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# **Association between endometriosis, infertility and autoimmune antiplatelet glycoprotein VI antibodies in two patients**

Running title: Endometriosis and antiplatelet GPVI antibodies

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## **Association between endometriosis, infertility and autoimmune antiplatelet glycoprotein VI antibodies in two patients**

### **Abstract**

The association between endometriosis and autoimmune diseases is well known, however no acquired platelet function defect has been described so far. We describe the case of 2 patients with endometriosis associated to an antiplatelet glycoprotein VI (anti-GPVI) antibody. The 2 women with deep pelvic endometriosis associated with secondary infertility presented a mild bleeding tendency, a deficient platelet aggregation response to collagen, convulxin or CRP and a severe GPVI deficiency. Immunoblot revealed a combined FcR $\gamma$  deficiency but no indication of GPVI cleavage. In the first case, platelet count was normal and an anti-GPVI IgG was detected in plasma. A first corticosteroids administration normalized *in vitro* platelet functions but further administrations were unsuccessful. Three IVF attempts failed. Conservative laparoscopic surgery was carried out after antifibrinolytic treatment without bleeding. The second case presented with a history of moderate thrombocytopenia and a weak anti-GPVI in a context of infertility and autoimmune disease, the Sjögren syndrome resolved after corticosteroids and hydroxychloroquine treatment. Acquired GPVI deficiencies are rare. It would be useful to determine whether the association with endometriosis is coincidental or not by more systematic investigations. It does not seem that in these patients, GPVI deficiency is associated with an increased risk of bleeding.

**Keywords:** endometriosis, auto-immune diseases, platelets, glycoprotein VI, infertility, autoantibodies

## **Plain language summary**

### **What is the context?**

- Evidence for an immune system dysfunction is reported in endometriosis and the association between endometriosis and autoimmune diseases is well known.
- No autoimmune platelet function defect has been described so far.

### **What is new?**

- We report 2 unrelated patients with endometriosis-associated infertility presenting an platelet glycoprotein VI deficiency due to an autoantibody.
- In both cases, a deficient platelet aggregation response to collagen, convulxin or CRP and a severe GPVI deficiency were observed.
- Immunoblot revealed no indication of GPVI cleavage.

### **What is the impact?**

- Our observation raises the question whether GPVI could be a preferential target for the development of anti-GPVI autoantibodies associated with endometriosis.
- It does not seem that in these patients, GPVI deficiency is associated with an increased risk of severe bleeding disorder.

## Introduction

Endometriosis affects 2 to 10% of reproductive age women and is defined as the presence of endometrial glands and stroma at extra-uterine sites.<sup>1,2</sup> This chronic inflammatory ovarian steroid hormone dependent disease is associated with pain and infertility. About two-thirds of adolescent girls with chronic pelvic pain or dysmenorrhea have laparoscopic evidence of endometriosis. Endometriosis is now considered as a rather more systemic disease than limited to pelvis.<sup>3</sup> The endometriosis etiology is largely unknown but the widely accepted Sampson's retrograde menstruation theory accounts for pelvic dissemination of endometrial cells leading to a chronic inflammatory response.<sup>4</sup> Evidence for a immune system dysfunction is also reported and an association between endometriosis and autoimmune diseases has been proposed by several studies with relatively good quality evidence supporting association between endometriosis and systemic lupus erythematosus (SLE).<sup>5,6</sup> In contrast, no clear relationship between hemostatic defects related to platelet disorders and endometriosis has been established. Glycoprotein (GP)VI is an platelet receptor triggering platelet activation by collagen and fibrin(ogen).<sup>7-9</sup> GPVI is member of the immunoglobulin receptors superfamily, and is coexpressed with the common gamma chain of Fc receptors (FcR $\gamma$ ) at a level of  $3730 \pm 453$  copies at the platelet surface.<sup>10</sup> GPVI has been found to be a target for autoantibodies in patients presenting autoimmune diseases, in particular immune thrombocytopenia (ITP) and in some cases in association with SLE.<sup>11,12</sup> The number of patients described in the literature to date with acquired immune GPVI deficiency is less than 20 but autoantibodies to GPVI are probably more frequent than reported because they usually are asymptomatic or associated with mild bleeding. A decreased expression of GPVI at the platelet surface leads to the search for an anti-GPVI autoantibody, that **usually induces activation-dependent shedding of the GPVI ectodomain by metalloprotease, even if, in a limited number of cases, internalization and intracellular processing of antibody-GPVI complexes has been proposed as a possible alternative pathway.**<sup>13</sup> Here, we report 2 unrelated patients with endometriosis-associated infertility presenting a GPVI deficiency due to an autoantibody.

## Case Reports

### Case 1

A 27-year-old patient presented to laboratory investigation with a 3-year history of pelvic endometriosis-associated infertility. As the patient's medical history revealed a history of moderate bleeding tendency (epistaxis, menorrhagia, easy bruising and post-operative bleeding, ISTH-BAT=4), platelet count and platelet aggregation assay were performed. The platelet count was 165 G/L with a normal mean platelet volume (MPV) of 12.8 fL. Platelet aggregation with citrated platelet rich plasma (PRP) showed an absence of response to collagen (5 µg/mL) with normal response to other agonists (ADP, arachidonic acid). Aggregation was also null in response to a GPVI-specific agonist, convulxin at a concentration that induces aggregation of normal platelets (600 pM), strongly in favor of a GPVI deficiency (figure 1a). Quantification of the major platelet surface glycoproteins by flow cytometry (GpScreen kit, Diagnostica Stago, France) indicated a decreased expression of GPVI at the platelet surface with 398 sites per platelet for normal values of 3730±453 (mean±SD).<sup>10</sup> Immunoblotting experiments were performed with different antibodies to GPVI on lysates of washed platelets<sup>14</sup>. First, we observed a combined deficiency of GPVI extracellular domain and of the FcRγ chain in platelets from patient compared to control (Figure 1b). Secondly, with an antibody against GPVI cytoplasmic domain, we confirmed the GPVI deficiency in platelets from patients (Figure 1b). Moreover, no sign of GPVI fragmentation was observed as incubation of control platelets with N-ethylmaleimide (NEM) induced the shedding of the ectodomain of GPVI as attested by the presence of low molecular weight GPVI intracellular remnant that was not observed in the patient's sample (figure 1b). All these observations are in favor of internalization and intracellular processing and degradation of GPVI and not GPVI shedding. The absence of GPVI shedding was confirmed by low soluble GPVI levels detected in the patient's plasma (0.3 ng/mL) (normal value 1.92±0.36 ng/mL, mean±SD, n=53). The presence of an anti-GPVI antibody was searched in the platelet poor plasma (PPP) of the patient using a home-made ELISA assay in which purified GPVI is immobilized on microtitration plates platelets.<sup>12,14</sup> Soluble GPVI was measured in the plasma using an ELISA- based assay using a Meso Scale Discovery Electroluminescence platform by Egan K, et al.<sup>15</sup>. Briefly a polyclonal capture anti-GPVI antibody GPVI AF3627, RnD systems) was immobilized in Multi-Array 96 plate. Diluted plasma was added to the wells and followed by a biotinylated anti GPVI antibody (BAF-3627, RnD systems). Revelation was performed using streptavidin SULFO-TAG. The concentration of sGPVI in the samples was determined using a calibration curve made with recombinant

GPVI (3627-GP, RnD systems), that was straight from 0.09 to 12.5 ng/mL. A positive signal was observed with an anti-IgG, A, M reagent (38 AU) and the presence of anti-GPVI IgG was confirmed using a specific antihuman IgG antibody (Figure 1c). Addition of soluble recombinant GPVI to the patient's PPP quenched the signal confirming the specificity of the antibody. Patient's plasmatic IgG were purified and analyzed for their capacity to activate platelets.<sup>12,14</sup> Flow cytometry experiments did not show an increased level of activation markers (P-selectin and GPVI dimers) when control platelets were incubated with the patient's IgG (300 µg/mL). Together, these observations were in favor of a GPVI deficiency caused by the internalization of the anti-GPVI-FcR $\gamma$  complexes without thrombocytopenia. No other argument in favor of clinical or biological dysimmunity disease was observed in the patient. Although the anti-GPVI was unlikely to increase the risk of bleeding, a corticosteroid response test (1 mg/kg/day for 15 days) was carried out in anticipation of the treatment of endometriosis and infertility. It resulted in a normalization of the platelet response to collagen (5 µg/mL) and convulxin (600 pM) (Figure 2a), and a decreased level of anti-GPVI (Figure 2b). A laparoscopic resection of an endometriosis cyst on the right ovary (50 mm) was then performed without bleeding complication. Eight months later, the aggregation response to collagen (5 µg/mL) was again null, the level of the anti-GPVI IgG elevated (63 AU) and the expression of GPVI at platelet surface lowered (334 sites/platelet). A 15 days course of prednisolone 1 mg/kg/24h was administered before oocyte puncture. It resulted in increased GPVI expression (2397 sites/platelet) and collagen-induced platelet aggregation. Oocyte puncture was achieved without hemorrhagic complications. Unfortunately, *in vitro* fertilization with intracytoplasmic microinjection (IVF/ICSI) did not result in pregnancy despite transfer of 2 fresh embryos. During the 4-following months, a gradual resurgence of GPVI deficiency and anti-GPVI was observed. A new corticosteroid treatment was carried out but was ineffective on GPVI deficiency and platelet response to collagen (Figure 2b). Interestingly, the oocyte puncture was nevertheless performed without hemorrhagic complications. Five immature oocytes have been collected but failed to obtain embryonic development. In the following months GPVI deficiency did not improve. The patient underwent a conservative laparoscopic surgery (hysteroscopy, laparoscopy with extensive adhesiolysis, bilateral uterolysis, exeresis of rectovaginal septum endometriosis nodule (13x6x3 mm) with colpectomy and rectal shaving), while receiving tranexamic acid (1g x3/day) the day before intervention and for 10 days. No bleeding was observed. After 7 months a new oocyte puncture was performed and IVF/ICSI with 2 fresh embryos transfer

succeed. However, a hemoperitoneum associated with an ectopic pregnancy required a treatment by methotrexate with tranexamic acid (1g x3/day) for 7 days.

### *Case 2*

A 31-year-old patient presented to laboratory investigation with an 8 years history of pelvic endometriosis-associated infertility. The history of the patient reveals the presence of ecchymosis since the age of 8, but platelet count was normal (400 G/L). Menorrhagia started at menarche (14 years old). Diagnosis of ITP was established at the age of 23, on the basis of an isolated and asymptomatic thrombocytopenia (platelet count 40 G/L). Initial assessment found positive antinuclear antibodies (1/640, speckled), anti SSA and anti SSB antibodies. Serum protein electrophoresis showed polyclonal hypergammaglobulinemia. Bone marrow smear confirmed the peripheral mechanism of thrombocytopenia. The thrombocytopenia was corticosteroid responsive. Four years later, the patient presented with inflammatory arthralgias, concerning wrists, elbows, then ankles and shoulders. The clinical examination found arthritis of the proximal interphalangeal joints of the second and third fingers of the left hand, as well as of the thumb spine, and a limitation of shoulder's amplitudes. Symptoms of the Sjögren syndrome (SS) resolved after short oral corticosteroid therapy. Treatment with hydroxychloroquine 200 mg x2 / day was initiated to control arthralgias.

At presentation, we observed a mild macro-thrombocytopenia with a platelet count of 130 G/L and a MPV of 13.1 fl. [Since patient presents ecchymosis since the age of 8, a platelet aggregation assay was performed.](#) Platelet aggregation with citrated PRP showed an absence of response to collagen (3.3 µg/mL) and to collagen-related peptide (CRP 9 µg/mL), a GPVI specific agonist. Response to other agonists (ADP, arachidonic acid) was normal (Figure 3a). Quantification of the major platelet surface glycoproteins indicated a decreased expression of GPVI at platelet surface with 133 sites per platelet. The GPVI deficiency was confirmed by immunoblot and combined with an important deficiency of the FcR $\gamma$  chain (Figure 3b). No GPVI degradation fragment was observed and the absence of GPVI shedding was confirmed by the low level of soluble GPVI in the patient's plasma (1.5 ng/mL) The presence of an anti-GPVI antibody was evidenced in the patient's PPP when detection was performed with the anti-IgG,A,M reagent (48 AU); but a very faint signal was obtained with the IgG specific reagent suggesting the anti-GPVI antibody is possibly an IgM as previously observed in another patient with SLE.<sup>12</sup>



## Discussion

This is the first time to our knowledge that an association between an acquired immune platelet defect, i.e an immune GPVI deficiency, and endometriosis is reported. The likelihood of such an association is low as GPVI deficiencies are rare.

One of the postulated origins of endometriotic tissue is retrograde menstruations. Heavy menstrual flow and increased volume of retrograde menstruation could favor the dissemination, in the pelvic cavity, of endometrial tissue.<sup>16</sup> Together with blood components, this process could induce the ectopic implantation of endometrial cells, contributing to inflammation and fibrosis. Guo et al.<sup>17,18</sup> made the assumption that platelets are implicated in the development of endometriosis through multiple mechanisms associated with platelet activation and proposed aspirin as treatment of endometriosis. However, endometriosis has been reported in 2 sisters with Glanzmann thrombasthenia, a bleeding disorder due to the complete congenital defect in platelet GPIIb/IIIa, a receptor required for platelet activation and aggregation.<sup>19</sup> This is not in favor of the importance of platelet activation to promote endometriosis. Conversely, patients suffering from congenital bleeding disorders such as von Willebrand disease (vWD) tend to have heavy menstruation. Nevertheless, there is only weak evidence that women with vWD are more likely to develop endometriosis.<sup>20,21</sup> On the other hand, association between endometriosis and autoimmune diseases is well known, and the association of endometriosis and SLE, SS, rheumatoid arthritis (RA), coeliac disease, multiple sclerosis, inflammatory bowel disease has been proposed by several studies.<sup>6,11,22-25</sup> In the cases we report, the isolated GPVI deficiency is unlikely to be the cause of heavy retrograde menstruations as patients do not suffer in any way of a bleeding disorder comparable to vWD or Glanzmann thrombasthenia. The fact that, in case 1, conservative laparoscopic surgery has been performed without bleeding while the GPVI was low (189 sites/platelet) reinforce the notion that GPVI deficiency is not a serious bleeding disorder.

Infertility is an important concern for women suffering of endometriosis. Our 2 patients were examined in the context of infertility. Chronic pelvic inflammation could impair fertility because of high elevated levels of cytokines, and of fibrosis altering the function of reproductive organs.<sup>26,27</sup> Otherwise, an increased prevalence of various autoantibodies and autoimmune diseases such as SLE, SS, RA, is reported in endometriosis women and raise the possibility of a link between autoimmunity and endometriosis -associated infertility.<sup>25,28</sup> The observation in 2 cases of endometriosis of an anti-platelet GPVI autoantibody, in a SS context for one of the cases, is consistent with the notion of autoimmune pathology associated with endometriosis. The fact that the rare cases described in the literature of acquired GPVI

deficiency mainly concern women suggests that recurrent bleedings of gynecological origin probably trigger hemostasis analysis and therefore confirms the poor impact of GPVI deficiency on hemostasis.<sup>13</sup> Whether the immune dysregulation precedes and favors implantation of endometrial cells at ectopic sites or is a consequence of chronic inflammation is an open question. Our observation does not provide answer to this question since autoantibodies to GPVI were detected at a time when patients already suffered from endometriosis.

ITP occurs with an incidence rate of 1.6 to 3.9 per 100,000 patient-years, which increases with age and has slight female preponderance. It has been proposed that patients with ITP have a state of immune dysregulation that extends beyond platelet autoantibodies.<sup>29</sup> The surface expressed major platelet receptors GPIIb/IIIa and GPIb are the most frequently identified targets of antibodies in ITP<sup>30</sup> and thrombocytopenia is explained by both an increased destruction and a decreased production. Few patients with an autoantibody to GPVI have been reported so far; most possibly because testing for antibody against GPVI in ITP is not generalized. Of note, the platelet count is relatively weakly affected in our 2 patients. To our knowledge there is no report describing the association of anti-GPIIb/IIIa or anti-GPIb with endometriosis. Our observation raises the question whether GPVI could be a preferential target for the development of anti-GPVI autoantibodies associated with endometriosis. A more systematic analysis of platelet GPVI expression in patients with endometriosis would be necessary to answer that question.

**Disclosure of Interest**

The authors report no conflict of interest.

**Ethics approval**

Written informed consents were obtained from the 2 patients for using their data for clinical research and publication.

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## Figures legends

**Figure 1. Case 1 at diagnosis.** (a) Platelet aggregation was measured by light transmission aggregometry (TA8V, Diagnostica Stago) with citrated PRP in response to the following agonists: collagen (5 µg/ml) and convulxin (600 pM), ADP (10, 7 and 3 µM), arachidonic acid (1 mM), (b) Western blot analysis of GPVI and FcR $\gamma$  expression: Platelet proteins (fixed amounts) were separated by SDS-PAGE and immunoblotted (IB) with either a monoclonal (1G5) or polyclonal anti-GPVI cytoplasmic domain (anti-GPVI-IC) or polyclonal anti-FcR $\gamma$ . Washed platelets from were incubated with N-ethyl maleimide (NEM) 2 mM for 20 min at 37°C, pelleted and lysed in the same conditions as patient's platelets. The protein concentration was measured simultaneously in all lysates using the BCA method. The amount of protein deposited for the NEM-treated sample was 20 µg. The method is sufficiently sensitive to detect the GPVI remnant at 20 kDa. Results indicate a combined GPVI/FcR $\gamma$  deficiency. (c) Detection of anti-GPVI antibodies in patient's plasma at presentation: Diluted PPP of control subjects and patient was incubated on immobilized soluble GPVI. PPP was also supplemented with soluble GPVI to assess the specificity of the binding. Bound antibodies were detected with either HRP coupled anti-human pan IgG-A-M (i) or specific anti-IgG antibodies (ii) and hydrolysis of Tetra Methyl Benzidine (TMB) was measured at 450 nm (AU: arbitrary units). . Results indicate the presence in the patient's PPP of an IgG anti-GPVI. NEM: N-ethylmaleimide. In (iii) the ELISA was performed as in (ii) but samples were purified IgGs (100 µg) from a control subject and patient 1 mixed or not with purified recombinant GPVI.

**Figure 2. Case 1 evolution.** Platelet aggregation in response to collagen (5 µg/ml) and convulxin (600 pM) before (a) and after (b) the first corticosteroid treatment. Collagen and convulxin-induced platelet aggregation were measured by light transmission aggregometry on PRP. (C) GPVI expression was quantified by flow cytometry. Anti-GPVI antibodies were detected by ELISA. Black arrows indicate corticosteroid treatments that restored GPVI expression/function 2 times but was ineffective the third time. Green arrows indicate oocyte puncture. AU: arbitrary unit, NEM: N-ethylmaleimide.

**Figure 3. Case 2 at diagnosis.** (a) Platelet aggregation was measured by light transmission aggregometry (TA8V, Diagnostica Stago) with citrated PRP and in response to the following agonists: collagen (3.3 µg/ml), CRP (9 µg/ml), ADP (10, 5 and 2.5 µM), arachidonic acid (1

mM), (b) Western blot analysis of GPVI and FcR $\gamma$  expression. Platelet proteins (fixed amounts) were separated by SDS-PAGE and immunoblotted (IB) with either an anti-GPVI full length or a polyclonal anti- FcR $\gamma$  or a polyclonal anti-GPVI cytoplasmic domain (anti-GPVI-IC). Results indicated a combined GPVI/FcR $\gamma$  deficiency in the platelets from case 2 patient.