Increased Plasma Concentrations of Antiprothrombin Antibodies in Women with Recurrent Spontaneous Abortions

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Background: Antiphospholipid antibodies are associated with recurrent fetal loss, but the clinical relevance of antiprothrombin (aPT) antibodies remains controversial. This study was designed to evaluate the relationship of plasma concentrations of aPT antibodies (IgG, IgM, and IgA isotypes) and recurrent spontaneous abortion (RSA) not associated with antiphospholipid-antibody syndrome.

Methods: In this retrospective case–control study, we measured plasma aPT antibodies in 100 pregnant women at 8–12 weeks of gestation who had histories of recurrent abortion not associated with antiphospholipid-antibody syndrome. The controls were 200 healthy gestational-age–matched women with uncomplicated gestations.

Results: The mean (SD) plasma aPT concentrations were significantly ($P < 0.001$) higher in women with histories of recurrent abortion than in healthy controls [7.97 (0.79) and 2.08 (0.07) kU/L]. Similarly, the concentrations of IgM aPT were significantly ($P < 0.001$) higher in patients than in controls [5.73 (0.85) and 1.83 (0.05) kU/L]. No differences were found for IgA aPT ($P = 0.358$).

Conclusions: High concentrations of aPT antibodies (IgG and IgM isotypes) are associated with pregnancy loss in women with RSA. We suggest that the antibodies may have a relevant role in the etiology and pathogenesis of the condition.

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Recurrent spontaneous abortion (RSA)3 is usually defined as the loss of ≥3 consecutive pregnancies at <20 weeks of pregnancy (1). Among the risk factors for RSA [chromosomal (2), genetic (3), anatomical (4), endocrinological (5), and placental anomalies (6); infection (7); and stress (8)], thrombophilic conditions attributable to venous thromboembolism, as in the case of antiphospholipid-antibody syndrome (APS) (9–12), have a relevant role. APS is a possible cause of pregnancy loss through the promotion of microvascular placental thrombosis, which is frequently associated with infarction, perivillous fibrin deposits, and chronic inflammatory lesions (13). Antiprothrombin (aPT) antibodies, which are present in ~50% of antiphospholipid-positive patients (14) and are frequently found in women with APS, show wide variation in immunological and functional properties, depending mainly on their affinity for human prothrombin (or factor II), a vitamin K–dependent glycoprotein that performs several anticoagulant activities (15). The clinical relevance in RSA of aPT antibodies has not been established despite increasing knowledge about their mechanism(s) of action and their presence in a number of conditions associated with venous thromboembolism and the hypercoagulable state of APS (15).

The aim of the present study was to measure plasma concentrations of aPT (IgM, IgG, and IgA isotypes) antibodies in women with histories of RSA unaffected by APS.

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Received May 5, 2006; accepted November 6, 2006.
Previously published online at DOI: 10.1373/clinchem.2006.073098

3 Nonstandard abbreviations: RSA, recurrent spontaneous abortion; APS, antiphospholipid-antibody syndrome; aPT, antiprothrombin antibody; LAC, lupus anticoagulant; aCL, anticardiolipin; β2-GPI, anti-β2-glycoprotein I; ANA, antinuclear antibody.
**Materials and Methods**

**STUDY PATIENTS**

A retrospective case–control study was performed with plasma samples collected from 300 pregnant women recruited from February 2003 to June 2005 at the Division of Obstetrics and Gynecology, University of Siena, a tertiary clinical care center. All the women were of Italian origin and lived in Tuscany. Patients with thyroid dysfunction, glucose intolerance, renal or liver disease, pre-existing hypertension, uterine anomalies, current infection or history of all types of infection, or preexisting autoimmune disease, such as systemic lupus erythematosus or APS, were excluded from the study.

All case patients (n = 100; median age, 34.5 years; range, 24–45 years) were patients with history of RSA (median, 3; range, 3–7) who were undergoing evaluation for vaginal bleeding at 8–19 weeks of gestation (median, 12 weeks; range, 8–19 weeks). In all of the case patients, pregnancy ended spontaneously within 20 weeks after the date of the last menstrual period (median, 12 weeks; range, 8–19 weeks; Table 1). All cases had a normal karyotype, and the fetuses were free of detectable anomalies.

Controls were gestational-age–matched pregnant women (n = 200; median age, 34 years; range, 24–45 years) with no history of RSA (median, 1 prior spontaneous abortion; range, 0–2) who were recruited at the same time as the study patients and who gave birth without complications to healthy full-term (>37 weeks) infants of appropriate weight for gestational age (Table 1).

Gestational age was evaluated on the basis of the last menstrual period as recorded by the referring physician and confirmed by ultrasound (real-time ultrasound scan equipment, Siemens Sonoline ELEGRA® Millennium Edition with a transvaginal probe at 4.5–7.0 MHz) at hospitalization.

All women gave written informed consent before participation, and the study was approved by the local Human Investigation Committee.

**COLLECTION OF SAMPLES**

Samples were collected at 8–10 AM from the antecubital vein, without venous stasis, in Vacutainer blood collecting tubes containing 10 mL/L of 38 mL/L sodium citrate. Blood samples were centrifuged at 4 °C, 1600 g for 10 min at room temperature. All plasma samples were kept at −80 °C until assay.

**ANTIBODY TESTS**

To exclude the presence of systemic lupus erythematosus or APS, we measured lupus anticoagulants (LACs), antiphospholipid (aCL), anti–β2-glycoprotein I (β2-GPI), and antinuclear antibodies (ANAs) with reagents purchased from Orgentec Diagnostika. Plasma samples were tested for the presence of LAC activity, according to recommended criteria from the International Society on Thrombosis and Hemostasis Subcommittee on Lupus Anticoagulants–Phospholipid-dependent Antibodies (16) with the use of a TEST™ LAC screen and TEST™ LAC confirm reagent set that employs the reagents of the dilute Russell viper venom test. The sample control ratio classified the test result as normal (ratio, 0.8–1.2), slightly positive (1.2–1.5), moderately positive (1.5–2), or heavily positive (>2).

Samples were tested for the presence of aCL antibodies (IgG, IgM, and IgA isotypes) with a standardized ELISA (17, 18), and results were expressed as GPLU, MPLU, and APLU for IgG, IgM, and IgA, respectively. The detection limits were 1000 APLU/L and 1000 GPLU/L for IgA and IgG, respectively, and 500 MPLU/L for IgM aCL. Positive results were defined as >10 000 GPLU/L for IgG, >7000 MPLU/L for IgM, and >10 000 APLU/L for IgA. The intraassay imprecision (CV; n = 24) was 3.8% for IgG, 3.4% for IgM, and 3.3% for IgA, and the interassay CVs (n = 5) were 5.4% for IgG, 3.7% for IgM, and 5.9% for IgA at mean concentrations of IgG, IgM, and IgA of ~3000 GPLU/L, 900 MPLU/L, and 190 (or 970 for interassay) APLU/L.

Serum anti–β2-GPI (IgG, IgM, and IgA) antibody concentrations were measured by ELISA (19, 20). Results <500 U/L were interpreted as negative, >8000 U/L as positive, and 5000–8000 U/L as borderline positive. The intraassay CVs were 5.0% for IgG, 3.8% for IgM, and 4.0% for IgA (n = 24), whereas the interassay CVs were 7.4% for IgG, 6.3% for IgM, and 5.2% for IgA (n = 3 different runs with 16 determination of each sample) at IgG, IgM, and IgA concentrations [expressed as antiphospholipid units for IgG (GPLU), IgM (MPLU), and IgA (APLU)] of ~1200 GPLU/L, 1500 MPLU/L, and 7200 APLU/L.

For APL antibody screening we used the Immuno- metric Enzyme Immunoassay (Orgentec Diagnostika GmbH) for the quantitative determination of the sum of autoantibodies against cardiolipin, phosphatidylserine,

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**Table 1. Summary of clinical data.**

<table>
<thead>
<tr>
<th></th>
<th>Study group (n = 200), mean (SE)</th>
<th>Control group (n = 200), mean (SE)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age, years</td>
<td>34.2 (0.3)</td>
<td>34.8 (0.4)</td>
<td>NS</td>
</tr>
<tr>
<td>Abortion, no.</td>
<td>3.4 (0.08)</td>
<td>0.5 (0.03)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Gestational age at sampling, weeks</td>
<td>12.15 (0.1)</td>
<td>12.15 (0.1)</td>
<td>NS</td>
</tr>
<tr>
<td>Parity, no.</td>
<td>0</td>
<td>1.53 (0.03)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Gestational age at pregnancy termination, weeks</td>
<td>12.6 (0.3)</td>
<td>38.9 (0.2)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*NS, not significant.*
phosphatidylinositol, and phosphatidic acid (IgG and IgM class). Results were considered negative when <10 000 U/L and positive when >10 000 U/L. The intraassay (n = 24) and interassay (n = 3) CVs were <3.0% at concentrations for IgG and IgM of 3500 GPLU/L and for IgM of 3700 MPLU/L.

ANAs were detected by immunofluorescence using Hep 2 cells as the antigen (Hep-2000 ANA Test System; Immunoconcepts). A titer >1/80 was classified as positive for the purpose of this study.

aPT antibody assay
aPT antibody measurement was performed with direct solid phase ELISA for quantitative determination of IgA, IgG, and IgM isotypes against prothrombin in human plasma, according to manufacturer instructions (Bouty).

The coating processes used in the manufacture of the ELISA microtiter plates allow retention of the native immunogenic structure of prothrombin after its immobilization. The lower detection limits for aPTs IgA, IgG, and IgM were determined at 100 U/L; the imprecision was <3.8% for intraassay (n = 25) and <6.9% for interassay (n = 5 different runs with 6 determinations of each sample) at concentrations of intraassay IgG 1000 GPLU/L, IgM 1400 MPLU/L, and IgA 1680 APLU/L and interassay IgG 1060 GPLU/L, IgM 1450 MPLU/L, and IgA 1670 APLU/L. The assay is specific only for autoantibodies directed against prothrombin, and no interference was observed with hemolysis (≤10 mg/L triglycerides), bilirubin (≤400 mg/L), or anticoagulants. Values >7000 U/L were considered positive.

Statistical analysis
Clinical data were expressed as mean (SD). Statistical significance of results was assessed by using the unpaired t-test and the Mann–Whitney U-test for data with gaussian distribution and the Fisher exact test for nongaussian data distribution. Statistical significance was assumed for P <0.05.

Results
In the present study we tested a total of 600 patients, of whom 226 (38%) were excluded from the study because of the presence of autoimmune diseases (177/226; 78%), antiphospholipid positivity (49/226; 22%), or other exclusion criteria (74/226; 33%). With respect to antiphospholipid-positive patients with RSA (n = 49) excluded from the study, 29 (59%) were positive for IgG, 17 (35%) had IgM, and 5 (10%) had IgA aPT antibodies. Therefore, the final evaluation was conducted on 300 patients, of whom 100 (33%) were case patients.

The clinical characteristics of patients enrolled are summarized in Table 1. All 100 women with a history of RSA experienced early spontaneous miscarriage before 20 completed weeks, and there were no significant differences with respect to maternal age, parity, or gestational age at sampling between groups. No LAC, aCL, β2-GPI, or ANA antibodies were detectable in control and case patients (data not shown). The mean (SD) aPT IgG isotype plasma concentrations were significantly (P <0.0001) higher in women with history of RSA [7.97 (0.79); median, 62 kU/L; range, 1–49 kU/L] than in healthy controls [2.08 (0.07); median, 1.8 kU/L; range, 1–5.3 kU/L] (Fig. 1. The mean (SD) APT IgM isotype concentrations were significantly (P <0.0001) higher in the group of patients with a history of RSA who experienced early spontaneous miscarriage [5.73 (0.85); median, 4.3 kU/L; range, 1–61.9 kU/L] than in healthy pregnant women [1.83 (0.05); median, 1.5 kU/L; range, 1–4.6 kU/L] (Fig. 2, whereas no differences were found for aPT IgA isotype concentrations (P = 0.358; Fig. 3.

The prevalences of aPT IgG (37/100; 37%) and IgM (18/100; 18%) isotypes were significantly higher (P <0.0001 and P <0.01) in patients with history of RSA than in controls (IgG: 2/200; 1% IgM: 0/200; 0%), whereas the prevalence of the aPT IgA isotype did not differ between groups (patient group, 4/100, 4%; control group, 1/200; P >0.05). The prevalences of aPT IgG, IgM, and IgA isotypes did not differ between case patients and antiphospholipid-positive patients with RSA (P = 0.127 for IgG; P = 0.11 for IgM; P = 0.27 for IgA; data not shown).

Discussion
Maternal–fetal immunity plays an important role throughout pregnancy, but mainly at early gestation, when the immune cross-talk between the embryo and the maternal decidua is fundamental for successful implantation (21). The impairment of this local network may cause defective placentation and/or early pregnancy loss, and autoimmune factors have been reported to be frequently correlated with spontaneous abortion (22).
In the present study we found that patients with RSA have higher mean concentrations of IgG and IgM, but not IgA and aPT, than do healthy pregnant women. For IgA, the lack of statistically significant differences between controls and RSA patients is attributable to the wide distribution of aPT IgA antibody values. The fact that IgA appears after IgG and IgM, however, may be a further possible explanation. Therefore, differences in aPT antibodies may be due to an immune phenomenon that is already working at the time of hospitalization and has allowed the appearance of IgG and IgM, but not IgA. Larger studies are required to elucidate the pathogenetic role of aPT IgA antibodies.

The association between aPT antibodies and RSA is not novel, because high concentrations have been reported in patients affected by APS (23). Moreover, some acute infectious diseases (syphilis, HIV, hepatitis C, leprosy, and malaria) may be associated with increased aPT antibodies (24). Our data, however, were collected from patients without APS and infectious diseases and therefore support the hypothesis that aPT antibodies play a role, independently from APS, in the events cascade leading to early RSA. Indeed, aPT antibodies are commonly found in women with APS and may lead to pregnancy loss through the promotion of microvascular placental thrombosis, which is frequently associated with infarction, perivillous fibrin deposits, and chronic inflammatory lesions (13). The development of the placental circulation is crucial to the establishment of pregnancy and is ensured by structural modifications of the spiral arteries (25) and establishment of a hypercoagulable state from an increase in procoagulant factors and a decrease in anticoagulant factors and fibrinolysis (26). Disturbances in this hemostatic balance may lead to adverse pregnancy outcomes. Although the mechanism by which aPT antibodies may cause recurrent abortion remains to be defined, placental thrombosis may have an etiologic role, because increased basal thrombogenic potential would enhance hypercoagulability during pregnancy, leading to placental thrombosis and fetal loss. The association of placental thrombosis with fetal loss is supported by findings of thrombotic changes and infaracts in pathological studies of the placentas obtained from pregnancies terminated by fetal loss (27).

Prothrombin seems to be important in the development of the embryo, particularly in regard to vascular integrity. Recent studies (28) have shown that prothrombin deficiency in prothrombin-deficient mice leads to partial embryonic fatality as a result of bleeding into the yolk sac cavity and tissue necrosis of the embryos. Thus aPT antibodies may lead to fatal changes in fetal development (22). Prothrombin plays a central role in the blood coagulation system by triggering the activation of platelets, converting soluble fibrinogen into insoluble fibrin polymer, and activating regulatory pathways that control the rate of further thrombin formation (29). Another target organ for aPT antibodies may be the endothelium; proposed effects of aPT antibodies on endothelial cells include inhibition of thrombin-mediated endothelial cell prostacyclin release and protein C activation (30). In addition, aPT antibodies may recognize the prothrombin–anionic phospholipid complex on the endothelial cell surface, thus activating endothelial cells and inducing procoagulant substances via prothrombin, leading to a hypercoagulable state (31). aPT antibodies may also promote thrombosis by facilitating prothrombin interactions with damaged blood vessel walls and promoting thrombin generation, leading to a hypercoagulable condition.

In conclusion, women with a history of RSA have high circulation concentrations of aPT antibodies (IgG and IgM isotypes). Further prospective studies are needed to confirm the causal association between aPT antibodies and pregnancy loss.
References