Haplotype M2 in the annexin A5 (ANXA5) gene and the occurrence of obstetric complications

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Summary
Inherited or acquired thrombophilias have been largely explored as a cause of pregnancy complications. However, pathogenesis of obstetric complications, as fetal loss and pregnancy-related hypertensive disorders is still partly unexplained. Recently, a common haplotype (M2) within the annexin A5 (ANXA5) gene has been described as a risk factor in recurrent fetal losses (RFL). It has been demonstrated to reduce the promoter activity of the ANXA5 promoter in luciferase reporter assays. Aim of this study was to investigate the prevalence of M2 haplotype in three different settings of women with previous obstetric complications: RFL, intra-uterine fetal death (IUFD) and pregnancy-related hypertension (gestational hypertension [GH] and pre-eclampsia [PE]). One hundred three patients with previous RFL, 54 with IUFD, 158 with hypertensive disease (67 GH, 91 PE) were investigated. As controls, 195 women from the same ethnic background with uneventful pregnancies were enrolled.

Logistic regression, correcting for age, gravidity and parity showed that the ANXA5 haplotype is significantly and independently associated with the occurrence of RFL (3.1; 95%CI: 1.1–9.5; p=0.047) and pregnancy-related hypertensive disorders (2.1; 95%CI: 1.2–3.5; p=0.008). The M2 haplotype might be a new and relevant risk factor for obstetric complications.

Keywords
Annexins, pregnancy, polymorphism, risk factors

Introduction
A growing body of evidence suggests that a placental vascular complication may increase the risk of an adverse pregnancy outcome, such as recurrent fetal loss (RFL), intra-uterine fetal death (IUFD), gestational hypertension (GH), pre-eclampsia (PE) and fetal growth restriction (FGR) (1, 2).

Pregnancy is an acquired hypercoagulable state and women predisposed to thrombophilia may present clinical symptoms of coagulation defects for the first time during pregnancy, or at the postpartum period (3–4). Prothrombotic inherited or acquired markers, known as thrombophilic factors, have been largely explored as a cause of pregnancy complications resulting in an inadequate feto-placental circulation (3–7).

There is substantial interest in examining whether heritable thrombophilias are also associated with the occurrence of obstetric complications, like RFL, IUFD or PE and FGR. Many studies have observed an association with homozygosity and heterozygosity for the factor V (FV) Leiden variant, heterozygosity for the prothrombin (FII) G20210A variant, the rare inherited protein C, protein S and antithrombin deficiencies, as well as presence of anticardiolipin antibodies and/lupus anticoagulant. However, few other studies resulted in contradicting findings, likely reflecting heterogeneity of study population, study design, sample size, inclusion criteria, population studied, outcome definition and thrombophilias studied (2–3, 5–16).

Although in the last years many pathogenetic aspects of these multifactorial conditions have been elucidated, they still remain incompletely understood; in about 30–40% of cases, RFL remains unexplained after standard gynaecological, hormonal and karyotype investigations (7).

The role of ANXA5 as natural anticoagulant factor has been investigated since 1987 (17). Annexins are proteins with a high Ca⁺⁺-dependent affinity for anionic phospholipids. The apical surface of placental syncytiotrophoblast exposes a phosphatidilserine layer. It has been hypothesised that during pregnancy...
ANXA5 molecules, interacting with this layer, form an anti-
thrombotic shield for pro-coagulant proteins; then, this may be 
disrupted by lupus-like anti-annexin antibodies, namely anti-
phospholipid (aPL) antibodies (18, 19). Previous studies re-
ported that women with RFL showed a resistance to ANXA5 
anticoagulant activity and a reduction of ANXA5 on placental 
villi in presence of a high titre of aPL (20, 21). In addition, 
ANXA5 could act as anticoagulant protein (22) down-regulating 
the cell surface presentation of tissue factor (23).

The ANXA5 gene spans 29 kb of genomic DNA and contains 
a complex 5’ region and 12 exons coding for a polypeptide of 319 
amino acids (24). Biochemical experiments reported that pro-
gressive 5’ deletion of the core promoter diminished transcriptional 
activity, demonstrating the importance of this region for 
expression of the gene (25). Recently, a common haplotype (M2) 
within the ANXA5 gene has been described as a risk factor for 
RFL (26). In addition, in luciferase reporter assays, the M2 hap-
лотype has been demonstrated to diminish the ANXA5 pro-
moter activity (26).

Further on, in placenta from patients with PE and FGR, we 
recently showed a reduced ANXA5 gene expression in those 
carrying the M2 haplotype (27). This was the first “ex vivo” dem-
onstration that ANXA5 gene expression in human placenta is de-
pendent on the M2 haplotype. Thus, it is possible that the carrier-
ship of this haplotype is, at least to some extent, responsible of a 
series of obstetric complications. Aim of this study was to inves-
tigate the M2 haplotype in three different settings of women with 
a history of obstetric complications: RFL, IUFD and pregnancy-
related hypertensive disorders (GH or PE).

Patients and methods

Patients and controls

This case-controlled study includes three different groups of pa-
tients: 103 women with previous RFL, 54 with IUFD, and 158 
with hypertensive disorders (67 GH, 91 PE). As controls, 195 
women from the same ethnic background with at least one une-
ventful pregnancy and no history of obstetric complications were 
enrolled.

All cases and controls were Caucasian women from Southern 
Italy, recruited at the Thrombosis and Haemostasis Centre of 
I.R.C.C.S. “Casa Sollievo della Sofferenza”, S. Giovanni Roton-

Median age (range) of the cases was 32 years (21–46) and 
that of controls was 32 years (17–55). A questionnaire on social-
-economic characteristics and obstetric history, week of gestation 
in which obstetric complications occurred, was filled out by the 
study participants.

RFL was defined as the occurrence of three or more fetal 
losses at ≤23 weeks. Among these, early fetal losses were de-
efined as those occurred at ≤15 weeks, whereas late as those oc-
curring at >15 and ≤23 weeks of pregnancy (28).

In order to define an unexplained pregnancy loss, each pa-
tient underwent: oral glucose tolerance test; evaluation for the 
presence of antibodies against toxoplasma, rubella, herpes, cyto-
megalovirus, chlamydia trachomatis, listeria monocytogenes; 
autoantibodies; hysterosalpingogram; karyotyping (including the 
partner) evaluation for the presence of an abnormal karyo-
type in spontaneous miscarriages; luteal-phase endometrial 
biopsies and hormonal profile. IUFD was defined as a fetal loss 
at ≥24 gestational weeks (28). GH was defined as the occurrence 
of blood pressure ≥140/90 mmHg on two different occasions 
more than 4 hours (h) apart in a previously normotensive woman. 
PE was defined as GH and significant proteinuria (≥0.3 g in a 
24-h specimen) (7). FGR was defined when biometric variables 
(head circumference, abdominal circumference, femur length) 
were below the 10th centile, according to fetal size charts for the 
Italian population (29).

Women with aPL antibodies were excluded in all groups, 
while those with inherited thrombophilia were excluded of the 
RFL or IUFD group.

The study was carried out after the approval of a local Ethics 
Commission. Informed consent was obtained from each case and 
control individual.

Materials and methods

Blood samples were collected in a 1:10 ratio in sodium citrate 
0.1M and centrifuged at 1,841 x g for 10 minutes (min) at room 
temperature. Plasma was separated and stored at −80°C until 
samples could be assayed for protein C, free protein S and anti-
thrombin levels, as well as aPL antibodies. Aliquot to test the 
presence of lupus-anticoagulant was pre-filtered through a 
0.22 µm disposable filter to obtain platelet-free plasma. Leuko-
cyte DNA was obtained from frozen blood by the use of standard 
techniques. FV Leiden and FII G20210A gene variants were 
tested as previously described (28, 30). Protein C, free protein S 
and antithrombin, as well as aPL antibodies, were investigated at 
least two months after the last pregnancy, as reported elsewhere 
(31).

The presence of M2 haplotype (a set of four consecutive 
nucleotide substitutions in the ANXA5 gene promoter −19G/A, 
+1A/C, 27T/C and 76G/A) was investigated, as described by 
Bogdanova et al. (26).

When only two of the four variants (+1A/C, 27T/C) were 
present, the haplotype was defined as M1 (26). Briefly, a 
polymerase chain reaction was carried out to amplify the appro-
riate region of the ANXA5 promoter. The amplification was 
achieved by priming with a “forward” (5’GCGAGGACA-
AGGTCTCC3’) and a “reverse” (5’CATGGCGACTACT-
GAGGTCTCC3’) oligonucleotide. Contents of each reaction were: 
0.1 µg of genomic DNA, 10 pmoles of each primer, 100 µM of 
dNTPs, 5mM Tris HCl pH 8.4, 25 mM KCl, 1.5mM MgCl2, 
DMSO 5% (v/v) and 1 unit of Platinum® Taq DNA Polymerase 
(Invitrogen Corporation, Carlsbad, CA, USA) in a volume 
sample of 50 µl. After a pre-denaturation at 95°C for 2 min, 
cycling conditions were: 94°C for 30 seconds (s), 62°C for 30 s, 
72°C for 1 min in 35 cycles using a Perkin Elmer-Cetus thermal 
cycler.

Amplified DNA fragments were subjected to direct sequenc-
ing analysis using an ABI PRISM 3100 genetic Analyzer Se-
cquer (PE biosystems, Foster City, CA, USA).

Statistical analysis

All the analyses were performed using SPSS version 11.0.(SPSS 
Inc., Chicago, IL, USA). Between groups, differences in means 
were evaluated by the Mann-Whitney U-test, whereas differ-
ences in frequencies were calculated using chi-squared statistics. Where appropriate, odds ratios (OR) and 95% confidence intervals (CI) were calculated. Adjusted OR and 95% CI were calculated by logistic regression models that controlled for potential confounding variables such as gravidity, parity and the presence of the M2 haplotype.

**Results**

Table 1 represents the clinical features of cases and controls. Among women with RFL (n=103), the vast majority (n=91) showed a history of early RFL, whereas 12 (11.7%) had previously both early and late episodes.

In the group of women with pregnancy-related hypertensive disorders (n=158), 67 (42.5%) had a diagnosis of GH and 91 (57.5%) of PE. Altogether, 46 FGR (26 males, 20 females) were recorded with a mean birth weight (± SD) of 1393.1 grams (± 520.9) and a gestational age of 35 weeks (median, range 27–39); of them, 33 (71.7%) were in the group of PE and 13 (28.1%) in that with GH.

A significant difference for gravidity (Mann Whitney U test: p <0.001) and parity (Mann Whitney U test: p <0.001) was observed between cases and controls, whereas no significant difference was observed (Mann Whitney U test: p >0.05) as far as the age is concerned.

As shown in Table 2, the M2 haplotype was recorded in 30 (15%) controls and in 35 (34%; chi-squared test: p<0.001; OR: 2.8; 95% CI: 1.6–4.9) women with RFL. Among these, 32/35 were in the group with early RFL and 3/35 in the group with both early and late events (n=12). Among women with IUFD, the M2 haplotype was present in 8/54 (15%; chi-squared test: p>0.05 vs. controls).

In the entire hypertensive group, 46/158 (29%; chi-squared test vs controls: p<0.002; OR: 2.2; 95% CI: 1.3–3.7) showed the M2 haplotype; of them, 25/46 (54.3%) were in the GH group and 21/46 (45.7%) in the PE group. Twenty-four (15.5%) of these patients had previously shown one fetal loss (median: 8 weeks; range: 6–34 weeks).

As far as the FGR sub-group is concerned, 23 out of 46 (50%; chi-squared test vs controls: p<0.001 OR: 5.5; 95% CI: 2.7–11.0)

**Table 1: Clinical features of cases and controls.**

<table>
<thead>
<tr>
<th></th>
<th>RFL (n=103)</th>
<th>IUFD (n=54)</th>
<th>GH+PE (n=158)</th>
<th>Controls (n=195)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, median (range)</td>
<td>33 (22–43)</td>
<td>31 (21–43)</td>
<td>32 (21–46)</td>
<td>32 (17–55)</td>
</tr>
<tr>
<td>Gravidity, median (range)</td>
<td>4 (3–12)</td>
<td>2 (1–7)</td>
<td>2 (1–3)</td>
<td>2 (1–4)</td>
</tr>
<tr>
<td>Parity, median (range)</td>
<td>0 (0–3)</td>
<td>0 (0–2)</td>
<td>1 (0–2)</td>
<td>2 (1–4)</td>
</tr>
<tr>
<td>No. of fetal losses, median (range)</td>
<td>4 (3–12)</td>
<td>2 (1–4)</td>
<td>1 (0–6)</td>
<td>/</td>
</tr>
<tr>
<td>Weeks of early fetal losses, median (range)</td>
<td>8 (5–15)</td>
<td>/</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>Weeks of late fetal losses, median (range)</td>
<td>19 (16–23)</td>
<td>/</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>Weeks of fetal death, median (range)</td>
<td>/</td>
<td>31.5 (24–40)</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>FGR n (%)*</td>
<td>/</td>
<td>/</td>
<td>46* (29.1)</td>
<td>/</td>
</tr>
<tr>
<td>FV Leiden, n (%)</td>
<td>0</td>
<td>0</td>
<td>6 (3.8)</td>
<td>10 (5.1)</td>
</tr>
<tr>
<td>FIIA20210, n (%)</td>
<td>0</td>
<td>0</td>
<td>11 (7.0)</td>
<td>11 (5.6)</td>
</tr>
</tbody>
</table>

RFL: recurrent fetal loss; IUFD, intrauterine fetal death; FGR, fetal growth restriction; GH+PE, gestational hypertension/pre-eclampsia; *13 in the GH group, 13 in the PE group.

**Table 2: Genotype frequencies of ANXA5 gene promoter haplotypes in different settings of patients and in controls.**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>RFL (n=%)</th>
<th>IUFD (n=%)</th>
<th>GH+PE (n=%)</th>
<th>Controls (n=)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N/N</td>
<td>63 (61%)</td>
<td>44 (81%)</td>
<td>103 (65%)</td>
<td>154 (79%)</td>
</tr>
<tr>
<td>N/M1</td>
<td>5 (5%)</td>
<td>2 (4%)</td>
<td>9 (5.5%)</td>
<td>11 (6%)</td>
</tr>
<tr>
<td>M1/M1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>N/M2, M1/M2*</td>
<td>31 (30%)</td>
<td>8 (15%)</td>
<td>43 (27.5%)</td>
<td>30 (15%)</td>
</tr>
<tr>
<td>M2/M2</td>
<td>4 (4%)</td>
<td>0</td>
<td>3 (2%)</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>103</td>
<td>54</td>
<td>158</td>
<td>195</td>
</tr>
</tbody>
</table>

N/N: wild type for all four of variants implicated in haplotypes; N/M1: heterozygote for only two variants 1A/C and 27T/C; M1/M1: homozygote for only two variants 1A/C and 27T/C; M1/M2: heterozygote for variants –19G/A and 76G/A and homozygote for 1A/C, 27T/C; N/M2: heterozygote for variants –19G/A, 1A/C, 27T/C and 76G/A; M2/M2: homozygote for variants –19G/A, 1A/C, 27T/C and 76G/A; *M1/M2 was observed in one RFL, one IUFD, four GH+PE and two controls.
women carried the M2 haplotype: 13 were in the PE group, 10 in the GH group.

The haplotype frequencies observed in controls were not different from those predicted from the Hardy-Weinberg equilibrium (chi-squared test).

Inherited common thrombophilias were observed in 21 (10.8%); 10 FV Leiden and 11 FIIA20210, all heterozygotes) controls; only two of them carried also the M2 haplotype. Among women with pregnancy-related hypertensive disorders, 17 (10.9%) (6 FV Leiden and 11 FIIA20210, all heterozygotes) carried common inherited thrombophilias; only two with FV Leiden and five with FIIA20210 carried the M2 haplotype. Natural anticoagulants deficiencies were not observed in all the groups investigated.

As shown in Table 3, logistic regression, correcting for age, gravidity and parity showed that the haplotype M2 was significantly and independently associated with the occurrence of RFL (chi-squared test p=0.047; OR: 3.1; 95% CI: 1.1–9.5) and hypertensive disorders (chi-squared test p=0.008; OR: 2.1; 95% CI: 1.2–3.5).

Discussion

Many obstetric complications, as fetal losses or pregnancy-related hypertension, are multifactorial diseases, with partly explained pathogenesis. The occurrence of fetal losses represents a social problem, affecting about 10–20% of the general population (32), while pregnancy-related hypertension affects 5–7% of all pregnancies (11).

It has been shown that ANXA5 plays a very important role for maintaining the placental integrity in a mouse model (33). Recently, Bogdanova et al. described the M2 haplotype in a small and heterogeneous group of women with RFL (36 with loss in the first or second trimester and 16 stillbirths) (26). In the present study, we show a significantly higher prevalence of the M2 haplotype in a large sample of women with a history of both early (n=91; 88%) or late (n=12; 12%) events. Moreover, we describe for the first time that the M2 haplotype represents an independent risk factor for pregnancy-related hypertension. Univariate analysis shows the presence of a significant association with RFL, FGR and pregnancy-related hypertension. Logistic regression, correcting for age, parity and gravidity, shows an independent association with RFL and pregnancy-related hypertension, but not with FGR. Due to the strong correlation between PE and FGR, it is conceivable that the simultaneous inclusion of these variables leads to the exclusion of the FGR, retaining the most powerful association in the logistic model. Actually, 33 (36%) women with FGR had also PE.

Interestingly, the M2 haplotype was not significantly more represented in the group of women who have experienced IUFD. It appears that, on contrast to the “classic” thrombophilias, ANXA5 plays a major role in the pathogenesis of early RFL events, as early occurring RFL (32/91, 35%), decreasing its effects in late events, as late RFL (3/12, 25%) and IUFD (8/54, 15%).

The M2 carrihership rate in our controls was very similar to that reported by Bogdanova et al. (26) for the German population. Both control groups, the German PopGen and the Italian controls, were in Hardy-Weinberg equilibrium for the M2 haplotype. Thus, our selected control group may be viewed as a representative sample of the Italian population.

A direct link between reduced ANXA5 expression and a prothrombotic placental environment has been established immunohistochemically in pre-eclamptic patients and in an animal model (34, 35). We investigated the possible role played by gene variants in the pregnancy-related hypertensive disorders and found that women carrying the M2 haplotype displayed a two-fold higher risk to develop this complication during pregnancy. The change from high- to low-resistance vessels normally occurs gradually during the first 24 weeks of pregnancy, but at a variable rate. It has been suggested that this decline is the result of trophoblastic invasion of the arcuate arteries leading to increased utero-placental blood flow (36).

Pregnancies with several complications have higher utero-placental resistance patterns than normal. Our data suggest that ANXA5 is crucial during the first phase of the pregnancy, characterised by trophoblast invasion, which is dependent on villous apical surface localization of the molecule, and plays an important role in ensuring a good placental function. Reduced expression of ANXA5 in the villi, due to the carrihership of the M2 haplotype, can be responsible for fetal death in the first part of pregnancy, whereas later it can determine, at least in part, the

### Table 3: Significant associations between different obstetric complications and M2 haplotype. Logistic regression.

<table>
<thead>
<tr>
<th></th>
<th>OR; 95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>RFL</td>
<td>3.1; (1.1–9.5)</td>
<td>0.047</td>
</tr>
<tr>
<td>GH+PE</td>
<td>2.1; (1.2–3.5)</td>
<td>0.008</td>
</tr>
</tbody>
</table>

CI, confidence interval. Potential confounding variables: age, gravidity and parity.

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### What is known about this topic?

- Annexin A5 (ANXA5) is a placental anticoagulant protein. It has been hypothesised that during pregnancy ANXA5 molecules form an antithrombotic shield for pro-coagulant proteins.
- The M2 haplotype within the ANXA5 gene has been showed to diminish the promoter activity in luciferase reporter assay.
- An association between the M2 haplotype and early fetal losses has been reported.

### What does this paper add?

- The M2 haplotype is not significantly more represented in the group of women who have experienced intrauterine fetal death (IUFD). It appears that, at variance with “classic” thrombophilias, ANXA5 plays a role in the pathogenesis of early RFL, but not IUFD.
- The M2 haplotype represents the first genetic independent risk with hypertensive disease of pregnancy.
- The M2 haplotype may be considered as new marker for the work-up of the obstetric complications.
phenotype of the pregnancy-related hypertensive disorders with or without FGR, having a far smaller effect in causing intrauterine death.

In conclusion, the high prevalence of the ANXA5 M2 haplotype in patient groups with two different obstetrics complications strongly suggests that it could be a new and relevant risk factor for adverse pregnancy outcomes.

Whether our findings will be confirmed by further studies, the investigation of this genetic marker could be considered as a diagnostic tool to identify women at higher risk of obstetric complications and to develop adequate pharmacological strategies for preventing following episodes.

References