Comparison of common platelet receptors between the chacma baboon (Papio ursinus) and human for use in pre-clinical human-targeted anti-platelet studies

Walter J. Janse van Rensburg

Department of Haematology and Cell Biology, Faculty of Health Sciences, University of the Free State, Bloemfontein, Free State, South Africa

Abstract

Anti-platelet agents play a central part in the treatment and prevention of acute thrombotic events. Discriminating animal models are needed for the development of novel agents. The chacma baboon has been extensively used as a model to evaluate anti-platelet agents. However, limited data exist to prove the translatability of this species to humans. We aimed to determine the suitability of the chacma baboon in preclinical human targeted GPIIb/IIa, GPIIbα and P2Y12 studies. Light-transmission platelet aggregometry (LTA), whole blood impedance aggregometry, receptor number quantification and genomic DNA sequencing were performed. Baboon ADP and arachidonic acid-induced LTA aggregation results differed significantly from human values, even at increased concentrations. LTA ristocetin-induced agglutination was comparable between species, but baboon platelets needed twice the concentration of ristocetin to elicit a similar response. Citrated baboon blood had significantly less aggregation than humans when evaluated with impedance aggregometry. However, hirudinised baboon whole blood gave similar aggregation as humans at the same agonist concentrations. GPIIb, GPIIaα and GPIIbα numbers were significantly more on the baboon platelets. None of the amino acids deemed vital for receptor function, ligand binding or receptor inhibition, were radically different between the species. However, a conservative change in a calcium-binding region of GPIIb may render the baboon platelets more sensitive to calcium-binding agents. The chacma baboon may be used for the evaluation of human-targeted GPIIb/IIα-, GPIIβα- and P2Y12-inhibiting agents. However, the best anticoagulant, optimal agonist concentrations, increase in receptor number and sequence differences must be considered for any future studies.

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. Anti-platelet drugs such as aspirin, clopidogrel (P2Y12-inhibitor) or a glycoprotein (GP) IIb/IIIa inhibitor, play a pivotal part in this treatment regimen [5]. Pre-clinically blockage of von Willebrand factor (VWF) and GPIIbα interaction has also shown much promise in the treatment of ACS [6–8]. 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 [9]. For thrombotic studies, animals need to have a similar haemostatic system to that of humans, which make non-human primates the most appropriate for this purpose [10]. In our environment, we have established a successful arterial thrombosis model in the chacma baboon (Papio ursinus) to evaluate the safety and efficacy of anti-platelet agents in preventing arterial thrombosis [6–11]. We also established a baboon model to evaluate putative treatments for thrombotic thrombocytopenic purpura (TTP) [12]. Using these models, our group showed that blocking the VWF-GPIIbα axis is effective in preventing arterial thrombosis and could treat TTP in the P. ursinus baboon [6, 7, 8, 13, 14]. We further confirmed the effectiveness of clopidogrel in arterial thrombosis prevention in this specific specie [7]. However, in a study performed in the arterial thrombosis model with the GPIIb/IIIa inhibitor tirofiban hydrochloride, it was found that in this specific animal model tirofiban hydrochloride was not effective in preventing arterial thrombosis at the recommended dose. It was found that between three and nine times the recommended dose was needed to
abolish platelet aggregation at high shear rates. However, abciximab was effective in abolishing arterial platelet deposition, albeit with an increase in blood loss [11]. Some reviewers had concerns regarding the translatability of the tirofiban results to humans, because 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 GPVI inhibiting anti-platelet agents due to genetic differences between this species and the human [15]. Molecular data does exist for other non-human primates regarding these receptors. However, P. ursinus is a much larger non-human primate than the commonly used monkeys, such as Macaca fasicularis, thus, more blood can be drawn from one animal at a time. This is a great advantage for preclinical anti-platelet studies where large quantities of blood are needed. Therefore, the aim of this study was to compare the GPIIb/IIIa, GPIbα and P2Y12 receptors between P. ursinus and the human to predict the suitability and translational value of P. ursinus for future anti-platelet 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. Taking the sensitive nature of non-human primate studies into consideration, 10 animals are the minimum amount that would give statistically interpretable results, but which is still ethically acceptable. 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. 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, Becton Dickinson, Woodmead, South Africa). Citrated blood was used for light-transmission aggregometry (LTA) and receptor number quantification using flow cytometry. Blood remaining in the collection tube was transferred onto FTA™ (Whatman™, Buckinghamshire, UK) for DNA extraction and storage. Five millilitres (1 x 5 ml tube) of venous blood were collected from 10 normal healthy volunteers in tubes containing 3.2% sodium citrate (1 part sodium citrate to 9 parts blood; BD Vacutainer, Becton Dickinson, South Africa). The volunteer blood was used for receptor number quantification using flow cytometry. On a separate occasion eight millilitres (1 x 5 ml sodium citrate tube and 1 x 3 ml hirudin tube) of venous blood was also drawn from five baboons and ten healthy volunteers to determine whole blood platelet aggregation. The hirudin containing tubes (Ref #: MP0600) are specially marketed for whole blood aggregometry using the Multiplate® instrument (Dynabyte, Munich, Germany). The number of animals was reduced, as we only wanted to test the proof of concept.

Platelet-rich plasma (PRP) for LTA was prepared by centrifuging the tubes for ten minutes at 200 x g. Platelet counts were performed on each of the PRP samples using the Sysmex XE-2100 full blood analyser (Roche, Johannesburg, South Africa). This instrument also provided the platelet size for each sample. Platelet counts were not adjusted to prevent inhibition of aggregation [16]. All platelet counts were between 190 and 430 x 10⁹/l. LTA was performed at 37°C on the Chrono-log platelet aggregometer (Chrono-log Corporation, Havertown, PA, USA) using different concentrations of adenosine diphosphate (ADP, Helena Biosciences, Gateshead, United Kingdom), arachidonic acid (Helena Biosciences, Gateshead, United Kingdom) and ristocetin (Helena Biosciences, Gateshead, United Kingdom). We started with the highest concentrations used in routine human LTA. We then compared the baboon data to historical control values of 38 normal human volunteers (males and females, ages 21–40) using the Student t-test, with p<0.05 taken as a significant difference. The agonist concentrations used for the baboons were as follows: ADP – 10, 15, 20 and 40 μM; ristocetin – 1.25, 1.875 and 2.5 mg/ml; arachidonic acid – 0.5, 0.75 and 1 mg/ml. The baboon results were compared to human control values at the following agonist concentrations: ADP – 10 μM; ristocetin – 1.25 mg/ml; arachidonic acid – 0.5 mg/ml. Every baboon sample was tested once with each of the different agonists. The results for all the baboons were tabulated and the mean and standard deviation (SD) was determined for the group. The human samples were tested individually with each agonist, results tabulated and the mean and SD determined for the group.

Whole blood impedance aggregometry was done on the Multiplate® whole blood aggregometer (Dynabyte, Germany) according to the manufacturer’s instructions. Baboon blood was analysed using the manufacturer’s recommended agonist concentrations and 1.5 times the recommended agonist concentrations on samples from both the citrated and hirudin containing tubes. The final agonist concentrations were as follows: ADP (ADPtest) – 6.5 and 9.75 μM; ristocetin (RISTOtest) – 0.77 and 1.155 mg/ml; arachidonic acid (ASPtest) – 0.5 and 0.75 mM. Results were compared to human results using the manufacturer’s recommended agonist concentrations, also using samples from both citrated and hirudin containing tubes. All reagents are specifically produced for the Multiplate® instrument (Dynabyte, Germany). As with the LTA method, individual baboon samples were tested once with each of the different agonists. The results for all the baboons were tabulated and the mean and standard deviation (SD) was determined for the group. And each human sample was tested separately with each agonist, the results were tabulated and the mean and SD were determined for the group. Results were analysed with the Student t-test, with p<0.05 taken as a significant difference. As this was only an additional test performed in order to establish if different anticoagulants may influence baboon aggregation, the amount of animals were limited to only five per group, in accordance with the “reduction” principle of animal research.

Baboon and human platelet receptor number quantification was done on unstimulated platelets by flow cytometry on a BD FACSCalibur® flow cytometer (Beckton Dickinson, Woodmead, South Africa). The Platelet Calibrator kit (Biocytex, Marseille, France) was used to quantify the expression. This kit can measure any platelet surface molecule, given that there is a monoclonal antibody existing against the molecule. A no wash indirect immunofluorescence technique was employed to stain platelets with specific monoclonal antibodies. Calibration beads, coated with increasing and accurately known numbers of mouse IgG, were 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 the manufacturer (Biocytex, France). The following monoclonal antibodies were used: anti-GPIIb/IIIa – anti-CD42b (BD Cat #: 555471) and 6B4 (Prof Karen Vanhooorebeke, KU Leuven); anti-GPIbα – anti-CD41a (BD Cat #: 555465); anti-GPIIIa – anti-CD61 (BD Cat #: 555752). Unfortunately, we were not able to quantify the P2Y12 receptor, as there was no commercial monoclonal antibody available at the time. All quantification results were analysed with the Student t-test, with p<0.05 taken as a significant difference. The same method with the same antibodies was used for both the baboon and human platelets to ensure that the results obtained are comparable.

Genomic DNA (gDNA) from one animal was sequenced to determine a wild type. Genomic DNA was extracted from FTA™ paper (Whatman™, UK) using a methanol extraction method [17]. Primers were designed with Primer3Plus online primer designing tool [18] using the human reference sequences as templates. 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. The PCR product was purified with the Illustra™ ExoStar™ 1-Step reagent (GE Healthcare, Buckinghamshire, UK).

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 [19]. Sequences were compared to the human reference sequences using the online alignment program Lalign [20]. This program uses the algorithm from Huang and Miller proposed in 1991 to calculate the percentage similarity between the two sequences [21]. The nucleotide sequence was translated to the amino acid sequence using the online translation tool from ExPASy [22]. We then compared the amino acid sequence of the baboon to the amino acid sequence of the human using the Lalign program [20]. Purified gDNA was also sent to a commercial laboratory, which performs human diagnostic genetic screening of GPIIb/IIIa (Glanzmann’s thrombasthenia) and GPIbα (Bernard-Soulier). However, they were not able to sequence all the exons of the three receptors; therefore, we had to design new amplification and sequencing experiments to enable us to sequence the baboon receptors (primer list supplied as supplemental data). Unfortunately, due to budgetary constraints during the study, we were not able to explore other techniques in identifying the missing sequences.

Results

Light-transmission aggregometry

The amount of aggregation was determined using the maximum amplitude of the aggregation graph (Figure 1). ADP-induced platelet aggregation results were significantly different between baboons and humans. Even at ADP levels four times (40 μM) the highest human concentration of 10 μM the baboon platelets failed to reach the amount of aggregation of human platelets, with only the primary wave of aggregation observed. ADP concentrations were not further increased, as a plateau of aggregation was reached after addition of 20 μM. 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. The arachidonic acid concentration was not increased further, as the physical reagent became very viscous at the high concentration. We previously

![Figure 1](image1.png)

Figure 1. Examples of aggregation tracings found with baboon platelets. (A) ADP-induced platelet aggregation using three different concentrations of ADP. (B) Ristocetin-induced agglutination using three different concentrations of ristocetin. Trace 3 of the second graph shows failure of aggregation at the maximum human ristocetin concentration. (C) Arachidonic acid induced aggregation using three different concentrations of arachidonic acid. The first graph shows a failed reaction at trace 1, which is twice the maximum human concentration. (D) A repeat of the failed arachidonic acid reaction found in (C), showing some aggregation at twice the maximum human concentration.
published that collagen-induced aggregation (at 8 μg/ml) differences were not clinically important when compared to humans [15] (Table I).

### Whole blood impedance aggregometry

The amount of aggregation was determined by the area under the curve (AU) of the aggregation graphs (Table II). All citrated baboon blood samples had significantly less \( p<0.05 \) aggregation than citrated human blood, regardless of the agonist concentrations used. However, when evaluating the samples from the hirudin containing tubes, none of the baboon samples showed any significant difference form the hirudinised human samples. Because of the smaller sample size, higher SDs were found in the baboons than in humans.

### Platelet receptor number quantification

The GPIIb, GPIIIa and GPIbα receptor numbers all differed significantly \( p<0.05 \) between baboon and human platelets (Table III). The baboon platelet had 37% more GPIIb, 27% more GPIIIa, and 25.5% more GPIbα on the platelet surface than humans. This is a significant finding when considering that the baboon platelets were found to be much smaller than human platelets (as determined in a concurrent human study done in our laboratory) \( 7.23 \pm 0.62 \) fL vs. \( 8.8–12.5 \) fL [23]. It must be noted that GPIIb and GPIIIa are not in a 1:1 ratio. It can be attributed to the fact that different antibodies were used directed against each of the receptors separately and not in complex with each other. Thus, the specific antibody affinities may differ, causing the discrepancy. However, as we used exactly the same antibodies,

| Table I. LTA results of ADP, ristocetin and arachidonic acid given as percentage aggregation. |
| ADP | Baboon (n=10) | 10 μM | 15 μM | 20 μM | 40 μM | Human 10 μM (n=38) |
| Mean | 28.2 | 31.6 | 38.3 | 37.9 | 71 |
| SD | 10.26 | 12.08 | 9.02 | 10.02 | 10 |
| p-Value | 0.00000001 | 0.00000043 | 0.00000004 | 0.00000019 |
| Ristocetin | Baboon (n=10) | 1.25 mg/ml | 1.875 mg/ml | 2.5 mg/ml | Human 1.25 mg/ml (n=38) |
| Mean | 34.2 | 57.9 | 74.6 | 78 |
| SD | 33.82 | 21.27 | 8.07 | 13 |
| p-Value | 0.0026 | 0.016 | 0.32 |
| Arachidonic Acid | Baboon (n=10) | 0.5 mg/ml | 0.75 mg/ml | 1 mg/ml | Human 0.5 mg/ml (n=38) |
| Mean | 35 | 38.1 | 38.7 | 67 |
| SD | 24.98 | 20.77 | 7.85 | 8 |
| p-Value | 0.0026 | 0.0015 | 0.00000005 |

\( p<0.05 \) is taken as a significant difference. The grey boxes indicate the maximum amount of aggregation seen for each agonist.

| Table II. Whole blood impedance aggregometry results given as area under the curve (AU). |
| Citrate tube | ADP (1x: 6.5 μM) | Mean ± SD |
| Baboon (n = 5) (1x) | 78.0 ± 72.6* |
| Baboon (n = 5) (1.5x) | 147.0 ± 105.9* |
| Human (n = 10) (1x) | 521.2 ± 122.2 |
| Arachidonic acid (1x: 0.5 mM) | 68.0 ± 88.1* |
| Ristocetin (1x: 0.77 mg/ml) | 352.4 ± 90.4* |
| Hirudin tube | ADP (1x: 6.5 μM) | 810 ± 321.1 |
| Baboon (n = 5) (1x) | 758.8 ± 290.7 |
| Baboon (n = 5) (1.5x) | 850.9 ± 387.8 |
| Human (n = 10) (1x) | 706.3 ± 203.8 |
| Arachidonic acid (1x: 0.5 mM) | 688.0 ± 167.4 |
| Ristocetin (1x: 0.77 mg/ml) | 1473.8 ± 445.2 |

\( 1x \) is the maximum agonist concentration used in human aggregations (indicated next to each agonist). \( 1.5x \) is 1.5 times the maximum agonist concentration used for human aggregations. \( p<0.05 \) is taken as a significant difference.

| Table III. Platelet receptor quantification given as numbers. P < 0.05 is taken as a significant difference. |
| Platelet receptor quantification | Baboon (n=10) |  |
| GPIIb | 76 677 |
| GPIIIa | 65 048 |
| GPIbα | 41 761 |
| Human (n=10) |  |
| GPIIb | Mean |
| GPIIIa | 55 801 |
| GPIbα | 51 142 |
| GPIIIa | 33 266 |
| GPIbα | 7013 |

\( p<0.05 \) is taken as a significant difference. The grey boxes indicate the maximum amount of aggregation seen for each agonist.

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with exactly the same method to determine the quantities, the subsequent comparison between baboon and human platelets are influenced.

Sequencing results

Seventeen single nucleotide differences were detected in the baboon P2Y12 sequence (Figure 2) when compared with the human reference sequence (P2RY12-001, transcript ID: ENST00000302632). Of the seventeen nucleotide changes, twelve did not cause an amino acid change. There were, however, five changes that caused changes in four amino acids. When evaluating the nucleotide sequence, the baboon P2Y12 has a 98.3% similarity to the human. The four amino acid differences between the baboon and human P2Y12 proteins equates to 98.8% similarity between the protein sequences. The amino acid changes were Val4Ile, Gly44Ser, Thr271Ala and Leu322Gln. Val4Ile, Gly44Ser and Thr271Ala are all conservative substitutions. Only Leu322Gln is a radical substitution.

After numerous attempts, exon 28 of GPIIb could not be sequenced. However, this exon does not contain any amino acid essential for ligand binding, conformational changes or signalling. Sixty-two single nucleotide differences were detected in the baboon GPIIb sequence (Figure 3) when compared with the human GPIIb reference sequence (ITGA2B-001, transcript ID: ENST00000262407). Forty-two nucleotide differences did not cause an amino acid change. Twenty nucleotide differences caused changes in eighteen amino acids. The nucleotide sequences are 97.9% similar between baboon and human GPIIb for all the exons sequenced. There is 98.2% similarity between the amino acid sequences of exons 1–27 and 98.3% similarity between the amino acid sequences of exons 29 to 30. The amino acid changes are Gln7His, Gln18His, Asn158His, Asp159Asn, Val200Ile, Leu221Ile, Asp271Gly, Tyr274Phe, Arg281His, Ala339Thr, Arg368Gln, Arg400Ser, Ala637Thr, Glu676Asp, Pro809His, Glu868Lys, Tyr915His and Trp952Arg.

The GPIIb amino acid sequences between P. ursinus and the olive baboon (P. anubis: Accession number: XP_003913168) were 99.4% similar, 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 and Ile843Thr were conservative substitutions between the two species. Three amino acid changes were
Figure 3. Comparison of the amino acid sequences of GPIIb between the baboon and human. The signal peptide is underlined with a double line. The β-propeller region is shaded in grey. The missing exon 28 is indicated by strikethrough. The transmembrane domain is underlined with a dashed line. The cytoplasmic domain is indicated in grey lettering. Conservative amino acid changes are indicated with an asterisk (*). Radical amino acid changes are indicated with a hash (#). All ligand binding amino acids are indicated with arrows (↑). The amino acid vital for interaction with GPIIIa (Arg995) is indicated with a cross (†). The hydrophobic region N-terminal to Arg995, also implicated in GPIIIb interaction is underlined with a dotted line. The GVLGG sequence that plays a role in the conformational change and subsequent GPIIb/IIIa activation is indicated by dollar signs ($). The calcium binding amino acids are in italics and underlined.

Figure 4. Comparison of the amino acid sequences of GPIIIa between the baboon and human. The signal peptide is underlined with a double line. The ligand binding region is underlined. The cysteine-rich tandem repeat region is shaded in grey. The transmembrane domain is underlined with a dashed line. The cytoplasmic domain is indicated in grey lettering. Conservative amino acid changes are indicated with an asterisk (*). Radical amino acid changes are indicated with a hash (#). Asp723, the amino acid vital for interaction with GPIIb and talin is indicated with a cross (†). The hydrophobic region N-terminal to Asp723, also implicated in GPIIIa interaction is underlined with a dotted line.
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 GPIIa. 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. ursinus* GPIIa is 99.2% similar to human, thus, *P. ursinus* is more similar to humans than *P. anubis*. Considering that *P. ursinus* also has less variation in GPIIb than *P. anubis*, it may be that targeted GPIIb/IIIa studies.

GPIbα was very challenging to sequence, with 249 bases (equating to 83 amino acids) failing to sequence. The non-sequenced region spans from bases 1186 to 1435 of the GPIbα translation region. Bases 1436–1959 of the sequence were successfully sequenced. The non-sequenced region is located between the VWF-binding domain and the transmembrane domain, in the extracellular portion of the protein. This region is notorious to be very polymorphic, with the presence of a variable number of tandem repeats (VNTR) in humans. The region was named the macroglycoprotein and also contained the PEST domain. The three different size variations of GPIbα in humans 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. Due to the differences detected in this region, it was decided to evaluate each domain within the baboon GPIbα sequence separately. Eighty-nine nucleotide differences were detected in the baboon GPIbα sequence (Figure 5) when compared with the human GPIbα reference sequence (GP1BA-001, transcript ID: ENST00000329380). Twenty-nine differences did not cause an amino acid change. There were, however, sixty differences that caused change in fifty-four amino acids. The baboon nucleotide sequences of the signal peptide, VWF-binding domain, PEST (Proline, Glutamic acid, Serine and Threonine) rich/macroglycoprotein region, transmembrane domain and cytoplasmic domain showed 95.8%, 94.7%, 60.8%, 96.8% and 96.0% similarity, respectively. The amino acid sequences were 93.8%, 89.4%, 65.2%, 90.5% and 95.0% similar, respectively. A comparison was also made between the complete sequences of *P. ursinus* and *P. anubis* (Accession number: XP_009187694). 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

![Figure 5. 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 underlined. Thrombin binding region is in italics and underlined with a waved line. VWF binding amino acids are indicated with arrows. The 6B4 binding amino acids are indicated with a cross (†). A conservative amino acid change is indicated with an asterisk (*). A radical amino acid substitution is indicated with a hash (#). The PEST/Macroglycoprotein domain is underlined with a dotted line. The non-sequenced amino acids are indicated with a strikethrough. The transmembrane region is in grey lettering. The beginning and end of the VNTR are indicated with dollar signs ($)．](image)
78.7% similarity to the human. Interestingly, *P. anubis* has 94 amino acids more than the human, all of them located within the PEST/macroglucoprotein 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-GPIIbα agents in than *P. anubis*.

**Discussion**

The decrease in ADP and arachidonic acid-induced aggregation in the citrated baboon blood was demonstrated in both LTA and whole blood impedance aggregometry. Ristocetin induced agglutination was comparable between baboon and human LTA, when using twice the maximum concentration of ristocetin used for humans. However, high concentrations of ristocetin may cause nonspecific agglutination, because of the interaction between ristocetin and fibrinogen, resulting in a “false correction” in the aggregation. One can hypothesise that it may be due to small sequence differences between the baboon and human receptors. One specific amino acid change in GPIIb, Arg368Gln, needs to be highlighted. The Arg368Gln is situated within a calcium binding site of GPIIb. Thus, a change in this this area may render the baboon platelet more sensitive to changes in calcium concentrations than human platelets. Taking into account that we used sodium citrate, a calcium chelator, as anticoagulant, it may be postulated that the baboon platelet is more sensitive to the presence of a calcium chelator. Therefore, baboon platelets may be less responsive to agonists in the presence of sodium citrate than human platelets. However, the use of hirudin as anticoagulant resulted in comparable results with whole blood impedance aggregometry. For the LTA component of the study, we tried to keep our methods as close as possible to the human diagnostic protocols used in our diagnostic laboratory, thus, the use of an alternative anticoagulant was not considered. This was a limitation of the study, and a follow-up study is planned to determine the optimal anticoagulant for baboon LTA. However, it is believed that the fact that all results using hirudin as anticoagulant were comparable between baboon and human whole blood impedance aggregometry sufficiently showed that the issue of decreased baboon aggregation is mostly negated when using hirudin instead of sodium citrate as anticoagulant. The fact that baboon and human platelets react similarly to the same agonist concentrations when evaluated in whole blood, may also be ascribed to the fact that with the increase in receptor numbers together with subtle sequence differences in all the receptors, the baboon platelets may actually be also less sensitive with less active signaling than human platelets when evaluated in isolation as seen with LTA. Whole blood contains a variety of other cell-derived substances that can attenuate platelet aggregation, thus LTA may be more sensitive in determining subtle functional changes in platelet receptors, as it evaluates platelets in isolation.

Considering the close relationship between platelet activation and subsequent GPIIb/IIIa mediated aggregation, an increase in GPIIb/IIIa may also have an effect on platelet aggregation with LTA. ADP-induced platelet activation is needed for fibrinogen to cause irreversible platelet aggregation [27–31], and GPIIb/IIIa is the receptor for fibrinogen on the platelet surface [32–34]. There is also a correlation between the velocity of ADP-induced platelet aggregation and the saturation of GPIIb/IIIa [35], with the velocity of ADP-induced aggregation directly proportional to the amount of receptor saturation, up to a certain saturation point, after which a plateau is reached. These findings were based on experiments using different fibrinogen concentrations. However, baboon and normal clinical human fibrinogen levels are comparable [36]. Therefore, it may be possible 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 when assessed with LTA. This may also be the case with arachidonic acid.

The amino acids Cys97 and Cys175 have been described as pivotal in P2Y12 receptor expression [37], as well as being putative binding sites for the active metabolites of prasugrel and clopidogrel [38, 39]. However, recently Cys97 has been implicated as the most likely target for P2Y12 inhibiting agents [40, 41]. Glu181, Arg256, Arg265 and Lys280 have been recognised as amino acids that are essential for the functional integrity of P2Y12. Lys280 has also been identified as the vital amino acid in the ligand-binding pocket of the receptor [42]. All these amino acids were present in the baboon sequence; therefore, the P2Y12 receptor expression and ligand and/or anti-P2Y12 agent binding should not be altered or influenced in the baboon. Nevertheless, the amino acid changes present may cause the baboon platelet to be less responsive to ADP when the platelets are evaluated with LTA.

Out of the eighteen amino acid changes found in GPIIb, only Arg281His, Arg400Ser, Pro809His and Trp952Arg, are radical amino acid changes. Arg281His is located within the RGD binding region of GPIIb (amino acids 1 to 334) [43]. From the forty amino acids identified as ligand binding regions [44], only one conservative amino acid change, Val200Ile, was found in the baboon. Arg368Gln is another noteworthy conservative amino acid change, discussed as possible reason for decreased baboon platelet aggregation when using sodium-citrate as anticoagulant. All three amino acid changes in GPIIa are conservative substitutions; therefore, they have limited impact on the folding of the protein. Amino acid residues 164–202 of GPIIIa were the minimum integrin domains required for specific RGD motif containing ligand binding to the GPIbIIIa receptor complex [43]. Thr195Ser is present in this region. All the amino acids involved in the conformational changes, GPIIb and GPIIIa interaction, as well as the talin and kindling binding sites, were intact in the baboon sequences. As is the case with P2Y12, although none of the amino acid changes were in functionally important areas, their presence may render the protein less sensitive to its ligands and agonist, causing a decrease in platelet reactivity when evaluated with LTA.

His15Arg, a conservative substitution, was found in the signal peptide of GPIbα. The N-Terminal globular region of GPIbα holds the entire capacity of the GPIb/IX/V complex to bind its ligands [45]. The VWF-binding amino acids within GPIbα are Asp235 and Lys262 [46], with the anti-GPIbα antibody, 6B4, binding to Asp235 and Lys237 [47]. 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 [46]. The residues directly in contact between the VWF-A1 domain and GPIbα were determined at Ser11, His12, Gln14, Asn16, His37, Glu128, Lys152, Asp175, Thr176, Phe199, Glu225, Asn226, Tyr228 and Ser241. Nonetheless, none of these amino acids are deemed vital for VWF-GPIbα interaction [48]. Within the VWF-binding domain there were thirty amino acid changes found within the baboon GPIbα. However, the VWF-binding amino acids Asp235 and Lys262, as well as the 6B4-binding Lys237, were all present in the baboon. Out of the twenty-five thrombin-binding amino acids only one conservative amino acid substitution, Glu225Asp, were found in the baboon sequence. These results compare well with functional studies where human-targeted 6B4 were able to successfully inhibit arterial thrombosis in a baboon model [7]. His37Gln and Glu225Asp were the only contact amino acids influenced. However, both the changes were conservative substitutions, and as stated earlier, these contact amino acids are not vital for VWF binding to GPIbα. Thus, these changes are unlikely to inhibit the binding of VWF to GPIbα. The thirty amino acid changes within the
N-terminal globular region may possibly explain why a commercial monoclonal antibody (anti-CD42b, BD Cat #: 555471) was not able to bind to baboon platelets. Due to the massive variance found in the PEST/macroglycoprotein regions, together with the lack of a functional description of this region, the results for this region were omitted from further discussion. The baboon transmembrane region had two conservative amino acid substitutions. Binding of 14-3-3ζ to GPIbα is essential for the signalling that ultimately leads to the activation of integrin GPIIb/IIIa [49]. Five amino acids Ser606, Gly607, His608, Ser609 and Leu610, were found to be critical within the 14-3-3ζ binding domain [50]. 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. The critical amino acids, Ser606, Gly607, His608, Ser609 and Leu610, were all intact in the baboon GPIbα sequence. Even though none of the amino acid changes were in vital areas for receptor function, it cannot be ruled out that these amino acid changes may cause a subtle conformational change in the baboon receptor protein to functionally alter its structure, possibly making the baboon receptor have less affinity for its agonist and ligands. 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 with LTA. Conversely, this correction may have been spurious because of the interaction between fibrinogen and the high concentration of ristocetin, and not VWF and GPIbα, as has been described in humans [51]. It can also not be excluded that differences exist between the baboon and human VWF causing variances in baboon ristocetin induced agglutination. The antithrombotic properties of the anti-human anti-GPIbα antibody, 6B4 [6, 7], and the anti-human anti-VWF antibody, ALX-0081 [8], showed that baboon and human GPIbα and VWF have similar properties. Consequently, it may 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. However, this does not explain why a 25.5% increase in receptor number requires 100% more ristocetin to elicit a response. Therefore, it can be hypothesised that the need for a higher ristocetin concentration in LTA may be due to a combination of the receptor number increase and the sequence differences observed in the human GPIbα sequence. Interestingly, ristocetin induced agglutination was comparable between baboon and human platelets at similar ristocetin concentrations when evaluated with whole blood impedance aggregometry using hirudin as anticoagulant. Thus, baboon ristocetin induced agglutination may also be more sensitive to low levels of calcium as seen with the ADP and arachidonic acid results. Nonetheless, it was reported that porcine platelets did not respond adequately to ristocetin, even at very high concentrations (3.6 mg/mL). It was speculated that this was due to species-specific differences between the human and porcine VWF and GPIbα [24]. Thus, the fact that baboon platelets do react with ristocetin, makes it a more suitable animal model for GPIbα studies than the pig.

Predictive protein modelling was done on the online platform created by the Swiss Institute of Bioinformatics (http://swissmodel.expasy.org/workspace). No drastic changes in the secondary protein structures were predicted; however, no predictive software can be 100% accurate in determining protein folding. Thus, we cannot exclude the possibility that some of the amino acid changes caused radical conformational or functional modifications responsible for the observed functional differences.

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 [11]. 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 GPIIa 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. The high similarity between the RGD binding regions with the human and baboon GPIIb/IIIa receptors also contributes to the fact that results from the baboon model may be used to predict the possible effect of these agents on humans with only limited interpretation and calculations needed. This illustrates how the current study will contribute to making relevant and more accurate conclusions and predictions from baboon model studies in the future. When re-evaluating this particular previous study, our current data can be used to better predict the potential outcome of pre-clinical human-targeted studies. An example is that the increase in receptor numbers would have made the increased need for inhibiting agents more predictable. However, results of that study is not disqualified by the current study, as the increased need of three to nine times the recommended human dose to elicit full inhibition of arterial thrombosis in the baboon model is not fully explained by the smaller increase in receptor numbers on the baboon platelets. Determining the exact receptor epitope to which an inhibiting agent is directed, and the availability of the baboon sequence, will also enable us to more accurately predict the interaction between baboon platelets and human-targeted agents, prior to commencement of any functional pre-clinical studies. Therefore, we will be reducing the possibility of expensive wastage of resources and animals.

Therefore, considering the GPIIb, GPIIIa, GPIbα and P2Y12 sequencing results, it is believed that due to the minimal changes observed between the human and baboon at vital amino acids, and the fact that this animal has successfully been used in previous functional studies, the chacma baboon (Papio ursinus) may be used as model to evaluate human-targeted anti-GPIIb/IIIa, anti-GPIbα and anti-P2Y12 agents in. However, the radical amino acid changes, optimal anticoagulant and agonist concentrations, and receptor number differences should be taken into consideration when evaluating molecular-based anti-platelet agents in the future.

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Declaration of interests

The authors have no conflict of interest to declare. The research was funded by a National Health Laboratory Services (NHLS) Research Trust grant.

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 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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