Med Clin (Barc). 2010;**134(10)**:433–438

MEDICINA CLINICA

www.elsevier.es/medicinaclinica

Original article

# Anti-annexin A5 antibodies in women with spontaneous pregnancy loss

Jaume Alijotas-Reig [a,*], Raquel Ferrer-Oliveras [b], María José Rodrigo-Anoro [c,d], Inmaculada Farran-Codina [b], Elisa Llurba-Olivé [b], Miquel Vilardell-Tarres [a], Manel Casellas-Caro [b,e]

[a] *Systemic Autoimmune Disease Unit, Department of Internal Medicine I, Hospital Universitari Vall d'Hebron, Department of Medicine, Universitat Autonoma, Barcelona, Spain*
[b] *Department of Obstetrics, Hospital Universitari Vall d'Hebron, Universitat Autonoma, Barcelona, Spain*
[c] *Immunology Unit, Clinical Laboratories, Hospital Universitari Vall d'Hebron, Universitat Autonoma, Barcelona, Spain*
[d] *Department of Cell Biology, Physiology and Immunology, Universitat Autonoma, Barcelona, Spain*
[e] *Department of Obstetrics and Gynecology, Universitat Autonoma, Barcelona, Spain*

ARTICLE INFO

*Article history:*
Received 10 June 2009
Accepted 16 September 2009
Available online 22 de diciembre de 2009

*Keywords:*
Anti-annexin A5 antibodies
Antiphospholipid antibodies
Antiphospholipid syndrome
Recurrent miscarriage
Unexplained fetal losses

ABSTRACT

*Background and Objective:* The aim was to evaluate the role of anti-annexin A5 (anti-ANXA5) antibodies as risk factor for recurrent miscarriage (RM) and unexplained fetal loss (UFL).

*Patients and methods:* Retrospective, cohort study. *Setting*: Vall d'Hebron University Hospital. *Subjects*: 122 women, in two groups: Study group: 54 women with RM/UFL and control group: 68 pregnant without RM/UFL. *Intervention*: Antiphospholipid, mainly anti-ANXA5 antibody analysis. Comparison of all antiphospholipid antibodies between groups.

*Results:* Antiphospholipid antibody (aPL) prevalence in the study group was 10/54 (14.8%) and 5/68 (7.3%) in control group ($p$=0.09). In the RM subgroup, it was 3/25 and 9/34 in UFL versus 5/68 in controls ($p$=0.013). Lupus anticoagulant (LA) was present in 4 cases, all belonging to the study group ($p$=0.011). Four out of 34 women with UFL were positive for anticardiolipin antibodies-IgG (IgG-aCL) versus 1/68 in controls ($p$=0.041). In RM subgroup, anti-ANXA5 antibodies were positive in 2/25 versus 3/68 in controls, and in UFL subgroup, 3/34 versus 3/68 cases ($p$=1.000).

*Conclusion:* According to our results, anti-ANXA5 antibodies should not be considered as a risk factor for RM/UFL.



## Anticuerpos anti-anexina A5 en mujeres con historia de abortos recurrentes

*Palabras clave:*
Anticuerpos anti-anexina A5
Anticuerpos anti-antifosfoolipido
Síndrome antifosfolípido
Aborto recurrente
Pérdida fetal inexplicada

RESUMEN

*Fundamento y Objetivo:* El objetivo principal fue evaluar el papel de los anticuerpos anti-anexina A5 (ac-anti-ANXA5) como factor de riesgo de abortos recurrentes (AR) y de la pérdida fetal inexplicada (PFI).

*Pacientes y Método:* Se trata de un estudio de cohortes retrospectivo. Se desarrolló en el Hospital Universitario Vall d'Hebron de Barcelona. Se estudiaron un total de 122 mujeres, en dos grupos: grupo de estudio, formado por: 54 mujeres con AR/PFI y grupo control, constituido por 68 gestantes sin historia de AR/PFI. Se estudiaron los anticuerpos antifosfolípido (aFL), con especial interés para los ac-anti-ANXA5. Se compararon los resultados entre ambos grupos, estudio y control.

*Resultados:* La prevalencia de positividad para los aFL fue de 10/54 (14,8%) en el grupo de estudio y de 5/68 (7,3%) en el grupo control (p=0,09). En el subgrupo de mujeres con AR, la prevalencia de los aFL fue de 3/25 y de 9/35 en el subgrupo afecto de PFI, versus 5/68 en el grupo control (p=0,013). El anticoagulante lúpico (AL) fue positivo en 4 casos, todos ellos pertenecientes al grupo de estudio (p=0,011). Cuatro de las 34 mujeres con historia de PFI tenían anticuerpos anti-cardiolipina isotipo IgG versus 1/68 en el grupo control (p=0,041). En el subgrupo de AR, los ac-anti-anexina A5 se detectaron en 2/25 casos versus 3/68 en el grupo control y 3/34 el subgrupo con PFI (p=1,000).

*Conclusiones:* De acuerdo con los resultados de nuestro estudio, los anticuerpos anti-anexina A5 no deberían ser considerados como factores de riesgo de AR y/o de PFI



* Corresponding author.
*E-mail address:* jalijotas@vhebron.net (J. Alijotas-Reig).

0025-7753/$ - see front matter © 2009 Elsevier España, S.L. All rights reserved.
doi:10.1016/j.medcli.2009.09.040

## Introduction

Pregnancy loss after implantation is frequent and idiopathic recurrent miscarriage is often seen in otherwise healthy women.[1] While several mechanisms have been proposed for early recurrent miscarriage, the exact causes of these and especially for late miscarriage are not completely understood.[2]

Various antiphospholipid and/or antiprotein (aPL and/or aP) antibodies, mainly anticardiolipin (aCL) and lupus anticoagulant (LA), have been suspected to be associated with recurrent pregnancy loss (RPL) in the absence of any common aetiology. However, the importance of anti-annexin A5 (anti-ANXA5) antibodies as risk factor for RPL remains unclear.[3–5] Insofar as they recognize many phospholipids, phospholipids-binding proteins, or both, aPL antibodies have been related to a specific hypercoagulable state, named antiphospholipid syndrome (APS).[6]

Although it was initially thought that aPL reacts directly with phospholipids, subsequent studies have shown that critical epitopes also involve a number of phospholipid-binding proteins. The most prevalent of these proteins is $Beta_2$-glycoprotein 1,[7] although in the 1990's many authors assumed that ANXA5 was a cofactor for aPL as well.[3] On the other hand, members of the workshop panel, that met in Sydney before the eleventh international congress on antiphospholipid antibodies agreed that immunoglobulin IgG and IgM antibodies anti-aNXA5 should not be added to the definition of antiphospholipid syndrome (APS).[8]

ANXA5 belongs to a large family of structurally related, calcium-binding proteins, the annexins. Annexin A5, a 36 kDa glycoprotein, is a potent anticoagulant both in placental and in systemic circulation.[3,9] In the placenta, ANXA5 regulates syncytiotrophoblast membrane fusion,[10] and has been implicated in RPL[4,11–13]; thus ANXA5 antibodies induce apoptosis in human umbilical vein endothelial cells (HUVECs).[14] However, other authors argued against the relation of anti-ANXA5 antibodies and RPL, since comparable prevalence rates of IgG and IgM anti-ANXA5 antibodies were obtained from study group versus control group.[15,16] In consequence, the importance of anti-ANXA5 antibodies remains controversial. The aim of this study was therefore to analyze the role of anti-ANXA5 antibodies as risk marker for pregnancy losses, including RM and UFL in otherwise healthy women.

## Patients and methods

*Study population*: We retrospectively studied a population that included 54 women with 59 episodes of pregnancy loss recruited in the High Obstetric Risk Unit of our institution, a tertiary teaching hospital, from January 2007 to December 2007. Pregnancy loss included recurrent miscarriage (RM) defined as three or more consecutive pregnancy losses before 10 weeks of gestation and unexplained fetal losses (UFLs) defined as one or more pregnancy losses at, or beyond, 10 weeks of pregnancy. These women were recruited within 4 weeks after their last loss. First blood test was performed at the same time that clinical inclusion was made. A second confirmative blood test for aPL was drawn within 12 weeks and 6 months at the latest. Only cases with embryonic or fetal losses after an ultrasound with fetal pulse were included in the study. Ethnicity, body mass index (BMI), and smoking habit were analyzed. Of these 54 women, 25 experienced early RM and 34 were affected by UFL. These women were found to have no anatomic uterine malformations, hormonal dysfunctions, or chromosomal abnormalities in the couple as a cause for pregnancy loss. Hormonal studies included TSH, free-T4, and progesterone levels. Karyotype analyses were performed using cellular cultures and optic microscopy study. Previous arterial or venous thrombosis and presence of any data related to any systemic autoimmune disease, mainly systemic lupus erythematosus (SLE), were ruled out. Women with past or present history of glucose metabolism alterations were also excluded.

*Control group*: 68 healthy pregnant women with at least one normal pregnancy were selected as a control group. These women had no history of PL and had a median age of 33.1 (range 18–48) years. These women were included over different gestational ages during normal pregnancy.

*Ethics approval*: We gave verbal information to the patients. Besides, this study was approved by the ethics committee of the Vall d'Hebron University Hospital. (CEIC-HUVH).

## Methods

### *Clinical assessment*

Patients were assisted by an obstetrician and by an internist/immunologist. All women were subsequently submitted to our protocol for the diagnosis of recurrent miscarriage as described before. Specific laboratory screening tests were: LA, aCL (IgG/IgM isotypes), and anti-ANXA5 antibodies (IgG/IgM isotypes) for all groups studied. Only patients with double positivity for aPL (positive on more than one occasion) were included in the study.

### *Sample collection/laboratory analysis*

Venous blood samples were collected with minimal venous stasis using a 19 gauge butterfly needle. Samples were collected in plain tubes for aCL and anti-ANXA5 antibodies, and into 0.109 mol/l trisodium citrate for LA testing. Sera IgG/IgM for aCL and for anti-ANXA5 antibodies were prepared by single centrifugation at 3.000 rpm for 15 min at room temperature. Platelet-poor plasma (PPP) for LA testing was prepared by double centrifugation at 3000 rpm for 20 min at room temperature. Samples were frozen and stored at -70 °C until assay.

### *Antiphospholipid antibody detection*

*Anti-cardiolipin antibodies*: *aCL* was assayed by using standardised immunometric quantitative ELISA according to Loizou and Harris recommendations,[17] defining the normal cut-off value in the assay as median IgG or IgM units plus three standard deviations of a healthy group. Reagents were supplied by ORGENTEC Diagnostika GmbH, Mainz, Germany. Results were expressed in either GPL or MPL (U/ml). Positive results were defined as $\geq$10 for IgG (GPL U/ml) and $\geq$7 for IgM (MPL U/ml).

*Anti-annexin A5 antibodies*: *Serum* anti-ANXA5 antibodies were measured using an indirect solid phase ELISA for the quantitative determination of IgG/IgM autoantibodies to annexin A5. Reagents were supplied by ORGENTEC Diagnostika GmbH, Mainz, Germany. All samples were run in duplicate and mean absorbance values calculated. Results were calculated by interpolation in a calibration curve using 4 logistic parameters (LP) and were expressed in arbitrary units (U/ml) since no international reference standard reagents are available. Positive results were defined as $\geq$8 U/ml for IgG and for IgM as well. Borderline values 5–8 U/ml were considered as negative.

The lower detection limit for anti-ANXA5 IgG/IgM antibodies was determined as 0.13 U/ml. Intra-day coefficient of variation (CV) at two levels of concentration varied from 6.2 and 5.5 to 9 and 5.4 to anti-ANXA5 IgG and IgM, respectively; inter-day CV

varied from 6 and 10.2 to 4.8 and 14.3 to anti-ANXA5 IgG and IgM respectively.

The statistical basis for significant differences between values was established by the criterion (cut-off) of mean plus 3 SD versus healthy women control group.

*Lupus anticoagulant*: LA was diagnosed according to the recommendations of the International Society of Thrombosis and Haemostasis.[18] Diluted Russell's time was performed using specific reagents supplied by Hemosl IL, Instrumentation Laboratory SpA, Milano, Italy.

### *Statistical analysis*

Descriptive statistics (mean ± SD) were calculated for continuous variables, and frequency statistics (number of cases and percentage) were calculated for categorical variables. Antiphospholipid antibodies positivity (LA, aCL, and anti-ANXA5) between miscarriages versus controls and foetal losses versus controls were examined using Fisher's Exact Test. All *p*-values were two sided, and statistical significance was assumed at $p<0.05$.

## Results

Finally, 54 cases (study group) and 68 pregnant women (control group) were analysed. Of these 54 women belonging to study group, 25 experienced RM and 34 were affected by UFL. Five women suffered from both RM and UFL. The age (mean ± SD) and number of miscarriage and UFL (mean ± SD) can be seen in Tables 1 and 2. The control group is younger than RM subgroup ($p$=0.009) but not younger than UFL subgroup. However, when we compare the mean age of whole study group with the mean age of control group, differences were not obtained. Only three women (study group) aged over 38 years old at the time of inclusion. Overall, IVF/ART has been used in 8 women, 4 of them belonging to study group and other 4 pertaining to controls (Table 1). None of them used donor oocytes. Ethnicity, BMI, and smoking habit were analyzed. Statistical differences between study group and control group were not found (Tables 1 and 2).

Laboratory results may be seen in Tables 3 and 4. Briefly, the overall prevalence of aPL positive in study group was therefore 10/54 (14.8%) and 5/68 (7.3%) in control group ($p$=0.09; Table 3). Lupus anticoagulant showed significant differences between the study group and controls (7.4% versus 0%; $p$=0.036).

**Table 1**
Demographic and general characteristics of study group versus control group

| | PL[a] (*n*=54) | Control (*n*=68) | *p*-value[b] |
|---|---|---|---|
| Age | 34.6 ± 4.1 | 33.1 ± 4.8 | 0.084 |
| Ethnicity | | | |
| Caucasian | 38/54 (70.37%) | 48/68 (70.50%) | |
| Hispanic | 13/54 (24.07%) | 18/68 (26.47%) | |
| African | 3/54 (5.55%) | 2/68 (2.94%) | |
| | | | 0.465 |
| Smoking | 16/54 (29.62%) | 21/68 (30.88%) | 1.000 |
| BMI[c] | | | |
| ≤24.9 | 30/54 (55%) | 49/68 (58.82%) | |
| 25–29.9 | 15/54 (27.7%) | 8/68 (26.47%) | |
| 30–34.9 | 6/54 (11.11%) | 8/68 (11.76%) | |
| >35 | 3/54 (5.5%) | 3/68 (4.42%) | |
| | | | 0.542 |
| IVF/ART[d] | 4/54 (7.41%) | 5/68 (7.35%) | 1.000 |

[a] PL: pregnancy losses (recurrent miscarriages plus unexplained fetal losses).
[b] PL versus control.
[c] BMI: body Mass Index (kg/m$^2$).
[d] IVF/ART: in vitro fertilization/assisted reproductive techniques.

**Table 3**
Antiphospholipid antibody comparison between in pregnancy loss whole and controls

| | PL (*n*=54) | Control (*n*=68) | *p*-value[a] |
|---|---|---|---|
| aPL | 10/54 (14.8%) | 5/68 (7.3%) | 0.090[b] |
| LA | 4/54 (7.4%) | 0/68 | 0.036[c] |
| ACL | | | |
| IgG | 4/54 (7.4%) | 1/68 (1.5%) | 0.169 |
| IgM | 2/54 (3.7%) | 1/68 (1.5%) | 0.583 |
| IgG/IgM | 4/54 (7.4%) | 2/68 (2.9%) | 0.404 |
| Anti-ANX A5 | | | |
| IgG | 2/54 (3.7%) | 3/68 (4.4%) | 1.000 |
| IgM | 3/54 (5.6%) | 0/68 | 0.084[b] |
| IgG/IgM | 4/54 (7.4%) | 3/68 (4.4%) | 0.698 |

aCL: anticardiolipin antibodies; aPL: antiphospholipid antibodies; anti-ANX A5: anti-annexin A5 antibodies; LA: lupus anticoagulant; PL: pregnancy losses (recurrent miscarriage+unexplained fetal loss).
[a] PL versus control.
[b] Borderline differences.
[c] Significant differences

**Table 2**
Age, obstetric background and general demographic characteristics of two subgroups of patients

| | RM (*n*=25) | Control (*n*=68) | *p*-value[a] | UFL (*n*=34) | Control (*n*=68) | *p*-value[b] |
|---|---|---|---|---|---|---|
| Age; mean (SD) | 36.1 ± 3.4 | 33.1 ± 4.8 | 0.009[c] | 33.7 ± 4.3 | 33.1 ± 4.8 | 0.559 |
| Ethnicity | | | | | | |
| Caucasian | 18/25 (72%) | 46/68 (67.6%) | | 24/34 (70.58%) | 46/68 (67.7%) | |
| Hispanic | 6/25 (24%) | 12/68 (17.6%) | | 8/34 (23.5%) | 12/68 (17.6%) | |
| African | 1/25 (4%) | 1/68 (1.47%) | 0.689 | 2/24 (5.88%) | 1/68 (1.47%) | 0.903 |
| Smoking habit | 7/25 (28%) | 21/68 (30.8%) | 1.000 | 9/34 (26.5%) | 21/68 (30.9%) | 0.818 |
| Miscarriage[d] n | 25/54 | – | – | – | – | – |
| Miscarriage; mean (SD) | 3.75 ± 1.2 (r:3–8) | – | – | – | – | – |
| Fetal loss[e]; n | – | – | – | 34/54 | – | – |
| Fetal loss; mean (SD) | – | – | – | 1.6 ± 0.8 (r:1–5) | – | – |

*r*: range; SD: standard deviation.
RM: recurrent miscarriage; UFL: unexplained fetal losses.
[a] Recurrent miscarriage versus control.
[b] Unexplained fetal losses versus control.
[c] Significant differences.
[d] Miscarriage: abortion suffered before 10 week of pregnancy.
[e] Fetal loss: miscarriage (abortion) suffered at 10 weeks or later of gestation.

**Table 4**
aPL antibody comparison in this series of patients

| | RM ($n$=25) | Control ($n$=68) | $p$-value[a] | UFL ($n$=34) | Control ($n$=68) | $p$-value[b] |
|---|---|---|---|---|---|---|
| aPL | 3/25 (12%) | 5/68 (7.3) | 0.440 | 9/34 (26.5%) | 5/68 (7.3%) | 0.013[c] |
| LA | 0/25 | 0/68 | – | 4/34 (11.7%) | 0/68 | 0.011[c] |
| aCL | | | | | | |
| IgG | 2/25 (8%) | 1/68 (1.4%) | 0.175 | 4/34 (11.8%) | 1/68 (1.5%) | 0.041[c] |
| IgM | 1/25 (4%) | 1/68 (1.4%) | 0.460 | 2/34 (5.9%) | 1/68 (1.58%) | 0.257 |
| IgG/IgM | 2/25 (8%) | 2/68 (2.9%) | 0.292 | 4/34 (11.8%) | 2/68 (2.9%) | 0.090[d] |
| Anti-ANX A5 | | | | | | |
| IgG | 1/25 (4%) | 3/68 (4.4%) | 1.000 | 1/34 (2.9%) | 3/68 (4.4%) | 1.000 |
| IgM | 1/25 (4%) | 0/68 | 0.269 | 2/34 (5.9%) | 0/68 | 0.109 |
| IgG/IgM | 2/25 (8%) | 3/68 (4.4%) | 1.000 | 3/34 (8.9%) | 3/68 (4.4%) | 1.000 |

aPL: antiphospholipid antibodies; aCL IgG/IgM: anticardiolipin antibody IgG/IgM isotypes; LA: lupus anticoagulant; anti-ANXA5: anti-annexin A5 antibodies; RM: recurrent miscarriage; UFL: unexplained fetal losses.
[a] Recurrent miscarriages versus control.
[b] Unexplained fetal losses versus control.
[c] Significant differences.
[d] Borderline differences.

In women with RM, the prevalence of aPL was 3/25 (12%) versus 5/68 (7.35%) in controls ($p$=0.440) and 9/34 (26.47%) versus 5/68 (7.35%) ($p$=0.013) in the cohort of women with unexplained fetal losses. LA was present in four women affected by UFL and in no cases in a control group ($p$=0.011). Only four out of 54 (7.41%) women pertaining to study group were also positive for IgG-aCL versus 1/68 in controls (1.5%) ($p$=0.404). However, significant differences were obtained when we compared IgG-aCL between UFL subgroup and controls (0.041; Table 4).

In women with RM, anti-ANXA5 antibodies (IgG/IgM) were found positive in 2/25 (8%) versus 3/68 (4.4%) in control group and in 3/34 (8.9%) versus 3/68 (4.4%) cases in women with UFL respectively ($p$=1.000). When IgG and IgM isotypes were compared separately for patients and controls, the results did not reach any statistical significance (Table 4). In the control group the positive anti-ANXA5 cases were not associated with other aPL positive tests. In whole study group (RM+UFL), three cases had only anti-ANXA5 antibody as an aPL marker. Besides, when the data of the whole study group were analyzed, significant results for anti-ANXA5 antibodies were not either obtained, although almost 6% in pregnancy loss versus 0% in controls for IgM anti-ANXA5 antibodies were obtained ($p$=0.084; Table 3).

## Discussion

The mechanism of fetal loss related to aPL is still poorly understood, although placental thrombosis may cause infarction and eventual fetal death.[1,2] In addition to up-regulated coagulation, placental inflammation and direct trophoblast damage by aPL antibodies during syncytium formation have been suggested.[19,20]

ANXA5 is the most abundant annexin among mammals, is mostly found intracellularly at vesicles and plasma membranes, and it is highly expressed on the apical surfaces of syncytiotrophoblast and human endothelial cells.[10,14] It may also be found in small amounts in blood, amniotic fluid, and seminal plasma.[3,20] ANXA5 anticoagulant properties are linked to its ability to bind phospholipids, especially phosphatidylserine (PS). Extracellular ANXA5 recognizes the exposure to PS on the outer membrane and binds to PS on the membrane. This complex forms a shield that prevents an excessive phospholipid-dependent coagulation reaction.[3,9]

When we compared study versus control group, only a small but significant difference was obtained when compared to the age of RM subgroup versus controls. This age difference may be explained, perhaps, because women in RM subgroup spent much time in the attempt to have a normal pregnancy, because previous pregnancies were unsuccessful (3,8; $r$=3–8). Another possible explanation could have relation to the size of the sample. Accordingly, if we had included more women, the age differences probably would have disappeared. When we analyzed the BMI, almost 45% of these women in both groups had some degree of overweight but with no differences within them. Thus, according to their age, ethnicity, smoking habit, and BMI, it seems that we compared homogeneous populations.

In our work, the prevalences of aPL were different in the study group and controls and these values reached near significant differences. This may be possibly explained by the size of study group or it may be due to the weight of the anti-ANXA5 antibodies in the whole results, because of their negative results. When the same parameter is separately focused in the two study subgroups, different results were obtained. No differences were seen in the case of the RM subgroup. However, when we analyze the prevalence of aPL in the UFL versus the control group a marked difference was seen (26.5 versus 7.3%), mainly for LA and aCL IgG isotype. This disagreement between the aPL prevalence in the two subgroups reinforces the hypothesis that different pathological mechanisms might be involved in this kind of obstetric pathology.[2,6,17]

Previous papers focusing on the role of anti-ANXA5 antibodies in pregnancy losses seem to demonstrate a relationship between them and early recurrent miscarriage, implantation failures, and fetal losses.[4,7–13]. In the same way, two papers deserve some comments. Zammiti et al.[21] obtained interesting results. They assessed anti-ANXA5 IgG and IgM antibodies as risk factor in 172 women with RM. Anti-ANXA5 IgG but not IgM antibodies were associated only with late RM, but not with early RM. Finally, when they studied these antibodies for combined early–late RM, neither anti-ANXA5 IgG nor anti-ANXA5 IgM antibodies were associated with early–late RM. On the other hand, Arnold et al.[22] showed that ANXA5 IgG antibody prevalence was significantly increased in aPL-positive women with RM compared with aPL-negative women with RM ($p$=0.01). However, ANXA5 antibody positivity was not an independent risk marker for RM.[22] In our study, RM and UFL were also taken separately because the etiology of both seemed to have different mechanisms. Nevertheless, when we analyzed the data of these two subgroups together, significant differences were not obtained, although IgM-anti-ANXA5 antibodies raised borderline differences (6% versus 0%). Once again, no significant results were seen when comparing

antiphospholipid-positive or -negative women with pregnancy losses with those who were anti-ANXA5 positive. Overall, these findings suggest that anti-ANXA5 antibodies do not constitute an independent risk factor or a biological marker for RM/UFL. Moreover, these findings agree with the results obtained in case-control studies performed by Wu et al.[16] and Siaka et al.,[23] which showed anti-ANXA5 antibodies were not associated with early or late RM, and they were not an independent risk marker for recurrent pregnancy losses as well.

In the same way, Bizzaro et al.[5] studied a large cohort of women that had 1038 pregnancies, concluding that IgG/IgM anti-ANXA5 antibodies measured in healthy women does not give a positive predictive lead towards the possibility of a miscarriage, and it is not useful to evaluate the risk of miscarriage at the beginning of pregnancy.

Recently, Galli et al showed that IgG but not IgM anti-ANXA5 antibodies may be related to miscarriage.[24] Despite the fact that ANXA5 concentration is reduced in isolated placenta from women with aPL associated with pregnancy losses[25,26] anti-ANXA5 antibodies may also interfere with syncytiotrophoblast function,[10] our results do not agree as expected according to ANXA5 physiology and anti-ANXA5 antibody pathophysiology. Apart from sample size and criteria population selection, technical pitfalls could explain the lack of positive results. The anti-ANXA5 antibody detection system has not been standardized yet, but ELISA assay prevailed. High binding plates (degree of saturation of plates), may provoke conformational changes of the antigen, modifying the results. Other factors to consider are calcium and antigen (annexin) concentrations, and both type of buffer used and dilutions. This is important in order to reduce non-specific bindings.[3] In the end, the cut off value had a major influence on the prevalence of anti-ANXA5. The number of standard deviations (from 2 to 5) above the mean value of small control groups (20–30 samples) could lead to incomparable results.[3] In the end, although the study group sample was small, it had been well defined and confounding factors had been excluded. The size of control group seems appropriate (68 individuals) in order to obtain reliable significant differences.

Although it may be expected that anti-ANXA5 antibodies played a similar role to that of antiβ2-GPI, even in LA and aCL negative patients,[6,8,27] our results are disappointing in this regard. Apart from the known anticoagulant properties related to ANXA5, it is interesting to note that, surprisingly, a transgenic ANXA5-deficient mouse was found to be fertile.[28] On the other hand, in our study, 4 cases of women affected by RM/UFL had only anti-ANXA5 antibodies as a unique and recurrent aPL marker but they could not be included within APS definition since anti-ANXA5 antibodies are not yet accepted by laboratory criterion for APS. In particular cases, anti-ANXA5 antibodies could act as a risk marker for RM/UFL. Definitive studies using more standardized laboratory detection assay are needed in order to clarify the exact role played for the anti-ANXA5 antibodies during pregnancy, and especially their relationship with recurrent pregnancy loss in both early and late recurrent miscarriage.

## Conflict of interest

The authors declare no conflicts of interest.

## Acknowledgements

We thank Montserrat Solans, biologist, for her laboratory assistance. We also thank Eduard Hermosilla for helping in the management of statistical data and Terence Carty for correcting the English grammar and style of the manuscript.

## References

1. Chouat G, Menu E, Delange G, Mareau JF, Khrishnan L, Hui L, et al. Immuno-endocrine interactions in early pregnancy. Human Reprod. 1995;10(Suppl. 2):55–9.
2. Mahjoub T, Mtiraoui N, Tamin H, Hizem S, Finan RR, Nsri B, et al. Assoxiation between adverse pregnancy outcomes and maternal factor V G1691A and prothrombin G20210A genotypes in women with a history of recurrent idiophatic miscarriages. Am J Hematol. 2005;80:12–9.
3. Bozic B, Irman S, Gaspersic N, Kveder T, Rozman B. Antibodies against annexin A5: detection pitfalls and clinical associations. Autoimmunity. 2005;38: 425–30.
4. Gris JC, Queré I, Sanmarco M, Boutiere B, Mercier E, Amiral J, et al. Antiphospholipid antibodies and antiprotein syndromes in non-thrombotic, non-autoimmune women with unexplained recurrent primary early fetal loss. Thromb Haemost. 2000;84:228–36.
5. Bizzaro N, Antico A, Musso M, Platzgummer S, Camogliano L, Tozzoli R, et al. A prospective study of 1038 pregnancies on the predictive value of anti-annexin V antibodies for fetal loss. Ann NY Acad Sci. 2005;1050:1–9.
6. Alijotas-Reig J. Towards the comprehension of heterogeneity of clinical manifestations related to antiphospholipid antibodies. Med Clin (Barc). 2005;125:187–9.
7. Galli M, Comfurius P, Maassen C, Hemker HC, de Baets MH, van Breda-Vriesman PJ, et al. Anticardiolipin antibodies directed not to cardiolipin but to a plasma protein cofactor. Lancet. 1990;335:1544–7.
8. Miyakis S, Lockshin MD, Atsumi T, et al. International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome. J Thromb Haemost. 2006;3:1–12.
9. Pigault C, Follenius-Wund A, Schmutz M, Freyssinet JM, Brisston A. Formation of two dimensional arrays of annexin V on phophatidylserine-containing liposomes. J Mol Bol. 1994;235:199–208.
10. Krikun G, Lockwood CJ, Wu XX, Zhou XD, Gulle S, Calandri C, et al. The expression of the placental anticoagulant protein, annexin V, by villous trophoblasts: immuno-localization and in vitro regulation. Placenta. 1994;15:601–12.
11. Matsubayashi H, Arai T, Izumi S, Sugi T, McIntyre M. Anti-annexin V antibodies in patients with early pregnancy loss or implantation failures. Fertil Steril. 2001;76:694–9.
12. Matsuda J, Gotoh M, Saitoh N, Gohchi K, Tsukamoto M, Yamamoto T. Anti-annexin antibody in sera of patients with habitual fetal loss or preeclampsia. Throm Res. 1994;75:105–6.
13. Gris JC, Perneger TV, Quere I, Mercier E, Fabbro-Peray P, Lavigne-Lissalde G, et al. Antiphospholipid antibodies/antiprotein antibodies, hemostasis-related autoantibodies, and plasma homocystein as risk factor for first pregnancy loss: a matched case-control study. Blood. 2003;102:3504–13.
14. Ailus K, Tulppala M, Palosou T, Ylikorkala O, Vaarala O. Antibodies to beta2-glycoprotein I and prothrombin in habitual abortion. Fertil Steril. 1996;66: 937–41.
15. Bizzaro N, Tonutti E, Villalta D, Tampoia M, Tozzoli R. Prevalence and clinical correlation of anti-phospholipid binding protein antibodies in anticardiolipin-negative patients with systemic lupus erythematosus and women with unexplained recurrent miscarriages. Arch Pathol Lab Med. 2005;129: 61–8.
16. Wu XX, Arslan AA, Wein R, Reutelingsperger CP, Lockwood CJ, Kucczynski E, et al. Analysis of circulating annexin A5 parameters during pregnancy: absence of differences between women with recurrent spontaneous pregnancy losses and controls. Am J Obstet Gynecol. 2006;195: 971–8.
17. Loizou S, McCrea JD, Rudge JC, Reynolds R, Boyle CC, Harris EN. Measurement of anticrdioipin antibodies by an enzyme-linked immunosorbent assay (ELISA). Standardisation and quantification of results. Clin Exp Immunol. 1985;62:738–45.
18. Brant JT, Triplett DA, Alving B, Scharrer I, On behalf of the Sub-committee on Lupus anticoagulant/Antiphospholipid Antibody of the Scientific and Standardization Committee of the ISTH. Criteria for the diagnosis of lupus anticoagulants: an update. Thrmb Haemost. 1995;74: 1185–90.
19. Di Simone N, Meroni PL, del Papa N, Raschi E, Caliandro D, de Carolis S, et al. Antiphospholipid antibodies affect trophoblast gonadotropin secretion and invasiveness by binding directly and through adhered B2-glycoprotein I. Arthritis Rheum. 2000;43:140–51.
20. Ruiz-Iratorza G, Martínez-Berriotxoa A, Egurbide MV. Síndrome antifosfolipídico en el siglo XXI. Med Clin (Barc). 2009, doi: 10.1016/j.medclin.2008.10.046.
21. Zammiti W, Mitiraoui N, Kallel C, Mercier E, Almawi WY, Mahjoub T. A case-control study on the association of idiophatic recurrent pregnancy loss with autoantibodies against beat2-glycoprotein I and annexin V. Reproduction. 2006;131:817–22.
22. Arnold J, Holmes Z, Pickering W, Farmer C, Regan L, Cohen H. Anti-beta2-glycoprotein I and anti-annexin V antibodies in women with recurrent miscarriages. Br J Haematol. 2001;113:911–4.
23. Siaka C, Lambert M, Caron C, Amiral J, Hachulla E, Hatron PY, et al. Low prevalence of nti-annexin V antibodies in antiphospholipid syndrome with fetal loss. Rev Med Intern. 1999;20:762–5.
24. Galli M, Borrelli G, Jacobsen EM, Marfisi RM, Finazzi G, Marchioli R, et al. Clinical significance of different antiphospholipid antibodies in the WASP study. Blood. 2007;110:1178–83.

25. van Heerde WL, de Groot PG, Reutelingsperger CPM. The complexity of the phospholipid binding protein annexin V. Thromb Haemost. 1995;73:172–9.
26. Rand JH, Wu XX, Quinn AS, Chen PP, McCrae KR, Bovill EG, et al. Human monoclonal antiphospholipid antibodies disrupt the annexin A5 anticoagulant crystal shield on phospholipid bilayers: evidence from atomic force microscopy and functional assay. Am J Pathol. 2003;163:1193–200.
27. Alijotas-Reig J, Casellas-Caro M, Ferrer-Oliveras R, Llurba-Olive E, Hermosilla E, Vilardell-Tarrés M. Are anti-Beta$_2$_Glicoprotein-I antibodies markers for recurrent pregnancy loss in lupus anticoagulant/anticardiolipin seronegative women? Am J Reprod Immunol. 2008;60:229–37.
28. Brachvogel B, Dikschas J, Moch H. Annexin A5 is not essential for skeletal development. Mol Cell Biol. 2003;23:2907–13.