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## **The $\beta$ 2-glycoprotein I/HLA-DR complex is the major autoantibody target in obstetric antiphospholipid syndrome**

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### **Potential Conflicts of Interest**

KT and HA have applied a related patent (WO2015008498). HA is a stockholder of Hula Immune Inc.

## **ABSTRACT**

### **Objectives**

The clinical manifestations of antiphospholipid syndrome (APS) include vascular thrombosis and pregnancy morbidity, including recurrent pregnancy loss (RPL). However, more than half of RPL patients never determine the cause of their RPL. Recently,  $\beta$ 2-glycoprotein I ( $\beta$ 2GPI) complexed with human leukocyte antigen class II molecules ( $\beta$ 2GPI/HLA-II) was found to be a major autoantibody target in APS. In this study, autoantibodies against the  $\beta$ 2GPI/HLA-II complex were analyzed in women with RPL, which is a clinical symptom of obstetric APS.

### **Methods**

Serum levels of antiphospholipid antibodies (aPL), including immunoglobulin (Ig) G/IgM anti-cardiolipin antibody, IgG/IgM anti- $\beta$ 2GPI antibody, and lupus anticoagulant, as well as anti- $\beta$ 2GPI/HLA-DR antibodies were measured in 227 women with RPL. In this prospective, multicenter, cross-sectional study, women with RPL and their partners underwent HLA-DR immunotyping and analysis to identify potential causes and risk factors associated with RPL. The normal range for anti- $\beta$ 2GPI/HLA-DR antibody levels was obtained from 208 fertile women used as controls.

### **Results**

aPL was detected in 19.8% of women with RPL. Fifty-two out of the 227 (22.9%) women with RPL tested positive for anti- $\beta$ 2GPI/HLA-DR antibodies, and 24 out of the 121 (19.8%) women with unexplained RPL were positive for anti- $\beta$ 2GPI/HLA-DR antibodies. Twenty-one out of 112 (18.8%) women, who had clinical symptoms of APS but not aPL of criteria, tested positive for anti- $\beta$ 2GPI/HLA-DR antibodies.

### **Conclusions**

The anti- $\beta$ 2GPI/HLA-DR antibody is frequently associated with RPL. Detection of these autoantibodies is useful in understanding the pathogenesis of RPL. Our findings will

potentially provide new therapeutic modalities for patients with obstetric APS.

**Keywords:**

antiphospholipid syndrome, autoantibody,  $\beta$ 2-glycoprotein I, HLA class II, recurrent pregnancy loss

## INTRODUCTION

The clinical manifestations of antiphospholipid syndrome (APS) are vascular thrombosis and pregnancy morbidity.(1) Unexplained fetal death beyond 10 gestational weeks (GW), premature births of normal neonates before 34 GW due to eclampsia, severe eclampsia or recognized features of placental insufficiency, and recurrent pregnancy loss (RPL) comprise pregnancy morbidity as defined in the revised Sapporo classification of APS.(1) The laboratory criteria for APS include antiphospholipid antibodies (aPL), lupus anticoagulant (LA), anti-cardiolipin antibody (aCL) and anti- $\beta$ 2-glycoprotein I antibody (a $\beta$ 2GPI).(1-4) aPL mainly targets  $\beta$ 2GPI, a phospholipid-binding molecule.(5, 6) It is thought that circular  $\beta$ 2GPI is converted to linear  $\beta$ 2GPI, exposing the major epitope for aPL when  $\beta$ 2GPI binds to anionic phospholipids.(7) aPL is usually detected by enzyme-linked immunosorbent assay (ELISA) methods, using plates containing solid-phase negatively charged phospholipids or plates with  $\beta$ 2GPI.(8, 9)

However, autoantibodies against  $\beta$ 2GPI bound to negatively charged phospholipids or plates are detected in less than half of patients with clinical manifestations of APS.(2-4) Additionally,  $\beta$ 2GPI is secreted from the cell and is usually not expressed on the cell surface; how aPL binds to vascular endothelial cells to induce thrombosis and/or pregnancy morbidity remains unclear.

A series of studies have demonstrated that misfolded proteins generated in the endoplasmic reticulum (ER), generally eliminated by ER-associated degradation, can be rescued from degradation and transported to the cell surface without being processed into peptides while associated with the peptide-binding groove of human leukocyte antigen (HLA) class II molecules in the ER.(10) In addition, misfolded proteins complexed with HLA class II molecules of disease-susceptible alleles have been found to be involved in the pathogenesis of several autoimmune diseases, serving as autoantibody targets. Immunoglobulin G (IgG) heavy chain/HLA-DR complexes in patients with rheumatoid

arthritis, myeloperoxidase/HLA-DR complexes in patients with microscopic polyangiitis, and  $\beta$ 2GPI/HLA-DR complexes in patients with APS were found to be major targets for autoantibodies.(11-13) The previous study used stored sera of 120 patients with APS, and the majority of them had a history of vascular thrombosis. The study found that 83% of the 120 patients had autoantibodies that targeted  $\beta$ 2GPI/HLA-DR complexes.(13) Approximately 50% of the APS patients with autoantibodies targeting  $\beta$ 2GPI/HLA-DR complexes tested negative for IgG aCL or IgG a $\beta$ 2GPI.(13) A recent study also found that 27% of 111 aPL-negative patients with idiopathic, chronic limb ulcers had autoantibodies targeting  $\beta$ 2GPI/HLA-DR complexes.(14) These results suggest that autoantibodies targeting  $\beta$ 2GPI/HLA-DR complexes are associated with APS manifestations, even in patients who lack aPL of criteria.

RPL is defined as the loss of two or more pregnancies(15, 16) and has a prevalence of 0.8%–1.4% of the population.(17) RPL dramatically impacts countries with declining birthrates and increasing aging populations, such as certain Western countries and Japan. The causes of RPL in more than half of patients are unknown.(18-20) RPL is one of the clinical manifestations of APS and, furthermore, we have found that the  $\beta$ 2GPI/HLA class II complexes existed in the vascular endothelial cells of placental decidual tissue obtained from APS women with spontaneous miscarriages.(13) Therefore, we hypothesize that the novel autoantibody targeting  $\beta$ 2GPI/HLA-DR complexes may be involved in the pathogenesis of not only vascular thrombosis but also RPL. We evaluated whether fertile women and women with RPL have autoantibodies targeting  $\beta$ 2GPI/HLA-DR complexes and extensively addressed whether women with unexplained RPL possess the novel autoantibodies in a prospective, multicenter, and cross-sectional study.

## **PATIENTS AND METHODS**

This study on RPL patients was approved by the institutional review boards of five medical centers (reference number 1566 at Kobe University Hospital), and all participants enrolled after providing written informed consent. In this study, RPL is defined as the occurrence of two or more consecutive pregnancy losses according to the definition of American Society for Reproductive Medicine.(15, 16) When the dead fetus had crown-rump length (CRL) exceeding the average CRL of 10 gestational weeks (GW), pregnancy losses at 10 or more GW were diagnosed. Women with RPL who visited one of the five centers between December 2016 and November 2018 underwent serum measurements for autoantibodies against the  $\beta$ 2GPI/HLA-DR complex (anti- $\beta$ 2GPI/HLA-DR antibody) and HLA-DR typing, as well as conventional assessments to identify cause and risk factors for RPL. Partners of the participating women also underwent HLA-DR typing. To establish normal range and cut-off value, serum levels of anti- $\beta$ 2GPI/HLA-DR antibody in 208 healthy, fertile women were measured, to be used as a control. All of the control population had a history of normal term delivery and no pregnancy losses or autoimmune diseases. The serum samples of fertile, control women were purchased from the National Center for Child Health and Development (NCCHD) BioBank (Tokyo, Japan). The serum samples of the control population were collected from women, who had already been known to be negative for all aPL of criteria, from July 2014 to July 2017 at the NCCHD. The samples were immediately frozen just after blood collection and stored at  $-80^{\circ}\text{C}$  until use for measuring anti- $\beta$ 2GPI/HLA-DR antibody. The association of anti- $\beta$ 2GPI/HLA-DR antibody with causes and risk factors of RPL were then assessed.

### **Methods for measuring serum levels of autoantibodies targeting the $\beta$ 2GPI/HLA-DR complex**

To analyze autoantibody levels specific to the  $\beta$ 2GPI/HLA-DR complex and not HLA-DR



alone, we modified a previously described method to determine anti- $\beta$ 2GPI/HLA-DR antibody levels.(13) Complementary DNA (cDNA) was prepared from pooled human peripheral blood mononuclear cells (3H Biomedical, Uppsala, Sweden) and cloned into pME18S or pCAGGS expression vectors. 293T cells were transiently transfected using Polyethylenimine Max (Polyscience, Illinois, USA). GFP-labeled cells expressing the  $\beta$ 2GPI/HLA-DR complex and DsRed labeled cells expressing HLA-DR were generated by transfecting GFP,  $\beta$ 2GPI, HLA-DRA\*01:01, and DRB1\*07:01, or DsRed, HLA-DRA\*01:01, and DRB1\*07:01 into 293T cells, respectively. A single lot of  $\beta$ 2GPI/HLA-DR transfected and HLA-DR transfected cells were aliquoted ( $3 \times 10^6$  cells per tube) with 500 $\mu$ l of the cryoprotectant medium (90% fetal bovine serum and 10% dimethyl sulfoxide), and stored at  $-80^{\circ}\text{C}$  until use. A serum sample from a woman with RPL in which anti- $\beta$ 2GPI/HLA-DR antibody was detectable after a  $10^6$ -fold dilution was used as a standard throughout this study. The anti- $\beta$ 2GPI/HLA-DR antibody levels of a standard serum was defined as 1,000 anti- $\beta$ 2GPI/HLA-DR antibody units (ABH-U).

The mean fluorescence intensity (MFI) of IgG binding to transfected cells in the sample sera were analyzed with flow cytometry. Specific IgG binding to the  $\beta$ 2GPI/HLA-DR complex was calculated by subtracting the MFI of IgG binding to cells transfected with HLA-DR alone from cells transfected with both  $\beta$ 2GPI and HLA-DR (Supplemental figure 1).

Anti- $\beta$ 2GPI/HLA-DR antibody levels in each serum sample were calculated from the standard curve generated by measuring specific IgG binding to the  $\beta$ 2GPI/HLA-DR complex in serially diluted standard sera. All measurements were performed in duplicate, and the mean values were defined as the anti- $\beta$ 2GPI/HLA-DR antibody level of the sample.

### **Clinical checkups to identify causes and risk factors for RPL**

All women with RPL enrolled in this study attended checkups to identify causes and risk

factors for RPL. Ultrasound examinations were performed to detect uterine malformations, including septate uteri, bicorporeal uteri, and uterine myomas that deform the uterine cavity. Magnetic resonance imaging (MRI) or hysterosalpingography were also performed if necessary. The serum levels of thyroid stimulating hormone (TSH) (normal: 0.449–3.809 mIU/mL) and free thyroxine (normal: 0.82–1.22 ng/dL) were measured using an Abbott ARCHITECT i2000SR Analyzer (Abbott Japan, Tokyo, Japan). Hyperthyroidism and hypothyroidism were diagnosed by the serum TSH levels of  $<0.4$  mIU/mL and  $\geq 2.5$  mIU/mL, respectively. Measurements of serum IgG aCL levels (normal:  $<10$  U/ml), IgM aCL levels (normal:  $<8$  U/mL),  $\beta$ 2GPI-dependent anti-cardiolipin antibody (aCL/ $\beta$ 2GPI) levels (normal:  $<1.8$  U/ml), and tests for LA based on diluted Russell's viper venom time (normal:  $<1.3$  Normalized ratio) were performed (Special References Laboratories, Tokyo, Japan). IgG a $\beta$ 2GPI levels (normal:  $<20$  U/ml) and IgM a $\beta$ 2GPI levels (normal:  $<20$  U/mL) were measured using a Quanta Flash Anti-phospholipid Assay Panel (Inova Diagnostics, CA, USA). In this study, the 99th percentile cutoff values of healthy controls established by diagnostic laboratories were used as an upper limit of normal range for each aPL, and aPL positivity was determined by repeated tests conducted 12 or more weeks apart. Hemostatic molecular markers, protein S (PS) activity (normal: 60%–150%), protein C (PC) activity (normal: 67%–127%), and coagulation factor XII (normal: 50%–150%) were also measured. Fresh blood specimens were used for testing aPL and hemostatic molecular markers. The serum samples for measuring anti- $\beta$ 2GPI/HLA-DR antibody levels, which were simultaneously collected with blood specimens for other tests, were immediately frozen just after blood collection and stored at  $-80^{\circ}\text{C}$  until use.

Women with RPL and their partners underwent chromosomal karyotyping of peripheral blood (Special References Laboratories, Tokyo, Japan), and HLA-DR typing using Luminex-based HLA class II single-antigen bead assays (HLA Laboratory, Kyoto, Japan).

## Statistics

Clinical characteristics were compared between women with RPL and fertile, control women. Differences between the two groups were analyzed using the Student *t* test, the Mann–Whitney U test, the chi-square test, and the Fisher exact test with a Bonferroni correction applied for multiple comparisons. Statistical significance was set at *P* value <0.05. All statistical analyses were performed using SPSS software, version 19 (SPSS Inc, Chicago, Illinois).

## RESULTS

### The study population

During the study period, 227 couples who experienced RPL were enrolled. One hundred seventeen couples were enrolled at Kobe University Hospital; 71 at University of Toyama Hospital; 26 at Okayama University Hospital; 10 at the University of Tokyo Hospital; and three at Hyogo College of Medicine College Hospital. One hundred eighty-five women with RPL (81.5%) had only histories of two or more pregnancy losses before 10 GW, and 11 (4.8%) had only histories of two or more pregnancy losses at 10 or more GW. Serum samples from 208 healthy fertile control women were purchased.

The age ( $p<0.05$ ) and parity ( $p<0.0001$ ) among women with RPL was significantly lower than among control women (**Table 1**). Gravidity ( $p<0.0001$ ), the number of spontaneous miscarriages ( $p<0.0001$ ), the number of stillbirths at 22 or more GW ( $p<0.001$ ), and the percentage of women who had a history of stillbirth at 22 or more GW ( $p<0.001$ ) in women with RPL was significantly higher than in fertile control women. However, this difference is not believed to significantly influence study results.

### The distribution of serum levels of anti- $\beta$ 2GPI/HLA-DR antibody in fertile control women

To establish the cut-off value for the anti- $\beta$ 2GPI/HLA-DR antibody, serum levels of autoantibody in 208 healthy fertile control women were measured as described in **Patients and Methods**. The normal range of serum anti- $\beta$ 2GPI/HLA-DR antibody levels ( $<52.6$  ABH-U) was set at the 99th percentile for the control women (**Figure 1**).

### Anti- $\beta$ 2GPI/HLA-DR antibody in women with RPL

To assess whether anti- $\beta$ 2GPI/HLA-II antibody is involved in the pathogenesis of RPL, the serum levels of anti- $\beta$ 2GPI/HLA-DR antibody in the 227 women with RPL were measured.

The median serum level of anti- $\beta$ 2GPI/HLA-DR antibody in women with RPL was 21.0 ABH-U (range, 0–1,952 ABH-U); significantly higher than the level in control women (2.9 ABH-U [0–308.2 ABH-U]) ( $p<0.00005$ ) (**Figure 2**). In total, 52 of the 227 (22.9%) women with RPL tested positive for anti- $\beta$ 2GPI/HLA-DR antibody.

The proportion of women with RPL tested positive for anti- $\beta$ 2GPI/HLA-DR antibody in 11 women with only histories of two or more pregnancy losses at 10 or more GW (54.5%) was significantly higher than that in 185 women with only histories of pregnancy losses before 10 GW (21.1%,  $p<0.05$ ); and tended to be higher than that in 31 women who experienced both pregnancy losses before 10 GW and at 10 or more GW (22.6%,  $p=0.07$ ).

Next, we assessed the relationship between anti- $\beta$ 2GPI/HLA-DR antibody and aPL (**Figure 3 and Supplemental table 1**). Forty-five of the 227 (19.8%) women with RPL possessed at least one of the five aPL of criteria, and positive rates of each aPL in this study (positive,  $\geq$  the 99th percentile of healthy controls) were as follows: IgG aCL ( $\geq 10$  U/ml), 8.8%; IgM aCL ( $\geq 8$  U/ml), 6.2%; IgG a $\beta$ 2GPI ( $\geq 20$  U/ml), 3.1%; IgM a $\beta$ 2GPI ( $\geq 20$  U/ml), 1.3%; LA ( $\geq 1.3$  Normalized ratio), 2.6%, and IgG aCL $\beta$ 2GPI, 1.3% ( $\geq 1.8$  U/ml). The positive rate of anti- $\beta$ 2GPI/HLA-DR antibody (22.9%) was substantially higher than that of each aPL of criteria. Of note, a total of 35 of the 52 (67.3%) RPL patients who were positive for anti- $\beta$ 2GPI/HLA-DR antibody displayed no aPL of criteria (**Figure 3**). On the other hand, there was no significant difference in the serum levels of anti- $\beta$ 2GPI/HLA-DR antibody between aPL-positive women with RPL ( $n=45$ ) and aPL-negative women with RPL ( $n=182$ ) (median [range], 29.4 [0–927.5] vs 20.4 [0–1952.0] ABH-U,  $p=0.12$ ) (**Supplemental figure 2**).

In addition, all three women with RPL who had double or triple positivity for aPL of criteria also tested positive for anti- $\beta$ 2GPI/HLA-DR antibody, and two women with triple positivity had anti- $\beta$ 2GPI/HLA-DR antibody levels of more than 100 ABH-U (**Supplemental table 1**). One woman with triple positivity for aPL of criteria and a high anti- $\beta$ 2GPI/HLA-DR antibody serum level (927.5 ABH-U), had a history of two miscarriages before 10 GW

and termination of pregnancy due to life-threatening HELLP (hemolysis, elevated liver enzymes, and low platelets) syndrome at 14 GW. Another woman with triple positivity for aPL of criteria and an anti- $\beta$ 2GPI/HLA-DR antibody level of 330.7 ABH-U, had a history of thromboembolism with cerebral infarction and two fetal deaths beyond 10 GW.

The association between anti- $\beta$ 2GPI/HLA-DR antibody and other commonly accepted risk factors of RPL may be reflected in causes and risk factors for RPL (**Figure 4**). Details of risk factors for 33 women with multiple RPL risk factors are shown in **Supplemental table 2**. The incidence of risk factors for all 227 women with RPL were: uterine malformation, 8.4%; thyroid dysfunction, 6.6%; chromosome karyotype abnormality (male and female), 4.8%; positive for aPL, 19.8%; and unexplained, 53.3%. Anti- $\beta$ 2GPI/HLA-DR antibody was most frequently detected in 22.9% of women with RPL. Further, proportions of women who tested positive for anti- $\beta$ 2GPI/HLA-DR antibody by risk factor were: uterine malformation, 26.3% (5/19); thyroid dysfunction, 26.7% (4/15); chromosome karyotype abnormality of couples, 18.2% (2/11); positive aPL, 37.8% (17/45); low factor XII activity, 28.6% (6/21); low PS activity, 20% (7/35); and low PC activity, 25% (1/4). In total, 121 of the 227 (53.3%) women with RPL had no risk factors, and 24 of the 121 (19.8%) women with unexplained RPL tested positive for anti- $\beta$ 2GPI/HLA-DR antibody. There was no significant difference in proportion of women who tested positive for anti- $\beta$ 2GPI/HLA-DR antibody among groups of each risk factor for RPL.

#### **Allele frequencies in couples with RPL**

To assess whether HLA-DR4, HLA-DR7, and HLA-DR 13 susceptible alleles for APS (21-24), are also associated with RPL, HLA-DR alleles of the couples were prospectively typed. HLA-DRB1 allele frequencies in fertile control women and couples with RPL are displayed in **table 2**. Frequencies of HLA-DR4 in the 52 women with RPL who tested positive for anti- $\beta$ 2GPI/HLA-DR antibody were significantly higher than in 175 women with RPL who tested

negative (31.5% vs 21.1%,  $p<0.05$ ), but frequencies of HLA-DR7 and HLA-DR13 were not significantly different between the two groups. Conversely, frequencies of HLA-DR4 among the 52 partners of women with RPL who tested positive for anti- $\beta$ 2GPI/HLA-DR antibody were significantly lower than in the 175 partners of women with RPL who tested negative (18.0% vs 31.7%,  $p<0.05$ ). Allele frequencies of HLA-DR7 and HLA-DR13 were not significantly different between the two groups.

The proportion of women who tested positive for anti- $\beta$ 2GPI/HLA-DR antibody in couples whose women had HLA-DR4 and partners had no HLA-DR4 was higher than that in couples whose women had no HLA-DR4 and partners had HLA-DR4 (37.3% vs 15.5%,  $p<0.01$ ), and higher than that in couples whose women and partners both had no HLA-DR4 (37.3% vs 19.4%,  $p<0.05$ ).

## DISCUSSION

Several clinical factors are known to be involved in pathophysiology of RPL. Frequencies of causes and risk factors for RPL are reported as: uterine malformation, 1.8%–27.0%; thyroid dysfunction, 1.7%–9.5%; chromosome karyotype abnormality of couples, 2.6%–7.7%; positive aPL, 8.7%–20.3%; and unexplained, >50%.(18, 19, 25-27). In the present prospective, multicenter, cross-sectional study, frequencies of causes and risk factors for RPL were almost identical to the previous reports, and 121 (53.3%) of 227 women with RPL had unexplained etiology, who did not have commonly accepted risk factors for RPL. This study demonstrated for the first time that 52 (22.9%) women with RPL tested positive ( $\geq 52.6$  ABH-U) for autoantibodies against the  $\beta$ 2GPI/HLA-DR complex, and suggested that anti- $\beta$ 2GPI/HLA-DR antibody may be the most frequently detected biomarker for RPL. Further, 24 (19.8%) of the 121 women with unexplained RPL tested positive for anti- $\beta$ 2GPI/HLA-DR antibody. Anti- $\beta$ 2GPI/HLA-DR antibody may thus be involved in the pathogenesis of RPL, and may be a major risk factor for RPL independently of other risk factors.

Anti- $\beta$ 2GPI/HLA-DR antibody coexisted with aPL of criteria as well as other risk factors. A previous study suggested that anti- $\beta$ 2GPI/HLA-DR antibody may damage vascular endothelial cells of the placental decidua (13). Therefore, anti- $\beta$ 2GPI/HLA-DR antibody may promote RPL in combination with other risk factors, such as thrombogenesis caused by aPL, low factor XII activity, low PS activity, and low PC activity, and impaired placentation due to thyroid dysfunction.

In the present study, 139 (61.2%) of 227 women with RPL had clinical manifestations of APS, including vascular thrombosis (n=1); three or more unexplained miscarriages before 10 GW (n=102); one or more unexplained fetal death beyond 10 GW (n=34), and premature births before 34 GW due to hypertensive disorders of pregnancy (HDP), fetal growth restriction (FGR), or recognized features of placental insufficiency (n=3). No aPL of criteria was positive in 112 (80.6%) of the 139 women with clinical manifestations



of APS. However, 21 (18.8%) of the 112 women were found to have anti- $\beta$ 2GPI/HLA-DR antibody, suggesting that measurement of anti- $\beta$ 2GPI/HLA-DR antibody may be more sensitive than aPL of criteria for diagnosing APS. There was no significant difference in the serum levels of anti- $\beta$ 2GPI/ HLA-DR antibody between women with RPL and aPL and those without aPL.  $\beta$ 2GPI is the main phospholipid-binding molecule recognized by aPL.(5, 6) Circular  $\beta$ 2GPI is converted to linear  $\beta$ 2GPI; which exposes the major epitope for aPL when  $\beta$ 2GPI binds to anionic phospholipids.(7) Therefore, aPL is detected by ELISA in clinical settings using plates containing solid-phase negatively charged phospholipids, i.e., cardiolipin (CL), and  $\beta$ 2GPI or negatively charged plates containing  $\beta$ 2GPI.(8, 9) However,  $\beta$ 2GPI/HLA-DR complexes express epitopes shared by  $\beta$ 2GPI/CL complexes as well as unique epitopes that are not present on these complexes.(13) The present study found that 17 (32.7%) of 52 women with RPL who tested positive for anti- $\beta$ 2GPI/HLA-DR antibody also had aPL of criteria, but the remaining 35 (67.3%) women did not. Therefore, the measurements of anti- $\beta$ 2GPI/HLA-DR antibody may contribute to improve diagnostic sensitivity for APS because of the recognition of unique epitopes which are not recognized by conventional aPL.

The proportion of women with RPL tested positive for anti- $\beta$ 2GPI/HLA-DR antibody in 11 women with only histories of two or more pregnancy losses at 10 or more GW was higher than that in 185 women with only histories of pregnancy losses before 10 GW; and tended to be higher than that in 31 women who experienced both pregnancy losses before 10 GW and at 10 or more GW. Anti- $\beta$ 2GPI/HLA-DR antibody might be more closely associated with pregnancy losses at 10 or more GW than those before 10 GW as conventional aPL was.(28)

The presence of multiple aPL of criteria and LA positivity is strongly associated with severity of thromboembolisms and pregnancy complications.(29-34) In the present study, four of six women with RPL and LA tested positive for anti- $\beta$ 2GPI/HLA-DR antibody.

Additionally, all three women with RPL and double or triple positivity for aPL of criteria had anti- $\beta$ 2GPI/HLA-DR antibody, and the two women with triple positivity for aPL had very high anti- $\beta$ 2GPI/HLA-DR antibody levels of 927.5 ABH-U and 330.7 ABH-U. The former (Case 1 in Supplemental table 1), who tested positive for IgG aCL, IgG a $\beta$ 2GPI, and LA, experienced early-onset HELLP syndrome at 14 weeks of gestation, and the latter (Case 5 in Supplemental table 1), who tested positive for IgG aCL, IgG a $\beta$ 2GPI, and LA suffered a thromboembolism with cerebral infarction. Multiple positivity for aPL of criteria may be associated with positive tests for high levels of anti- $\beta$ 2GPI/HLA-DR antibody, and these conditions may be causally associated with severity of APS manifestations.

Anti- $\beta$ 2GPI/HLA-DR antibody is likely involved in the pathogenesis not only of RPL, but also of vascular thrombosis, another clinical manifestation of APS. A previous study demonstrated that autoantibodies against  $\beta$ 2GPI/HLA class II complexes mediated complement-dependent cytotoxicity for cells expressing the  $\beta$ 2GPI/HLA class II complexes on the vascular endothelial cells of placental decidual tissue obtained from women with APS.(13) Thus, autoantibodies against  $\beta$ 2GPI/HLA class II complexes may cause spontaneous miscarriages and vascular thrombosis by damaging vascular endothelial cells of the placental decidua or systemic vessels expressing  $\beta$ 2GPI/HLA class II complexes in a complement-dependent manner.

Alleles of HLA-DR4, HLA-DR7, and HLA-DR13 carry susceptibility to APS.(21-24) Previous study demonstrated that the binding ability of HLA-DR to  $\beta$ 2GPI differed depending on HLA-DR alleles. Alleles, HLA-DRA\*01:01/DRB1\*07:01, HLA-DRA\*01:01/DRB1\*04:02, HLA-DRA\*01:01/DRB1\*03:04, and HLA-DRA\*01:01/DRB1\*13:01, showed strong binding to  $\beta$ 2GPI; and antiphospholipid monoclonal antibodies derived from an APS patient (EY2C9) also strongly bound to  $\beta$ 2GPI complexed with these alleles.(13) These results indicated that a binding affinity of  $\beta$ 2GPI to each allele of HLA-DR is important for autoantibody recognition of  $\beta$ 2GPI/HLA-DR

complexes. On the other hand, HLA-DR allele itself does not affect the autoantibody binding to  $\beta$ 2GPI/HLA-DR complexes unlike T cell receptor. Because EY2C9 recognized  $\beta$ 2GPI complexed with HLA-DR7 the most efficiently, an assay system for detecting autoantibodies against  $\beta$ 2GPI/HLA-DR7 complex can be a highly sensitive method to diagnose APS. For this reason, in the present study, the  $\beta$ 2GPI/HLA-DR7 complex was used as an antigen to detect autoantibodies against  $\beta$ 2GPI/HLA-DR complex in women with RPL.

Alleles of HLA-DR3, HLA-DR4, HLA-DR10, and HLA-DR15 carry susceptibility to RPL.(35-37) A systematic review and meta-analysis reveals that HLA-DR4 significantly increases the risk for RPL.(35) In the present study, the allele frequencies of HLA-DR4 among 52 women with a positive test for anti- $\beta$ 2GPI/HLA-DR antibody were significantly higher than those among 175 women with a negative test (31.5% vs 21.1%,  $p<0.05$ ).  $\beta$ 2GPI/HLA class II complexes are localized to vascular endothelial cells in the placental decidua derived from mothers with APS, that directly contact with the villi derived from fetuses.(13) HLA-DR4 has strong binding ability to  $\beta$ 2GPI, and EY2C9 also strongly bind to  $\beta$ 2GPI/HLA-DR4 complex.(13) If a woman carry HLA-DR4, her vascular endothelial cells of the placental decidua may express HLA-DR4. Inflammation caused by infection or physiological stress may induce the formation of  $\beta$ 2GPI/HLA-DR4 complexes on vascular endothelial cells, so that anti- $\beta$ 2GPI/HLA-DR antibodies may damage vascular endothelial cells expressing  $\beta$ 2GPI/HLA-DR4 complexes, ultimately leading to pregnancy losses. Women who carry HLA-DR4 may have increased susceptibility to RPL with a positive test for anti- $\beta$ 2GPI/HLA-DR antibody.

In contrast, allele frequencies of HLA-DR4 among 52 partners of women with a positive test for anti- $\beta$ 2GPI/HLA-DR antibody were significantly lower than those among 175 partners of women with a negative test for anti- $\beta$ 2GPI/HLA-DR antibodies (18.0% vs 31.7%,  $p<0.05$ ). Couples of women with HLA-DR4 and partners with no HLA-DR4 may have high susceptibility to RPL with production of anti- $\beta$ 2GPI/HLA-DR antibody in women.

This suggests that not anti-HLA-DR4 antibodies but anti- $\beta$ 2GPI/HLA-DR4 antibodies cause RPL.

The standard treatment for pregnant women with APS is combination therapy of heparin and low-dose aspirin,(15) and the heparin/aspirin therapy may be also effective for women with RPL and anti- $\beta$ 2GPI/HLA-DR antibody. A cohort study to assess efficacy of antiplatelet and anticoagulant therapy for these women is already underway.

The limitations of this study are that the strict specificity of anti- $\beta$ 2GPI/HLA-DR antibody for diagnosis of APS cannot be determined. Therefore, prospective observational case-control studies, in which individuals who tested positive for anti- $\beta$ 2GPI/HLA-DR antibody are defined as cases, are required. Histories of vascular thrombosis and obstetric complications including HDP and FGR were not evaluated. Future studies assessing whether anti- $\beta$ 2GPI/HLA-DR antibody is causally associated with thrombosis, HDP and FGR are also necessary.

## **AUTHOR CONTRIBUTIONS**

All listed authors meet the criteria for authorship and have contributed to the study design, data generation, data analysis, manuscript writing and manuscript review.

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**Table 1.** The clinical characteristics of the 227 women with RPL and the 208 control women

Characteristics	Women with RPL n=227	Control n=208	<i>P</i> -value
Age, years old	35.0 $\pm$ 4.6	36.1 $\pm$ 4.3	<0.05
Gravidity	3.6 $\pm$ 1.8	1.4 $\pm$ 0.6	<0.0001
Parity	0.6 $\pm$ 0.8	1.4 $\pm$ 0.6	<0.0001
The number of spontaneous miscarriages	3.0 $\pm$ 1.6	0.0 $\pm$ 0.0	<0.0001
The number of stillbirths at 22 or more GW	0.1 $\pm$ 0.3	0.0 $\pm$ 0.0	<0.001
Women who have a history of stillbirths at 22 or more GW	5.7%	0.0%	<0.001

Data are expressed as the average  $\pm$  standard deviation.

Abbreviations: RPL, recurrent pregnancy loss; GW, gestational weeks.

**Table 2.** HLA-DRB1 allele frequencies in the 208 fertile control women and the 227 couples with RPL

Subjects	HLA-DR alleles													
	DR1		DR3	DR4										DR7
	01:01	01:02	03:01	04:01	04:02	04:03	04:04	04:05	04:06	04:07	04:08	04:10	04:11	07:01
Fertile control women (n=208)	4.7%	0%	0.2%	1.5%	0%	2.2%	0.7%	11.2%	3.5%	0%	0%	1.7%	0%	0.5%
Women with RPL (n=227)	6.5%	0%	0.4%	1.1%	0%	4.7%	0.7%	12.9%	1.8%	0%	0%	2.2%	0%	0.7%
Anti-β2GPI/HLA-DR antibody-positive women with RPL (n=52)	3.9%	0%	0%	1.0%	0%	6.9%	1.0%	15.7%	4.9%	0%	0%	2.0%	0%	1.0%
Anti-β2GPI/HLA-DR antibody-negative women with RPL (n=175)	7.2%	0%	0.6%	1.2%	0%	4.0%	0.6%	12.1%	0.9%	0%	0%	2.3%	0%	0.6%
Partners of RPL women (n=227)	5.3%	0%	0.5%	1.6%	0%	3.3%	0%	18.0%	3.8%	0.8%	0%	1.1%	0%	0.5%
Partners of anti-β2GPI/HLA-DR antibody-positive women with RPL (n=52)	6.7%	0%	1.0%	1.0%	0%	1.0%	0%	13.0%	2.0%	0%	0%	1.0%	0%	1.0%
Partners of anti-β2GPI/HLA-DR antibody-negative women with RPL (n=175)	4.9%	0%	0.4%	1.8%	0%	4.0%	0%	19.4%	4.3%	1.1%	0%	1.1%	0%	0.4%

Subjects	HLA-DR alleles														
	DR8				DR9	DR10	DR11			DR12			DR13		
	08:02	08:03	08:09	08:23	09:01	10:01	11:01	11:06	11:08	12:01	12:02	12:05	13:01	13:02	13:07
Fertile control women (n=208)	3.7%	6.5%	0%	0%	16.4%	1.5%	2.5%	0%	0%	3.5%	1.7%	0%	0.5%	6.7%	0%
Women with RPL (n=227)	3.6%	6.0%	0%	0%	15.6%	0.7%	2.0%	0%	0%	4.2%	2.0%	0%	0.4%	5.1%	0%
Anti-β2GPI/HLA-DR antibody-positive women with RPL (n=52)	5.9%	6.9%	0%	0%	14.7%	1.0%	2.0%	0%	0%	2.0%	1.0%	0%	0%	6.9%	0%
Anti-β2GPI/HLA-DR antibody-negative women with RPL (n=175)	2.9%	5.8%	0%	0%	15.9%	0.6%	2.0%	0%	0%	4.9%	2.3%	0%	0.6%	4.6%	0%
Partners of RPL women (n=227)	2.8%	8.7%	0.5%	0%	14.7%	1.4%	2.1%	0%	0%	2.1%	1.8%	0%	0.5%	5.1%	0%
Partners of anti-β2GPI/HLA-DR antibody-positive women with RPL (n=52)	5.0%	8.7%	1.0%	0%	24.0%	2.0%	2.9%	0%	0%	2.9%	1.0%	0%	1.0%	6.7%	0%
Partners of anti-β2GPI/HLA-DR antibody-negative women with RPL (n=175)	2.5%	8.9%	0.4%	0%	12.0%	1.4%	1.7%	0%	0%	1.7%	2.0%	0%	0.4%	4.6%	0%

Subjects	HLA-DR alleles														
	DR14										DR15			DR16	
	14:54	14:02	14:03	14:04	14:05	14:06	14:07	14:12	14:29	15:01	15:02	15:04	15:11	16:02	
Fertile control women (n=208)	3.2%	0%	2.2%	0%	2.0%	1.7%	0.2%	0%	0%	8.0%	11.2%	0%	0%	1.7%	
Women with RPL (n=227)	3.8%	0%	0.7%	0%	2.5%	2.5%	0.0%	0%	0%	8.3%	10.7%	0%	0%	0.9%	
Anti-β2GPI/HLA-DR antibody-positive women with RPL (n=52)	4.9%	0%	0.0%	0%	3.9%	0.0%	0.0%	0%	0%	4.9%	9.8%	0%	0%	0%	
Anti-β2GPI/HLA-DR antibody-negative women with RPL (n=175)	3.5%	0%	0.9%	0%	2.0%	3.2%	0.0%	0%	0%	9.2%	11.0%	0%	0%	1.2%	
Partners of RPL women (n=227)	2.1%	0%	1.2%	0%	1.9%	0.9%	0.2%	0%	0%	6.4%	8.4%	0%	0%	0.9%	
Partners of anti-β2GPI/HLA-DR antibody-positive women with RPL (n=52)	1.0%	0%	0%	0%	1.0%	1.0%	0%	0%	0%	3.8%	11.5%	0%	0%	2.0%	
Partners of anti-β2GPI/HLA-DR antibody-negative women with RPL (n=175)	2.3%	0%	1.5%	0%	2.5%	1.1%	0.4%	0%	0%	7.1%	7.4%	0%	0%	0.7%	

HLA, human leukocyte antigen; RPL, recurrent pregnancy loss;  $\beta$ 2GPI,  $\beta$ 2-glycoprotein I; anti- $\beta$ 2GPI/HLA-DR antibody, autoantibody against  $\beta$ 2GPI/HLA-DR complex.

## Figure legends

### **Figure 1. Distribution of serum levels of autoantibody against $\beta$ 2GPI/HLA-DR complex in the 208 control women.**

The control women enrolled in this study were as follows: 1) women who had delivered at least one term living infants; 2) women who did not have histories of miscarriages and stillbirths; 3) women who did not have autoimmune diseases; 4) women who tested negative for aPL.

Abbreviations:  $\beta$ 2GPI,  $\beta$ 2-glycoprotein I; HLA, human leukocyte antigen; aPL, antiphospholipid antibody.

### **Figure 2. Distribution of serum levels of autoantibody against $\beta$ 2GPI/HLA-DR complex in the 227 women with RPL.**

The normal upper limit for autoantibody against  $\beta$ 2GPI/HLA-DR complex established by the 208 control women was 52.6 ABH-U. Fifty-two of the 227 (22.9%) women with RPL tested positive for anti- $\beta$ 2GPI/HLA-DR antibody.

Abbreviations:  $\beta$ 2GPI,  $\beta$ 2-glycoprotein I; HLA, human leukocyte antigen; RPL, recurrent pregnancy loss; ABH-U, anti- $\beta$ 2GPI/HLA-DR antibody units.

### **Figure 3. Autoantibody against $\beta$ 2GPI/HLA-DR complex and aPL antibody positivity in the 227 women with RPL.**

Autoantibodies against  $\beta$ 2GPI/HLA-DR complex were positive in 52 women with RPL. IgG aCL and IgM aCL were positive in 20 and 14 women with RPL, respectively. IgG a $\beta$ 2GPI, IgM a $\beta$ 2GPI, and IgG aCL $\beta$ 2GPI were positive in 7, 3, and 3 women with RPL, respectively. LA were positive in 6 women with RPL. Thirty-five of the 52 (67.3%) women with RPL who were positive for autoantibodies against  $\beta$ 2GPI/HLA-DR complex had no aPL of criteria.

All three women with RPL who had autoantibodies against  $\beta$ 2GPI/HLA-DR complex and

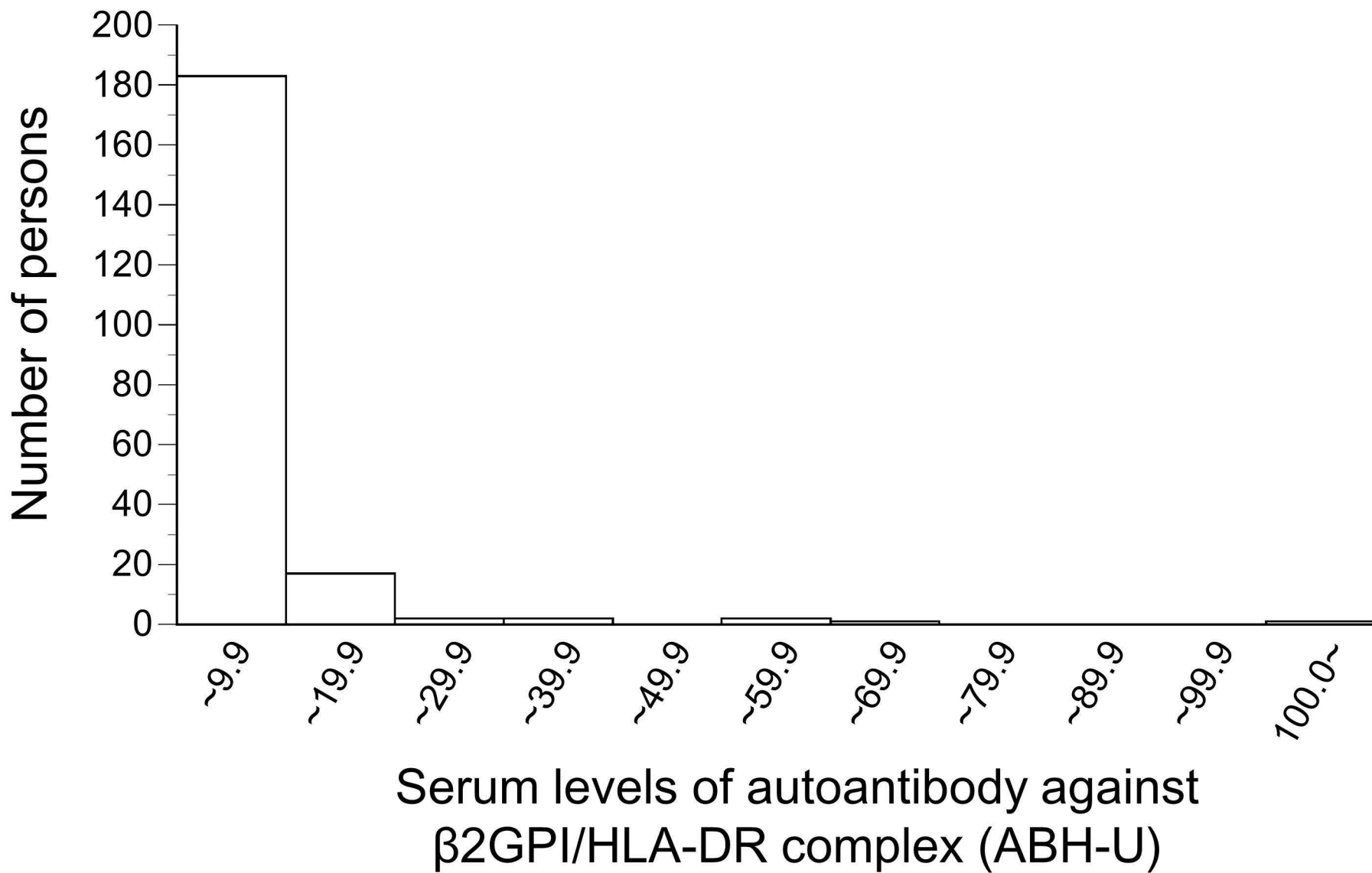
IgG aCL $\beta$ 2GPI tested positive for IgG a $\beta$ 2GPI and IgG aCL, and two of the three women also tested positive for LA.

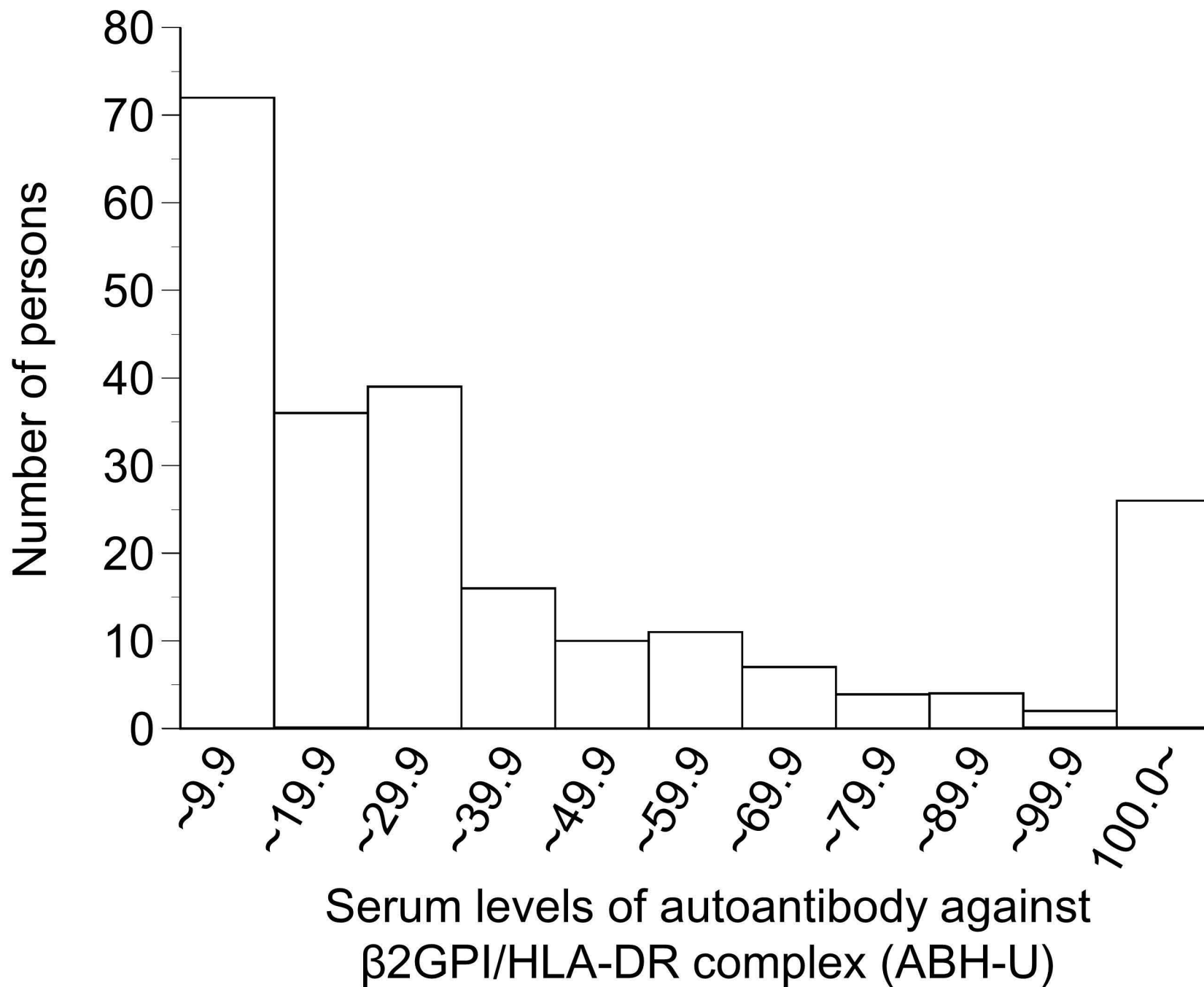
Abbreviations:  $\beta$ 2GPI,  $\beta$ 2-glycoprotein I; HLA, human leukocyte antigen; IgG, immunoglobulin G; IgM, immunoglobulin M; aCL, anti-cardiolipin antibody; a $\beta$ 2GPI, anti- $\beta$ 2-glycoprotein I antibody; aCL/ $\beta$ 2GPI,  $\beta$ 2GPI-dependent anti-cardiolipin antibody; LA, lupus anticoagulant.

**Figure 4. Risk factors or causes of RPL among the 227 women with RPL enrolled in this study.**

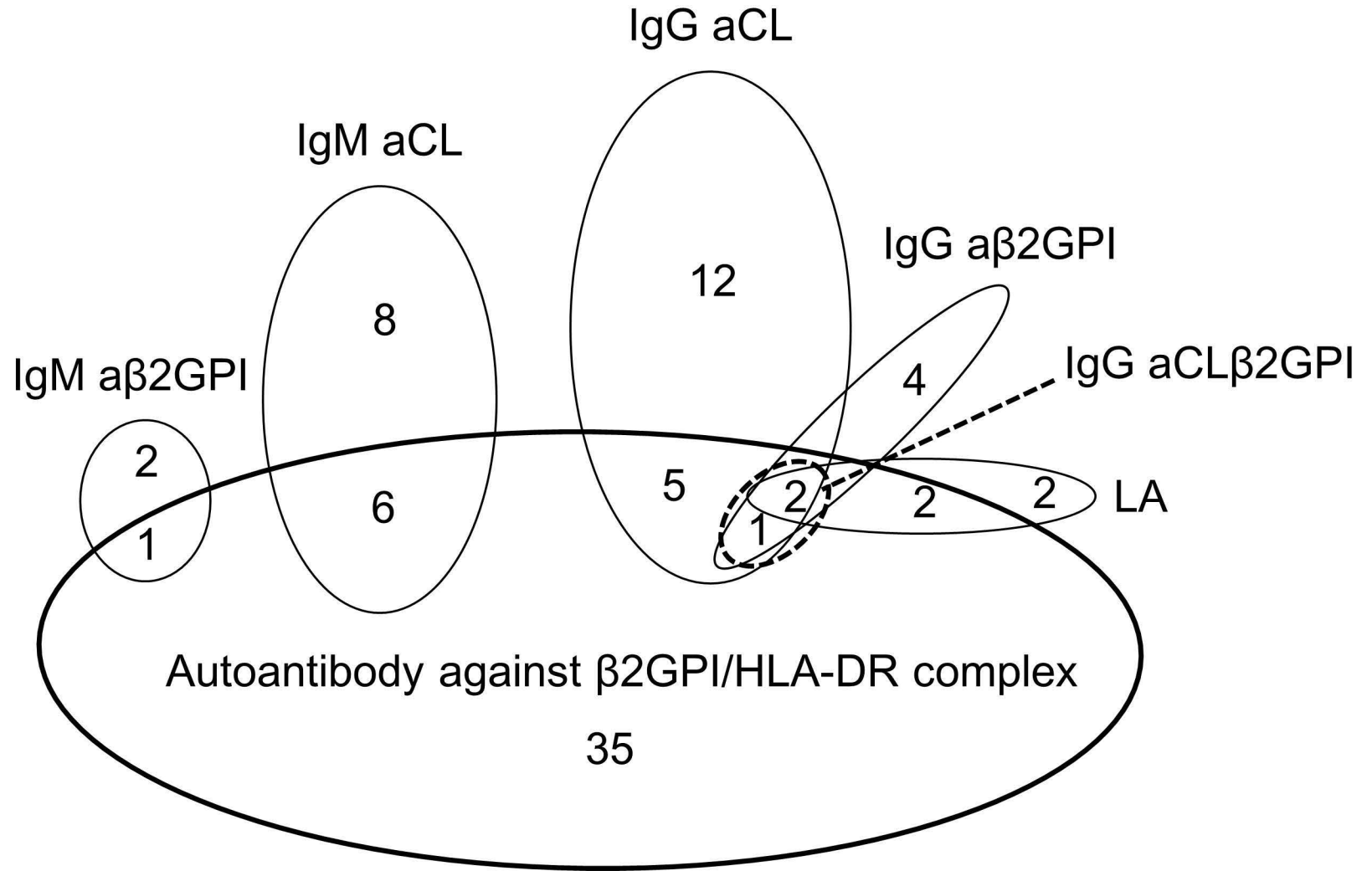
Among the 227 women with RPL, 19 women (8.4%) had uterine malformations, 15 women (6.6%) had thyroid dysfunction, 11 women (4.8%) had chromosomal abnormalities, 45 women (19.8%) had aPL, 21 women (9.3%) had low factor XII activity, 35 women (15.4%) had low protein S activity, 4 woman (1.8%) had low protein C activity, 33 women (14.5%) had multiple risk factors, and 121 women (53.3%) in whom risk factors or causes of RPL were unexplained. The black column indicates women with RPL who tested positive for autoantibody against  $\beta$ 2GPI/HLA-DR complex (n=52).

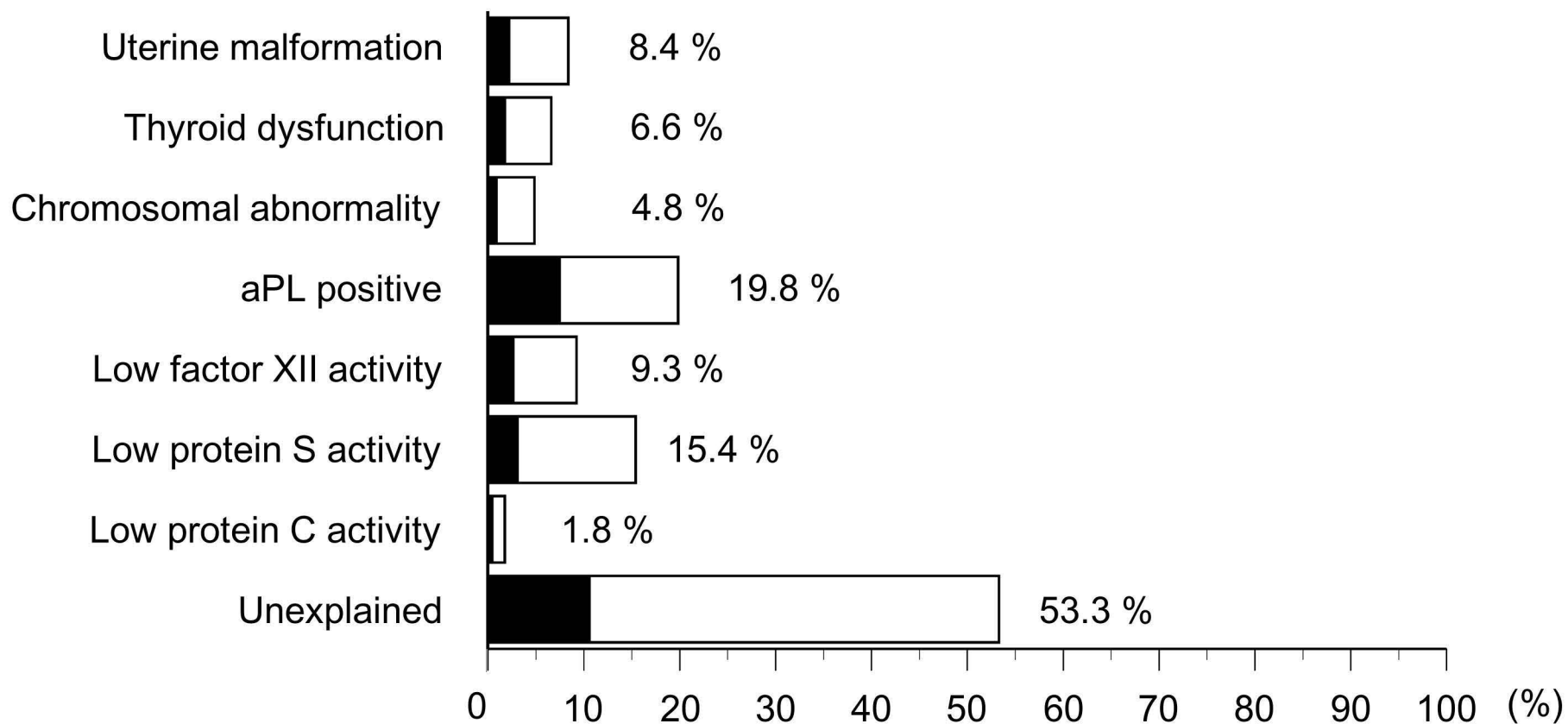
Abbreviations: RPL, recurrent pregnancy loss; aPL, antiphospholipid antibodies;  $\beta$ 2GPI,  $\beta$ 2-glycoprotein I; HLA, human leukocyte antigen.











**Supplemental table 1.** Serum levels of anti- $\beta$ 2GPI/HLA-DR antibodies and aPL in the 45 aPL-positive women with RPL

Case	aCL		a $\beta$ 2GPI		aCL/ $\beta$ 2GPI	LA	anti- $\beta$ 2GPI/HLA-DR
	IgG (U/ml)	IgM (U/ml)	IgG (U/ml)	IgM (U/ml)	-IgG (U/ml)	(Normalized ratio)	antibody (ABH-U)
1	360.0	—	6100.0	—	224	29.8	927.5
2	—	9.0	—	—	—	—	623.5
3	—	20.0	—	—	—	—	446.4
4	11.0	—	—	—	—	—	386.3
5	50.0	—	1466.1	—	44.8	27.7	330.7
6	—	17.0	—	—	—	—	220.1
7	11.0	—	—	—	—	—	211.5
8	—	10.0	—	—	—	—	122.2
9	27.2	—	—	—	—	—	102.5
10	—	—	—	—	—	1.6	99.9
11	14.0	—	53.3	—	4.9	—	73.0
12	—	—	—	27.9	—	—	65.5
13	—	—	—	—	—	1.4	65.5
14	14.0	—	—	—	—	—	64.3
15	—	10.0	—	—	—	—	57.1
16	—	12.0	—	—	—	—	54.8
17	16.0	—	—	—	—	—	53.7
18	—	—	—	—	—	12.2	49.3
19	—	—	—	144.0	—	—	45.3
20	—	15.0	—	—	—	—	44.6
21	14.0	—	—	—	—	—	44.6
22	—	—	29.4	—	—	—	38.2
23	24.0	—	—	—	—	—	29.6
24	—	—	21.8	—	—	—	25.6
25	—	11.0	—	—	—	—	23.8
26	—	8.0	—	—	—	—	20.4
27	—	—	48.3	—	—	—	19.7
28	—	26.4	—	—	—	—	14.9
29	59.0	—	—	—	—	—	14.1
30	15.0	—	—	—	—	—	13.2
31	—	—	—	—	—	1.3	10.5
32	17.5	—	—	—	—	—	8.9
33	18.0	—	—	—	—	—	5.9
34	—	—	29.0	—	—	—	5.1
35	—	17.0	—	—	—	—	0
36	28.9	—	—	—	—	—	0
37	21.0	—	—	—	—	—	0
38	26.0	—	—	—	—	—	0
39	—	9.0	—	—	—	—	0
40	12.0	—	—	—	—	—	0
41	10.0	—	—	—	—	—	0
42	23.1	—	—	—	—	—	0
43	—	17.0	—	—	—	—	0
44	—	42.0	—	—	—	—	0
45	—	—	—	34.1	—	—	0

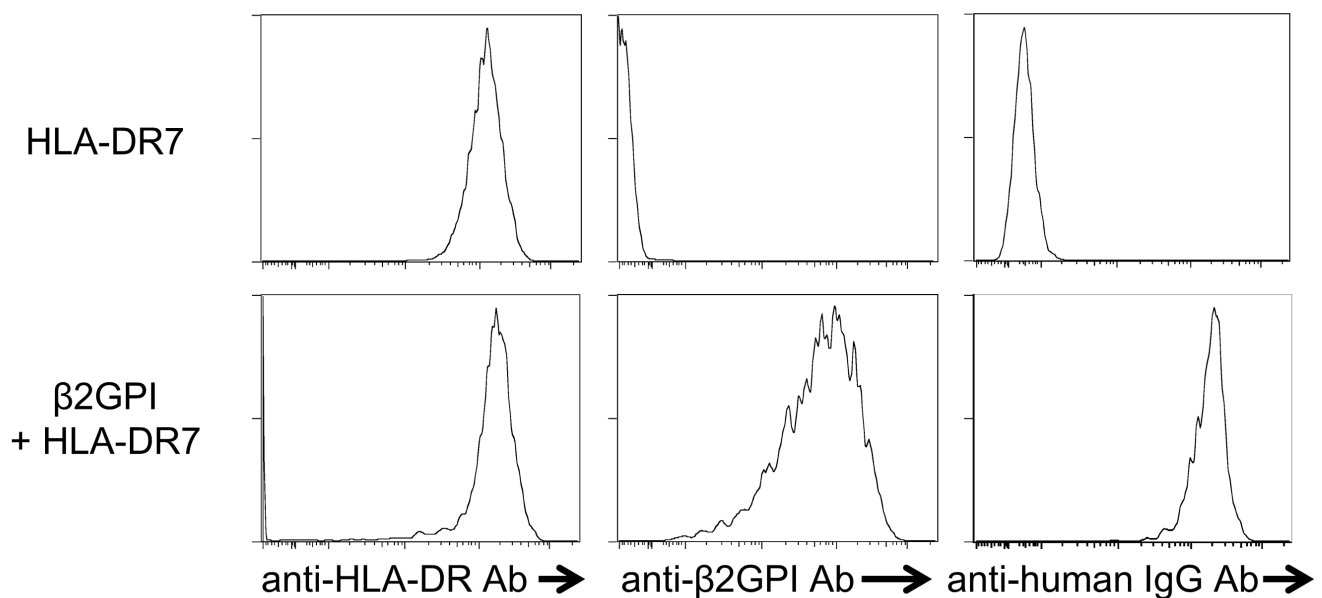
— indicates a negative result for each aPL.

Abbreviations:  $\beta$ 2GPI,  $\beta$ 2-glycoprotein I; HLA, human leukocyte antigen; aPL, antiphospholipid antibodies; ABH-U, anti- $\beta$ 2GPI/HLA-DR antibody units; RPL, recurrent pregnancy loss; IgG, immunoglobulin G; IgM, immunoglobulin M; aCL, anti-cardiolipin antibody; a $\beta$ 2GPI, anti- $\beta$ 2-glycoprotein I antibody; aCL/ $\beta$ 2GPI,  $\beta$ 2GPI-dependent anti-cardiolipin antibody; LA, lupus anticoagulant.

**Supplemental table 2.** The details of overlapping factors among the 33 couples with RPL and multiple risk factors

Risk factors or causes of RPL				n
Thyroid dysfunction	+ aPL positivity	+ Low FXII activity	+ Low PS activity	1
aPL positivity	+ Low FXII activity	+ Low PS activity		2
Uterine malformation	+ aPL positivity	+ Low PS activity		2
Uterine malformation	+ aPL positivity	+ Low FXII activity		1
Uterine malformation	+ Chromosomal abnormality	+ aPL positivity		1
Uterine malformation	+ Thyroid dysfunction	+ Low PS activity		1
Chromosomal abnormality	+ aPL positivity	+ Low PS activity		1
aPL positivity	+ Low PS activity	+ Low PC activity		1
aPL positivity	+ Low PS activity			4
Thyroid dysfunction	+ Chromosomal abnormality			2
Thyroid dysfunction	+ Low PS activity			2
Chromosomal abnormality	+ aPL positivity			2
Chromosomal abnormality	+ Low PS activity			2
Uterine malformation	+ aPL positivity			2
aPL positivity	+ Low FXII activity			2
Uterine malformation	+ Low PS activity			1
Uterine malformation	+ Thyroid dysfunction			1
Uterine malformation	+ Low FXII activity			1
Chromosomal abnormality	+ Low PC activity			1
Thyroid dysfunction	+ aPL positivity			1
aPL positivity	+ Low PC activity			1
Low FXII activity	+ Low PS activity			1

Abbreviations: RPL, recurrent pregnancy loss; aPL, antiphospholipid antibody; FXII, factor XII; PS, protein S; PC, protein C.

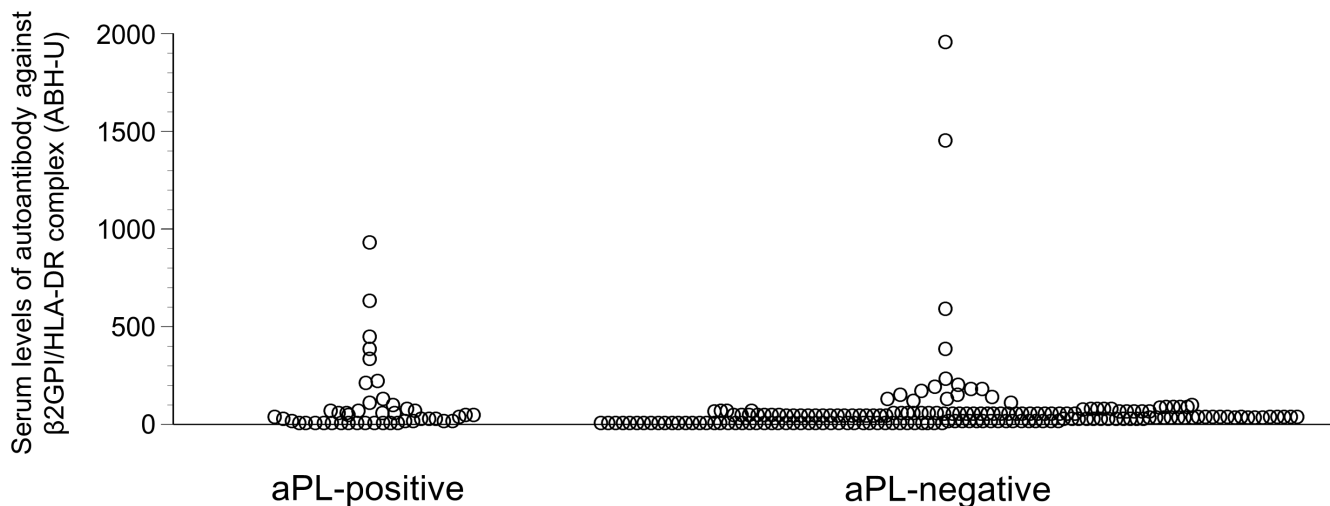


**Supplemental figure 1. The expressions of HLA-DR and β2GPI on the cell surface of the transfected cells and IgG binding to these transfected cells in the 10<sup>2</sup>-fold diluted standard serum**

GFP-labeled cells expressing the β2GPI/HLA-DR complex and DsRed labeled cells expressing HLA-DR were stained with anti-HLA antibody or anti-β2GPI antibody. IgG binding to cells expressing HLA-DR alone and cells expressing the β2GPI/HLA-DR complex were stained with APC-labeled anti-human IgG Fc antibody.

DsRed labeled HLA-DR expressing cells express HLA-DR alone, and autoantibodies against HLA-DR in the serum bind to the transfected cells (upper three histograms). GFP-labeled β2GPI/HLA-DR complex expressing cells express both HLA-DR and β2GPI, and autoantibodies against HLA-DR or those against β2GPI/HLA-DR complex bind to the transfected cells (lower three histograms).

Abbreviations: β2GPI, β2-glycoprotein I; HLA, human leukocyte antigen.



**Supplemental figure 2. Comparison of serum levels of anti-β2GPI/HLA-DR antibody in aPL-positive women with RPL with those in aPL-negative women with RPL.**

All women with RPL enrolled in this study underwent aPL measurements, including IgG aCL (positive,  $\geq 10$  U/ml), IgM aCL (positive,  $\geq 8$  U/ml), IgG aβ2GPI (positive,  $\geq 20$  U/ml), IgM aβ2GPI (positive,  $\geq 20$  U/ml), and LA (positive,  $\geq 1.3$  Normalized ratio). aPL-positive women had at least one positive result for these aPL.

There was no significant difference in the serum levels of anti-β2GPI/ HLA-DR antibody between aPL-positive women with RPL (n=45) and aPL-negative women with RPL (n=182) (median [range], 29.4 [0–927.5] vs 20.4 [0–1952.0] ABH-U,  $p=0.12$ ). Difference was analyzed by the Mann-Whitney *U* test.

Abbreviations: β2GPI, β2-glycoprotein I; HLA, human leukocyte antigen; aPL, antiphospholipid antibodies; ABH-U, anti-β2GPI/HLA-DR antibody units; RPL, recurrent pregnancy loss; IgG, immunoglobulin G; IgM, immunoglobulin M; aCL, anti-cardiolipin antibody; aβ2GPI, anti-β2-glycoprotein I antibody; aCL/β2GPI, β2GPI-dependent anti-cardiolipin antibody; LA, lupus anticoagulant.