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Prostaglandin E₂-EP₃ receptor subtype gene deletion in mother and son impairs platelet aggregation

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Prostaglandin E2 (PGE2) is an important inflammatory mediator produced in large quantities by atherosclerotic plaques and smaller quantities in healthy arterial walls (Gross et al, 2007). Interestingly, PGE2 has a biphasic effect on platelets, determined by the receptor to which it binds (EP1-EP4): while PGE2 itself does not induce platelet aggregation (Fabre et al, 2001), low concentrations of PGE2 sensitize platelets to its activators and enhance platelet aggregation via EP3 activation (Gross et al, 2007; Fabre et al, 2001), whereas high PGE2 levels inhibit aggregation, mainly through EP4 receptors (Iyu et al, 2010). Altogether, the stimulatory effect on platelets by EP3 receptor activation overweigh the inhibitory effect (Gross et al, 2007; Iyu et al, 2010), suggesting that PGE2 facilitates platelet aggregation.

In transgenic mice carrying a null mutation of the gene encoding the EP3 receptor (PTGER3), a variety of clinical symptoms has been observed, e.g. obesity, increased insulin, and inhibition of platelet aggregation (Ceddia et al, 2016). To the best of our knowledge the effect of a heterozygous deletion of PTGER3 has not been described in humans before, and is described here in a mother and her son. The patients had no bleeding history, but platelet aggregation testing was chosen to measure a functional effect of the mutation.

*Patient 1* was a 43-year-old woman referred for suspicion of Ehlers-Danlos syndrome. As a child she was diagnosed with psychomotor retardation and hypermobility. Lymphoedema of the legs was diagnosed in her early twenties, insulin-dependent diabetes mellitus was diagnosed at the age of 24 years, and the patient also developed obesity. The patient had eight early spontaneous abortions, but at the age of 30 years one pregnancy (supported by progesterone treatment) resulted in the birth of a child (Patient 2). Patient 1 was diagnosed with autoimmune hyperthyroidism at 32 years of age. Her diabetes mellitus was dysregulated and bilateral proliferative retinopathy developed. The patient had no bleeding history with a bleeding score of 2 points. Family history only included hypermobility on the maternal site.

*Patient 2* was the 13-year-old son of Patient 1. Due to delayed development (IQ 68) and dysmorphic features he was 5 years old when referred for paediatric examination.
Array-comparative genomic hybridisation (CGH) was performed by an external laboratory (Rigshospitalet, Copenhagen, Denmark) for Patient 1, while array-CGH for Patient 2 was performed in house with OGT 105Kx2 oligo-array-CGH (Oxford Gene Technology, Oxford, UK). In order to identify variants on the remaining allele, *PTGER3* was also Sanger sequenced.

For platelet aggregation tests, venous blood was drawn into vacutainer sodium citrate tubes (Becton Dickinson, Franklin Lakes, NJ, USA), while Terumo vacuette EDTA tubes (Terumo, Eschborn, Germany) were used for the platelet count.

To observe alterations in platelet aggregation platelets were pre-incubated at 37°C for 3 min with PGE₂ at final concentrations of 0.3 nmol/l, 3 nmol/l, 30 nmol/l and 3 µmol/l, respectively. PGE₂ (5 mg; Sigma-Aldrich, St Louis, MO, USA) was dissolved in 250 µl ethanol to a stock solution of 56.7 mmol/l. Platelet response was evaluated with method of Born and Cross (1963) performed in a lumi aggregometer (model 409-x, Chrono-log Corp., Havertown, PA USA), which measures the increase in light transmission through platelet-rich plasma (PRP) relative to platelet-poor plasma (PPP). PRP was prepared by centrifugation at 1500 g for 2.5 min at room temperature, while the residual blood was centrifuged at 3200 g for 15 min to obtain PPP. High platelet counts were standardized to 250 x 10⁹/l by adding autologous PPP. After pre-incubation, platelet aggregation was induced by addition of an agonist: Collagen was used at very low concentrations to allow evaluation of the PGE₂ effect on platelet aggregation. In a normal setting, 3.2 µg/ml collagen is used, while here collagen concentrations of 0.4, 0.8 and 1.25 µg/ml were applied.

A deletion of 130 kb at chromosome 1p31.1 was detected in Patient 1. The extension of the deletion was chr 1: 71318475-71465448 (hg19). The same deletion was found in Patient 2. The deletion encompasses the last exons (3-4, 3-5 or 3-6 dependent on the isoform) from most isoforms of *PTGER3*. No mutations were found in the remaining allele by Sanger sequencing. The father (mother was deceased), maternal aunt and sister of Patient 1 did not have the mutation.
Platelet counts were 309 x 10^9/l and 313 x 10^9/l in Patients 1 and 2, respectively. Microscopically, no morphological alterations were observed. When pre-incubated with different concentrations of PGE₂, no platelet response was seen when stimulated with 0.4 µg/ml collagen (Figure 1) (equivalent results were obtained with 0.8 and 1.25 µg/ml). In contrast, platelets from the healthy control responded to PGE₂ in a dose-dependent manner despite the low collagen concentrations. Platelets pre-incubated with 3 µmol/l PGE₂ exhibited no aggregation in any of the individuals.

This study aimed to elucidate the physiological impact of a mono-allelic expression of PTGER3 on platelet aggregation. The clearly reduced response to collagen after pre-incubation with PGE₂ in our patients indicate that the heterozygous PTGER3 deletion indeed does affect the function of PGE₂ on platelets, and interestingly, this was in patients with no bleeding history. This is in concordance with recent studies: In a clinical trial on patients with peripheral arterial disease, DG041, a potent EP₃ antagonist, had a favourable effect on inflammatory biomarkers as well as on ankle-brachial index measurements without increasing bleeding risk (deCODE Genetics, 2017). These results were consistent with a previous study showing that DG041 inhibited platelet function and did not alter haemostatic function (Tilly et al, 2014). Furthermore, in vivo studies on mice have shown that Ptger3 deletion has beneficial effects on stroke outcomes in terms of smaller infarct volume, reduced oedema and reduced neurological dysfunction (Ahmad et al, 2007; Saleem et al, 2009). Similar results are seen in models utilizing an EP₃ antagonist (Ikeda-Matsuo et al, 2011).

In conclusion, we report a large heterozygous deletion of PTGER3 in a mother and son with decreased platelet aggregation, but with no bleeding tendency. These results support the capability of a EP₃ antagonist to prevent arterial thrombosis. Whether this prevention already exists as a natural protection warrants further clarification through a larger cohort study.

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Author contributions

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C.R. Fagerberg and B. Jensen conceived the study; C.R. Fagerberg arranged patient interviews; M. Nybo, J. Graakjær and C.B. Andersen performed the analyses. Data was interpreted by M. Nybo and T.S. Goharian. The manuscript was drafted by M. Nybo, T.S. Goharian and C.R. Fagerberg, and all authors critically reviewed the manuscript.

REFERENCES


**FIGURE LEGEND**

**Figure 1.** Aggregation of platelets in response to PGE$_2$ stimulation

Platelets were pre-incubated with PGE$_2$ at various concentrations prior to stimulation with collagen. While platelets from the healthy control responded in a dose-dependent fashion, platelets from the two patients did not respond to stimulation with 0.4 µg/ml collagen (with equivalent results for 0.8 and 1.25 µg/ml). phosphate-buffered saline (red), 0.3 nmol/l PGE$_2$ (blue), 3 nmol/l PGE$_2$ (green), 30 nmol/l PGE$_2$ (black). 3 µmol/l PGE$_2$ resulted in no response at all for all persons tested (not shown). A: Patient 1; B: Patient 2; C: Control.