Dear Sirs,

The prostaglandin endoperoxide synthase 1 (PTGS1, COX1) that is deposited in the Online Mendelian Inheritance in Man (OMIM) database (accession no. 176805) catalyses the conversion of arachidonic acid (AA) into prostaglandin H$_2$ intermediates and thromboxane A$_2$ (TxA$_2$). Polymorphisms of genes involved in AA metabolism are potential modifiers in platelet function (1-4). The functional consequences of most allelic variants, in terms of their influence on basal PTGS1-mediated AA metabolism and bleeding risk, are not well understood (5).

We here describe a 36-year-old male German patient without bleeding history prior surgical interventions but with post-operative recurrent disseminated bleeding complications after hemorrhoidectomy and atherectomy. The patient was neither on any medication nor physiotherapeutica and there was no known history of bleeding disorder. Prompt and detailed coagulation studies were performed that showed normal platelet count (mean: 187 x10$^9$/l), normal PT value (111 %), normal aPTT (38 seconds [sec]) and a normal fibrinogen concentration (mean 267 mg/dl). The lupus-sensitive PTT and dilute Russell’s viper venom time (dRVVT) revealed no pathological findings (PTT-LA 31.7 sec; dRVVT 0.9). Broad factor assay analysis was performed to rule out a clotting factor deficiency (all results revealed normal activities between 68 % - 119 %), including Ristocetin Cofactor (51 %), von Willebrand factor antigen (57 %) and normal von Willebrand multimer patterns.

Initial PFA-100$^\text{®}$ test revealed significant impaired platelet function compared to healthy controls (collagen/ADP: 150 sec vs 83 ± 21 sec; p=0.161 and collagen/epinephrine 226 sec vs 126 ± 44 sec; p=0.016; unpaired t-test). Moreover, different platelet function tests such as light transmission aggregometry (LTA) revealed impaired platelet function compared to healthy controls (collagen/ADP: 150 sec vs 83 ± 21 sec; p=0.161 and collagen/epinephrine 226 sec vs 126 ± 44 sec; p=0.016; unpaired t-test). Moreover, different platelet function tests such as light transmission aggregometry (LTA) revealed impaired platelet function compared to healthy controls (collagen/ADP: 150 sec vs 83 ± 21 sec; p=0.161 and collagen/epinephrine 226 sec vs 126 ± 44 sec; p=0.016; unpaired t-test).

For the PTGS1 gene we found heterozygosis of two known SNPs 22C>T (R8W; rs1236913) and 50C>T (P17L; rs5757) respectively, and showed no further alteration. For the PTGS1 gene we found heterozygosis of two known SNPs 22C>T (R8W; rs1236913) and 50C>T (P17L; rs5757) respectively, and showed no further alteration.

Gene expression analyses were then focused on the PTGS1 mRNA levels in platelets of the patient and of healthy controls with different PTGS1 genotypes. Total RNA was extracted from leukocyte-depleted platelets as described before (11). Relative quantification of PTGS1, TXBAS1, TXB2A2R and P2RY12 gene expression was done by reverse transcriptase real-time polymerase chain reaction (qRT-PCR) using Universal Probe assays and a LightCycler 480 system (Roche, Basel, Switzerland). All mRNA levels were quantified relative to the levels of the reference genes GAPDH and YWHAZ using the deltaCt method. Primers and probes for qRT-PCR were: PTGS1 forward 5’-tcgtcgggctctgttatgg-3’, reverse 5’-gttcattggctctgcctggc-3’ and P2RY12 were sequenced according to standard procedures. PCR with sequence-specific primers (PCR-SSP) for genotyping the PTGS1 22C>T and 50C>T single nucleotide polymorphisms (SNPs) was performed using standard protocols as described before (9) and primers as follows: forward PTGS1-22C 5’-tgccaggaaggctttgcc-3’; forward PTGS1-22T 5’-ctgaggagtctttgctct-3’; reverse PTGS1-50C 5’-agggacgggacg-3’; reverse PTGS1-50T 5’-cagggagggacgca-3’; internal control (HBB) forward 5’-ggttggcaactctactccagg-3’; reverse 5’-gcactactggttggcaag-3’. PCR products were separated on 3% agarose gels.

Molecular DNA analysis of the ALD relevant genes in the patient revealed wild-type sequence of the TXBAS1 gene. The TXB2A2R and P2RY12 genes were homozygous for the minor 1026A (silent; rs5757) and 18T (silent; rs6785930) variants, respectively, and showed no further alteration. For the PTGS1 gene we found heterozygosis of two known SNPs 22C>T (R8W; rs1236913) and 50C>T (P17L; rs5842787). These SNPs are located in the coding region and affect both alternative spliced variants of the PTGS1 gene that were reported previously (10). Using PCR-SSP for both SNPs we could confirm the compound heterozygous genotype and exclude a transconstellation of the 22T and 50T variants (Figure 1). However, in vitro function studies showed no significant effect on the PTGS1 metabolic activity for the two variants (1).

Gene expression analyses were then focused on the PTGS1 mRNA levels in platelets of the patient and of healthy controls with different PTGS1 genotypes. Total RNA was extracted from leukocyte-depleted platelets as described before (11). Relative quantification of PTGS1, TXBAS1, TXB2A2R and P2RY12 gene expression was done by reverse transcriptase real-time polymerase chain reaction (qRT-PCR) using Universal Probe assays and a LightCycler 480 system (Roche, Basel, Switzerland). All mRNA levels were quantified relative to the levels of the reference genes GAPDH and YWHAZ using the deltaCt method. Primers and probes for qRT-PCR were: PTGS1 forward 5’-tcgtcgggctctgttatgg-3’, reverse 5’-gttcattggctctgcctggc-3’.

PTGS1 compound heterozygosity impairs gene expression and platelet aggregation and is associated with severe bleeding complications

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The patient’s PTGS1 gene expression was significantly lower compared to healthy controls carrying each the major alleles (five-fold lower level: 19.7 % vs 90.5 %, \( p=0.0002 \)) or those that carry one copy of the 50C>T allelic variant (two-fold lower level: 19.7 % vs 44 %, \( p=0.0193 \)) (Figure 1B). Moreover, PTGS1 50C>T heterozygotes showed two-fold lower level of mRNA expression compared to wild-type controls (44 % vs 90.4 %, \( p=0.0034 \)). Gene transcripts for TBXA2R showed similar levels in all PTGS1 genotype groups including the patient.

We also investigated PTGS1 protein expression in relation to the expression of GAPDH as reference protein by Western blot analysis. Extraction, electrophoresis, blotting and detection of platelet proteins were performed according to standard Western blotting procedures. The anti-PTGS1 rabbit polyclonal antibody H-62 and the anti-GAPDH mouse monoclonal antibody 0411 (both Santa Cruz Biotechnology; Heidelberg, Germany) were used for specific protein detection.

Evaluation of the PTGS1:GAPDH ratio based on the optical density (OD) of the specific protein bands confirmed the sig-

**Figure 1:** PTGS1 genotyping and relative quantification of PTGS1 gene expression in regard to the PTGS1 genotype. A) Four primer combinations were used in PCR-SSP to identify the cis/trans constellation of the PTGS1 22C>T and 50C>T alleles. As expected, control individuals were either wild-type (contr01: homozygous 22C-50C) or single heterozygotes (contr02: 22T-50C or contr03: 22C-50T) individuals. The patient revealed compound heterozygosity proven by positive reactions #2 and #4 (22T-50C and 22C-50T), whereas reactions #1 (wild-type) and #3 (22T-50T) were negative. B) mRNA levels of the PTGS1 (left panel) and the TBXA2R (right panel) genes were determined by qRT-PCR in platelet RNA relative to the mean mRNA levels of the reference genes GAPDH and YWAHZ. Control individuals without bleeding or platelet deficiency phenotype included PTGS1 wild-type (wt; \( n=9 \)), 22C>T heterozygotes (\( n=2 \)) and 50C>T heterozygotes (\( n=3 \)). The compound heterozygous patient revealed significantly lower PTGS1 mRNA levels than the controls. C) Expression of PTGS1 and GAPDH proteins in platelets of healthy controls (7 wild-type and 4 heterozygous individuals) and the patient (2 blood samples) was investigated by Western blot analysis. The blot images (representative results shown on the left) were used to determine the optical density (OD) of the PTGS1 and GAPDH bands and to calculate the PTGS1:GAPDH OD ratio. Statistical analysis of the OD ratio (right panel) indicated significantly lower PTGS1 expression in the compound heterozygous patient compared to the wild-type controls. The heterozygous controls showed trends towards lower PTGS1 expression that was not significant.
significant downregulation of PTGS1 in the compound heterozygous patient compared to wild-type controls (mean OD ratio ± SD: 0.53 ± 0.05 vs 1.42 ± 0.48; p=0.039) (Figure 1C). Control individuals with one variant allele 22C>T (0.91 ± 0.13; p=0.198) or 50C>T (0.74 ± 0.02; p=0.095) tended to have lower PTGS1:GAPDH ratios compared to wild-type controls, however, not significant. Future studies confirming the in vivo relevance of the PTGS1 variants, including their influence on gene expression, disease susceptibility and risk assessment, are warranted.

To our knowledge, this study represents the first investigation that correlates a compound heterozygosity in PTGS1 to bleeding complication. Although there is no definite proof, the most plausible explanation for the patient’s recurrent severe bleeding incidents appears to be the compound heterozygosity within the coding region of the PTGS1 gene. From our mRNA and protein data we assume that both SNPs affect gene expression. Interestingly, for the European/Caucasian population a complete linkage disequilibrium of the 50C>T SNP with a number of SNPs in the 5’ region including the promoter of the PTGS1 gene was reported (1). Data deposited by the International HapMap Project (http://hapmap.ncbi.nlm.nih.gov/) suppose that the frequencies of respective variants in Utah residents with Northern and Western European ancestry from the CEPH collection are 0.054 and 0.080 for the minor T alleles in rs1236913 and rs3842787, respectively. Based on these values, the frequency of the compound heterozygote in this descent is supposed to be approximately 0.00432, meaning that about one in 24000 persons could be affected by this genetic constellation. Therefore, the clinical knowledge of existing compound heterozygosity of the PTGS1 gene might be helpful to estimate the bleeding risk prior surgical interventions.

Conflicts of interest
None declared.

References

Erratum to McEwen et al. "Diurnal changes and levels of fibrin generation are not altered by continuous positive airway pressure (CPAP) in obstructive sleep apnoea (OSA). A randomised, placebo-controlled cross-over study" (Thromb Haemost 2012; 108: 701–709)

In the original article by McEwen BJ, Phillips CL, et al. "Diurnal changes and levels of fibrin generation are not altered by continuous positive airway pressure (CPAP) in obstructive sleep apnoea (OSA). A randomised, placebo-controlled crossover study. (Thromb Haemost 2012; 108: 701–709) the authors recently realised that a statistical analysis error has resulted in minor data errors in Table 1. They also realised that no data on the Epworth Sleepiness Score (ESS) was listed despite the table legend listing the meaning of ESS.

The correct (mean ± SD) CPAP vs Placebo CPAP data are: AHI (events/h): 5.7 ± 1.7 vs 40.2 ± 5.2, p<0.00001; ODI (Events/h): 4.2 ± 1.7 vs 39.6 ± 4.4, p<0.00001; SaO₂-T90 (%TST): 0.6 ± 0.3 vs 9.8 ± 2.3, p<0.001; Min SaO₂ (%) 90± 1.2 vs 77 ± 1.4, p<0.00001; Treatment Compliance (h/night) 4.3 ± 0.4 vs 3.4 ± 0.4, p<0.05; Epworth Sleepiness Score (ESS) 7.4 ± 0.8 vs 8.7 ± 0.7, p<0.05.

Although this new data is very similar to the original data, the authors felt it important to correct. Importantly, these errors had no influence on the main coagulation outcomes and study interpretation.