GPIbα. Figure 73 show the comparison between the amino acid sequences of the GPIbα protein of the baboon and human. There is 93.8% similarity between

the protein sequences of the signal peptides. An 89.4% similarity between the protein sequences of the VWF-binding domains was detected. There is 57.9% similarity between the protein sequences of the PEST / macroglycoprotein region. A 90.5% similarity between the protein sequences of the transmembrane regions was found. There is 95.0% similarity between the protein sequences of the cytoplasmic domains.

A comparison was also made between the complete sequences of *P. ursinus*, *P. anubis* (Accession number: XP_009187694) and the human. When evaluating the whole sequence *P. ursinus* is 79.1% similar to the human and 84.3% similar to *P. anubis*. *Papio anubis* showed a 78.7% similarity to the human. Interestingly, *P. anubis* has 94 amino acids more than the human, all of them located within the PEST/macroglycoprotein region, this translated into a 62.1% similarity in this region. This is in contrast to the 65.2% similarity seen between *P. ursinus* and the human. Therefore, *P. ursinus* seems to be a more suitable candidate to test human targeted anti-GPIbα agents in than *P. anubis*.

Table 13. Amino acid changes found in the baboon GPIbα protein.

Region / Domain	Amino acid change
Signal peptide	His15Arg
VWF-binding domain	Arg20Ser
	Thr23Ala
	His37Gln
	Ser39Gly
	Glu40Lys
	Thr50Ser
	Met52Val
	Tyr54Phe
	Asn61Tyr
	Arg64Lys
	Gln88Lys
	Val104Ile

	Arg111Gln
	Arg121His
	Glu125Lys
	Asn159His
	Glu162Gln
	Asn169Asp
	Glu172Gly
	His195Pro
	Glu225Asp
	Ala244Thr
	Phe254Thr
	Tyr257His
	Gly271Asp
	Thr273Pro
	Asp274Gly
	Tyr278Asp
	Pro280Thr
	Glu281Asp
PEST / macroglycoprotein domain	Lys288Glu
	Val295Glu
	Tyr311His
	Thr315Ala
	Ser320Gly
	Met322Thr
	Asn346Asp
	Met351Thr
	Glu352Gly
	Ile354Ala
	Pro366Leu
	Thr478Asn
	Leu482Phe
	Pro485Gln

	Leu496Phe
	Ser499Tyr
Transmembrane domain	Phe523Leu
	Leu535Met
Cytoplasmic domain	Ser537Ile
	Gly540Arg
	Ala554Pro
	Thr556Ala
	Ser641Gly

The signal peptide of GPIbα contains 16 amino acids for both the baboon and human. Only one conservative amino acid substitution, a His at position 15 to an Arg, was found in this region in the baboon. Because it is a conservative substitution, it is unlikely to have an effect on the baboon GPIbα.

The N-Terminal globular region of GPIbα, spanning amino acids 1 to 282 of the GPIbα-protein, holds the entire capacity of the GPIb/IX/V complex to bind its ligands (Vanhoorelbeke *et al*., 2007). It is named the VWF-binding domain. The VWF-binding amino acids within GPIbα are Asp235 and Lys262 (Katagiri *et al*., 1990), with the anti-GPIbα antibody, 6B4, binding to Asp235 and Lys237 (Fontayne *et al*., 2007). The thrombin-binding domain is located between Phe216 and Alanine Ala274, with the region between Phe216 and Thr240 probably being the centre of the thrombin-GPIbα interaction (Katagiri *et al*., 1990). Andrews *et al.* (2003) determined the contact residues between the VWF-A1 domain and GPIbα at Ser11, His12, Glu14, Asn16, His37, Glu128, Lys152, Asp175, Thr176, Phe199, Glu225, Asn226, Tyr228 and Ser241.

Within the VWF-binding domain (figure 77) there were 30 amino acid changes found between the human and baboon GPIbα proteins. However, the VWF-binding amino acids Asp235 and Lys262, as well as the 6B4-binding Lys237, were all intact in the baboon sequence. Out of the 25 thrombin-binding amino acids between Phe216 and Thr240, only one conservative amino acid

substitution, Glu225Asp, were found in the baboon sequence. These results correlate well with previous functional studies where human-targeted 6B4 were able to successfully inhibit thrombosis in a baboon model (Fontayne *et al.*, 2008). When considering the contact amino acids described by Andrews *et al.* (2003), His37Gln and Glu225Asp were the only changes possibly influencing the VWF-GPIbα interaction. However, both the changes were conservative substitutions, and unlikely to inhibit the binding of VWF to GPIbα. On the other hand, the 30 amino acid changes within the N-terminal globular region may possibly explain why a commercial monoclonal antibody was not able to bind to baboon platelets within our study. The amino acid changes described by Li *et al.* (1995) and Kuijpers *et al.* (1992b) were not found in the baboon sequence.

Due to the massive variance found in the PEST / macroglycoprotein regions together with the lack of a functional description of this region, the investigator considered an attempt to fully discuss and interpret the results for this region as a futile exercise. Therefore, the results for this region were omitted from further discussion.

The baboon transmembrane region had two conservative amino acid substitutions, namely Phe523Leu and Leu535Met. Due to the fact that they are both conservative substitutions, they are unlikely to have an effect on the function of the baboon GPIbα.

Binding of 14-3-3ζ to GPIbα is essential for the signalling that ultimately leads to the activation of integrin GPIIb/IIIa (Gu *et al.*, 1999). 14-3-3ζ binds to the C-terminus of the cytoplasmic domain within GPIbα. The binding region is between Asp596 and Leu610. Five amino acids Ser606, Gly607, His608, Ser609 and Leu610, were found to be critical within this binding domain (Du *et al.*, 1996).

Five amino acid changes were found in the cytoplasmic domain. Three changes, Ser537Ile, Gly540Arg and Ala554Pro, were radical substitutions. However, they were not located in an area of this domain that has been

described as functionally important. Thus, it is improbable that they will have an effect on the function of baboon GPIbα. Two conservative substitutions, Thr556Ala and Ser611Gly, were also found. Because they are conservative substitution, they are not expected to cause an effect on baboon GPIbα. It must, however, be noted that Ser611Gly is situated within the 14-3-3ζ-binding area. Nevertheless, the critical amino acids, Ser606, Gly607, His608, Ser609 and Leu610, as found by Du *et al.* (1996), were all intact in the baboon sequence.

Because of the variance found within this receptor the predicted secondary structures, as well as predicted disorders of the baboon and human GPIbα proteins, were omitted from the results and discussion, as it would only cause confusion.

When taking all the GPIbα results into consideration, together with the fact that this specie has been successfully used in human targeted anti-GPIbα studies in the past, the *Papio ursinus* baboon is considered to be a suitable model to evaluate human targeted anti-GPIbα agents. The statement is made despite the fact that complete sequencing was unsuccessful, since sequencing of the functionally active regions was successful. The region that could not be sequenced is also known to be extremely variable between species, and the function of it is still to be described. Even though the sequence of the *Papio ursinus* baboon was incomplete for the PEST / macroglycoprotein region, it was still more similar to humans than other commonly used animals. It can also not be ruled out that the unsequenced fragment may actually not be present in the baboon sequence.

Special care should, nonetheless, be taken when interpreting results found with the *Papio ursinus* baboon, as sequence differences my cause interference in anti-GPIbα agent binding. Sequence differences can furthermore not be excluded as the reason more ristocetin is needed for platelet function studies in baboons. However, the increase in GPIbα receptor numbers is more likely the underlying cause.

```
Baboon MPLLLLLLLPSPLRPHPICEVSKVASHLEVNCDKSNLAALPPDLPKDTTILQLGKNLLY
                 ©               ® ©              © ©©     44
Human  MPLLLLLLLPSPLHPHPICEVSKVASHLEVNCDKRNLTALPPDLPKDTTILHLSENLLY

       Signal peptide [vWF-binding domain
--------------------------------------------------------------------
Baboon TFSLASLVPFTRLTQLYLDKCELTKLQVDGTLPVLGTLDLSHNKLQSLPLLGQTLPALTI
          © © ©   ® ©                        ©                © 104
Human  TFSLATLMPYTRLTQLNLDRCELTKLQVDGTLPVLGTLDLSHNQLQSLPLLGQTLPALTV

--------------------------------------------------------------------
Baboon LDVSFNQLTSLPLGALHGLGKLQELYLKGNELKTLPPGLLTPTPKLEKLSLANNHLTQLP
          ©         ©   ©                                   © ©  164
Human  LDVSFNRLTSLPLGALRGLGELQELYLKGNELKTLPPGLLTPTPKLEKLSLANNNLTELP

--------------------------------------------------------------------
Baboon AGLLDGLGNLDTLLLQENSLYTIPKGFFGSPLLPFAFLHGNPWLCNCEILYFRRWLQDNA
         © ®                   ®                                 224
Human  AGLLNGLENLDTLLLQENSLYTIPKGFFGSHLLPFAFLHGNPWLCNCEILYFRRWLQDNA

                                                          [Thrombin
--------------------------------------------------------------------
Baboon DNVYVWKQGVDVKAMTSNVTSVQCDNSDKTPVHKYPGKGCPTLGDEDDPGLYDDYTDEDT
       ©               ©        ® ©              ® ®®   ® ®©     284
Human  ENVYVWKQGVDVKAMTSNVASVQCDNSDKFPVYKYPGKGCPTLGDEGDTDLYDYYPEEDT
                ↑                           ↑
       binding         ]                     vWF-binding domain][
--------------------------------------------------------------------
Baboon EGDEVRATRTEVKFPTKAHTTPWGLFHSWSAASLDGQTPSSLHPTQESTKEQTTFPPRWT
         ©     ®             ©   ©   © ®                         344
Human  EGDKVRATRTVVKFPTKAHTTPWGLFYSWSTASLDSQMPSSLHPTQESTKEQTTFPPRWT

       PEST/Macroglycoprotein region
--------------------------------------------------------------------

Baboon PDFTLHTGSATFSKTPKSTTELTPSPTTSEPVPEP************************ 379
        ©   ®® ®          ®            [Non-sequenced fragment
Human  PNFTLHMESITFSKTPKSTTEPTPSPTTSEPVPEPAPNMTTLEPTPSPTTPEPTSEPAPS 404

--------------------------------------------------------------------
Baboon *********************************************************FLT 382
                                     Non-sequenced fragment]
Human  PTTPEPTSEPAPSPTTPEPTSEPAPSPTTPEPTPIPTIATSPTILVSATSLITPKSTFLT 464

--------------------------------------------------------------------
Baboon TTKPVSLLESTKKNIPEFDQQPKLRGVLQGHFESYRNDPFLHPDFCCLLPLGFYVLGLLW 442
                 ©  © ®        © ®                          ©
Human  TTKPVSLLESTKKTIPELDQPPKLRGVLQGHLESSRNDPFLHPDFCCLLPLGFYVLGLFW 524

                        PEST/Macroglycoprotein region][Transmembrane
--------------------------------------------------------------------
Baboon LLFASVVLILMLIWVRHVKPQALDSGQGAPLATATQTTHLELQRGRQVTVPRAWLLFLRG 502
                 © ®  ®          ® ©
Human  LLFASVVLILLLSWVGHVKPQALDSGQGAALTTATQTTHLELQRGRQVTVPRAWLLFLRG 584

       Region    ][Cytoplasmic region
--------------------------------------------------------------------
Baboon SLPTFRSSLFLWVRPNGRVGPLVAGRRPSALSQGRGQDLLGTVSIRYSGHSL 554
                                             ©
Human  SLPTFRSSLFLWVRPNGRVGPLVAGRRPSALSQGRGQDLLSTVSIRYSGHSL 636

                                       Cytoplasmic region]
```

Figure 76: Comparison of baboon and human GPIb alpha amino acid sequences. Green lettering indicates the signal peptide. The VWF-binding region is shaded in grey. VWF contact amino acids are in red lettering. Thrombin binding region is in italics. VWF binding amino acids are indicated with arrows. The 6B4 binding amino acids are in purple lettering. A conservative amino acid change is indicated with ©. A radical amino acid substitution is indicated with ®. The PEST/Macroglycoprotein domain is highlighted in yellow. The non-sequenced amino acids are shown with an asterisk (*). The transmembrane region is in blue lettering. The cytoplasmic region is in orange lettering.

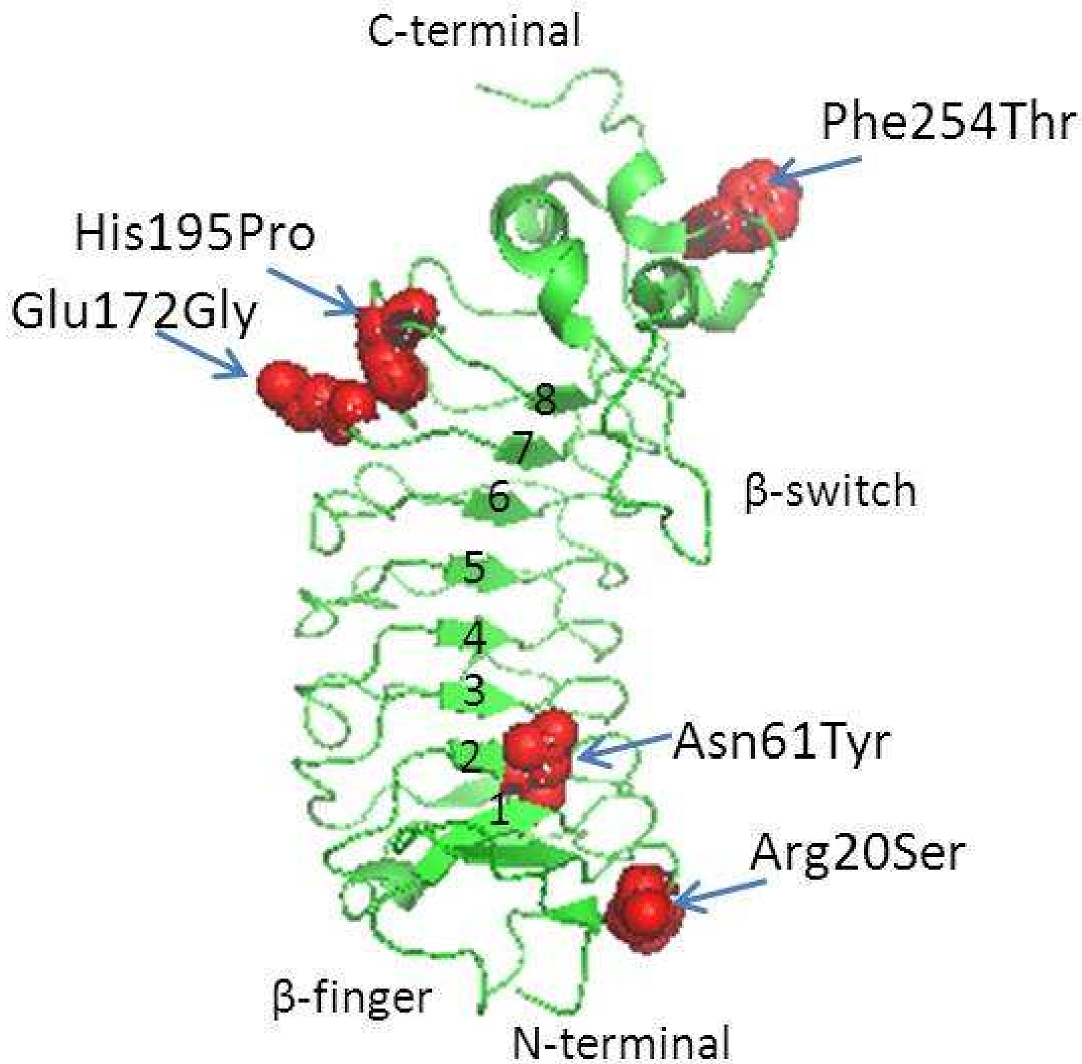


Figure 77. Crystal structure of the GPIb alpha VWF binding region as published by Huizinga *et al.* (2002), depicting radical amino acid changes found in the *P. ursinus* sequence (shown in red dots). Cartoon drawn with PyMol. Crystal structure available from RCSB Protein Data Bank (accession # 1QYY). The crystal structure only contained the VWF binding sequence, thus only some of the amino acid changes found in the baboon sequence could be indicated. Only Asn61Tyr is located near a Leu-rich repeat (repeat 2), however, this area is not implicated in VWF binding.

Chapter 5 – Conclusion

It is concluded that the Cape chacma baboon (*Papio ursinus*) is a suitable animal model for the evaluation of human-targeted anti-platelet agents directed against the receptors P2Y12, GPIIb/IIIa and GPIbα. The findings of this study correlated well with previously published studies where human-targeted P2Y12, GPIIb/IIIa and GPIbα-inhibiting agents were successfully evaluated in an arterial thrombosis model in these animals.

However, the receptor number differences between human and baboon platelets, regarding GPIIb/IIIa and GPIbα, should be carefully considered in any future studies, especially in dose-finding studies. The amino acid differences in all three of these receptors should also be considered when evaluating molecular-based inhibiting agents of these receptors, prior to commencement of a study. It is to ensure the compatibility of the baboon receptor and study agent. This issue was highlighted by the fact that a commercial monoclonal antibody did not bind to baboon GPIbα, but that the anti-human anti-GPIbα monoclonal antibody F(ab) fragment 6B4, whose complementary binding amino acid was intact in the baboon GPIbα, was able to bind to the baboon platelets. This study also emphasised the need for the use of higher agonist concentrations in evaluating platelet aggregation in baboons and should be kept in mind when planning future studies.

It was established that the Cape chacma baboon is not a suitable animal model for the evaluation of human-targeted anti-GPVI agents. The amino acid differences at strategic locations within the baboon GPVI sequence may be the reason behind non-reactivity of previously tested anti-human anti-GPVI agents in the Cape chacma baboon. Even though human- and methodological-error could not be excluded as reason for the non-binding of anti-GPVI monoclonal antibodies to the baboon platelets during quantification, it is believed that the lack of antibody-binding to the baboon platelets may rather be attributed to the sequence differences between the species. This hypothesis is made in the light of the fact that antibodies directed against the other receptors on the same platelets were able to successfully bind to the baboon platelets. The findings of this study also correlated well with findings from

previous functional-studies, where the baboon platelets were non-reactive to human-targeted anti-GPVI agents.

It was the first formal study undertaken to establish the molecular make-up of platelet receptors on the Cape chacma baboon platelets. The fact that we established that the Cape chacma baboon is a suitable model for evaluation of P2Y12, GPIIb/IIIa-and GPIbα-inhibiting agents, together with the unsuitability of the animal for anti-GPVI evaluation, greatly contributes to the translational value of the Cape chacma baboon as animal model for anti-thrombotic agent evaluation. The Cape chacma baboons' P2Y12, GPIIb/IIIa and GPIbα sequences have also shown to be more comparable to humans than the olive baboon, and thus a more suitable model to use in human targeted trials.

Limitations and future studies

This study had some limitations. The fact that antibodies against baboon platelet receptors were not freely available was a big limitation. Therefore, binding of antibodies could not be ensured prior to testing. The only antibody-binding epitope on a receptor we were certain of was for the 6B4 monoclonal antibody whose antibody-binding epitope was previously published. None of the commercial manufacturing companies were able or willing to divulge the antibody-binding epitopes of their commercial antibodies. It was especially restrictive in the case of GPIbα. The commercial antibody did not show binding to the baboon platelets, but because the company could not provide the antibody-binding epitope, the reason for non-binding could not be fully attributed to a definite amino-acid change between the human and baboon GPIbα sequences. The specific drawback was limited by using 6B4. However, the non-binding of the commercial anti-GPIbα monoclonal could still not be explained, due to lack of evidence.

Experiments studying platelet activation were not included in the flow cytometry experiments. Therefore, although measures were taken to limit platelet activation before platelet receptor quantification, platelet activation could not be excluded as the reason for the increased platelet numbers in the baboon experiments. However,

as we performed the human experiments at the same time using the same method, it is believed that the results obtained in this study are interpretable.

The variability within the PEST / macroglycoprotein region of the GPIbα gene made sequencing of this part of the gene extremely troublesome. It caused confusion in respect of the fragment size needed for sequencing and lead to numerous failed sequencing attempts. It was ultimately decided to abandon any further attempts to sequence the missing 249 bases in this region, for this study. Fortunately, the regions that were successfully sequenced gave enough information to make reasonable deductions from the results.

The lack of an anti-P2Y12 monoclonal antibody prevented the quantification of the P2Y12 receptor. Therefore, limiting the discussion and consequent conclusions to only the ADP-induced platelet aggregation and P2Y12 sequencing results. A possible future study is to develop a method to successfully quantify the number of P2Y12 receptors on the platelet surface. It could contribute to the explanation behind the limited aggregation seen in baboon platelets in response to ADP as the sequencing results proved inconclusive. Full sequence analysis of the intronic and exonic regions of P2Y12 could also be important for future studies, as it was found in another study that not only changes in amino acids may cause functional changes in the protein.

A further limitation of the study regarded the anti-GPVI monoclonal antibodies. No commercial antibodies are available against GPVI. The six antibodies we received as gifts did not react to the baboon platelets. Human and methodological error could not be excluded as reason for the non-binding of anti-GPVI monoclonal antibodies to the baboon platelets during quantification, because after non-binding to the baboon platelets, quantification using human platelets was not done. Therefore, it cannot be excluded that the antibodies were non-reactive due to degeneration during transportation since they were shipped at room temperature between France and Australia to South Africa, respectively. These antibodies are theoretically stable at room temperature; however, stability was not guaranteed. The antibody-binding epitopes were not known; therefore, non-reactivity could not conclusively be attributed to receptor sequence differences.

Another limiting factor which influenced the study was that primers had to be designed using the human reference sequence as a template. Numerous PCR attempts failed due to non-reactive primers. The commercial company that assisted in sequencing GPIIb, GPIIIa and GPIbα were not able to sequence ten exons (exons 1, 2, 3, 4, 14, 15, 19, 20, 25, 26 and 28) from GPIIb, three exons (exons 10, 11 and 12) from GPIIIa, and over a thousand bases from exon 2 of GPIbα. They used human targeted primers and had successfully sequenced human control samples concurrently with the baboon samples. Primers were redesigned for those specific fragments. For some fragments only one new primer pair was needed, while for other fragments (such as parts of exon 2 of GPIbα) numerous different primers and different cycling conditions were needed to successfully sequence that specific fragment. Sequencing of exon 28 of GPIIb continued to be unsuccessful after numerous attempts. Due to the minimal amount of vital amino acids contained within exon 28, it was decided to leave it for a future study.

Future studies planned after completion of this study is the determination of the ideal anticoagulant for LTA testing, sequencing of exon 28 of GPIIb, the complete sequencing of GPIbα, the complete molecular characterisation of VWF, ADAMTS13, cyclooxygenase, thromboxane, as well as the coagulation proteins of the Cape chacma baboon. Determination of crystal structures for the receptors and their ligands may also be a valuable future study. Taking into account that the University of the Free State recently acquired a next-generation sequencer, the University could make a valuable contribution to the Baboon Genome Project of BCM-HGSC.

Impact of study

The results of the study have been published in or submitted to peer reviewed journals. It is believed that the study has greatly enhanced the knowledge of the *Papio ursinus* haemostatic system. The fact that agonist concentrations were formally established for baboon studies filled an important void in pre-clinical studies by taking the guessing out of baboon platelet function studies. Establishment of receptor numbers also gave vital information regarding the need for different agonist concentrations. The vast amount of genetic material gathered is central for any future use of *Papio ursinus* as model in platelet studies. Collectively, the results from

this study contribute to the translatability of *Papio ursinus* in human targeted anti-platelet studies. It was the first formal study on the genetic make-up of platelet receptors in baboons, and has opened up the field of molecular exploration into the haemostatic system of the species.

Summary (English)

Keywords: *Acute coronary syndrome • Anti-platelet agents • Platelet receptors • P2Y12 • Glycoprotein VI • Glycoprotein IIb/IIIa • Glycoprotein Ibα • Cape chacma baboon*

Background: Acute coronary syndrome is globally a major cause of morbidity and mortality. Treatment and prevention involve the use of an anti-platelet agent. The current available agents have either side-effects or are relatively ineffective. Therefore, there exists a need to develop safer and more effective agents. Platelet receptors are a target for anti-platelet agents and new generation agents function on a molecular level. The Cape chacma baboon (*Papio ursinus*) has been a popular model for the pre-clinical evaluation of anti-platelet agents. However, limited molecular data are available for these animals, restricting its translational value. The aim of this study was to characterize four common platelet receptors in the Cape chacma baboon and compare the results to human data.

Methods: The platelet receptors P2Y12, glycoprotein (GP) VI, GPIIb/IIIa and GPIbα were selected for this study. Light transmission platelet aggregometry was performed to assess baboon platelet function; receptor number quantification was performed by flow cytometry; and Sanger sequencing was done on genomic baboon DNA. All results were compared to normal human data.

Results: Baboon ADP-induced platelet aggregation results were significantly different from normal human results, even at ADP levels four times (40 µM) the highest human concentration of 10 µM. Baboon collagen-induced aggregation remained significantly different at twice (8 µg/ml) the highest human concentration of 4 µg/ml. However, the differences in collagen-induced aggregation results were not clinically relevant from the human results, because all except one result (at 8 µg/ml) fell within the normal human reference range. At double the highest human concentration for ristocetin (2.5 mg/ml) baboon platelets gave statistically similar

results. At double the highest human concentration (1 mg/ml) arachidonic acid results remained significantly different between baboons and human.

Baboon quantification results showed a 37% increase in GPIIb, 27% increase in GPIIIa and 25.5% increase in GPIbα. GPVI quantification failed due to non-reactive monoclonal antibodies. P2Y12 quantification was not possible, as no commercial monoclonal antibodies exist for it.

The P2Y12 protein sequence was 98.8% similar. It differed by only four amino acids, none of which have been described as functionally essential. The GPVI protein sequence showed 95% similarity. It included a 14 amino acid difference and a three amino acid deletion. One change was at a region where an amino acid change has been implicated in reduced collagen-induced platelet aggregation in humans. Two differences were directly adjacent to a collagen-binding amino acid. The deletion was within the signalling region of GPVI. Exon 28 of GPIIb could not be sequenced. The GPIIb protein sequence for exon 1-27 was 98.2% similar and for exons 29-30 there was 98.3% similarity. There was an 18 amino acid difference. One amino acid change was in the ligand-binding region. The GPIIIa protein sequence was 99.6% similar, with three amino acid changes. One change was in the ligand-binding region. 54 amino acid changes were found in GPIbα. The protein sequences of the signal peptide, VWF-binding-, PEST / macroglycoprotein-, transmembrane- and cytoplasmic domains showed 93.8%, 89.4%, 57.9%, 90.5% and 95.0% similarity, respectively. 246 bases of GPIbα failed to sequence.

Discussion and Conclusion: Sequentially and functionally baboon P2Y12, GPIIb/IIIa and GPIbα is comparable to humans. The higher agonist-levels needed for baboon platelet aggregation may be attributed to the increase in surface receptor numbers. However, receptor-number, optimal agonist concentrations and potentially inhibiting amino acid changes should be noted for future studies. Non-reactive antibodies and changes in critical amino acids caused the baboon GPVI to be not comparable to humans. The Cape chacma baboon (*Papio ursinus*) is therefore, deemed a suitable animal model for the evaluation of human-targeted anti-platelet agents directed against the receptors P2Y12, GPIIb/IIIa and GPIbα, but not for the evaluation of human-targeted anti-GPVI agents.

Opsomming (Afrikaans)

Sleutelwoorde: *Akute koronêre sindroom • Anti-plaaitjiemiddels • Plaaitjiereseptore • P2Y12 • Glikoproteïen VI • Glikoproteïen IIb/IIIa • Glikoproteïen Ibα • Kaapse bobbejaan*

Agtergrond: Akute koronêre sindroom is wêreldwyd ‘n belangrike oorsaak van morbiditeit en mortaliteit. Anti-plaatjiemiddels word vir voorkoming en behandeling gebruik, maar die beskikbare produkte het newe-effekte en is nie ewe effektief nie. Daarom is daar 'n behoefte om veiliger en meer doeltreffende middels te ontwikkel. Plaatjieoppervlak-reseptore is 'n teiken vir anti-plaatjiemiddels en die nuwe generasie middels funksioneer op 'n molekulêre vlak. Die Kaapse bobbejaan (*Papio ursinus*) is 'n gesogte model vir die pre-kliniese evaluering van nuwe anti-plaatjiemiddels. Die beskikbare molekulêre data vir hierdie diere is egter onvoldoende en beperk die oorplasingswaarde van die spesie. Die doel van hierdie studie was dus om vier algemene plaatjiereseptore in die Kaapse bobbejaan te karakteriseer en die resultate met beskikbare menslike data te vergelyk.

Metodes: Die plaatjiereseptore P2Y12, glikoproteïen (GP) VI, GPIIb/IIIa en GPIbα is vir hierdie studie geselekteer. Lig-oordrag-plaatjie-aggregometrie is uitgevoer om bobbejaan-plaatjiefunksie te bepaal; reseptorkwantifisering is deur middel van vloeisitometrie gedoen; en Sanger DNS-volgordebepaling is op genomiese bobbejaan-DNS gedoen. Alle resultate is met normale menslike data vergelyk.

Resultate: Bobbejaan-ADP-geïnduseerde plaatjieaggregasie resultate het beduidend van dié by die mens verskil, selfs met ADP-vlakke vier maal hoër as die hoogste menslike konsentrasie (40 mM). Bobbejaan-kollageen-geïnduseerde aggregasie het ook beduidend van die mens verskil teen twee keer die hoogste menslike konsentrasie (8 mg/ml). Die verskille was egter nie klinies relevant nie aangesien almal behalwe een bobbejaan se resultate (teen 8 mg/ml) binne die normale menslike verwysingsreikwydte geval het. Teen dubbel die hoogste menslike konsentrasie vir ristocetin (2.5 mg/ml) het bobbejaanplaatjies statisties soortgelyke agglutinasieresultate getoon. Teen dubbel die hoogste menslike konsentrasie vir aragidoonsuur (1 mg/ml) het resultate aanduidend verskillend gebly.

Bobbejaan-kwantifiseringresultate het in vergelyking met mensplaatjies 'n 37% toename in GPIIb, ‘n 27% toename in GPIIIa en ‘n 25.5% toename in GPIbα getoon. GPVI kwantifisering het misluk as gevolg van nie-reaktiewe monoklonale teenliggaampies. P2Y12 kwantifisering was nie moontlik nie, aangesien daar geen kommersiële monoklonale teenliggaampies teen P2Y12 bestaan nie.

Die P2Y12-aminosuurvolgorde was 98.8% soortgelyk. P2Y12 het met net vier aminosure verskil, waarvan geeneen as funksioneel noodsaaklik beskou word nie. Die GPVI-aminosuurvolgorde het 'n 95% ooreenstemming. Dit sluit 'n 14-aminosuurverskil en 'n drie-aminosuurdelesie in. Een verandering was in 'n gebied waar 'n aminosuurverandering in mense beskryf is wat tot verminderde kollageen-geïnduseerde plaatjieaggregasie lei. Twee verskille was ook direk aangrensend aan ‘n kollageen-bindende aminosuur. Die delesie is geleë in die seinstreek van GPVI. Ekson 28 van GPIIb se volgordebepaling was onsuksesvol. Die GPIIb-aminosuurvolgorde vir ekson 1-27 was 98.2%, en vir eksons 29-30, 98.3% ooreenstemmend met 'n 18-aminosuurverskil. Slegs een aminosuurverandering was in die ligandbindingstreek. Die GPIIIa-aminosuurvolgorde was 99.6% ooreenstemmend, met drie aminosuurveranderinge. Een verandering was in die ligandbindingstreek. 54-aminosuurveranderings is in die GPIbα opeenvolging gevind. Die aminosuurvolgorde van die seinpeptied, VWF-bindingsdomein, PEST / makroglikoproteïen-, transmembraan- en sitoplasmiese-domein het onderskeidelik 93.8%, 89.4%, 57.9%, 90.5% en 95.0% ooreenstemming getoon. 246 basisse van GPIbα se basisopeenvolgingbepaling het misluk.

Bespreking en Gevolgtrekking: Op basisopeenvolging- en funksionele vlak is die bobbejaan P2Y12, GPIIb/IIIa en GPIbα vergelykbaar met mense. Die hoër agonisvlakke wat vir bobbejaan-plaatjieaggregasie benodig word kan aan ‘n toename in oppervlakreseptor toegeskryf word. Reseptor-hoeveelheid, die optimale agonis konsentrasies en moontlike inhiberende aminosuurverskille moet egter in gedagte gehou word met toekomstige studies. Nie-reaktiewe teenliggaampies en veranderinge in kritiese aminosure het veroorsaak dat GPVI nie vergelykbaar met mense is nie. Die Kaapse bobbejaan (*Papio ursinus*) word dus as ‘n geskikte dieremodel vir die evaluering van mensgerigte antiplaatjiiemiddels teen die reseptore

P2Y12, GPIIb/IIIa en GPIbα beskou, maar nie vir die evaluering van mensgerigte anti-GPVI middels nie.

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Appendix A

Animal welfare sheet

Baboon: ____

Date	Day	Feeding	Bleeding	Haematomas	Vomiting	Abnormal behaviour	Other
	0						
	1						
	2						
	3						
	4						
	5						
	6						
	7						

Appendix B

Informed Consent Form

Quantification of platelet receptors with flow cytometry

To Project Participant:

You are invited to take part in a research project conducted by Mr. Wattie van Rensburg, a doctoral student at the Department of Haematology & Cell Biology, University of the Free State, Bloemfontein, South Africa. In this study, we will quantify the number of platelet receptors on human platelets and compare the percentage of receptors on human platelets to the percentage of the same receptors on baboon platelets. The receptors that will be quantified are glycoprotein (GP) Ibα, GPIIb, GPIIIa and GPVI. These receptors play vital roles in blood platelets adhering to, and clumping at a site of blood vessel wall injury. This process helps to limit blood loss at the site of injury by forming a platelet plug. We hope that our research will establish the differences in receptor numbers on human and baboon platelets. This might in future assist with the better selection of animal models for platelet research and lead to the development of better anti-platelet drugs used to help treat and prevent heart-attack and stroke.

You were selected to participate in this study by a process of random selection.

We need one tube (5 ml) of blood and your time involvement will approximately be five (5) minutes as the sample is drawn. There will be no further engagements, remunerations or costs involved. Your blood will not be used for any other purposes than the one named above.

The risks should not exceed those that are normally expected in donating blood samples for scientific purposes. You may experience a slight degree of pain and discomfort during the procedure and adverse effects like a slight bruise or swelling at the site of puncture.

We do not expect any of these adverse medical effects to occur. However, if you do experience any medical problems as a result of your participation, the

necessary medical treatment within the scope of the Haematology Clinic will be provided.
Reports resulting from this study might in future be used for scientific publication purposes, but will not identify you as a participant. All information gathered in this study will remain confidential and be given out only with your permission or as required by law. If you give us permission by signing this consent form, we will protect your confidentiality.

If you have any questions about this research at any time, please call Mr. Wattie van Rensburg at 083 507 2659 or contact the Dept of Haematology and Cell Biology at Universitas Hospital.

By signing this consent form, you indicate that you have read the form and agree voluntarily to participate in the study.
If you choose not to take part there will be no penalty or loss of benefits to which you are entitled. If you agree to take part, you are free to withdraw from it at any time. Likewise, no penalty or loss of benefits to which you are otherwise entitled will occur.

I agree to participate in the study involving the quantification of platelet receptors by flow cytometry, as set out above.

………………………….   ……………….

Signature   Date

THIS PROJECT HAS BEEN REVIEWED BY THE ETHICAL REVIEW COMMITTEE OF THE UNIVERSITY OF THE FREE STATE, BLOEMFONTEIN, SOUTH AFRICA FOR THE PROTECTION OF HUMAN SUBJECTS IN RESEARCH. ADDITIONAL CONCERNS, COMPLAINTS OR QUESTIONS REGARDING YOUR RIGHTS AS A RESEARCH PARTICIPANT, SHOULD BE DIRECTED TO THE RESEARCH ADMINISTRATION DEPARTEMENT OF THE UNIVERSITY OF THE FREE STATE.

Appendix C

Ingeligte Toestemmingvorm

Kwantifisering van plaatjiereseptore deur middel van vloeisitometrie

Aan projek-deelnemer:

U word hartlik uitgenooi om deel te neem aan ‘n navorsingsprojek onderneem deur Mnr. Wattie van Rensburg, ‘n doktorale student by die Departement van Hematologie en Selbiologie, Universiteit van die Vrystaat, Bloemfontein, Suid-Afrika. Hy beoog om bloedplaatjiereseptore op mensplaatjies te kwantifiseer en dit met die hoefeelheid ooreenstemmende reseptore op bobbejaan plaatjies te vergelyk. Die reseptore ter sprake is glikoproteïen (GP) Ibα, GPIIb, GPIIIa en GPVI. Dié reseptore speel ‘n belangrike rol in die klomping en klewing van bloedplaatjies by die plek van bloedvatwandbeskadiging. Dié proses help om bloedverlies te beperk deur ‘n plaatjie-prop te vorm. Ek hoop dat die projek die verskille in reseptorgetalle tussen mens- en bobbejaanplaatjies sal vestig. Dit kan lei tot die beter seleksie van diere-modelle vir plaatjienavorsing, en sodoende tot die ontwikkeling van beter anti-plaatjiemiddels wat gebruik word in die voorkoming en behandeling van beroertes en hartaanvalle.

U is deur ‘n lukrake seleksie proses gekies om deel te neem aan die projek.

Ons benodig een buis (5 ml) van u bloed en die versameling daarvan sal ongeveer vyf (5) minute van u tyd in beslag neem. Daar sal geen verdere verpligtinge of kostes vir u betrokke wees nie. U bloed sal vir geen ander doeleindes as die genoemde een gebruik word nie.

Die risiko’s behoort nie die wat normaalweg met bloedskenk vir wetenskaplike doeleindes gepaard gaan, te oorskry nie. U kan ‘n klein bietjie pyn en ongemak gedurende die prosedure ervaar, asook newe-effekte soos ‘n klein kneusplek of swelling by die punksiemerk.

Ons verwag egter geen newe-effekte nie, maar as u enige mediese probleme as gevolg van u deelname ervaar, sal u die nodige mediese behandeling binne die vermoë van die Hematologiekliniek ontvang.

Die resultate van die studie kan in die toekoms vir wetenskaplike publikasiedoeleindes gebruik word, maar u sal nie as 'n deelnemer geïdentifiseer word nie. Alle informasie wat in die studie versamel word sal vertroulik bly en slegs met u toestemming bekend gemaak word of soos deur die wet verlang word.
Indien u ons toestemming gee deur die vorm te teken, sal ons u identiteit beskerm.

Indien u enige vrae het in verband met die navorsing, kan u gerus vir Mnr. Wattie van Rensburg by 083 507 2659 skakel, of die Departement van Hematologie en Selbiologie by die Universitas Hospitaal kontak.

Deur die toestemmingsvorm te teken bevestig u dat u die vorm gelees het en vrywillig instem om aan die studie deel te neem. Indien u instem om deel te neem, is u geregtig om enige tyd te onttrek. Dienooreerkomstig, sal daar ook geen straf of benadeling wees nie.

Ek stem in om aan projek wat die kwantifisering van plaatjiereseptore deur middel van vloeisitometrie behels deel te neem, soos hierbo uiteengesit.

………………………… …………………

Handtekening Datum

Appendix D

Article: Janse van Rensburg WJ, Badenhorst PN & Roodt JP. The Cape Chacma baboon is not suitable for evaluating human targeted anti-GPVI agents. Platelets 2014; Early Online: 1-6, DOI: 10.3109/09537104.2014.952224.

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ORIGINAL ARTICLE

The Cape Chacma baboon is not suitable for evaluating human targeted anti-GPVI agents

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Abstract

An effective and safe anti-platelet drug is central to the management of patients with acute coronary syndrome (ACS). Glycoprotein VI (GPVI) is currently regarded as a potential target for novel anti-platelet agents due to its collagen-binding potential. Development of anti-thrombotics is associated with testing in animals. We have previously successfully evaluated anti-platelet drugs in the Cape Chacma baboon (*Papio ursinus*). However, various anti-GPVI agents did not have an effect on baboons when evaluated in our arterial thrombosis model. To evaluate the suitability of baboons for GPVI studies, we performed collagen-induced platelet aggregation, GPVI quantification and DNA sequencing. Baboon platelets needed double the amount of collagen compared to human platelets to illicit proper aggregation. GPVI quantification was unsuccessful due to non-binding of monoclonal antibodies. Sequencing of the GPVI gene revealed 36 SNPs leading to 14 amino acid changes, as well as a 9 bp deletion causing a 3 amino acid deletion. Several of the amino acid changes were within the ligand binding region of GPVI, causing limited binding of humanized anti-GPVI antibodies to the baboon platelets. Therefore, the baboon was deemed not suitable to evaluate human targeted anti-GPVI agents.

Keywords

Animal model, anti-GPVI Basic

History

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Introduction

Acute coronary syndrome (ACS) is caused by occlusion of the coronary arteries of the heart. ACS includes conditions such as unstable angina, ST-elevation myocardial infarction (STEMI) and non-STEMI [1].

The American College of Cardiology Foundation and the American Heart Association recommend the use of a multi-drug treatment regimen in managing ACS because of the complexity involved with thrombosis. Central to the treatment are effective and safe anti-platelet drugs, such as aspirin, clopidogrel or a glycoprotein (GP) IIb/IIIa inhibitor [2]. The search for new anti-platelet drugs presents an ongoing necessity for safe and more discriminating animal models to evaluate these drugs. Usually, novel anti-platelet drugs are initially tested in lower mammals (such as rats, mice, rabbits). It is recommended that larger animals, and if possible non-human primates, be used as translational proof of concept prior to commencing clinical trials in humans [3]. The type of animal used in thrombotic studies is selected by comparing its blood coagulation, platelet adhesion and aggregation and fibrinolytic systems with that of humans. Non-human primates are considered the most appropriate animals when taking these requirements into consideration [4].

Anti-platelet drugs usually target specific receptors on the platelet surface. These receptors play a part in platelet adhesion (e.g. GPIb/V/IX complex, GPIa/IIa, GPVI), activation (e.g. P2Y12, P2Y1) and aggregation (e.g. GPIIb/IIIa). Various drugs have been developed against some of these receptors; however, the search for a drug that is both effective and safe is still ongoing [5]. In recent years the collagen receptor, GPVI, has been identified as a very promising target for anti-platelet drugs, as patients with GPVI deficiency only have mild bleeding tendencies [6–12]. Several different anti-GPVI agents have been tested in the past with mixed results, some being very effective and safer than current drugs, while others caused worrisome adverse effects, such as thrombocytopenia [13–19]. Therefore, taking the conflicting evidence and the absence of a commercial anti-GPVI agent into account, GPVI still remains an avenue for exploration.

In our setting we have successfully used the Cape Chacma baboon (*Papio ursinus*) to evaluate the safety and efficacy of other anti-platelet drugs, such as the GPIIb/IIIa inhibitor, tirofiban hydrochloride [20], the GPIb antagonist, 6B4 [21, 22], and the Von Willebrand factor (vWF) nanobody ALX-0081 [23], in arterial thrombosis models. This species has also been used successfully in the evaluation of the efficacy of an anti-vWF antibody [24], GBR600, and an anti-vWF nanobody, ALX-0681 [25], in the treatment of thrombotic thrombocytopenic purpura (TTP) in a TTP model in baboons. However, after numerous failed attempts to evaluate the efficacy and safety of different anti-GPVI agents in the baboon model, we decided to evaluate the GPVI receptor on baboon platelets at a molecular level. The aim of this study was to quantify the number of GPVI receptors on the baboon platelet, to do collagen-induced platelet aggregometry,

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and perform DNA sequencing to compare the baboon receptor to the human reference sequence for GPVI.

Materials and methods

Human ethics approval was granted by the Ethics committee at the Faculty of Health Sciences, University of the Free State, Bloemfontein, South Africa (ECUFS #133/2011). Animal ethics approval was granted by the Interfaculty Control Committee on Animal Experimentation of the University of the Free State, Bloemfontein, South Africa (Animal Experiment #18/2011). The guidelines and requirements of this committee are in accordance with the South African National Standard for the care and use of animals for scientific research (document SANS 10386 of 2008).

Ten Cape Chacma baboons were used to quantify the receptors and do platelet aggregation, and one baboon was used to determine the wild-type of the baboon GPVI sequence. All animals were housed at the primate facility on the campus of the University of the Free State, and were monitored for 30 days prior to taking blood, and all were deemed healthy and without any disease or infection. Animals were anaesthetized by injection of 0.1 ml/kg Ketamine intramuscularly. Twenty milliliters (4 × 5 ml tubes) of venous blood were collected in tubes containing 3.2% sodium-citrate (1 part sodium citrate to 9 parts blood; BD Vacutainer, Becton Dickinson, Woodmead, South Africa) and 5 ml of blood were collected in a tube containing EDTA (BD Vacutainer, Becton Dickinson, Woodmead, South Africa). Citrated blood was used for platelet aggregometry and the EDTA blood was used for flow cytometry. Accurately 500 µl of the EDTA whole blood was blotted onto FTA™ paper (Whatman™, Buckinghamshire, UK) for DNA extraction and storage.

All laboratory work, except the final sequencing reactions, was done at the Department of Hematology and Cell Biology at the University of the Free State. Light-transmission collagen-induced platelet aggregometry was performed on a Chrono-log platelet aggregometer (Chrono-log Corporation, Havertown, PA) at 37 °C using platelet-rich plasma (PRP). PRP were prepared by centrifuging the samples at 200 *g* for 20 minutes at room temperature. We used three different concentrations (4, 6 and 8 µg/ml) of HORM® collagen (Takeda, Linz, Austria) to induce aggregation. The lowest concentration we used (4 µg/ml) is the highest concentration used for human platelet function aggregometry in our diagnostic laboratory. We used HORM® collagen (type I equine tendon fibrils) as we found previously that baboon platelets do not always react to other types of collagen. We compared our results to historical data from normal human volunteers used during routine platelet function aggregometry, with $p < 0.05$ taken as a significant difference. Unfortunately we did not perform platelet counts on the baboons, but considered all samples as having normal platelet counts. The normal platelet count for *Papio* species is $233–399 \times 10^9$/L [26], which correlates well with the normal human reference range of $150–450 \times 10^9$/L [27].

GPVI quantification was done with the Platelet Calibrator kit (Biocytex, Marseille, France) according to the manufacturer's method, using the BD FACSCalibur® flow cytometer (Becton Dickinson, Woodmead, South Africa). Six different anti-GPVI monoclonal antibodies (all IgG_1 subtype) were used (one of these monoclonal antibodies, 3J24.2, was supplied by Dr. Martine Jandrot-Perrus from The French Institute of Health and Medical Research, Unit of Hemostasis, Bio-Engineering, Immunopathology and Cardiovascular Remodelling, Hôpital Bichat-Claude-Bernard, Paris, France; the other five (lA12, 4B8, 12A5, 12C9, 12H1) were supplied by Prof. Rob Andrew from Monash University, Melbourne, Australia). Antibodies were used at a concentration of 10 µg/ml. The Platelet Calibrator kit contains a secondary monoclonal antibody directed against mouse IgG to detect binding.

Sequencing was done on one animal to determine a wild type. When big variations were detected, the specific exon was sequenced for two extra animals, to determine if it is animal or species specific. Genomic DNA was extracted from FTA™ paper (Whatman™, Buckinghamshire, UK) using a methanol extraction method [28]. Primers were designed with Primer3Plus online primer designing tool [29] using the human reference sequence as template. We performed a polymerase chain reaction (PCR) using a Phusion® Blood Direct PCR Kit (Thermo Scientific, available from Inqaba Biotec, Pretoria, South Africa). The PCR product was then run on a 2% agarose gel at 150 volts for between 40 and 60 minutes to determine if we have produced a DNA fragment and if it was of the right fragment size. Most of the reactions gave more than one fragment on electrophoresis. The fragment of the correct size was then removed from the gel and purified with a Zymoclean™ Gel DNA Recovery Kit (Zymo Research, available from Inqaba Biotec, Pretoria, South Africa).

We sent our purified samples together with our primers we used for PCR, to Inqaba Biotec, Pretoria, South Africa, for sequencing on their ABI 3130XL sequencer (Applied Biosystems®, available from Life Technologies™, Johannesburg, South Africa). Sequence data were analyzed using Chromas Lite [30]. Sequences were compared to the human reference sequence [31] using the online alignment program Lalign [32]. The Lalign program uses the algorithm from Huang and Miller (1991) to calculate the percentage similarity between the two sequences [33]. We translated the nucleotide sequence to the amino acid sequence using the online translation tool from ExPASy [34] and also compared the amino acid sequence of the baboon to the human amino acid sequence human using the Lalign program [33].

Results

Collagen-induced platelet aggregation

Table I contains the results for collagen-induced platelet aggregation at the different concentrations. We found that at 4 µg/ml half of the baboon samples (5/10) did not reach 5% aggregation. At 6 µg/ml, 40% of the baboon samples (4/10) also did not reach 5% aggregation. At double the maximum human concentration (8 µg/ml), all of the samples showed substantial aggregation. Statistically there was a significant difference between baboon aggregation at 8 µg/ml and maximum human aggregation results (baboon: 56.7% ± 5.46%; human: 66% ± 16%). Nevertheless, this difference may not be of clinical importance, since all the baboon samples did demonstrate substantial aggregation at this concentration, with a minimum aggregation of 48% (Baboon 7), only 2% less than the lower limit of the human reference range. The mean percentage aggregation ± standard deviation for baboon platelets at 8 µg/ml (56.7% ± 5.46%), also fell within the normal range for humans (50–76%). Therefore, the aggregation of baboon platelets at double the maximum human collagen concentration is comparable to human platelet aggregation at the maximum concentration.

Quantification results

GPVI could not be quantified on any of the baboons' platelets, as none of the six monoclonal antibodies were able to bind to any of the animals' platelets, even though the antibodies were able to bind to human platelets in previous studies [9, 35]. Glycoproteins Ibα, IIb and IIIa were all successfully quantified using this quantification technique on the same samples. It must,

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Table I. Collagen-induced platelet aggregation expressed as percentage.

	Collagen-induced platelet aggregation			
Baboon	4 μg/ml	6 μg/ml	8 μg/ml	Human 4 μg/ml
1	20	50	53	
2	1*	1*	56	
3	2*	50	59	
4	30	49	53	
5	8	53	55	$N = 38$
6	1*	3*	56	
7	2*	2*	48	
8	1*	3*	56	
9	53	53	66	
10	49	49	65	
Mean	16.7	31.3	56.7	66.0
Standard deviation	20.54	25.05	5.46	16.0
p Value	0.000014	0.00167	0.0065	

Results marked with an * had less than 5% aggregation.

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however, be noted that we cannot exclude artefact of sample preparation for our GPVI findings, as we did not perform GPVI quantification on human platelets following the failure of the baboon platelets to react. GPVI is known to be shed from the platelet surface, and thus, a failure to successfully protect the platelets from shedding GPVI could have resulted in our experiments not working. However, we added 20 mM EDTA to all our flow cytometry buffers to prevent shedding.

Sequence results

Figure 1 shows the comparison of the nucleotide sequence of the baboon and human, and Figure 2 shows the comparison of the predicted amino acid sequences. Several single nucleotide polymorphisms (SNPs) were detected between the human and baboon. A total of 36 SNPs were detected in the baboon GPVI sequence when compared with the human GPVI reference sequence. Of the 36, 23 SNPs were silent mutations, which did not cause an amino acid change. There were, however, 14 SNPs which caused a change in 14 amino acids. A 9 bp deletion was also found from position 942 to 950 (after position 166 in exon 8). When evaluating the nucleotide sequence, the baboon GPVI has a 95.6% similarity to the human GPVI receptor [36]. There was a 95% similarity in the amino acid sequence. The 14 amino acid changes detected were Arg46Ser (R46S), Arg60Lys (R60K), Ser61Arg (S61R), Leu78Pro (L78P), Val181Met (V181M), Pro199Ser (P199S), Ser204Thr (S204T), Phe214Leu (F214L), Tyr244His (Y244H), Ile262Leu (I262L), Gly282Val (G282V), Asn302His (N302H), Gly304Asp (G304D) and Gln310Pro (Q310P). Leu295 (L295), Pro296 (P296) and Leu297 (L297) were deleted from the baboon sequence.

Discussion

The collagen binding regions on GPVI are Lys59, Val34 and Leu36 [37, 38]. It can, therefore, be postulated that for a monoclonal antibody to directly inhibit collagen binding to GPVI, it needs to bind to Lys59, Val34 or Leu36. The monoclonal antibodies used for flow cytometry receptor quantification should bind to these epitopes, as these antibodies should only bind to and not cause depletion of GPVI. It was found that Lys59, Val34 and Leu36 are intact in the baboon sequence, however, the two amino acids directly following Lys59 are different between the baboon and human (Arg60Lys – conservative amino acid change, and Ser61Arg – radical amino acid change). Radical amino acid substitutions Arg46Ser and Leu78Pro are also present in the vicinity of the binding epitopes. The average number of linear amino acids needed as contact residues for proper antibody-antigen interaction is 50–79 amino acids [39]. It can, therefore, be hypothesized that the presence of the radical amino acid changes within the nearby vicinity (within 50–79 amino acids) of the ligand binding regions of GPVI interferes with the binding of monoclonal antibodies to GPVI to such an extent that limited or no antibody binding takes place. Thus, the reason we could not quantify baboon GPVI using human targeted anti-GPVI antibodies may lay in the amino acid sequence differences between the two species.

The reason only HORM® collagen works on the baboon platelets to elicit aggregation may be because the longer fibrils in HORM® collagen is able to bind more of the different collagen binding amino acids than shorter types of collagen, and is thus more effective in binding to GPVI. The fact that more collagen is needed for induction of collagen-induced platelet aggregation in baboons may also be explained by the sequencing results. A mutation in Arg60 of GPVI has previously been implicated in reduced collagen affinity [40]. Therefore, the Arg60Lys mutation within the baboon sequence could be the cause of the reduced collagen induced platelet aggregation seen in baboons. GPVI also contains a Proline-rich (RPLPPLPPLP) domain inside the cytoplasmic tail from Arg287 to Pro296 [36], and Fyn and Lyn activate Syk by means of the phosphorylation of the ITAM in FcRγ by binding to this motif within GPVI [41]. The baboon GPVI does not contain part (Leu295 and Pro296) of this domain due to the 9 bp omission within the baboon GPVI gene. We, therefore, propose that these missing amino acids could cause the signal to be weaker in the baboons than in humans, thus, more collagen is needed to elicit the same amount or strength of aggregation in the baboon platelets as in the human platelets.

When evaluating the crystal structure of GPVI [42] it was found that both the Ser61Arg and Arg46Ser mutations form part of the periphery of the putative binding groove within GPVI, and the Arg60Lys mutation is directly adjacent to collagen-related peptide (CRP) when bound to GPVI. These mutations are unlikely to cause major structural changes within the GPVI protein, however, they may contribute to the fact that collagen does not bind as effectively to baboon platelets as to human platelets.

We subsequently compared the baboon sequence with the mouse, chimpanzee and cynomolgus monkey sequences. The mouse and baboon sequences have a 62.9% similarity, and the chimpanzee sequence shows an 85% similarity. Comparison with the cynomolgus monkey sequence (*Mucaca fascicularis*; NCBI accession # XP_005590417) was very interesting. Even though the cynomolgus monkey GPVI has 18 amino acids less than the baboon, it showed a 91.1% similarity. The cynomolgus monkey has the same amino acids as the human at R46 and R60, but contains a Ser61His (S61H) mutation where the baboon has a S61R mutation at the same place. The cynomolgus monkey also has the L78P, V181M, F214L, Y244H, I262L, G282V, N302H, G304D and Q310P mutations, as well as the deletions of L295, P296 and L297, as seen in the baboon. The cynomolgus monkey has been successfully used to study human-targeted anti-GPVI antibodies in the past [19]. Therefore, we hypothesize that the R46S and R60K mutations are the major changes responsible for the functional differences seen between human and baboon GPVI studies.

It must, however, be noted that the baboons we use in our facility are all purpose bred animals from our breeding colony. However, we have two separate breeding colonies at our facility, and used animals from both colonies in this study, to limit the amount of any founder effect. Conversely, we have females in both colonies that are related. Therefore, it can not be excluded that our findings may be due to a founder effect in our colony and not necessarily a specie-wide problem.

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Baboon  ATGTCTCCATCCCCGACCGCCCTCTTCTGTCTTGGGCTGTGTCTGGGGCGTGTGCCAGCG
                                                                        60
Human   ATGTCTCCATCCCCGACCGCCCTCTTCTGTCTTGGGCTGTGTCTGGGGCGTGTGCCAGCG

Baboon  CAGAGTGGCCCACTGCCTAAGCCCTCCCTCCAGGCTCTGCCCAGCTCCCTGGTGCCCCTG
                #  #  #  #                                              120
Human   CAGAGTGGACCGCTCCCCAAGCCCTCCCTCCAGGCTCTGCCCAGCTCCCTGGTGCCCCTG

Baboon  GAGAAGCCAGTGACCCTCCGGTGCCAGGGACCTCCGGGCGTGGACCTGTACCGCCTGGAG
                                                                        180
Human   GAGAAGCCAGTGACCCTCCGGTGCCAGGGACCTCCGGGCGTGGACCTGTACCGCCTGGAG

Baboon  AAGCTGAGTTCCAGCAGTTACCAGGATCAGGCGGTGCTCTTCATCCCGGCCATGAAGAAA
                         $             #  #                       $     240
Human   AAGCTGAGTTCCAGCAGGTACCAGGATCAGGCAGTCCTCTTCATCCCGGCCATGAAGAGA

Baboon  CGTCTGGCCGGACGCTACCGCTGCTCCTACCAGAACGGAAGCCTCTGGTCCCCGCCCAGC
        $       #                                           $           300
Human   AGTCTGGCTGGACGCTACCGCTGCTCCTACCAGAACGGAAGCCTCTGGTCCCTGCCCAGC

Baboon  GACCAGCTGGAGCTCGTTGCCACCGGAGTTTTTGCCAAGCCGTCGCTCTCAGCCCAGCCC
                               #              #  #                      360
Human   GACCAGCTGGAGCTCGTTGCCACGGGAGTTTTTGCCAAACCCTCGCTCTCAGCCCAGCCC

Baboon  GGCCCGGCGGTGTCCTCAGGAGGGGACGTAACCCTACAGTGTCAGACCCGGTATGGCTTT
                      #                                #                420
Human   GGCCCGGCGGTGTCGTCAGGAGGGGACGTAACCCTACAGTGTCAGACTCGGTATGGCTTT

Baboon  GACCAATTTGCTCTGTACAAGGAAGGGGACCCTGCGCCCTACAAGAATCCCGAGAGATGG
                                                                        480
Human   GACCAATTTGCTCTGTACAAGGAAGGGGACCCTGCGCCCTACAAGAATCCCGAGAGATGG

Baboon  TACCGGGCTAGTTTCCCCATCATCACGGTGACCGCTGCCCACAGCGGAACCTACCGGTGC
           #          #                    #                    #       540
Human   TACAGGGCTAGTTTTCCCATCATCACGGTGACCGCCGCCCACAGCGGAACCTACCGATGC

Baboon  TACAGCTTCTCCAGCAGGGACCCATACCTGTGGTCGGCTCCCAGCGACCCCCTGGAGCTC
                                           #  #                    #    600
Human   TACAGCTTCTCCAGCAGGGACCCATACCTGTGGTCAGCCCCCAGCGACCCCCTGGAGCTT

Baboon  ATGGTCACAGGAACCTCTGTGACCCCCAGCCGGTTACCAACAGAACCACCTTCCTCGGTA
        $                                                      $        660
Human   GTGGTCACAGGAACCTCTGTGACCCCCAGCCGGTTACCAACAGAACCACCTTCCCCGGTA

Baboon  GCAGAATTCACAGAAGCCACCGCTGAACTGACCGTCTCACTCACAAACGAAGTCTTCACA
                 $                             $                        720
Human   GCAGAATTCTCAGAAGCCACCGCTGAACTGACCGTCTCATTCACAAACGAAGTCTTCACA

Baboon  ACTGAGACTTCTAGGAGTATCACCGCCAGTCCAAAGGAGTCAGACTCTCCAGCTGGTCCC
                                                                   #    780
Human   ACTGAGACTTCTAGGAGTATCACCGCCAGTCCAAAGGAGTCAGACTCTCCAGCTGGTCCT

Baboon  GCCCGCCAGCACTACACCAAGGGCAACCTGGTCCGGATATGCCTCGGGGCTGTGATCCTA
                 $                                                      840
Human   GCCCGCCAGTACTACACCAAGGGCAACCTGGTCCGGATATGCCTCGGGGCTGTGATCCTA

Baboon  ATACTCCTGGCAGGGTTTCTGGCAGAGGACTGGCACAGCCGGAGGAAGCGCCTGCGGCAC
           $       #                                                    900
Human   ATAATCCTGGCGGGGTTTCTGGCAGAGGACTGGCACAGCCGGAGGAAGCGCCTGCGGCAC

Baboon  AGGGTCAGGGCTGTGCAGAGGCCGCTCCCGCCCCTCCCGCC---------GACCCGGAAA    951
            $                     #              $$$$$$$$$
Human   AGGGGCAGGGCTGTGCAGAGGCCGCTTCCGCCCCTCCCGCCCCTCCCGCTGACCCGGAAA    960

Baboon  TCACACGGGGATCAGGATGGAGGCCGACCGGATGTTCACAGCCGCGGGTTATGTTCATGA    1011
           $      $                 $
Human   TCAAACGGGGGTCAGGATGGAGGCCGACAGGATGTTCACAGCCGCGGGTTATGTTCATGA    1020
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Figure 1. Comparison of baboon and human GPVI nucleotide sequences. Differences not leading to an amino acid change are indicated with a hash (#), and nucleotide changes causing an amino acid change are indicated with a dollar sign ($).

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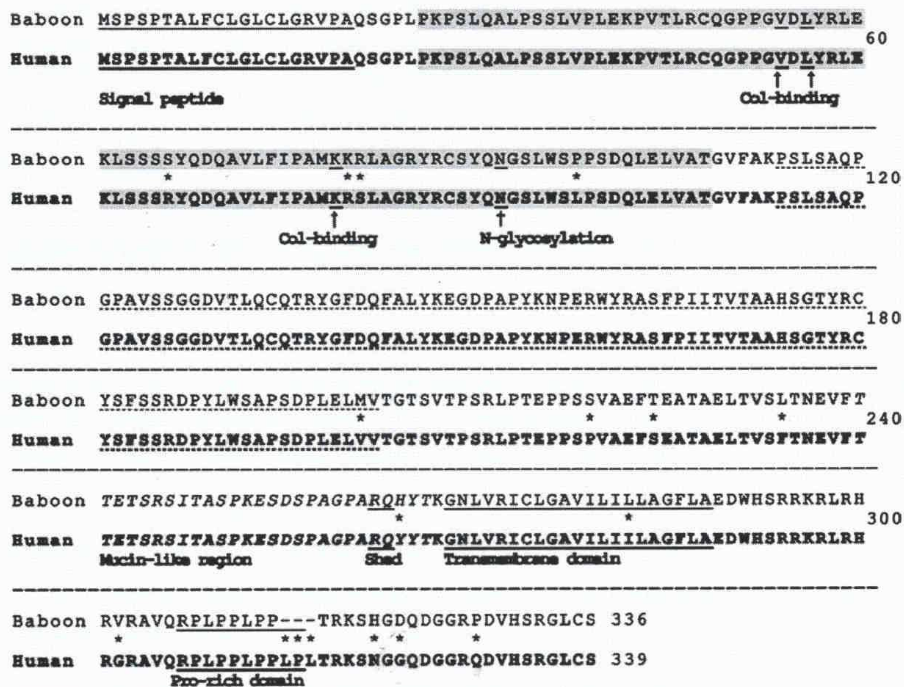

Figure 2. Comparison of baboon and human GPVI amino acid sequences. A difference in amino acid is indicated with an asterisk (*). The signal peptide, sheddase cleaving site, transmembrane domain and proline-rich domain is underlined. The ligand-binding sites are indicated with arrows. The N-glycosylation site is indicated with a cross (†). The D1 immunoglobulin-like domain is shaded, and the D2 immunoglobulin-like domain is underlined with a dotted line. The mucin-like domain is in italics.

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Conclusion

The GPVI receptor on the platelets of the Cape Chacma baboon (*Papio ursinus*) differs to such an extent from its human counterpart that it makes this species unsuitable as animal model for evaluating anti-GPVI anti-platelet agents.

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Declaration of interest

The authors have no conflict of interest to declare. The research was funded by an NHLS Research Trust grant.

Author contributions

W.J.J.v.R. primarily designed and performed the research, collected, analyzed and interpreted data, performed the statistical analysis, and wrote the manuscript.

P.N.B. assisted with the study design, interpreted data, and critically read the manuscript.

J.P.R. assisted with the study design, interpreted data, and critically read the manuscript.

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Appendix E

Draft Article: Janse van Rensburg WJ & Badenhorst PN. Translational value of the Cape Chacma baboon (*Papio ursinus*) in human targeted P2Y12-inhibiting anti-platelet studies.

Translational Value of the Cape Chacma Baboon (*Papio ursinus*) in Human Targeted P2Y$_{12}$-inhibiting Anti-platelet Studies

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Short title: Translational value of the Cape chacma baboon

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Abbreviations

ACS	Acute coronary syndrome
ADP	Adenosine diphosphate
Ala	Alanine
CHD	Coronary heart disease
gDNA	Genomic DNA
Gln	Glutamine
Gly	Glycine
GP	Glycoprotein
Ile	Isoleucine
Leu	Leucine
PCR	Polymerase chain reaction
Ser	Serine
Thr	Threonine
USA	United States of America
Val	Valine
WHO	World Health Organisation

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Abstract

Anti-P2Y$_{12}$ antiplatelet agents play a central part in the treatment and prevention of acute thrombotic events. These agents have disadvantages ranging from patient resistance to potentially fatal side-effects. Discriminating animal models are needed for development of agents that overcome these disadvantages. The Cape chacma baboon (*Papio ursinus*) has been extensively used as a model to evaluate anti-platelet agents. We aimed to determine whether the Cape chacma baboon is a suitable model to use in human targeted P2Y$_{12}$ studies. Platelet function analysis (PFA-100™), adenosine diphosphate (ADP)-induced whole blood impedance platelet aggregation, and DNA sequencing were performed. Results were compared to available healthy human data. Four amino acid changes were detected between the human and baboon P2Y$_{12}$ sequences. However, none of these amino acids was deemed vital for P2Y$_{12}$ receptor function or inhibition. The Cape chacma baboon is considered to be a suitable animal for the evaluation of human targeted anti-P2Y$_{12}$ agents.

Keywords: Cape chacma baboon, P2Y$_{12}$-inhibitors, P2Y$_{12}$-receptor

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Introduction

Coronary heart disease (CHD), which includes acute coronary syndrome (ACS), is the underlying cause of more than half of the cardiovascular disease related deaths annually [1]. This amounts to roughly one in every six deaths in the United States of America (USA). ACS alone produced approximately 1 141 000 hospital admissions in 2010 in the USA [2]. According to the World Health Organisation (WHO), in 2011 seven million deaths were caused by ischaemic heart disease globally, which is a staggering 11.2% of all deaths recorded [3]. The WHO estimated that by 2030, approximately 23.6 million people will die each year of cardiovascular disease. Therefore, ACS is a primary cause of morbidity and mortality in the world [4].

With the incidence of ACS remaining high, development of safe and effective drugs to treat and prevent ACS is a constant process. A multi-drug treatment regimen is recommended for the management of ACS. Effective and safe anti-platelet drugs [such as aspirin, clopidogrel or a glycoprotein (GP) IIb/IIIa inhibitor] play a pivotal part in this treatment regimen [5]. The G-protein-coupled adenosine diphosphate (ADP) platelet receptor, $P2Y_{12}$, has an essential role in platelet activation [6], and consequently is a favourite target for anti-platelet agents. Clopidogrel bisulphate is the $P2Y_{12}$ inhibitor of choice at the Department Haematology, at the University of the Free State, Bloemfontein, South Africa. However, it has some disadvantages. It has to be metabolised, and therefore, displays delayed activity. It binds irreversibly to platelets and has prolonged effects, and furthermore, inter-individual variability is seen due to variances in metabolism. Clopidogrel bisulphate also elicits potentially fatal side-effects such as bone marrow aplasia and thrombotic thrombocytopenic purpura [7].

Newer anti-$P2Y_{12}$ agents, such as prasugrel and ticagrelor, have emerged, but with limited success when evaluated in a clinical setting [8, 9]. Therefore, the need remains to develop safer and more effective anti-$P2Y_{12}$ agents, as well as safe and more discriminating animal models to test them. Typically, new anti-platelet agents are initially tested in lower mammals (for example, rats, mice, rabbits). However, for translational proof of concept before proceeding to human clinical trials, it is recommended that larger animals, and if possible non-human primates, be used to test these novel agents [10]. For thrombotic studies, animals need to have a similar haemostatic system to

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that of humans, which make non-human primates the most appropriate for this purpose [11]. In our environment we have established a successful arterial thrombosis model in the Cape chacma baboon (*Papio ursinus*) to evaluate the safety and efficacy of anti-platelet agents [12-15]. However, limited translational evidence exists for this particular species, which limits the suitability of results obtained using this model. Recently it was found that *P. ursinus* was not a suitable animal model for evaluating human targeted glycoprotein VI inhibiting anti-platelet agents due to genetic differences between this species and the human [16]. Therefore, the aim of this study was to compare the $P2Y_{12}$ receptors between *P. ursinus* and the human to predict the suitability and translational value of *P. ursinus* in future $P2Y_{12}$ studies.

Materials and methods

Ten purpose-bred male *P. ursinus* baboons were randomly selected from the primate breeding colony of the Animal Experimentation Unit of the University of the Free State in Bloemfontein, South Africa. The baboons were monitored for 30 days prior to the study to screen for any visible signs of infections or defects. Animals were anaesthetised by injection of 0.1 mL/kg ketamine intramuscularly. Three millilitres (1 x 3 mL tube) of venous blood were collected in heparinised tubes and five millilitres (1 x 5 mL tube) collected in tubes containing 3.2% sodium citrate (1 part sodium citrate to 9 parts blood; BD Vacutainer, Becton Dickinson, Woodmead, South Africa). Citrated blood was used for platelet function analysis, and the heparinised blood was used for whole blood impedance platelet aggregometry (Multiplate®). Blood remaining in the collection tube was transferred onto FTA™ paper (Whatman™, Buckinghamshire, UK) for DNA extraction and storage.

Platelet function was analysed with the Platelet Function Analyzer (PFA)-100™ (Siemens, Munich, Germany). Aperture closure times were measured using citrated whole blood on both the collagen/ADP (CADP) and collagen/epinephrine (CEPI) cartridges supplied by the manufacturer. We evaluated our results against the normal ranges for humans, which are 55–137 seconds for CADP and 78–199 seconds for CEPI [17].

ADP-induced whole blood impedance platelet aggregometry was performed on the Multiplate® aggregometer (Multiplate, Munich, Germany). We used 20 µL of 0.2 mM ADP (ADPtest kit, Multiplate, Munich, Germany, Cat. # MP0120) as an

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agonist, together with 300 µL of heparinised whole blood, as per manufacturer's instructions. We compared our results to normal human values previously determined in our laboratory using the Student T-test, with $p<0.05$ taken as a statistically significant difference.

Genomic DNA (gDNA) from one animal was sequenced to determine a wild type. Genomic DNA was extracted from FTA™ paper (Whatman™, Buckinghamshire, UK) using a methanol extraction method [18]. Primers were designed with Primer3Plus online primer designing tool [19] using the human reference sequence (P2RY12-001, ENST00000302632) as a template [20]. We performed a polymerase chain reaction (PCR) using a Phusion® Blood Direct PCR Kit (Thermo Scientific, available from Inqaba Biotec, Pretoria, South Africa). The PCR product was subjected to electrophoresis on a 2% agarose gel at 150 volts for between 40 and 60 minutes to determine if we have produced a DNA fragment and if it was of the right fragment size. All reactions gave a single fragment. The PCR product was purified with the Illustra™ ExoStar™ 1-Step reagent (GE Healthcare, Buckinghamshire, UK). This reagent enzymatically eliminates excess primers and nucleotides from the PCR product to avoid interference of these components with the consequent sequencing reaction. Four µL of the Illustra™ ExoStar™ 1-Step reagent was added to 10 µL of PCR product. The mixture was incubated for 15 minutes at 37°C for optimal enzyme activity, followed by 15 minutes at 80°C to deactivate the enzyme.

The purified samples, together with the PCR primers, were sent to Inqaba Biotec, Pretoria, South Africa, for sequencing on an ABI 3130XL sequencer (Applied Biosystems®, available from Life Technologies™, Johannesburg, South Africa). Sequence data were analysed using Chromas Lite [21]. Sequences were compared to the human reference sequence [20] using the online alignment program Lalign [22]. This program uses the algorithm from Huang and Miller proposed in 1991 to calculate the percentage similarity between the two sequences [23]. The nucleotide sequence was translated to the amino acid sequence using the online translation tool from ExPASy (*24*). We then compared the amino acid sequence of the baboon to the amino acid sequence of the human using the Lalign program [22].

Results

Platelet Function Analysis

A mean of 64 ± 5 seconds was obtained for the CADP cartridges,which falls within the

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normal range of 55–137 seconds for healthy humans. We obtained a mean of 85 ± 16 seconds for the CEPI cartridges, also within the normal range of 78–199 seconds for healthy humans [17].

Whole blood impedance aggregometry

We determined the average aggregation (AU) for baboons at 710.7 ± 169.8. We compared our results to healthy human values (795.3 ± 204) previously determined in our lab. The p-value was found to be 0.43, indicating a non-significant difference between the human and baboon results.

Sequencing results

Figure 1 shows a comparison between the nucleotide sequence of the baboon and human $P2Y_{12}$, and Figure 2 shows the comparison of the predicted amino acid sequences. Seventeen single nucleotide differences were detected in the baboon $P2Y_{12}$ sequence when compared with the human reference sequence. Of the 17 nucleotide changes, 12 were silent mutations (shown with * in Figure 1) and did not cause an amino acid change. There were, however, five changes (indicated with + in Figure 1) that caused a change in four amino acids. When evaluating the nucleotide sequence, the baboon $P2Y_{12}$ has a 98.3% similarity to the human. The four amino acid differences between the baboon and human $P2Y_{12}$ proteins equates to 98.8% similarity between the protein sequences. The amino acid changes were valine 4 to isoleucine (Val4Ile), glycine 44 to serine (Gly44Ser), threonine 271 to alanine (Thr271Ala), and leucine 332 to glutamine (Leu322Gln).

Discussion

We determined that the platelet function analysis with the PFA-100™ and the whole blood impedance aggregometry with the Multiplate® aggregometer, baboon and human platelets gave similar results. The amino acids cysteine 97 (Cys97) and cysteine 175 (Cys175) have been described as pivotal in $P2Y_{12}$ receptor expression [25], as well as being putative binding sites for the active metabolites of prasugrel and clopidogrel (indicated with = in Figure 2) [26, 27]. However, recently Cys97 has been implicated as the most likely target for $P2Y_{12}$ inhibiting agents [28, 29]. Glutamic acid 181 (Glu181), arginine 256 (Arg256), arginine 265 (Arg265) and lysine 280 (Lys280) have been recognised as amino acids that are essential for the functional integrity of $P2Y_{12}$ (indicated with **X** in Figure 2). Lys280 has also been identified as the vital amino acid in the ligand-binding pocket of the receptor [30]. All these amino acids were present in the baboon sequence; therefore, the $P2Y_{12}$

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receptor expression and ligand and/or anti-$P2Y_{12}$ agent binding should not be altered or influenced in the baboon.

The algorithm of Huang and Miller can calculate whether an amino acid change is a conservative or a radical substitution [23]. A conservative substitution is defined as "the substitution of an amino acid by another with similar chemical properties." A conservative substitution, therefore, has a negligible effect on the protein structure [31]. In contrast, a radical substitution is defined as "the substitution of an amino acid by another with markedly different chemical properties," which may potentially impact the protein structure [32]. Val4Ile, Gly44Ser and Thr271Ala are all conservative substitutions (indicated with © in Figure 2). Only Leu322Gln is a radical substitution (indicated with ® in Figure 2). The main difference between Leu and Gln is that Leu is a neutral nonpolar amino acid, and Gln is a neutral polar amino acid [33]. However, none of these amino acids have been described as having an effect on $P2Y_{12}$ function. Therefore, these changes are not seen as clinically relevant.

Conclusion

Because platelet function analysis and whole blood impedance aggregometry were comparable to healthy human results and no major amino acid changes were observed in the baboon protein sequence, we were able to show that *P. ursinus* is a suitable model to evaluate human targeted $P2Y_{12}$ antagonists. Therefore, results obtained using *P. ursinus* have a very high translation value when evaluating human targeted anti-$P2Y_{12}$ agents.

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Conflict of interest

The authors have no conflict of interest to declare.

Author contributions

W.J.J.v.R. designed and performed the research, collected, analysed and interpreted data, performed the statistical analysis, and wrote the manuscript. P.N.B. assisted with the study design, interpreted data, and critically read the manuscript.

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Human subject statement

Ethics approval was granted by the Ethics Committee at the Faculty of Health Sciences of the University of the Free State, Bloemfontein, South Africa (ECUFS #134/2011). All procedures were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from all participants for being included in the study.

Animal subject statement

Animal ethics approval was granted by the Interfaculty Control Committee on Animal Experimentation of the University of the Free State, Bloemfontein, South Africa (Animal Experiment #18/2011 and #21/2013). The guidelines and requirements of this committee are in accordance with the South African National Standard for the care and use of animals for scientific research (document SANS 10386 of 2008).

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Figure 1

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BABOON ATGCAAGCCATCGACAACCTCACGTCTGCGCCTGGGAACACCAGTCTGTGCACCAGAGAC
                +             *           *                        60
HUMAN  ATGCAAGCCGTCGACAACCTCACCTCTGCGCCTGGTAACACCAGTCTGTGCACCAGAGAC
-----------------------------------------------------------------------
BABOON TACAAAATCACCCAGGTCCTCTTCCCACTGCTCTACACTGTCCTGTTTTTTGTTGGACTC
                                                                  *   120
HUMAN  TACAAAATCACCCAGGTCCTCTTCCCACTGCTCTACACTGTCCTGTTTTTTGTTGGACTT
-----------------------------------------------------------------------
BABOON ATCACAAATAGCCTGGCGATGAGGATTTTCTTTCAAATTCGGAGTAAATCAAACTTTATT
                +                            *                     180
HUMAN  ATCACAAATGGCCTGGCGATGAGGATTTTCTTTCAAATCCGGAGTAAATCAAACTTTATT
-----------------------------------------------------------------------
BABOON ATTTTTCTTAAGAACACAGTCATTTCCGATCTTCTCATGATTCTGACTTTTCCATTCAAA
                                 *                                 240
HUMAN  ATTTTTCTTAAGAACACAGTCATTTCTGATCTTCTCATGATTCTGACTTTTCCATTCAAA
-----------------------------------------------------------------------
BABOON ATTCTTAGTGATGCCAAACTGGGAACAGGACCACTGAGAACTTTTGTGTGTCAAGTTACC
                                                                   300
HUMAN  ATTCTTAGTGATGCCAAACTGGGAACAGGACCACTGAGAACTTTTGTGTGTCAAGTTACC
BABOON TCCGTCATATTTTATTTCACAATGTATATCAGTATTTCATTCCTGGGACTGATAACTATC
                                                                   360
HUMAN  TCCGTCATATTTTATTTCACAATGTATATCAGTATTTCATTCCTGGGACTGATAACTATC
-----------------------------------------------------------------------
BABOON GATCGCTACCAGAAGACCACCAGGCCATTTAAAACATCCAACCCCAAAAATCTCTTGGGG
                                                                   420
HUMAN  GATCGCTACCAGAAGACCACCAGGCCATTTAAAACATCCAACCCCAAAAATCTCTTGGGG
-----------------------------------------------------------------------
BABOON GCTAAGATTCTCTCTGTTGTCATCTGGGCATTCATGTTCTTACTCTCTTTGCCTAACATG
                                                                   480
HUMAN  GCTAAGATTCTCTCTGTTGTCATCTGGGCATTCATGTTCTTACTCTCTTTGCCTAACATG
-----------------------------------------------------------------------
BABOON ATTCTGACCAACAGGCAGCCGAGAGACAAGAATGTGAAGAAATGCTCTTTCCTTAAATCA
                                                                   540
HUMAN  ATTCTGACCAACAGGCAGCCGAGAGACAAGAATGTGAAGAAATGCTCTTTCCTTAAATCA
-----------------------------------------------------------------------
BABOON GAGTTCGGCCTAGTCTGGCATGAAATAGTAAATTACATCTGTCAAGTCATTTTCTGGATT
               *                                                   600
HUMAN  GAGTTCGGTCTAGTCTGGCATGAAATAGTAAATTACATCTGTCAAGTCATTTTCTGGATT
-----------------------------------------------------------------------
BABOON AATTTCTTAATTGTCATTGTATGTTACACACTCATTACAAAAGAACTGTACCGGTCATAT
                     *           *                                *   660
HUMAN  AATTTCTTAATTGTTATTGTATGTTATACACTCATTACAAAAGAACTGTACCGGTCATAC
-----------------------------------------------------------------------
BABOON GTAAGAACAAGGGGTGTAGGTAAAGTCCCCAGGAAAAAGGTGAACGTCAAAGTTTTCATT
               *                                                   720
HUMAN  GTAAGAACGAGGGGTGTAGGTAAAGTCCCCAGGAAAAAGGTGAACGTCAAAGTTTTCATT
-----------------------------------------------------------------------
BABOON ATCATTGCTGTATTCTTTATTTGTTTTGTTCCTTTCCATTTTGCCCGAATTCCTTATACC
                                                               *   780
HUMAN  ATCATTGCTGTATTCTTTATTTGTTTTGTTCCTTTCCATTTTGCCCGAATTCCTTACACC
-----------------------------------------------------------------------
BABOON CTGAGCCAAACCCGGGATGTCTTTGACTGCGCCGCTGAAAATACTCTGTTCTATGTGAAA
                                     + +                           840
HUMAN  CTGAGCCAAACCCGGGATGTCTTTGACTGCACTGCTGAAAATACTCTGTTCTATGTGAAA
-----------------------------------------------------------------------
BABOON GAGAGTACTCTGTGGTTAACTTCCTTAAATGCATGCCTGGATCCGTTCATCTATTTTTTC
            *                                                      900
HUMAN  GAGAGCACTCTGTGGTTAACTTCCTTAAATGCATGCCTGGATCCGTTCATCTATTTTTTC
-----------------------------------------------------------------------
BABOON CTTTGCAAGTCCTTCAGAAATTCCTTGATAAGTATGCTGAAGTGCCCCAATTCTGCAACA
                                                                   960
HUMAN  CTTTGCAAGTCCTTCAGAAATTCCTTGATAAGTATGCTGAAGTGCCCCAATTCTGCAACA
-----------------------------------------------------------------------
BABOON TCTCAGTCCCAGGACAATAGGAAAAAAGAACAGGATGGTGGTGACCCAAATGAAGAGACT
           +                                                       1020
HUMAN  TCTCTGTCCCAGGACAATAGGAAAAAAGAACAGGATGGTGGTGACCCAAATGAAGAGACT
-----------------------------------------------------------------------
BABOON CCAATGTAA
                                                                   1029
HUMAN  CCAATGTAA
-----------------------------------------------------------------------
```

Figure 1. Comparison of the human and baboon $P2Y_{12}$ nucleotide sequences. Differences not leading to an amino acid change are indicated with (*), and nucleotide changes causing an amino acid change are indicated with (+).

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Figure 2

```
BABOON  MQAIDNLTSAPGNTSLCTRDYKITQVLFPLLYTVLFFVGLITNSLAMRIFFQIRSKSNFI
           ©                                       ©                60
HUMAN   MQAVDNLTSAPGNTSLCTRDYKITQVLFPLLYTVLFFVGLITNGLAMRIFFQIRSKSNFI
---------------------------------------------------------------------
BABOON  IFLKNTVISDLLMILTFPFKILSDAKLGTGPLRTFVCQVTSVIFYFTMYISISFLGLITI
                                            =                       120
HUMAN   IFLKNTVISDLLMILTFPFKILSDAKLGTGPLRTFVCQVTSVIFYFTMYISISFLGLITI
---------------------------------------------------------------------
BABOON  DRYQKTTRPFKTSNPKNLLGAKILSVVIWAFMFLLSLPNMILTNRQPRDKNVKKCSFLKS
                                                              =     180
HUMAN   DRYQKTTRPFKTSNPKNLLGAKILSVVIWAFMFLLSLPNMILTNRQPRDKNVKKCSFLKS
---------------------------------------------------------------------
BABOON  EFGLVWHEIVNYICQVIFWINFLIVIVCYTLITKELYRSYVRTRGVGKVPRKKVNVKVFI
        X                                                           240
HUMAN   EFGLVWHEIVNYICQVIFWINFLIVIVCYTLITKELYRSYVRTRGVGKVPRKKVNVKVFI
---------------------------------------------------------------------
BABOON  IIAVFFICFVPFHFARIPYTLSQTRDVFDCAAENTLFYVKESTLWLTSLNACLDPFIYFF
                       X        X     ©        X                    300
HUMAN   IIAVFFICFVPFHFARIPYTLSQTRDVFDCTAENTLFYVKESTLWLTSLNACLDPFIYFF
---------------------------------------------------------------------
BABOON  LCKSFRNSLISMLKCPNSATSQSQDNRKKEQDGGDPNEETPM
                             ®                                      342
HUMAN   LCKSFRNSLISMLKCPNSATSLSQDNRKKEQDGGDPNEETPM
---------------------------------------------------------------------
```

Figure 2. Comparison of the human and baboon $P2Y_{12}$ amino acid sequences. The conservative amino acid substitutions are indicated with (©) and the radical change is indicated with (®). The putative agonist binding amino acids are indicated with (=). The functionally vital amino acids are indicated with (X).

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Draft

Appendix F

Primers used for PCR reactions.

All primers were tested with human DNA to test reactivity. Primers shaded in grey were primers subsequently designed and used, after previous efforts to amplify the specific fragments failed with other primers, using different conditions.

Primers GPIIb		
Exon	**Name**	**Sequence**
1	1SIIBF	CCT GTG GAG GAA TCT GAA
	1ASIIBR	TCC TGC TCT CTC CCA ATA C
2	2SIIBF	CTC ACC CAG CTT TCC TAT GC
	2SASIIBR	CTC TCC TTG CCT GGG ACT C
3	3SIIBF	CTT CAG CGC CCC ACC CCT T
	3ASIIBR	GCG AGA CTT GGG CTC CTC CTG
4	4SIIBF	CAA TCG GGG GCA GGG ACA C
	4ASIIBR	CAA GCC GTC GCG AGT GGG
5-6	5/6SIIBF	CTG ACC CCT CCT CCT TGT CT
	5/6ASIIBR	GGA TGG GCA CGT ACC TAA GA
7	7SIIBF	CGT GCC CAT CCG TAC ACC TCC C
	7ASIIBR	GCA GGA CCT GAC CGT CTG CGG
8-12	8-12IIBSF	GAC GGG GAT CTC AAC ACT ACA G
	8-12IIBASR	CAT TGT AGC CAT CCC GGT C
13-18	13-18IIBSF	GGG CCA AGT GCT GGT GTT C
	13-18IIBASR	CTC CAT GCA GCA CGA CAG
19-21	19-21IIBSF	GAC TGT GGG GAA GAT GAC GT
	19-21IIBASR	CAC CAC CCT GGT CTC ATT CT
22-26	22-26IIBSF	GTG AGC GTG GGG AAT CTG
	22-26IIBASR	CGA GAA CTG GAT CCT GAA GC
16-17	16/17SIIBF	CCT GAG CCC CAC TTA CGT
	16/17ASIIBR	CTC CCA GCC CTG CCA ATC
26	26SIIBF	CCA ACC ACC GGG GCA CCT CTG T
	26ASIIBR	TCC CTC CTC CCA TCC CCT CTG C
27	27SIIBF	TGG GGA CTG ACG ATG CTT
	27ASIIBR	CCT CCC AGA GCA AAG TGG T
28	28SIIBF	CGG GTG TGG GAC CTG GAC
	28ASIIBR	CTT TTC TGG CTG GGC ACT G
29	29SIIBF	TGA GGG GTG GTA CGG GTG GG
	29ASIIBR	CAG AGC CAA GCC TGT GCC CC
30	30SIIBF	CAG GGA GGT GCT CAT ATG CT
	30ASIIBR	CCC AAC CCT CCT GCT AGA AT
2-3	GPIIb Ex2-3_F	CTC ACC CAG CTT TCC TAT GC
	GPIIb Ex2-3_R	GTT CCC TGT GCC CTG TAC C
8-9	GPIIb Ex8-9_F	GAC TAA TTT GCG CCC TTG TC
	GPIIb Ex8-9_R	CAG TGG TGG GGG CAC TTA C
10-11	GPIIb Ex10-11_F	TGG GTA CAA GAA TGA TGC TCT C
	GPIIb Ex10-11_R	GGG ACC CAA CTG GGT AGG
12	GPIIb Ex12_F	CAC TCA TCT GGC CCA CAG
	GPIIb Ex12_R	GGC TCC TCT CTT CCC TCA C
13	GPIIb Ex13_F	CAA TCA GCC ACT TCC TTT CTG
	GPIIb Ex13_R	TTT CTA GCT GGA GGC AGT CC
14-16	GPIIb Ex14-16_F	CCT AAT CGC CAA TTC TGA CC
	GPIIb Ex14-16_R	CCC CTT CAT GCC ACT CAC
17-18	GPIIb Ex17-18_F	CCA GGG AGG TCC TGA CTC TT
	GPIIb Ex17-18_R	TGG CAC TAA CCC TAA TCC TGA
19	GPIIb Ex19_F	CCA AGC CCA CTG TTT TCC TA
	GPIIb Ex19_R	GGG ACT AAG GTG TGG AGC AG
21	GPIIb Ex21_F	TTG GCA CAC AAA TCT TTC CA

	GPIIb Ex21_R	TTA TTC ATG AGC CCC TGG TG
22	GPIIb Ex22_F	CAC TTG GGC AGT GAC CTT G
	GPIIb Ex22_R	GCC TCG AAA GAC CCT TCT GT
23-24	GPIIb Ex23-24_F	GCC CTG TTT CTC CTC ATC C
	GPIIb Ex23-24_R	AGT TCT GAG GAC CCG CTC AC
25-26	GPIIb Ex25-26_F	GTG AGC GGG TCC TCA GAA CT
	GPIIb Ex25-26_R	GGC CAG AGA CCA GAG AGC
27	GPIIb Ex27_F	TGG GGA CTG ACG ATG CTT
	GPIIb Ex27_R	CCT CCC AGA GCA AAG TGG T
28-29	GPIIb Ex28-29_F	GAC TCC TGC AAC CAA TAG GC
	GPIIb Ex28-29_R	GAC TCC ACC GTC CTT CAC AC
30	GPIIb Ex30_F	GTC CAG GGA GGT GCT CAT AG
	GPIIb Ex30_R	CCA AAA CAT TCC TTC GGT CA
2-3	GPIIb Ex2-3F	GCT CAC CCA GCT TTC CTA TG
	GPIIb Ex2-3R	ATT GTT CCC TGT GCC CTG TA
14-15	GPIIb Ex 14-15F	AAC CCC TGG GAA GAT GAG AT
	GPIIb Ex 14-15R	AGA TCC TTT AAG GCC CAT GC
19	GPIIb Ex 19F	CCA AGC CCA CTG TTT TCC TA
	GPIIb Ex 19R	GGG ACT AAG GTG TGG AGC AG
25-26	GPIIb Ex25-26F	GTG AGC GGG TCC TCA GAA CT
	GPIIb Ex25-26R	GGC CAG AGA CCA GAG AGC
28	GPIIb Ex28F	GAC TCC TGC AAC CAA TAG GC
	GPIIb Ex28R	GAC TGG TCT CTG CTC CAT CC

Primers GPIIIa		
Exon	**Name**	**Sequence**
1	1IIIASF	ACC TTC ACT GAG CCA AAT CTG GTC
	1IIIAASR	CAA GGT CTG GTA TCT GCT CC
2	2IIIASF	GAG AGT CGC CAT AGT TCT GA
	2IIIAASR	ACC TCC ACC TTG TGC TCT AT
3	3IIIASF	AAG TAA CAT CTT TCT GCC TTC CA
	3IIIAASR	GCG TCT GGA GGA GGG ACT TAC
4	4IIIASF	GAA GAC CAC CTG CTT GCC CAT G
	4IIIAASR	GAC TGT AGC CTG CAT GAT GGC
5	5IIIASF	TAT CTC CCA TCC CTC CCC AG
	5IIIAASR	CAA GCT GAA ACC GAG CCC TGC A
6	IIIA-6AF	TTT GTT TTG TCT CCT CTG CCT
	IIIA-6BR	ATG AAT AGA AAC CAT AGG TGG T
7	7-SIIIAF	AGC CCG AAG CAA GAT AAG TTC TAA GC
	7-ASIIIAR	GGG GAG TGG ATT AGG GGA GAC TCT T
8	GPIIIA8SF	TGA ACT GCT CTT GCA TTT CCC G
	GPIIIA8ASR	TTG GGT GCC CAC AGA TGC TCC
9	GPIIIA9SF	AGC TGG AGT GTT AAC TGG GC
	GPIIIA9ASR	ATG TTT CCC AGT GGT TGC AGG
10	IIIA-10AF	GGG CCC AAC TGT GTC TAA AT
	IIIA-10BR	AAG GGC GAT AGT CCT CCT C
10	IIIA-10'AF	GTA TGC GGT TGT GGG CCT G
	IIIA-10'BR	TAT ATG AGG GGG TGT GGG TT
12	GPIIIA12SF	TGG AGT GGT CCC ATC TTC CAG
	GPIIIA12ASR	TCC TGC CTA ACA TGG TTC TCC
13	GPIIIA13SF	TTC ATAGGC CAG GTT CAA GTG
	GPIIIA13ASR	TGG AAA GAC GAT GGT ACT GGC
14	GPIIIA14SF	TTC CAG AGA ACG GTG CCT TGG
	GPIIIA14ASR	TCC ACA TAC TGA CAT TCT CCC
15	15SIIIAF	TAT TCC AGA GAA CGG TGC CTT G
	15ASIIIAR	TAA ACA TGA TGG CAG GGA CTC C
11	GPIIIa Ex 11_F	TAC AAG GGG GAG ATG TGC TC
	GPIIIa Ex 11_R	CCC CGG TCA AAC TTC TTA CA
12	GPIIIa Ex 12_F	ATG GGG ACA CCT GTG AGA AG
	GPIIIa Ex 12_R	ACA ATT CAC TGC ATC CTT GC
11	GPIIIa Ex 11_F.1	CTT CCT GGG CTG TGT GTT TT
	GPIIIa Ex 11_R.1	GGC TCT CTC CAG ACT CCA CA
12	GPIIIa Ex 12_F.1	GGA GAT CAG AGC TGG ACT GG

	GPIIIa Ex 12_R.1	TAG AAC CTG GGT GTG TGC AA

Primers GPIb alpha		
Exon	**Name**	**Sequence**
1	GPIba-Ex1F	TTC CTT GCC ACT GGC TTA GT
	GPIba-Ex1R	CCT CCT GTC CTC CCT ACT CC
2	GPIba-32F	TCC ACT CAA GGC TCC CTT GC
	GPIba1349R	GCT TGT GGC AGA CAC CAG GAT GG
2	GPIba1133F	CCA CTA CTG AAC CAA CCC CAA GC
	GPIba2273R	CAC AAC GAG TGC TCA CAT CC
2	GPIba-Ex2_3F	GGA TGT GAG CAC TCG TTG TG
	GPIba-Ex2_3R	GCT GGT CTC GAA CTC CCT AC
2	GPIba Ex2CF	CCT CCA AGA GAA CTC GCT GT
	GPIba Ex2CR	TGT CAC ACT GCA CAC TGG TC
2	GPIba Ex2EF	TAC TGA ACC AAC CCC AAG C
	GPIba Ex2ER	CAA ATG CCC TTG GAG CAC
2	GPIba Ex2FF	CTT TTC TCC ACC CCG ACT TT
	GPIba Ex2FR	CTG TGT TCC TTA CCC CTC CA
2	GPIb alpha Ex2 Rest_F	TCT CCA AAA CTC CAA AAT CCA
	GPIb alpha Ex2 Rest_R	CTG TGT TCC TTA CCC CTC CA
2	GPIba 2.1F	CTA CTG AAC CAA CCC CAA GC
	GPIba 2.1R	TTG TGG CAG ACA CCA GGA T
2	GPIba 2.2F	CAC TCC AAG CCC GAC CAC
	GPIba 2.2R	GCT TTG GTG GCT GAT CAA GT
2	GPIba 2.3F	CAC AAG CCT GAT CAC TCC AA
	GPIba 2.3R	GAG GAC CAC AGA GGC AAA GA
2	GPIba 2.4F	CTT TTC TCC ACC CCG ACT TT
	GPIba 2.4R	GAA CCT CGA AGG AAG AGC AG
2	GPIba 2.5F	CAC AAA CCA CAC ACC TGG AG
	GPIba 2.5R	TCC AAT AGG AGA GCC CAC AG
2	GPIba 1_F	CTC ATA GCC CGT CCC AGA G
	GPIba 1_R	TTG GAG TGA TCA GGC TTG TG
2	GPIba 2_F	CTG CTC TTC CTT CGA GGT TC
	GPIba 2_R	TCC AAT AGG AGA GCC CAC AG
2	GPIba 2X.1 F	ACG AGG ACA CTG AGG GTG AT
	GPIba 2X.1 R	GGT GGA TTC TAA GAG TGA TAC GG
2	GPIba 2X.2_F	CCC TGG GCT TCT ATG TCT TG
	GPIba 2X.2_R	CTG TGT TCC TTA CCC CTC CA
2	Iba Final F	CAC TAA GGA GCA GAC CAC ATT C
	Iba Final R	CAG AGG AGA CCC AAG ACA TAG A

Primers GPVI		
Exon	**Name**	**Sequence**
1	GPVI-Ex1F	CCA GGC TCC CAC CAC TTC
	GPVI-Ex1R	CCA GAT CTG ACC CTC AGG AC
2-3	GPVI-Ex2_3F	TAA AAG CGA GTC CTG GCA TC
	GPVI-Ex2_3R	GAT CCC CCT TCC TTT ACC C
4	GPVI-Ex4F	GCT CCT GCC TTC AAC ATC AG
	GPVI-Ex4R	TGG TCT GCA CTA CCC CTA CC
5	GPVI-Ex5F	ATT TGT TCA GGA CCC ACA GC
	GPVI-Ex5R	GGG TCC GTG TAC CTC ATA CG
6	GPVI-Ex6F	AAC CAC GGG GAA GAC CTA AC
	GPVI-Ex6R	AGA GCT CCG TCC TCA CAC TC
7	GPVI-Ex7F	GGC GGG AAT ATG GTT TCA TT
	GPVI-Ex7R	GGA GTT GGC TTT GGT GAA GA
8	GPVI-Ex8_FR1F	AGG TGG GCT CTT TCA CCT G
	GPVI-Ex8_FR1R	GGA GGA TTT TGC ACA GAG GA
8	GPVI-Ex8_FR2F	CCA GCT CTC AGG GTT GAC TC
	GPVI-Ex8_FR2R	AAA GTG CCG GGA TTA CAG G
2	GPVI-Ex2F	GCA TGC AAA TGT CTT ATC ACC
	GPVI-Ex2R	CAG GAG GGA AGG GGT CTG
3	GPVI-Ex3F	AGC CCT GCT CCT CTT CCA G
	GPVI-Ex3R	CCG ATC CCC CTT CCT TTA C

8	GPVI-Ex8_Fr1.1F	GGA TCT CTG AGA AGC CCA GAT
	GPVI-Ex8_Fr1.1R	ATC CCT CCC TTG GAT ACG AC

Primers P2Y12		
Exon	**Name**	**Sequence**
1	P2Y12-Ex1F	CAC AAC AAA CAT CAT AGT GCT GAA
	P2Y12-Ex1R	CCA AAT AAA TCT GAT GCA TTA CCA
2	P2Y12-Ex2F	TGC ATA GCT TTG AGT CCA GTG
	P2Y12-Ex2R	CAA TTT TAA GAG AAA CTT GGA AAA A
3	P2Y12-Ex3FR1F	GGC TGA AAA TAA CCA TCC TCT C
	P2Y12-Ex3FR1R	AAG GAA AGA GCA TTT CTT CAC A
3	P2Y12-Ex3FR2F	GGC AGC CGA GAG ACA AGA
	P2Y12-Ex3FR2R	TGG CAA AAC TCT GCA AAA CA

Appendix G

Calculation template for receptor number quantification.

Available from Biocytex in Microsoft Excel format.

PLATELET CALIBRATOR (Ref. 7011)

Date :

Experimentation :

Opérateur / Operator :

Lot N° :

Date Exp. :

Echantillon / Sample :

Identification	...
	...

Anticorps / Antibody:

	Dénomination / Description	Isotype	Lot	Concentration de saturation / Saturating concentration (µg/ml)
AcM 1 / MAb1				
AcM 2 / MAb 2				
AcM 3 / MAb 3				
AcM 4 / MAb 4				
AcM 5 / Mab 5				

Courbe de calibration / Calibration curve

Billes / Beads	Nombre de sites / Number of sites	MFI FL1
A	1	0.000
B	2	0.00
C	3	0.00

Données de la droite / Curve data (y=ax+b)

Pente / Slope (a)	#VALUE!
Origine / Intercept (b)	#VALUE!
r	#NUM!
r²	#NUM!

Courbe de Calibration / Calibration Curve

Sites par cellule / Sites per cell: 1,000,000; 100,000; 10,000; 1,000; 100

MFI FL1 (a.u.): 0,1; 1; 10; 100; 1000; 10000

Résultats Anticorps / Antibody Results :

Anticorps / Antibody	Isotype	IgG1 MFI FL1	IgG1 Sites / cell.	IgG2a MFI FL1	IgG2a Sites / cell.	IgG2b MFI FL1	IgG2b Sites / cell.
Ctl IgG1	IgG1						
Ctl IgG2a	IgG2a						
Ctl IgG2b	IgG2b						

Observation :

#VALUE!

MFI : Mean Fluorescence Intensity / Intensité moyenne de fluorescence.

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"*Deo Gratias!*" – Thanks be to God!