


Assessment of M2/ANXA5 haplotype as a risk factor in couples with placenta-mediated pregnancy complications

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Abstract

Purpose The aim of this study was to confirm the associated M2/ANXA5 carrier risk in women with placenta-mediated pregnancy complications (PMPC) and to test their male partners for such association. Further analysis evaluated the influence of maternal vs. paternal M2 alleles on miscarriage. **Methods** Two hundred eighty-eight couples with preeclampsia (PE), intrauterine growth restriction (IUGR), or premature birth (PB) were recruited ($n = 96$ of each phenotype). The prevalence of the M2 haplotype was compared to two control cohorts. They included a group of women with a history of normal pregnancy without gestational pathology (Munich controls, $n = 94$) and a random population sample (PopGen controls, $n = 533$).

Results Significant association of M2 haplotype and pregnancy complications was confirmed for women and for couples, where prevalence was elevated from 15.4 to 23.8% ($p < 0.001$). Post hoc analyses demonstrated an association for IUGR and PB individually. A strong link between previous miscarriages and M2 carrier status was identified which may explain the predisposition to placental pregnancy complication. M2/ANXA5 appears to be a risk factor for adverse

pregnancy outcomes related, but not limited to miscarriages, with similar prevalence in women and their male partners.

Conclusion These findings support the proposed physiological function of ANXA5 as an embryonic anticoagulant that appears deficient in contiguous specter of thrombophilia-related pregnancy complications culminating more frequently in miscarriage in a maternal M2 carrier background.

Keywords M2/ANXA5 · Annexin A5 · Pregnancy · Placenta-mediated pregnancy complications · Miscarriage

Introduction

Hereditary thrombophilia is considered an etiological factor for adverse pregnancy outcome and evidence of impeded placental perfusion links to an increased risk of obstetric complications [1–4]. Initial clinical research concentrated mostly on the low thrombotic risk genetic variants of Factor V, Factor V Leiden (FVL, G1691A, R506Q, rs6025) and prothrombin G20210A (PTm, G20210A, rs1799963) [5, 6]. In the last decade, genetic and functional studies characterized a constellation of four consecutive single nucleotide variants comprised of the minor alleles of SNPs rs112782763, rs28717001, rs28651243, and rs113588187 in the proximal promoter region of ANXA5 gene, c.-467G>A, c.-448A>C, c.-422T>C, and c.-373G>A. This constellation, inherited as a haplotype confirmed through molecular cloning and termed M2 in 2007 [7], was associated with increased risk for adverse pregnancy outcome in patient cohorts of European [3, 7–10], Asian [11, 12], and Austronesian origin [13, 14]. M2/ANXA5, a risk factor for susceptibility to recurrent pregnancy loss (RPL) with a prevalence of about 15% in the general European population [3, 7, 9, 10], was designated RPRGL3, OMIM entry 614391 and concomitant expression studies evaluated its influence on

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ANXA5 levels. Gene reporter assays confirmed lowered read-out signal from constructs harboring the M2 allele in HeLa cells [7] and recently in constructs carrying the c.-467G>A SNP of M2 in BeWo cell line [15]. Reduced ANXA5 mRNA abundance was demonstrated in chorion samples carrying the M2 allele [16], where this reduction proved to be haplotype specific [17] and congruent decrease of ANXA5 protein levels has been detected in placental tissue of M2 carriers with pre-eclampsia (PE) [12].

Since ANXA5 is mostly enriched on the surface of chorion villi of embryonic origin, where it is proposed to act as an anticoagulant [18, 19], studies were initiated to assess the contribution of the paternal M2 allele as a risk factor for adverse pregnancy outcome. The first indication of such a contribution came from an expression study of ANXA5 mRNA, where lowered M2 allele specific levels were confirmed independent of parental origin in chorion of intrauterine growth restriction (IUGR) patients [17]. An initial pilot study in RPL couples gave primary independent confirmation of a similar risk attributed to paternal M2 alleles [20], followed by studies in larger European cohorts [9, 10] and Malaysian research [14]. Considering the potential importance of paternal M2 allele in the diagnostic work up of adverse pregnancy outcomes, this study was carried out where the aim was to test the associated risk contribution in couples with PE, IUGR, and premature births (PB).

Patients and methods

Study populations

The present genetic association study complied with the ethical guidelines of the institutions involved and was approved of the Review Board of the University Munich (IRB 209-12). Informed consent was obtained from all subjects examined. The criteria of strengthening the reporting of genetic association studies (STREGA) were observed as far as applicable.

Three hundred nineteen couples gave their consent for participation and were consecutively recruited between 2010 and 2015 in the Division of Gynecological Endocrinology and Reproductive Medicine and the Clinic and Polyclinic for Gynecology and Obstetrics, Ludwig-Maximilians University Munich. Because of format compliance with gene sequencing platforms, 288 couples with age well matched to the controls were selected. All female patients from the study group were between 18 and 40 years old, had a body mass index (BMI) between 19 and 24 kg/m² and presented with a history of PE, IUGR, or preterm birth (PB). Criteria for PE according to the ACOG Committee were gestational hypertension (≥ 140 Hg systolic/90 mmHg diastolic in at least two measurements ≥ 6 h interval) with proteinuria (≥ 300 mg/24 h urine) after the 20th week of gestation [21]. Neonates with birth weight below the

5th percentile and delayed growth in the progress of pregnancy according to the percentile curve were considered IUGR [22, 23]. PB was defined as delivery between 23 + 5 and 36 + 5 gestational weeks [24]. Only patients without identifying any reasons for PB (such as fetal defects or malformations, multiple pregnancies, placental anomalies, or clinical signs of infection such as premature rupture of membranes, preterm labor, chorioamnionitis, cervicitis with cervical insufficiency, and preterm cervical dilatation, as well as IUGR and PE) were recruited [25]. All patients were prescreened negative for hypertension, diabetes, kidney disease, pulmonary and cardiac disorders, collagenosis, anemia, abuse of alcohol, drugs and smoking, uterine anomalies (uterine fibroids, muellerian anomalies), endocrine dysfunctions (polycystic ovary syndrome according to the Rotterdam criteria [26], hyperprolactinemia, hyperandrogenemia, thyroidal dysfunctions such as hypo-/hyperthyreosis, and thyroid autoantibodies), deficiencies of protein C, protein S, antithrombin. Inherited thrombophilia (factor V Leiden mutation (FVL), prothrombin (PTm) 20210G>A substitution) were ruled out. Antiphospholipid syndrome according to the international consensus statement regarding the classification criteria for antiphospholipid syndrome [27] was excluded as well as fetal and parental chromosomal disorders. Further criteria for exclusion of pregnancy and placental anomalies were as follows: (a) for pregnancy anomalies, multiple pregnancies and pregnancies after assisted reproductive technology were excluded, as well as intrauterine infections tested positive for cytomegalovirus, malaria, parvovirus, rubella, toxoplasmosis, herpes simplex virus, and human immunodeficiency virus (HIV). Clinical signs of infections, such as premature rupture of membranes, preterm labor, chorioamnionitis, cervicitis with cervical insufficiency and preterm cervical dilatation were also considered as exclusion criteria. (b) Placental anomalies were considered so that patients with pathologic trophoblast invasion, placenta previa, and vascular anomalies/insertio velamentosa were excluded from this study.

The male partners were between 18 and 49 years old with normal BMI range for males, i.e., 20–25 kg/m² and were negative for any toxins such as alcohol or drugs. Clinically relevant features of the couples and two control groups are provided in Table 1.

STREGA compliant control cohorts consisted of a group of fertile women ($n = 94$; $n = 89$ thereof of European descent) with a history of normal pregnancies, one or more normal-term deliveries of healthy, normal-weight singletons, and without gestational pathology from the Division of Gynecological Endocrinology and Reproductive Medicine, University of Munich [20], and a population control cohort drafted from the PopGen bio-bank at UKSH Kiel ($n = 533$) [28]. The PopGen control group was a sample of healthy individuals, identified through official population registers and comprised of about equal numbers men and women that

Table 1 Clinical features of women with obstetric complications and the parous control group from Munich

	PE (n = 96)	IUGR (n = 96)	PB (n = 96)	Munich controls (n = 89)
Age, mean ± sd	29.2 ± 6.0	28.0 ± 5.6	29.1 ± 6.3	33.9 ± 4.6
Gravidity, mean ± sd	2.2 ± 1.2	2.0 ± 0.9	2.0 ± 1.1	1.4 ± 0.6
Parity, mean ± sd	1.9 ± 1.0	1.6 ± 0.7	1.6 ± 0.9	1.4 ± 0.6
Miscarriages, n (%)				
0	76 (79%)	70 (73%)	78 (81%)	89 (100%)
1	16 (17%)	18 (19%)	14 (15%)	0 (0%)
≥ 2	4 (4%)	8 (8%)	4 (4%)	0 (0%)
Gestational age at delivery, mean ± sd	37.1 ± 2.1	36.6 ± 1.6	28.4 ± 3.3	–
Female neonates, n (%)	46 (48%)	50 (52%)	48 (50%)	–
New-borns' growth percentiles				
≤ 3th	0 (0%)	31 (32%)	0 (0%)	–
3th–5th	0 (0%)	65 (68%)	0 (0%)	–

PE preeclampsia, IUGR intrauterine growth restriction, PB premature birth

distributed along three age groups (18–30, 30–50, 50–80 years).

DNA of participating subjects was extracted from white blood cells, using the QIAmp DNA blood mini kit (Qiagen, Hilden, Germany) and stored in 100-µl aliquots at 4 °C for further analyses.

Genotyping and statistical analysis

Genotyping of extracted DNA was performed on PCR amplified genomic DNA via Sanger sequencing as previously described [7]. Genotypes were scored in table format and 4-digit coded for further processing.

The statistical software package R (version 3.3.1, <http://www.R-project.org>) was used. Logistic regression was employed to determine odds ratios (OR) and 95% confidence intervals (CI) after taking into account age and parity for subjects with complications and those without separately. Gravidity was considered to be too strongly co-linear with parity to be included as a covariate as well. Post hoc linear multiple comparisons were made based on the methods in the multcomp package [29]. Contingency tables were tested using the chi-square test or the Fisher exact test if counts were low.

Results

Basic characteristics

Table 1 lists the essential clinically relevant features of each female cohort. The three groups with pregnancy complications had similar distributions regarding previous miscarriages and differences from the control populations resulted from the cohort definitions.

Association of M2/ANXA5 carrier status with obstetric complications in pregnant women

Women with obstetric complications had M2 carrier rates varying between 25 and 27% compared to 15% in women from the control group in Munich (Table 2). A logistic model yielded an OR of 2.2 (95% CI 1.2 to 4.7, *p* = 0.026) for carrying the M2 haplotype given pregnancy complications. Age was not found to be significantly associated for women with pregnancy complications (OR = 0.78 per decade, 95% CI 0.5 to 1.3, *p* = 0.36), nor was parity (OR = 1.4 per increase by one, 95% CI 1.0 to 2.1, *p* = 0.062), though there was a trend. Post hoc tests comparing each complication group with the Munich control subjects all yielded similar ORs slightly greater than 2, but these were not significant (Table 3).

In women with pregnancy complications, a very strong dependence was observed between the number of previous miscarriages and M2 carrier status (Table 4). The M2 carrier rates were 13% in women without previous miscarriages compared to 71 and 75% for those with one or more than one previous miscarriage, respectively, *p* < 0.001. The breakdown of rates did not differ substantially for each particular pregnancy complication. Upon adjusting for RPL (≥ 2 miscarriages), there was no substantial change in the prevalence of M2 carriers of the three pregnancy complication categories. However, when analyzing the subset of women without a single previous miscarriage, there was no longer an association between M2 carrier status and pregnancy complications.

M2/ANXA5 carrier status in male partners

With an M2 carrier rate of 21.5%, the male partners of women with pregnancy complications had a carrier status only 4.5 percentage points lower (95% CI – 2.8 to 11.8 percentage

Table 2 Genotype frequencies of ANXA5 promoter haplotypes in obstetric complication couples and two different control groups

Cohort Genotype	IUGR		PB		PE		PopGen	Munich
	Women, % (n)	Men, % (n)	Women, % (n)	Men, % (n)	Women, % (n)	Men, % (n)	Both sexes, % (n)	Women, % (n)
<i>N/N</i>	64.6 (62)	59.4 (57)	67.7 (65)	67.7 (65)	65.6 (63)	68.8 (66)	77.9 (415)	68.5 (61)
<i>N/M1</i>	10.4 (10)	15.6 (15)	5.2 (5)	7.3 (7)	7.3 (7)	13.5 (13)	6.6 (35)	16.9 (15)
<i>M1/M1</i>	0 (0)	3.1 (3)	1.0 (1)	0 (0)	0 (0)	0 (0)	0.2 (1)	0 (0)
<i>N/M2, M1/M2^a</i>	24.0 (23)	21.9 (21)	26.0 (25)	22.9 (22)	25.0 (24)	17.7 (17)	14.4 (77)	12.4 (11)
<i>M2/M2</i>	1.0 (1)	0	0 (0)	2.1 (2)	2.1 (2)	0 (0)	0.9 (5)	2.2 (2)
<i>Total</i>	96	96	96	96	96	96	533	89
<i>M2 carrier rate, %</i>	25.0	21.9	26.0	25.0	27.1	17.7	15.4	14.6

IUGR intrauterine growth restriction, *PB* premature birth, *PE* preeclampsia, *PopGen* Muenster and Munich control groups, % percentage, *n* count

^a Genotype *M1/M2* was only observed in one PB, one PE, and in two IUGR couple partners, three Munich and five PopGen controls

points, $p = 0.24$) than the women from this study. In fact, only in the preeclampsia group did the M2 carrier status of women (27.1%) and men (17.7%) differ meaningfully, though the numbers were too small to exclude the possibility that the difference arose by chance (95% CI for difference – 3.4 to 22.2 percentage points, $p = 0.17$).

In male partners of women with pregnancy complications, there was no evidence for a possible association between the number of previous miscarriages and M2 carrier status (Table 4). The M2 carrier rates were 22% in partners of women without previous miscarriages compared to 19 and 19% for those with one or more than one previous miscarriage, respectively, $p = 0.89$.

M2/ANXA5 carrier status in couples

Compared to the M2 carrier rate in the general population (15.4%), couples with pregnancy complications had a much higher prevalence (23.8%), with an OR of 1.7 (95% CI 1.3 to 2.4, $p < 0.001$). Post hoc tests comparing each obstetric complication to the PopGen control sample all had OR values close to 1.7 and the IUGR and PB groups differed significantly from the general population (Table 3).

Discussion

Women with obstetric complications had a higher prevalence of the M2 haplotype than women with normal deliveries and this prevalence was very similar among IUGR, PB, and PE patients. The haplotype was strongly associated with the number of previous miscarriages. Their male partners had similarly elevated prevalence and taken together, couples with pregnancy complications had considerably higher prevalence of M2 carriers than the general population.

The results for the women from this study are in agreement with previously obtained results for these obstetric complications [3, 8]. The estimated odds ratios were close to 2. The elevated risk of placental obstetric complications because of inherited thrombophilia has been extensively reviewed [1–3] and the findings here could be understood in accordance with pathological aspects of these gestational anomalies [30].

Fetal involvement of the M2 allele of ANXA5 in placental obstetric complications was first documented for IUGR patients [17], followed by a report on PE [12], whereby reduced expression of the natural anticoagulant ANXA5 was described in chorion of IUGR and PE patients, independent of M2 parental origin. Genetic evidence substantiated the role of the paternal ANXA5 promoter genotype with elevated M2

Table 3 Associations between obstetric complications and the M2 haplotype in comparison to the control groups in post hoc analysis

Index category	IUGR		PB		PE	
	Women	Women + men	Women	Women + men	Women	Women + men
Reference category	Munich control	PopGen	Munich control	PopGen	Munich control	PopGen
OR	2.0	1.7	2.1	1.9	2.5	1.6
95% CI	0.8 to 5.2	1.0 to 2.8	0.8 to 5.4	1.2 to 3.1	1.0 to 6.2	1.0 to 2.6
<i>p</i> value	0.16	0.035	0.14	0.0057	0.058	0.078

IUGR intrauterine growth restriction, *PB* premature birth, *PE* preeclampsia, *OR* odds ratio, *CI* confidence interval

Table 4 Associations between previous miscarriage rates and the M2 haplotype

		0 miscarriages		1 miscarriage		> 1 miscarriage	
		Women	Men	Women	Men	Women	Men
All pregnancy complications	Non-M2 carrier	195 (87%)	174 (78%)	14 (29%)	39 (81%)	4 (25%)	13 (81%)
	M2 carrier	29 (13%)	50 (22%)	34 (71%)	9 (19%)	12 (75%)	3 (19%)
IUGR	Non-M2 carrier	64 (91%)		7 (39%)		1 (12%)	
	M2 carrier	6 (9%)		11 (61%)		7 (88%)	
PB	Non-M2 carrier	66 (85%)		3 (21%)		2 (50%)	
	M2 carrier	12 (15%)		11 (79%)		2 (50%)	
PE	Non-M2 carrier	65 (86%)		4 (25%)		1 (25%)	
	M2 carrier	11 (14%)		12 (75%)		3 (75%)	

IUGR intrauterine growth restriction, *PB* premature birth, *PE* preeclampsia

carrier rates of male partners of RPL women, first in a pilot research [20] and further in following larger scale population investigations [9, 10, 14].

The aim of the current study was to confirm the role of M2 in *ANXA5* as a risk factor in patients with obstetric complications and to test the paternal *ANXA5* allele for possible involvement in such pathology. M2 carrier rates among the male partners from groups of IUGR and PB patients were comparable to those in females, whereas for PE, the carrier rates were lower, but inconclusively so. When compared with the general population, the rates for couples with the first two complications were significantly lower, but only showed a trend in the case of PE.

Although a reduced *ANXA5* expression has been found in chorion of PE women, where the M2 allele was obviously of paternal origin and an enrichment of fetal, compared to maternal M2 alleles was noted in PE placental samples [12], this possible involvement could not be confirmed by genetic evidence from this study. On the other hand, the cited PE study [12] did not find enrichment of maternal M2 carriers compared to controls, thus differing with earlier research on obstetric complications including PE [3]. This difference might be explained by the small sample size of the study suggesting insufficient power [12].

One of the pathologic mechanisms often discussed involves defective placentation resulting from either extensive or shallow trophoblastic invasion as a key factor in PE among other obstetric complications [31]. According to this hypothesis, a change of hemostatic parameters would be expected to play a subordinate role in the manifestation of the particular condition. Since invading extravillous trophoblasts are of embryonic origin, a paternal genetic factor would have potential bearing on the process of placentation failure.

Another pathologic mechanism points at predisposition to endothelial dysfunction causing altered coagulation parameters among others, supposedly having a crucial role in the pathogenesis of PE [32], where it is hard to make a clear

distinction between maternal and fetal factors. An early large epidemiological study that used records of the Norway Medical Birth Registry covering 1.7 million births confirmed about equal involvement of fetal (paternally inherited) and maternal genes in PE pathology and excluded possible maternally singular (mitochondrial genome) genetic factors [33]. A later Norwegian study found no evidence for fetal contribution of inherited thrombophilia (Factor V Leiden and MTHFR C677T variant alleles) to PE risk and confirmed the exposure of maternal carriers [34]. From the current study results, it would appear logical to propose the influence of paternal M2 alleles in the pathology of embryonic development disorders, such as IUGR and PB that appears in accordance with biological function, since this developmental pathology would be largely dependent on chorion supply and PE in contrast may be more of a maternal phenomenon with lesser involvement of extra-embryonic membranes.

A striking feature observed in this study is the strong maternal link between the M2 haplotype and previous miscarriages, but lack of such relation to the paternal carrier status. This cannot be explained consistently by proposing a causal connection for miscarriages only and assuming that pregnancy complications are merely a secondary feature, since such model does not account for the observed prevalence of M2 haplotype among male partners. Moreover, a statistical interaction between miscarriages and complications seems likely, but could not be analyzed with these data since the Munich control group was selected not to have miscarriages. Since 10 to 15% of all women suffer at least one miscarriage [35], the ideal control population for such an analysis would be comprised of women without pregnancy complications, but without restriction regarding miscarriages. In addition, the mean maternal age of women with pregnancy complications from this study was below 30, largely excluding chromosomal aberrations as trivial reason for miscarriage. For this age group general miscarriage rates are 8% and below [36] and aneuploidies are 25% and below of all miscarried embryos [37].

A plausible model that can be proposed, relevant to biological function, would perceive thrombophilia-related obstetric complications and ultimately miscarriage as manifestations of a contiguous disorder, where combination of maternal and paternal *RPRGL3* (*M2/ANXA5*) alleles would determine the severity of phenotypic expression with modulator function of the maternal factor. In other words, maternal M2 carrier status might modulate the clinical presentation of the thrombophilia predisposition in pregnancy. The recently reported assisted conception trial with low-molecular-weight heparin (LMWH) in M2 carrying couples may lend support to such notion [38]. According to their results, LMWH-treated couples in which only the male was an M2 carrier benefited significantly more regarding live births than those with only female M2 carriers. This could mean that LMWH supplementation of ANXA5 anticoagulant function in villous trophoblasts would perform potentially better for paternally heterozygous chorion on the background of a normal maternal genotype in blood.

There is clearly much more to be learned about the role of *M2/ANXA5* in this setting and doing so may well help to better understand the pathological mechanisms of thrombophilia-related pregnancy complications.

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Conflict of interest The authors declare that they have no conflict of interest.

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