# Interleukin-17/Interleukin-21 and Interferon-γ producing T cells specific for β2 Glycoprotein I in atherosclerosis inflammation of systemic lupus erythematosus patients with antiphospholipid syndrome

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## Supplemental Methods

### Reagents

Human β2GPI was purified by perchloric acid treatment of pooled normal human sera obtained from blood donors followed by affinity purification on Heparin column (HiTrap Heparin HP, GE Healthcare, Milan, Italy) and by ion-exchange chromatography (Resource-S, GE Healthcare)[1]. We also ruled out the presence of contaminants by a limulus test. The human β2GPI used has been tested with a limulus test and resulted negative throughout the whole study. Human recombinant (hr) interleukin (IL)-2 and tetanus toxoid (TT) were provided by Novartis, Siena, Italy. PHA was purchased from Life Technologies (Carlsbad, CA). Fluorochrome-conjugated human monoclonal antibodies (mAbs) anti-CD3, anti-CD4, anti-CD8, anti-IFNγ and isotype-matched control mAb were purchased from BD Biosciences (San Jose, CA, USA). The fluorochrome-conjugated anti-IL-17 mAb was obtained from eBioscience (San Diego, CA, USA). PMA, ionomycin and brefeldin A were purchased from BD Biosciences (San Jose, CA, USA).

### Patients

Upon approval of the local Ethical Committee, 10 patients (10 females, mean age 51; range 42-56 years) with SLE-APS, 10 aPL negative patients (10 females, mean age 51, range 43-55), 5 SLE aPL-positive patients (5 females, mean age 49, range 44-53), and 5 SLE aPL-negative patients (5 females, mean age 50, range 44-56), all affected by carotid atherosclerotic arteriopathy were included in the study. The carotid plaques were obtained by endoarterectomy from each patient. The clinical informations of each patient are reported in Table S1, S2, S3, and S4.

All patients studied (SLE-APS, SLE aPL-positive, SLE aPL-negative and aPL negative patients) were eligible for vascular surgery. SLE-APS patients were triple positive for aPL, with high titers serum anti-β2GPI, anti-cardiolipin (aCL) antibodies and with positivity for Lupus Anticoagulant (LA). All SLE-APS patients in this study satisfied the Myiakis's criteria for APS, and they were on oral anticoagulation with vitamin K antagonists, then switched to low molecular weight heparin few days before surgery[2]. None of them displayed traditional risk factors for atherosclerosis and they were not receiving any anti-lipidemic drugs. All the SLE aPL-positive patients were affected by SLE but not by APS, although they were positive for aPL, with serum anti-β2GPI, anti-cardiolipin antibodies or with positivity for LA. All SLE aPL-neg patients were affected by SLE but not by APS, and they were triple negative for serum aPL, such as anti-β2GPI, anti-cardiolipin antibodies and with negativity for Lupus Anticoagulant.

**Anti-phospholipid antibody detection**

For the detection of aCL and aβ2GPI in patient sera, commercially available solid-phase ELISA employing purified human β2GPI in complex with CL and human β2GPI were used (Inova, Ca, USA). Sera were considered positive when their concentration exceeded the cut-off of 10 U/mL for IgG and IgM. All samples were tested by the respective in-house assay as described elsewhere[3]. The results of the two techniques were comparable.

Analysis of LA was performed in accordance with the international recommendations as described recently[4].

**Generation and characterization of T cell clones from atherosclerotic plaques' inflammatory infiltrates**

Carotid specimens, obtained by endoarterectomy, were investigated in both SLE-APS and in aPL negative patients under the same experimental conditions. Plaque fragments were cultured for 7 days in RPMI 1640 medium supplemented with IL-2 (50 units/ml) to expand *in vivo*-activated T cells. Specimens were then disrupted, and single T cells were cloned under limiting dilution, as described[5]. To assess their phenotype profile, T cell clones were screened by flow cytometry with fluorochrome-conjugated anti-CD3, anti-CD4, anti-CD8 on a BD FACSCanto II (BD Bioscience), using the FACS Diva 6.1.3. software. The repertoire of the TCR Vβ chain of β2GPI-specific Th clones was analyzed with a panel of mAbs specific to the following: Vβ1, Vβ2, Vβ4, Vβ5.1, Vβ5.2, Vβ5.3, Vβ7, Vβ8, Vβ9, Vβ11, Vβ12, Vβ13.1, Vβ13.2 and Vβ13.6, Vβ14, Vβ16, Vβ17, Vβ18, Vβ20, Vβ21.3, Vβ22, and Vβ23 (Beckman Coulter); Vβ6.7 (Gentaur) and Vβ3.1 (In Vitro Gen). Isotype-matched nonspecific Ig were used as negative control. Vβ10, Vβ15, and Vβ19 T cell receptor typing were investigated by Clontech kit, according to the manufacturer's instructions. Each β2GPI-reactive $CD4^+$ T cell clone was stained by only one of the TCR-Vβ chain–specific monoclonal antibodies, showing a single peak of fluorescence intensity (Fig. S1) .

T cell clones were then analyzed for their responsiveness to β2GPI by measuring [$^3$H]thymidine uptake after 60 h of co-culture with irradiated autologous PBMCs in the presence of medium, or β2GPI (10 nM). The mitogenic index (MI) was calculated as the ratio between mean values of counts per minute (cpm) obtained in stimulated cultures and those obtained in the presence of medium alone. MI >5 was considered as positive.

**Assessment of T cell clones cytokine profile.**

To assess the cytokine production of β2GPI-specific T cell clones upon antigen stimulation, $5 \times 10^5$ T cell blasts of each clone were co-cultured for 48 h in 0.5 ml of serum-free medium with $5 \times 10^5$ irradiated autologous PBMCs in the absence or presence of β2GPI (10 nM). At the end of the

culture period, duplicate samples of each supernatant were assayed for their IFN-γ, TNF-α, IL-4, IL-21 and IL-17 (BioSource International, Camarillo, CA) production by ELISA[5]. For further investigation, T cell blasts from each β2GPI-specific T cell clone were stimulated with medium or β2GPI (10 nM) in the presence of autologous APCs for 48 h in ELISPOT microplates coated with anti-IFN-γ or anti-IL-17 antibody, respectively (eBioscience, Inc., San Diego, Ca, USA). At the end of culture period, the number of IFN-γ and IL-17 SFCs were counted as described[5].

**T cell clone-mediated cytotoxicity and Fas-Fas Ligand (L) mediated proapoptotic activity.**

T cell clones cytolytic activity was assessed as reported[5]. T cell blasts of β2GPI-specific T cell clones were incubated at ratios of 10, 5, and 2.5 to 1 with $^{51}$Cr-labeled autologous Epstein-Barr virus transformed (EBV)-B cells pre-incubated with β2GPI (10 nM) or medium alone. After centrifugation, microplates were incubated for 8 h at 37° C, and 0.1 ml of supernatant was removed for the measurement of $^{51}$Cr release, as reported[16]. The ability of β2GPI-specific T cell clones to induce Fas-FasL mediated apoptosis was assessed using Fas$^{+}$ Jurkat cells as target. T cell blasts from each clone were co-cultured with $^{51}$Cr-labeled Jurkat cells at an effector/target (E:T) ratio of 10, 5, and 2.5 to 1 for 18 h in the presence of PMA (10 ng/ml) and ionomycin (1 μmol/ml), as reported[5].

**T helper assay to assess their ability to induce Tissue Factor (TF) production and procoagulant activity (PCA) in autologus monocytes.**

T cell blasts ($8 \times 10^5$ / ml) of β2GPI-specific T cell clones were co-cultured for 16 hrs with autologous monocytes ($4 \times 10^5$ / ml) in the presence of serum-free medium or β2GPI (10 nM). At the end of the culture period, the amount of TF protein was quantitated by a specific ELISA (American

Diagnostica, Greenwich, CT) in duplicate samples of supernatants obtained from cell suspensions after solubilization of membrane proteins with Triton X-100 and ultracentrifugation, as reported[5]. At the end of culture period, cell suspensions consisting of monocytes alone, or monocytes plus activated T cells were disrupted by repeated freezing and thawing followed by sonication. Total cellular content of PCA was determined in a one-stage clotting assay and expressed in arbitrary units ($U/10^5$ monocytes) assigned by comparison with a standard curve derived from rabbit brain thromboplastin standard (Manchester Comparative Reagents, Manchester, UK), as reported[5]. Our log-log plot was linear up to 200 seconds clotting time. Values less than 10 $U/10^5$ monocytes corresponded to clotting times ranging from 170 to 80 seconds. One thousand units corresponded to approximately 22 seconds clotting time. PCA was characterized as factor VII-dependent procoagulant activity by evaluating its sensitivity to phospholipase C (Calbiochem, San Diego, CA), concanavalin A, and cysteine protease inhibitor (HgCI2), and by using factor VII- and factor X-deficient plasma samples[6].

**T cell clones' helper assay to evaluate the induction of immunoglobulin (Ig) production by autologous B cell**

T cell blasts of each clone were co-cultured at ratios of 0.2, 1, and 5 to 1 with autologous PBMCs in the absence or presence of β2GPI and, on day 10, IgM, IgG, and IgA levels in cell free culture supernatants were measured as previously described[7].

**Statistical analysis**

Statistical analyses were performed using Student's *t* test; data were considered significant if p values $\geq 0.05$.

**Study approval**

Prior written informed consent was received from SLE patients and controls according to the Helsinki Declaration. Experiments were approved by the local Ethics Committee.

**Supplemental references**


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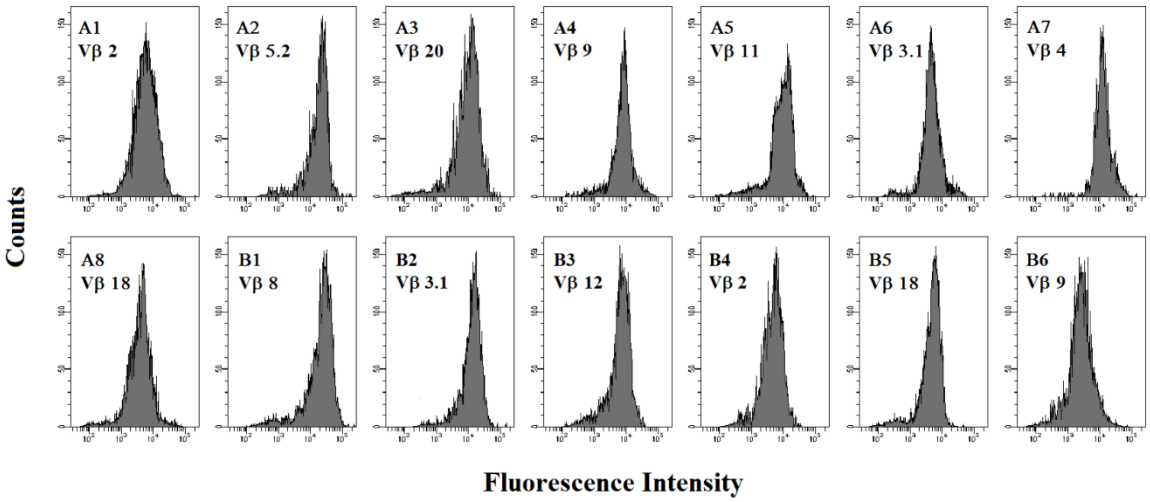
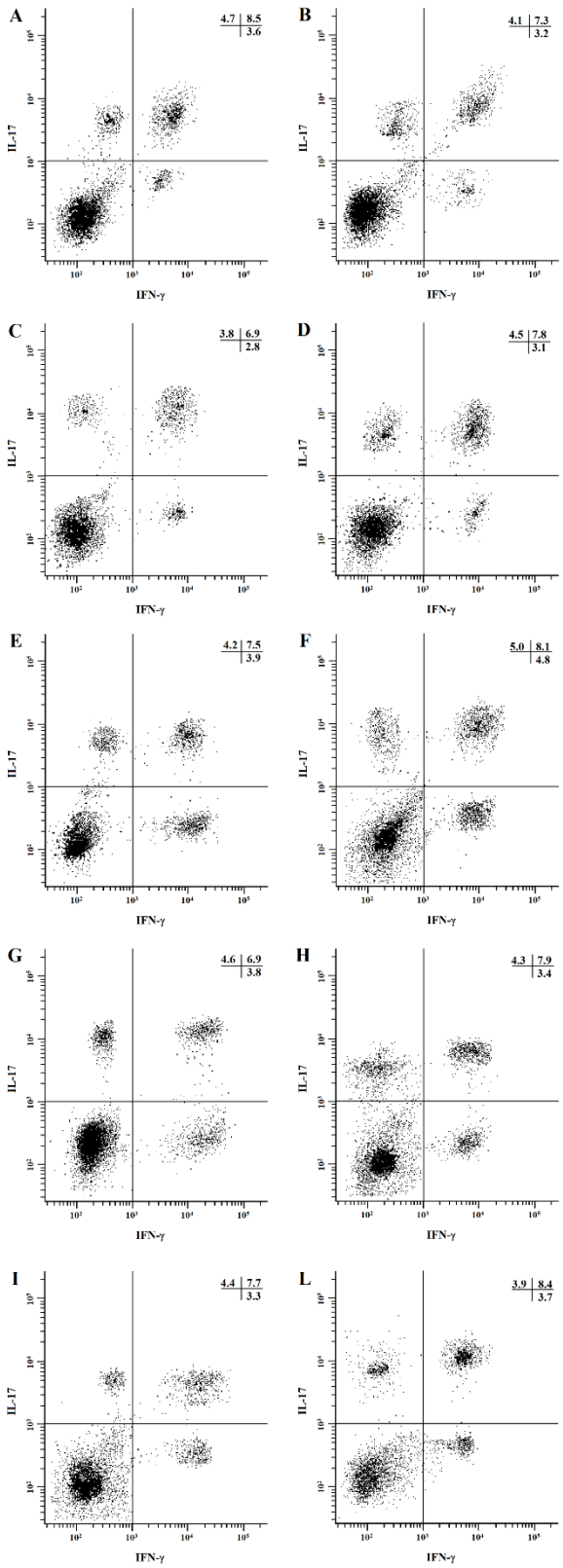
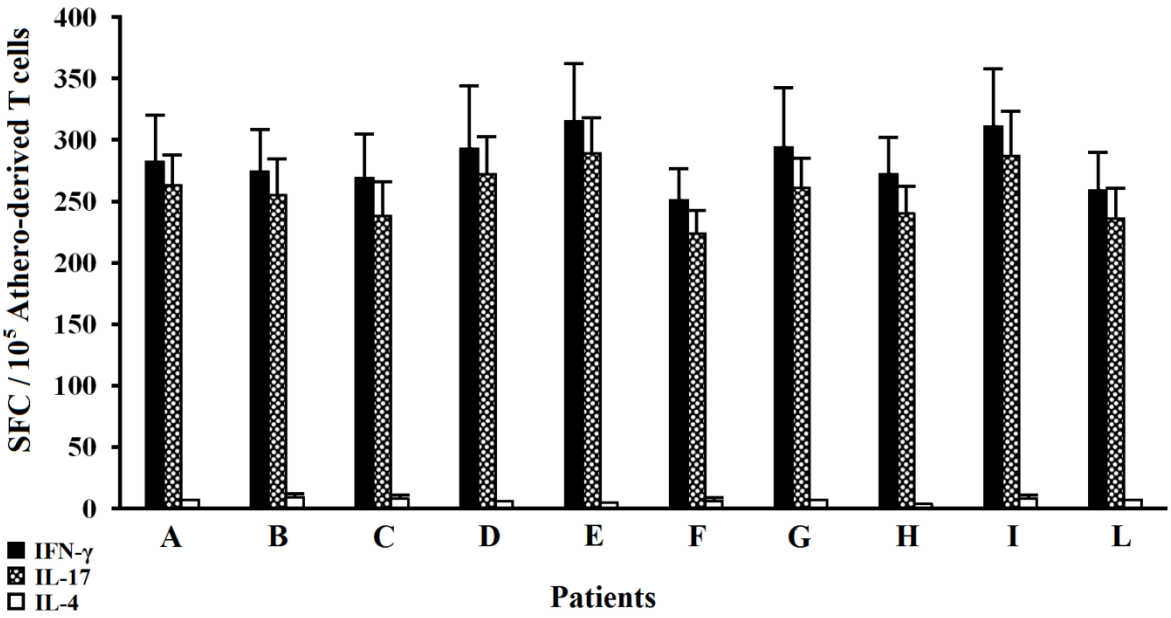



**Supplemental Figure S1**




**Supplemental Figure S2**




**Supplemental Figure S3**

**Legends to Supplementary figures**

**Figure S1. TCR Vβ chain repertoire of β2GPI-specific T cell clones derived from the atherosclerotique plaques of SLE-APS patients**. The cloality of T cell clones specific for β2GPI was analysed by a panel of monoclonal antibodies specific for human TCR Vβ families, as detailed in Material and Methods. T cell blasts from each clone were divided in aliquots and stained with each of the monolonaly antibody and the appropriate controls. All clones obtained from two representative patients, named "A" and "B", are represented in the figure.

**Figure S2. IFN-γ and IL-17 intra-cellular cytokine staining of plaque-infiltrating T cells of SLE-APS patients.** Cells were stained for surface and intracellular markers with the following mAbs for flow cytometry: anti-CD4-PerCP, anti-IL-17-PE, and anti-IFN-γ-FITC (Becton Dickinson). Samples obtained from the atherosclerotic lesions of each of the SLE-APS patients were stimulated with PMA (25 ng/ml) plus 1 μg/ml ionomycin in the presence of brefeldin A (1 μg/ml). The analysis was performed using FACS Canto II (BD), by the acquisition software FACSDiva 6.1.3. For each sample 5000 events were acquired. Dot plots expression of IFN-$\gamma^+$ and IL-$17^+$ on $CD4^+$ T cells of the 10 T-cell lines obtained from the 10 SLE-APS patients (named A, B, C, D, E, F, G, H, I, L) are shown.

**Figure S3. β2GPI driven IFN-γ and IL-17 secretion by β2GPI-specific atherosclerotic plaque derived T-cells from SLE-APS patients.** Atherosclerotic plaque-derived T-cell lines were expanded from SLE-APS patients by addition of IL-2. At day 7, T-cell blasts from each line were stimulated for 48 h with β2GPI or medium, in the presence of irradiated autologous APCs in ELISPOT microplates coated with anti-IFN-γ, anti-IL-17, or anti-IL-4 antibodies. After specific stimulation with β2GPI, a significant proportion of SLE-APS atherosclerotic plaque-derived Th cells produced IL-17 and IFN-γ, but not IL-4. Values are the mean ± SD number of SFCs per $10^5$ cells over background levels.

| Patients | Age | Sex | Treatment | SLEDAI | Autoantibodies | Total clones | Total No. of $CD8^+$ clones | No. of $CD8^+$ clones reactive to β2GPI | Total No. of $CD4^+$ clones | No. of $CD4^+$ clones reactive to β2GPI |
|---|---|---|---|---|---|---|---|---|---|---|
| A | 42 | F | a+b | 7 | c+d+e | 38 | 5 | 0 | 33 | 8 |
| B | 56 | F | a+b | 8 | c+d+e+f | 34 | 4 | 0 | 30 | 6 |
| C | 51 | F | b | 7 | c+d | 33 | 6 | 0 | 27 | 6 |
| D | 50 | F | b | 5 | e | 31 | 1 | 0 | 30 | 8 |
| E | 50 | F | b | 8 | c+d+e | 32 | 4 | 0 | 28 | 7 |
| F | 54 | F | a+b | 6 | c+d+g | 34 | 3 | 0 | 31 | 7 |
| G | 55 | F | b | 7 | c+d-h | 35 | 3 | 0 | 32 | 8 |
| H | 48 | F | a+b | 7 | c+d+e | 30 | 3 | 0 | 27 | 7 |
| I | 49 | F | b | 8 | c+d+e+h | 36 | 4 | 0 | 32 | 8 |
| L | 55 | F | a+b | 6 | c+d | 31 | 4 | 0 | 27 | 6 |

**Table S1. Clinical and lab information of the 10 SLE-APS patients.** All the 10 patients shown in this table were affected by SLE-APS and were triple positive for aPL, with high titers serum anti-β2GPI, anti-cardiolipin antibodies and with positivity for Lupus Anticoagulant. a: treated with hydroxychloroquine; b: treated with glucocorticoids; c: ANA-positive; d: anti-SSA-positive; e: anti-ds DNA-positive; f: anti-SSB-positive; g: anti-U1 RNP-positive; h: anti-Sm-positive.

| Patients | Age | Sex | Treatment | SLEDAI | Autoantibodies | Total clones | Total No. of $CD8^+$ clones | No. of $CD8^+$ clones reactive to β2GPI | Total No. of $CD4^+$ clones | No. of $CD4^+$ clones reactive to β2GPI |
|---|---|---|---|---|---|---|---|---|---|---|
| M | 43 | F | none | 0 | none | 39 | 5 | 0 | 36 | 0 |
| N | 55 | F | none | 0 | none | 37 | 4 | 0 | 33 | 0 |
| O | 51 | F | none | 0 | none | 29 | 7 | 0 | 22 | 0 |
| P | 50 | F | none | 0 | none | 28 | 4 | 0 | 24 | 0 |
| Q | 50 | F | none | 0 | none | 36 | 4 | 0 | 32 | 0 |
| R | 54 | F | none | 0 | none | 31 | 3 | 0 | 28 | 0 |
| S | 55 | F | none | 0 | none | 38 | 5 | 0 | 33 | 0 |
| T | 48 | F | none | 0 | none | 26 | 3 | 0 | 23 | 0 |
| U | 49 | F | none | 0 | none | 34 | 5 | 0 | 27 | 0 |
| V | 55 | F | none | 0 | none | 32 | 2 | 0 | 30 | 0 |

**Table S2. Clinical and lab information of the 10 aPL-neg patients.** All the 10 patients shown in this table were affected by carotid atherosclerotic arteriopathy but not by SLE-APS and were triple negative for serum aPL, such as anti-β2GPI, anti-cardiolipin antibodies and with negativity for Lupus Anticoagulant, and they were all negative for any autoantibody.They were not treated with any steroids, nor other immune-suppressants.

| Patients | Age | Sex | Treatment | SLEDAI | Autoantibodies | Total clones | Total No. of $CD8^+$ clones | No. of $CD8^+$ clones reactive to β2GPI | Total No. of $CD4^+$ clones | No. of $CD4^+$ clones reactive to β2GPI |
|---|---|---|---|---|---|---|---|---|---|---|
| Y | 44 | F | a+b | 6 | c+d | 32 | 4 | 0 | 28 | 4 |
| YA | 53 | F | b | 9 | c+e | 28 | 3 | 0 | 24 | 3 |
| YB | 52 | F | b | 8 | c+d+e | 25 | 5 | 0 | 20 | 5 |
| YC | 48 | F | a+b | 7 | c+d | 34 | 3 | 0 | 31 | 6 |
| YD | 49 | F | a+b | 5 | c+d+e+f | 38 | 6 | 0 | 32 | 7 |

**Table S3. Clinical and lab information of the 5 SLE aPL-pos patients.** All the 5 patients shown in this table were affected by SLE but not by APS, although they were positive for aPL, with serum anti-β2GPI, anti-cardiolipin antibodies or with positivity for Lupus Anticoagulant. a: treated with hydroxychloroquine; b: treated with glucocorticoids; c: ANA-positive; d: anti-SSA-positive; e: anti-ds DNA-positive; f: anti-SSB-positive; g: anti-U1 RNP-positive; h: anti-Sm-positive.

| Patients | Age | Sex | Treatment | SLEDAI | Autoantibodies | Total clones | Total No. of $CD8^+$ clones | No. of $CD8^+$ clones reactive to β2GPI | Total No. of $CD4^+$ clones | No. of $CD4^+$ clones reactive to β2GPI |
|---|---|---|---|---|---|---|---|---|---|---|
| Z | 52 | F | b | 7 | c+d+e | 29 | 5 | 0 | 26 | 0 |
| ZA | 49 | F | a+b | 9 | c+e+g | 34 | 4 | 0 | 28 | 0 |
| ZB | 51 | F | a+b | 8 | c+e+h | 31 | 7 | 0 | 24 | 0 |
| ZC | 44 | F | b | 6 | c+h+g | 39 | 6 | 0 | 33 | 0 |
| ZD | 56 | F | b | 7 | c+e+h | 33 | 8 | 0 | 25 | 0 |

**Table S4. Clinical and lab information of the 5 SLE aPL-neg patients.** All the 5 patients shown in this table were affected by SLE but not by APS, and they were triple negative for serum aPL, such as anti-β2GPI, anti-cardiolipin antibodies and with negativity for Lupus Anticoagulant. a: treated with hydroxychloroquine; b: treated with glucocorticoids; c: ANA-positive; d: anti-SSA-positive; e: anti-ds DNA-positive; f: anti-SSB-positive; g: anti-U1 RNP-positive; h: anti-Sm-positive.