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Characterization and Comparison of Two Known Mutations in Protease-Activated Receptors (PAR4 Ala120Thr and PAR2 Ala118Thr)

MASTERARBEIT

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Contents

1. Abstract

Introduction: Protease-activated receptors (PARs) play an essential role in the human bloodstream. They are involved in coagulation and barrier function as well as in upregulation of inflammation. Four PARs are known in humans, namely PAR1-4. A well-known mutation located in PAR4, Ala120Thr, seems to be important in coagulation as it was shown that people with this single nucleotide polymorphism have an increased activation of blood platelets by the protease thrombin and leading to more cardiovascular events. Interestingly, a similar mutant is known in PAR2 Ala118Thr that is in contrast to PAR4 not expressed on platelets. Both receptors appear to be involved in vascular inflammation and thus increased or decreased vascular inflammation is suspected in carriers of the mutations. The PAR4 mutation has already been studied thoroughly regarding platelet activation, while its effect on vascular inflammation is not fully understood. On the contrary, the PAR2 mutant has not been studied at all. Accordingly, the main focus in this paper lies on PAR2 and PAR4.

This thesis examined whether the mutations PAR4 Ala120Thr and PAR2 Ala118Thr lead to an increased or decreased proteolytic cleavage efficiency by thrombin of the receptors compared to the wild-type (PAR4 Ala120, PAR2 Ala118). Additionally, the aim of the study was to find out what effect the variation of thrombin concentrations and the addition of the cofactor thrombomodulin (TM) have on the cleavage efficiency by thrombin.

Methods: For this comparison of the cleavage efficiency by thrombin an experimental study design was chosen. In order to construct the mutations Ala120Thr in PAR4 and Ala118Thr in PAR2, the corresponding primers were prepared first. Cloning took place via site-specific mutagenesis followed by transformation in *E. coli (Escherichia coli)*. In a further step, the cleavage assay was carried out. Human 293T cells (human embryonic kidney cells) were transfected with the wild-type and mutated plasmids via lipid-droplets. The cleavage efficiency of the receptors by thrombin was made visible using a secreted alkaline phosphatase (SEAP) assay. Receptor expression was quantified by counting the cells and measuring the cell activity by a cell viability assay. With the help of various antibodies, the expression of the receptors into the cell membrane was made visible by using a cell surface enzyme-linked immunosorbent assay (ELISA).

Results: The PAR4 mutation did not reveal any different cleavage efficiency by thrombin compared to the wild-type receptor. Quite unexpectedly, there was no direct cleavage by thrombin in the mutated PAR2, although the receptors were expressed and carried to the cell membrane, which was confirmed by the cell surface ELISA. Hence, the PAR2 mutation showed a lower cleavage efficiency by thrombin than the wild-type PAR2. A higher cleavage efficiency by thrombin was measured in both receptors, PAR4 and PAR2, with an increased concentration of the protease thrombin as well as when adding the cofactor TM.

Conclusion: The results of the PAR4 examinations lead to the assumption that the observed clinical effects in carriers of the PAR4 mutation may be caused by different expression or activation on platelets rather than by the cleavage efficiency by thrombin. Furthermore, the present study on PAR2 revealed quite unexpectedly no direct cleavage by thrombin of the PAR2 mutation compared to the wild-type and thereby provides a new basis for approaching further research. An even better understanding of the cleavage and activation of the PARs would be essential to develop, for example, new treatment methods of PAR-related coagulation and inflammatory diseases.

2. Abbreviations

AP	Activation peptide
BSA	Bovine serum albumin
CCK8	Cell counting kit 8
cDNA	Complementary deoxyribonucleic acid
DGA	Diacylglycerol
DMEM	Dulbecco's modified eagle's medium
dNTP	Deoxynucleotide triphosphates
E. coli	*Escherichia coli*
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
GPCRs	G-protein-coupled receptors
HBSS	Hank's balanced salt solution
HEK cell	Human embryonic kidney cell
HRP	Horseradish peroxidase
IL-8	Interleukin-8
IP3	Inositol triphosphate
LB	Lysogeny broth
MOPS	3-(N-morpholino) propane sulfonic acid
NAD(H)	Nicotinamide adenine dinucleotide (hydrogen)
NCBI	National Center for Biotechnology Information
NEAA	Non-essential amino acid
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
OD	Optical density
PAR	Protease-activated receptors
PBS	Phosphate buffer saline

PCR	Polymerase chain reaction
PLC	Phospholipase C
PNPP	P-nitrophenol phosphate
PFA	Paraformaldehyde
SEAP	Secreted alkaline phosphatase
SNP	Single nucleotide polymorphism
SOC	Super optimal broth with catabolite repression
TM	Thrombomodulin
TMB	3,3',5,5'-tetramethylbenzidine
YD-3	1-benzyl-3(ethoxycarbonylphenyl)-indazole

3. Introduction

Cardiovascular diseases are among the most common disorders in today's society and they are a leading cause of death, especially in the older generations, but they also shape the lives of younger people (1, 2). In the bloodstream, PARs play an important role at a molecular level (3, 4). The four known human PARs are membrane bound G-protein-coupled receptors (GPCRs) which consist of seven-transmembrane domains (5). The genes F2RL, F2RL1, F2RL2 and F2RL3 encode PAR1-4. They are particularly common on platelets, but depending on the PAR, they are also expressed on other body cells as for example on human keratinocytes, fibroblasts, smooth muscle cells or on the endothelium. The N-terminus of the amino acid chain is directed extracellularly and the carboxy end is directed intracellularly. It is unique that these receptors are activated via an intramolecular ligand generated by proteolytic cleavage at the N-terminus. A part of the amino end of the protein is split off specifically by various proteases, creating a new tethered ligand. This newly created N-terminus now binds to the second extracellular loop of the receptor and thereby activates it. All four receptors are activated by the same mechanism via GPCRs. The differences are that they are expressed on variable cells, that they are cleaved by diverse proteases and different G-proteins are involved and so do not perform the same function. They also differ in the activation of the intracellular pathways and the biochemical kinetics vary widely. Moreover, PAR 1-4 differ in many other aspects: potentially multiple cleavage sites, involvement of coreceptors and cofactors, multiple dimerizations with other PARs, varying spatial organization, differing glycosylation and phosphorylation patterns (5, 6, 7, 8, 9). Many functions and properties of the PARs have been researched in the last 20 years, but much is still unknown. PAR1, 2 and 3 are located in the human genome on chromosome 3 and PAR4 on chromosome 19 (10). PAR1 and PAR4 are largely expressed on the blood platelets and, therefore, they are involved in the activation or inactivation of the platelets. PAR3 is mainly found on the platelets in mice, while it is secondary in the human body (11). PAR2 is located, for example, in the cells of the dorsal root ganglia, in the epithelial cells, nerve fibres and myocytes of the respiratory tract and in various cells of the digestive tract. But it is not expressed on the platelets and therefore not involved in the platelet activation or inactivation (8, 12, 13, 14). PAR4 and PAR2 are both supposed to be involved in vascular inflammation (15, 16).

PARs are cleaved by various proteases found in different tissues, but the most important protease is the serine protease thrombin (6, 7, 17). PAR4 is cleaved between position 47 and 48 (arginine and glycine) (18). In contrast to PAR1, it was thought for a long time that a 100-fold higher thrombin concentration was needed to activate human PAR4 (0.01nM for PAR1 and 1 nM for PAR4 measured on COS7 fibroblast cells) because PAR4 is less sensitive to thrombin (19, 20, 21). Only recently this assumption has been refuted by conducting the experiments on platelets (22). It is generally agreed, however, that PAR1 is important in the first rapid activation of the platelets and that PAR4 rather leads the slower and stronger platelet aggregation that follows after (23). PAR2 is mainly cleaved by trypsin between the amino acid position 36 and 37, where an arginine and a serine are located (17). In 2019 it was shown that PAR2 is also cleaved between the same amino acids by physiological concentrations of thrombin if thrombin is recruited to the cell surface and bound to the co-factor TM (7). TM is encoded by the gene THBE.

In this thesis the focus lies on PAR4 and PAR2. The cleavage of the receptors and the subsequent binding of the new N-terminus to the second extracellular loop leads to the activation of a certain G-protein subunit depending on which protease the receptors are cut by. For example, intracellular activation pathway via G12/13 subunit in PAR4 is stimulated through the cleavage by the protease thrombin and in PAR2 through the cleavage by the protease trypsin (7, 24). Another intracellular pathway leading via the G-protein subunit Gq is promoted by the protease thrombin in PAR4 and by the protease matriptase in PAR2 (24, 25). This recruits phospholipase C (PLC) that activates inositol triphosphate (IP3) and diacylglycerol (DAG). In PAR4 the two molecules produce a release of Ca^{2+} from the endoplasmic reticulum which leads to platelet activation (26). In contrast, these pathways lead to a release of pro-inflammatory signals in PAR2 expressing cells (25). A different pathway over the gene regulation protein nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) that migrates into the cell nucleus and binds there to the DNA response element is involved in PAR4 and PAR2 signalling (16, 27). This enables the cells to synthesize various chemokines such as interleukin-8 (IL-8) and cause inflammation (8).

It is known that platelet aggregation via the activation of PAR4 is higher in dark-skinned people than in white people. This could therefore lead to an increased cardiovascular risk and a worse outcome after a cardiovascular occasion (4, 28). Molecularly a single

nucleotide polymorphism (SNP) in the genetic code for the PAR4 could explain this increased risk. The specific point mutation Ala120Thr was found to be the critical mutation in this context. The amino acid position 120 is located in the second transmembrane loop of PAR4. This is exactly that part of the receptor to which the cleaved N-terminus binds and activates it. A carrier of the mutation has a threonine instead of an alanine at this position. The prevalence of this mutation is higher in black people than in white people. 63% of the black people and 19% of the white population are carriers of this specific point mutation. Inhibition by a synthetically produced PAR4 antagonist (YD-3) also presented a lower antithrombotic effect *in vitro* on platelets with the mutated receptor (29). It was shown that the Ala120Thr mutation in PAR4 leads to a higher activity of the receptor at thrombin concentrations lower than 1nM. No difference was seen with neither a longer exposition time to thrombin nor at higher thrombin concentrations in the experiments which were carried out with platelets. It was also shown that PAR4 Ala120Thr is not cleaved more compared to the wild-type (22). In one of the latest studies on this SNP flow cytometry revealed that the mutation does not show any increased expression of the receptor on the surface of platelets (30). Whether PAR4 Ala120Thr has an influence on vascular inflammation, in which PAR4 also appears to be involved, has not yet been researched.

A similar point mutation in PAR2 is already known. The mutation in PAR2 is located at amino acid position 118. At this position the amino acid alanine has been replaced by a threonine. The effect of the comparable mutation Ala118Thr on PAR2 has not yet been considered. In this study, these two mutations were explored in more detail. *In vitro* it was investigated whether these two specific mutations lead to an increased proteolytic cleavage efficiency by thrombin of the receptors compared to the wild-type. In addition, it was evaluated whether the increased activation of PAR4 in carriers of the Ala120Thr mutation can be explained by increased cleavage efficiency by thrombin. In the case of the similar mutation Ala118Thr in PAR2, it was researched whether this SNP increases or decreases the cleavage efficiency by thrombin of the receptor towards prolonged inflammatory signalling. These changes could play an important medical role for carriers of the mutation. Since the receptor is expressed on various cells (see above) and represents an essential part of our immune system through the expression of IL-8. If the receptor, as had already been researched similarly in PAR4, is cleaved more by the SNP, then the carriers of the mutation could, for example, suffer

an excessive and prolonged inflammatory reaction of their tissue. In the long term, further investigation on this specific point mutation in PAR2 could lead to a deeper understanding of the pathophysiology of some diseases.

4. Materials and methods

4.1. Reagents

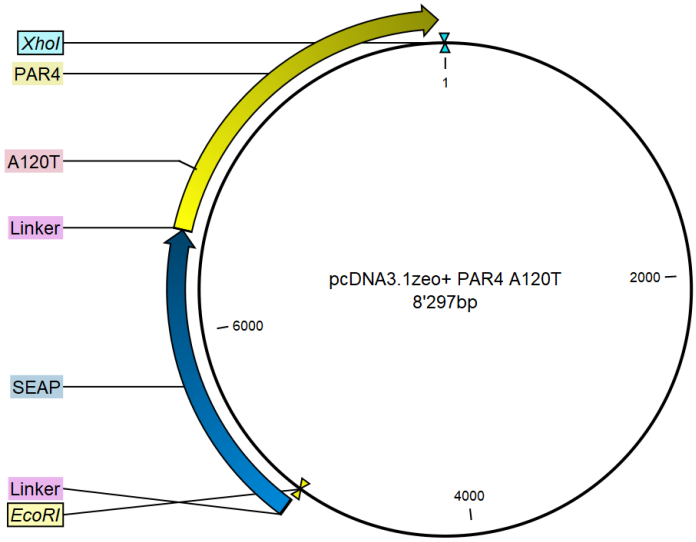
The two specific primers containing the mutations were produced custom-made by Microsynth AG (Belgach, Switzerland). The Phusion Polymerase and the associated Phusion GC Buffer as well as the deoxynucleotide triphosphates (dNTP) were ordered from New England Biolabs (Frankfurt am Main, Germany). The SeaKem GTG Agarose for the agarose gel was from FMC Bioproducts (Uffholtz, France). The dye EZ-Vision Two DNA Dye and its buffer for the detection of the DNA in the agarose gel were ordered from vwr (Dietikon, Switzerland). The reagents 3-(N-morpholino) propane sulfonic acid (MOPS), Ca_2Cl and $MgCl_2$ were purchased from Sigma (Buchs SG, Switzerland). To maintain and harvest the human 293T cells, phosphate buffer saline (PBS) from Gibco, fetal bovine serum (FBS) from Corning, Dulbecco's modified eagle's medium (DMEM) from Gibco, non-essential amino acid solution (NEAA) from Gibco, L-Glutamine and dissociation buffer both from Gibco were used. Opti-MEM, Hank's balanced salt solution (HBSS) and trypan blue stain were important for the transfection, SEAP assay and cell counting. All of them were from Gibco and were ordered from Fisher Scientific (Reinach, Switzerland). To digest the template DNA, DpnI and the buffer were ordered from Promega (Dübendorf, Switzerland). All components of super optimal broth with catabolite repression (SOC) and lysogeny broth (LB) medium for the transformation into *E. coli* bacteria and the bacterial growth were purchased from Sigma (Buchs SG, Switzerland). Ampicillin was bought from Sigma (Buchs SG, Switzerland). The Miniprep kit for obtaining the mutated plasmids from the bacteria was supplied by Macherey-Nagel (Oensingen, Switzerland). Lipofectamine 2000 from Invitrogen for the transfection, p-nitrophenol phosphate (PNPP) and its buffer for the SEAP assay were all bought at Thermo Fisher Scientific (Reinach, Switzerland). L-polylysine for the transfection was purchased from vwr (Dietikon, Switzerland). The protease thrombin was ordered from Haematologic Technologies, (Esses Junction, VT, USA). Human albumin was supplied by Sigma (Buchs SG, Switzerland). The cell counting kit 8 (CCK8) from Sigma (Buchs SG, Switzerland) was used to measure the cell viability. Paraformaldehyde (PFA) and bovine serum albumin (BSA) for the cell surface ELISA were purchased from Sigma (Buchs SG, Switzerland). The monoclonal PAR1 ATAP2 antibody was used as in previous studies (31). The PAR2 SAM11 antibody from

Thermo Fisher Scientific (Reinach, Switzerland), PAR2 ECL antibody (MAB3949; R&D Systems, Inc., MN, USA) were used as stated by the manufacturer. Tetramethylbenzidine (TMB) was purchased from Thermo Fisher Scientific (Reinach, Switzerland). Sulfonic acid was from Sigma (Buchs SG, Switzerland). For the polymerase chain reaction (PCR) and the digestion by the enzyme DpnI the thermocycler from vwr (Dietikon, Switzerland) was needed and the centrifuge was also acquired from vwr (Dietikon, Switzerland). The lab shaker was from Adolf Kühner AG (Birsfelden, Switzerland). The NanoDrop spectrophotometer, the photometer multiskan labsystem MCC 346 and the tilt platform were bought from Thermo Fisher Scientific (Reinach, Switzerland).

4.2. Construction of the mutated PAR4 and PAR2

The vector pcDNA3.1zeo+ (complementary deoxyribonucleic acid) served as a structural framework in which the sequence of the wild-type receptors PAR4 and PAR2 were inserted by restriction enzymes upstream the basepair 6519 (supplementary Table 1). In addition, the sequence of the secreted alkaline phosphatase (SEAP) was inserted upstream of the receptor sequence. The substrate of the SEAP was used to measure the cleavage by thrombin (32). The vector pcDNA3.1zeo+ was cleaved at base pair 6519 by the restriction enzyme EcoRI for the insertion of the wild-type PAR4. For the insertion of the sequence of the wild-type PAR2 the pcDNA3.1zeo+ was cleaved at position 6519 with the restriction enzyme EcoRI and, additionally, at position 6552 with the restriction enzyme XhoI. PcDNA3.1zeo+-SEAP-PAR4 was named pI26 and with the insertion of the PAR2 sequence pI331. The total length of the constructed pI26 was 8297 base pairs and the pI331 was 7641 base pairs long. The two wild-type plasmids had already been constructed for the purpose of previous research on the wild-type receptors PAR4 and PAR2 by Reto Schüpbach's laboratory and therefore it was not necessary to carry out this process again (8). The related plasmid maps are shown in Figure 1. The vector pcDNA3.1zeo+ served as a template to clone the mutated PAR4 and PAR2 by site specific mutagenesis.

A



B

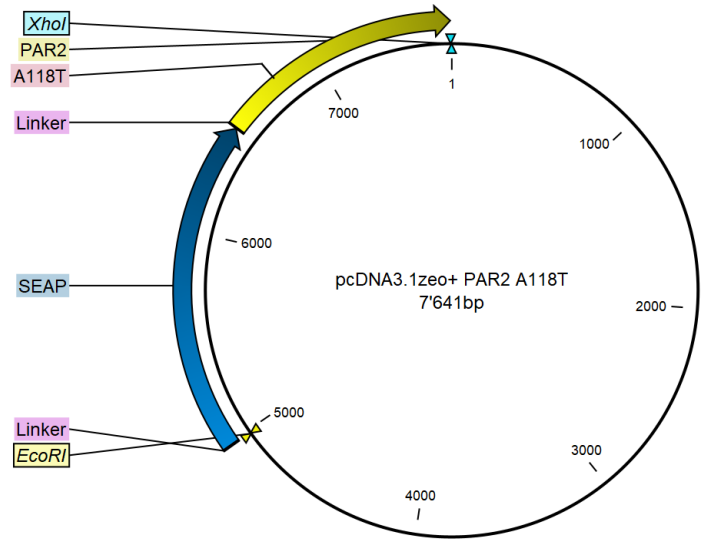


Figure 1: Illustration of plasmid pI26 with the mutation PAR4 Ala120Thr and plasmid pI331 with the mutation PAR2 Ala118Thr. The vector structure is marked uncoloured and the base pair position of the mutations are written in pink. The location of the SEAP sequence is shown in blue. The length and position of the two receptors are shown in yellow. The linkers between the different structures are marked in purple. The abbreviation bp means base pair and A and T stands for the amino acids alanine and threonine. (A) Representation of plasmid pI26 and the corresponding position of PAR4 Ala120Thr. (B) Scheme of plasmid pI331 and the corresponding position of the mutation PAR2 Ala118Thr.

The primers for the two receptor mutants were designed so that the nucleotide exchange was exactly in the right position. In PAR4 the amino acid position 120 and in PAR2 the amino acid position 118 were changed. The associated primers are listed in Table 1. The highlighted nucleotide indicates the location of the mutation. Both point mutations replace the amino acid alanine to a threonine in the transcription of the receptors. Once the primer had been generated, the plasmids were cloned by site specific mutagenesis.

Table 1: Overview of the primers for PAR4 Ala120Thr and PAR2 Ala118Thr. The bold bases indicate the mutated sites. The forward and the reverse primers are listed.

Receptors	**Primers**
PAR4 Ala120Thr	forward 5'CTGATGAACCTCGCG**A**CTGCTGACCTCCTGC'3 reverse 5'GCAGGAGGTCAGCAG**T**CGCGAGGTTCATCAG'3
PAR2 Ala118Thr	forward 5'GATTTACATGGCCAATCTG**A**CCTTGGCTGACC'3 reverse 5'GGTCAGCCAAGG**T**CAGATTGGCCATGTAAATC'3

The plasmids pl26 and pl331 served as template sequences in the polymerase chain reaction PCR. For the PCR, H_2O, 10mM dNTP, 5x Phusion GC Buffer, Phusion Polymerase, the template plasmids (pl26 or pl331) and the associated reverse and forward primers from PAR4 or PAR2 were mixed (Table 2). It was important to always vortex the substances well before mixing and, of course, they were stored refrigerated on ice; otherwise, the polymerase would have been activated too early.

Table 2: The reaction substances and the associated quantities for the PCR.

PCR reaction substances	**amount**
H_2O	18.5 µL
5x Phusion GC buffer	5 µL
Phusion Polymerase	0.25 µL
10 mM dNTP	0.5 µL
Plasmid (pl26 or pl331)	0.25 µL
Primer forward and reverse	0.5 µL

The PCR took place in the thermocycler. First, the thermocycler was heated up to 98 ℃ for 30 seconds. Then, a cycle consisting of three steps was repeated 30 times. In the first step of the cycle, the reaction mixture was heated to 98 ℃ for 30 seconds to separate the two strands of the DNA. This was followed by the annealing of the mutated primers to the DNA for PAR4 at 66.7 ℃ and for PAR2 at 70 ℃ for 30 seconds. In the last step of the cycle, the Phusion Polymerase transcribed the new DNA strand with the desired mutation in four minutes at 72 ℃. These three steps mentioned were always repeated in the same order (33). The schematic representation of the three repeated steps of the PCR is shown in Figure 2. The final step of the PCR was the elongation of the DNA during 10 minutes at 72 ℃.

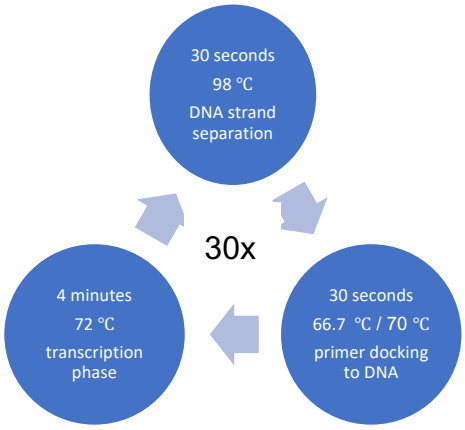


Figure 2: This diagram illustrates the PCR cycle with the corresponding temperatures and durations. The three steps were repeated 30 times in the thermocycler.

The PCR product mixture still contained the amount of template wild-type DNA that was added at the beginning. To eliminate the circular wild-type DNA it had to be digested by the DnpI enzyme. This specific enzyme can cut DNA in the palindromic sequence 5'GATC'3 between the bases adenine, which is methylated and thymine (34). In general, this adenine methylation can only be found in genetic material that is naturally generated in cells and not created artificially by a PCR. This step allowed the purification of the PCR product by cutting the non-mutated DNA (35, 36). The PCR reaction mixture, the enzyme DnpI, and its buffer remained in the thermocycler at 37 ℃ for one hour. Next, the enzyme was inactivated for 20 minutes at 80 ℃. A gel electrophoresis from agarose was then used to check whether the plasmids with the mutated sequence were present. The PCR products were linear-shaped compared to the annular template plasmids. After that, the plasmids pl26 and pl331, which contained the sequence of the mutated receptors, were transferred into *E. coli* bacteria to replicate the DNA.

4.3. Preparation of *E. coli*, transformation and extraction of the plasmids

E. coli are commensals of our intestines (37). On the one hand, in unfavourable situations they can turn out to be a pathological agent. On the other hand, they can be a very useful tool in the research laboratory. Thus, these bacteria were used to replicate the mutated plasmid which had been generated by the PCR. First, the *E. coli* were

multiplied to obtain enough bacteria for the following transformation step. For this reason, 1 ml *E. coli* culture was mixed with 99 ml LB and left for two hours at 37 ℃ in a shaking incubator. The bacteria divided in this process. In the incubator the bacterial multiplication was measured every 30 minutes with the photo spectrometer until a level of 0.250 optical density (OD) was reached. This minimum value of the OD clearly indicated that there were enough bacteria in the medium. As soon as this minimum level of bacteria was reached, they were centrifuged for five minutes at 4 ℃ at 5000 rpm. Next, 50 ml $MgCl_2$ 0.1 M was added, and the mixture was centrifuged again for five minutes at 4 ℃ at 5000 rpm. After discarding the supernatant, 50 ml $CaCl_2$ 0.1 M was added and incubated on ice for another 30 minutes. In the meantime, a solution was made to preserve the bacteria. This solution contained 0.1 M MOPS, 0.005 M $CaCl_2$, 20% glycerol and was adjusted to a pH of 6.5. To produce the solution, first 4.1852 g MOPS was dissolved in 20 ml H_2O and then titrated with 9 ml NaOH until a pH value of 6.5 was reached. After that, MOPS was placed in a 0.5 ml bulb and then mixed with 0.0368 g $CaCl_2$ dissolved in 1 ml glycerol. Finally, the flask was filled with 3.5 ml H_2O to a total volume of 5 ml. After the *E. coli* had been cooled on ice for 30 minutes, they were mixed with 5 ml of the solution. In a next step, 100 µl portions of *E. coli* were put into tubes and then immediately shock frozen at -196 ℃ and stored at -80 ℃.

The bacteria were then ready for the heat shock transformation to multiply the wild-type and mutated plasmids. In a first step, the plasmids were transformed into the bacteria *E. coli* as explained above. The bacteria were cooled on ice for 30 minutes and afterwards they were shocked with heat for 90 seconds at 42 ℃. The bacteria were then incubated for 1 hour at 37 ℃. This heat shock enabled the cell wall of the gram negative bacterium to become permeable to the plasmid (38). The bacteria were spread on LB-agarose plates treated with ampicillin so that they would grow optimally at a temperature of 37 ℃. The following day some colonies were sent to Microsynth AG for the sequencing, where the colonies were checked for the specific mutations via the Sanger Sequencing by the primer p91, which bound to the sequence of the upstream located SEAP sequence and by the primer BGH reverse. This sequencing confirmed that the mutations were present on the plasmids. A liquid culture had to be produced from the bacteria which were still on the agarose plate. A liquid of 10 ml LB and 10 µl ampicillin was prepared and then the positive (containing the mutation) colony was picked from the agarose plate and added to the media. The bacteria in the

liquid culture were able to grow overnight at 37 ℃ in the shaking incubator. In a next step, the plasmids that carried the mutated DNA for the receptors had to be extracted from the *E. coli* to be purified. A miniprep kit was used according to the manufacturing instructions for the extraction out of the bacteria so that the plasmids were obtained in pure form within an hour.

4.4. Cleavage assay of PAR4 and PAR2

The cloning and transformation into the bacteria were followed by the expression in human embryonic kidney cells (HEK), also known as human 293T cells, and the measurement of the thrombin cleavage efficiency of PAR4 and PAR2, which delivered the results that enabled a comparison of the wild-type and the mutation (7). The cleavage efficiency by thrombin was made visible by a SEAP assay. SEAP stands for secreted alkaline phosphatase. The DNA sequence of the SEAP was linked by Reto Schüpbach's laboratory on the pcDNA3.1zeo+ vector upstream of the DNA sequence of the receptor as already mentioned earlier (8). Hence, the construct carried an extracellular alkaline phosphatase tag (39). This allowed to test the cell surface expression of the construct via simply measuring alkaline phosphatase activity of the cell surface. Consequently, it enables to test whether thrombin cleaves the extracellular N-terminus of the construct by measuring the decline of cell surface alkaline phosphatase activity or measuring alkaline phosphatase activity in the cell supernatant. Alkaline phosphatase activity was measured using p-nitrophenol phosphate (PNPP) acted as a substrate for the enzyme alkaline phosphatase and was therefore important in the cleavage assay. PNPP carries a phosphate group. This phosphate group was cleaved by the enzyme alkaline phosphatase and the resulting p-nitrophenol showed a yellow colour at a wavelength of 405 nm. The interaction of PNPP with the alkaline phosphatase is shown in Figure 3. This whole system was used to test whether the protease thrombin cleaves the corresponding receptor.

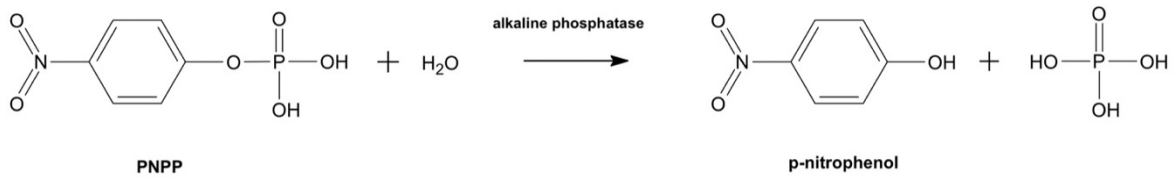


Figure 3: The chemical reaction of the PNPP dephosphorylation to p-nitrophenol and phosphate. Alkaline phosphatase cleaves the phosphate group from the PNPP and forms p-nitrophenol in the process. This reaction results in a yellow colour change, which was quantified at a wavelength of 405 nm.

Cell culturing conditions and lipofection, which were important for the cleavage assay, will be explained now. In order to measure the cleavage efficiency by thrombin, the receptors had to be expressed on the surface of cells. For this purpose, human 293T cells were used. They have the special characteristic that they grow adherent to the bottom of the culture flask and can be kept relatively easily although they have to be cultivated in an incubator under certain conditions. They grew in an environment of 37 °C, 5% CO_2 and a humidity of at least 95%. The cells were cultured in a nutrient solution (DMEM + 10% FBS + 1x NEAA + 2x L-Glu) the whole time.

The human 293T cells were transfected with the plasmids carrying either the mutated receptor sequence or the wild-type sequence in order to express the receptor genes and to integrate them into their cell membrane. Human 293T cells had to grow in the incubator for two days in order to have a confluence rate of 70-90% for the transfection via lipofection. The transfection process with lipofectamine of the human 293T cells was done according to the Thermo Fisher Scientific protocol. Lipofectamine 2000 was required to transfect the human 293T cells with 1.6 mg/ml plasmid. Lipofectamine 2000 is a substance that allows forming a chemical complex with the negatively charged nucleic acids in order to permeate the cell membrane more easily (40, 41). Lipofectamine 2000 and the plasmids were mixed and stored for 20 minutes at room temperatures. The mixture was then applied to the human 293T cells over a period of four hours enabling the DNA to be taken up by the cell. A 96-well plate was treated with L-polylysine so that the transfected cells would better adhere to the bottom of the well plate. The human 293T cells were harvested from the 6-well plate (on which the transfection took place) using cell dissociation buffer and carefully placed on the 96-well

plate prepared with L-polylysine. The cells (transfected with the plasmids) were then incubated for two days so that they had sufficient time to express the receptors and transport them into the cell membrane.

Two days after transfection the cleavage efficiency by thrombin of the PARs on the cell surface were made visible with a SEAP assay. The serine protease thrombin was used to cleave the receptors. Four different concentrations of thrombin (0, 3, 10, 30 nM) diluted in HBSS were used to compare the cleavage efficiency by thrombin of the receptors. For the SEAP 50µl HBSS or corresponding protease concentrations was prewarmed in a 96-well plate at 37 ℃ for 20 minutes. HBSS is a solution that maintained the pH and osmotic gradient and guaranteed an optimal environment for the cells. After 20 minutes the medium was removed from the well plate with the transfected cells. The well plate with the cells was then washed carefully with HBSS. Preheated HBSS or proteases were added onto the cells and the plate was placed in the incubator for exactly 20 minutes. In the meantime, a 96-well filter plate was washed twice with distilled H_2O and then centrifuged. Another non-sterile 96-well plate was prepared with 50 µl PNPP in each reaction compartment and the filter plate was placed on top of that PNPP plate. After the cells had been in the incubator for 20 minutes, the supernatant of the cells was pipetted into the filter plate. The filter plate was centrifuged and the supernatant thus passed into the 96-well plate below with 2x PNPP. In the meantime, 50 µl 1x PNPP was applied to the 96-well plate which carried only the cells. The absorption of the PNPP in the 96-well plate containing the cells was first measured in the plate photo spectrometer after 2.5 minutes at a wavelength of 405 nm. The supernatant of the cells was then measured in the same way after 3, 6 and 12 minutes. The supernatant contained all the alkaline phosphatases that came from cleaved PARs. The receptors on the cells were measured to compare the receptor expression in each well of the 96-well plate. The principle behind this is explained in more detail in the discussion.

4.5. Cell viability and counting of PAR4 and PAR2 transfected cells

Finally, the cell viability of PAR4 was measured in transfected human 293T cells. It was done with a cell counting kit 8 (CCK8). CCK8 is a substance that contains tetrazolium salt. Only living cells reduce the tetrazolium salt by obtaining the electron from the nicotinamide adenine dinucleotide hydrogen (NADH), which results in an orange colour change at a wavelength of 450 nm. The height of the absorption at 450 nm is directly proportional to the number of living cells since only living cells have the energy to produce NADH from $NAD^+ + H^+$ via a dehydrogenase. 200 µl media (DMEM + 10% FBS + 1x NEAA + 2x L-Glu) and 2 µl CCK8 were mixed and applied to the cells, which had been transfected and seeded on a 12-well plate two days before. The colour change was measured and quantified in the photo spectrometer at a wavelength of 450 nm after 30 minutes of incubation (37 ℃ , 5% CO_2, >95 % humidity).

To rule out that there were different numbers of cells in the various compartments of the 96-well plate, the cells carrying PAR4 and PAR2 were counted after the transfection. In a Neubauer counting chamber there are four big squares each containing 4x4 smaller squares for counting. One big square has an area of 1 mm^2 and measures 0.1 mm in height, which results in a volume of 0.1 µl. In this research, the cells were mixed in a ratio of 1:1 with trypan blue, and therefore the average had to be multiplied by 2 and 10^4 to get the number of cells per ml. A sample calculation is shown in Table 3. For this procedure, the cells were harvested with cell dissociation buffer and resuspended with 400 µl media. 25 µl of it was mixed with 25 µl trypan blue. 30 µl of this cell suspension was placed in a Neubauer counting chamber and then the cells were counted.

Table 3: Calculation steps of the cell count. The calculation is shown using the example of the counting of PAR2 Ala118Thr.

Steps	**Calculations for PAR2 Ala118Thr**
Number of cells in the four squares	1. big square: 11 / 0.05 µl 2. big square: 12 / 0.05 µl 3. big square: 9 / 0.05 µl 4. big square: 10 / 0.05 µl
Average calculation	11 / 0.05 µl + 12 / 0.05 µl + 9 / 0.05 µl + 10 / 0.05 µl = 42 / 0.05 µl 42 / 0.05 µl ÷ 4 = 10.5 / 0.05 µl
Extrapolation to 1 µl	10.5 / 0.05 µl × 2 → 21 / 1 µl
Extrapolation to 1 ml	21×10^4 / ml

4.6. Detection of PAR2 with a cell surface ELISA

In addition to the previously described methods further research about the structure and its incorporation into the cell membrane of the PAR2 was done. The aim was to confirm the hypothesis that the PAR2 mutation Ala118Thr could structurally change the receptor and its expression into the cell membrane. This was checked by antibodies which specifically bound to the membrane-bound receptor. Two different antibodies were used, which bound to various amino acids on the PAR2 receptor. The PAR2 ECL antibody bound extracellularly to the second loop of the transmembrane receptor. The second antibody was PAR2 SAM11 antibody and was able to bind at the N-terminus where the receptor was cleaved. An antibody for PAR1 was used, as PAR1 was natively expressed on human 293T cells. This PAR1 ATAP2 called antibody bound to the PAR1 cleavage site. It showed whether there were the same number of cells in the

different well plate wells. This made it possible to make an assertion about the PAR2 expression. The binding to the surface of the receptor was made visible via a cell surface ELISA. In order to make the binding of the just mentioned three antibodies visible, the goat anti-mouse antibody was applied to the cells. This anti-mouse antibody bound to the two variable ends to the fixed domain of the first antibodies. It is called anti-mouse antibody since the first antibodies were produced in mice and thus this antibody is directed against the constant domain of the first antibodies. A horseradish peroxidase (HRP) is located on the constant domain of the goat anti-mouse antibody. The enzyme HRP is able to convert TMB so that a colour change to yellow can be measured at a wavelength of 450 nm. With this method more precise statements could be made about the expression of PAR2 on the cell surface of the human 293T cells. For reasons of clarity, the goat anti-mouse antibody is named secondary antibody in this paper.

The human 293T cells transfected with the mutated and the wild-type PAR2 were first washed with 125 µl HBSS containing 0.1% human albumin. Then 50 µl of 2% paraformaldehyde (PFA) was added for 20 minutes at 4 °C to fix the cells. It was then washed twice again with 125 µl HBSS/human albumin each time. Then 1% BSA dissolved in PBS was added to the cells and the cells were incubated at room temperature for one hour. After the incubation, the washing process with HBS/human albumin was repeated twice. Now the addition of the antibodies followed. To some cells only the incubation agent (HBSS/human albumin) was applied in order to measure the basic absorption level. The PAR2 ECL antibody was applied to other human 293T cells in a ratio of 1:100. The PAR2 SAM11 antibody was also applied to the cells in a ratio of 1:100. The third antibody, PAR1 ATAP2 antibody, was applied to the cells in a ratio of 1: 500. 50 µl of each antibody was given to separate wells. The cells were left on the tilting table for one hour at room temperature and protected from light. This allowed the antibodies to bind regularly to the receptors on the cell surface. The washing process was repeated twice with 125 µl HBSS/human albumin. In each well 50 µl secondary antibody in a ratio of 1: 5000 (mixed with HBSS/human albumin) was applied to the cells. The cells were left covered on the tilting table again for one hour at room temperature. This was followed by a double washing step as described above for the last time. After the incubation time of one hour, 50 µl of TMB in a ratio of 1:1 was applied

to the cells. After ten minutes, the reaction was terminated with 50 µl sulfic acid. The absorption was measured at 450 nm.

4.7. Statistics

The evaluations and analyses of the results were done with Microsoft Excel. A two-sampled, two-tailed t-test was used to calculate the p-values and to determine the significant levels. For the statistics, the mean values +/- SEM of the gained results were compared. Where indicated the values were normalized if the baseline of the compared results were significantly different.

4.8. Ethics

No ethics application had to be made for this research, as neither laboratory animals nor components from animals or humans were used.

5. Results

5.1. Transfection of empty plasmid, TM, PAR4 and PAR2

The cleavage efficiency by thrombin of PAR4 and PAR2 was quantified by a SEAP assay. As described in chapter 4.4., two different measurements were made. On the one hand, alkaline phosphatase activity was measured in the cell supernatant, which was proportional to the number of cleaved receptors. On the other hand, the alkaline phosphatase activity of the cell surface was determined. The cell signal measured resulted from uncleaved receptors since not every single receptor in the cell membrane of the human 293T cells was cleaved by the protease thrombin. In addition, it had to be well considered that the human 293T cells are human embryonic kidney cells. Since kidney cells produce alkaline phosphatase, they also contributed to a certain extent to the chemical reaction with PNPP measured via the absorption (42). The strength of the signal from the kidney cell own alkaline phosphatase was first defined and later used as a baseline. This value was determined using cells which had the sequence neither of a receptor nor of the secreted alkaline phosphatase or of TM. These cells were named “empty plasmids”. It is important to note that empty cells still contained the pcDNA3.1zeo+ vector. If the pcDNA3.1zeo+ vector had been missing, the term “untransfected cells” would have been used.

The determination of the baseline values was carried out using a SEAP assay. The measured absorption from the empty cells was 0.115 OD +/- SEM regardless of the thrombin concentrations. This value could therefore be assumed to be the baseline absorption value occurring in human 293T cells. When adding TM to the empty cells no significant difference had been measured at all thrombin concentrations. Accordingly, TM does not increase the baseline absorption. As expected, the transfection with the wild-type PAR4 and PAR2 with and without TM revealed a significantly higher absorption level compared to the empty cells. The comparison between the wild-type PAR4 and the wild-type PAR2 with and without TM showed a significantly higher value for PAR2. An absorption value of 0.225 OD +/- SEM was determined for all PAR2 wild-type measurements. The only concentrations at which no significant differences were measured between wild-type PAR4 and wild-type PAR2 were at 30 nM thrombin independent of TM and at 3 nM thrombin with TM.

Furthermore, the individual receptors were each compared at different thrombin concentrations. Thrombin cleaved the PAR4 wild-type more efficiently starting with concentrations of 30 nM. The addition of TM the PAR4 wild-type cleavage efficiency increased already at a concentration of 10 nM. The PAR2 wild-type showed no better cleavage efficiency at thrombin concentrations up to 30 nM, however, the cleavage efficiency significantly rose at 30 nM thrombin when adding TM. The corresponding results are shown in Figure 4. The detailed p-values are listed in Table 2A-2C in the appendix.

To summarize, the baseline was determined at 0.115 OD +/- SEM in empty transfected cells. TM does not change that level of absorption and the PAR2 wild-type resulted in a higher absorption in general.

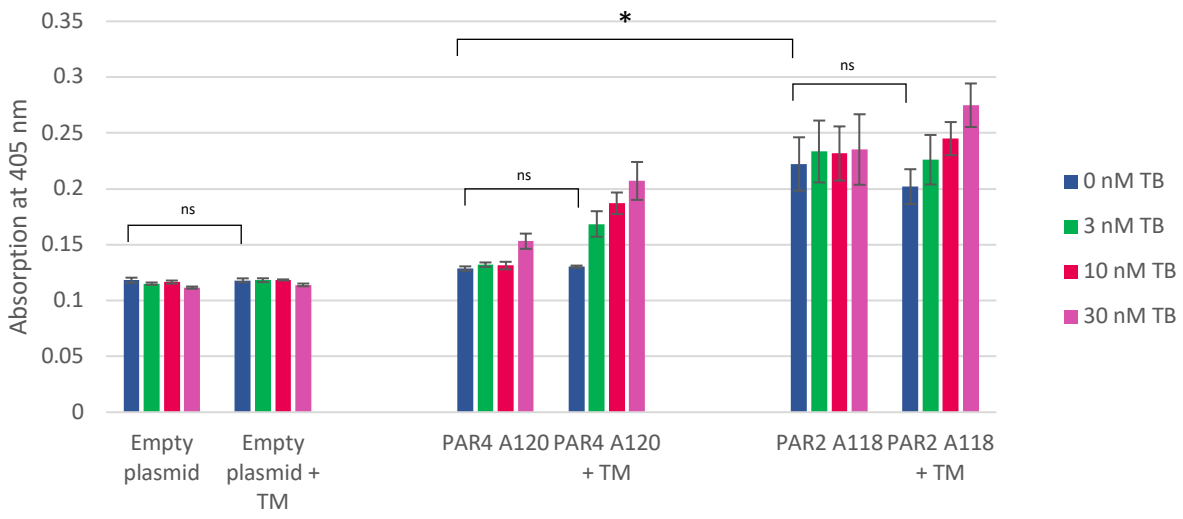


Figure 4: Determination of the baseline absorption value of the SEAP assay and no change of the absorption value by TM. Human 293T cells were either transfected with the vector pcDNA3.1zeo+ (empty plasmid), wild-type PAR4 or PAR2 in absence or presence of TM and the cleavage efficiency by thrombin was then determined by means of a SEAP assay. The cleavage efficiency by thrombin was proportional to the absorption at 405 nm. In all six variants of transfected human 293T cells, different thrombin concentrations were used, which are presented in different colours. For reasons of clarity, the amino acid Ala is abbreviated to A and thrombin to TB. The results were measured after twelve minutes of incubation with PNPP. The data show the mean +/- SEM; three independent experiments were performed as biological replicates with three identical measurements each as a technical replicate; ns = not significant, $p>0.05$, *$p<0.05$, **$p<0.01$, ***$p<0.001$; the p-values were determined with a student's *t*-test.

5.2. Comparison of the PAR4 wild-type and mutation cleavage efficiency by thrombin

The results of the alkaline phosphatase in the cell supernatant are presented in the following. The results described refer to the measuring of the cleavage efficiency by thrombin in absence of TM. The cleavage efficiency by thrombin of the wild-type PAR4 increased with a thrombin concentration at 10 nM and higher compared to zero thrombin. In comparison, the mutated PAR4 already showed a significantly higher cleavage efficiency starting at 3 nM thrombin. When adding TM, cleavage efficiency by thrombin rose in both receptors (wild-type and mutated PAR4) starting at 3 nM and higher. When comparing the cleavage efficiency by thrombin of the wild-type with the mutated PAR4, no significantly higher or lower cleavage efficiency by thrombin of the receptor could be noted. This statement could be made for all thrombin concentrations and independent of TM. The graphical representation of the data just described are shown in Figure 5 and the corresponding p-values are attached in supplementary Tables 2D-2F in the appendix.

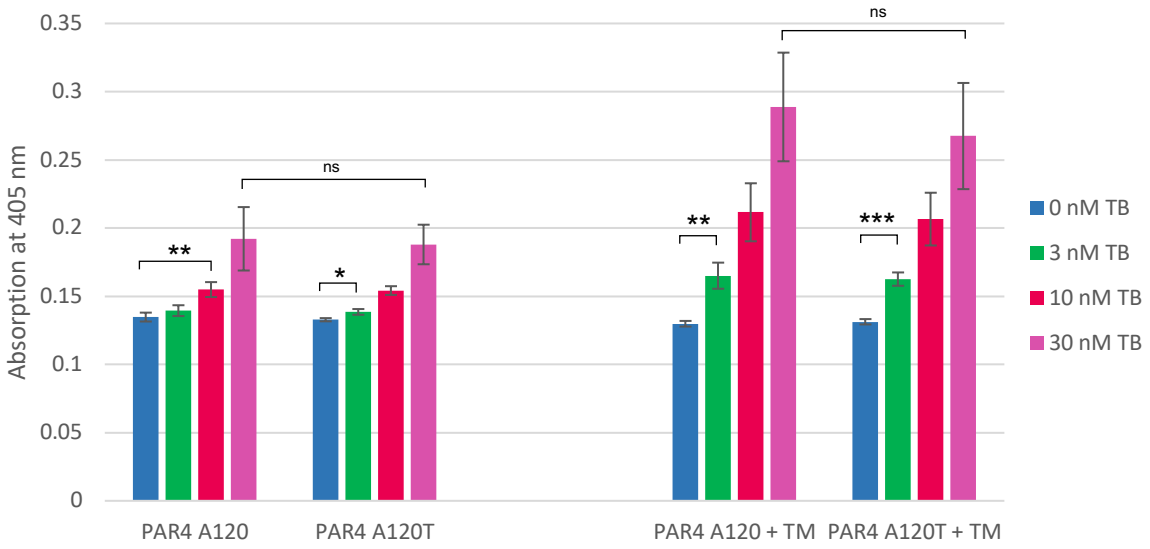


Figure 5: Same cleavage efficiency by thrombin of the PAR4 Ala120Thr compared to the wild-type. The figure shows the cleavage efficiency by thrombin of the wild-type and mutated PAR4 measured in the supernatant of the human 293T cells with a SEAP assay. All measurements were made after twelve minutes of contact with PNPP. The diagram represents the cleavage efficiency by thrombin of the wild-type and mutated receptor without and with TM. The different thrombin concentrations are marked in the respective colours. For reasons of clarity, the amino acid Ala and Thr are abbreviated to A and T and thrombin to TB. Six independent experiments were performed as biological replicates and each was measured three times as a technical replicate; the values indicate the mean +/- SEM in each case; ns = not significant, $p>0.05$, *$p<0.05$, **$p<0.01$, ***$p<0.001$; the p-values were determined with a student's *t*-test.

The absorption of the PNPP on the cells with and without TM became smaller the higher the concentration of the protease thrombin was. More receptors were cleaved at higher thrombin concentrations and thus less p-nitrophenol (dephosphorylated PNPP) was measured on the cells. Consequently, the absorption rate decreased. However, these results should be viewed with caution as the individual measurements vary greatly, leading to large standard errors of the mean and are therefore not significant (Figure 6). The corresponding p-values are listed in Table 2G in the appendix.

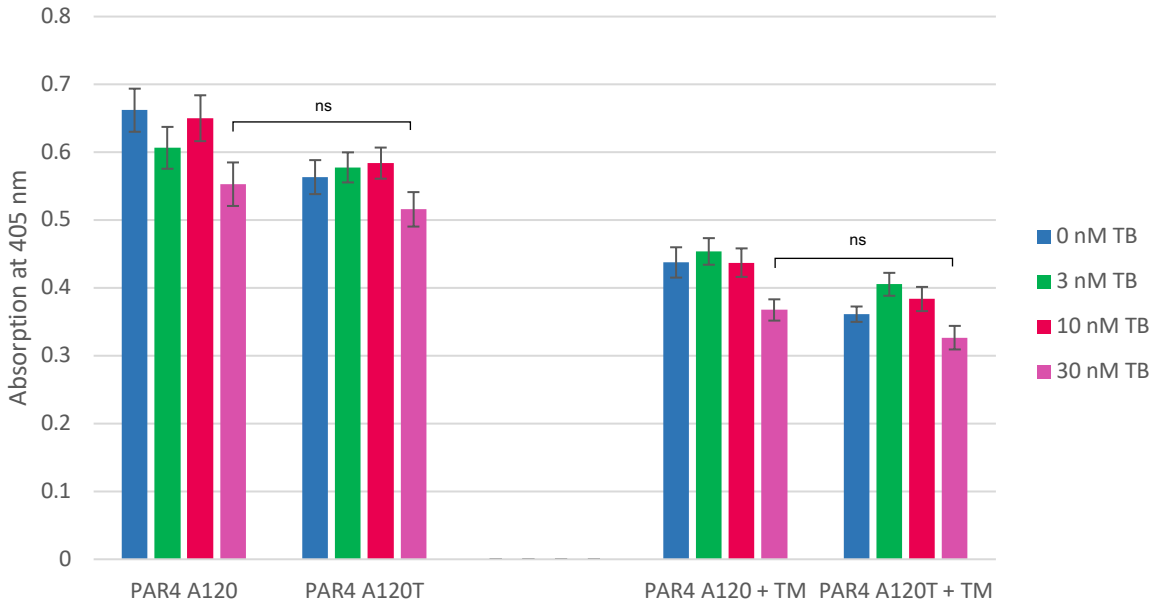


Figure 6: The same PAR4 wild-type alkaline phosphatase activity of the cell surface compared to the PAR4 Ala120Thr. All measurements were made after 2.5 minutes of contact with PNPP. The different thrombin concentrations are marked in the respective colours. For reasons of clarity, the amino acid Ala and Thr are abbreviated to A and T and thrombin to TB. Six independent experiments were performed as biological replicates and each was measured three times as a technical replicate; the values indicate the mean +/- SEM in each case; ns = not significant, $p>0.05$, *$p<0.05$, **$p<0.01$, ***$p<0.001$; the p-values were determined with a student's *t*-test.

By counting the cells, it was possible to indirectly determine whether there was a different number of receptors on a single cell. First, the cells on the 96-well plate had to be counted. Thus, it could be ruled out that there were different amounts of human 293T cells in the individual wells of the 96-well plate in the SEAP assay. The results of the counts can be found in Table 4. There were no significant differences in the transfections used for the SEAP assays.

As the SEAP measurement of the cells showed the same absorption values and no significant difference in the amount of human 293T cells was counted in the wells for the wild-type and the mutated PAR4, it can be concluded that the mutation did not change the number of receptors expressed on the cell surface.

Table 4: Same number of cells in PAR4 Ala120 and PAR4 Ala120Thr with and without TM. The cells were counted each from the same plasmid transfections used for the SEAP assay. The results are from four different transfections and performed twice each. The countings from the same transfection are listed in the same line. The different countings are numbered in the column “counting”. For reasons of clarity, the amino acid Ala and Thr are abbreviated to A and T.

Counting	PAR4 A120	PAR4 A120T	PAR4 A120 + TM	PAR4 A120T + TM
1	315 000 / ml	285 000 / ml	200 000 / ml	115 000 / ml
2	290 000 / ml	345 000 / ml	150 000 / ml	55 000 / ml
3	325 000 / ml	335 000 / ml	180 000 / ml	295 000 / ml
4	460 000 / ml	295 000 / ml	240 000 / ml	120 000 / ml
5	340 000 / ml	525 000 / ml	625 000 / ml	555 000 / ml
6	375 000 / ml	390 000 / ml	435 000 / ml	630 000 / ml
7	505 000 / ml	490 000 / ml	460 000 / ml	425 000 / ml
8	820 000 / ml	550 000 / ml	765 000 / ml	560 000 / ml

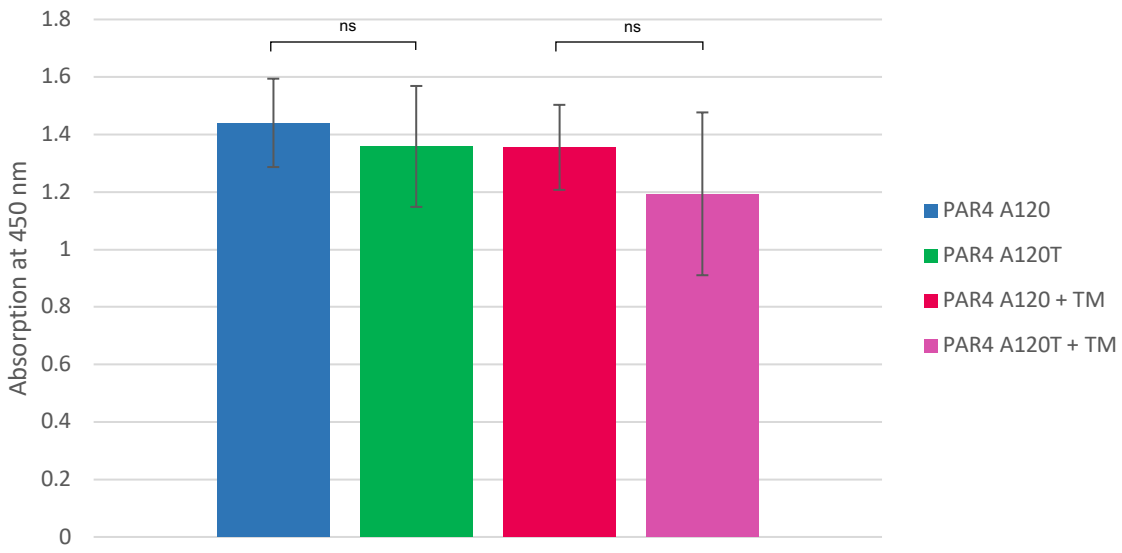


Figure 7: The same cell viability of PAR4 wild-type and mutant with and without TM by CCK8. The diagram shows the absorptions of the reduced tetrazolium salt at 450 nm. The colours illustrate the genotype of the receptors and the addition or absence of TM. The amino acids Ala and Thr have been replaced by the abbreviations A and T for a clear presentation. The values indicate the mean +/- SEM of two measurements in each case; ns = not significant $p>0.05$, *$p<0.05$, **$p<0.01$, ***$p<0.001$; the p-values were determined with a student's *t*-test.

These findings were also confirmed by the cell viability assay. However, the CCK8 did not measure the total number of cells but only the viability of the cells. The test demonstrated that the cell viability of the wild-type and the mutant were the same. The associated graphs can be found in Figure 7 and the p-values for the cell viability in the supplementary Table 2H in the appendix.

In summary, there was no difference in the cleavage assay nor the cell count or the cell viability between the wild-type receptor and the receptor with the point mutation Ala120Thr in PAR4. The cleavage efficiency by thrombin increased in the wild-type and the mutant with rising thrombin concentrations. TM enabled a more efficient cleavage by thrombin than in absence of it.

5.3. Comparison of the PAR2 wild-type and mutation cleavage efficiency by thrombin

The cleavage efficiency of PAR2 by thrombin was also determined by a SEAP absorption assay. Thus, a conclusion could be drawn about the cleavage efficiency by thrombin via the measured absorption level. The evaluation was therefore carried out according to the same principle as for PAR4.

In a first step, it was evaluated how the cleavage efficiency of the non-mutated receptor behaved at different thrombin concentrations when measuring the alkaline phosphatase in the supernatant. The results without TM showed no significantly higher cleavage efficiency at different thrombin concentrations for the wild-type receptor, not even at a high thrombin concentration of 30 nM. The measurement with TM increased the absorption for the wild-type receptor significantly at thrombin concentrations of 10 nM or higher (Figure 8).

In a next step, it was analysed how the PAR2 cleavage efficiency by thrombin changed due to the Ala118Thr mutation. Unexpectedly, there was absolutely no direct cleavage seen with the mutated receptors measuring the supernatant, neither with nor without TM. Nevertheless, the baseline absorption value of 0.18 OD +/- SEM was detected. These values did not differ between the various thrombin concentrations. The addition of TM increased the cleavage efficiency starting at 10 nM thrombin concentrations in the mutated receptors. Compared to the wild-type PAR2, the mutated PAR2 showed a significantly lower cleavage efficiency from thrombin concentrations of 10 nM and higher. By adding TM, this effect could already be made visible at 3 nM of thrombin (Figure 8). The associated p-values are shown in the supplementary Table 2I-2K in the appendix.

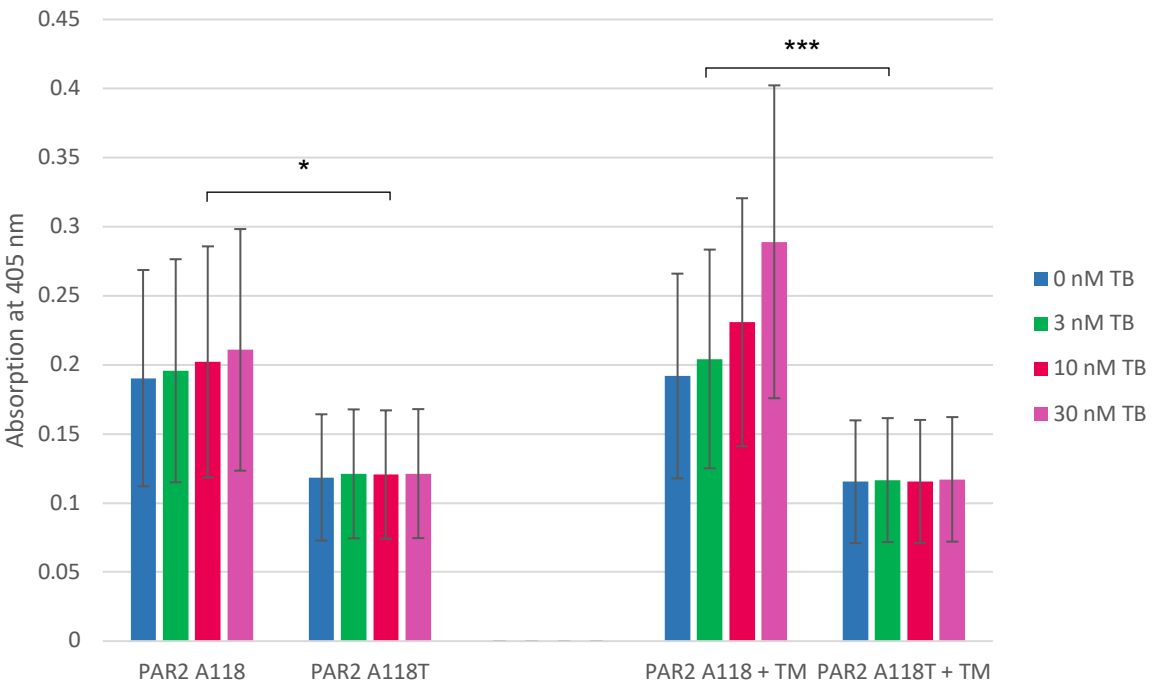


Figure 8: Lower cleavage efficiency by thrombin of the PAR2 mutation compared to the wild-type PAR2. The figure shows the cleavage efficiency by thrombin of the wild-type and mutated PAR2 measured in the supernatant of the human 293T cells with a SEAP assay. All measurements were made after twelve minutes of contact with PNPP. The different thrombin concentrations are marked in the respective colours. For reasons of clarity, the amino acid Ala and Thr are abbreviated to A and T and thrombin to TB. Three independent experiments were performed as biological replicates and each was measured three times as a technical replicate; the values indicate the mean +/- SEM in each case; ns = not significant, $p>0.05$, *$p<0.05$, **$p<0.01$, ***$p<0.001$; the p-values were determined with a student's *t*-test.

Figure 9 shows the alkaline phosphatase activity of the cell surface of PAR2. The absorption seen with the wild-type PAR2 decreased with higher thrombin concentrations. In the mutated receptors the baseline absorption of 0.18 OD +/- SEM was measured at all thrombin concentrations. However, these measurements of the cells must be viewed with caution since the standard errors of the mean were large and therefore not significant (Figure 9). The corresponding p-values are presented in appendix Table 2L.

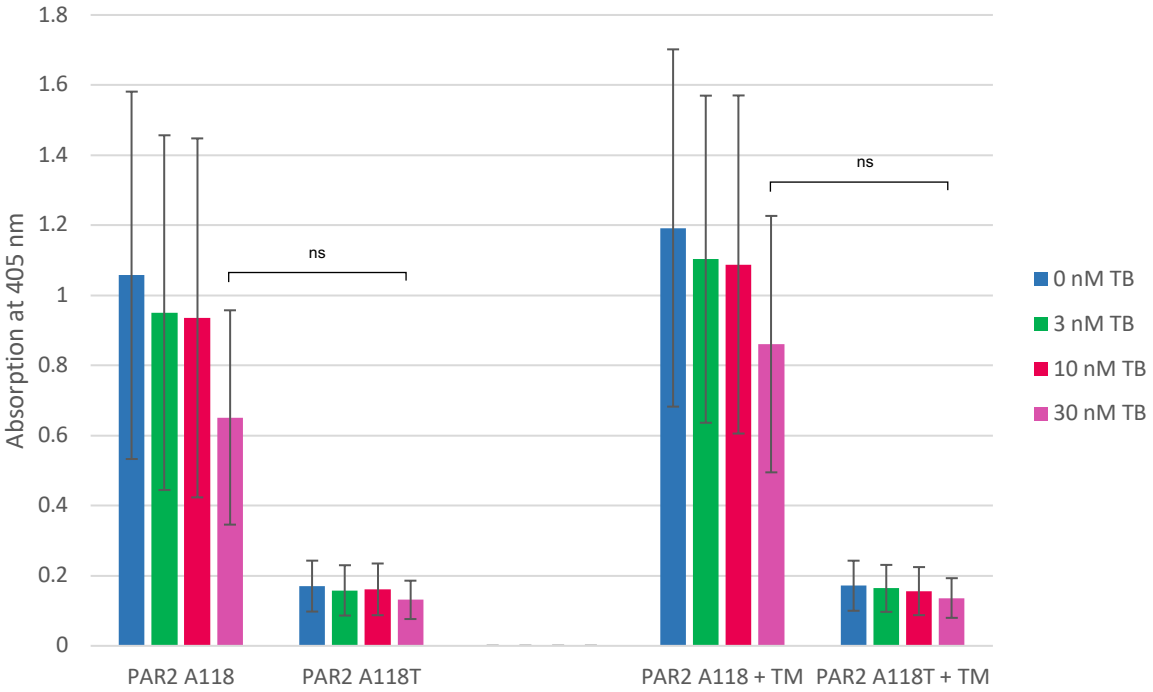


Figure 9: No significant change in the alkaline phosphatase activity of the cell surface in PAR2 wild-type and mutation. The figure shows the cleavage efficiency by thrombin of the wild-type and mutated PAR2 by measuring the alkaline phosphatase activity of the cell surface of the human 293T cells with a SEAP assay. All measurements were made after 2.5 minutes of contact with PNPP. The different thrombin concentrations are marked in the respective colours. For reasons of clarity, the amino acid Ala and Thr are abbreviated to A and T and thrombin to TB. Three independent experiments were performed as biological replicates and each was measured three times as a technical replicate; the values indicate the mean +/- SEM in each case; ns = not significant, $p>0.05$, *$p<0.05$, **$p<0.01$, ***$p<0.001$; the p-values were determined with a student's *t*-test.

The cells were also counted in the PAR2 similar to PAR4. No significant difference could be seen between the mutation and the wild-type receptor (Table 5). By counting the same quantity of cells expressing the wild-type or the mutated receptor, it could be excluded that the varying results were produced by a different number of cells.

Table 5: Same amount of PAR2 Ala118 and PAR2 Ala118Thr expressing cells with and without TM. The cells were all counted from the same plasmid transfections used for the following SEAP assay. The results are from two different transfections and performed twice each. The countings from the same transfection are listed in the same line. The different countings are numbered in the column "counting". For reasons of clarity, the amino acids Ala and Thr are abbreviated to A and T.

Counting	PAR2 A118	PAR2 A118T	PAR2 A118 + TM	PAR2 A118T + TM
1	235 000 / ml	310 000 / ml	405 000 / ml	210 000 / ml
2	315 000 / ml	360 000 / ml	245 000 / ml	245 000 / ml
3	500 000 / ml	355 000 / ml	355 000 / ml	325 000 / ml
4	390 000 / ml	230 000 / ml	540 000 / ml	235 000 / ml

Based on the unexpected results in the cleavage assay of the mutation Ala118Thr in PAR2, a cell surface ELISA was made to see whether the receptors were expressed or not on the cell surface of the human 293T cells by the point mutation. All results were measured with TM. The addition of the secondary antibody resulted in a significantly higher absorption with the wild-type receptors than with the colony two mutation in PAR2. Furthermore, the signal from the binding of the secondary antibody in colony three was higher than that of colony two. All three mutated colonies showed a lower signal than the wild-type receptor when adding the PAR2 ECL antibody. The PAR2 SAM 11 antibody showed differences between the three mutation colonies but not in comparison to the wild-type PAR2. As expected, the PAR1 ATAP2 antibody was found to bind to the cells regardless of the transfection. The absorption signal was lower in the colonies one and two of the mutation compared to the wild-type PAR2. There was no significant difference of the signal when comparing the wild-type to the colony three of the mutation (Figure 10). The corresponding p-values are listed in the appendix Table 2O.

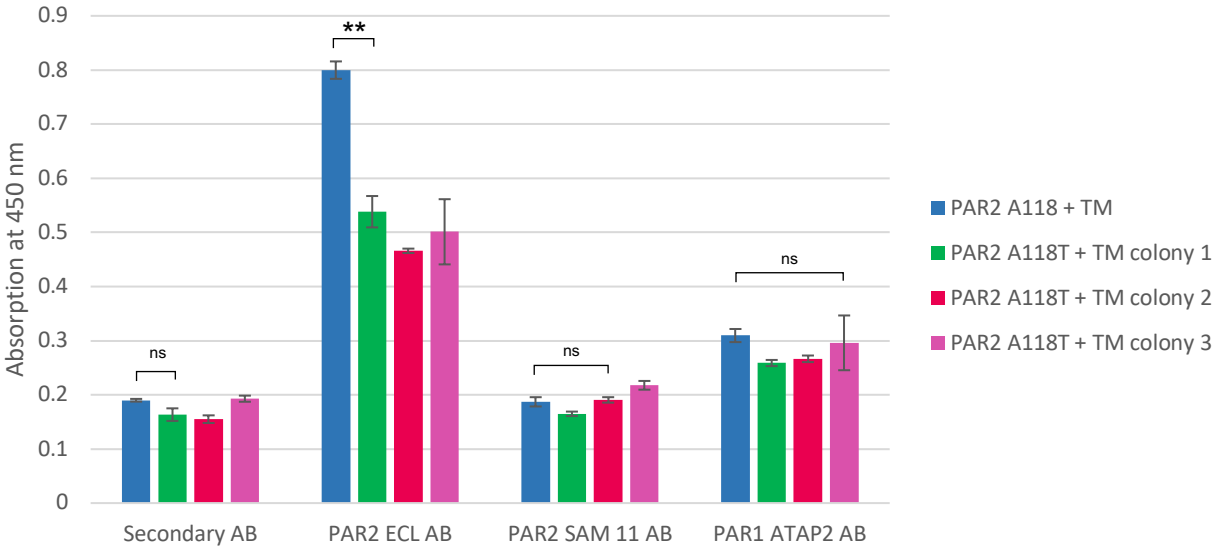


Figure 10: Equivalent antibody binding in the PAR2 wild-type and mutation. The figure shows the absorptions at 450 nm of the cell surface ELISA. The different receptors are marked in the respective colours. For reasons of clarity, the amino acid Ala and Thr are abbreviated to A and T and antibody to AB. Each experiment was measured three times as a technical replicate; the values indicate the mean +/- SEM in each case; ns = not significant, $p>0.05$, *$p<0.05$, **$p<0.01$, ***$p<0.001$; the p-values were determined with a student's *t*-test.

In summary, the measurements of PAR2 revealed unexpected results: the mutation PAR2 Ala118Thr showed no direct cleavage by thrombin. TM did not change these findings either. With different PAR2 antibodies it was confirmed that the wild-type and the mutated receptors were expressed in the cell membrane.

6. Discussion

In the present thesis I studied the specific mutation in PAR4 Ala120Thr and I compared it with the wild-type receptor. The mutation Ala120Thr in PAR4 showed no better cleavage efficiency by thrombin compared to the wild-type receptor although the mutant is better activated by thrombin in platelets. The cofactor TM increased the cleavage efficiency by thrombin in the wild-type and mutated PAR4 but between them no difference was observed. Furthermore, I was able to show that an increased concentration of the protease thrombin cleaved PAR4 better, regardless of whether the 120th amino acid was an alanine or a threonine. The experiments also revealed that this SNP does not cause any increased expression of the receptor on the surface of the transfected cells. To compare the results with the current state of scientific knowledge, a brief overview of the last years of research regarding this mutation in PAR4 is given below.

At the beginning of the 21st century, the point mutation Ala120Thr in PAR4 appeared in the database of the NCBI found by whole genome sequencing. In 2013 and 2014 it was discovered that the aggregation of platelets in black people was higher than in white people due to an increased incidence of mutant PAR4 at amino acid 120 (28, 29). Two years later they examined which different SNPs were represented in the PAR4 in a cohort of cardiac patients. It was then confirmed that the SNP Ala120Thr is also more frequently in cardiac patients than other point mutations (43). Over the years, the idea of using protease-activated receptors as carriers in the antithrombotic therapy became more and more important. In 2014 Vorapaxar, the first active PAR-related drug, was launched. It blocks PAR1 and thus the first, rapid phase of platelet activation. Vorapaxar proved to be an unsuitable drug in antithrombotic therapy because it showed severe bleeding as a side effect, promting researchers to look for new approaches (44). Thus, the idea to block PAR4 evolved. It was postulated, however, that carriers of the Ala120Thr mutation might not respond to this therapy or respond less well (29, 45). The first monoclonal antibody that blocked PAR4 was developed in 2018 but only used *in vitro*. The blocking was equally effective in the wild-type and in the mutation. These results were promising in terms of an antithrombotic target independent of the genotype of PAR4 (46). Researchers hoped to achieve a greater therapeutic range with a new antithrombotic target on PAR4 than with Vorapaxar, the only medicine which attacked a protease-activated receptor and had been used for therapy so far (44). In 2018, several research teams from all over the world dealt with this

mutation and the associated platelet aggregation. In those studies, a higher incident of the wild-type compared to the mutation of PAR4 was observed in the Japanese population. Nevertheless, 5.9% of the Japanese people examined turned out to be carriers of the mutation Ala120Thr. Platelets of individuals caring the PAR4 mutant and human 293T cells transfected with the mutation showed an increased release of Ca^{2+} compared to the cells expressing the wild-type PAR4 (47). Further research in this mutation found that the frequency of the mutation among Somali residents depends on their actual origin. Against this background it became clear that there where large differences within the black population and not all black people belonged to the same group (48, 49). Recently, the occurrence of the Thr120 variant in the indigenous Australian population of the Tiwi islands has been researched. In percentage terms, the occurrence of the mutation can be compared with those from Europe and Japan (50). In 2018, a study investigated the intensity of bleeding after an acute coronary syndrome and the subsequent treatment with Vorapaxar. The Thr120 carriers were bleeding less than the wild-type population with Vorapaxar treatment. This study indirectly showed that the mutation caused increased platelet activity (51). In the last few months, there has been intensive and more specific research into the mutation. For example, in a comparative study of platelet aggregation inhibitors, it was found that Prasugrel, an antiplatelet drug, is less effective if the thrombocytes are carriers of the Ala120Thr mutation (52). More molecular knowledge has also been gained over time. This knowledge is essential for a better understanding and, if necessary, for the future development of a PAR4-specific antithrombotic drug. The two different intracellular activation pathways already mentioned in the introduction, which are activated during the cleavage of PAR4, were therefore also specifically addressed. The mutation showed increased activity through both the Gq and the G13 route (53). The increased activation of PAR4 Ala120Thr was only demonstrated with the AYPGKF activation peptide (AP). It is an artificially produced AP that cannot be compared to the protease thrombin found in human blood, as it can activate the receptors without cleavage. Another very important study was conducted in 2018 on the SNP Ala120Thr and found that 0.3 nM thrombin is required for PAR4 activation on platelets. The level of the thrombin concentration, which causes half of the thrombocytes to aggregate, was significantly lower due to the mutation. In addition, it was shown that the mentioned above effect was greater with AP than with thrombin and that the same number of receptors were expressed on the surface of the blood platelets regardless of genetics. With a thrombin

concentration below 0.6 nM, an increased aggregation was measured in the gene variant Thr120. The difference between the mutation and the wild-type disappeared through stimulation over a longer period of time. Moreover, there was no difference in the cleavage of the two gene variants. In order to successfully desensitize the receptor, a three-fold higher AP concentration was required with the mutation. With the addition of Vorapaxar it was be shown that a concentration of 1 nM thrombin led to a 1.8 times higher aggregation for the platelets with the mutation (22).

In summary, my results regarding the mutation in PAR4 are similar to those of Whitley MJ et al.. However, an important difference is that I measured the cleavage efficiency at lower thrombin concentrations, i.e. 3, 10 and 30 nM. In 2018 Whitley MJ et al. measured at concentrations of 10, 50 and 100 nM. In addition, I transfected human 293T cells whereas Whitley MJ et al. worked with COS7 cells. My data show that the cleavage efficiency by thrombin is not influenced by the cells used. Whitley MJ et al. did some further research and extracted mutated platelets from the blood of carriers and measured the activity of these platelets. A thrombin concentration-dependent effect was discovered. Only at a protease concentration below 0.6 nM a significantly higher activity was seen compared to the wild-type (22).

As for my results, some differences emerge when comparing the efficiency of cleavage by thrombin of the PAR4 wild-type (Figure 4 and Figure 5). For example, Figure 4 shows a significantly better cleavage efficiency starting at a thrombin concentration of 30 nM. With the addition of TM, this effect has already been seen at concentrations of 10 nM. However, Figure 5 already shows this effect at a lower protease concentration than in Figure 4. What could be the underlying cause? These findings can be explained by the extremely sensitive technique of pipetting. To reduce these fluctuations, the measurements would have to be repeated several times more. With the cell count and the additional confirmation of the results by measuring the cell viability, I was able to demonstrate that the number of receptors in the cell membrane does not change due to the mutation and between the different transfections. In August 2020 the Australian Centre for Blood Diseases, Monash University, Melbourne, Australia came to exactly this conclusion (30).

Further research could examine specific elements of the activation pathways in more detail. This would be performed by the use of specific antibodies targeting single elements of these molecular pathways. Such investigations would differentiate the intracellular functions of the wild-type and the corresponding mutation more clearly. If we

were to find out where exactly the origin of the difference in platelet activation lies, we would be much closer to individualised therapy specialised to a patient's genotype.
In addition, I also focused on a similar mutation which is located on PAR2. The mutation Ala118Thr is in the second loop of the seven-transmembrane protein in PAR2 like the mutation discussed in PAR4 (29). Genetically, the two mutations are extremely similar. Against this background, the hypothesis of my work was that they behave in the same or similar way. To date, this mutation of PAR2 has not been researched and there is no literature on it. The mutation was discovered by genome-wide sequencing and can be found in the database of the NCBI. The biggest difference to PAR4 certainly is that platelets play no role in this mutation as PAR2 is not expressed. It has been known for several years that PAR2 plays a role in various inflammatory cells of the innate and adaptive immune system (54). Surprisingly, the mutated PAR2 in my experiments showed absolutely no direct cleavage by thrombin although the receptor was expressed on the cell surface, shown by ELISA. Similar to the PAR4 wild-type, a discrepancy in the wild-type measurements between Figure 4 and Figure 8 can also be seen in PAR2. Figure 4 shows a significantly better cleavage efficiency with 30 nM thrombin in presence of TM. In Figure 8 this effect is lost due to the extremely high standard errors of the mean. For control purposes, my experiments with PAR2 were repeated by a team member of Reto Schüpbach's laboratory. This experiment resulted in a lower and different signal which was consistent with the literature (8). In fact, no plausible reason for the different values was found. The reason for the discrepancy might have its origin in the laboratory work itself. Therefore, it would certainly be necessary to repeat the experiments before final conclusions could be drawn.
Since no direct cleavage by thrombin was found in the mutation, all expressed PAR2 and thus also the alkaline phosphatase were still located in the cell membrane. Against this background, I expected a high absorption signal of the cell surface-bound receptors. However, the evaluation showed a much lower absorption value in the mutation than in the wild-type, which does not match with the not cleaved receptors in the mutation. It is likely that a mistake in the laboratory, as mentioned above, led to this result. To verify the number of receptors, however, a more precise method such as a flow cytometry would be necessary. Thus, if my findings were to be confirmed in further research, it would be interesting to carry out an activity assay as NF-κB activity assay

in the next step to see whether the molecular pathway is inhibited as well as the cleavage. A cleavage-independent activation of the receptor caused by the mutation in PAR2 could be the result of further investigation.

It is already known that PAR2 plays an important role in the body's pro-inflammatory processes (55, 56). The aim of further research could be to analyse whether the mutation leads to increased inflammation in the human body. Moreover, it would be interesting to study the mutation in patients both on a molecular and clinical level in the coming years. Perhaps a correlation can be established between the inflammatory response and the mutation.

In summary, the specific SNP in PAR4 does not appear to influence cleavage efficiency by thrombin. In PAR4, despite the increased aggregation of platelets shown in vivo, no significant changes were found in relation to cleavage efficiency by thrombin (47). In PAR2, no definitive link between the mutation and its influence on cleavage by thrombin can yet be identified. Although my results on Ala118Thr in PAR2 must be viewed with caution, they do give an idea of how this mutation might affect cleavage by thrombin. To get a deeper insight into what the mutations of PAR4 and PAR2 do, the focus of further studies should shift more towards understanding the intracellular activation pathways. An intracellular pathway activation assay such as the NF-κB activity assay could be performed with different concentrations of thrombin and activating peptides to see if the mutations alter receptor activation. It is undisputed that many mechanisms are still unknown in these two mutant receptors and that further research needs to be done to gain a deeper understanding.

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7. Appendix

Supplementary Table 1: cDNA Sequence of pcDNA3.1zeo+ with PAR4 A120/PAR2 A118.
For reasons of clarity, the amino acid Ala is abbreviated as A.

Name	Sequence
pI26 (PAR4 A120 + pcDNA3.1zeo+) **PAR4 A120 = yellow**	5′GACGGATCGGGAGATCTCCCGATCCCCTATGGTCGACTCTCAGTACAATCTGCTCT GATGCCGCATAGTTAAGCCAGTATCTGCTCCCTGCTTGTGTGTTGGAGGTCGCTGAGT AGTGCGCGAGCAAAATTTAAGCTACAACAAGGCAAGGCTTGACCGACAATTGCATGAA GAATCTGCTTAGGGTTAGGCGTTTTGCGCTGCTTCGCGATGTACGGGCCAGATATACG CGTTGACATTGATTATTGACTAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTC ATAGCCCATATATGGAGTTCCGCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTG ACCGCCCAACGACCCCCGCCCATTGACGTCAATAATGACGTATGTTCCCATAGTAACG CCAATAGGGACTTTCCATTGACGTCAATGGGTGGACTATTTACGGTAAACTGCCCACT TGGCAGTACATCAAGTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAATGACGG TAAATGGCCCGCCTGGCATTATGCCCAGTACATGACCTTATGGGACTTTCCTACTTGG CGTACATCTACGTATTAGTCATCGCTATTACCATGGTGATGCGGTTTTGGCAGTACAT CAATGGGCGTGGATAGCGGTTTGACTCACGGGGATTTCCAAGTCTCCACCCCATTGAC GTCAATGGGAGTTTGTTTTGGCACCAAAATCAACGGGACTTTCCAAAATGTCGTAACA ACTCCGCCCCATTGACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATATAAG CAGAGCTCTCTGGCTAACTAGAGAACCCACTGCTTACTGGCTTATCGAAATTAATACG ACTCACTATAGGGAGACCCAAGCTGGCTAGCGTTTAAACTTAAGCTTGGTACCGAGCT CGGATCCACTAGTCCAGTGTGGTGGAATTCGCCCACCATGCTGCTGCTGCTGCTGCTG CTGGGCCTGAGGCTACAGCTCTCCCTGGGCATCATCCCAGTTGAGGAGGAGAACCCGG ACTTCTGGAACCGCGAGGCAGCCGAGGCCCTGGGTGCCGCCAAGAAGCTGCAGCCTGC ACAGACAGCCGCCAAGAACCTCATCATCTTCCTGGGCGATGGGATGGGGGTGTCTACG GTGACAGCTGCCAGGATCCTAAAAGGGCAGAAGAAGGACAAACTGGGGCCTGAGATAC CCCTGGCCATGGACCGCTTCCCATATGTGGCTCTGTCCAAGACATACAATGTAGACAA ACATGTGCCAGACAGTGGAGCCACAGCCACGGCCTACCTGTGCGGGGTCAAGGGCAAC TTCCAGACCATTGGCTTGAGTGCAGCCGCCCGCTTTAACCAGTGCAACACGACACGCG GCAACGAGGTCATCTCCGTGATGAATCGGGCCAAGAAAGCAGGGAAGTCAGTGGGAGT GGTAACCACCACACGAGTGCAGCACGCCTCGCCAGCCGGCACCTACGCCCACACGGTG AACCGCAACTGGTACTCGGACGCCGACGTGCCTGCCTCGGCCCGCCAGGAGGGGTGCC AGGACATCGCTACGCAGCTCATCTCCAACATGGACATTGACGTGATCCTAGGTGGAGG CCGAAAGTACATGTTTCGCATGGGAACCCCAGACCCTGAGTACCCAGATGACTACAGC CAAGGTGGGACCAGGCTGGACGGGAAGAATCTGGTGCAGGAATGGCTGGCGAAGCGCC AGGGTGCCCGGTATGTGTGGAACCGCACTGAGCTCATGCAGGCTTCCCTGGACCCGTC TGTGACCCATCTCATGGGTCTCTTTGAGCCTGGAGACATGAAATACGAGATCCACCGA GACTCCACACTGGACCCCTCCCTGATGGAGATGACAGAGGCTGCCCTGCGCCTGCTGA GCAGGAACCCCCGCGGCTTCTTCCTCTTCGTGGAGGGTGGTCGCATCGACCATGGTCA TCATGAAAGCAGGGCTTACCGGGCACTGACTGAGACGATCATGTTCGACGACGCCATT GAGAGGGCGGGCCAGCTCACCAGCGAGGAGGACACGCTGAGCCTCGTCACTGCCGACC ACTCCCACGTCTTCTCCTTCGGAGGCTACCCCCTGCGAGGGAGCTCCATCTTCGGGCT GGCCCCTGGCAAGGCCCGGGACAGGAAGGCCTACACGGTCCTCCTATACGGAAACGGT CCAGGCTATGTGCTCAAGGACGGCGCCCGGCCGGATGTTACCGAGAGCGAGAGCGGGA GCCCCGAGTATCGGCAGCAGTCAGCAGTGCCCCTGGACGAAGAGACCCACGCAGGCGA GGACGTGGCGGTGTTCGCGCGCGGCCCGCAGGCGCACCTGGTTCACGGCGTGCAGGAG CAGACCTTCATAGCGCACGTCATGGCCTTCGCCGCCTGCCTGGAGCCCTACACCGCCT GCGACCTGGCGCCCCCCGCCGGCACCACCGACGCCGCGCACCCGGGTTACTCTAGGGC CCAGACCCCCAGCGTCTACGACGAGAGCGGGAGCACCGGAGGTGGTGATGACAGCACG CCCTCAATCCTGCCTGCCCCCCGCGGCTACCCAGGCCAAGTCTGTGCCAATGACAGTG ACACCCTGGAGCTCCCGGACAGCTCACGGGCACTGCTTCTGGGCTGGGTGCCCACCAG GCTGGTGCCCGCCCTCTATGGGCTGGTCCTGGTGGTGGGGCTGCCGGCCAATGGGCTG GCGCTGTGGGTGCTGGCCACGCAGGCACCTCGGCTGCCCTCCACCATGCTGCTGATGA ACCTCGCGGCTGCTGACCTCCTGCTGGCCCTGGCGCTGCCCCCGCGGATCGCCTACCA CCTGCGTGGCCAGCGCTGGCCCTTCGGGGAGGCCGCCTGCCGCCTGGCCACGGCCGCA CTCTATGGTCACATGTATGGCTCAGTGCTGCTGCTGGCCGCCGTCAGCCTGGATCGCT

ACCTGGCCCTGGTGCACCCGCTGCGGGCCCGCGCCCTGCGTGGCCGGCGCCTGGCCCT
TGGACTCTGCATGGCTGCTTGGCTCATGGCGGCCGCCCTGGCACTGCCCCTGACACTG
CAGCGGCAGACCTTCCGGCTGGCGCGCTCCGATCGCGTGCTCTGCCATGACGCGCTGC
CCCTGGACGCACAGGCCTCCCACTGGCAACCGGCCTTCACCTGCCTGGCGCTGTTGGG
CTGTTTCCTGCCCCTGCTGGCCATGCTGCTGTGCTACGGGGCCACCCTGCACACGCTG
GCGGCCAGCGGCCGGCGCTACGGCCACGCGCTGAGGCTGACCGCAGTGGTGCTGGCCT
CCGCCGTGGCCTTCTTCGTGCCCAGCAACCTGCTGCTGCTGCTGCATTACTCGGACCC
GAGCCCCAGCGCCTGGGGCAACCTCTATGGTGCCTACGTGCCCAGCCTGGCGCTGAGC
ACCCTCAACAGCTGCGTGGATCCCTTCATCTACTACTACGTGTCGGCCGAGTTCAGGG
ACAAGGTGCGGGCAGGGCTCTTCCAACGGTCGCCGGGGGACACCGTGGCCTCCAAGGC
CTCTGCGGAAGGGGGCAGCCGGGGCATGGGCACCCACTCCTCTTTGCTCCAGTGACAC
AAAGTGGGGAAGGCTGTACTGGGTCGAACAGGGTCCCTTCCCCCACTTCACGTCCTTC
CTGGGACCTCAGAATGTGACCTTATTTGGAAATAGGGTTGTTACAACTGTCACTAGCG
GAGGTCACTTTGGAGAAGGGTGGGCCTTACATCCAGTGTGGGTGGTGTCCTCATAAGA
TAAGGAGAGGCCAGGCCTGGTGGCTCACGCCTGTAATCCCAGCACTTTAAGAGGCCAA
GGCGGATGGATCACTTGAGCCCAGGAGTTCAACACCAGCCTGAGCAACATGGTAAAAC
CCCATCTCTACCAAAAATACAAAAATTAGCTGGGCTTGGTGGCTGGCGCCTGTAATCC
CAGCTACTCAGGAGACTGAGGCAGAAGGATCGCTTGAACCTGGGAGGCAGAGGTTGCA
GTGAGCCGAGATTGCGCCACTGGACTCCAGCCTGCGTGACAGAGAGCCTGTCTCTAAA
TTAATTAATTAATTAATTTAATTCAATTTTAAAAAGACGAAAAGTGACGGCCAGGTGC
AGTGGCTCACGCCTATAATCTCAGCACTCTGGGAGGCCAAGATGGAGGATTGCTTGAA
GCCAGGAGTTTGGGACCAGCCTGGGCAACATAGGGGGATCCCATCTCTACACACAAAA
AAATTCTGCAGATATCCAGCACAGTGGCGGCCGCTCGAGTCTAGAGGGCCCGTTTAAA
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CCCCGTGCCTTCCTTGACCCTGGAAGGTGCCACTCCCACTGTCCTTTCCTAATAAAAT
GAGGAAATTGCATCGCATTGTCTGAGTAGGTGTCATTCTATTCTGGGGGGTGGGGTGG
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GCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGTTACGCGCAGCGTGACCG
CTACACTTGCCAGCGCCCTAGCGCCCGCTCCTTTCGCTTTCTTCCCTTCCTTTCTCGC
CACGTTCGCCGGCTTTCCCCGTCAAGCTCTAAATCGGGGCATCCCTTTAGGGTTCCGA
TTTAGTGCTTTACGGCACCTCGACCCCAAAAAACTTGATTAGGGTGATGGTTCACGTA
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TAATAGTGGACTCTTGTTCCAAACTGGAACAACACTCAACCCTATCTCGGTCTATTCT
TTTGATTTATAAGGGATTTTGGGGATTTCGGCCTATTGGTTAAAAAATGAGCTGATTT
AACAAAAATTTAACGCGAATTAATTCTGTGGAATGTGTGTCAGTTAGGGTGTGGAAAG
TCCCCAGGCTCCCCAGGCAGGCAGAAGTATGCAAAGCATGCATCTCAATTAGTCAGCA
ACCAGGTGTGGAAAGTCCCCAGGCTCCCCAGCAGGCAGAAGTATGCAAAGCATGCATC
TCAATTAGTCAGCAACCATAGTCCCGCCCCTAACTCCGCCCATCCCGCCCCTAACTCC
GCCCAGTTCCGCCCATTCTCCGCCCCATGGCTGACTAATTTTTTTTATTTATGCAGAG
GCCGAGGCCGCCTCTGCCTCTGAGCTATTCCAGAAGTAGTGAGGAGGCTTTTTTGGAG
GCCTAGGCTTTTGCAAAAAGCTCCCGGGAGCTTGTATATCCATTTTCGGATCTGATCA
GCACGTGTTGACAATTAATCATCGGCATAGTATATCGGCATAGTATAATACGACAAGG
TGAGGAACTAAACCATGGCCAAGTTGACCAGTGCCGTTCCGGTGCTCACCGCGCGCGA
CGTCGCCGGAGCGGTCGAGTTCTGGACCGACCGGCTCGGGTTCTCCCGGGACTTCGTG
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CGTC 3′

pl331 (PAR2 A118 + pcDNA3.1zeo+)

PAR2 A118 = yellow

5′GACGGATCGGGAGATCTCCCGATCCCCTATGGTCGACTCTCAGTACAATCTGCTCT
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TAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTC 3′

Supplementary Table 2: P-values determined in a student's t-test. (A) Comparison of the baseline absorptions of the SEAP assay with and without TM to determine the effect of TM at different thrombin concentrations. (B) Empty plasmid compared to PAR4 and PAR2 wild-type and comparison of PAR4 and PAR2 wild-type at different thrombin concentrations. (C) P-values at different thrombin concentrations in the baseline transfection. (D) Thrombin concentration comparison of the PAR4 wild-type, mutant with and without TM. (E) P-values of the comparison of PAR4 wild-type to the PAR4 mutation. (F) Comparison of PAR4 cleavage efficiency by thrombin in presence and absence of TM. (G) P-value calculations of PAR4 at various thrombin concentrations of the alkaline phosphatase cell surface activity. (H) Comparison of the cell viability of the wild-type PAR4 and the mutated receptor and the corresponding p-value after the addition of TM. (I) Thrombin concentration comparison of the PAR2 wild-type, mutant with and without TM. (J) P-values of the comparison of PAR2 wild-type to the PAR2 mutation. (K) Comparison of PAR2 cleavage efficiency by thrombin in presence and absence of TM. (L) P-value calculations of PAR2 at various thrombin concentrations of the alkaline phosphatase cell surface activity. (O) P-values of the cell surface ELISA PAR2 wild-type and mutation. The calculated p-values are listed in each table. For reasons of clarity, the amino acid Ala is abbreviated as A; $p<0.05$ = significant; the p-values were determined with a student's *t*-test.

A

Plasmids	Thrombin	P-value
Empty plasmid / Empty plasmid + TM	0 nM	0.96865234
	3 nM	0.18069961
	10 nM	0.42384314
	30 nM	0.19117999
PAR4 A120 / PAR4 A120 + TM	0 nM	0.5146687
	3 nM	0.03489797
	10 nM	0.00544436
	30 nM	0.08798569
PAR2 A118 / PAR2 A118 + TM	0 nM	0.48181741
	3 nM	0.84638352
	10 nM	0.66341922
	30 nM	0.34523146

B

Plasmids	Thrombin	P-value
Empty plasmid / PAR4 A120	0 nM	0.02422524
	3 nM	0.00168964
	10 nM	0.01503585
	30 nM	0.01944385
Empty plasmid + TM / PAR4 A120 + TM	0 nM	0.00204564
	3 nM	0.01208119
	10 nM	0.00216146
	30 nM	0.02098241
Empty plasmid / PAR2 A118	0 nM	0.01216686
	3 nM	0.01298705
	10 nM	0.00905179
	30 nM	0.01731439
Empty plasmid + TM / PAR2 A118 + TM	0 nM	0.00584577
	3 nM	0.00830966
	10 nM	0.00105394
	30 nM	0.00118356
PAR4 A120 / PAR2 A118	0 nM	0.01728718
	3 nM	0.0218358
	10 nM	0.01488983
	30 nM	0.06382295
PAR4 A120 + TM / PAR2 A118 + TM	0 nM	0.01011154
	3 nM	0.0822503
	10 nM	0.03060778
	30 nM	0.17521174

C

Thrombin	Plasmids	P-values
0 nM / 3 nM	Empty plasmid	0.27871764
	Empty plasmid + TM	0.96108379
	PAR4 A120	0.28274696
	PAR4 A120 + TM	0.15869329
	PAR2 A118	0.01958132
	PAR2 A118 + TM	0.01247563
0 nM / 10 nM	Empty plasmid	0.55959925
	Empty plasmid + TM	0.88514344
	PAR4 A120	0.52849021
	PAR4 A120 + TM	0.00422252
	PAR2 A118	0.7973312
	PAR2 A118 + TM	0.1161298
0 nM / 30 nM	Empty plasmid	0.05320227
	Empty plasmid + TM	0.09150654
	PAR4 A120	0.02608099
	PAR4 A120 + TM	0.03568438
	PAR2 A118	0.76087709
	PAR2 A118 + TM	0.04321596
3 nM / 10 nM	Empty plasmid	0.44773427
	Empty plasmid + TM	0.92738163
	PAR4 A120	0.85194852
	PAR4 A120 + TM	0.28249664
	PAR2 A118	0.96381776
	PAR2 A118 + TM	0.5177031
3 nM / 30 nM	Empty plasmid	0.07012091
	Empty plasmid + TM	0.102738
	PAR4 A120	0.0418092
	PAR4 A120 + TM	0.17728775
	PAR2 A118	0.96827269
	PAR2 A118 + TM	0.17375446
10 nM / 30 nM	Empty plasmid	0.04046766
	Empty plasmid + TM	0.09382788
	PAR4 A120	0.04570989
	PAR4 A120 + TM	0.3536224
	PAR2 A118	0.9331315
	PAR2 A118 + TM	0.28915062

D

Thrombin	Plasmids	P-values
0 nM / 3 nM	PAR4 A120	0.378221242
	PAR4 A120T	0.039118987
	PAR4 A120 + TM	0.004895343
	PAR4 A120T + TM	0.000618353
0 nM / 10 nM	PAR4 A120	0.009979904
	PAR4 A120T	9.12837×10^{-5}
	PAR4 A120 + TM	0.003342229
	PAR4 A120T + TM	0.002421727
0 nM / 30 nM	PAR4 A120	0.034574756
	PAR4 A120T	0.003534644
	PAR4 A120 + TM	0.002579011
	PAR4 A120T + TM	0.004922018
3 nM / 10 nM	PAR4 A120	0.045039127
	PAR4 A120T	0.002167317
	PAR4 A120 + TM	0.074117393
	PAR4 A120T + TM	0.048396568
3 nM / 30 nM	PAR4 A120	0.0495663
	PAR4 A120T	0.00707375
	PAR4 A120 + TM	0.01289155
	PAR4 A120T + TM	0.02103208
10 nM / 30 nM	PAR4 A120	0.15015954
	PAR4 A120T	0.046213
	PAR4 A120 + TM	0.11798256
	PAR4 A120T + TM	0.17961509

E

Thrombin	Plasmids	P-Values
0 nM	PAR4 A120 / PAR4 A120T	0.586609988
	PAR4 A120 + TM / PAR4 A120T + TM	0.618586851
3 nM	PAR4 A120 / PAR4 A120T	0.837264042
	PAR4 A120 + TM / PAR4 A120T + TM	0.830078001
10 nM	PAR4 A120 / PAR4 A120T	0.911429706
	PAR4 A120 + TM / PAR4 A120T + TM	0.863070043
30 nM	PAR4 A120 / PAR4 A120T	0.882162962
	PAR4 A120 + TM / PAR4 A120T + TM	0.706037045

F

Thrombin	Plasmids	P-Values
0 nM	PAR4 A120 / PAR4 A120 + TM	0.23366642
	PAR4 A120T / PAR4 A120T + TM	0.51785676
3 nM	PAR4 A120 / PAR4 A120 + TM	0.03302658
	PAR4 A120T / PAR4 A120T + TM	0.00387507
10 nM	PAR4 A120 / PAR4 A120 + TM	0.02735115
	PAR4 A120T / PAR4 A120T + TM	0.0196297
30 nM	PAR4 A120 / PAR4 A120 + TM	0.06248983
	PAR4 A120T / PAR4 A120T + TM	0.0783922

G

Thrombin	Plasmids	P-values
0 nM / 3 nM	PAR4 A120	0.71977092
	PAR4 A120T	0.90195554
	PAR4 A120 + TM	0.87537806
	PAR4 A120T + TM	0.53733922
0 nM / 10 nM	PAR4 A120	0.94180479
	PAR4 A120T	0.86156735
	PAR4 A120 + TM	0.99792154
	PAR4 A120T + TM	0.7610163
0 nM / 30 nM	PAR4 A120	0.49259994
	PAR4 A120T	0.7028749
	PAR4 A120 + TM	0.46677182
	PAR4 A120T + TM	0.63320384
3 nM / 10 nM	PAR4 A120	0.78420814
	PAR4 A120T	0.95487018
	PAR4 A120 + TM	0.86887599
	PAR4 A120T + TM	0.79998067
3 nM / 30 nM	PAR4 A120	0.72984318
	PAR4 A120T	0.60052076
	PAR4 A120 + TM	0.33472358
	PAR4 A120T + TM	0.36081917
10 nM / 30 nM	PAR4 A120	0.55191798
	PAR4 A120T	0.57040493
	PAR4 A120 + TM	0.45058657
	PAR4 A120T + TM	0.51469242

H

	Plasmids	P-Values
Cell viability	PAR4 A120 / PAR4 A120T	0.78272417
	PAR4 A120 + TM / PAR4 A120T + TM	0.66305711
	PAR4 A120 / PAR4 A120 + TM	0.72820791
	PAR4 A120T / PAR4 A120T + TM	0.68625476

I

Thrombin	Plasmids	P-values
0 nM / 3 nM	PAR2 A118	0.8916534
	PAR2 A118T	0.52979664
	PAR2 A118 + TM	0.28640553
	PAR2 A118T + TM	0.38920245
0 nM / 10 nM	PAR2 A118	0.77281038
	PAR2 A118T	0.52558186
	PAR2 A118 + TM	0.04181606
	PAR2 A118T + TM	0.84889058
0 nM / 30 nM	PAR2 A118	0.62920425
	PAR2 A118T	0.41768268
	PAR2 A118 + TM	0.00825154
	PAR2 A118T + TM	0.11978531
3 nM / 10 nM	PAR2 A118	0.87746317
	PAR2 A118T	0.86430444
	PAR2 A118 + TM	0.14627049
	PAR2 A118T + TM	0.43688721
3 nM / 30 nM	PAR2 A118	0.72516475
	PAR2 A118T	0.94331772
	PAR2 A118 + TM	0.01574054
	PAR2 A118T + TM	0.54518765
10 nM / 30 nM	PAR2 A118	0.84237817
	PAR2 A118T	0.63856169
	PAR2 A118 + TM	0.06220385
	PAR2 A118T + TM	0.09873467

J

Thrombin	Plasmids	P-Values
0 nM	PAR2 A118 / PAR2 A118T	0.04796412
	PAR2 A118 + TM / PAR2 A118T + TM	0.00014317
3 nM	PAR2 A118 / PAR2 A118T	0.05047608
	PAR2 A118 + TM / PAR2 A118T + TM	0.00089533
10 nM	PAR2 A118 / PAR2 A118T	0.04506797
	PAR2 A118 + TM / PAR2 A118T + TM	0.0006713
30 nM	PAR2 A118 / PAR2 A118T	0.04021452
	PAR2 A118 + TM / PAR2 A118T + TM	0.00086813

K

Thrombin	Plasmids	P-Values
0 nM	PAR2 A118 / PAR2 A118 + TM	0.95514403
	PAR2 A118T / PAR2 A118T + TM	0.36761463
3 nM	PAR2 A118 / PAR2 A118 + TM	0.77553403
	PAR2 A118T / PAR2 A118T + TM	0.14442559
10 nM	PAR2 A118 / PAR2 A118 + TM	0.40343658
	PAR2 A118T / PAR2 A118T + TM	0.01112842
30 nM	PAR2 A118 / PAR2 A118 + TM	0.09268304
	PAR2 A118T / PAR2 A118T + TM	0.01099569

L

Thrombin	Plasmids	P-values
0 nM / 3 nM	PAR2 A118	0.82132436
	PAR2 A118T	0.79954121
	PAR2 A118 + TM	0.75845882
	PAR2 A118T + TM	0.82723953
0 nM / 10 nM	PAR2 A118	0.80120654
	PAR2 A118T	0.85174926
	PAR2 A118 + TM	0.74571252
	PAR2 A118T + TM	0.71537865
0 nM / 30 nM	PAR2 A118	0.30289134
	PAR2 A118T	0.31952734
	PAR2 A118 + TM	0.2534003
	PAR2 A118T + TM	0.33007731
3 nM / 10 nM	PAR2 A118	0.9757797
	PAR2 A118T	0.9557184
	PAR2 A118 + TM	0.95999316
	PAR2 A118T + TM	0.84108182
3 nM / 30 nM	PAR2 A118	0.4517092
	PAR2 A118T	0.53641406
	PAR2 A118 + TM	0.34754685
	PAR2 A118T + TM	0.38549497
10 nM / 30 nM	PAR2 A118	0.48655669
	PAR2 A118T	0.50948652
	PAR2 A118 + TM	0.43458018
	PAR2 A118T + TM	0.61380158

o

Antibody	Plasmids	P-values
Secondary AB	PAR2 A118 +TM/ PAR2 A118T + TM colony 1	0.08915031
	PAR2 A118 +TM/ PAR2 A118T + TM colony 2	0.00956414
	PAR2 A118 +TM/ PAR2 A118T + TM colony 3	0.66325448
	PAR2 A118T +TM colony 1/ PAR2 A118T + TM colony 2	0.57032253
	PAR2 A118T +TM colony 1/ PAR2 A118T + TM colony 3	0.08477078
	PAR2 A118T +TM colony 2/ PAR2 A118T + TM colony 3	0.01386649
PAR2 ECL AB	PAR2 A118 +TM/ PAR2 A118T + TM colony 1	0.001393637
	PAR2 A118 +TM/ PAR2 A118T + TM colony 2	3.60881E-05
	PAR2 A118 +TM/ PAR2 A118T + TM colony 3	0.008699306
	PAR2 A118T +TM colony 1/ PAR2 A118T + TM colony 2	0.068960732
	PAR2 A118T +TM colony 1/ PAR2 A118T + TM colony 3	0.608280314
	PAR2 A118T +TM colony 2/ PAR2 A118T + TM colony 3	0.591646193
PAR2 SAM 11 AB	PAR2 A118 +TM/ PAR2 A118T + TM colony 1	0.08004013
	PAR2 A118 +TM/ PAR2 A118T + TM colony 2	0.73989841
	PAR2 A118 +TM/ PAR2 A118T + TM colony 3	0.06062812
	PAR2 A118T +TM colony 1/ PAR2 A118T + TM colony 2	0.01679235
	PAR2 A118T +TM colony 1/ PAR2 A118T + TM colony 3	0.0043804
	PAR2 A118T +TM colony 2/ PAR2 A118T + TM colony 3	0.04716198
PAR1 ATAP2 AB	PAR2 A118 +TM/ PAR2 A118T + TM colony 1	0.01917151
	PAR2 A118 +TM/ PAR2 A118T + TM colony 2	0.03373638
	PAR2 A118 +TM/ PAR2 A118T + TM colony 3	0.80798493
	PAR2 A118T +TM colony 1/ PAR2 A118T + TM colony 2	0.4018254
	PAR2 A118T +TM colony 1/ PAR2 A118T + TM colony 3	0.50462346
	PAR2 A118T +TM colony 2/ PAR2 A118T + TM colony 3	0.59395098

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9. Curriculum vitae

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2017 - 2020	Bachelor Humanmedizin
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2012 - 2016	Matura, Schwerpunktfach Biologie und Chemie
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10. Declaration

Masterarbeit

Ich erkläre ausdrücklich, dass es sich bei der von mir im Rahmen des Studiengangs Humanmedizin eingereichten schriftlichen Arbeit mit dem Titel

Characterization and Comparison of Two Known Mutations in Protease-Activated Receptors (PAR4 Ala120Thr and PAR2 Ala118Thr)

um eine von mir selbst und ohne unerlaubte Beihilfe sowie *in eigenen Worten* verfasste Masterarbeit* handelt.

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Ich bestätige mit meiner Unterschrift die Richtigkeit dieser Angaben.

Datum: 29.05.22

Name: von der Lage Vorname: Paula

Unterschrift: P. v. d. Lage

* Falls die Masterarbeit eine Publikation enthält, bei der ich Erst- oder Koautor/-in bin, wird meine eigene Arbeitsleistung im Begleittext detailliert und strukturiert beschrieben.