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Letter to the Editors-in-Chief

Elevated annexin A5 plasma levels in term pregnancies of M2/ANXA5 carriers

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Annexin A5 (ANXA5) is a protein most abundantly expressed in placenta, with well characterized anticoagulant function [1]. A promoter haplotype of the ANXA5 gene termed M2, a risk factor for recurrent pregnancy loss (RPL) susceptibility, RPRGL3, OMIM entry 614,391, reduces gene expression [2]. This placental anticoagulant has been proposed to form a protective shield on the surface of villous syncytiotrophoblasts thus maintaining the necessary hemodynamic balance in pregnancy [3]. Reported ANXA5 levels in plasma of healthy volunteers are usually below 5 ng/ml and may vary extensively (up to 20-fold higher) in amniotic fluid, for example [4]. An early comparative study of ANXA5 plasma levels in non-pregnant RPL cases vs. non-pregnant women without gestational pathology demonstrated reduced circulating ANXA5 in subjects with 3 or more recurrent miscarriages [5]. A subsequent study, conducted in pregnant women with history of 3 or more losses compared to pregnant control subjects without gestational pathology showed no significant difference in circulating ANXA5 of cases vs. controls [6]. However, measured ANXA5 plasma levels in pregnant cases or controls were significantly higher than their non-pregnant counterparts. In addition, a higher ANXA5 plasma concentration range (non-significant) was recorded in pregnant cases vs. pregnant controls. Finally, a third study measuring coagulation parameters and ANXA5 and ANXA4 plasma levels in non-pregnant, maternal (first, second, third trimester and upon delivery), and post-partum women did not detect any significant distinctions in circulating ANXA5 among these groups [7]. In order to address the unclear diagnostic and predictive value of ANXA5 plasma measurements in pregnant and non-pregnant subjects, this study aimed to answer two major questions: 1. Are there any significant differences in circulating ANXA5 levels between a) non-pregnant women, b) term pregnant with a history of RPL and c) term pregnant women without any gestational pathology; and 2. Are there any significant differences in circulating ANXA5 levels of M2/ANXA5 carriers vs. normal genotype subjects of these clinically defined groups.

Ethical board approval of participating institutions was obtained. Peripheral blood was collected for genotyping and for ANXA5 plasma measurements from 160 Malaysian Malay pregnant women shortly after term delivery. The pregnant women group consisted of RPL women ($n = 100$) with a previous history of ≥2 pregnancy losses before or at the 17th week of gestation and the parous women group comprised of women with 1 or more term pregnancies without gestational pathology ($n = 60$). All the RPL subjects were diagnosed with idiopathic RPL after an extensive screening protocol excluding trivial reasons for repeated miscarriage [8]. Non-pregnant women without history of pregnancies ($n = 52$) served as control group. Clinically relevant features are summarized in Table 1.

Additional ($n = 6$) German idiopathic RPL women with a previous history of ≥2 pregnancy losses before the 15th week of gestation, experiencing spontaneous abortions at the University of Muenster Obstetrics and Gynecology Clinic donated peripheral blood and placental tissue. Genotyping of DNA extracted of peripheral blood and chorion samples followed established protocols [3,8]. The concentrations of ANXA5 in plasma were determined using a human ANXA5 platinum ELISA kit (eBioscience, Vienna, Austria). The coefficients of variation (CV) of this assay were CV < 10% in intra-assay, and CV < 11% in inter-assay comparisons, as reported by the manufacturer. Detection limit was 0.15 ng/ml, as determined on serial dilutions of ANXA5 purified from human placenta (Sigma-Aldrich, Darmstadt, Germany). ANXA5 in chorion villi was measured in 1:10 diluted cleared lysates after tissue weight normalized lysis in T-PER (Pierce/Thermo Scientific, Rockford, IL). Since measured circulating ANXA5 levels in all clinical groups showed non-normal distribution, non-parametric statistical evaluation using the Kruskal-Wallis test (SPSS version 22.0, Chicago, USA) was performed to compare plasma ANXA5 among the non-pregnant, RPL and parous groups. Adjustment for age and gravidity was made using ANCOVA test. Next, these groups were subdivided into 'Normal' and M2 haplotype carriers; further comparison was made by Mann-Whitney *U* test with post-hoc Bonferroni correction (adjusted significant *p*-value ≤0.017). In order to relate measured levels of circulating ANXA5 obtained under the conditions of the ELISA test to the genotype information of clinical cohorts ('Normal' vs. M2 haplotype carriers), patients were divided in 'high' vs. 'low' ANXA5 concentration groups. Receiver Operating Characteristic (ROC) curves and areas under the curves (AUCs) were used to define the cutoff value for division of all samples and it was determined as 0.2 ng/ml. Further, multiple logistic regression was used to model the association of circulating ANXA5 levels treated categorically (i.e. 'high' vs 'low' ANXA5 concentrations) with M2 carrier status adjusted for age and gravidity. Significance level was set at $p \leq 0.05$.

In this study, the distribution of M2 carriers with median ANXA5 concentrations and interquartile ranges (IQR) in the clinically defined subgroups were as follows: 25 carriers (48%) in the non-pregnant group (0.34 ng/ml, IQR: 0.03–0.73); 19 (32%) in the pregnant parous women (0.01 ng/ml, IQR: 0.01–0.49) and 49 (49%) in the pregnant RPL women group (0.34 ng/ml, IQR: 0.07–0.86).

There was no significant difference in circulating ANXA5 levels between subjects of the non-pregnant, RPL and parous groups (median, 0.25 ng/ml vs. 0.21 ng/ml vs. 0.01 ng/ml, $p = 0.14$) (Fig. 1A). Similarly, no significant differences in circulating ANXA5 levels were observed across these three clinical subgroups among M2-carriers or non-carriers, respectively. However, in the within-group analysis, (Fig. 1B), there was a trend of significant difference in measured plasma ANXA5 between M2-carriers and non-carriers ($p = 0.02$, with post-hoc Bonferoni correction).

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Table 1
Clinical features of cases and control groups.

	Pregnant		Non-pregnant	*p*-Values*
	RPL	Parous		
Age	32.3 ± 5.5	29.5 ± 4.6	19.5 ± 2.6	< 0.001
Gravidity	5.3 ± 1.7	2.2 ± 1.4	0	<0.001
History of RPL				
2	76	–	–	
>2	24	–	–	
Gestational losses of the most recent RPL				
≤15 weeks	92	–	–	
>15 weeks	8	–	–	

* ANOVA test across three groups.

In the RPL group, M2 carriers had 2.67 times the odds compared to non-M2 carriers (95% CI 1.19 to 5.99, $p = 0.017$) to have higher circulating ANXA5 levels. This odd ratio remained almost unchanged, 2.65 (95% CI 1.18 to 5.96, $p = 0.018$) when adjusted for age and gravidity. For the rest of clinical groups (non pregnant and parous women), circulating ANXA5 levels of M2 carriers vs. non-carriers did not differ significantly, for the non pregnant-group crude OR: 1.915 (95% CI 0.630 to 5.822), $p = 0.252$; adjusted OR: 1.915 (95% CI 0.630 to 5.822) and for the parous group crude OR: 0.911 (95% CI 0.296 to 2.804), $p = 0.872$; adjusted OR: 1.206 (95% CI 0.344 to 4.227, $p = 0.770$.

Of all 6 pregnant RPL women followed up at the University Clinic Muenster one was M2 carrier. Chorion samples were obtained from 4 of the 6 women, all 4 with normal maternal genotypes. Measured plasma ANXA5 levels ranged from 0.86 (normal maternal genotype) to 1.03 ng/ml (single M2 carrier) without substantial differences. Two of the chorion samples were with normal ANXA5 genotype and had estimated ANXA5 levels of 10.4 and 11.08 μg/g tissue. The rest of two were heterozygous M2 carriers (obviously with paternal origin of the M2 allele) and had 7.8 and 4.96 μg/g ANXA5, 73% and 46% of normal genotype levels accordingly.

The current study did not detect any significant differences of ANXA5 plasma levels among the non pregnant, RPL and parous groups. The distribution of M2/ANXA5 carriers in these groups was indicative of the haplotype's proposed recurrent miscarriage predisposition role [3, 8]. Surprisingly, M2 carriers of the RPL group had significantly higher ANXA5 plasma levels (median: 0.34 ng/ml) compared to non-carriers (median: 0.02 ng/ml).

Since ANXA5 is most abundantly expressed in syncytiotrophoblasts [9], release of villous ANXA5 depots into the circulation of term pregnant M2 carriers might possibly indicate chorion of normal genotype, able to sustain healthy pregnancy. Conversely, M2 carrying chorion deficient of ANXA5 expression [3], would be nevertheless expected to release more ANXA5 carrying membrane microparticles in the blood circulation. Whatever the mechanism of increased ANXA5 circulation levels in pregnant M2 carriers might be, ANXA5 seems required for term, non-complicated pregnancies with a critical threshold that must be apparently reached in chorion.

Independent of mutual covariance and possible regulation strategies of ANXA5 at the feto-maternal interface and in maternal circulation ANXA5 plasma levels do not appear as a confident diagnostic criterion for successful pregnancy outcome. Since the haplotype M2/ANXA5 has been proposed as a biomarker for LMWH supplementation of anticoagulant function [10], this finding has a direct bearing on the genetic vs. biochemical diagnostic approaches.

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