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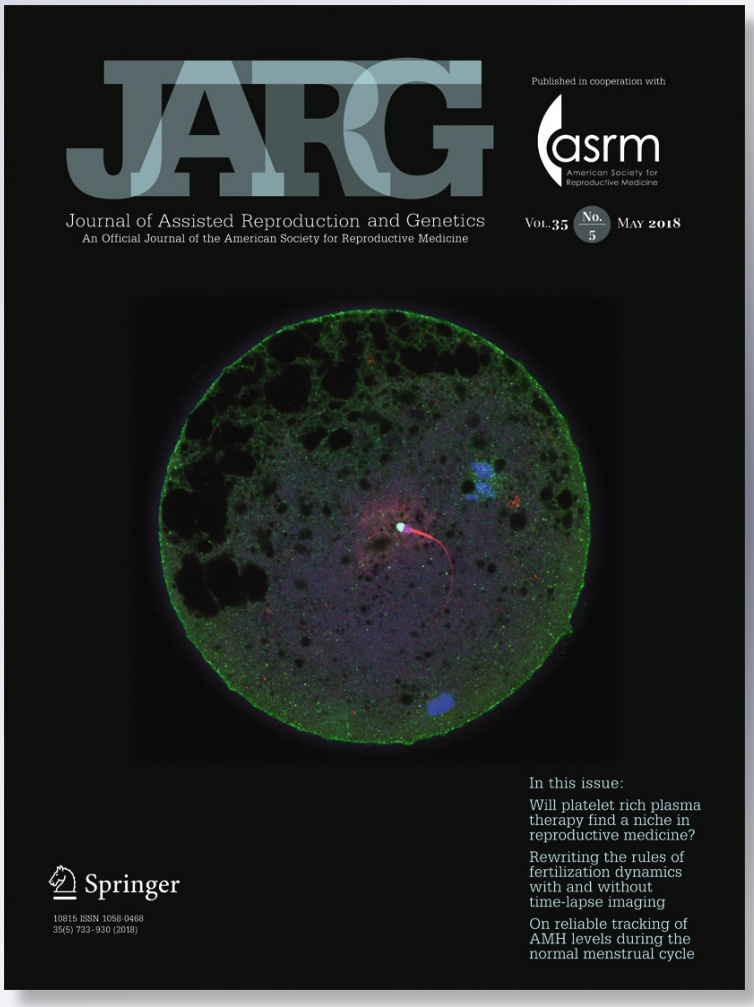

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GENETICS

Maternal carriers of the ANXA5 M2 haplotype are exposed to a greater risk for placenta-mediated pregnancy complications

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Abstract

Purpose Annexin A5 (ANXA5) is a protein abundantly expressed in normal placenta where it contributes to the healthy outcome of a pregnancy. Lower ANXA5 levels have been observed in *M2/ANXA5* haplotype carrying chorion. Consequently, this study aimed to assess the potential association of M2 maternal carrier status with the risk of recurrent pregnancy loss (RPL), the timing of miscarriages, and other obstetric complications, for the first time in a population from Latin America.

Methods This study was designed as a prospective recruitment of RPL patients with post hoc analysis. The distribution of the *M2/ANXA5* haplotype was compared between a group of 229 Argentine women with RPL and 100 parous controls, and was further analyzed in subgroups of patients stratified according to the timing of miscarriages and in relation to other obstetric complications.

Results No significant differences were found in the distribution of M2 haplotype among either RPL patients or the subgroups with embryonic, early fetal, or late fetal losses compared to parous controls. Notwithstanding, maternal *M2/ANXA5* was found to be independently associated with a higher risk of suffering intrauterine growth restriction (IUGR) and/or preeclampsia (PE). Simultaneously, the presence of inherited and/or acquired thrombophilia also proved to be an independent risk factor for these.

Conclusions The association found between the maternal carriage of the *M2/ANXA5* haplotype and an elevated risk of IUGR and/or PE supports the hypothesis that carrier status of this haplotype and the consequently reduced placental ANXA5 expression might be responsible, at least partially, for the onset of these gestational vascular complications.

Keywords Annexin A5 · M2/ANXA5 · Recurrent pregnancy loss · IUGR · Pre-eclampsia · Risk factor

Introduction

Among women attempting to conceive, 1–3% of them suffer from recurrent pregnancy loss (RPL). Although there are some well-described risk factors for RPL, such as parental chromosomal anomalies, anatomical uterine abnormalities, endocrine dysfunctions, and high levels of antiphospholipid antibodies, up to 50% of the cases still remain unexplained [1, 2]. Inherited thrombophilia has been also associated with RPL and other obstetric complications, and it has been proposed that the increased obstetric risk might be mediated by an impairment of placental perfusion [3–6].

Annexin A5 (ANXA5) is a protein that is abundantly expressed in normal placenta where it was proposed to exert an anticoagulant function [7]. Upon calcium loading, ANXA5 avidly binds to phosphatidylserine (PS) that is naturally exposed on the apical surface of placental syncytiotrophoblasts, setting up a bidimensional shield that interferes with phospholipid dependent clotting reactions [8–10]. Furthermore, it has been also proposed that ANXA5 displays an essential role promoting membrane repair that might be crucial for the integrity of a healthy placenta and very recent research ascribes

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facilitating function in the fusion of villous trophoblasts [11]. In pathophysiological scenarios, like the presence of antiphospholipid antibodies, anti-annexin antibodies, or even when ANXA5 protein levels are relatively decreased, this ANXA5 shield undergoes a disruption that has been suggested to be a considerable risk factor affecting the normal outcome of a pregnancy due to the occurrence of a hypercoagulable state in the intervillous space [12–14].

Almost a decade ago, Bogdanova et al. described the presence of different ANXA5 promoter haplotypes that, in reporter gene assays, showed to accordingly affect the expression levels of this protein. Among them, the carrier status of the M2 haplotype resulted in 60% reduction of ANXA5 promoter activity making it a logical candidate to have an impact on the etiology of RPL and/or other obstetric complications [15]. Further studies demonstrated that the M2 haplotype was responsible for a reduced expression of ANXA5 in chorionic placenta compared to the normal haplotype [16, 17], and that M2 carriers have an increased risk of suffering RPL and placenta-mediated pregnancy complications (PMPCs) in various European, Asian, and an Austronesian populations [15, 18–25].

The aims of this study were to assess the potential association of M2 maternal carrier status with the risk of RPL, to analyze its proposed influence according to the timing of miscarriages and to trace the link with other PMPC, for the first time in a population from Latin America.

Patients and methods

This field study to verify possible association of *M2/ANXA5* was designed as a prospective recruitment of RPL patients with post hoc analysis that was approved by the ethical committees of the institutions involved and was performed according to the principles of the Declaration of Helsinki. Informed consent was obtained from all participants.

Initially, the prevalence of the ANXA5 M2 haplotype was estimated in groups of 100 RPL patients and 50 strictly selected controls accordingly. Since the issuing comparison lacked on statistical power, recruitment continued until 329 subjects, including 229 cases and 100 controls, were selected for this study after meeting the corresponding inclusion criteria. Patients were enrolled between June 2010 and March 2016 in the Autoimmune, Thrombophilic Diseases and Pregnancy Section, Acute Care Hospital "Dr. Carlos G. Durand," Buenos Aires, Argentina. During this time, 229 women who were presented with RPL, fulfilled all inclusion criteria, and agreed to participate in the study were selected from a large cohort of 1185 consecutive miscarriage couples that attended to the medical center. RPL was defined, according to a slight modification of the statement of the Practice Committee of the American Society for Reproductive Medicine, as two or more unexplained failed pregnancies with the same partner [26]. Pregnancies were confirmed by sonography showing vital heartbeat, and pregnancy losses were identified when transvaginal ultrasound showed no heartbeat of an embryo with more than 7-mm crown to rump length, no embryo in a gestational sac having a mean sac diameter of more than 25 mm, or no appearance of an embryo within 7–10 days after the primary examination [27]. Patients were divided into three different groups according to fetal development at the time of pregnancy loss: embryonic (between 5 and 10 weeks of gestation), early fetal (between 10 and 15 weeks of gestation), and late fetal losses (after 15 weeks) [28–30].

Study participants were additionally stratified to the status of placental-mediated pregnancy complications: preeclampsia (PE), placental abruption, and intrauterine growth restriction (IUGR). PE was defined as high blood pressure (systolic blood pressure > 140 mmHg or diastolic blood pressure ≥ 90 mmHg) and 24-h proteinuria ≥ 0.3 g [31]. Placental abruption was defined as bleeding associated with partial or total separation of the placenta from its normal insertion site corresponding to the uterine fundus, this occurring from the 20 weeks of gestation until before delivery [32]. Fetuses with an estimated fetal weight < 3rd percentile or fetuses with a combination of estimated fetal weight < 10th percentile and with abnormal umbilical Doppler sonography were classified as IUGR [33].

RPL patients who presented with either endocrine disorders (clinical hypothyroidism [34], diabetes mellitus, polycystic ovary syndrome according to the Rotterdam criteria [35] and hyperprolactinemia), related infections, immunologic alterations (except from obstetric antiphospholipid syndrome (APS)), anatomic abnormalities of the uterus (detected by ultrasonography), premature rupture of membranes, or a history suggestive of cervical incompetency were excluded. Carrier status of fetal and/or parental chromosomal abnormalities, diagnosed through cytogenetic analyses, was also considered as an exclusion criterion.

Inherited thrombophilia, deficiencies in circulation levels of either Antithrombin (AT), Protein C (PC), or free Protein S (PS) and carrier status of Factor V Leiden or Prothrombin G20210A and acquired thrombophilia were evaluated in all patients. Factor V Leiden (FVL) and Prothrombin G20210A (PTm) genotypes were determined by real-time polymerase chain reaction (RT-PCR) using FV Leiden and FII G20210A specific reagents (Roche Diagnostics GmbH). PC, AT, and free PS levels were measured on a Destiny Max Coagulometer (Tcoag, Ireland). PC and AT were determined through a chromogenic method (Stago, France, and Xa-Chromogenix, Mölndal, Sweden, respectively) and free PS was determined through an immunoturbidimetric method (Liatest Stago, France). PC < 70%, AT < 80%, and free PS < 60% were considered as deficiencies in their circulation levels. As free PS levels exhibit variations during pregnancy,

the free PS cutoff levels according to week of gestation were considered, if it was necessary, as suggested by Szecsi et al. [36]. PC and AT levels do not show significant changes during pregnancy.

Obstetric APS was diagnosed according to updated international consensus classification criteria [37]. Clinical criteria included only obstetric morbidity (three pregnancy losses before the 10th week, and/or one pregnancy loss at or after the 10th week, and/or premature delivery before the 34th week because of PE or placental insufficiency). Laboratory criteria included positive test results for antiphospholipid antibodies, represented by positive test for lupus anticoagulant, and/or moderated or high titers for IgG and/or IgM anti-β2 glycoprotein I antibodies, and/or moderated or high titers for IgG and/or IgM anticardiolipin antibodies. Laboratory criteria were confirmed positive on two or more occasions at least 12 weeks after first positivity. Clinical characteristics of RPL patients are summarized in Table 1.

The parous control group consisted of 100 women with a history of normal pregnancies, at menopausal or post menopausal age (in order to be able to obtain complete obstetric history for each one of them), with two or more normal term deliveries of healthy and normal weight singletons and with no gestational pathology in any of their pregnancies.

All of the subjects were of Argentinian descent and shared common ethno-geographic and social origins, thus representative of the urban admixed population of Buenos Aires, Argentina. This population is the result of genetic admixture processes principally involving Europeans (mainly Spaniards and Italians) and Native Americans [38–40].

Genomic DNA was extracted from peripheral leukocytes using the High Pure PCR Template Preparation Kit for genomic DNA (Roche Diagnostics, GmbH, Mannheim, Germany). Genotyping of the proximal core promoter region of the *ANXA5* gene was conducted by amplicon sequencing as described by Bogdanova et al. [15].

Statistical analyses were performed using SPSS 15.0 for Windows (SPSS, Chicago, IL, USA). Genotype distributions among patients and control groups were assessed as carrier rates (percentage), according to a dominant model. Odds ratios (ORs) with corresponding 95% confidence intervals (CIs) were estimated by binary logistic regression to evaluate the strength of the association between maternal carrier status of *M2/ANXA5* and the risk of IUGR and/or PE; $p < 0.05$ was considered statistically significant.

Results

The proximal core promoter region of the *ANXA5* gene was genotyped in 229 Argentine women with RPL and in 100 parous controls. Both patient and control groups fulfilled Hardy-Weinberg equilibrium (HWE), and in fact, the parous women group was in perfect HWE with $p = 1$, as estimated through complete enumeration of genotypes (Table 2).

Table 1 Clinical characteristics of patients with recurrent pregnancy loss

Age	32 years [26–35][a]
BMI	24.2 kg/m^2 [21.9–27.1]
Number of losses	3 [2–4]
Embryonic losses	55 (24.0%)
Early fetal losses	63 (27.5%)
Late fetal losses	111 (48.5%)
IUGR	33 (14.4%)
Placental abruption	14 (6.1%)
Pre-eclampsia	16 (7.0%)
Obesity	9 (3.8%)
Obstetric APS	37 (16.1%)
FVL carriers	6 (2.6%)
PTm carriers	12 (5.2%)

[a] Values are expressed as median [interquartile range] or number (percentage)

In order to assess the potential RPL association in maternal *M2/ANXA5* carriers, its distribution was compared among the RPL and parous control groups and no significant difference was found ($p > 0.05$) (Fig. 1). Next, aiming to relate a possible influence to the timing of the losses, the M2 carrier rates were individually assessed in RPL subgroups stratified according to fetal development at the time of pregnancy loss (Table 2). There were no significant differences ($p > 0.05$) in the distribution of the M2 haplotype among RPL subgroups with either embryonic, early fetal or late fetal losses compared to parous controls. A higher M2 carrier rate in the subgroup of patients with early fetal losses was noted, although not of statistical significance (22.2%; 14/63).

Subsequently, a subgroup of patients with PMPC was tested for M2 association. Consequently, the potential impact of *M2/ANXA5* was assessed in the subgroups of patients that suffered from either IUGR and/or PE ($n = 41$). These patients had significantly higher M2 carrier rates as compared to parous controls, who have not experienced any of these gestational vascular complications (34.1%; 14/41 vs. 17.0%; 17/100; $p = 0.026$) (Fig. 1). Next, in order to exclude the RPL phenotype from the statistical analysis, PMPC patients were compared to the rest of RPL patients without obstetric complications (RPL - NO PE+IUGR). This comparison also yielded a significant difference in the distribution of *M2/ANXA5* (34.1%; 14/41 vs. 14.9%; 28/188; $p = 0.004$) (Fig. 1). Moreover, when evaluating "miscarriages" but not RPL as phenotype category, similar results were obtained when comparing RPL patients with IUGR and/or PE to a subgroup of RPL patients with at least one healthy delivery without experiencing any gestational vascular complications (RPL+HD) (34.1%; 14/41 vs. 17.4%; 19/109; $p = 0.047$) (Fig. 1).

Table 2 Genotype frequencies of ANXA5 promoter haplotypes in control groups and RPL patients

Genotype	Parous controls, $n = 100$		RPL, $n = 229$		Embryoniclosses, $n = 55$	Early fetal losses, $n = 63$	Late fetal losses, $n = 111$	IUGR/PE, $n = 41$	RPL-healthy delivery, $n = 109$	RPL NO IUGR/PE, $n = 188$
	Observed	Expected[a]	Observed	Expected						
N/N	71 (71.0)	72 (72.0)	159 (69.5)	161 (70.3)	46 (83.6)	41 (65.1)	74 (66.7)	22 (53.7)	74 (67.9)	137 (72.9)
N/M1	12 (12.0)	11 (11.0)	27 (11.8)	24 (10.5)	6 (10.9)	7 (11.1)	14 (12.6)	5 (12.2)	16 (14.7)	22 (11.7)
M1/M1	0 (0.0)	0 (0.0)	1 (0.4)	1 (0.4)	0 (0.0)	1 (1.6)	0 (0.0)	0 (0.0)	(0.0)	1 (0.5)
N/M2; M1/M2[b]	17 (17.0)	16 (16.0)	39 (17.0)	41 (17.9)	4 (7.3)	13 (20.6)	22 (19.8)	14 (34.1)	18 (16.5)	25 (13.3)
M2/M2	0 (0.0)	1 (1.0)	3 (1.3)	2 (0.8)	1 (1.8)	1 (1.6)	1 (0.9)	0 (0.0)	1 (0.9)	3 (1.6)
M2 carriers	17 (17.0%)	17 (17.0%)	42 (18.3%)	43 (18.3%)	6 (10.9%)	14 (22.2%)	23 (20.7%)	14 (34.1%)	19 (17.4%)	28 (14.9)

Values are expressed as number (percentage)

RPL recurrent pregnancy loss, *IUGR/PE* intrauterine growth restriction/pre-eclampsia

[a] Genotype frequencies expected under Hardy-Weinberg equilibrium

[b] A genotype M1/M2 was observed only once in the parous control group

Hence, in order to assess the strength of the association between maternal *M2/ANXA5* with a higher IUGR and/or PE risk in RPL patients with greater confidence, two different logistic binary regression analyses were performed considering the presence of either hereditary and/or acquired thrombophilia as covariates. The analyses were conducted in comparison to the RPL patients without obstetric complications (RPL - NO PE+IUGR) and to the RPL patients with healthy delivery (RPL+HD) subgroups. Among the 41 RPL with IUGR and/or PE patients, 10 presented with obstetric APS, 3 with FVL, and 1 with obstetric APS+FVL. Despite that approximately 35% of these patients already presented

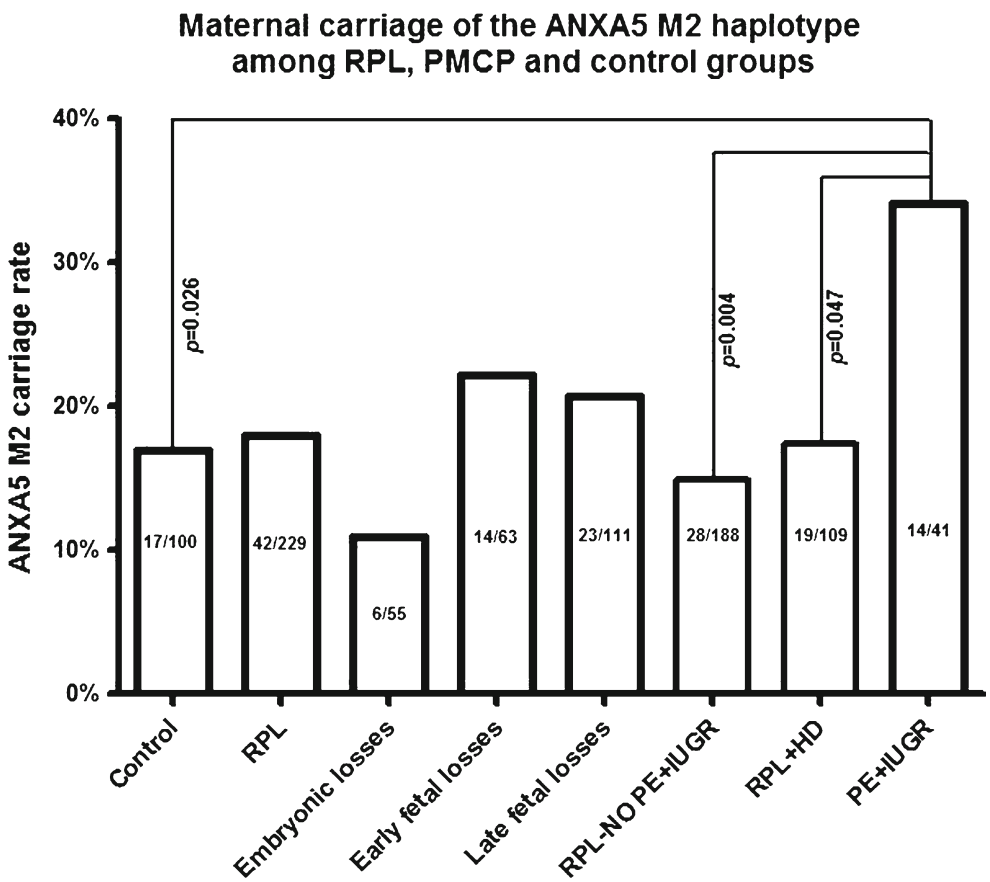

Fig. 1 Distribution of *M2/ANXA5* carriers (%) in fertile controls, RPL patients, and additionally in clinical subgroups of patients stratified according to the timing of losses (embryonic, early fetal and late fetal), with or without obstetric complications, pre-eclampsia and/or intrauterine growth restriction (PE+IUGR, RPL-NO PE+IUGR) and a subgroup of RPL patients with healthy delivery (RPL+HD). Patients with PE+IUGR have significantly higher *M2/ANXA5* carrier rate compared to fertile controls, to RPL patients without PE+IUGR and to RPL patients with healthy deliveries

with a known risk factor, the logistic analysis indicated maternal *M2/ANXA5* is independently associated, OR = 2.84 (1.31 to 6.16, $p = 0.008$) and OR = 2.38 (1.04 to 5.45, $p = 0.040$, respectively), with a higher risk of these gestational vascular complications. Parallel to this, the presence of inherited and/or acquired thrombophilia was also an independent risk factor for IUGR and/or PE in RPL patients with OR = 2.51 (1.20 to 5.26, $p = 0.015$) and OR = 2.58 (1.10 to 6.05; $p = 0.03$, respectively).

Finally, the association between maternal carriage of the *M2/ANXA5* haplotype and the risk of obstetric APS was assessed, and there was no significant difference ($p > 0.05$) in M2 distribution among RPL patients who presented with obstetric APS (24.3%; 9/37) and those who did not (17.1%; 33/193).

Discussion

In previous studies from various European and Asian, and an Austronesian population, the *M2/ANXA5* haplotype was found to be associated with an increased risk of RPL as well as of other PMPCs like PE, IUGR, and small for gestational age newborns [15, 18–24]. Superficially, the results of this study, the first from a Latin-American population so far, would indicate that maternal carriers of *M2/ANXA5* would not suffer a greater RPL risk. Nevertheless, the findings indicate that maternal carriage of this haplotype would be associated with a higher risk of IUGR and/or PE, which are gestational vascular complications partly with underlying thrombotic pathology among other etiologies that have been described [41].

As previously stated, RPL is of multifactorial etiology but it has been fairly well documented that certain risk factors are more strongly associated with pregnancy loss at different gestational times [42]. From this point of view, despite having excluded those patients who presented with known risk factors for RPL (except of APS), the resulting sample of RPL women might still represent an etiologically heterogeneous group. This could be one of the main reasons for the discrepancies between results published so far and the results of this study, regarding the role of *M2/ANXA5* as RPL predisposition. Furthermore, different studies have suggested that the placental rather than the maternal genotype would be determinant in the ANXA5 placental expression levels [16, 17]. Besides, as it is well known, the genetic footprints can have an impact in the understanding of population-level differences in biomedical traits [43]. Therefore, the lack of data on paternal genotype and the ethnic genetic footprints might also help to explain the discrepancies with regard to the implication of the *M2/ANXA5* haplotype as a risk factor responsible, at least in part, for RPL.

As a consequence of the possible etiological heterogeneity in the composition of the RPL group, maternal carriage rates of the M2 haplotype in RPL subgroups stratified according to the timing of losses were compared. Obtained results indicated that the M2 haplotype would not be an independent risk factor associated with a higher predisposition to either embryonic, early fetal or late fetal losses. The highest rate of maternal *M2/ANXA5* carriage was detected in patients with early fetal losses though its difference was not significant with regard to parous controls. In conclusion, the solely maternal *M2/ANXA5* carrier status did not appear independently associated with the timing of losses.

Among placental-mediated pregnancy complications, IUGR and PE are two separate gestational vascular pathologies that might share similar pathophysiological mechanisms. It is well known that obstetric APS and maternal carrier status of FVL have been associated with a higher risk of PE and, in addition, other inherited thrombophilia has also been associated with the risk of suffering IUGR [44–47]. Based on the literature, carrier status of the *M2/ANXA5* haplotype might well be a risk factor for the occurrence of these gestational vascular pathologies. Consequently, three different analyses were performed to address this possibility. The results from the first, where M2 carrier rates of RPL patients with IUGR and/or PE were compared to parous controls superficially, suggested that the M2 haplotype might be a risk factor for IUGR and/or PE, excluding RPL, but not miscarriage, which may be a compound of a contiguous specter of thrombophilia related placental complications including PE, IUGR and, ultimately, miscarriage. As there were no complete data on thrombophilia for the parous control group, two additional analyses were performed. The results obtained from both analyses further confirmed the association between maternal *M2/ANXA5* carriage and PMPC after adjusting for the presence of either hereditary and/or acquired thrombophilia.

Consequently, even though approximately 35% of the patients with IUGR and/or PE already presented with a known risk factor, the results of performed comparisons confirmed that maternal carriage of *M2/ANXA5* would be independently associated with a more than two times higher risk of suffering from these gestational vascular complications. Notwithstanding, the results also indicated that the maternal presence of inherited and/or acquired thrombophilia would be an independent risk factor for IUGR and/or PE in RPL patients, being in agreement with previously performed studies. It could be argued that in this logistic model the assumption that APS, FVL, or PTm carrier status would have a similar impact on the risk of PMPC was taken. After all, this might not have been the most accurate analysis but based on sample sizes in subgroups, it has been the most informative statistical approach. Therefore, making this assumption and considering the results of the regression analysis, maternal carrier status of either the *M2/ANXA5* haplotype or acquired and/or inherited thrombophilia would be independent risk factors for IUGR and/or PE with a similar strength of association. This is in line

with the notion about the proposed thrombophilic role of *M2/ANXA5*.

The results of this study are in agreement with previous findings about the role of *M2/ANXA5* in gestational vascular complications. Ota et al. found that the carrier rate of this haplotype was significantly higher in placentae from pre-eclamptic patients than in controls, the placental expression of ANXA5 mRNA was lower in M2 carriers, the ANXA5 placental protein levels were also slightly but significantly lower and the placental M2 carrier status correlated with the severity of perivillous fibrin deposition [25]. They also found that these effects were determined by the placental genotype, suggesting that placental carriage of the M2 haplotype, whether transmitted maternally or paternally, might play a key role increasing the risk of PE contributing to localized thrombosis at the feto-maternal interface. In this regard, we acknowledge that it would have been important to further analyze the paternal M2/ANXA5 carrier status to better assess its association with a higher risk of suffering from gestational vascular complications. Moreover, Tiscia et al. had previously documented that maternal *M2/ANXA5* carrier status would be associated with a twofold higher risk of pregnancy-related hypertensive disorders [18]. Meanwhile, Sifakis et al. found decreased ANXA5 mRNA expression in placentas from IUGR-affected compared to uncomplicated pregnancies. However, they found similar placental ANXA5 protein levels in both groups [48]. Considering the afore mentioned evidence, results of this study are consistent with a model proposing that the reduction in ANXA5 placental expression due to M2 carrier status negatively affects its anticoagulant capacity promoting fibrin/fibrinoid deposition both in the intervillous and perivillous space that are responsible, at least in part, for the onset of either IUGR and/or PE (Fig. 2). Notwithstanding, there cannot be certainty that the enhancement of local thrombosis at the feto-maternal interface is the only way the reduction of ANXA5 levels exerts its pathophysiological mechanism. Impairment in trophoblastic invasion, a process involved in the etiology of both gestational vascular complications during embryonic implantation, has been suggested as another plausible opportunity [41, 49–51].

Those infants that suffered from IUGR are at increased risk of fetal/neonatal morbidity and mortality and during child and adulthood they might be still at risk of experiencing neurosensory disability, cognitive impairment, short stature, hypertension, diabetes, and long-term cardiovascular disease [52, 53]. Besides, PE still remains as a major complication in pregnancy that poses a severe risk to both the mother and the fetus and often requires preterm delivery. As previously mentioned, different etiologies and risk factors for both IUGR and PE have been identified but few contribute significantly to risk prediction. In this context, the results of this study might be of practical importance for the management of PMPC with similar impact to the successful IVF outcome management after proper diagnostic workup [54].

In conclusion, this study generated evidence of an association between the maternal carrier status of *M2/ANXA5* haplotype and an elevated risk of IUGR and/or PE, supporting the hypothesis that carrier status of this haplotype and the

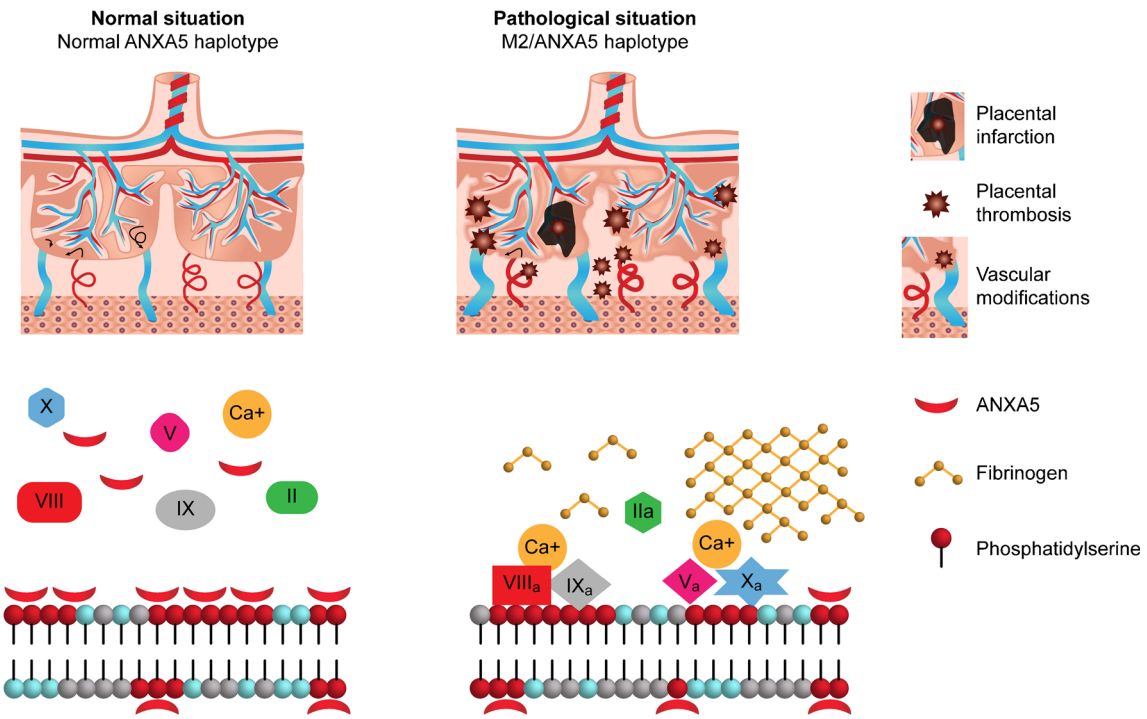

Fig. 2 A model of pathophysiological expression of ANXA5 deficiency in placentae carrying the *M2/ANXA5* haplotype

consequently reduced placental ANXA5 expression might be responsible, at least in part, for the onset of these PMPC. If further analyses confirm these initial findings, genotyping for *M2/ANXA5* could be considered as a suitable prognostic marker for any one of these gestational vascular complications.

Compliance with ethical standards

This field study to verify possible association of *M2/ANXA5* was designed as a prospective recruitment of RPL patients with post hoc analysis that was approved by the ethical committees of the institutions involved and was performed according to the principles of the Declaration of Helsinki. Informed consent was obtained from all participants.

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