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COMMENTARY

Annexin A5 haplotype M2 is not a risk factor for recurrent spontaneous abortion in Northern Europe: is there sufficient evidence?

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Abstract The M2 haplotype of the annexin A5 gene is a well-recognized predisposition factor for recurrent spontaneous abortion (RSA). A recent publication by Nagirnaja et al. (2015) in *PLoS One* discusses the risk role of the M2 haplotype for RSA in cases compared with controls of North European extraction and arrives at a negative result. As a number of previous and fairly recent studies have supported the proposed involvement of the M2 haplotype in the cause of idiopathic RSA, this commentary aims to highlight problematic issues in the above publication. It is the opinion of the authors that the study by Nagirnaja et al. (2015) does not generate adequate proof of the absence of RSA risk, attributable to carriage of the M2 haplotype.

KEYWORDS: annexin A5, ANXA5, M2/ANXA5, recurrent miscarriage, risk factor

Hereditary thrombophilia is considered to be one of several causes of recurrent spontanous abortion (RSA), supported by evidence of impaired placental perfusion caused by thrombotic events. This cause is reflected in increased risk of adverse pregnancy outcomes, reviewed in clinical studies and initially limited to genetic variants of blood coagulation factors II (prothrombin) and V. In 2007, another proposed hereditary factor for thrombophilia related RSA was identified, termed "M2", a haplotype in the proximal core promoter region of the annexin A5 (*ANXA5*) gene, defined as a constellation of four single nucleotide polymorphisms (SNP), rs112782763, rs28717001, rs28651243 and rs113588187 (Bogdanova et al., 2007). The haplotype, an RSA predisposition factor, RPRGL3, OMIM entry 614391, was confirmed through molecular cloning and direct sequencing of the relevant amplicon clones, so the minor alleles' action of all four SNP comprising it manifested in reduced expression of a reporter gene compared with a background construct harbouring all the major (normal) alleles, when using a functionally representative cell line. Since then, more than 20 studies have discussed and delivered evidence on the risk role of this common *ANXA5* genetic variant from retrospective and prospective clinical studies on thrombophilia-related obstetric complications in various ethnic backgrounds, and on its pathophysiological expression as embryonic anticoagulant.

A recent study by Nagirnaja et al. (2015) describes a case-control approach to the association of *M2/ANXA5* with RSA comparing patient and control groups of Northern European extraction. Although the authors agree on the congruity of the M2 haplotype, as constellation of the four SNP

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rs112782763, rs28717001, rs28651243 and rs113588187 from a wealth of sequencing and microarray data, this information is not new. They basically compare the M2 incidence in 313 patients with unexplained RSA to a fertile women control group of 214 participants from Estonia and Denmark and arrive at the conclusion that *M2/ANXA5* is not a risk factor for RSA in Northern Europe. After thorough analysis of the results reported, several major and a minor issues in this publication remain that appear problematic in relation to the conclusions reached.

Major issues

Patient groups

The exclusion criteria for "unexplained recurrent miscarriage" as defined by the Nagirnaja et al. (2015) did not take into account fetal chromosomal abnormalities, known to be the most common cause of early pregnancy losses. According to a fairly recent review on the subject, chromosomal and submicroscopic genetic abnormalities on average are prevalent in about 45% of early RSA samples. Early idiopathic RSA is caused mostly by chromosomal abnormalities and, as shown recently, with only a residual spontaneous abortion rate of 7% (Hodes-Wertz et al., 2012). Information on chromosomal aberrations in the pregnancy losses of patients who have experienced RSA in this study was not available, so it is hard to accurately discriminate the effect of the *ANXA5* M2 haplotype from an elevated risk of chromosomal abnormalities in general. A recent study of RSA cases from Estonia and Denmark has demonstrated genomic copy number variations significantly enriched in this particular patient group and conferring an increased risk of RSA (Nagirnaja et al., 2014).

Indeed, the risk of RSA attributed to M2 carriers from both retrospective and prospective clinical cohorts and estimated in Germans, for example, has been rather consistent. Results were from a total of 600 patients who have experienced RSA, largely pre-screened negative for fetal chromosomal abnormalities among other well-known risk factors, with 1123 control participants included in these studies (Bogdanova et al., 2007; Rogenhofer et al., 2012, 2013a, 2013b, 2014; Tüttelmann et al., 2013).

A recent study examining the risk role of *M2/ANXA5* in the most sizeable European RSA cohort (500 couples), confirms the risk role of the haplotype and sheds more light on allelic dependence and interaction with additional pro-thrombotic risk factors (Demetriou et al., 2015). This recent study, affirming the association of the *ANXA5* risk haplotype with early, but not late, RSA (as noted previously by Tüttelmann et al., 2013), raises the question of whether 10.5% of Estonian women and 22.9% of Danish women who had experienced both early and late RSA, respectively (according to Table 4 in Nagirnaja et al., 2015), should have been analysed separately from the other patient cohorts that only experienced early spontaneous abortions.

Control groups

To arrive at a greater statistical power, without even mentioning population structure comparative genotyping, the authors used imputation of *M2/ANXA5* SNP data from the German KORA S3 cohort (Holle et al, 2005), composed of 1644 participants. Despite the fact that controls in the KORA study have been used in many disease association studies, they have some limitations. First, the controls do not adhere to the definition of random population controls, as they comprise of DNA samples from blood donors, defined as "healthy", recruited in the Augsburg area of Bavaria, South Germany. This presents methodological age, gender and status bias. In contrast, PopGen controls are true random population controls as defined by Krawczak et al. (2006). Second, there is a problem with the haplotype reconstruction. Statistical derivation of haplotypes from KORA genotype data usually results in incidence overestimates, which are even greater when common haplotypes are considered, owing to phase reconstruction errors that are inherent to array genotyping (Heid et al., 2005). Third, from all *M2/ANXA5* published research that confirms the haplotype as RSA risk factor, three original studies use random population controls, according to their strict definition: seminal publication on the risk role of *M2/ANXA5* (Bogdanova et al., 2007) (and all subsequent publications utilizing the PopGen cohort); the study by Tüttelmann et al. (2013), using a population sample from the National Genetic Laboratory in Sofia, Bulgaria; and the recent study by Thean Hock et al. (2015), with a Malaysian random population sample. The varying incidences reported for the M2 haplotype among world populations are to be considered, but even the very recent Malaysian study that posits a 23.6% genetic incidence of M2 with a corresponding 42.2% carriage rate for Malays, agrees on the risk role of the haplotype in idiopathic women and couples who have experienced RSA. In addition, several studies on European populations (Bogdanova et al., 2007; Demetriou et al., 2015; Rogenhofer et al., 2012; Tiscia et al., 2009; Tüttelmann et al., 2013), which in total included 944 parous female controls with at least one uneventful pregnancy and without pregnancy loss, reported incidences of M2 haplotype ranging from 10 to 17% among these controls, with correspondingly elevated carriage rates among the clinically selected groups, thus confirming the risk role in patient cohorts with idiopathic RSA.

Discussion of expression studies

Historically, reporter gene assays in a functionally representative cell line delivered the first evidence on the physiological action of the M2 haplotype (Bogdanova et al., 2007). In later studies on chorions carrying the M2 haplotype, reduced *ANXA5* mRNA abundance was shown, which was confirmed to be haplotype specific, and concomitantly lowered ANXA5 protein levels have been detected in placental tissue of M2 carriers with a thrombophilic placental complication. Nagirnaja et al. (2015) discuss a counter example of a single study that, in their opinion, demonstrates increased ANXA5 plasma levels depending on M1 haplotype in healthy individuals (Hiddink et al., 2012). The "significant impact" of *M1/ANXA5* in Dutch control paraticipants discussed by Hiddink et al. (2012), is based on only six homozygous samples in total, with two of them showing twice as high plasma levels as the other four. One should exercise caution in making a justified conclusion from these data.