

A case-control study on the association of idiopathic recurrent pregnancy loss with autoantibodies against β 2-glycoprotein I and annexin V

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Abstract

Whereas antiphospholipid antibodies (aPL) are associated with thrombotic events and recurrent spontaneous abortion (RSA), the contribution of anti- β 2 glycoprotein 1 (β 2GPI) and anti-annexin V antibodies as risk factors for RSA remain poorly understood. We investigated anti- β 2-GPI and anti-annexin V IgM and IgG antibodies as potential risk factors for RSA in 200 women with more than three consecutive idiopathic RSA, and 200 age-matched, healthy, parous women. Pearson's chi squared test analysis showed that while anti- β 2-GPI IgG ($P = 0.416$) and IgM ($P = 0.72$) were comparable between patients and controls, elevated anti-annexin V IgG ($P = 0.006$), but not IgM ($P = 0.084$), was more pronounced in patients. Higher frequencies of elevated IgG-only ($P = 0.005$), but not IgM-only ($P = 1.000$; OR = 6.66), anti-annexin V antibodies were noted among patients. Multinomial regression analysis showed that body-mass index (overweight and obesity; $P = 0.008$), education status ($P < 0.001$) and anti- β 2-GPI IgM ($P = 0.033$), but not IgG ($P = 0.723$), were associated with early abortion, while anti- β 2-GPI IgG ($P = 0.030$) and anti-annexin V IgG ($P = 0.004$) were associated with late RSA. For combined early-late RSA, the only variable selected was education status ($P < 0.001$), and neither anti-annexin V nor anti- β 2-GPI IgM and IgG was associated with early-late RSA. Accordingly, anti-annexin V and anti- β 2-GPI should be regarded as independent risk markers of RSA.

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Introduction

Pregnancy loss after implantation is frequent (Wilcox *et al.* 1988, Chaouat *et al.* 1995), and idiopathic recurrent spontaneous abortion (RSA) is often seen in otherwise healthy women (Mahjoub *et al.* 2005, Quenby *et al.* 2005). While several mechanisms have been proposed for early RSA (Huang *et al.* 2005), the exact causes of late miscarriage remain poorly understood. Inherited thrombophilia, including point mutations in factor V (Leiden) and prothrombin (G20210A) genes (Mahjoub *et al.* 2005), and antiphospholipid (aPL) antibodies, including anticardiolipin (aCL) antibodies and lupus anticoagulant (LA) (Mtiraoui *et al.* 2005), constitute significant risk factors for late RSA (Gris *et al.* 1999, Norwitz *et al.* 2001), since women who are carriers of prothrombotic gene polymorphisms or aPL are predisposed to pregnancy failure due to placental thrombosis (Gris *et al.* 1999, Martinelli *et al.* 2002).

Insofar as they recognize many phospholipids, phospholipid-binding proteins, or both, aPL antibodies have been associated with a hypercoagulable state – antiphospholipid syndrome (APS) – which arises from platelet activation, impairment of the endothelial system and facilitation of blood coagulation (Mercanoglu *et al.* 2004), and have been implicated in pregnancy complications (Gris *et al.* 2003, Levine *et al.* 2002, Rand *et al.* 2005). The precise mechanism of aPL in precipitating fetal loss is unclear, and may include precipitation of a prothrombotic state in the uteroplacental vasculature (Matsubayashi *et al.* 2001). β 2-Glycoprotein I (β 2-GPI), a single-chain 50 kDa glycoprotein and a target of aPL (Galli *et al.* 1990), was found to be involved in RSA, as its reduced expression was reported in women with APS (Miyakis *et al.* 2004). Antibodies to β 2-GPI (anti- β 2-GPI) were described as prothrombotic in individuals with APS (Falcon *et al.* 1997), and were suggested to play a role in RSA, since they inhibit trophoblast proliferation (Norwitz *et al.* 2001), and

enhance uteroplacental thrombosis and abnormal placentation (Gris *et al.* 2003).

In addition to β 2-GPI, annexin V is a potent anticoagulant that regulates exocytosis and syncytiotrophoblast membrane fusion (Arai *et al.* 2003), and has been implicated in RSA, as shown by findings that anti-annexin V antibodies induce thrombosis in human umbilical vein endothelial cells (HUVEC) (Arnold *et al.* 2001), and that their concentrations are reduced in isolated placenta from women with aPL-associated RSA (Gris *et al.* 1999, Matsubayashi *et al.* 2001). Antibodies against annexin V may interfere with syncytiotrophoblast function (Rand *et al.* 1994, 2005), resulting in altered expression of anionic lipids on the plasma membrane, and induction of apoptosis in HUVEC (Ailus *et al.* 1996). Others argue against involvement of anti-annexin V with RSA, since comparable prevalence rates of IgG (25% vs 23%) and IgM (27% vs 28%) anti-annexin V were seen in RSA vs control women respectively (Bizzaro *et al.* 2005).

We previously reported on the association of aCL antibodies and LA with idiopathic RSA, where elevated aCL antibody-only, positive LA only, or combined elevated aCL-positive LA were consistently higher in RSA patients (Mtiraoui *et al.* 2005). Since the exact role and clinical significance of anti- β 2-GPI and anti-annexin V IgG and IgM antibodies as risk factors for RSA are unclear, this study aimed to determine the prevalence of anti-annexin V and anti- β 2-GPI IgG and IgM antibodies in women with idiopathic RSA, and to evaluate their contribution with other confounding variables in RSA.

Subjects and Methods

Study subjects

From 2002 to 2004, 200 women with three or more unexplained consecutive pregnancy losses 5–30 weeks post-

gestation (mean age 28.6 ± 5.6 years), and attending the hospital maternity service of Sousse (Tunisia), were recruited (Table 1). Pregnancy losses were classified as early (5–10 weeks) and late (11–30 weeks). Exclusion criteria included induced abortions, infections, systemic disease, uterine structural abnormalities, and personal or family history of thrombosis. As control, 200 age-matched, healthy women with uncomplicated pregnancies (mean 28.2 ± 5.5 years) were recruited (Table 1). Patients and controls were asked to complete a questionnaire detailing demographic details, number and outcome of pregnancies, and RSA risk factors, and to sign an informed, consent form. The study was conducted after all institutional ethics requirements were met. Blood samples were obtained from study participants 8–12 weeks after the last pregnancy.

Antibody measurements

Serum anti- β 2-GPI IgG and IgM levels were measured by quantitative ELISA, using the Zymutest kit according to the manufacturer's instructions (Hyphen Biomed, Neuville-Sur-Oise, France). Results of <20 U/ml were interpreted as negative, while specimens with values of ≥ 20 U/ml were considered positive. Serum samples were also tested for anti-annexin V IgG and IgM by quantitative ELISA, using the Zymutest kit, according to manufacturer's instructions (Hyphen Biomed). Results of <20 U/ml were interpreted as negative and results of ≥ 20 U/ml as positive.

Statistical analysis

First, we examined means and standard deviation of all biomarkers in case and control patients. Statistical analysis was performed on SPSS v. 11.5 statistics software (SPSS, Chicago, IL, USA). Data were expressed as percentages of the mean. The Mann–Whitney nonparametric U-test, regression multinomial logistic and Pearson's chi-square test were used to assess intergroup significance. In

Table 1 Clinical characteristics of patients and controls.

	Patients ¹	Controls ¹	P	OR	95% CI
Age	28.68 $\pm$ 5.61	28.24 $\pm$ 5.51	0.434 ²		
Regional	49:50:41:60	42:43:43:72	0.531 ³		
Smokers	9/191	9/191	1.000 ³	1.000	0.389–2.574
Education			$<0.001^3$		
Intermediate	41	69			
Secondary	81	92			
University	78	39			
Alcohol	5	5	1.000 ³	1.000	0.285–3.509
BMI ⁴	25.78 $\pm$ 4.01	24.62 $\pm$ 3.75	0.360 ²	1.159	0.395–1.923
Oral contraceptives	51	46	0.641 ³	1.146	0.725–1.811
Pregnancy outcomes					
Live	0.51 $\pm$ 0.72	3.81 $\pm$ 1.40	$<0.001^2$	3.300	3.081–3.519
Early loss	2.29 $\pm$ 1.30	0.02 $\pm$ 0.14	$<0.001^2$	2.270	2.088–2.452
Late loss	1.34 $\pm$ 1.30	0.04 $\pm$ 0.184	$<0.001^2$	1.305	1.122–1.488

¹ A total of 200 patient and 200 controls were included.

² Fisher's exact test.

³ Pearson's chi-square test.

⁴ Body-mass index, weight (kg)/(height (m))².

addition, the odds ratios (OR) and 95% confidence intervals (CI) were calculated. Statistical significance was set at $P < 0.05$.

Results

Patients and controls

The characteristics of patients and controls are summarized in Table 1. Both patients and controls had similar age ($P = 0.434$) and body-mass index (BMI; $P = 0.360$), and were from different parts of Tunisia ($P = 0.531$). While there was no significant difference between patients and controls in terms of alcohol consumption ($P = 1.000$) or smoking ($P = 1.000$), differences in terms of education background were noted among study participants ($P < 0.001$), exemplified by the higher proportion of patients having a university-level education, while controls generally had an intermediate level education.

Prevalence of anti- β_2 -glycoprotein I (anti- β_2 -GPI) IgG and IgM antibodies

The status of anti- β_2 -GPI IgG and IgM, and that of anti-annexin V IgM and IgG antibodies were assessed. Anti- β_2 -GPI IgG and IgM, and anti-annexin V IgM and IgG were categorized as normal or elevated according to a cutoff value of 20 U/ml. Anti- β_2 -GPI IgG ($P = 0.416$) and IgM ($P = 0.72$) were comparable between patients and controls (Table 2). In contrast, elevated anti-annexin V IgG ($P = 0.006$), but not IgM ($P = 0.084$), was more pronounced in patients than control parous women (Table 2).

Analysis of anti-annexin V antibodies

In view of the association of anti-annexin V, but not anti- β_2 -GPI antibodies with RSA, we tested the anti-annexin V IgM and IgG status (normal, elevated) in patients and controls. Higher frequencies of elevated IgG-only ($P = 0.005$), but not IgM-only ($P = 1.000$), were noted among patients (Table 3). Only one patient presented with elevated IgM-elevated IgG.

Risk factors for pregnancy loss

Predictors of early, late and early-late abortions were determined by performing three logistic regression analysis models with the dependent variable being early, late, and early-late RSA, and the independent potentially confounding variables being age, BMI, use of oral contraceptives, education, anti-annexin V IgM and IgG, and anti- β_2 -GPI IgM and IgG (Table 4). The only variables that were selected by this technique for the first outcome (early RSA) were BMI of > 25 ($P = 0.008$), education status ($P < 0.001$) and anti- β_2 -GPI IgM ($P = 0.033$) (Table 4). For the second outcome (late RSA), it was anti- β_2 -GPI IgG ($P = 0.030$) and anti-annexin V IgG ($P = 0.004$). For the third outcome (early-late), the only variable selected was education status ($P < 0.001$). Adjusting for the variables selected, anti- β_2 -GPI IgM and IgG and anti-annexin V IgM and IgG were not associated with combined early-late RSA (Table 4).

Discussion

Autoimmune factors involving production of antibodies targeting phospholipids (cardiolipin and phosphatidylserine) or phospholipid-binding proteins (β_2 GPI and annexin V) were previously described as risk factors for RSA (Martinelli *et al.* 2002, Gris *et al.* 2003, Rand *et al.* 2005). Unlike the well-documented involvement of aCL and LA, the role of anti-annexin V and anti- β_2 -GPI antibodies in idiopathic RSA has yet to be established, although it is presumed that it involves precipitation of a prothrombotic state that favors altered placentation, poor fetal circulation and possibly induction of apoptosis (Arai *et al.* 2003, Rand *et al.* 2005).

Results from this study demonstrated that anti-annexin V, but not anti- β_2 -GPI, antibodies were elevated in women with idiopathic RSA. Added to our previous finding on association of aCL and LA with RSA (Mtiraoui *et al.* 2005), these results indicate that the presence of these autoantibodies is closely related to RSA, via a mechanism which requires participation of immune and nonimmune factors in precipitating a prothrombotic state that favors

Table 2 Prevalence of anti- β_2 -GPI and anti-annexin V IgG and IgM antibodies.

Antibody	Status	Patients ¹	Controls ¹	<i>P</i>	OR	95% CI
Anti- β_2 -GPI IgG ²	Normal	191 (49.5%)	195 (50.5%)	0.416 ³	1.84	0.61–5.58
	Elevated	9 (64.3%)	5 (35.7%)			
Anti- β_2 -GPI IgM ²	Normal	195 (49.7%)	197 (50.3%)	0.72 ⁴	1.68	0.397–7.14
	Elevated	5 (62.5%)	3 (37.5%)			
Anti-annexin V IgG ²	Normal	187 (48.6%)	198 (51.4%)	0.006 ⁴	6.88	1.53–30.91
	Elevated	13 (86.7%)	2 (13.3%)			
Anti-annexin V IgM ²	Normal	184 (48.8%)	193 (51.2%)	0.084 ³	2.4	0.96–5.96
	Elevated	16 (69.6%)	7 (30.4%)			

¹ Study comprised 200 patients and 200 controls.

² Determined according to normal < 12.5 UA/ml; elevated > 12.5 UA/ml.

³ Pearson's Chi Squared test.

⁴ Fisher's exact test.

Table 3 Isotype distribution of anti-annexin V antibodies.

IgM state	IgG state	Patients	Controls	P ²	OR	95% CI
Normal	Normal	172 (93.5) ¹	191 (99.0)	0.005	6.66	1.47–30.19
	Elevated	12 (6.5)	2 (1.0)			
Elevated	Normal	15 (93.8)	7 (100.0)	1.000	0.68	0.51–0.91
	Elevated	1 (6.3)	0 (0.0)			

¹ Percentage of total within IgM state subcategory.² Chi-square test.

fetal loss. Multivariate regression analysis demonstrated that anti- β_2 -GPI IgM was associated with early RSA, while anti-annexin V IgG was a significant risk factor for (exclusively) late RSA. Neither anti- β_2 -GPI nor annexin V IgG or IgM was associated with combined early-late RSA. Interestingly, university education was associated with early and early-late RSA. While the cause of this remains speculative, it is possible that sociocultural habits (Tan *et al.* 1995), together with career-related factors (Bocciolone *et al.* 1989, Tan *et al.* 1995), may have contributed to RSA in university-educated women, the majority of whom are working women.

Results on the role of anti- β_2 -GPI in RSA are controversial, and some reports described an association of anti- β_2 -GPI IgG with RSA (Lee *et al.* 1999, Gris *et al.* 2000), and others implicated IgM (Foratseiro *et al.* 1997), but not IgG (Lynch *et al.* 1999, Arnold *et al.* 2001) anti- β_2 -GPI, as potential risk factor for idiopathic RSA, as also shown here. In our hands, the presence of anti- β_2 -GPI IgM was associated with (exclusively) early RSA, while IgG was associated with (exclusively) late RSA, in apparent agreement with previous studies which also found no significant association of anti- β_2 -GPI antibodies in women with three or more consecutive pregnancy losses (Lynch *et al.* 1999, Arnold *et al.* 2001), and in antiphospholipid-positive patients with obstetric complications (two or more spontaneous fetal losses) (Foratseiro *et al.* 1997). This may be explained by differences in assay sensitivity, and the low number of patients (21–44) included in some studies

(Ailus *et al.* 1996, Foratseiro *et al.* 1997). Insofar as aCL antibodies target plasma proteins bound to anionic phospholipids, including β_2 -GPI (Marai *et al.* 2005), it was suggested that the association of anti- β_2 -GPI with RSA was dependent on aCL antibody status, since anti- β_2 -GPI antibody was elevated in aCL-negative patients (Lee *et al.* 1999). This does not appear to be the case here, since comparable prevalence rates of anti- β_2 -GPI were seen in patients and controls, irrespective of aCL isotype ($P = 0.569$) or status ($P = 0.495$).

In accordance with previous findings (Gris *et al.* 2000, 2003, Matsubayashi *et al.* 2001), anti-annexin V antibodies were significantly higher in RSA patients than in fertile women. Logistic regression analysis confirmed the association of anti-annexin V IgG in late RSA, which was reminiscent of the findings of the Nimes group that anti-annexin V IgG is an independent risk factor for unexplained RSA (Gris *et al.* 2000, 2003), and in apparent disagreement with other reports, which disputed such an association (Siaka *et al.* 1999, Arnold *et al.* 2001, Arai *et al.* 2003, Bizzaro *et al.* 2005). However, the small sample size (Siaka *et al.* 1999, Arnold *et al.* 2001), coupled with poor assay sensitivity (Siaka *et al.* 1999), and ethnic origin (Arai *et al.* 2003, Bizzaro *et al.* 2005) employed in the latter studies warrants scrutiny in evaluating their conclusion.

While RSA may be partly explained by placental thrombosis precipitated by endothelial cell activation and annexin V displacement, this does not account for all miscarriages, thus pointing to an antibody-directed mechanism. Accordingly, the presence of antibodies that bind trophoblast-associated β_2 -GPI may interfere with trophoblast cell maturation, resulting in defective placentation. In addition, as annexin V acts as an inhibitor of lipid-dependent blood coagulation, antibodies against it may serve a procoagulant function. This was supported by the findings that administration of annexin V inhibited thrombus formation (Romisch *et al.* 1991) and fibrin accretion (Van Ryn-McKenna *et al.* 1993), and administration of anti-annexin V induced placental thrombosis, necrosis

Table 4 Regression analysis.

Variable	Stage of pregnancy loss		
	Early	Late	Early + late
Age	1.30 (0.67–2.51) ¹	1.64 (0.87–3.11)	0.96 (0.64–1.42)
BMI ²	0.46 (0.26–0.82)*	0.98 (0.56–1.70)	0.73 (0.51–1.03)
Oral contraceptives	1.77 (0.58–5.36)	0.47 (0.22–1.01)	0.91 (0.52–1.60)
Education ³	0.33 (0.18–0.61)*	0.63 (0.39–1.02)	0.52 (0.38–0.73)*
Anti-annexin V			
IgG	3.94 (0.39–39.94)	17.47 (2.43–125.49)*	5.02 (0.98–25.68)
IgM	2.03 (0.39–10.68)	2.10 (0.46–9.63)	1.90 (0.66–5.43)
Anti- β_2 GPI			
IgG	1.51 (0.16–14.58)	4.90 (1.17–20.54)*	1.06 (0.26–4.40)
IgM	9.00 (1.20–67.72)*	2.22 (0.21–24.00)	1.40 (0.21–9.30)

¹ OR (95% CI).² Reference being normal BMI (20–25).³ Reference being university education.* $P < 0.05$ vs controls.

and fetal loss (Wang *et al.* 1999). Mechanistically, it was hypothesized that anti-annexin V antibodies can disrupt or compromise the antithrombotic shield, thereby contributing to reproductive failure. Furthermore, by inducing trophoblast apoptosis and significantly reducing trophoblast gonadotropin secretion, anti-annexin V antibody was found to induce defective placentation (Di Simone *et al.* 2001).

As with similar studies, a limitation of our study is that anti-annexin V and anti- β 2GPI antibodies were measured in the patients after event establishment (pregnancy failure), thereby raising the possibility that these antibodies were the consequence, and not necessarily the cause of RSA, as was also suggested elsewhere (Gris *et al.* 2003). This can be resolved only by a prospective study that monitors changes in pregnancy outcomes in antibody-positive versus antibody-negative patients.

In conclusion, our study proposes a role for anti- β 2GPI IgM (but not IgG) and anti-annexin V IgG antibodies in mediating exclusively early and late RSA respectively. This may serve as a diagnostic tool as part of the routine workup for women with idiopathic RSA, and also has therapeutic implications, evidenced by the utility of combining intravenous immunoglobulin in the standard therapy regimen, which allegedly resulted in excellent fetal and maternal outcome (Branch *et al.* 2000), in particular in cases of APS (Diejomaoh *et al.* 2002). However, the definitive utility of this treatment modality awaits the outcome of international randomized trials, which are currently in progress.

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