# New insight into antiphospholipid syndrome: antibodies to $\beta_2$glycoprotein I-domain 5 fail to induce thrombi in rats

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## Supplementary Methods

### Animal models

An *in vivo* model of antibody-induced thrombus formation was established in male Wistar rats (270-300 g) (Envigo, S. Pietro al Natisone, Italy) and kept under standard conditions in the Animal House of the University of Trieste, Italy as previously reported in details.[1] Briefly, the animals received an intraperitoneal injection of lipopolysaccharide (LPS) from Escherichia coli O55:B5 (2.5 mg/kg body weight) (Sigma-Aldrich) 4 hours before general anesthesia. After infusion of Rhodamine 6G (Sigma-Aldrich) into the femoral vein, serum IgG (10 mg/rat) from patients and controls were slowly administered into the carotid artery. For some experiments the protocol was slightly changed and the animals received intraperitoneal injection of IgG 15 hours before general anesthesia followed by the injection of LPS by the same route. Clot formation and partial or complete occlusion of blood vessels were monitored by intravital microscopy and analyzed in at least 5 microvascular areas. The microvasculature was examined using a BX50WI microscope (Olympus, Center Valley, USA), equipped with CCD camera model SensiCam and SensiCam digital converter (PCO). The in vivo procedures were performed in compliance with the guidelines of European (86/609/EEC) and Italian (D.L.116/92) laws and were approved by the Italian Ministry of University and Research and the Administration of the University Animal House. This study was conducted in accordance with the Declaration of Helsinki.

### Antibody binding assays

The interaction of IgG with phospholipid-bound $\beta_2$GPI was evaluated by coating the wells of 96-well polystyrene plates (Polysorp Immunoplate, Nalge Nunc International) with cardiolipin (50 µg/ml) (Sigma-Aldrich) overnight at 4°C. After blocking the free binding sites with 1% ultrapure BSA (Sigma-Aldrich) in PBS (PBS/BSA), increasing concentrations of purified $\beta_2$GPI (1,5,75 µg/ml) were added and left to incubate for 2 hours at room temperature. Free $\beta_2$GPI was removed by washing with the blocking buffer and phospholipid-bound $\beta_2$GPI was allowed to react with IgG (50 µg/ml) from patients and controls for additional 2 hours at room temperature. Bound antibodies were detected with alkaline phosphatase-conjugated goat anti-human IgG (Sigma-Aldrich).

The interaction of IgG with soluble $\beta_2$GPI was evaluated by incubating patients' and controls' IgG (50 µg/ml) with increasing concentrations (50,100,200 µg/ml) of human purified $\beta_2$GPI or BSA as unrelated antigen for 1 hour at 37°C followed by overnight incubation at 4°C in a tube rotator. The samples were centrifuged at 3,000 g for 5 min at room temperature and the residual un-complexed

antibodies were tested using γ-irradiated polystyrene plates (Combiplate EB, Labsystems) directly coated with purified human $\beta_2$GPI (10 µg/ml) as previously described.[2]

**Supplementary Table 1.** Clinical and laboratory characteristics of the patients and controls

| Sample ID | Age /sex | Diagnosis | aCL IgG, GPL* | aβ2GPI IgG, OD† | aD1 IgG AU‡ | aD4,5 IgG AU‡ | LA | AT | VT | PM |
|---|---|---|---|---|---|---|---|---|---|---|
| P1 | 35/F | APS non-criteria | 0 | 1.45 | 9 | 59 | neg | No | No | 2EM |
| P2 | 56/F | aPL carrier | 20 | 1.10 | 13 | 39 | neg | No | No | No |
| P3 | 29/F | APS non-criteria | 4 | 0.93 | 24 | 49 | neg | No | No | 2EM |
| P4 | 36/F | APS non-criteria | 3 | 0.98 | 15 | 63 | neg | No | No | FGR |
| P5 | 60/M | APS non-criteria | 10 | 0.76 | 6 | 44 | neg | No | SVT | *NA* |
| P6 | 34/F | PAPS | 88 | 1.73 | 69 | 11 | pos | Yes | No | No |
| P7 | 51/F | PAPS | 155 | 1.56 | 78 | 13 | pos | Yes | No | Yes |
| P8 | 34/F | SAPS | 67 | 0.62 | 104 | 10 | pos | No | Yes | No |
| P9 | 46/M | PAPS | 131 | 1.68 | 60 | 7 | pos | Yes | Yes | *NA* |
| P10 | 46/F | PAPS | 181 | 1.72 | 66 | 15 | pos | No | No | Yes |
| NHS1 | 39/F | healthy ctrl | 6 | 0.00 | 0 | 7 | neg | No | No | No |
| NHS2 | 27/F | healthy ctrl | 5 | 0.04 | 4 | 6 | neg | No | No | No |
| NHS3 | 43/F | healthy ctrl | 8 | 0.03 | 13 | 11 | neg | No | No | No |
| NHS4 | 42/F | healthy ctrl | 10 | 0.00 | 16 | 18 | neg | No | No | No |
| NHS5 | 34/F | healthy ctrl | 6 | 0.00 | 0 | 6 | neg | No | No | No |

aCL indicates anti-cardiolipin antibodies; aβ2GPI, anti-β2glycoprotein I antibodies; LA, lupus anticoagulant; AT, arterial thrombosis; VT, venous thrombosis; PM, pregnancy morbidity, as defined by Miyakis et al[1]; EM, early miscarriages; FGR, fetal growth retardation; SVT, superficial venous thrombosis; PAPS, primary antiphospholipid syndrome; aPL carrier, antiphospholipid-positive asymptomatic subject; SAPS, secondary antiphospholipid syndrome; ctrl, control; D4/5, β2GPI domains 4/5; D1, β2GPI domain 1; and NA, not applicable. *aCL IgG cut-off 20 GPL; † aβ2GPI IgG cut-off 0.170 OD; ‡Anti-β2GPI D4/5 cut-off 19 AU; and anti-β2GPI D1 cut-off 25 AU

**Supplementary Figure 1. Sequences of peptides obtained by trypsin degradation of recombinant domains 4 and 5.** The amino acid sequences of domains 4 and 5 are underlined and included in the published sequence of β2GPI.[3]

**β2glycoprotein I precursor**

1 mispvlilfs sflchvaiag rtcpkpddlp fstvvplktf yepgeeitys ckpgyvsrgg
61 mrkficpltg lwpintlkct prvcpfagil engavryttf eypntisfsc ntgfylngad
121 sakcteegkw spelpvcapi icpppsiptf atlrvykpsa gnnslyrdta vfeclpqham
181 fgndtitctt hgnwtklpec revk*cpfpsr pdngfvnypa kptlyykdka tfgchdgysl*
*241 dgpeeiectk lgnwsampsc k***asckvpvkk atvvyqgerv kiqekfkngm lhgdkvsffc**
**301 knkekkcsyt edaqcidgti evpkcfkehs slafwktdas dvkpc**

Italics: Domain 4

**Bold: Domain 5**

Underlined: identified sequence

## References


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